EFFECT OF VOLUNTARY EXERCISE ON C-FOS EXPRESSION IN THE HIPPOCAMPUS OF RATS EXPOSED TO ALCOHOL NEONATALLY

by

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ABSTRACT

Our lab uses a rodent model of Fetal Alcohol Spectrum Disorder to observe how neonatal alcohol exposure effects neuroplasticity during development, as well as behavioral therapies that might ameliorate such damage. Using a rodent model of third trimester binge drinking on postnatal days (PD) 4-9, our lab observed decreased the number of adult-born, mature neurons surviving in the adult hippocampal dentate gyrus (DG) (Klintsova et al., 2007). Rats with access to voluntary exercise show enhanced cell proliferation at PD42 in dentate gyrus (DG) in control and alcohol exposed (AE) animals. However at PD72 AE animals show no long-term benefit of wheel running in looking at the newly generated cells (Helfer et al., 2009). To investigate mechanisms underlying decreased cell survival in AE animals after wheel running, we use an immediate early gene, c-Fos, to capture subtle changes in activation patterns within the hippocampal regions CA1, CA3, and DG at PD42. C-Fos induction, which past studies have shown to be previously impaired following AE, increases with bouts of stimulation such as wheel running (Clark et al., 2011). C-Fos identifies active neurons, which may have a functional role in the hippocampal circuit. On PD4, rats are assigned to either the suckle control (SC), sham intubated (SI) or alcohol exposure (AE) treatment group. On PD4-9 AE rats are exposed to a milk/ethanol solution (5.25 g/kg ethanol per day) via intragastric intubation in a binge-like manner. During PD30-42, rats are placed in either standard social housing (SH) or voluntary wheel running (WR; 24 hr access). Tissue was stained using a c-Fos antibody and analyzed on a light microscope using unbiased stereological techniques.

By using c-Fos, we hoped to gain further insight into how AE affects hippocampal activation and the maturation of newly generated cells in the hippocampus following WR to further develop interventions to mitigate effects of alcohol exposure. This study found a significant effect of housing and a significant effect of neonatal treatment in area CA1. However no significant differences were found in either CA3 or DG. Such unexpected findings led to further analysis using correlations to determine whether there was a relationship between number of c-Fos+ cells and time since lights on at tissue harvest. These correlations revealed that due to methodological obstacles, it would be necessary and beneficial to reproduce this exact study to overcome any confounding variables affecting the results presented in this thesis.

Chapter 1

INTRODUCTION

1.1 Fetal Alcohol Spectrum Disorders

Amongst the innumerable medical conditions and diagnoses that the people of the world face today exist those that are one hundred percent preventable. Fetal Alcohol Spectrum Disorders (FASD) is an umbrella term that encompasses a variety of avoidable diagnoses that result from a mother drinking alcohol during her pregnancy, otherwise known as prenatal alcohol exposure. The spectrum of FASD includes the diagnoses of Fetal Alcohol Syndrome (FAS), partial FAS, alcohol related neurodevelopmental disorder (ARND), and alcohol related birth defects (ARBD). These diagnoses may differ based on documented alcohol use, physical features, cognitive features, neuroanatomical correlates, and the presence of symptoms throughout life (Stratton *et al.,* 1996). Since the first publication describing FASD (Jones and Smith, 1973), many different diagnostic criteria has been proposed, and there is still no single definitive diagnostic criteria that physicians and health professionals currently rely on.

The Centers for Disease Control and Prevention published a comprehensive guideline for referral and diagnosis of FAS and related conditions, which included detailed reference to the 1996 report by the Institute of Medicine (IOM) and the 1997 4-digit Diagnostic Code published at the University of Washington (Bertrand *et al.,* 2004; Stratton *et al.,* 1996; Astley *et al.,* 1997). In 1996, the IOM published detailed criteria for the diagnoses that fall within the FASD spectrum. Fetal Alcohol Syndrome

(FAS) and Alcohol-Related effects are cited as the most prevalent cause of mental retardation and most easily observed forms of FASD (Chudley *et al.,* 2005). Included in the diagnosis of FAS are cases of confirmed maternal alcohol exposure, cases without confirmed maternal alcohol exposure, as well as partial FAS without confirmed maternal alcohol exposure (Stratton *et al.,* 1996). FAS can include a variety of the following: confirmed maternal alcohol exposure, a wide array of facial anomalies (i.e. smooth philtrum, short palpebral fissures), one form of evidence for growth retardations, such as low birth weight, and one form of evidence for central nervous system (CNS) neurodevelopmental abnormalities, such as microcephaly (Stratton *et al.,* 1996). Partial FAS diagnosis uses similar diagnosis as FAS, but also includes necessary evidence that exhibits "complex pattern of behavior or cognitive abnormalities" that is unable to be explained by environment or home life (Stratton *et al.,* 1996). Other diagnoses that fall under the term of Alcohol Related Effects are known as Alcohol-Related birth defects (ARBD) and Alcohol-related Neurodevelopmental disorder (ARND) (Stratton *et al.,* 1996). Within ARBD is a plethora of varying congenital anomalies that range from defects of the heart, skeletal system, renal system, ocular system, and auditory function. ARND can be diagnosed via the presence of CNS neurodevelopmental abnormalities, as described in FAS diagnosis criteria, as well as the presence of complex behavior (Stratton *et al.,* 1996).

A year after the IOM published the aforementioned guidelines for diagnosis of conditions within FASD came another set of diagnostic criteria in 1997. The FAS Diagnostic and Prevention Network of the University of Washington published a 4-Digit Diagnostic Criteria that focuses on four key diagnostic markers of FASD. These include growth deficiency, FAS facial phenotype, CNS abnormalities, and prenatal

alcohol exposure. A Likert scale is used to rank each symptom on a 4-point scale, with 4 being the most pronounced of a symptom and 1 being the least. (Astley and Clarren, 2004). Although diagnoses of FASD rely on a combination of many published guidelines, three main aspects, growth, morphology, and CNS dysfunction, are used to assess all cases (Streissguth *et al.,* 1994).

The prevalence and incidence of FASD is difficult to quantify based on the diagnostic system that is in place, however, in reports of such epidemiological data, many researchers focus on incidences of FAS due to the severity and easily distinguishable characteristics (Abel and Sokol, 1987). In a report done by Abel and Sokol in 1987, estimates for the incidence of FAS and its costs provided the public with startling numbers and facts that would hopefully make an impact. It was found that FAS is the overall number one cause of mental retardation in the Western World, its prevalence is 1.9 per 1000 births world wide as seen in studies performed in Australia, Canada, Finland, France, Sweden, Switzerland, U.K., and U.S.A, and the cost of treating those with FAS in the U.S. alone is about $321 million per year. More recently, the total incidence of all diagnoses within FASD in the U.S. was found to be a startling 10 of every 1000 live births (1%) (May and Gossage, 2001).

In the United States alone, 130,000 women put their fetuses at risk for developing an alcohol related disorder per year by drinking while pregnant (Lupton *et al.,* 2004). Such exposure may go unreported and undetected, and therefore the reported prevalence, incidence, and costs of FASD may be an underestimation of actual numbers. High incidence of alcohol use while pregnant can be attributed to a plethora of risk factors. Such risks may relate to socioeconomic standings where the presence of poverty, unstable education, and surroundings filled with abusive drinking

may have a major impact on the development of FAS in offspring (Sampson *et al.,* 1997). Preventing FASD is a public health concern that encompasses change not only within the mother, but also from the spouse, close friends and family, as well as community members (Stratton *et al.,* 1996).

1.2 Cognitive-Behavioral Dysfunction and Neurodevelopmental Outcomes

Diagnosis of conditions within FASD based on purely physical measures may become more difficult as an affected offspring begins to grow and develop. Longitudinal studies found that from birth through 14 years of age, an offspring's weight, height, and cranial circumference became insignificant parameters to define prenatal alcohol exposure (Sampson *et al.,* 1994). Furthermore, distinguishing facial anomalies that were once used for diagnosis were no longer as pronounced after puberty as they had once been (Streissguth *et al.,* 1991). Of the parameters used for diagnosis of FASD conditions, the consistent and long lasting effects of prenatal alcohol exposure have always been seen through measures of both cognitive-behavioral dysfunction and negative neurodevelopmental outcomes. For instance, it has been seen that children with FAS exhibit deficits in verbal learning and memory, lower IQ, as well as deficits in executive functioning as measured by tasks such as planning ability and concept formation (Mattson *et al.,* 1996; Mattson *et al.,* 1997; Mattson *et al.,* 1999).

The effect of prenatal alcohol exposure on cognitive-behavioral functions and neurodevelopment can be seen via numerous structural and functional effects on the developing brain. Affected areas include, but are not limited, to the cortex, corpus callosum, cerebellum, basal ganglia, and hippocampus (Roebuck *et al.,* 1998). Structural and functional effects of prenatal alcohol exposure may include decreased

brain growth, occurrence of agenesis (absence/incomplete development of a structure), and deficits in motor and balance abilities following alcohol insult to the cortex, corpus callosum, and cerebellum respectively (Roebuck *et al.,* 1998; Riley *et al.,* 2005; Connor *et al.,* 2006) Of particular importance to the current study is damage on the hippocampus. The hippocampus is important for learning and memory processes, which links it to cognitive and neurodevelopmental impairments that prenatal alcohol exposure produces in offspring.

1.3 Animal Models

Several animal studies and human studies have linked hippocampal damage caused by developmental alcohol exposure to behavioral deficits. In order to assess the affects of alcohol on the hippocampus and processes such as learning and memory, many animal studies focus on hippocampal-associated tasks that utilize measures such as spatial memory, spatial navigation, and trace fear conditioning. Prenatal alcohol exposure of rats causes deficits in spatial memory tasks as measured by performance of the Morris Water Maze. This task, which requires rats to locate a platform amidst opaque water and in relation to spatial cues, measures spatial memory by recording swim time required to discover the platform. Alcohol exposed animals spent a longer time to find the platform and also used greater distances to accomplish the task in contrast with control animals (Gianoulakis, 1988). Similarly, in a learning task in mice that aimed at differentiating impairments in performing delay fear conditioning and trace fear conditionings after alcohol exposure, it was seen that alcohol-exposed animals were impaired in only the trace fear conditioning tasks. Unlike the hippocampal independent task of delay conditioning, trace fear conditioning is a hippocampal-dependent task that measures learning by the act of freezing. In

comparison with control animals, the alcohol-exposed animals froze less, and therefore learned less, when exposed to the conditioned stimulus (tone) that was associated with a fearful unconditioned stimulus (foot shock) (Weitemier and Ryabinin, 2003). Human studies have shown similar hippocampal deficits as children with FAS exhibited deficits in spatial memory and spatial organization as well as inability to perform delayed object recall in an object memory task (Uecker and Nadel, 1996).

The widespread incidence of cognitive deficits caused by developmental alcohol exposure calls for a dire need for interventions that may be able to ameliorate some symptoms of FASD. For children with FASD, such interventions may include social skills training, counseling, occupational therapy, and education interventions (Stratton *et al*., 1996). Physical exercise may also have potential in improving cognition in children with FASD. In a meta-analysis that looked at effects of physical activity on children's cognition, there were significant and positive effects of physical activity on cognitive performance in children as measured by improvements in total math and reading achievement, grade point average, and intellectual quotient (Fedewa and Ahn, 2011).

However, there exists much variability within the human population that not only makes the quantification of the deficits associated with FASD difficult, but also makes the development of successful treatment plans difficult as well. Relevant variability within the human population includes, but is not limited to, the timing of alcohol exposure while pregnant, the amount of alcohol consumed while pregnant, and the participation in prescribed treatment plans in those affected with FASD. Additional limitations of using human fetuses and offspring not only include the

obvious ethical issues, but also such studies would be impractical due to the complexity and time consuming process of human development (Dobbing and Sands, 1978). Therefore, in order to further explore the effects of prenatal alcohol exposure on the brain, human patterns of alcohol exposure must be translated to appropriate animal models. Of the many types of animals that can be used for experimentation, rodents are commonly chosen for animal models due to the widespread and well-understood knowledge of their CNS and behavior, as well as the fact that they are cost effective and have a short gestational period (O'leary-Moore, 2011).

When developing an animal model for FASD, it is imperative to consider the timing of alcohol consumption during pregnancy and how this translates between species in question (Maier and West, 2001). A commonly observed period of alcohol exposure occurs during a developmental event in the brain known as the 'brain growth spurt'. The brain growth spurt, as seen across all species, is a "transient period of rapid brain growth" (Dobbing and Sands, 1973). A defining characteristic of this developmental event in species is an increased vulnerability to various insults, such as nutritional deficits, x-ray exposure, or other teratogens in the fetal environment. In the early parts of the growth spurt there is tremendous multiplication of cells, which is then followed by the development of myelination. The brain growth spurt hypothesis ignores the differential timing of birth between species and instead indicates age by events of brain growth and how exposure to detrimental events at different times correlates to developmental age in species (Dobbing and Sands, 1978). Dobbing and Sands created a curve comparing the brain growth spurt of multiple species, which allows classification of brain development into prenatal, perinatal, and postnatal periods. For humans, this stage of development will begin prenatally in the third

trimester and end around 3-4 years of age, and for rodents, the development will begin with birth and end by about 3 weeks of postnatal life. When offspring is exposed to ethanol during a human's third trimester and a rodent's early postnatal life, changes to brain structure and function are seen, including fewer cells in the hippocampus and decreased brain weight and head circumference (Coles, 1994).

Another crucial aspect that is necessary to consider in formulating an appropriate animal model includes the variables of maternal drinking pattern, such as the amount of alcohol consumed as well as the pattern in which this amount is consumed (Maier and West, 2001). It has been seen that the type of maternal drinking patterns that have the most devastating results on the developing fetus are those that are classified as "binge-drinking". In a study that looked at the impact of maternal drinking pattern on cognitive impairments in human offspring, it was found that when a mother drank 5 drinks on occasion (binge-drinking), the most serious consequences in the offspring resulted. Functional deficits in offspring birthed from mothers who binge drank include impairments in development, simple fine motor coordination, walking and balance, and an inability to successfully engage in imitative play (Jacobson, 1998).

In order to observe maternal drinking pattern in animals, researchers control for binge-like behavior by measuring blood alcohol concentration (BAC) to accurately observe how varying levels of alcohol present in the blood at a given time affect the developing brain of the offspring. In a study that looked at the effect of varying alcohol doses on rats exposed to alcohol postnatally, it was seen that as the peak BAC of 420 mg/ml was approached after an ethanol dose of 7.5 g/kg/day there was a simultaneous increase in the severity of microencephaly (small brain), a sign of CNS

damage (Bonthius and West, 1988). Furthermore, when binge-like doses were mimicked and a higher BAC was achieved, there was subsequent neuronal loss. Rats that were given smaller doses of alcohol (4.5 g/kg/day) in a more condensed, binge-like fashion (two or four feedings) presented with significant cell loss due to the achievement of a peak BAC of 190.7 mg/dl. This contrasted with rats that were exposed to higher doses of alcohol (6.6 g/kg/day) in less-condensed fashion (continuous administration), which achieved a peak BAC of 39.2 mg/dl; these animals did not present with any significant neuronal loss (Bonthius and West, 1990).

1.4 Neuroplasticity

Due to the damaging effects of both ethanol exposure during the brain growth spurt and binge-like alcohol exposure on the brain, many animal models incorporate both phenomena in order to observe the effects of developmental alcohol exposure on the brain. By using a third trimester binge-like equivalent model of alcohol exposure, the current study focuses on the impact that developmental alcohol exposure has on neuroplasticity in the brain. The phenomenon of plasticity in the brain describes the ability of the nervous system to change its structure and function throughout an organism's lifetime in response to certain experiences, drugs, disease, genetics, stress, injury, and diet for example (Kolb, 2003). Changes in the brain may include alterations in learning in memory, which are tasks that are widely accomplished by the hippocampal environment (Kolb, 2003). When investigating the effects of postnatal alcohol exposure on hippocampal neuroplasticity, it is possible to examine both structural changes, such as synapse density and number, dendritic length and density and number of cell bodies, as well as functional changes, such as LTP, receptor expression, and induction of transcription factors.

Numerous studies have investigated the effect of developmental alcohol exposure on plasticity of the developing brain in rodents using postnatal models. When compared to control animals, animals exposed postnatally to alcohol were seen to have significantly decreased dendritic spine density in the medial prefrontal cortex (mPFC), a frontal lobe brain structure that is vital in complex cognitive functioning (Whitcher and Klintsova, 2008). Animals exposed to early postnatal ethanol showed a significant decrease in the number of mature granule cells in the dentate gyrus (DG) of the hippocampus compared to control animals. This decrease of cell number indicated impairment in neurogenesis, the birth of new adult neurons, in the developing brain (Klintsova *et al.,* 2007). It has also been found that animals exposed postnatally to ethanol have decreased dendritic complexity in the medial prefrontal cortex (mPFC) when compared to control animals (Hamilton *et al.,* 2010). Developmental alcohol exposure, therefore, produces many detrimental changes in the neuroplasticity of the brain. Since neuroplasticity is a crucial component of processes such as learning and memory, insults to neuroplasticity may contribute to the cognitive and neurodevelopment deficits that are evident in patients of FASD.

1.5 Wheel Running

The current study employs the behavioral intervention of voluntary wheel running, in an attempt to ameliorate the negative effect that developmental alcohol exposure has on brain neuroplasticity and on cognition, In comparison with control standard housed animals, mice that were given access to a running wheel exhibited enhanced performance on the Morris Water Maze task, increased cell number, and enhanced synaptic plasticity in the dentate gyrus (DG) of the hippocampus. The running-wheel mice also demonstrated increased induction of long-term potentiation,

a correlate of learning, in the DG (Van Praag *et al.,* 1999). Furthermore, alcohol-exposed animals showed deficits in performance of Morris Water Maze task in comparison with controls (Thomas *et al.,* 2008). However, when given access to wheel running as a form of exercise intervention, performance on the Morris Water Maze was significantly improved. Using previous findings that exemplify the observed benefits that wheel running has on structural and functional plasticity in the hippocampus, this lab has attempted to further investigate the effect that postnatal alcohol exposure has on neuroplasticity in the hippocampus following wheel running. As expected, animals given access to behavioral stimuli such as wheel running show enhanced cell proliferation at postnatal day (PD) 42 in the DG in control and alcohol exposed animals (Helfer *et al.,* 2009). However at PD72, while control animals show long-lasting benefits of wheel running on cell survival as indicated by a continued increase in cell number, alcohol-exposed animals have impaired survival of new cells. Alcohol-exposed animals no longer show an increase in cell number at PD72 which suggests that alcohol-exposed animals do not gain the long-term benefit of wheel-running intervention that was seen in control animals.

Wheel running does have an effect on neuroplasticity in the developing hippocampus; however, there is no evidence of a long-term benefit of voluntary exercise in this area. In order to continue to elucidate information regarding hippocampal neuroplasticity, the current study focuses on molecular and cellular mechanisms occurring within the hippocampal environment. Changes in processes involving gene expression are thought to contribute to the plasticity that is seen in the brain (McClung and Nestler, 2008). By investigating changes in gene expression through the evaluation of transcription factors (DNA-binding proteins), the current

investigation hopes to gain further insight into the changes that postnatal alcohol exposure causes on neuroplasticity within the hippocampal environment.

1.6 Transcription Factors and Neuroplasticity

The proteins within the Fos family of transcription factors are useful in studying activity in the brain following certain stimulation. Members of the Fos family are able to join with other proteins to regulate the mechanisms of action of certain genes (Nestler, 2008). One transcription factor that is useful in the study of neural plasticity is a member of the Fos family, delta (Δ) fosB. Delta FosB, a splice variant of FosB, is a marker of neuronal activation and is upregulated after chronic stimulation, such as drug exposure (Nestler, 2001). The high stability of the ΔFosB molecule allows its to accumulate with repeated stimulation for several days, which is optimal for observing changes in gene expression that continue to occur once the chronic stimulation, or drug exposure stops (Nestler, 2001). Such long lasting influences of ΔFosB also may hint to its role in inducing and maintaining LTP and other forms of learning and memory (McClung and Nestler, 2008). In a study that looked at the induction ΔFosB following wheel running in brain areas specific to reward and addiction, it was seen that just like chronic use of addictive drugs, the chronic stimulation of wheel running did up regulate the expression of ΔFosB (Werme *et al.,* 2002). A recent unpublished study in our lab investigated ΔFosB expression in the hippocampal areas of CA1, CA3, and DG in rats prenatally exposed to alcohol and given the behavioral intervention of voluntary wheel running. In observing ΔFosB expression in alcohol exposed wheel-running animals, there was no effect of alcohol treatment, but there was differential activation in the differing areas of the

hippocampus. Wheel running upregulated ΔFosB in hippocampal areas CA1 and CA3, but there was no change in the DG.

Another member of the Fos family is the immediate early gene c-Fos. In contrast with ΔFosB, c-Fos induction is very transient and unstable, with expression peaking at about 2 hours following stimulation (Nestler, 2001). C-fos exhibits controlled expression, for it is almost undetectable in inactive cells, but it is quickly expressed in stimulated cells. Such characteristics of c-Fos provide insight for its underlying role in cell response to external stimuli an organism may experience (Sheng and Greenberg, 1990). Animal studies that have shown c-Fos induction following stimulation to the nervous system may also suggest its role in not only metabolic responses of the cell but also its role in neuronal plasticity (Sheng and Greenberg, 1990). The current study uses c-Fos to capture the subtle changes of excitability in the hippocampal environment that cumulative marker ΔFosB may not have been sensitive to detect. Analysis of c-Fos will provide us a more complete picture of neuronal activation following wheel running in that it identifies cells that are transcriptionally reprogramming and as a result, are engaging in important changes in cell activity (Ryabinin *et al.,* 1998).

Neonatal rats prenatally exposed to alcohol show decreased basal c-Fos expression in various areas of the hippocampus, suggesting hippocampal dysfunction (Jang *et al.,* 2005). However, it has been shown that c-Fos induction is increased in comparison to basal levels following bouts of stimulation such as wheel running (Clark *et al.,* 2011). Furthermore, it was also seen that previously suppressed levels c-Fos expression in the hippocampus of rats born to alcohol-intoxicated mothers were restored once rat rups were exposed to treadmill exercise (Sim *et al.,* 2008). Similarly,

in a recent study that looked at c-Fos expression in CA1 following neonatal alcohol exposure and following the context pre-exposure facilitation effect (CPFE) behavioral task, researchers saw that there was an increased c-Fos expression in control sham-intubated animals that were intubated, but received no alcohol treatment. There was also increased c-Fos expression in alcohol-exposed animals of two doses (4.00 g & 5.25 g) in comparison to Home Cage controls. However, in comparison to control sham-intubated animals, alcohol-exposed animals showed reduced c-Fos immunoreactivity in CA1 (Murawski *et al.,* 2012). If we consider CPFE as a hippocampal dependent stimulation, just as wheel running is also hippocampal dependent stimulation, we can hypothesize that following WR, we may see an increased c-Fos expression in both control and alcohol-exposed animals. However, based on previous findings, the effect of WR on alcohol-exposed animals may not be as great compared to the control animals.

1.7 Current Study

The current study will observe hippocampal activation patterns in rats exposed to alcohol neonatally at PD 42 following wheel running intervention, as wheel running causes preferential activation of newly generated cells in the hippocampus (Helfer *et al.,* 2009). We are investigating how active the hippocampus is as a whole in the three areas CA1, CA3, and DG in hopes to gain an idea of hippocampal activity following wheel running, as well as to gain an understanding regarding the hippocampal environment during this time of heightened activity. This study also aims to observe the effects that postnatal alcohol-exposure may have on long-term basal and reactive c-Fos expression in the hippocampus. In looking at neuronal activity throughout the hippocampus following wheel running, we can use these findings to further

understand the neuroplastic changes that we have observed in our lab, including adult neurogenesis and dendritic complexity. In comparing c-Fos expression in alcohol-exposed/social-housed animals to control/social-housed animals, we expect to see decreased c-Fos expression in alcohol-exposed animals. We also expect to see enhanced c-Fos expression in control wheel running animals as compared to control social-housed animals.

Chapter 2

METHODS

2.1 Animals

Adult Long Evans dams were supplied from Harlan (Indianapolis, IN), and were then bred in the University of Delaware's Office of Laboratory Animal Medicine Facility. All rats were kept on a 12-hour light/dark cycle (9AM/9PM) and had access to food and water *ad libitum*. On postnatal day (PD) 3, litters were culled to 8 pups that consisted of 6 males and 2 females. On PD3, using a 4 paw numbering system as a means for identification, pups were paw marked via injection of Black Magic Ink. On PD4, rat pups were pseudo-randomly assigned to one of three of our experimental groups, suckle control (SC), sham-intubated (SI), or alcohol-exposed (AE). SC animals received no experimental treatments except that they were weighed daily; SI animals were intragastrically intubated, but not administered any alcohol formula, and AE animals were intragastrically intubated and administered an alcohol/milk formula. All pups were weighed daily during the intubation procedure to assess health. Thirty-eight pups were used in this study (11 females; 27 males). The amount of animals per condition was 10 AE, 13 SC, and 15 SI. All protocols abide by National Institute of Health's animal care guidelines and was approved by the University of Delaware's Institutional Animal Care Use Committee (IACUC).

2.2 Postnatal Alcohol Treatment

On PD 4-9, AE pups were exposed to a milk/alcohol solution via intragastric intubations using polyethylene tubing. Twice daily, two hours apart (9am and 11am), AE pups were intubated with alcohol (11.3% v/v in milk formula) to total 5.25g/kg of alcohol exposure per day. On PD4, pups were given 2 doses of milk-only formula after the alcohol doses (1pm and 3pm). On PD5-9, pups were given 1 dose of milk-only formula 2 hours after the second alcohol dose (1pm). Milk-only doses are administered as a caloric supplement to ensure nutrient deprivation does not occur due to the fact that after alcohol administration, pups are unable to retrieve sufficient nourishment from the dam. To eliminate the occurrence of stress or neglect, pups were separated from the mother for no more than 15 minutes per session.

2.3 Blood Alcohol Concentrations

To ensure that a proper blood alcohol concentration (BAC) was obtained, blood samples from tail clips of AE pups were obtained 1.5 hours after the second alcohol dose on PD4. Blood was centrifuged for 15 minutes at 1500 revolutions per minutes, and the plasma was then collected and stored at -20°C until analysis. BACs for each AE pup were evaluated from blood plasma using an Analox GL5 Alcohol Analyzer (Analox Instruments, Boston, MA).

2.4 Wheel Running

On PD23, rats were counterbalanced for litter and neonatal treatment and housed three per cage, which allowed for exposure to social housing for a week until PD30. On PD30, animals were assigned to either standard social housing (SH) or voluntary wheel running (WR) and were kept in such conditions in the groups pups were weaned with until perfusions on PD42. WR animals had 24-hour access to

stainless steel running wheels that were capable of housing multiple rats and the wheels were also equipped with counters. Wheel rotations were recorded every 24 hours. All postnatal conditions were housed together during this time.

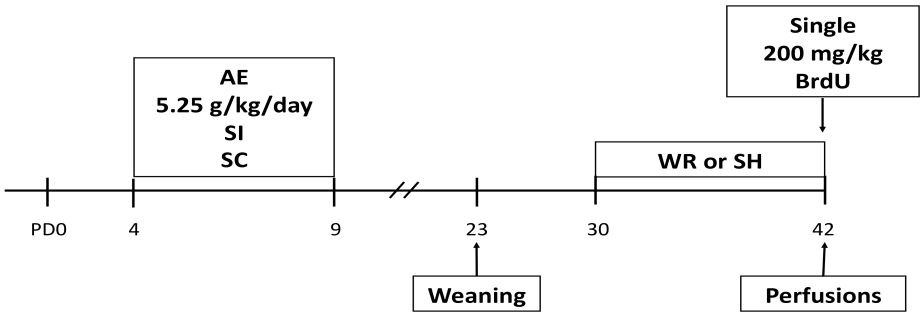

Figure 1: Timeline of Exposure to Wheel Running

2.5 Tissue Preparation

On PD42, animals were injected with a single injection of bromodeoxyuridine (BrdU; 200 mg/kg) at the onset of light cycle (9:00AM). Two hours later, animals were deeply sedated with a cocktail of ketamine/xylazine and transcardially perfused using heparanized 0.1 M phosphate-buffered saline (PBS; pH 7.2), followed by 4% paraformaldehyde in PBS (pH 7.2). For 24 hours, animal brains were stored in 4% paraformaldehyde and then were transferred to 30% sucrose in 4% paraformaldehyde for two days. The brains were then transferred to another 30% sucrose in 4% paraformaldehyde solution for another two to three days. Brains were serial sectioned horizontally through the entire extent of the hippocampus at 40μm sections using a cryostat. Sectioned brain tissue was then stored at -20°C in cryoprotectant solution.

2.6 Immunocytochemistry

Tissue processed for immunocytochemistry were chosen by systematic random sampling to include every 16th section (640 μm apart) to include the entire extent of the hippocampus. To assess the amount of neuronal activity via number of c-Fos+ cells throughout the hippocampal regions of CA1, CA3, and DG, tissue was immunolabeled using a c-Fos antibody protocol. Brain tissue was first washed with 0.1M TBS to rinse off cryoprotectant, and then tissue was incubated in 0.6% H2O2 in TBS for 30 minutes in order to eliminate endogenous peroxidases. Sections were washed again in 0.1M TBS to rinse off H_2O_2, and then, to block the non-specific binding sites, the tissue was incubated in blocking solution (3% normal goat serum and 0.1% Triton X100 in 0.1M TBS) for 1 hour. Brain sections were placed in primary antibody solution (anti-Fos, 1:500; s-52 [Santa Cruz Biotechnology]) diluted in washing solution (3% normal goat serum), and incubated for 48 hours at 4°C. On Day 3, tissue was rinsed in 0.1M TBS to remove primary antibody residue, then were washed in washing solution. Sections were then incubated in secondary antibody for 1 hour at room temperature (biotinylated goat anti-rabbit IgG, 1:2000; BA-1000 [Vector Laboratories]) made in washing solution. Tissue was rinsed in TBS, and then washed in washing solution. Tissues were incubated in ABC solution mixed in washing solution for 1 hour at room temperature (Vectastain ABC Kit, Standard; PK-400 [Vector Laboratories, Burlingame, CA]) and then rinsed with 0.1M TBS. The stain was visualized using nickel-enhanced Diaminobenzidine (DAB) solution in which the reaction was monitored under the light microscope. Once the reaction was complete, and the sections were rinsed again with TBS, slides were mounted on gelatin-covered slides and left to dry for 24-48 hours. At this point a 0.1% PyroninY counterstain was

used to further highlight the different cell layers of the hippocampus. Coverslips were applied using DPX mounting medium.

2.7 Image Analysis

C-Fos immunopositive cells in CA1, CA3, and DG were counted and analyzed using unbiased stereology on a light microscope using a lens magnification of 40x. The entirety of the pyramidal cell layers (PCL) of CA1 and CA3 and the granule cell layer (GCL) of the DG were traced and analyzed. Labeled cells were analyzed using the Optical Fractionator Workflow programs in StereoInvestigator (Version 10, MBF Bioscience, VT, USA). Counting frame and grid size used measured 200μm x200μm.

Chapter 3

RESULTS

3.1 Weights

Animals were weighed on: PD4, first day of alcohol exposure; PD9, last day of alcohol exposure; PD30, first day of voluntary wheel-running treatment; and PD42 last day of voluntary wheel running treatment. A repeated measures ANOVA was used to take into account the relationship between neonatal treatment and day. There was a significant difference in weight for day only, meaning that all neonatal treatments gained weight across days (F=(1.269,46.946)=2598.033, p<0.001)(Table 1). One-way ANOVA was used to observe differences in weight between each day and neonatal treatment. It was found that on PD4 and PD30 there was no significant difference between weights by neonatal treatment (p>0.05). On PD9, the difference between weights by neonatal treatment was trending with AE animals weighting less than SC or SI animals (F(2,37)=2.821, p=0.072). A two-way ANOVA was used to observe the effect of housing and neonatal treatment on weight on PD42. No significant difference found between neonatal treatments or animals housed in WR or SH (p>0.05).

Table 1: Weights of animals in neonatal groups SC, SI, AE as measured on PD4, PD9, PD30, and PD42. Data is expressed as g±SEM

Neonatal Treatment	Alcohol Dosing			
	PD4	PD9	PD30	PD42
SC	10.71±0.340	18.88±0.592	95.00±2.504	183.00±5.299
SI	10.23±0.508	18.65±0.930	94.13±3.582	177.33±6.188
AE	10.67±0.364	16.46±0.654	95.33±2.362	178.42±4.788

3.2 Blood Alcohol Concentrations

The mean BAC (± SEM) as measured 1.5 hours after the second milk/ethanol dose on PD4 was 357.07 ± 12.73 mg/dl. Data is comparable to studies using similar protocol (Helfer *et al.,* 2009; Klintsova *et al.,* 2007).

3.3 Wheel Running

The number of wheel revolutions was recorded every 24 hours. The mean number of revolutions as measured in meters (± SEM) per 24-hour period was 5430.70 ± 765.85 m (approximately 3.5 miles).

3.4 c-Fos Expression in CA1

An independent-samples *t*-test was used to compare the two control groups (SC and SI) used in this study. *T*-tests were performed between SI/WR and SC/WR as well as between SI/SH and SC/SH. There was no significant difference of c-Fos+ cells ($p > 0.05$) in any of the comparisons. Therefore, the two neonatal treatment groups, SC and SI, were combined and analyzed as one collective control group, which is referred to as “Con” throughout the remainder of the data.

A two-way ANOVA found a significant effect of housing in CA1 (F (1,27) = 11.924, p=0.002). There was also a significant effect between wheel running and neonatal treatment in CA1 (F (1,27) = 5.924, p=0.022). (Figure 2A)

An independent-samples t-test was performed to determine if there was any effect of alcohol exposure on the number of c-Fos+ cells in CA1 between animals of the two neonatal treatments while housed in standard housing. In looking at AE/SH versus Con/SH, there was a significant effect of alcohol on cell number in CA1 with AE/SH animals having fewer cells than Con/SH animals (t (11.51)=-2.967, p=0.012).

An independent-samples t-test was also performed to determine if there was any effect of housing condition on the number of c-Fos+ cells in CA1 within animals exposed to alcohol. In looking at AE/WR versus AE/SH there was a significant effect of housing on cell number in CA1 with AE/SH animals having fewer cells than AE/WR animals (t (5.060)=-2.990, p=0.030). Images representing the average number of c-Fos+ cells in CA1 of each neonatal treatment and housing treatment are expressed in Figure 2B-E.

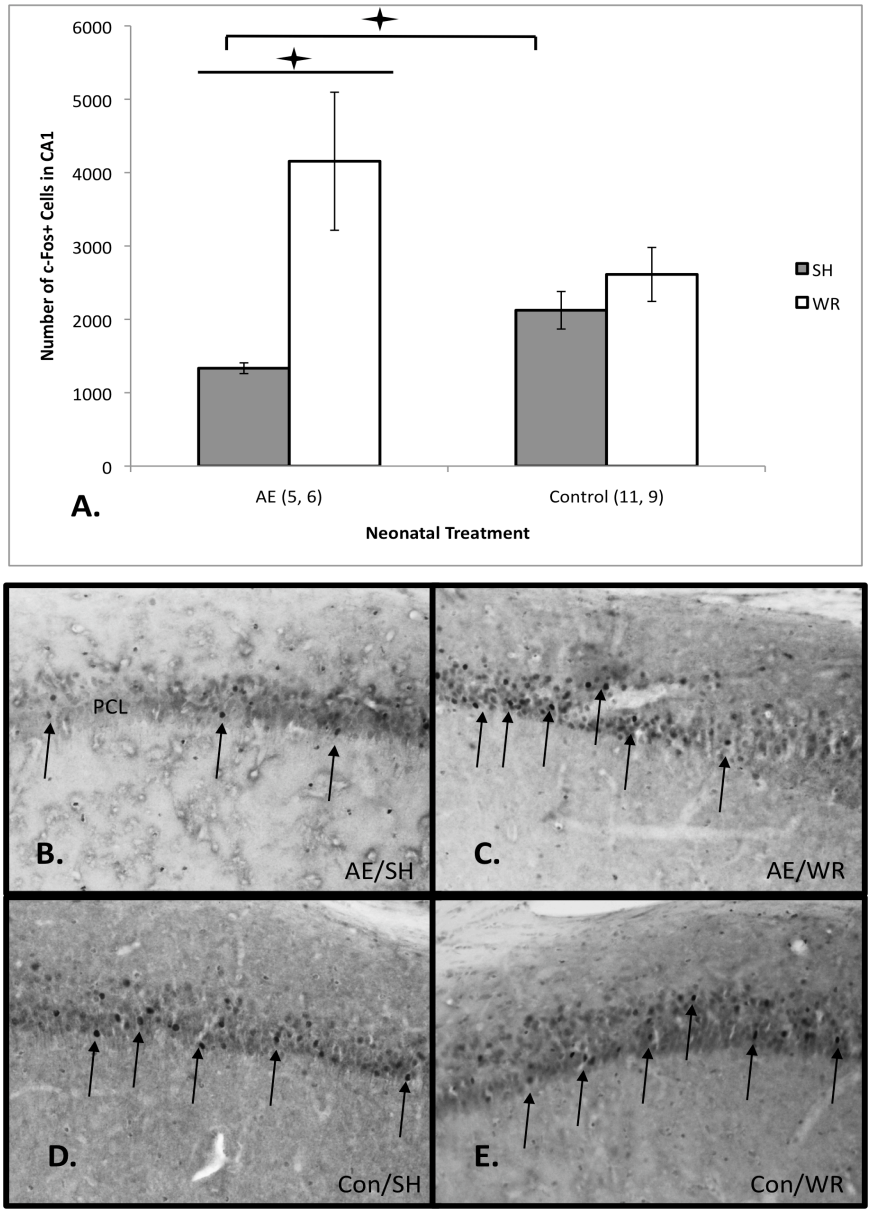

Figure 2: Number of c-Fos+ cells in CA1 at PD42. A: A significant effect of housing and significant effect of neonatal alcohol treatment were found (p=0.002; p=0.022). B: Arrows point to c-Fos+ cells in AE/SH animals. C: Arrows point to c-Fos+ cells in AE/WR animals. D: Arrows point to c-Fos+ cells in Con/SH animals. E: Arrows point to c-Fos+ cells in Con/WR animals.

3.5 c-Fos expression in CA3

A two-way ANOVA found no significant differences in the effect of housing or neonatal treatment in CA3 ($p>0.05$) (Figure 3A).

An independent-samples *t*-test was performed to determine if there was any effect of alcohol exposure on the number of c-Fos+ cells in CA3 between animals of the two neonatal treatments while housed in standard housing. In looking at AE/SH animals versus Con/SH, there was no significant effect of alcohol on cell number in CA3 ($p > 0.05$). Images representing the average number of c-Fos+ cells in CA3 of each neonatal treatment and housing treatment are expressed in Figure 3B-E.

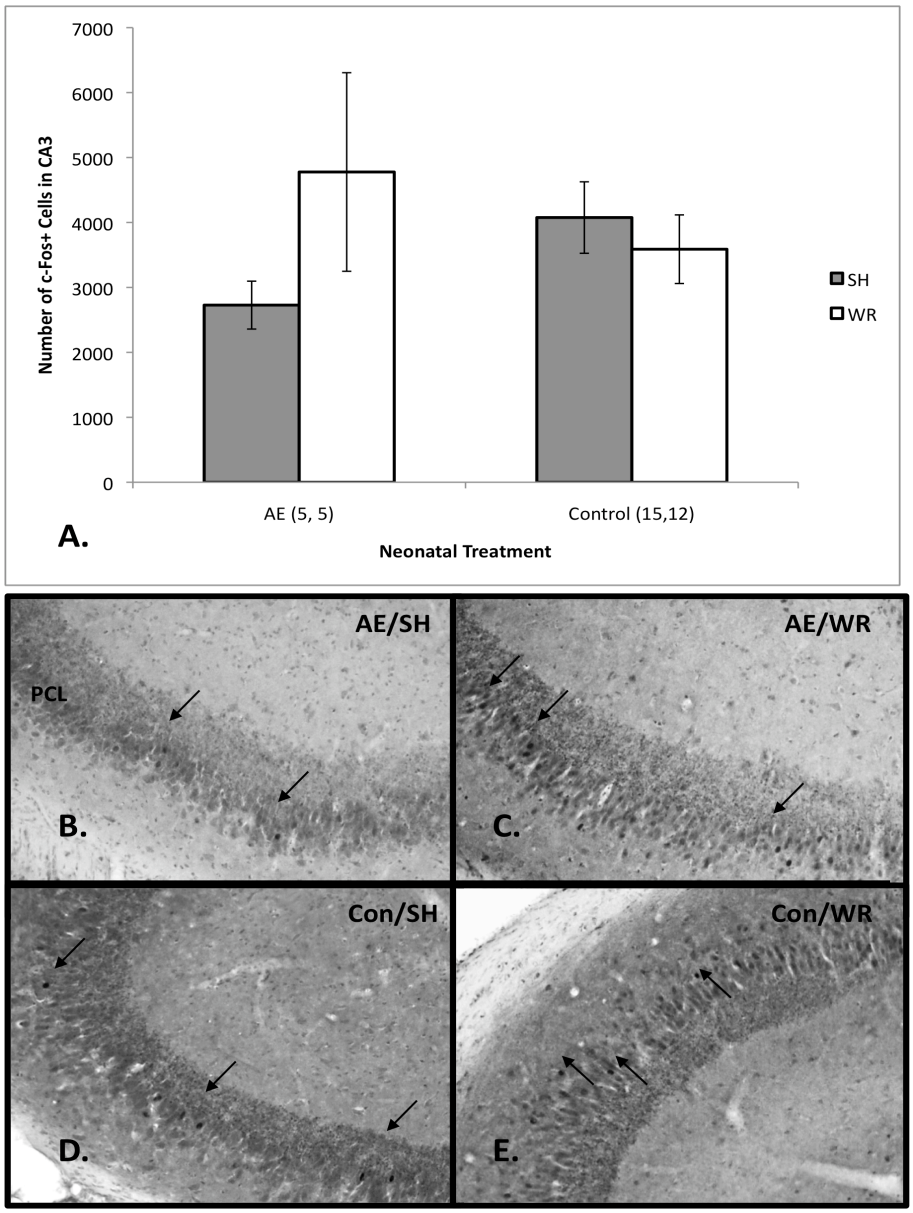

Figure 3: Number of c-Fos+ cells in CA3 at PD42. A: No significant differences in the effect of housing or neonatal treatment were found. B: Arrows point to c-Fos+ cells in AE/SH animals. C: Arrows point to c-Fos+ cells in AE/WR animals. D: Arrows point to c-Fos+ cells in Con/SH animals. E: Arrows point to c-Fos+ cells in Con/WR animals.

3.6 c-Fos Expression in Dentate Gyrus

A two-way ANOVA found no significant differences in the effect of housing or neonatal treatment in DG (p>0.05) (Figure 4A).

An independent-samples t-test was performed to determine if there was any effect of alcohol exposure on the number of c-Fos+ cells in Dentate Gyrus (DG) between animals of the two neonatal treatments while housed in standard housing. In looking at AE/SH animals versus Con/SH, there was no significant effect of alcohol on cell number in DG (p > 0.05). Images representing the average number of c-Fos+ cells in DG of each neonatal treatment and housing treatment are expressed in Figure 4B-E.

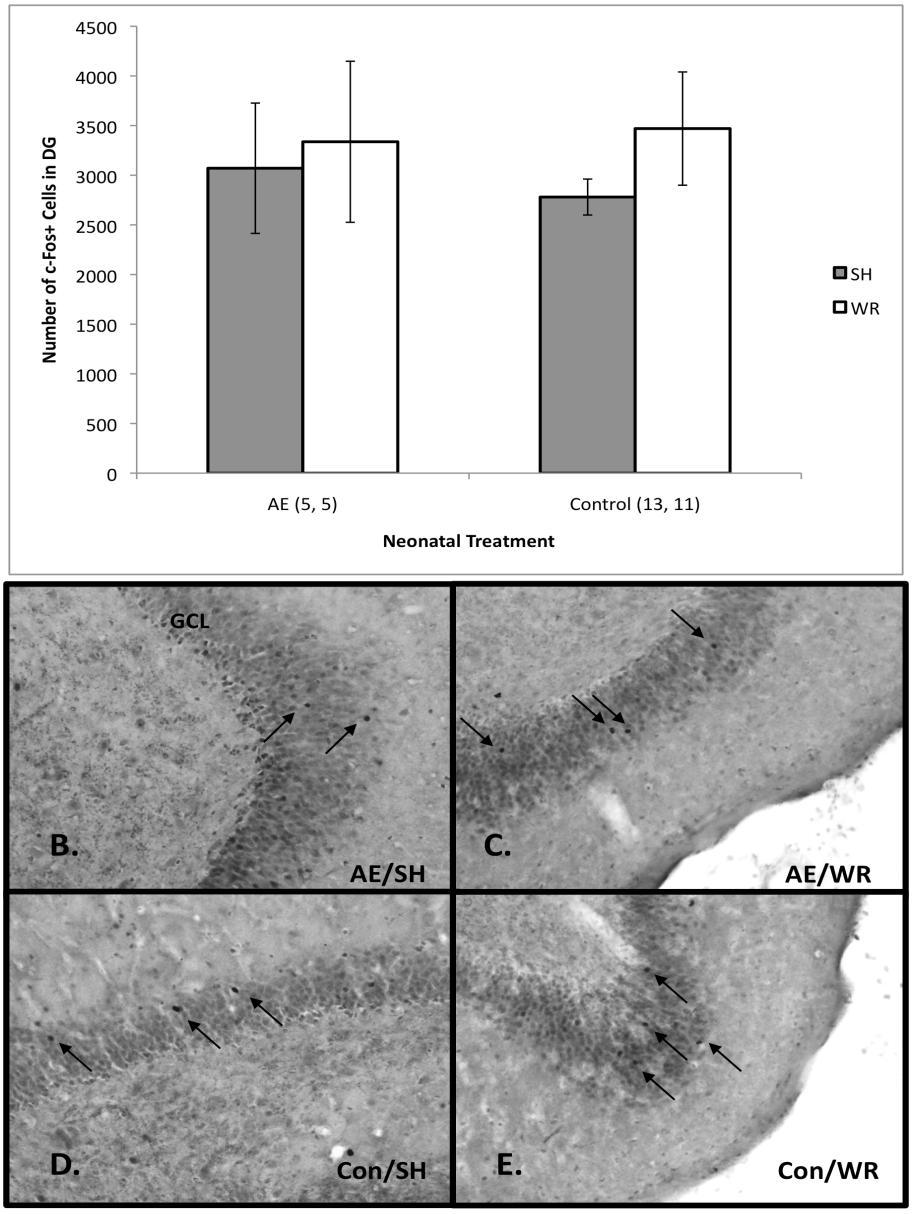

Figure 4: Number of c-Fos+ cells in DG at PD42. A: No significant differences in the effect of housing or neonatal treatment were found ($p>0.05$). B: Arrows point to c-Fos+ cells in AE/SH animals. C: Arrows point to c-Fos+ cells in AE/WR animals. D: Arrows point to c-Fos+ cells in Con/SH animals. E: Arrows point to c-Fos+ cells in Con/WR animals.

3.7 Correlation Data

Due to the uncharacteristic results of this study, Pearson Correlations were performed to analyze the relationship between the number of c-Fos+ cells and the time since lights on (9:00 a.m.) by brain region. Tests were performed to assess correlations between the number of c-Fos+ cells within the specific neonatal treatments in the different brain areas (AE/WR and Con/WR analyzed separately). The number of animals per brain area in each condition are as follows: CA1 (AE/WR N=6; Con/WR N=9); CA3 (AE/WR N=7; Con/WR N=12); DG (AE/WR N=6; Con/WR N=11). It was found that there was a trending negative correlation in AE/WR animals in CA1 (r^2=-0.780; p=0.067; two tails) and there was a significant negative correlation in groups Con/WR in CA1 (r^2=-0.875; p=0.002; two-tails) (Figure 5A). Interestingly, there was no significant correlation found in AE/WR or Con/WR in CA3 (p>0.05) (Figure 5B). There was no significant correlation in AE/WR in DG (p>0.05); however, there was a significant negative correlation in Con/WR animals in DG (r^2=-0.0651; p=0.030; two tails) (Figure 5C).

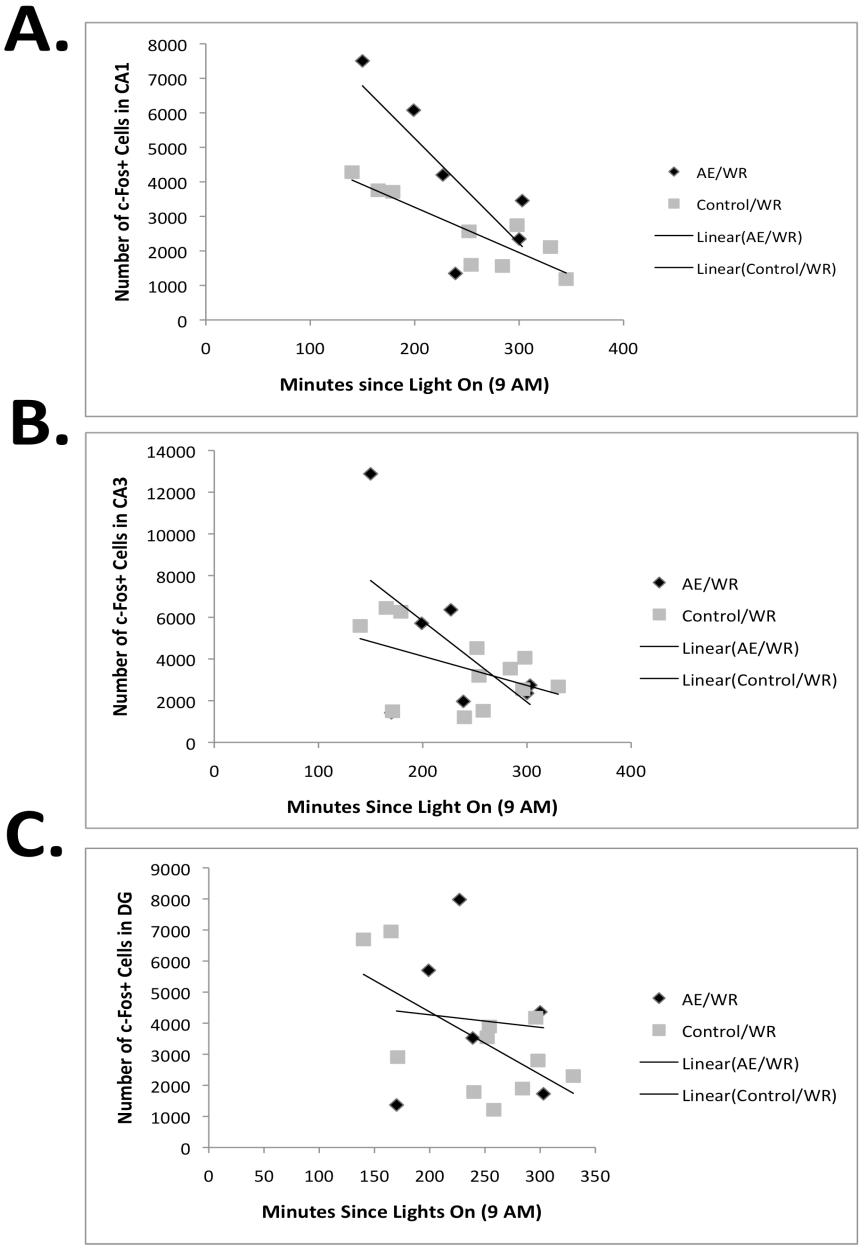

Figure 5: Correlations between number of c-Fos+ cells and minutes since lights on by brain region and neonatal treatment. A. CA1: AE/WR (r^2=-0.780; p=0.067), Con/WR (r^2=-0.875; p=0.002); B: CA3: AE/WR (r^2=-0.571; p>0.05), Con/WR (r^2=-0.469; p>0.05); C. DG: AE/WR (r^2=-0.087; p>0.05), Con/WR (r^2=-0.651; p=0.030)

Chapter 4

DISCUSSION

4.1 Effect of Alcohol Exposure on the Number of c-Fos+ Cells

The present study showed that there was a significant effect of alcohol on number of c-Fos+ cells in CA1, however, there was no alcohol effect seen in the DG or in CA3. Similarly, an unpublished study from our lab that looked at ΔFosB expression as a marker of neuronal activity and neuroplasticity using the same experimental timeline, alcohol dosage, and wheel running exposure saw no effect of alcohol treatment in any of the three brain areas. These inconclusive results raise speculation regarding the differences between the expression of the different proteins that are being compared as well as speculations regarding the nature of the three types of hippocampal areas that are being examined. Numerous other studies support the notion that c-Fos expression is affected by alcohol exposure, suggesting that c-Fos basal expression is more sensitive to alcohol exposure. A recently published study by Murawski *et al*. (2012) for instance, found reduced c-Fos expression in CA1 of animals exposed to 5.25g/kg alcohol over PD4-9.

Furthermore, in looking at the critical periods for alcohol induced cell loss in the three hippocampal areas in question, Tran and Kelly (2003) subjected rats to alcohol exposure using experimental models that resembled first, second, and third trimester alcohol exposure in humans. Following the varying time points of alcohol exposure, the number of c-Fos+ cells was quantified in CA1, CA3, and DG. Similar to the results reported in this thesis, Tran and Kelly found that only in CA1 was there a significant reduction of cell number due to the effect of alcohol. Therefore, it is during

all three trimesters when the cells in CA1 are most vulnerable to the effects of alcohol exposure, whereas the cells in CA3 and DG were not seen to be at risk for alcohol insult during any of these times of development.

Other studies that observed third-trimester binge like alcohol exposure also found no significant reductions in cell number in CA3 or DG, but significant reductions in CA1 cell number (Bonthius and West, 1990; Bonthius *et al.,* 2001). It would be useful in the future for our lab to further explore this differential vulnerability to alcohol exposure within the three hippocampal areas and assess reasons as to why the three areas are impacted in different ways when exposed to alcohol insult. Similarly, it may be valuable to employ different alcohol exposure paradigms that target each area in order to more effectively observe and understand the effects that alcohol exposure has on neuroplasticity in the hippocampal environment as a whole.

4.2 Effect of Wheel Running on the Number of c-Fos+ Cells

In the current study it was found that there was a significant effect of housing on c-Fos expression in CA1 as well as a significant interaction between wheel running and neonatal treatment in CA1. It was found that AE/WR animals had a significantly greater amount of c-Fos+ cells in CA1 as compared to AE/SH animals. In both AE/WR and Con/WR groups, WR animals had more c-Fos+ cells as compared social housed animals, however AE/WR animals had the most increase. Additionally, in comparison to Con/SH animals, AE/SH animals had significantly less c-Fos+ cells in CA1; such significant differences were not detected in CA3 and DG. This data highlights the negative effects that alcohol has on the number of c-Fos+ cells in CA1

as well as provides further evidence that WR has a role in enhancing neuroplasticity and in impacting overall hippocampal activation.

In a recent unpublished study on our lab, we observed an upregulation of ΔFosB in CA1 and CA3 in animals exposed to wheel running. The lack of c-Fos induction throughout the hippocampus in rats exposed to wheel-running was surprising, for expression of c-Fos and other transcription factors has been seen to be induced by similar vigorous activities (Clark *et. al.,* 2011). There have also been observed increases in c-Fos expression in DG, CA3, and CA1 in rat pups exposed to postnatal treadmill exercise that were born from alcohol-intoxicated mothers as compared to the deficit seen in c-Fos induction in pups without any exercise exposure (Sim *et al.,* 2008).

Possible explanations for the unexpected pattern of c-Fos induction seen in the current study may have to do with the intensity and the duration of voluntary wheel running to which each rat is exposed. In a study that looked at the effect of intensity and duration of treadmill running on c-Fos expression in the hippocampus, it was found that as the intensity of running increased, c-Fos induction increased as well (Lee *et al.,* 2003). Additionally, in looking at a duration of running for either 1 day, 3 days, 7 days, 14 days, or 28 days, peak c-Fos expression occurred at 7 days; after this time point, c-Fos expression began to subside (Lee *et al.,* 2003). The current model of voluntary wheel running does not take into account the individual intensity for each rat. Instead, data that is presented is the average distance run per cage. Therefore, it is not known whether certain rats run for a greater duration than others or whether certain rats run at a greater intensity. Rats are also exposed to voluntary wheel running from PD30-42, which exceeds the optimal duration of 7 days as cited by Lee and

colleagues (2003). Future investigations in our lab may want to explore not only the actual amount of wheel running per animal, but also the number of days of wheel running exposure in order to more effectively observe gene expression throughout the hippocampus.

4.3 Correlation between time since lights on and time of sacrifice on c-Fos Induction

The unforeseen results that were obtained from this study led us to believe that there may have been a confounding variable to take into account. Therefore, Pearson Correlations were performed to analyze the relationship between c-Fos expression and the time since lights on (9:00 am) at the time of tissue harvest. Previous research has conclusively found that c-Fos is a transient immediate early gene, and unlike other members of the Fos family that show cumulative expression, c-Fos protein expression peaks at about two hours following stimulation (Nestler, 2001; Zangenehpour and Chaudhuri, 2002). The methods of the current study did not allow for sacrifice immediately following wheel running; in some cases the light cycle had been underway for up to 6 hours prior to sacrifice. Since rats are most active during the night it is possible that the animals sacrificed later would not have run on the wheel for a few hours at the time of tissue harvest. Therefore, c-Fos expression in the current study may have been subdued and significantly impaired due to longer time periods between the end of wheel running at lights on and the time of sacrifice.

Correlations were performed to assess the relationship between c-Fos expression and time since lights on within the specific neonatal treatments in all brain areas (AE/WR and Con/WR). It was found that there was a trending negative correlation in AE/WR animals in CA1 and there was a significant negative correlation

in groups Con/WR in CA1, such that as more time since lights on passed, the number of c-Fos+ cells decreased. There was no significant correlation found in AE/WR or Con/WR in CA3. There was also no significant correlation in AE/WR in DG; yet, there was a significant negative correlation in Con/WR animals in DG.

At this point, it is not understandable why there exist such discrepancies in correlations between the brain areas and within neonatal groups. Clearly, there are pronounced methodological implications for these correlation results, which require completion of subsequent identical studies that will more conscientiously take the time-course of c-Fos peak activity into account.

4.4 Future Studies of Neuroplasticity within the Hippocampus

While the current study resulted in unanticipated methodological errors, we still expect that if these errors are corrected and the study reproduced, the results will show decreased c-Fos expression in AE animals, as well as enhanced c-Fos expression in Con/WR animals as compared to Con/SH animals.

In looking at neuronal activity via c-Fos induction throughout the hippocampus following behavioral interventions such as wheel running, we are still dedicated to the task of further elucidating the components of hippocampal neuroplasticity and how this affects the connections, incorporation and maturation of new cells. Our explorations of hippocampal neuroplasticity will not only continue to investigate c-Fos induction, but may also begin to investigate the activation patterns of other immediate early genes of interest. Such immediate early genes that have been seen to be involved in hippocampal neuroplasticity include the proteins Arc and Zif268. In a study that looked at the expression of c-Fos, Arc, and Zif268 in rats subjected to hippocampal dependent tasks such as spatial water task training, similar to the aforementioned

Morris Water Maze task, it was seen that all three immediate early genes were expressed following the task. Additionally, it was seen that Arc was most sensitive to expression following the hippocampal-dependent learning tasks (Guzowski *et al.,* 2001). Therefore, in future studies it may be more beneficial to use Arc to further explore hippocampal neuroplasticity using our exposure to voluntary wheel running. Further evidence that justifies the use of other transcription factors involved in neuroplasticity includes a study conducted by Clark and associates that observed expression of c-Fos, Zif268, and Arc in mice following 31 days of voluntary wheel running. Following wheel running, an induction in each of the three immediate early genes' expression was significantly evident.

4.5 Conclusions

The aim of this study was to better understand the hippocampal environment during periods of heightened activity following the behavioral intervention of wheel running in rats exposed to alcohol neonatally. In order to look at neuroplasticity throughout the hippocampus, c-Fos was used to mark cells in DG, CA1, and CA3 that were involved in neuronal activity. We hoped that by comparing c-Fos expression across neonatal treatments and housing conditions, we would better understand how hippocampal cells were making connections, incorporating into the network, as well as maturing to adulthood.

However, we encountered a crucial methodological issue that we will resolve in future studies. It is imperative to replicate this study to resolve the correlation that presently exists between time since lights on at time of sacrifice and the number of c-Fos+ cells. This study will be done again in the immediate future. Only after we

analyze such results will we have a more concrete idea of where to look next. Other immediate early genes, whose expression is indicative of neuronal activity, are of high interest in our task of studying neuroplasticity of the hippocampal environment. Through the observation of immediate early gene expression we hope to better understand the state of the hippocampal environment following behavioral intervention, and how this environment affects the fate of newly generated cells.

We are hoping that future studies help to further understand the hippocampal environment and the mechanisms that occur within this area after a developing brain is exposed to alcohol insult. Using wheel running as a behavioral intervention, these studies are aimed at providing valuable and practical therapies for those affected with the diagnoses of FASD. With continued exploration of hippocampal neuroplasticity using this experimental paradigm, we hope to be able to extrapolate such findings to the human population to fulfill the ultimate goal of providing effective therapies to children with FASD to ameliorate the damage caused by maternal drinking.

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