Behavioral Therapy to Ameliorate the Effects of Neonatal Alcohol Exposure on Dendritic Organization of Pyramidal Neurons in the Rodent mPFC

by

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ABSTRACT

During the third trimester of pregnancy in humans, a brain goes through the phase of rapid growth known as "brain growth spurt." A comparable event in brain development occurs in the first ten postnatal days of a rat's life. Third trimester alcohol exposure (or its equivalent) results in impaired cognition in adulthood and permanent structural changes in the brain, including the prefrontal cortex. The present study uses a rat model to examine the effect of neonatal alcohol exposure on dendritic morphology of pyramidal neurons in Layer III of the medial prefrontal cortex (mPFC), one of the last brain areas to develop in both humans and rats. Specifically, the density and phenotype of dendritic spines were analyzed. The present study also explores the impact of physical activity (wheel running WR) on dendritic morphology of mPFC neurons. Rat pups were randomly assigned to three groups: intubated with alcohol (5.25 g/kg/day; AE), sham intubated (SI), or suckle controls (SC) on PD 4–9. In order to study the effect of voluntary exercise, animals were placed in cages where they had 24-hour access to wheels during PD30-42. On the final day treatment, rats were anesthetized and perfused with 0.9% saline. Brains were subsequently processed for Golgi-Cox staining. Spine density and spine phenotypes were analyzed for basilar dendrites of Layer III mPFC neurons. The present study found that neonatal exposure to alcohol results in reduced spine density in both proximal and distal branches of basilar dendrites. This outcome was ameliorated following exposure to WR. In contrast, no differences in spine phenotype ratios were observed, but it was found that

that regardless of housing and postnatal treatment, proximal branches had significantly more mature spines than distal branches.

Chapter 1

INTRODUCTION

1.1 Fetal Alcohol Disorders

Despite the substantial research in both humans and animals that demonstrates consumption of alcohol by a mother during pregnancy can be harmful to the developing fetus, alcohol consumption during pregnancy is still a prevalent phenomenon. A study conducted by the Centers for Disease Control and Prevention between 1995 and 2001 found the average annual percentage of any alcohol use among pregnant women to be 12.2%, with 1.9% of women engaging in binge drinking, defined as 4 glasses or more at one time (Denny et al., 2009). Furthermore, in a study of 4,088 women who gave birth to children with birth defects, 30.3% of women reported drinking alcohol at some time during pregnancy, and 8.3% reported binge drinking (Ethen et al., 2009). Thus, maternal consumption of alcohol is an important and widespread problem with serious consequences.

Fetal Alcohol Spectrum Disorder (FASD) is an umbrella term that encompasses a wide range of physical, behavioral, cognitive, and learning deficits associated with prenatal alcohol exposure (Chudley et al., 2005). Recent findings suggest that the current prevalence of FASD in young children may be as high as 2-5% in the United States and Western European countries (May et al., 2009). The detrimental effects caused by early exposure to alcohol depend on the time, duration, and amount of exposure during gestation. For example, the amount of maternal alcohol consumption has also been correlated with decreases in frontal lobe size in the

developing child (Wass et al., 2001). Fetal alcohol syndrome (FAS) describes the most severe manifestation of alcohol exposure *in utero*. It is estimated to occur in approximately 0.5 to 2 out of 1000 live births (May & Gossage, 2001). FAS is characterized by craniofacial abnormalities, such as a thin upper lip, an indistinct philtrum, and a short nose. Other hallmark physical signs include growth deficiencies, abnormal brain size and shape, noteably in the frontal lobes, and dysfunction of the central nervous system (Sowell et al., 2002; Sampson, 1997).

In addition to physical manifestations, developmental alcohol exposure causes a number of cognitive and behavioral deficits. Children exposed to alcohol *in utero* often exhibit deficits in tasks requiring cognitive flexibility, inhibition, some measures of verbal fluency, abstract thinking, deductive reasoning, hypothesis testing, problem solving, and concept formation (Rasmussen & Bisanz, 2009). Even moderate consumption of alcohol may cause deficits that do not manifest until the child begins formal education: for example, maternal consumption of one or two drinks per day resulted in a 7 point decrease in IQ in seven year olds (Streissguth et al., 1990). Furthermore, adolescents with FASD do not perform as well in school, have difficulty developing normal relationships with peers, and are more prone to engage in substance abuse (Banakar et al., 2009). Thus, the effects and behavioral manifestations of alcohol exposure *in utero* are considerable and long-lasting.

1.2 Alcohol and the brain

Significant damage on the cellular level is caused by exposure to alcohol. Alcohol easily crosses the placenta and can cause apoptosis and necrosis of cells, which in turn impacts developing cell lineages. Cells of the central nervous system

are susceptible to its harmful effects. For example, it has been demonstrated that exposure to alcohol *in vitro* slows the cell cycle and increases the incidence of cell death of neocortical neurons obtained from sixteen-day old rats (Jacobs & Miller, 2001). In addition, alcohol negatively affects glial cells by inducing their transformation into astrocytes prematurely, which inhibits normal neuronal migration (Miller & Robertson, 1993). Alcohol also affects various neurotransmitter systems. For example, neonatal exposure to alcohol reduces the number of N-methyl-D-aspartate (NMDA) receptors, which bind the excitatory transmitter glutamate (Ikonomidou et al., 2000). This affects other neurotransmitter systems in turn, as glutamate and its NMDA receptors are important for the formation of synapses as well as plasticity. Conversely, alcohol increases the activity of neurons producing Gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter (Goodlett & Horn, 2001). It has been shown that ethanol administration on PD7 causes widespread apoptosis (physiological cell death) due to ethanol's dual antagonist effect of NMDA receptors and agonistic effect on $GABA_A$ receptors (Ikonomidou et al., 2000). Thus, the effects of alcohol on neurons are numerous and especially devastating during development.

Several brain structures including, but not limited to, the prefrontal cortex (PFC) undergo significant development during the brain growth spurt, which occurs in the third trimester of human gestation. Consequently, these structures are highly susceptible to neonatal alcohol exposure at this time. While a substantial proliferation of neurons occurs prior to the brain growth spurt, increases in dendritic complexity and synaptogenesis take place during this time period, accompanied by a significant increase in the number of glial cells and myelination of neurons (Dobbing & Sands, 1973). The brain growth spurt is a phenomenon that is observed in all species.

However, different species are born at different developmental stages, and consequently the time at which the brain growth spurt is observed varies from prenatal to postnatal by species. For example, the brain growth spurt take place in humans during the third trimester of gestation, and this corresponds to the first ten days of postnatal rat life. Consequently, this period of rodent development serves as a good model for examining the effects of alcohol exposure on the developing brain.

1.3 Effect of alcohol exposure on the Prefrontal Cortex (PFC)

The PFC is a brain area known to be important for executive functioning, which involves higher-order processing under conscious control as well as goal-oriented behavior (Welsh, Pennington, & Grossier, 1991). This encompasses planning, attention, decision-making, inhibition, and working memory. It is widely accepted that medial PFC area in rodents shares at least some of the higher cognitive functions encountered in primates. The ventral portion, which receives input from autonomic areas, is associated with adaptive behaviors, while the more dorsal region is important for temporal shifting during behavioral sequences (Rotaru et al., 2005). The PFC is one of the last brain areas to develop in both humans and rodents. In humans, the basic structure of the PFC is fully formed at birth and continues to mature into adolescence, as connections with other areas of the brain are refined and pruning and myelination take place (Simpson, 2008). The development of the PFC begins just prior to birth in the rat brain and does not reach completion until the first few weeks of postnatal life. The more superficial layers, including Layer III, mature more slowly and at a later time than deeper layers of cortex, as the cortex is formed from the inside-outside; in other words, the deeper layers (V and VI) are formed first (Fuster 2008).

Pyramidal neurons of the cortex have apical and basilar dendrites that stem from the apex and base of the soma, respectively. Apical dendrites of Layer III neurons of the PFC are important sites of cortico-cortical connections, while basilar dendrites receive input from the mediodorsal nucleus of the thalamus (Heidbreder and Groenewegen, 2003; Rotaru et al., 2005). Furthermore, it is important to distinguish between the proximal and distal branches of basilar dendrites. Spines in the proximal regions receive excitatory inputs from local areas, while inhibitory input from the mediodorsal nucleus of the thalamus is exerted by parvocellular interneurons on the soma and proximal dendrites (Rotaru et al., 2005). In contrast, distal branches mainly receive input from more distal cortical as well as direct connections from the thalamus (Spruston, 2008).

Few studies have examined the impact of neonatal alcohol exposure on the rodent medial PFC (mPFC). Prenatal alcohol exposure via a liquid diet decreased cell numbers in mPFC of male rats, and this was correlated with deficits in executive function (Mihalick et al., 2001). Inhalation of alcohol during PD2-6 resulted in a shorter duration and lower number of dendritic spikes in Layer V pyramidal neurons from the somatosensory cortex in adulthood (PD30-60) (Granato et al., 2012). Furthermore, similar experiments demonstrated that alcohol-exposed rats had simplified complexity of basilar dendrites of Layer II/III neurons (Granato et al., 2003). Previous work by the Klintsova lab found that binge-like alcohol exposure during PD 4-9 decreased basilar dendritic complexity in adolescent rats; however, a similar effect was not found for apical dendrites. Furthermore, while basilar spine density did not appear to be impacted by postnatal alcohol exposure, apical dendrites were found to have a decreased number of spines. Despite the fact that a significant

change in basilar spine density was not found, an increase in the number of mature spines was evident, although a similar effect was not observed in apical dendrites (Whitcher & Klintsova, 2008; Hamilton, Whitcher & Klintsova, 2010). This outcome suggests that the impact of alcohol on pyramidal neurons in the mPFC is specific and differs even across parts of the neuron. In turn, afferent inputs are selectively impacted. For example, unchanged spine density accompanied with decreased dendritic length of proximal basilar dendrites of layer II/III pyramidal neurons could result in a decrease of inhibitory inputs from the mediodorsal nucleus of the thalamus conveyed by interneurons (Hamilton, Whitcher & Klintsova, 2010). Furthermore, increased ratio of mature to immature spines suggests a decrease in plasticity. This overall decrease in inhibitory input could account for deficits observed in FASD patients, namely hyperactivity and lack of inhibition.

1.4 Importance of dendritic spines

Pyramidal neurons are studded with thousands of dendritic spines. It is estimated that only 4% of spines of pyramidal neurons in the neocortex do not have a functional synapse, and the majority of these connections are excitatory. However, inhibitory synapses can occur on the neck or head of a spine in addition to a primary excitatory connection (Yuste, 24). Furthermore, spines differ greatly in terms of shape and size. It is thought that immature spines are highly plastic and play a significant role in learning, while mature, stable spines are associated with established memories (Spruston, 2008). The size and complexity of the postsynaptic density (PSD), an electron-dense thickening that contains important proteins and receptors, has been

correlated with the size of the spine as well as the number of AMPA receptors (Yuste, 23).

In both humans and rats, it has been shown that spines are subject to external influence, including sensory deprivation, amphetamine and cocaine administration, and alcohol exposure (Zuo et al., 2005; Robinson & Kolb, 1999; Whitcher & Klintsova, 2008; Hamilton, Whitcher & Klintsova, 2010). During an autopsy of a four month old child born to a chronic alcoholic mother, staining using the Golgi method revealed a decrease in the number of dendritic spines on apical dendrites of Layer V cortical pyramidal neurons. Furthermore, there were a greater number of long, thin spines (Ferrer & Galofré, 1987). Interestingly, dendritic anomalies, including spine dysgenesis, have consistently been correlated with mental retardation (Kauffman et al., 2000). Thus, changes in spine density and phenotype as a result of alcohol exposure could explain deficits observed in children with FASD. In addition, decreases in spine density were also found for apical and basilar dendrites of Layer III pyramidal neurons in the dorsolateral prefrontal cortex (area 46) of schizophrenic patients when compared with normal controls and psychiatric subjects (Glantz, 2000).

Additionally, work in rodents shows that perinatal alcohol exposure decreased spine density in the proximal region of both apical and basilar dendrites and increased soma size in layer II/III neurons of the PFC when observed at PD90 (Lawrence et al., 2011). In contrast, studies in the Klintsova lab demonstrated that basilar spine density was not altered by postnatal alcohol exposure, while apical dendrites had fewer spines. However, there was an increase in the number of mature spines on basilar dendrites (Whitcher & Klintsova, 2008; Hamilton, Whitcher & Klintsova, 2010). Chronic exposure of hippocampal neurons to alcohol in culture resulted in an increase in

clustering of NMDA receptors on the synapse and consequently spine size, as evidenced by increases in PSD-52, a synaptic scaffolding protein, and filamentous actin (F-actin). Observed changes in the homeostasis of the synapse were thought to impact synaptic efficacy; since larger spines provide greater stability, future modification of the synapse could be limited (Carpenter-Hyland et al., 2007). Decreased plasticity hinders the ability to form new connections and to adapt as a result of change. Thus, it is ideal to maintain a balance between larger, more stable spines and long, thin spines.

1.5 Voluntary exercise as behavioral remedy

The beneficial effects of voluntary exercise on the brain are well researched. In humans, aerobic exercise has been correlated with increased volume of both gray and white matter in elderly subjects (Colcombe et al., 2006). Highly fit elderly individuals as well as those who did aerobic training for 6 months performed better on tests of executive functioning and showed greater task-related activity in regions of the prefrontal and parietal cortices that are involved in spatial selection and inhibitory functioning (Colcombe et al., 2004). In mice, exposure to WR for 3 weeks was associated with increases in cerebral blood flow following an infarct (Endres et al., 2003). Wheel running and treadmill running increased angiogenesis and cerebral blood flow in rats (Swain et al., 2003). These cardiovascular benefits provide the brain with increased levels of oxygen and nutrition factors.

In addition, human and animal research has demonstrated that exercise enhances levels of neurotrophic factors, including brain-derived neurotrophic factor (BDNF). BDNF promotes the differentiation, neurite extension, and survival of

neurons in culture, including, but not limited to, cortical neurons. It also has been shown to protect against ischemic damage in the cortex and hippocampus and to enhance plasticity (Cotman & Berchtold, 2002). Exposing rodents to wheel running (WR) for three days resulted in an increase in synapsin I and synaptophysin, two groups of molecules essential for synaptic transmission. However, blocking BDNF receptors in the hippocampus during this time period eliminated this effect, thereby confirming that exercise enhances BDNF and implicating this neurotrophic factor as a vital player in synaptic transmission (Vaynman et al., 2006).

Increases in blood flow, oxygenation, and neurotrophic factors as a result of voluntary exercise contribute to various observed structural benefits. For example, in rats, exposure to WR in adulthood has been correlated with increases in dendritic length, complexity, and spine density of cells in the dentate gyrus region of the hippocampus in rats (Eadie, Redila & Christie, 2005). Similarly, increases in the density of dendritic spines were observed in granule neurons of the dentate gyrus, CA1 pyramidal neurons, and Layer III pyramidal neurons of the entorhinal cortex of adult males rats singly housed in cages with free access to wheels for two months. In the CA1 region, changes in dendritic spine density were accompanied by changes in dendritic arborization and alterations in the morphology of individual spines, such that there more significantly longer spines on basilar dendrites and more spines with smaller heads (i.e. filopodia or immature spines) on both apical and basilar dendrites (Stranahan et al., 2007). A study performed by Helfer et al. (2009) showed that adolescent exposure to WR increases the number of proliferating cells in the hippocampus in both alcohol-exposed and control animals.

Exercise has also been shown to improve performance on behavioral tasks, both in normal subjects and those with impaired cognitive function. For example, exposure of rats to WR for one week before forced swimming attenuated down-regulation of hippocampal BDNF mRNA and improved behavioral measures of stress (Russo-Neustadt, A. et al. 2001). WR also enhanced performance on the Morris water maze task, a test of spatial learning, in both ethanol-treated and control animals (Thomas et al., 2008). Improvements in learning and memory subsequent to physical activity can be attributed to enhanced plasticity due to increases in BDNF. The various benefits of voluntary exercise will hopefully be shown to ameliorate the damaging effects of alcohol exposure on neurons in the mPFC.

1.6 Purpose

The purpose of this thesis is to expand upon previous work of the Klintsova lab by examining the long-term impact of binge-like alcohol exposure during PD4-9 on the density and maturity of dendritic spines on Layer III mPFC pyramidal neurons in adolescent (PD42) rodents. In addition, this thesis explores the impact of WR on the detrimental effects associated with neonatal alcohol exposure on mPFC dendritic morphology. Based on previous work, it is expected that spine density will not change while the ratio of mature vs. immature spines will increase as a result of neonatal alcohol exposure. Such an outcome would also confirm that the effects of neonatal alcohol exposure in rodents are severe and persistent. In addition, it is anticipated that WR will ameliorate the effects of alcohol on dendritic organization. The current research will serve as an important contribution to FASD literature. Few studies have

examined the morphological effects of early life alcohol exposure on the mPFC. Furthermore, this study will provide new information related to the benefits of behavioral interventions for FASD.

Chapter 2

METHODS

2.1 Animals

The subjects of the study were male Long Evans offspring of animals bred at the University of Delaware animal breeding colony. On PD23, pups were weaned and placed in standard cages with two or three rats to a cage. Rats were given access to food *ad libitum* for the duration of the study. The housing facility was maintained on a 12:12-hr light-dark cycle, with lights on at 0900hr.

2.2 Alcohol Exposure

Eight litters were culled to eight pups—six males and two females, whenever possible—on PD4. The pups were randomly assigned to one of three dosing conditions and were given an injection of ink in their paws for identification purposes. Between PD4-PD9, alcohol exposed (AE) animals received an intragastric intubation every two hours. The first two intubations of each day delivered a daily dose of 5.25g/kg of ethanol in milk formula. A third intubation of milk without ethanol was given two hours after the second alcohol dose. On the first day of intubations (PD4), a fourth intubation of milk without ethanol was administered four hours after the second alcohol dose in order to compensate for reduced milk intake by AE pups. Sham-intubated (SI) pups received intragastric intubations without milk or ethanol to control for the stress of the intubation procedure. A second control group, suckle control (SC), was left undisturbed; though, the animals were weighed daily during the dosing period

to check for proper development. Blood samples were collected from the tail vein (by cutting the tail end) of AE and SI pup 90 minutes after the second alcohol dose on PD4. Samples from AE animals were centrifuged (15000rpm for 15 minutes) and then the plasma was collected and frozen (-20C) for future analysis using an Analox GL5 Alcohol Analyzer (Analox Instruments, Boston, MA). Samples from SI animals were discarded after collection.

2.3 Wheel Running

Between PD30 and PD 42, half of the animals were pseudo randomly assigned to cages with attached stainless steel running wheels, allowing them to have 24-hour access to voluntary exercise. Each cage consisted of one animal from each of the three groups (AE, SI, and SC), because previous studies have shown that there is no difference in running activity between these three groups (Helfer et al., 2009). A counter attached to each of the wheels tallied the number of revolutions of the wheel, which served to determine the rats' daily running activity. At the onset of the light cycle (0900h) each day, this number was recorded. The remaining half of the animals from all three conditions (AE, SC, and SI) were housed in groups of three in standard cages (45×24×21cm).

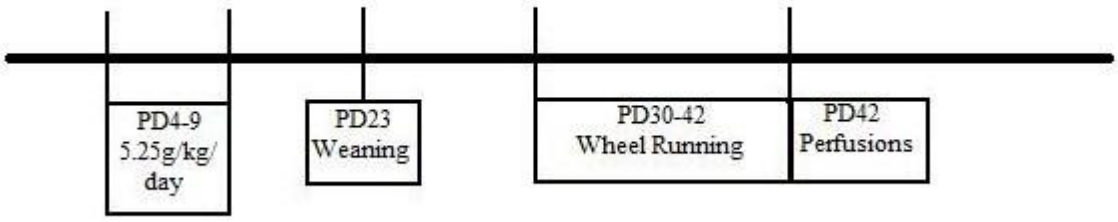


Figure 1: Timeline of exposure to WR

2.4 Golgi-Cox Staining

The procedure used for this study was based on previous methods used by the Klintsova lab (Whitcher & Klintsova, 2008; Hamilton, Whitcher & Klintsova, 2010). Animals were perfused first with heparinized 0.9% saline, then 0.9% saline without heparin. Then, brains were placed in Golgi-Cox solution, which is composed of 1% potassium dichromate, 1% mercury (II) chloride and 0.8% potassium chromate in distilled water. The solution was filtered after 5 days of storage in the dark, and then brains were placed in the solution for 2 weeks. Two days before sectioning of the tissue, the solution was replaced two times with 30% sucrose in 0.9% saline. Sectioning was performed using a vibratome. The prefrontal cortex was sectioned coronally 200μm thick and mounted on 2% gelatinized double subbed slides. After being kept in cool, high humidity environment overnight, slides were dipped in distilled water for 1 minute followed by 30 minutes of immersion in 2:1 ammonium hydroxide: distilled water solution in the dark. Slides were then rinsed in distilled water for 1 minute and placed in a 1:1 Kodak rapid fix: distilled water solution for 30 minutes in the dark. Again, the slides were placed in distilled water and then run through a series of alcohol solutions (70%, 95%, 100%) in order to dehydrate the

tissue. Finally, the slides were placed in 1:1 100% ethanol: histoclear solution followed by histoclear for 15 minutes each. Immediately following this procedure, slides were coverslipped using permount and stored in a cool dark place.

2.5 Spine Analysis

Dendritic trees of neurons in Layer III of mPFC were traced using the computer-based neuron tracing system, NeuroLucida—Version 8.10.1; MBF, Bioscience, Williston, VT. An experimenter blind to conditions identified fully impregnated spines on one second and one fourth order dendritic branch per neuron. The branches that were analyzed were selected based on the following criteria: 1) fully impregnated; 2) not blocked by any debris or neighboring branches; 3) over 20μm in length. Spine density was calculated per 10μm of dendritic length. The first ten spines of each branch were classified based the characteristics outlined by Irwin and colleagues (2002) and McKinney et al (2005). Spine analysis was performed on five to seven cells per animal.

2.6 Data Analysis

Using SPSS Statistics, the influence of postnatal treatment on body weights was determined using a repeated-measures ANOVA with postnatal condition (AE, SI, SC) as the between subjects factor and postnatal day as a within subjects factor, followed by post hoc analysis (Tukey's test). One-way ANOVA followed by Tukey post hoc test were used to evaluate the effect of postnatal condition on both the basilar dendrites' spine density in mPFC and the ratio of mature:immature spine phenotypes. The level of significance was set at $p < 0.05$ for all tests. 29 male animals were used

for this study. Three animals were lost during intubations. All groups had five animals per group except SC/SH, for which there were only four animals.

Chapter 3

RESULTS

3.1 Weights

Animal weights were recorded every day during alcohol administration (PD4 and 9) and WR (PD30 and 42). Weights from the neonatal period (PD 4 and 9) versus adolescence (PD 30 and 42) were analyzed separately. For neonatal body weights, a day by postnatal treatment interaction was evident [$F_{2,29}$=25.993, p = .000] as was a main effect of day [$F_{1,29}$=1278.506, p = .000]. A univariate ANOVA of PD4 weights revealed a main effect of postnatal treatment [$F_{2, 29}$=3.605, p = .042]. Post hoc tests showed that AE animals were significantly larger than SC, but not SI, animals. The same type of analysis on PD9 weights also revealed a significant main effect of postnatal treatment [$F_{2,29}$=8.300, p = .002]. Post hoc tests demonstrated that AE animals weighed significantly less than both SI (p = .008) and SC (p = .003), suggesting a powerful effect of alcohol. A repeated-measures ANOVA of adolescent body weights (PD30 and 42) showed a main effect of day [$F_{1, 29}$=583.258, p = .000] but neither a main effect of postnatal treatment nor a treatment by day interaction.

Table 1: Recorded animal weights throughout treatment. * indicates P<.05 versus SC, ** for P<.01

	Alcohol Dosing			
	PD4	PD9	PD30	PD42
SC	10.22±.441	20.00±1.323	110.20±30.865	100.56±8.509
SI	10.50±.927	19.60±.2.119	104.30±8.138	185.90±13.144
AE	11.20±.917*	17.20±1.317**	100.56±4.216	181.89±7.897

3.2 Blood Alcohol Concentration (BAC)

BAC was measured 1.5 hours after the second alcohol dose on PD4, the first day of exposure. The mean BAC (± SEM) was 403.75 ± 13.185 mg/dl for the WR only study.

3.3 Wheel Running

For the WR only study, the numbers of wheel revolutions (±SEM) per 24-hour period was 3594± 416 (approximately 2.49 miles) on average for all animals.

3.4 Spine Density of Basilar Dendrites

Spine density was calculated per 10μm of dendritic length. Univariate ANOVA postnatal condition and housing revealed no significant differences in spine density for Order 2 branches. However, we had reason to believe that there could be a significant effect of postnatal condition, because post hoc analysis suggested a trending difference in the number of spines in AE animals of both housing conditions (WR and SH) as compared to SC animals ($p = .088$). Separate analysis was then performed for SH and WR animals. Univariate ANOVA examining the effect of postnatal condition in SH animals confirmed a significant decrease in spine density ($F_{2,29} = 4.726$, $p = .033$), and post hoc tests indicated that Order 2 branches of AE animals had significantly fewer spines than SC animals ($p = .031$). Conversely,

univariate ANOVA of postnatal treatment effect on spine density in WR animals yielded no significant differences (Figure 2).

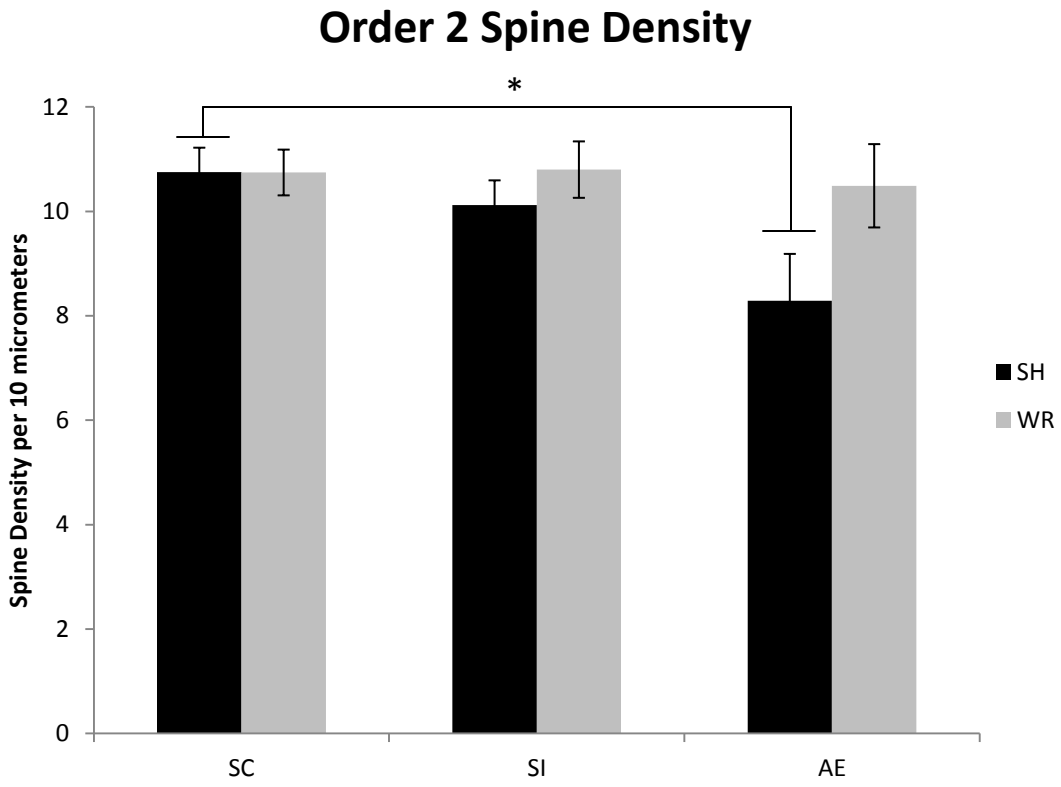


Figure 2: Comparison of spine density of Order 2 basilar dendritic branches. A significant decrease in spine density of AE animals is evident after SH but not WR.

For Order 4 branches, univariate ANOVA of postnatal condition and housing also revealed a trend towards a main effect of postnatal condition ($F_{2,29} = 3.240$, $p = .058$). Post hoc analysis indicated a reduction in spine density of Order 4 branches for AE animals compared with SC animals ($p = .051$). Univariate ANOVA for SH animals only revealed significant differences between Order 4 spine density for AE

and SC animals ($F_{2,29} = 4.748$, $p = .033$). Furthermore, post hoc tests indicated that this is due a reduction in spine density of AE animals compared to SC animals ($p = .026$). A univariate ANOVA was also performed for WR animals and revealed no significant differences of Order 4 spine density as a result of postnatal condition (Figure 3).

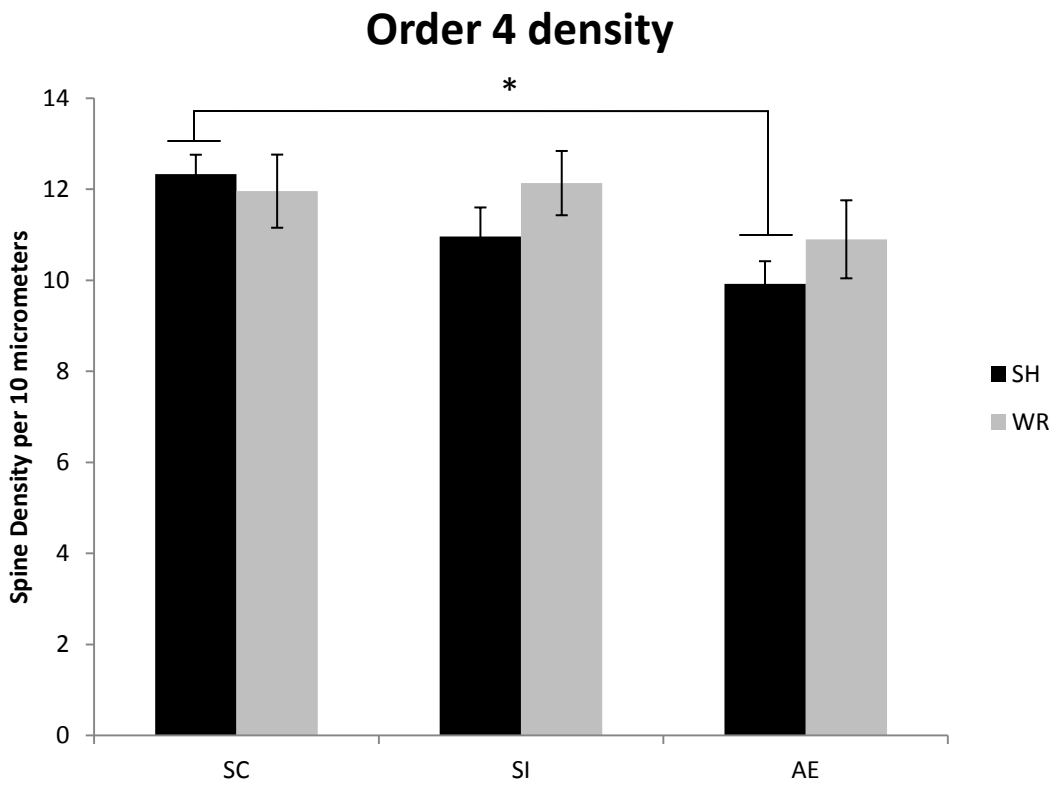
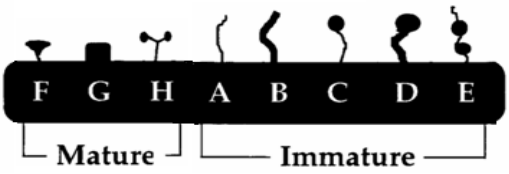


Figure 3: Comparison of spine density of Order 4 basilar dendritic branches. Exposure to alcohol resulted in a significant decrease in spine density for SH animals only.

3.5 Spine Phenotype of Basilar Dendrites

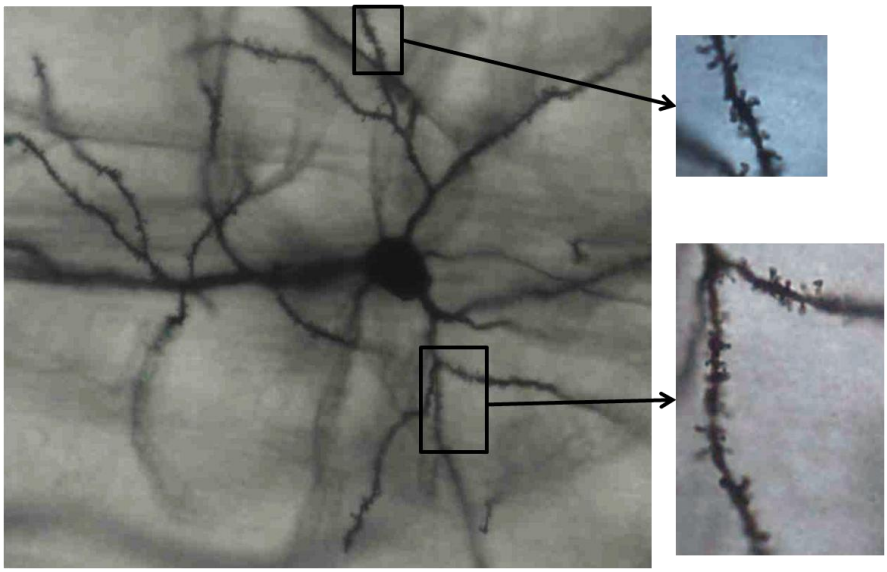
The first ten spines of one Order 2 and one Order 4 branch were classified based on characteristics outlined by Irwin and colleagues (2002) and McKinney et al (2005) (Figure 4a). For Order 2 branches, univariate ANOVA of postnatal condition and housing revealed no main effect of either parameter (Figure 5). Similar outcomes were seen for Order 4 branches (Figure 6). Interestingly, a main effect of order was found for both WR ($F_{2,29} = 15.232$, $p = .001$) and SH ($F_{2,29} = 49.928$, $p = .000$) animals, such that there was a significantly higher percentage of immature spines on Order 4 than Order 2 branches.

A)



MCKinney et al. (2005)

B)



C)

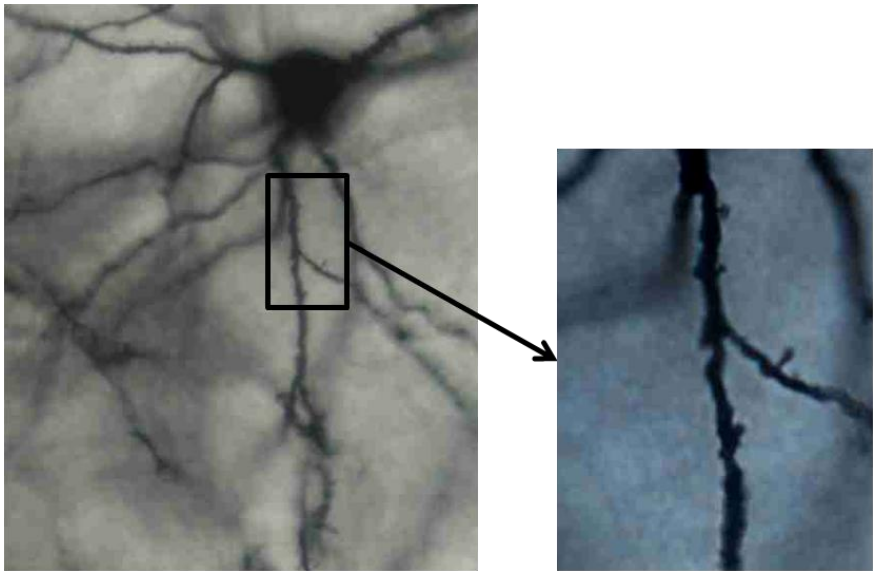


Figure 4: Spine Analysis. A) Spines were categorized based on previously established criteria. Cartoon obtained from McKinney et al., 2005. B) and C) Examples of Golgi impregnated Layer III pyramidal neurons in the mPFC that were analyzed. Images on the left are magnified with a 40x objective. Images on the right are magnified with a 100x objective. This high magnification allows determination of spine density as well as the classification of spine phenotypes.

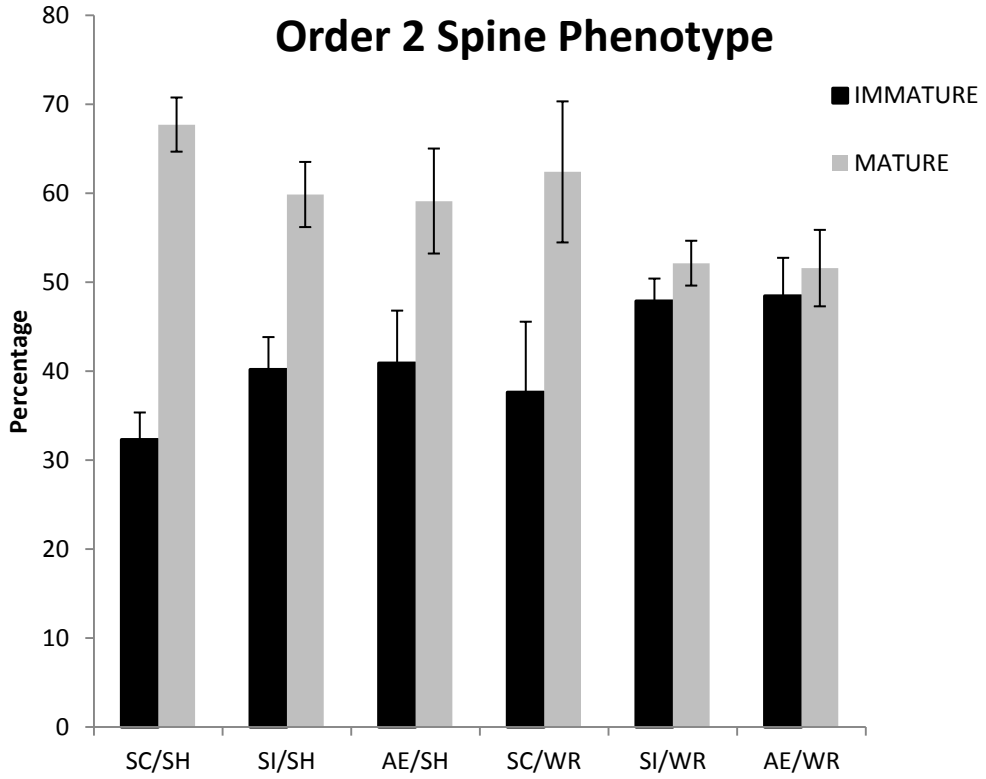


Figure 5: Spine Phenotype ratios for Order 2 branches. No significant differences were found as a result of postnatal condition or housing.

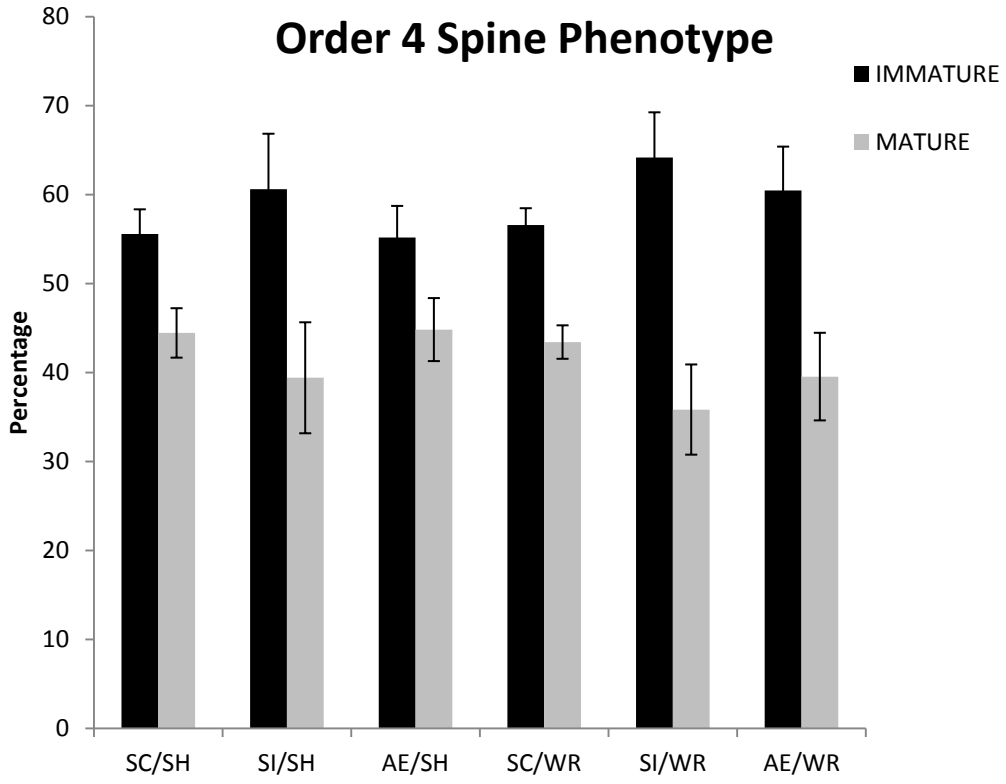


Figure 6: Spine phenotype ratios for Order 4 branches. No significant differences were found as a result of postnatal condition or housing.

Chapter 4

DISCUSSION

The results of the present study indicate that neonatal alcohol exposure impacted spine density of basilar dendrites of Layer III mPFC neurons such that a decrease in spine density was observed at PD42. Furthermore, there were no differences in spine density between AE and SC animals after WR, which suggests an ameliorative effect of voluntary exercise. Spine phenotype ratios were neither affected by exposure to alcohol nor housing. However, it was found that Order 4 branches have significantly more immature spines than Order 2 branches across conditions.

4.1 Effects on spine density

Previous studies in our lab showed that similar exposure to alcohol did not affect spine density of basilar dendrites but did cause an increase in the number of mature spines (Whitcher & Klintsova, 2009; Hamilton, Whitcher & Klintsova, 2010). However, these effects were observed at PD26-30 while the present study examined PD42. Typically, spines appear and disappear within a period of days or weeks, but the average density remains stable. In an *in vivo* imaging study of 6-10 week old rats, 60% of spines were observed during eight days of imaging, and about 85% of spines classified as stable persisted 30 days after the first day of imaging (Trachtenberg et al., 2002). The decrease in spine density observed at PD42 can be explained by the fact that, following an initial increase, spine density begins to decrease naturally after the fourth week of postnatal life (Miller, 1988). This pruning occurs due to an initial overproduction of spines and is important for the "sculpt[ing] of circuits that are

efficiently matched to the computational tasks that particular cortical areas need to solve" (Yuste, 102). Spine density is particularly susceptible during the period of overproduction to sensory deprivation and experimental paradigms (Yuste, 83). Thus, postnatal alcohol exposure may accelerate this natural pruning effect since it has been shown, for example, to increase the incidence of death of neocortical cells both *in vitro* and *in vivo* (Jacobs and Miller, 2001; Ikonomidou et al., 2000). In fact, an outcome similar to that found in the present study was seen when the effects of perinatal alcohol exposure were explored in rats at PD90. Most notably, AE animals were found to have decreased spine density in the proximal region of basilar dendrites in II/III neurons of the mPFC at this later time point (Lawrence et al., 2011).

Exposure to WR increased spine density in AE animals such that density in AE and SC animals was no longer significantly different. However, there was no effect of housing on the control animals themselves. The latter finding is in line with previous studies. For example, exposure of normal rats to WR for 4 weeks was not associated with increases in spine density in the PFC (Robinson and Kolb, 1999). Furthermore, while sensory deprivation by trimming whiskers increased the number of transient spines in the barrel cortex, the number of stable spines also decreased, resulting in a comparable spine density (Trachtenberg et al., 2002). However, there is also evidence that voluntary exercise can increase spine density. As previously mentioned, increases in spine density in the dentate gyrus of adult rodents were observed following a two-week exposure to wheel running (Eadie, Redila & Christie, 2005). Similarly, increased spine density as observed the dentate gyrus, CA1, and Layer III of the entorhinal cortex of adult rats housed in cages with wheels. Increased spine density was accompanied by a greater number of longer spines with small heads; in other

words, immature spines (Stranahan et al., 2007). Since the present study found a selective effect of WR on AE exposed animals, this suggests that the decreased spine density in AE animals was abnormal. Moreover, the therapy was able to bring spine density levels back to baseline in the PFC of the damaged brain, but it was not powerful enough to alter the density of spines in normal animals. This is supported by the finding that WR also does not impact dendritic complexity (length, number of bifurcations, number of endings) in the mPFC (Hamilton et al., in preparation). Alternatively, it is possible that there is something about animals exposed to alcohol that makes them better able to benefit from WR, in which case no changes would be observed in SC and SI animals, or that the exposure was not long enough to have an impact on the PFC of a normal brain. Further studies are needed to answer these questions.

4.2 Effects on spine phenotype

Overall, spine phenotype ratios for both Order 2 and Order 4 branches were not altered as a result of exposure to alcohol. However, there did appear to be fewer immature spines (and more mature spines) on Order 2 branches of SC/SH animals compared with AE/SH and SI/SH animals. This could reflect an impact of a stressful event during early development on the pruning of spines, which is vital for the refinement of connections, as discussed earlier. In addition, while no significant change in the ratio of mature:immature spines was observed as a result of WR, a trend was observed in Order 2 branches such that there was a more even ratio of mature to immature spines. All three conditions exhibited this change to an extent, but it was most pronounced in AE and SI animals, with a noteworthy contrast between the mature:immature spine ratio of SC/SH (70:30) and AE/WR animals (55:45). In AE

animals, who exhibited an increase in spine density as a result of exposure to WR, it is likely that spines that were added as a result of wheel running were of an immature phenotype, while preexisting spines were converted to mature spines through reinforcement of connections. Consequently, the number of immature spines increased slightly. Such an effect was observed in CA1 pyramidal neurons of adult male rats after two months of wheel running (Stranahan et al., 2007). On the other hand, an increased number of immature spines could signify that connections were not stabilizing. A greater number of immature spines in AE/WR rodents suggests enhanced plasticity, as immature spines are thought to be more plastic and important for learning. Phenotype ratios in SC and SI animals were relatively stable, and no differences were observed for distal branches. The selective effect observed in the proximal branches can be explained by the aforementioned difference in afferent input.

The observed variance in spine phenotype ratios of Order 2 and Order 4 branches is very interesting and also can be explained by the inputs for proximal versus distal regions. While proximal branches receive excitatory inputs from local areas and inhibitory connections from the mediodorsal nucleus of the thalamus via interneurons, distal branches mainly receive input from more distal cortical afferents as well as direct connections from the thalamus. Thus, at this time point (PD42), direct connections with the thalamus are much more plastic, while inputs from inhibitory interneurons are much more stable. This effect is independent of postnatal treatment and housing. The difference in phenotype ratios as a result of order is likely due to the fact that inhibitory connections suppress activity in the cell while excitatory

connections enhance activity and demand a greater workload on the postsynaptic neuron.

4.3 Conclusions and future directions

The findings of the present study provide evidence for the neuroanatomical basis of behavioral deficits exhibited by FASD patients and contributes to the literature of the differential effects of alcohol on the mPFC and on dendritic spines. Neonatal alcohol exposure resulted in a reduced spine density in both proximal and distal branches of Layer III pyramidal neurons when examined at PD42. This deficit was ameliorated following a twelve days of WR. In contrast, spine phenotype was unaltered as a result of alcohol exposure and WR. However, distal branches were predominated by immature spines, while proximal spines had a greater number of stable spines.

Currently, our lab is examining the combined effect of WR followed by housing in an enriched environment (EC) on Layer III neurons in the mPFC. Since spine density and phenotype will be examined in adulthood (PD72) as opposed to adolescence, decreases in spine density are expected to be more evident. In addition, it is anticipated that a combined paradigm of WR followed by EC will have a more powerful ameliorative outcome on the detrimental effects associated with postnatal alcohol exposure in rodents than WR alone, since studies suggest that EC provides additional benefits that exceed the effects of voluntary exercise (Bernstein, 1973). Another interesting area of investigation would be to determine whether alcohol directly impacts spines themselves or the afferent inputs that synapse on the spines, since there appear to be differential effects on proximal versus distal branches. Finally, the impact of alcohol exposure and WR on dendritic morphology within the

various regions of the mPFC as well as other areas of the brain, such as the hippocampus, should be explored.

REFERENCES

Banakar MK, Kudlur NS, George S. (2009). Fetal alcohol spectrum disorder (FASD). Indian Journal of Pediatrics 76:1173-5.

Carpenter-Hyland EP, Chandler LJ. (2007). Adaptive plasticity of NMDA receptors and dendritic pines: implications for enhanced vulnerability of the adolescent brain to alcohol addiction. Pharmacology Biochemical Behavior 86: 200–208.

Chudley AE, Conry J, Cook JL, Loock C, Rosales T, and LeBlanc N. (2005). Fetal alcohol spectrum disorder: Canadian guidelines for diagnosis CMAJ 172:1–21.

Cotman CW and Berchtold NC. (2002). Exercise: a behavioral intervention to enhance brain health and plasticity. Trends in Neuroscience 25(6):295-301.

Denny CH, Tsai J, Floyd, RL, and Green PP (2009). Alcohol Use Among Pregnant and Nonpregnant Women of Childbearing Age --- United States, 1991—2005. Div of Birth Defects and Developmental Disabilities, National Center on Birth Defects and Developmental Disabilities, CDC 58(19);529-532.

Dobbing, J. and Sands, J. (1973). The quantitative growth and development of the human brain. Archives of Disease in Childhood 48, 757-767.

Eadie BD, Redila VA, and Christie BR. (2005). Voluntary exercise alters the cytoarchitecture of the adult dentate gyrus by increasing cellular proliferation, dendritic complexity, and spine density. The Journal of Comparative Neurology 486(1):39-47.

Endres M, Gertz K, Lindauer U, Katchanov J, Schultze J, Schrock H, Nickenig G, Kuschinsky W, Dirnagl U, Laufs U. (2003). Mechanisms of stroke protection by physical activity. Annals of Neurology 54: 582–590.

Ethen MK, Ramadhani TA, Scheuerle AE (2009). Alcohol consumption by women before and during pregnancy. Maternal Child Health Journal 13(2):274-285.

Ferrer I, Galofré E. (1987). Dendritic spine anomalies in fetal alcohol syndrome. Neuropediatrics 18(3):161-3.

Fuster JM. The prefrontal cortex. New York: Raven Press, 2008.

Glantz LA, Lewis DA. (1998) Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. Archives of General Psychiatry 57: 65–73.

Goodlett CR and Horn KH. (2001). Mechanisms of alcohol-induced damage to the developing nervous system. Alcohol Research & Health 25:175–184.

Granato A, Di Rocco F, Zumbo A, Toesca A, Giannetti S. (2003). Organization of cortico-cortical associative projections in rats exposed to ethanol during early postnatal life. Brain Research Bulletin 60(4):339-344.

Granato A, Palmer LM, De Giorgio A, Tavian D, and Larkum ME(2012). Early exposure to alcohol leads to permanent impairment in neocortical pyramidal neurons. The Journal of Neuroscience 32(4): 1377-1382.

Hamilton GF, Whitcher LT, Klintsova AY. (2010). Postnatal binge-like alcohol exposure decreases dendritic complexity while increasing the density of mature spines in mPFC Layer II/III pyramidal neurons. Synapse 64(2):127-35.

Heidbreder CA, Groenewegen HJ. (2003). The medial prefrontal cortex in the rat: evidence for a dorso-ventral distinction based upon functional and anatomical characteristics. Neuroscience & Biobehavioral Reviews 27(6):555-79.

Helfer JL, Goodlett CR, Greenough WT, Klintsova AY. (2009). The effects of exercise on adolescent hippocampal neurogenesis in a rat model of binge alcohol exposure during the brain growth spurt. Brain Research. 1294:1-11.

Ickes BR, Pham TM, Sanders LA, Albeck DS, Mohammed AH, Granholm AC. (2000). Long-term environmental enrichment leads to regional increases in neurotrophin levels in rat brain. Experimental Neurology 164:45–52.

Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovska V, Horster F, Tenkova T, Dikranian K, Olney JW. (2000). Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. Science 287:1056–1060.

Irwin, S.A. et al. (2002). Dendritic spine and dendritic field characteristics of layer V pyramidal neurons in the visual cortex of fragile-X knockout mice. American Journal of Medical Genetics 111, 140–146.

Jacobs JS and Miller MW. (2001). Proliferation and death of cultured fetal neocortical neurons: Effects of ethanol on dynamics of cell growth. Journal of Neurocytology. 30, 391–401.

Kaufmann, W.E. and Moser, H.W. (2000) Dendritic anomalies in disorders associated with mental retardation. Cereb. Cortex 10, 981–991

Klintsova AY, Cowell RM, Swain RA, Napper RM, Goodlett CR, Greenough WT. (1998). Therapeutic effects of complex motor training on motor performance deficits induced by neonatal binge-like alcohol exposure in rats: I. Behavioral results. Brain Research 800(1):48-61.

Klintsova AY, Scamra C, Hoffman M, Napper RM, Goodlett CR, Greenough WT. (2002). Therapeutic effects of complex motor training on motor performance deficits induced by neonatal binge-like alcohol exposure in rats: II. A quantitative stereological study of synaptic plasticity in female rat cerebellum. Brain Research 937(12):83-93.

Klintsova AY, Helfer JL, Calizo LH, Dong WK, Goodlett CR, Greenough WT. (2007) Persistent impairment of hippocampal neurogenesis in young adult rats following early postnatal alcohol exposure. Alcoholism: Clinical and Experimental Research. 31(12):2073-82.

Kolb B and Gibb R. (1991). Environmental enrichment and cortical injury: behavioral and anatomical consequences of frontal cortex lesions. Cerebral Cortex. 1(2):189-98.

Lawrence RC, Otero NKH, and Kelly SJ. (2011). Selective effects of perinatal ethanol exposure in medial prefrontal cortex and nucleus accumbens. Neurotoxicology and Teratology 34(1): 128-135.

May, PA and Gossage JP. (2001). Estimating the prevalence of fetal alcohol syndrome: A summary. Alcohol Research & Health 25(3):159-167.

May PA, Gossage JP, Kalberg WO, Robinson LK, Buckley D, Manning M, Hoyme HE. (2009). Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. Dev Disabil Res Rev 15:176–192.

Mihalick SM, Crandall JE, Langlois JC, Krienke JD, Dube WV. (2001). Prenatal ethanol exposure, generalized learning impairment, and medial prefrontal cortical deficits in rats. Neurotoxicology and Teratology 23:453–462.

Miller MW. Development of projection and local circuit neurons in neocortex. New York: Plenum Press, 1998.

Miller MW and Robertson S. (1993). Prenatal expo-sure to ethanol alters the postnatal development and transformation of radial glia to astrocytes in the cortex. Journal of Comparative Neurology 337: 253 266.

Mrzljak L, Uylings HB, Van Eden CG, Judás M. (1990). Neuronal development in human prefrontal cortex in prenatal and postnatal stages. Progressive Brain Research 85:185-222.

Rasmussen C, Bisanz J. (2009). Executive functioning in children with fetal alcohol spectrum disorders: profiles and age-related differences. Child Neuropsychology. 15:201–215.

Robinson TE and Kolb B. (1999). Repeated exposure to either amphetamine or cocaine alters the morphology of neurons in the nucleus accumbens and prefrontal cortex. European Journal of Neuroscience 11:1598–1604.

Rotaru DC, Barrionuevo G, Sesack SR. (2005). Mediodorsal thalamic afferents to layer III of the rat prefrontal cortex: synaptic relationships to subclasses of interneurons. Journal of Comparative Neurology. 490(3):220-38.

Russo-Neustadt A, Ha T, Ramirez R, Kesslak JP. (2001). Physical activity-antidepressant treatment combination: impact on brainderived neurotrophic factor and behavior in an animal model. Behavioral Brain Research 120: 87–95.

Sampson PD, Streissthguth AP, Bookstein FL, Little RE, Clarren SK, Dehaene P, Hanson JW, Graham JM. (1997). Incidence of fetal alcohol syndrome and prevalence of alcohol-related neurodevelopmental disorder. Teratology 56:317-326.

Simpson RA. (2008). Young Adult Development Project. MIT.edu. Retrieved March 15, 2012 from http://hrweb.mit.edu/worklife/youngadult/index.html

Sowell ER, Thompson PM, Mattson SN, Tessner KD, Jernigan TL, Riley EP, Toga AW. (2002). Regional Brain Shape Abnormalities Persist into Adolescence after Heavy Prenatal Alcohol Exposure. Cerebral Cortex 12(8):856-865.

Spruston N. (2008). Pyramidal neurons: Dendritic structure and synaptic integration. National Review of Neuroscience 9:206–221.

Stranahan AM, Khalil D, Gould E. (2007). Running induces widespread structural alterations in the hippocampus and entorhinal cortex. Hippocampus. 17(11):1017-22.

Streissguth. AP, Barr HM, and Sampson PD. (1990). Moderate prenatal alcohol exposure: effects on child IQ and learning problems at age 7112 years. Alcoholism:

Clinical and Experimental Research 14. 662-669.

Swain RA, Harris AB, Wiener EC, Dutka MV, Morris HD, Theien BE, Konda S, Engberg K, Lauterbur PC, Greenough WT. (2003). Prolonged exercise induces angiogenesis and increases cerebral blood volume in primary motor cortex of the rat. Neuroscience 117: 1037–1046.

Thomas JD, Sather TM, and Whinery LA. (2008). Voluntary exercise influences behavioral development in rats exposed to alcohol during the neonatal brain growth spurt. Behavioral Neuroscience 122(6):1264-73.

Trachtenberg, J.T., Chen, B.E., Knott, G.W., Feng, G., Sanes, J.R., Svoboda, K. (2002). Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. Nature 420, 788–794.

van Praag H, Christie BR, Sejnowski TJ, and Gage FH. (1999). Running enhances neurogenesis, learning, and long-term potentiation in mice. Proceedings of the National Academy of Sciences 96(23):13427-31.

van Praag H, Kempermann G, and Gage FH. (2000). Neural consequences of environmental enrichment. National Review of Neuroscience. 1(3):191-8.

Vaynman S, Ying Z, and Gomez-Pinilla F. (2004). Hippocampal BDNF mediates the efficacy of exercise on synaptic plasticity and cognition. European Journal of Neuroscience 20:2580–2590.

Wass TS, Persutte WH & Hobbins JC. (2001). The impact of prenatal alcohol exposure on frontal cortex development in utero. American Journal of Obsteterics Gynecology 185(3): 737-742.

Welsh, M.C., Pennington, B.F. & Grossier, D.B. (1991). A normative-developmental study of executive function. Developmental Neuropsychology 7, 131–149.

Whitcher L, Klintsova A. (2008). Postnatal binge-like alcohol exposure reduces spine density without affecting dendritic morphology in rat mPFC. Synapse 62(8):566-573.

Yuste, R. Dendritic Spines. MIT Press, 2010.

Zuo Y, Lin A, Chang P, and Gan WB. (2005). Development of long-term dendritic spine stability in diverse regions of cerebral cortex. Neuron 46:181–189.