EFFECTS OF NEONATAL ALCOHOL EXPOSURE ON HIPPOCAMPAL NEURONAL ACTIVATION IN RESPONSE TO PHYSICAL EXERCISE

by

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ABSTRACT

Neurogenesis has been shown to be negatively affected by postnatal alcohol exposure in a rat animal model of fetal alcohol spectral disorders. Therapeutic interventions such as wheel running have been shown to rescue cellular proliferation in the dentate gyrus (DG) of the hippocampus. However, wheel running is not enough to rescue cellular survival. We hypothesize that survival of newly born cells is impaired because the cells are not being properly integrated into the hippocampal circuit. Rat pups were assigned to one of three treatment groups: suckle control, sham intubated, or alcohol exposed (AE). Animals were then placed in either wheel running or social housed conditions. Neuronal marker FosB/ΔFosB was used to label cells activated in DG and CA1 subfields of the hippocampus in response to wheel running. Although we expected AE to decrease FosB/ΔFosB expression, no effect of postnatal alcohol exposure on FosB/ΔFosB + cell number was found. However, twelve days of wheel running was found to induce FosB/ΔFosB expression in CA1 of all animals, while no effect was found in DG. Overall, these results suggest that AE has no long-term effect on FosB/ΔFosB expression within the hippocampus and wheel running's differential activation could be due to the distinct physiological and functional role of the hippocampal subfields.

Chapter 1

INTRODUCTION

1.1 Fetal Alcohol Spectral Disorders

"Of all the substances of abuse (including cocaine, heroin, and marijuana), alcohol produces by far the most serious neurobehavioral effects in the fetus" (Institute of Medicine Report to Congress, 1996).

Despite knowledge of alcohol's detrimental effects on prenatal development, in the United States alone, Fetal Alcohol Spectral Disorders (FASD) may affect up to 1% of the entire population (Sampson *et al.*, 1997; Stratton *et al*., 1996). This figure accounts for more than children affected by Down's syndrome, spina bifida, autism, cystic fibrosis, cerebral palsy and sudden infant death syndrome combined (NOFAS, 2004). A study by the Center for Disease Control and Prevention (CDC) states that 10% of pregnant women reported alcohol use and 2% reported binge-drinking (Tsai *et al*., 2002). Binge drinking qualifies as drinking five or more consecutive drinks in two hours.

Fetal Alcohol Spectral Disorders (FASD) is an umbrella term used to describe the diverse teratogenic effects that alcohol has on the developing fetus. Several disorders fall under this continuum: Fetal Alcohol Syndrome (FAS), Partial Fetal

Alcohol Syndrome (PFAS), Alcohol-Related Birth Defects (ARBD) and Alcohol-Related Neurodevelopmental Disorder (ARND). Alcohol's effects can range from mental and behavioral deficits to physical impairments. Studies have shown that learning and memory are impaired in both verbal and nonverbal memory tasks (Mattson and Roebuck 2002, Roebuck and Mattson, 2004). Behaviorally, children with FASD have been shown to have impaired social skills, which could be due to an inability to solve social dilemmas (Stevens *et al.,* 2012). This social deficit could also be a result of facial memory recognition problems because of its importance for the development of social skills (Wheeler *et al*., 2011). Physically, a child can present with one or more of the following FASD features: facial anomalies (thin upper lip, smooth philtrum and a small eye opening), growth deficits, heart, and lung and kidney defects (Wattendorf *et al*., 2005). Additionally, children with FASD are characterized by developmental motor delays, which are especially apparent in fine motor skills (Kalberg *et al*., 2006). Other highly associated problems with FASD children are hyperactivity and attention problems (Wattendorf *et al*., 2012).

As research has shown, effects extend beyond the well being of children. Mothers of children born with FASD are at a 44.82 fold increase in mortality risk (Li *et al*., 2011). Not only does FASD affect the health of both woman and child, but it can have serious financial consequences. Amendah and colleagues reported in 2011 that in the United States of America, the average amount of money spent on medical care for children with FAS was $16,782 in 2005. This figure is nine times higher than children without FAS (Amendah *et al.,* 2011). Over a lifetime this accumulates to

approximately $1.4 million (National Institute on Alcohol Abuse and Alcoholism, 2011). Although there is no cure to FASD, it can be prevented 100% by abstaining from alcohol consumption during pregnancy.

The wide array of problems that arise as a result of neonatal alcohol exposure suggests that it affects virtually all areas of the brain. One site in particular, the hippocampus, located in the medial temporal lobe, has a major role in learning, the consolidation of memory and spatial navigation. In addition, the hippocampus is a major site of adult neurogenesis, the generation of new brain cells, during adulthood. It was previously thought that the generation of new neurons throughout adulthood was not possible as every human was born with a fixed amount of neurons. However, research in the past two decades has shown that there are two areas that undergo adult neurogenesis: the subventricular zone (SVZ) located laterally to the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus. Exposing the DG to radiation, thus inhibiting neurogenesis, is responsible for apoptosis of newly born cells (Peissner *et al*., 1999). Following the radiation-induced inhibition of neurogenesis, animals display deficits in hippocampal-dependent tasks involving the use of learning, memory and spatial navigation (Rola *et al*., 2004). Results from these animal studies are consistent with signs and symptoms produced after brain radiation exposure in humans (Marazziti *et al*., 2012). The aforementioned results provide a causal link between the function of neurogenesis and the ability to successfully perform on hippocampal associated tasks.

1.2 Neurogenesis

Neurogenesis is a process that refers to the birth, migration, and differentiation of new neurons within the brain circuitry. This process is similar during hippocampal development in embryogenesis as it is into adulthood, and is known as adult neurogenesis (AN) (Stone *et al*., 2010). Progenitor cells in the SGZ of the DG enter the cell cycle and undergo several divisions. These divisions mark the first stage of neurogenesis, proliferation. After these undifferentiated cells have exited the cell cycle, the second stage of neurogenesis, migration, begins as the cells begin receiving initial synaptic input. The newly born cells migrate from the SGZ into the granule cell layer (GCL) of the DG. The final stage of neurogenesis then occurs when these multipotent cells differentiate into one of two specific cell phenotypes: neuron or glial, and begin to mature.

During development, newly generated granule cells exhibit a period of hyperexcitability, during which young, immature neurons have a low threshold for long-term potentiation (LTP) (Ge *et al.*, 2007). LTP is the process by which cells strengthen connections, or synapses, and the low threshold allows for connections to be formed easier, making introduction into the circuitry possible (Kempermann, 2006). Furthermore, the degree to which LTP is inducted is increased (Ge *et al*., 2007) Decreasing the threshold and increasing the magnitude of LTP could be responsible, at least in part, for allowing newly born granule cells to compete for the formation of new connections in an environment where already well established connections with

mature granule cells exist. Lack of integration, as suggested by low levels of excitability, could account for the decreased survival of newly born granule cells.

Functionally, DG GC's are responsible for relaying input from the entorhinal cortex into the hippocampus through the trisynaptic pathway. The trisynaptic pathway begins with information brought to the dendrites of granule cells in DG from the medial entorhinal cortex via the perforant pathway. From the DG, the axons of granule cells - mossy fibers - relay the signal to CA3. CA3, in turn, stimulates CA1 via pyramidal cells' axons known as Schaffer collaterals (Ramon y Cajal 1995).

More recently, it has been determined that DG GC's have an even more specialized role. To determine the role of newly born GC's, the Nakashiba group (2012) used transgenic mice that displayed inhibited output of old GCs, but the output of new GCs was left intact. Transgenic mice still showed pattern separation, but were deficient in pattern completion tasks (Nakashiba *et al*., 2012). Pattern separation is the ability of GC's to discriminate between the spatio-temporal relationships of similar events, which allows for the formation of distinct memories. Whereas pattern completion is the ability of GC's to reactivate whole representations using environmental cues, which allows for the recall of memories. Complementary to the study done by Nakashiba (2012), X-Ray irradiation of neurogenesis, which targets the process of cell division thus affecting newly born GC's, impairs pattern separation (Clelland *et al*., 2009). The opposite effect is seen during enhancement of neurogenesis via voluntary physical exercise. Animals that underwent exercise were capable of discriminating between similar contexts, or pattern separation (Sahay *et al*.,

2011). The data suggests that DG GC's differ in their function depending on their time of development such that younger GC's play a role in pattern separation, while the older GC's play a role in rapid pattern completion.

1.3 Animal Models of FASD

Although there are many studies that report the detrimental effects alcohol has on the developing brain, it is important to use an animal model that focuses on the development of the hippocampus for the purposes of this study. The hippocampus undergoes a significant period of growth and synaptogenesis during the third trimester, known as the brain growth spurt. In rats, the third trimester is equivalent to the first two weeks after birth (Dobbing and Sands, 1979).

Human data has provided us with a great deal of information on the effects of neonatal alcohol exposure. Animal models give the ability to control for the variables, such as dose and developmental timing of exposure, allowing the objective interpretation of data.

Microencephaly is a condition in which the brain is abnormally small. In a study conducted by Bonthius and West (1988), only alcohol doses of 4.5 g/kg/day or higher during the brain growth spurt were sufficient to result in significant microencephaly, as compared to controls. However, blood alcohol concentration (BAC) is a more accurate predictor of cerebral damage. The peak BACs produced by a dose of 4.5 g/kg/day were greater than 150 mg/dl. Anything less than 150 mg/dl

resulted in no significant microencephaly (Bonthius and West, 1988). The dependence of brain damage on BAC further reflects the importance of pattern or duration of alcohol exposure. Condensing or elongating the time during which alcohol is administered will yield higher or lower BACs, respectively.

Another variable that must be controlled for is the developmental timing of exposure. Among the three trimesters in pregnancy, the one that incurs the most serious effects on the hippocampus is the third trimester equivalent. A study done by the Livy group (2003), showed that during the third trimester equivalent, CA1 volume, pyramidal cell density and cell numbers were all reduced. Additionally, the number and density of granule cells within the DG was significantly reduced (Livy *et al.*, 2003).

1.4 Effects of Neonatal Alcohol Exposure on Hippocampal Neuroanatomy and Neurogenesis

Neonatal alcohol exposure has persistent and deleterious consequences affecting AN. The proliferative ability of newly born granule cells in the DG is significantly decreased following alcohol exposure as compared to controls (Redila *et al*., 2006). In a study done by the Klintsova group, the proliferative ability of the progenitor cells in a rodent model was not affected in any group (Klintsova, 2007). The discrepancy in proliferation can be explained by the nutritional effects of prenatal alcohol exposure. Pair fed animals did not display a significant decrease in proliferation (Redila *et al*., 2006). However, in the latter study, the survival of the non-

affected proliferative cells in the hippocampus was significantly decreased by 15% when measured thirty days later, as compared to non-alcohol exposed controls (Klintsova, 2007).

In addition, alterations in hippocampal neuroanatomy have been shown. Abnormal branching patterns are present in mossy fibers of alcohol exposed animals (West *et al*., 1981). Dendritic spine density is significantly decreased in CA1 hippocampal region of alcohol exposed rodents (Abel, 1983). Spine density is a measure of dendritic communication. The greater the spine density, the more connections it can form within the circuit. Neonatally alcohol exposed adult rodents had CA1 pyramidal neurons with decreased complexity, indicative of immature development (Smith and Davies, 1990). All of these studies support the idea that alcohol exposure has long-lasting effects on neurogenesis.

1.5 Behavioral Animal Data

AN is intimately involved with the hippocampal-affiliated processes of learning and memory. Blocking AN using the NR2A subunit containing N-methyl-D-Aspartate (NMDA) receptor antagonist, NVP-AM077, impedes learning and memory in behavioral testing of hippocampal-dependent tasks (Hu *et al.*, 2009). However, enhancing neurogenesis using the neurotrophic factor, Peptide 6, resulted in improvement in these hippocampal-dependent tasks as reported (Cohan *et al*., 2009).

The negative effects caused by blocking AN on behavioral testing are similar to the behavioral deficits seen in neonatally alcohol exposed animals. The radial arm

maze task, used to assess spatial learning and memory, showed evidence of impaired learning in prenatally alcohol exposed rats. The animals showed deficits in choosing the correct arms of the maze in order to retrieve the hidden food at the end of different arms (Reyes *et al*., 1989).

In another hippocampus-dependent task, the Morris Water Maze, performance is also impaired in alcohol exposed animals. This paradigm, developed by Richard Morris, involves placing a rodent in a pool of opaque water during which it must swim and find a hidden platform and remember where the platform is using the various spatial clues given. The ability to learn and have memory of a task when given spatial clues requires an intact and functional hippocampus. Alcohol exposed animals have increased latency to reach the hidden platform and take more roundabout paths (Blanchard *et al*., 1987) suggesting a damaged hippocampus.

Furthermore, interruptions in spatial learning on the Morris Water Maze task was accompanied by a downregulation in glutamate transmission related genes, such as the vesicular glutamate transporter 1, excitatory amino acid transporter (EAAT) 1-4, and NMDA receptor subunits NR-1 and 2A-D. A study by the Zink lab, reported that neonatally alcohol exposed rats displayed a significantly greater latency to enter the platform zone and significantly less time in the platform zone when tested during adulthood (Zink *et al.*, 2011). This is indicative of impaired spatial learning. In this same study, it was found that there was also a dysregulation of glutamate transmission-related gene expression, specifically the genes mentioned above, within the adult hippocampus (Zink *et al.*, 2011). Because of glutamate's essential role in

LTP induction, abnormal glutamate transmission could lead to low levels of cellular excitability accompanied by decreased plasticity.

1.6 Voluntary Physical Exercise

Voluntary physical exercise, such as wheel running, has consistently shown positive results in ameliorating the deficits in neurogenesis associated with the processes of FASD (Redila *et al*., 2006, Helfer *et al*., 2009, Boehme *et al*., 2011). A significant upregulation of cellular proliferation within the DG has been observed across several studies in response to wheel running in all groups, including both alcohol exposed and control animals (Redila *et al*., 2006; Helfer *et al.*, 2009; Boehme *et al*., 2011).

Wheel running has increased survival of newly born granule cells in control animals, but is not sufficient to rescue cell survival in a rodent model of FASD (Helfer *et al*., 2009). In this study, newly born granule cells were labeled with bromodeoxyuridine (BrdU), a measure of cellular proliferation, during adolescence (PD 42) directly after the last day (of 12 days) of wheel running. At PD 42, proliferation of granule cells is directly influenced by the previous 12 days of wheel running. Another group of animals underwent the same treatment of wheel running, but the newly born granule cells were measured with BrdU 30 days after wheel running (PD 72) to see how many newly generated cells survived. The results indicate that cell proliferation is significantly increased in wheel running animals of all

treatment groups. However, at PD 72, cell survival was only significantly increased in suckle control animals (Helfer *et al*., 2009).

1.7 The Study

Diminished neuronal survival in alcohol exposed animals can be a result of impaired synaptic plasticity mechanisms. Interestingly, long-term potentiation (LTP) is impaired in alcohol exposed animals (Berman and Hannigan, 2000). LTP is the process by which cells strengthen connections, or synapses. Impaired LTP suggests that the ability of neurons to become excitable and form synapses is impaired and could potentially be a cause of decreased cell survival.

The current study investigates the hypothesis that the newly born granule cells in alcohol-exposed animals are compromised due to impaired integration into the hippocampal circuit. The animal model of FASD used mimics a binge-like alcohol exposure during the third trimester equivalent (PD 4-9), which coincides with the brain growth spurt and hippocampal development.

During this rapid growth, if the neurons are to integrate and survive, they must form synapses in a process known as synaptogenesis, thus allowing neurons to send and receive signals from one another. Neonatal alcohol exposure could be making the cells less excitable and therefore affecting the process of integration and ultimately synaptogenesis, making it difficult for the cell to survive.

As previously mentioned, during AN, newly born granule cells go through a period of hyperexcitability, during which integration within the circuitry is made

easier. To see whether or not excitation of cells within the hippocampus is affected in response to alcohol exposure, cells can be labeled with an activity marker such as FosB/ΔFosB. FosB and ΔFosB are transcription factors upregulated in response to a repetitive stimulus. Specifically, the ΔFosB protein is cleaved from the larger protein, FosB, removing 110 amino acids from the C-terminus (Nakabeppu and Nathans, 1991).

In this study, FosB/ΔFosB was used because it is a long-term measure of cellular activity (Chen *et al*., 1997). Repeated activation of neurons result in a constant expression of FosB/ΔFosB (McClung *et al*., 2004). FosB/ΔFosB can last days after the last stimulus has disappeared because of the high stability of the protein (Ziolkowska *et al*., 2002). Because lack of expression of FosB/ΔFosB does not necessarily indicate an absence of cellular activity, it is important to note that it is the relative distribution of FosB/ΔFosB between animals that will be quantified. FosB/ΔFosB expression will be measured across different zones of the hippocampus. These areas of measurement include the following subregions of hippocampus: dentate gyrus (DG) and Cornu Ammonis (CA) 1. This study will provide valuable information as to the relative cellular activation of neonatally alcohol exposed animals in response to wheel running.

Chapter 2

METHODS

2.1 Animals

The subjects of the study were Long Evans offspring of animals bred at the University of Delaware animal breeding colony. The birth almost always occurred on gestational day (GD) 22, which was also considered postnatal day (PD) 0. On PD3, litters were culled to eight pups containing four males and four females. Forty-nine animals (36 males, 12 females) were used to measure the effects of alcohol and physical exercise on cellular activity. All animals were maintained in an animal lab at 22º C with ad libitum food and water. The housing facility was maintained on a 12:12-hr light-dark cycle, with lights on at 0900hr. University of Delaware Institutional Animal Care and Use Committee approved all animal-handling protocols.

2.2 Experimental Conditions

On PD4, Long Evans rat littermates were randomly separated into three experimental groups: alcohol exposed (AE, n=16), sham intubated (SI, n=16) and suckle control (SC, n=16). Pups' paws were tattooed with black ink for identification of pups within litters on PD4.

AE animals were given two alcohol doses per day (5.25 g/kg/day) separated by two hours on PD4-9. Alcohol was diluted (11.3% v/v) in a milk formula according to a previously described method (West et al., 1984). Alcohol and milk were delivered via intragastric intubation (Goodlett and Johnson, 1997). Two extra doses of milk were given (separated by two hours) after the last alcohol dose on PD4 as a caloric supplement. A single milk dose was given daily from PD 5-9 two hours after the last alcohol dose. SI animals were intubated, but no alcohol or milk was given as the dam provided the necessary nutrition. SC animals were left undisturbed except for daily weight measurements taken on PD4-9. All pups were left with the dam until PD23 for weaning.

2.3 Blood Alcohol Concentration Analysis

Blood samples were collected from AE and SI animals from a tail clip into heparinized capillary tubes 90 min after the second dose was given on PD 4. Samples were centrifuged at 15,000 rpm and plasma was assayed for blood alcohol concentration (BAC) using an Analox GL-5 Analyzer (Analox Instruments, Boston, MA).

2.4 Housing Conditions

Animals were weaned on PD 23 and housed with same-sex littermates. Animals from same litters and different conditions were mixed as much as possible to avoid litter effects. On PD 30, animals were assigned to one of two different housing

conditions: wheel running (WR, n=24) or social house (SH, n=24). All animals were placed three per cage whenever possible. WR animals were placed in a cage with access to a stainless steel running wheel and allowed 24/7 access for 12 days (PD30-42) for voluntary exercise. Social house (SH) animals were placed in standard cages from PD30-42.

2.5 Tissue Collection

On PD 42, animals were anesthetized via an intraperitoneal injection of ketamine mixed with xylazine. Animals were then transcardially perfused with heparanized 0.1 M phosphate buffer (PBS, pH=7.4) followed by a 4% paraformaldehyde solution in PBS with a pH of 7.4. Brains were post-fixed in 4% paraformaldehyde for 2 days and then transferred to 30% sucrose in 4% paraformaldehyde. Brains were frozen and placed in a cryostat to collect 40 µm horizontal sections of the entire hippocampus. Sections were stored in cell culture well plates filled with cryoprotectant solution made up of glycerol and ethylene glycol in a Tris-buffered solution (TBS), and placed in a -20 ºC freezer. To begin processing, sections were chosen using a systematic random sampling procedure such that starting around Bregma -2.30; every 16^{th} section (about 7-8 per animal) was chosen for immunohistochemistry. Bregma is a defined anatomical location on the skull used as a reference point to accurately locate a region of the brain. This sampling procedure allows analysis of the entire extent of the dentate gyrus (DG).

2.6 Immunohistochemistry

To analyze the number of FosB/ΔFosB+ cells, sections were first washed in TBS, then incubated in 0.6% H_2O_2 in TBS for 30 minutes to eliminate endogenous peroxidases and then washed in TBS again. Tissue was incubated in blocking solution (0.5% Triton X-100 and 3% normal goat serum in TBS) to block the non-specific binding sites. Sections were then transferred to primary antibody solution (FosB sc-48, 1:400; Santa Cruz Biotechnology) diluted in washing solution (2% goat serum in TBS) for 48 hours at 4 ºC. Following incubation, tissue was washed in TBS and then transferred to secondary antibody solution (anti-rabbit IgG made in goat antibody, 1:500; Sigma) diluted in washing solution for 1 hour at room temperature (RT). After, tissue was washed in TBS and then washing solution for 2 hours. Sections were then incubated in avidin-biotin-peroxidase complex solution (ABC Elite Kit, BA-9400; Vector Laboratories) for 1 hour at RT and then washed in TBS. Reaction of nickel-enhanced diaminobenzedine (DAB) was monitored under a light microscope. After the DAB reaction was detected, sections were washed in TBS and mounted onto gelatin-covered slides and left overnight to dry. The following day, slides were counterstained with 0.1% pyronin Y and coverslips were placed on the slides using DPX mounting medium.

2.7 Quantification

Images representative of the entire DG and CA1 were taken using a light microscope at 200x magnification. Specifically, images were taken from a lateral and medial segment of the DG and CA1 from each of eight sections of every animal. Images were then uploaded and analyzed using ImageJ to determine the relative expression of FosB/ΔFosB+ cells across the experimental conditions. The experimenter was blind to the animal conditions.

ImageJ was used to quantify the number of FosB/ΔFosB+ cells per given area by comparing the pixel luminescence of each individual stained cell against the background counter stain. The areas of interest (DG and CA1) were traced in every image to minimize the amount of background labeling that was counted, which would over estimate the cell counts. Images were then converted to a 16-Bit gray scale to threshold the image. A threshold of the image was then placed to differentiate between the pixel luminescence of labeled and non-labeled cells. The computer program would then input the threshold to determine which cells are stained, FosB/ΔFosB+ cells. Every threshold was set by the experimenter due to the uncontrollable differences in darkness of staining. However, all images were taken with identical light intensities to avoid any extraneous light effects. In the binary image, nearby cells were occasionally merged together. This merge would then be registered as a single cell instead of two. However, to avoid the amount of undercounting that resulted, the image analysis software was set to create a divide between neighboring cells using a Watershed tool.

Finally, all processed images were analyzed for cell counts. ImageJ reported the number of FosB/ΔFosB+ cells present in the traced area of the image.

2.8 Statistical Analysis

Two-way analysis of variances (ANOVAs) was used to analyze the effects of postnatal treatment and housing condition on number of FosB/ΔFosB+ cells in the CA1 and DG separately.

Chapter 3

RESULTS

3.1 Weights

Animals were weighed on the first and last day of alcohol exposure (PD4 and 9) and at the beginning and end of wheel running (PD30 and 42). While no significant difference was found between control and AE animals at the beginning of alcohol treatment, there was a significant difference in total body weight on PD9 such that AE animals displayed significantly lower weights as compared to SC (Table 1). No significant difference in body weights was found from the beginning of wheel running (PD30) and the end of wheel running (PD42) between AE and SC animals. All animal body weights steadily increased throughout all treatments (Table 1).

Table 1. Animal weights of SC, SI, AE (g, ± SEM) on PD4, PD9, PD30 and PD42. * indicates p<.05.

	Postnatal Treatment		
	SC	SI	AE
Weight (g)			
PD4	11.16 ± .32	10.04 ± .49	10.63 ± .37
PD9	19.8 ± .57	17.02 ± 1.41	16.64 ± .64*
PD30	98.44 ± 2.06	93.56 ± 3.35	95.12 ± 2.44
PD42	186.81 ± 4.71	177 ± 5.78	179.63 ± 4.24

3.2 Blood Alcohol Concentration

The mean BAC (± SEM), measured on PD4 after the second alcohol dose was 375.82 ± 10.30 mg/dl. This value follows previously published data using similar alcohol dosages (Helfer *et al*., 2009; Klintsova *et al*., 2007).

3.3 Wheel Running

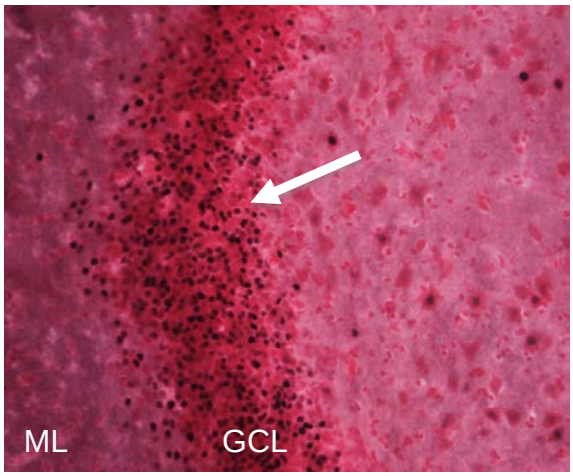
The amount of wheel revolutions (± SEM) per 24-hour period was 4366.37 ± 823.06 (approximately 5 km). Wheel revolutions per 24-hour period are indicative of the amount of voluntary physical exercise the animals underwent while being housed with a running wheel for twelve days.

3.4 FosB/ΔFosB expression in Dentate Gyrus

Seven 40 μm sections of each animal were stained with anti-FosB sc-48 antibody and the DG was analyzed to determine the relative amount of cellular activity

at PD42. Representative images of DG in control animals between housing conditions (SH vs. WR) provide a visual qualitative difference and are shown below (Figure 1). Interestingly, a two-way ANOVA found no effect of postnatal treatment or housing on expression of FosB/ΔFosB+ cells in DG ($p>.05$) (Figure 2). Values for the number of FosB/ΔFosB+ cells from all six conditions are displayed in Table 2 (appendix A).

A.



B.

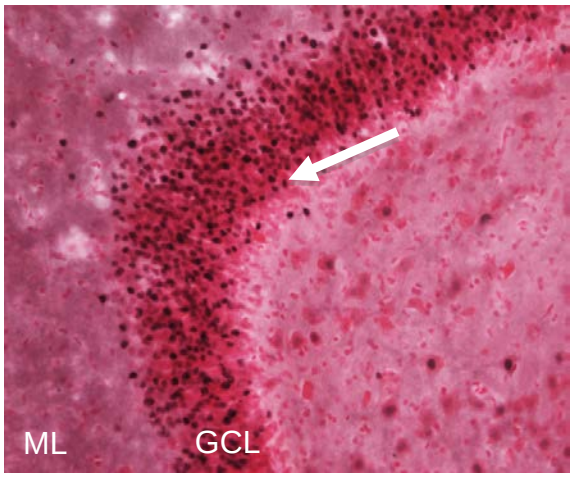


Figure 1. Qualitative differences between hippocampal FosB/ΔFosB expression in DG of SH and WR animals, respectively. FosB/ΔFosB+ cells are darkly stained (arrows). GCL is the granule cell layer and ML refers to the molecular layer. No visible difference between the numbers of FosB/ΔFosB+ cells is seen between (A.) SC/SH condition and (B.) SC/WR condition. Magnification at 200X.

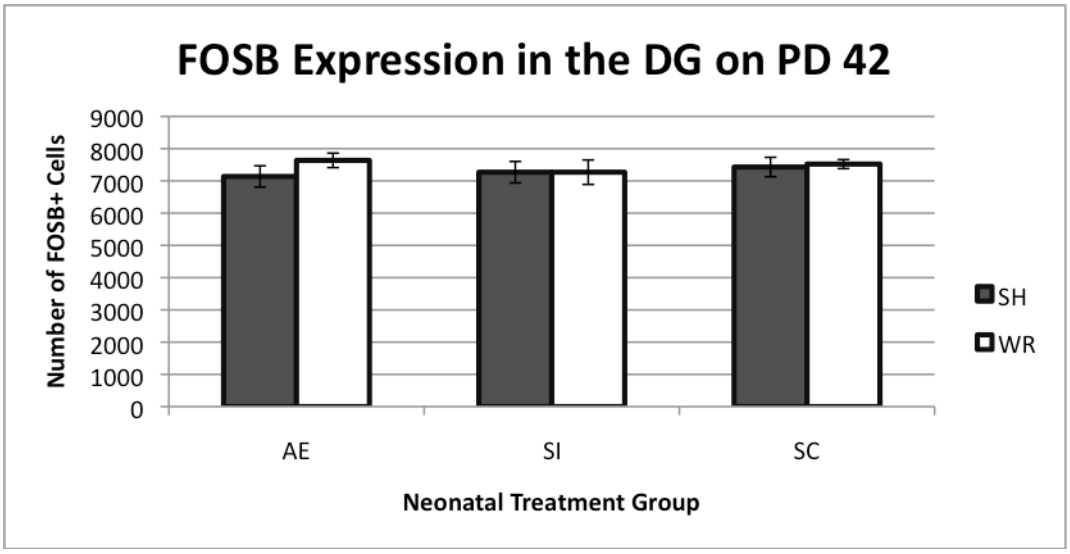
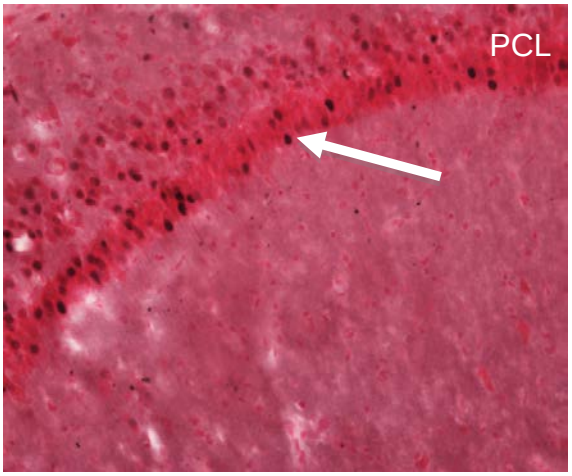


Figure 2. **Number of FosB/Δ FosB+ cells/mm^2 in DG of PD42 rodents across experimental conditions. No significant difference was found between postnatal treatment and housing conditions on FosB/Δ FosB expression (p>.05). AE= alcohol exposed, SI= sham intubated, SC= suckle control, SH= social housed, WR= wheel running.**

3.5 FosB/ΔFosB expression in CA1

The number of immuno-labeled FosB/ΔFosB+ cells were measured in seven 40 μm sections of the CA1. Representative images of CA1 in control animals between housing conditions (SH vs. WR) provide a visual qualitative difference and are shown below (Figure 3). A main effect of housing in CA1 was found using a two-way ANOVA ($F(1,35)=22.198$, $p<.001$), but no effect of postnatal treatment was found ($p>.05$). Overall, wheel running animals had more FosB/ΔFosB+ expression than social housed animals in all three postnatal conditions (Figure 4). Values for the number of FosB/ΔFosB+ cells from all six conditions are displayed in Table 3 (appendix B).

A.



B.

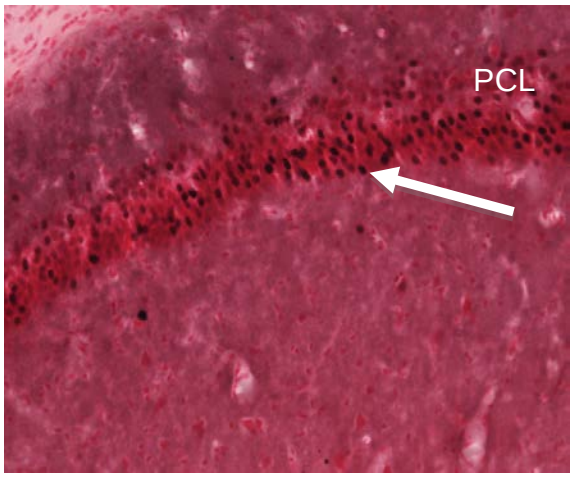


Figure 3. Qualitative differences between hippocampal FosB/ΔFosB expression in CA1 of SH and WR animals, respectively. FosB/ΔFosB+ cells are dark (arrows) in comparison to background. PCL is the pyramidal cell layer. (A.) SC/SH condition shows qualitatively less FosB/ΔFosB+ cells than (B.) SC/WR condition. Magnification at 200X.

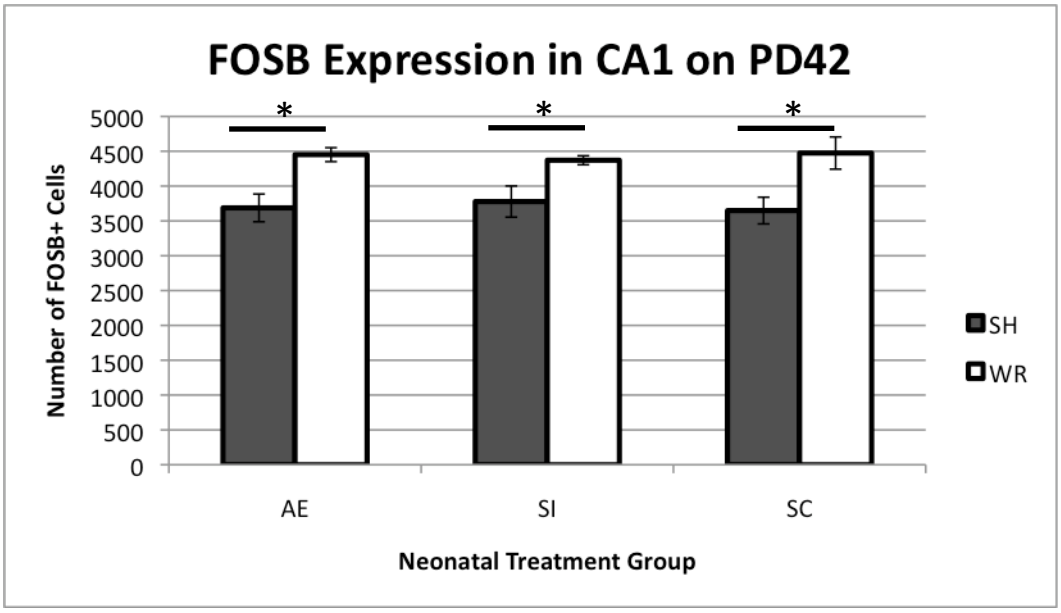


Figure 4. Number of FosB/Δ FosB+ cells/mm^2 in CA1 of PD42 rodents across experimental conditions. A significant difference of housing condition was found between all treatment groups. AE= alcohol exposed, SI= sham intubated, SC= suckle control, SH= social housed, WR= wheel running. * indicates p<.05.

Chapter 4

DISCUSSION

4.1 Effects of Third Trimester-Equivalent Alcohol Exposure on FosB/ΔFosB labeling

The present study found that neonatal alcohol exposure had no effect on FosB/ΔFosB expression in the hippocampal DG or CA1. Animals from all three treatment groups (AE, SI, SC) exhibited similar levels of FosB/ΔFosB expression within a specific area of hippocampus.

I expected to see reduced amount of FosB/ΔFosB+ labeling after neonatal alcohol exposure, as previous studies have shown a downregulation of other proteins that derive from the Fos gene family, such as c-Fos. c-Fos is present in cells in response to short-term activation. The Sim group (2008) reported decreased c-Fos expression in both the DG and CA1 subfields of the hippocampus of prenatally alcohol exposed rat pups at PD 22. Decreased c-Fos expression was then reversed after seven days of postnatal treadmill exercise at PD22 (Sim *et al.*, 2008). Other studies have also reported reductions of c-Fos and another activity-related IEG, Egr-1, in the hippocampus after acute and chronic alcohol exposure in rodents (Ryabinin *et al.*, 1998, Depaz *et al.*, 2000, Ryabinin *et al.*, 2003, Sharpe *et al.*, 2005).

Furthermore, a study done by Murawski and colleagues *(2012)*, showed that behavioral induction of c-Fos was significantly decreased in CA1 of alcohol exposed animals in response to a 5 minute exposure to a novel environment. In the same study, the decrease in c-Fos+ cells was accompanied by a significant reduction in the number of CA1 pyramidal cells (Murawski *et al.*, 2012).

Electrophysiology studies have supplemented and supported the role that neonatal alcohol exposure alters neuronal activity in the hippocampus. Because long-term potentiation (LTP) is a precursor to the formation of stable synapses, LTP is often used as a measure for synaptic plasticity. Although LTP studies have reported variable findings on the effects caused by alcohol, these differences arise from different alcohol administration protocols. Inhibition of LTP from *in vivo* recordings of CA1 (PD9) was reported in PD9 animals after a third trimester-equivalent alcohol exposure (Puglia *et al*., 2010). Puglia's third trimester-equivalent alcohol exposure (PD2-9) overlaps with our timing of exposure (PD4-9), and the BAC's achieved are similar to those previously published by our lab (Klintsova *et al.*, 2007, Helfer *et al*., 2009). Other studies have shown similar findings in response to acute alcohol exposure, which could be due to alcohol enhancing GABAergic-mediated hyperpolarization, thus leading to impaired LTP (Swartzwelder *et al*., 1995, Puglia *et al*., 2010). However, chronic alcohol exposure has shown no effect on LTP induction in CA1 neurons *in vivo*. Chronic alcohol exposure administration consisted of an ethanol-containing liquid diet everyday for 12 weeks (Fujii *et al.*, 2008). Given the

evidence, it is therefore reasonable to assume that LTP is inhibited after acute alcohol exposure.

The findings show no effect of alcohol exposure on FosB/ΔFosB labeling in either DG or CA1. Not many studies have looked at FosB/ΔFosB expression in response to alcohol exposure and it is possible that the protein is not as sensitive to alcohol's effects as c-Fos. A study by the Chen group reported that ΔFosB is far more stable and long-lasting than c-Fos (Chen *et al*., 1997).

4.2 Effects of Voluntary Physical Exercise on FosB/ΔFosB labeling in Neonatally Alcohol Exposed Animals

Wheel running animals had a significant increase of FosB/ΔFosB+ cells in CA1 as compared to the non-wheel runners. Surprisingly, twelve days of wheel running had no effect on the amount of FosB/ΔFosB+ granule cells in the DG. Voluntary physical exercise has been shown to induce the expression of various IEGs (Clark *et. al* 2011).

Wheel running was expected to increase FosB/ΔFosB expression in both CA1 and DG of the hippocampus. One study reported an increase in c-Fos, Egr-1 and Arc within newly generated hippocampal granule cells in response to voluntary wheel running (Clark *et al*., 2011). Thus, wheel running could be contributing to cell survival. Although the increase we saw in CA1 is consistent with previous findings, the amount of FosB/ΔFosB+ cells in the DG is in conflict with the previous study by

the Rhodes group. The discrepancy could be a result of differences in our approach in measuring FosB/ΔFosB expression. This study aimed to determine the relative FosB/ΔFosB expression of the entire hippocampal subfields, CA1 and DG, while the study by Clark (2011) looked for IEG expression in a more specific population of neurons, namely the newly born granule cells. Similar to Clark (2011), the Sim group (2008) reported suppression of c-Fos expression in both the DG and CA1 following prenatal alcohol exposure, but this effect was reversed when pups exercised on a treadmill for seven days.

Wheel running has a differential effect on the subfields of the hippocampus. FosB/ΔFosB expression increased in CA1 in response to WR, while there was no effect on the DG. One possible explanation is that there is a ceiling effect present within the DG, such that WR was not capable of further inducing FosB/ΔFosB expression as most of the neurons in DG already expressed this transcription factor. Another explanation is that differential activation could be reflective of the distinct physiological and functional roles of the cell make up within the subfields. CA1 consists of mostly pyramidal cells (PC's), while DG consists of granule cells (GC's). The literature, however, has minimal information on the differential effects between the two cell types in response to alcohol exposure and physical exercise.

In addition to wheel running, several conditions have been shown to increase IEG expression such as seizures. Animal seizure studies report exaggerated increases in c-Fos and FosB after amygdala kindling (Madsen et al., 2005). Because seizures

result in near maximal cell excitability, findings from epileptogenesis studies may be insightful in understanding of the differences in cell physiology between PCs and GCs. A study by the Lothman group (1995) reported hyperresponsiveness from *in vivo* slices in both CA1 and DG subfields in post-status epileptic rats as compared to controls. Hyperresponsiveness or hyperexcitability was defined as the production of three or more population spikes with maximal amplitude. Further investigation revealed that half of the CA1 tissue slices displayed a greater number of population spikes as compared to one-fifth of DG tissue (Rempe *et al*., 1995). The hyperexcitability of both subfields was attributed to an enhancement of glutamatergic excitation via NMDA-R activation (Lothman *et al*., 1995). A follow-up study showed CA1 to be more excitable than DG due to a decrease in GABAergic inhibition within CA1, but not in DG (Mangan *et al*., 1995). Similarly, a study found that LTP induction within the DG was not possible due to $GABA_A$ inhibiton. However, this effect was reversed when rodents were treated with bicuculline, a $GABA_A$ antagonist (Wang *et al*., 1999).

Another study reported differences in sodium currents during chronic activation between CA1 and DG in an animal model of post- status epilepticus. Influxes of sodium currents through neurons are responsible for depolarization and action potential formation, and thus are important for LTP induction and synaptic plasticity. Sodium currents were greater in CA1, while no change was revealed in DG as compared to non-epileptic control animals (Ketelaars et al., 2001). These findings

are consistent with the previous reported result that CA1 is more hyperexcitable in response to chronic activation to cells.

Furthermore, GC dendrites display strong voltage attenuation of incoming signals from the entorhinal cortex (Nevian et al., 2007), and the resting membrane potential is relatively hyperpolarized to pyramidal cells as can be seen by a greater voltage difference between resting and threshold potentials (Kress et al., 2008). Therefore, greater input is needed to generate an output from GCs. These studies suggest that wheel running might not have been sufficient to overcome the voltage attenuation caused by GCs, but was enough to increase activity in CA1 as supported by the significant increase in FosB/ΔFosB expression in response to WR.

Voluntary wheel running has many beneficial effects related to neuroplasticity besides increasing neuronal activation. The study by van Praag et al. (2005), reported enhanced learning in aged running mice in the Morris Water Maze, such that they had decreased latency and path length to reach the platform as compared to the old non-runners (van Praag *et al*., 2005). Data from this study showed that in addition to the cognitive benefits in the aged running mice, adult hippocampal neurogenesis was enhanced. There was a significant increase in the number of BrdU+ cells in the DG of aged runners, as compared to the aged non-runners. Significantly, more BrdU+ cells in DG matured into a neuronal phenotype as was evidenced by an increase in BrdU/NeuN+ double labeling in the running mice.

Furthermore, the beneficial effects of WR expand to other mechanisms responsible for proper brain development. A reduction in vascular endothelin growth

factor (VEGF), a marker of angiogenesis, and an upregulation of astrocyte and myelin formation were observed in middle aged sedentary mice. The aforementioned effects are all indicative of unhealthy brain aging. However, after six weeks of voluntary physical exercise during adulthood, the unhealthy brain aging effects were all reversed (Latimer *et al*., 2011). In another study, brain derived neurotrophic factor (BDNF) expression was significantly enhanced when prenatally alcohol exposed animals were allowed access to a wheel for 12 days (Boehme *et al*., 2011). Research has shown that BDNF is involved in synaptic plasticity, as rodents with a mutated BDNF gene show impaired synaptic transmission and plasticity (Pattwell *et al*., 2012).

4.3 A Potential Mechanism Through Which Alcohol Affects Cell Survival

One potential mechanism for the deficits seen in cell survival seen in previous studies (Klintsova *et al.*, 2007, Hamilton *et al*., 2012) is oxidative stress. Oxidative stress arises from the production of free radicals via metabolism and could be responsible for alcohol's affects (Ramachandran *et al*., 2003, Sun *et al*., 1997). The teratogenous effects of alcohol could be affecting the progenitor pool at the DNA level. Thus, replications made from progenitor cells carrying mutated DNA caused by alcohol could be responsible for the long-term down regulation seen in anti-oxidative enzymes. Studies have reported that oxidative stress markers such as lipid peroxidation are increased in the hippocampus of adult animals after neonatal alcohol exposure (Petkov *et al.*, 1997, Johnsen-Soriano *et al.*, 2007,). Also, decreases in

glutathione (GSH), an anti-oxidant, have been reported in the adult hippocampus after neonatal alcohol exposure (Brocardo *et al*., 2012).

However, the Christie group (2012) reports that voluntary physical exercise produces beneficial effects via increasing the production of anti-oxidants such as GSH. The previous study also showed that the beneficial effects of wheel running were concomitant with a decrease in anxiety-like behavior in FASD animals (Brocardo *et al.,*2012). Therefore, when taking the animals out of the wheel running condition and placing them in regular cages for 30 days, it is possible that anti-oxidant production decreases and cells become incapable of dealing with free radical production. This could eventually be responsible, in part, for the decrease in cell survival after 12 days of wheel running followed by 30 days of relative inactivity (Klintsova *et al*., 2007). Future studies should look at the effects of oxidative stress on cellular survival.

4.4 Conclusion

The goal of this study was to determine if neuronal integration was affected by neonatal alcohol exposure, and if so could voluntary physical exercise be used a therapeutic strategy to reverse alcohol's effects. Alcohol exposure during fetal development was expected to down regulate neuronal activity as measured by FosB/ΔFosB expression, however, no such effect was found. Thus it cannot be concluded that alcohol exposure decreases cell survival by decreasing cellular activation. Furthermore, physical exercise induced differential activation of the two

subfields, DG and CA1 such that exercise increased FosB/ΔFosB expression in CA1, but not in DG. This differential effect could be due to the differences in the role and function of the two subfields. By using the analysis of FosB/ΔFosB expression we did not distinguish between newly added and "old", existing cells in DG. Thus, additional study is needed to determine if AE affects expression of these transcription factors in the subpopulation of adult-generated neurons.

Future work will include a FosB/ΔFosB expression analysis of CA3 to gain an understanding of the chronic activation profile in the trisynaptic circuit. Preliminary data suggests a similar FosB/ΔFosB expression profile as CA1. This would not be surprising as CA3 consists of pyramidal cells just as CA1 does. In addition, it would be interesting to look at other IEG expression, such as c-Fos and Arc, across the subfields of the hippocampus and compare them to the results found in this study.

Finally, information gathered from this and future studies will allow greater understanding of alcohol's effects on the developing brain. Further research can be tailored to targeting the problem of synaptic plasticity and towards providing new therapeutic interventions for the cognitive behavioral deficits seen in children with FASD.

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Appendix A

Average number (± SEM) of total FosB/ΔFosB+ cells in the Dentate Gyrus of the hippocampus at PD42 across all conditions.

Postnatal Treatment	SH (cells/mm^2)	WR (cells/mm^2)
AE	7138.88 ± 330.74	7636.60 ± 225.58
SI	7269.81 ± 332.45	7269.81 ± 379.41
SC	7429.75 ± 301.58	7522.17 ± 139.59

Appendix B

Average number (± SEM) of total FosB/ΔFosB+ cells in the CA1 of the hippocampus at PD42 across all conditions.

	Housing Condition	
Postnatal Treatment	SH (cells/mm^2)	WR (cells/mm^2)
AE	3687.16 ± 199.52	4451.19 ± 100.26
SI	3777.62 ± 223.35	4371.23 ± 64.67
SC	3648.49 ± 191.34	4473.61 ± 231.33