IMPACT OF BINGE-LIKE ALCOHOL EXPOSURE DURING THE THIRD TRIMESTER EQUIVALENT ON CELL CYCLE KINETICS OF PROGENITOR CELLS IN THE DENTATE GYRUS OF PD10 RATS

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Neuroscience

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ABSTRACT

The subgranular zone (SGZ) of the dentate gyrus (DG) is one of two areas of the brain where neurons are generated into adulthood; the other is the subventricular zone. This unique cell population undergoes significant growth during the brain growth spurt (a postnatal event in rodents) and continues to develop throughout the lifespan. Several studies have examined the impact of neonatal alcohol exposure (AE) on postnatal neurogenesis in the developing DG. For example, a significant decrease in the number of granule cells was observed on PD10 in rats that received a daily binge-like dose of alcohol during the third trimester equivalent (PD4-9) or the equivalent of entire human gestation (G1-20 + PD4-9) (1). Furthermore, the impact of developmental AE on these precursors and their progeny is long lasting. Previous work in our lab demonstrated that binge-like AE on PD4-9 did not affect cell proliferation on PD42 or PD50 (2; 3) but decreased the number of adult-born granule cells that matured and survived for 30 days (3). Observed changes in postnatal neurogenesis could possibly reflect an enduring impact of neonatal AE on the progenitors' cell cycle. Indeed, several studies have demonstrated that alcohol alters cell cycle kinetics. For example, a high dose of alcohol *in vitro* (400 mg/dl) lengthened the cell cycle and increased the incidence of cell death in neocortical cell cultures obtained from rodent fetuses on G16 (4). Prenatal AE lengthened the cell cycle of proliferating cells in the ventricular zone due to an elongation of the G1 phase, while exposure did not impact cell cycle length of cells proliferating in the subventricular zone (5). The current study examined the impact of binge-like AE

during the third trimester equivalent on the cell cycle kinetics of neural precursors (NPCs) in the SGZ of DG of the rodent hippocampus. Rat pups were randomly assigned to three groups on PD4: intubated with alcohol (5.25 g/kg/day; AE), sham-intubated (SI), or suckle control (SC). Following sacrifice on PD10, cumulative labeling with bromodeoxyuridine (BrdU) was performed. Future work will examine the impact of developmental alcohol exposure on two other cell populations, Sox2+ and Ki67+ cells. Then, the total length of the cell cycle and the S phase can be calculated. This study was undertaken to establish if a change in duration of granule cell precursors' cell cycle at PD10 could be a cellular mechanism leading to alterations in new neuron survival.

Chapter 1

INTRODUCTION

1.1 Fetal Alcohol Exposure

According to the American Academy of Pediatrics Committee on Substance Abuse and Committee on Children with Disabilities, exposure to alcohol *in utero* is a leading cause of birth defects and mental retardation (49). The Surgeon General released a public health advisory in 1981 regarding alcohol use by pregnant women as well as those who could become pregnant and reissued this warning in 2004. In addition, Congress mandated in 1989 that warning labels be put on all products containing alcohol (6). Despite the known dangers and the fact that deficits caused by alcohol consumption during pregnancy are 100% preventable, a recent report from the CDC found that 1 in 13 pregnant women drank within a specified 30-day period. Furthermore, 1.4% of these women engaged in binge drinking, which amounted to approximately 6 drinks at a time on three or more occasions within the time surveyed (50). Clearly, maternal consumption of alcohol is a widespread phenomenon with serious consequences. Therefore, the study of the effects of alcohol exposure *in utero* as well as possible therapies for alcohol-induced deficits is important.

Fetal Alcohol Syndrome (FAS) is the most severe manifestation of alcohol exposure during gestation. It is estimated to occur in 2-7 of 1,000 live births in typical, mixed-racial, mixed-socioeconomic US populations (7). Children with FAS often exhibit growth deficiencies, neurobehavioral effects, and a distinct pattern of craniofacial abnormalities, including a smooth philtrum, thin upper lip, short nose, and

flattened midface (8). While diagnostic criteria have been outlined for FAS (6), other cases of prenatal alcohol exposure (PAE) often go undiagnosed due to the absence of characteristic facial features, comorbidity with other clinical conditions, and severity of exposure and symptoms (9). Misdiagnosis can have severe repercussions on a child's future, because early diagnosis is critical to ensuring the best outcome for the child. Fetal Alcohol Spectrum Disorders (FASD) is a non-diagnostic term that encompasses the wide range of physical, cognitive, and behavioral deficits that can result from alcohol exposure *in utero*. Diagnoses that fall under the umbrella term FASD include FAS, alcohol-related birth defects (ARBD), and alcohol-related neurodevelopment disorder (ARND) (6). In several Western European countries and the United States, as many as 2-5% of children are born with an FASD each year (7).

The detrimental effects caused by PAE depend on the time, duration, and amount of exposure during gestation. For example, the amount of maternal alcohol consumption was correlated with decreases in frontal lobe size in the developing child (10). One of the most common findings in MRI studies of PAE children is reduced brain volume. The corpus callosum appears to be highly susceptible to alcohol exposure, and deep gray matter structures, including the hippocampus, consistently exhibit volume reductions (reviewed in 11). A recent longitudinal study found that youth who were heavily exposed to alcohol *in utero* (more than 4 drinks per occasion at least once per week or more than 13 drinks per week) had a different trajectory of brain development, especially in posterior brain regions. While unexposed subjects exhibited an increase in cortical volume and a comparable loss in volume during adolescence due to pruning, AE subjects experienced a steady decrease in volume over time, which the authors suggest signifies impaired cortical plasticity. Overall, children

who exhibited a more quadratic trajectory of brain growth and plasticity (i.e., initial cortical volume increases followed by synaptic pruning and myelination, which manifested as volume reduction on MRI scans) within posterior cortices processed information more efficiently. Furthermore, smaller volume changes between scans were correlated with lower intelligence (IQ) and worse facial morphology in both groups; interestingly, the opposite was true for the frontal cortex (12).

Patients with an FASD often exhibit cognitive, learning, and behavioral deficits. The mean IQ of children diagnosed with FAS is in the low 70s, as compared to the average/normal IQ range of 90-109 (see 13 for review). Furthermore, seven-year-old children who had been exposed to a "moderate" amount of alcohol during pregnancy (two or more drinks per day) exhibited a 7-point decrease in IQ, and children born to mothers who drank more than 5 drinks per occasion ("binge" pattern) had impaired learning (14). PAE children exhibit deficits in tasks requiring cognitive flexibility, inhibition, some measures of verbal fluency, abstract thinking, deductive reasoning, hypothesis testing, problem solving, and concept formation (15). In addition, PAE children are at a higher risk for childhood psychiatric disorders, especially attention-deficit/hyperactivity disorder (ADHD) (16). Furthermore, adolescents with FASD do not perform as well in school, have difficulty developing normal relationships with peers, and are more prone to engage in substance abuse (17). Thus, the effects and behavioral manifestations of alcohol exposure *in utero* are considerable and long lasting.

Recently, researchers have begun to correlate neurobiological changes caused by developmental alcohol exposure to cognitive and behavioral deficits. For example, Willoughby et al. uncovered an association between impaired performance on select

memory indices and reductions in left hippocampal volume in children with FASDs (18). In a study of young African-American adults, "dysmorphic" subjects (those who had documented PAE and exhibited a relatively high number of physical characteristics associated with PAE in adulthood) performed significantly worse on several memory tests than control subjects and had significant decreases in total brain volume and regional volumes, namely in the hippocampus. Furthermore, an effect was found of dysmporphic status on memory that was mediated by hippocampal volume, suggesting a direct connection between alcohol-induced neurobiological deficits and memory that lasts into the second decade of life (19).

1.2 Animal Model of Developmental Alcohol Exposure

During development, all species experience a period known as the brain growth spurt, during which time there is a transient and rapid growth of the brain. Beginning at midgestation in human development, the brain growth spurt is characterized by proliferation of glial cells, myelination, increased dendritic complexity, and synaptogenesis (20). This period coincides with the first ten days of postnatal life in rodents (Figure 1). Consequently, this period of rodent development serves as a good model for examining the effects of alcohol exposure on the developing brain.

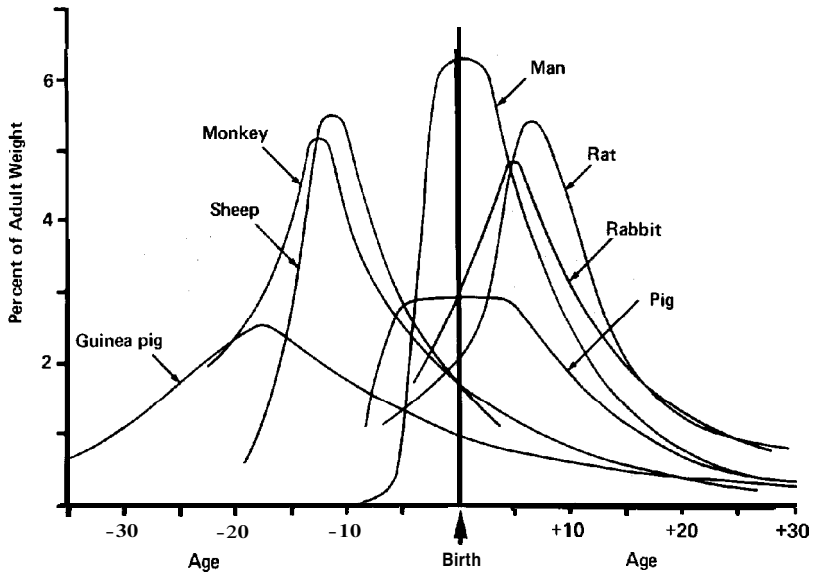


Figure 1: Brain Growth Spurt in seven mammalian species. Units of time are represented in months for humans and days for rats. The brain growth spurt begins during the third trimester of pregnancy in human development, while it occurs during the first two postnatal weeks in rodents. Image obtained from Dobbing and Sands, 1979.

Furthermore, this time period is crucial in terms of hippocampal development. In rodents, cells migrate from the primary germinative zone, located in the lateral ventricles, to the secondary matrix in the subventricular region. This secondary matrix then splits. In the first migration wave, cells form the outer granule cell layer, while a second migration forms the tertiary dentate matrix (which later becomes the hilus), which is highly active during PD3-10. The inner shell of the granule cell layer is formed from this matrix and, in turn, so is the greatest number of total granule cells. By PD20-30, this matrix becomes confined to the SGZ of the DG, and this region becomes the source of newly generated granule cells in adult animals (21; 22).

1.3 Alcohol and the Hippocampus

The SGZ of the DG is one of two areas of the brain (the other being the subventricular zone, SVZ) where neurons are generated for the duration of the life span from neural progenitor cells (NPCs). For the most part, NPCs differentiate into granule cells and integrate into the preexisting circuitry, but some instead become astrocytes. In fact, the majority of granule cells in the DG (85-90%) is generated postnatally and contributes to the hippocampal structure (23). An increasing amount of evidence indicates that these granule cells are essential for at least some forms of hippocampal-dependent learning and memory (reviewed in 24). For example, one study found that rodents injected for two weeks with methylazoxymethanol acetate (MAM), an antimitotic agent that was shown to reduce the number of proliferating cells in the hippocampus, performed poorly on a trace fear-conditioning task. However, MAM did not affect contextual fear conditioning or exploration of a novel environment (25).

Alcohol is both an NMDA receptor antagonist and a GABAa receptor agonist, and studies that examine the distinct impact of these two main effects of alcohol have also demonstrated negative outcomes following developmental exposure. For example, following treatment with the NMDA antagonist, MK-801, or GABAa agonist, phenobarbital, on PD6 and PD8, the number of cells positive for bromodeoxyuridine (BrdU+), a thymidine analog that marks cell proliferation, was reduced on PD9 in several "higher" areas of the brain, including the SGZ and the granule cell layer of the DG. In addition, administration of either of these two drugs on PD6, PD8, and PD10 caused a decrease in the number of cells positive for doublecortin (DCX+), BrdU+, and DCX+/BrdU+ on PD15. Together, these data

suggest that developmental exposure of these two drugs reduced the number of newly generated neurons in the DG (26).

Numerous studies have examined the impact of PAE on the hippocampus. For example, a significant decrease in the number of granule cells was observed on PD10 in rats that received a binge-like dose of alcohol during the third trimester equivalent (PD4-9) or for the equivalent of human gestation (G1-20 and PD4-9) (1). In addition, a single binge-like dose of alcohol on PD7 preferentially impacted the pool of neural stem/progenitor cells in mice hippocampi; this was determined by evaluating neurospheres and demonstrating co-localization of GFAP and nestin with caspase-3, a marker for apoptosis (27). Furthermore, the impact of developmental alcohol exposure on NPCs and their progeny is long lasting. For example, Ieraci and Herrera also found that a binge-like exposure to alcohol during early postnatal life decreased cell proliferation in 4-month-old mice (27). In a study by Miller, rats that achieved a high BAC during the dosing period (PD4-12) had a decreased volume of the DG and fewer granule cells on PD30-35 (1995). Previous work in the Klintsova lab demonstrated that binge-like alcohol exposure on PD4-9 did not affect cytogenesis between PD30 and PD50 but decreased the number of newly generated granule cells that matured and "survived" on PD50 and PD80 (3).

Animals exposed to alcohol prenatally, postnatally, and perinatally exhibit behavioral deficits on tasks such as the Morris water maze, the radial arm test, and T-mazes (see 28 for review). For example, binge-like exposure to alcohol on PD4-9 or PD7-9 impaired place learning in the Morris water maze task on PD26-31. In addition, rodents that received a higher dose (5.25g/kg/day) on PD7-9 had a longer escape latency compared with controls and animals exposed to a lower dose of alcohol

(4.5g/kg/day). However, both alcohol-exposed groups showed significantly impaired performance on a probe task (29). Stefovska et al. demonstrated that at 6 months of age, rats that had been exposed to phenobarbital during the first 2 weeks of postnatal life did not perform as well on the water maze task and had reduced numerical densities in the granule cell layer (26). In addition, administration of isoflurane, an anesthetic which purportedly acts on GABA and NMDA receptors, on PD7 decreased cell proliferation in the rodent hippocampus up to four days after administration and also resulted in impaired performance on the water maze task and fear conditioning several months after exposure (30).

1.4 The Cell Cycle

In the first twelve days of postnatal life, the cell cycle length of dividing cells in the rodent DG is between 15.1 and 17.7 hours, with the G1 phase ranging from 1.1 to 2.4 hours (31). During corticogenesis, cell cycle length of cortical precursors increases due to a lengthening of the G1 phase. This lengthening has been correlated with the transition from proliferation of stem cells to neurogenesis (reviewed in 32). In 5-week-old male rats, it was determined that the length of the cell cycle of proliferating cells in the DG is 24.7 hours, with the S phase constituting 9.5 hours of that time frame and G1 roughly 11 hours (33).

The G1 phase is an important stage of the cell cycle. It is during the time, for example, that there is a "checkpoint" that commits the cell to completing a full cycle. Thus, the decision between quiescence, cell division, and cell cycle exit is made at this time (34). Lengthening of the cell cycle during corticogenesis has been correlated with a transition from proliferation of NPCs to neurogenesis. While traditionally it was assumed that this alteration in the G1 phase was a consequence of differentiation, it

has also been suggested that it is in fact the lengthening that provides the impetus for neurogenesis. Furthermore, recent evidence supports a "cell cycle length hypothesis," which postulates that the ability of cell fate determinants to act on a progenitor is dependent on the temporal window of opportunity. More specifically, the length of G1 has been implicated as an important factor, such that a slow progression of G1 leads to consumption of the precursor pool while fast progression expands the pool. Accordingly, Salomoni and Calegari argue that instead of the traditional definition of neurogenesis as the "process by which NPCs exit the cell cycle and become postmitotic," it should instead be regarded as a decision by the mother cell (32). In other words, cell cycle exit is not causal to neurogenesis, but rather the cycling mother determines the fate of its daughter cells.

Cell cycle progression is regulated by cyclin-dependent kinases. Activation of cdk2, for example, pushes the cell from the G1 to S phase and initiates DNA replication in the S phase. Cyclins bind to and activate kinases at various stages of the cell cycle. In addition, cdk inhibitors slow the cell cycle and mediate entry into quiescence, a decision that is made during the G1 phase (32). Disruption of these various regulators can have profound effects both on the cell cycle and neurogenesis. For example, downregulation of cdk4/cyclinD1 by RNA interference lengthened the G1 phase and caused a shift from proliferation to differentiation of NPCs in the developing mouse brain (35).

1.5 Alcohol and the Cell Cycle

Several studies have demonstrated that alcohol alters cell cycle kinetics; however these effects differ greatly depending on the time of administration, the dose, and the region examined. For example, using an adolescent model of binge-like

exposure to alcohol, McClain et al. reported an acceleration of the cell cycle of NPCs in the DG due to a shortening of the S phase (36). In contrast, a high dose of alcohol *in vitro* (400 mg/dl) lengthened the cell cycle and increased the incidence of cell death in neocortical cell cultures (obtained from rodent fetuses on G16) harvested 1-4 days after plating (4). Similarly, prenatal administration of alcohol resulted in a lengthening of the cell cycle of proliferating cells in the ventricular zone due to an alteration of the G1 phase, while exposure did not impact cell cycle length of cells proliferating in the subventricular zone (5). Miller and Kuhn found that alcohol administration during gestation (G6-G22) did not alter the total length of the cell cycle of cortical neurons in rodents over time (37). However, since the cell cycle typically lengthens during this time frame in rodents, the cell cycle of neurons of alcohol-exposed rats was actually longer than controls at earlier time points but comparable to controls on G19 and 21. This change was attributed to the differential effect of alcohol on the ventricular and subventricular zones. Alcohol administration during the most proliferative period of the ventricular zone (G12-15) resulted in a decrease in cells labeled with BrdU, but an increase in these cells was observed when alcohol was administered later (G18-21) when the subventricular zone is more active (38). This outcome accounts for the initial lengthening of the cell cycle followed by a return to baseline found in the aforementioned study. Since the length of the S phase of the cell cycle of cortical precursors was unaffected by prenatal alcohol exposure (37), and other studies suggest that the G2 and M phases are also not impacted by alcohol (5; 39), it was hypothesized that prenatal exposure to alcohol impacted the G1 phase of cycling cells (37). In fact, according to Luo and Miller, "the G1-phase of the cell cycle is most susceptible to the action of ethanol" (40).

The effect of alcohol on the cell cycle may at least in part be caused by alterations to cyclin-dependent kinases and other regulators. For example, exposure of cerebellar granule progenitors to a high dose of ethanol in culture downregulated Cyclin A—an activator of cdk2—and CyclinD2—which aids in the transition from G1 to S. In turn, the expression and activity of cdk2 was suppressed, resulting in a lengthening of the S phase and total cell cycle (41). A recent study of cultures of dorsal root ganglion precursors obtained from E14 mice found a dose-dependent relationship between ethanol concentration and increased expression of CyclinD1, D2, and E2F1, all three of which are vital G1 to S phase regulators. This resulted in premature induction of DNA replication and apoptosis (43).

1.6 Alcohol and NPC Migration and Differentiation

Alcohol has been shown to alter later stages of neurogenesis, namely migration and differentiation. For example, a single "high" dose of ethanol on PD10 (3g/kg) that increased proliferation of neurons in the DG also increased the number of immature postmitotic neurons without altering the number of mature granule cells. The investigators proposed that these neurons might have an increased risk of ectopic migration, formation of aberrant connections, and enhanced excitation, conditions which had previously been correlated with increases in immature postmitotic neurons following ischemic damage and seizures (43). Furthermore, postnatally-generated cells that were heavily labeled with H3 thymidine on PD12 (in other words, cells that did not divide more than once) were distributed throughout the granule cell layer in animals exposed to alcohol on PD4-12. In contrast, labeled cells remained within the internal half of the granule cells layer in control animals, suggesting aberrant maturation of newly-generated cells following exposure to alcohol (23). Cultures of

hippocampal NPCs obtained from adult rats who had been exposed to alcohol during gestation expressed a greater number of glial markers than NPCs from normal rats. In addition, NPCs from PAE animals contained fewer cells positive for βIItubulin, which induces differentiation of NPCs into immature neurons, and synapsinIII, which regulates progression of neural cells. Overall, these results suggest that developmental alcohol exposure delayed differentiation of NPCs (44). Alcohol exposure has also been shown to alter the cell fate of neural stem cells and progenitor cells. Using neural stem cells (NSCs), neural-committed precursor cells (NCPs), and glial-committed precursor cells (GPCs) obtained from second trimester human fetuses, it was demonstrated that exposure to alcohol increased expression of neuronal marker map2a in NSC and GCP. In addition, neurosphere size was increased for NCPs, which the researchers suggest could be due to abnormal adhesion. This, in turn, could impact migration of these cells (45).

1.7 Purpose and Rationale

This goal of this project is to assess the short-term impact of neonatal alcohol exposure (PD4-9) on the cell cycle kinetics of postnatally generated granule cells in the DG of the hippocampus. We hypothesize that following a binge-like exposure to alcohol on PD4-9, the duration of NPC cell cycle will be lengthened when examined at PD10, likely due to a change in the G1 phase. This thesis will focus on the effect of developmental alcohol exposure on cells labeled with the thymidine analog Bromodeoxyuridine (BrdU), which incorporates into the DNA during the S phase of the cell cycle. Examination of this cell population will provide insight into the immediate impact of alcohol on dividing NPCs in the hippocampus and will ultimately enable us to calculate the length of both the S phase and total cell cycle.

As previously mentioned, work from our lab has shown that proliferation during PD30-50 was unaffected by binge-like exposure to alcohol on PD4-9, but the maturation and survival of newly generated neurons was negatively impacted when examined on PD50 and 80 (3). The outcome of this study posed an important question: what could impair the survival of these neurons without affecting cytogenesis? During neurogenesis, there are two critical periods of survival: one occurs when neural progeny become neuroblasts and the second when immature neurons become integrated into the preexisting network (reviewed in 46). Thus, the observed decrease in survival could be due to a long-lasting impact on one or both of these stages.

An increase in the length of the cell cycle of neural progenitors was proposed as a possible explanation for the observed decrease in survival and could have an impact on one or both of the two critical periods. An alteration in the cell cycle shortly following alcohol exposure, which we expect to observe in the present study, could continue into adolescence. Furthermore, lengthening of the G1 phase specifically could have a long-term impact on adult neurogenesis by fostering neurogenesis over proliferation of the precursor pool and generating postmitotic cells that either have impaired migration or differentiation.

Chapter 2

METHODS

2.1 Animals

The subjects of the study were male Long Evans pups of animals bred at the University of Delaware breeding colony. The pregnant dams were given access to food ad libitum for the duration of the study. The housing facility was maintained on a 12:12-hr light-dark cycle, with lights on at 0900hr.

2.2 Alcohol Exposure

13 litters were culled to eight pups—six males and two females—on PD3. Pups were randomly assigned to one of three dosing conditions (see description below) and were paw-marked with India ink for identification purposes. Between PD4-PD9, alcohol exposed (AE) animals received several intragastric intubations two hours apart. The first two intubations of each day delivered a combined dose of 5.25g/kg of ethanol in milk formula (11.9% v/v ethanol). A third intubation of milk without ethanol was given two hours after the last alcohol dose. On the first day of intubations (PD4), a fourth intubation of milk without ethanol was administered four hours after the last alcohol dose to compensate for reduced milk intake by AE pups. Sham - intubated (SI) pups received intragastric intubations without milk or ethanol to control for the stress of the intubation procedure. A second control group, called suckle control (SC), was left undisturbed. The animals were weighed daily during the dosing period to ensure proper development. Blood samples were collected from the

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

2.3 Cumulative Labeling with BrdU

The protocol for determining the cell cycle kinetics of NPC cells in the DG was modeled off of that used by McClain et al. in an adolescent model of binge-like exposure to alcohol (36). Cumulative labeling with BrdU, a method first described by Nowakowski et al. (47), was performed to calculate the length of the S phase. On PD10, pups from all three treatment groups were injected with BrdU (50 mg/kg) at the onset of the light cycle. One third of the pups were sacrificed 2 hours after the first injection. The remaining animals received a second injection of BrdU at this same time point (i.e. two hours after the initial injection). One third of these animals were sacrificed 2 hours later. Finally, the last third of the animals were injected four hours after the first injection and sacrificed 2 hours later (Figure 2).

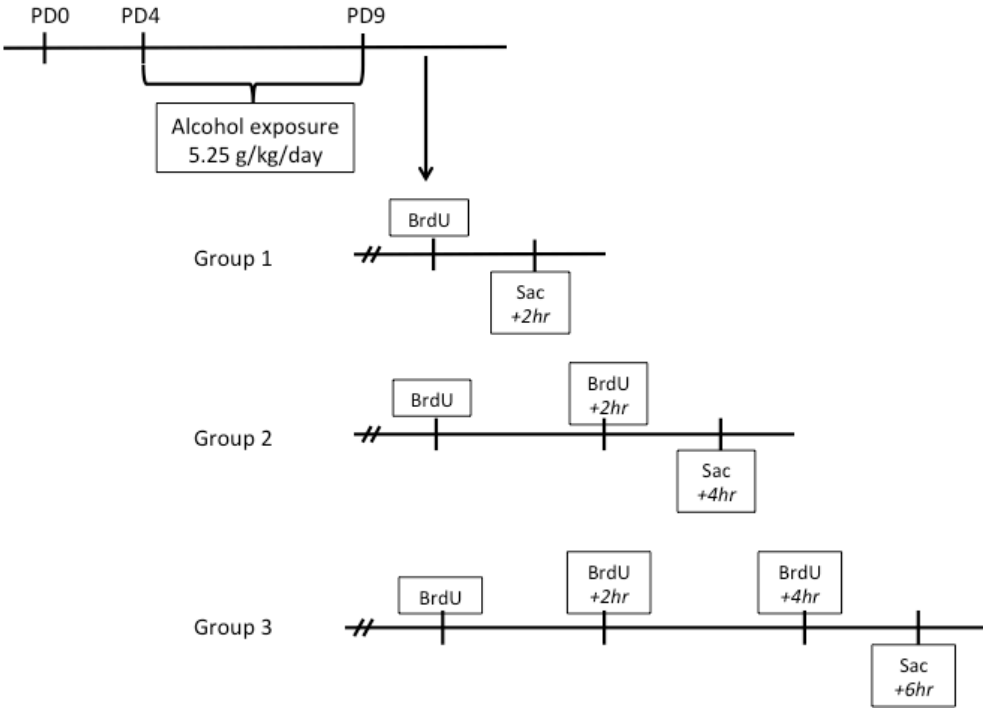


Figure 2: Alcohol and cumulative BrdU administration timelines

2.4 Cell Cycle Length Calculation

Future work in conjunction with the findings of the current study will examine the impact of developmental alcohol exposure on the length of the S phase and total cell cycle. Staining for Ki67, an endogenous protein that exists in cells that are actively dividing (all stages of the cell cycle except G0), as well as Sox-2, which labels all NPC cells, will be performed. The ratio of Ki67 to Sox-2 labeled cells is known as the growth fraction. Similarly, the S-phase fraction (also known as the BrdU labeling index) is the number of BrdU labeled cells divided by the size of the precursor pool. Over time, this value approaches the growth fraction as all of the dividing cells in the pool eventually pass through the S phase and incorporate BrdU. The total length of the cell cycle as well as the length of the S phase can be calculated

using these two fractions (Figure 4). In addition, a Hoescht stain will be performed to evaluate ethanol-induced neurodegeneration in the DG.

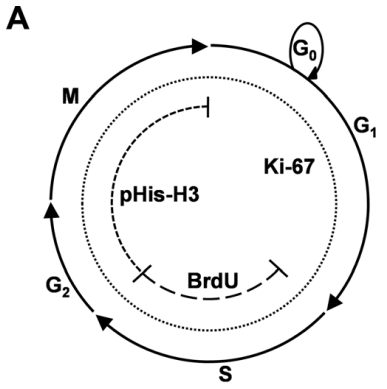


Figure 3: Cell cycle stages and their immunohistochemical markers. Image obtained from McClain et al., 2011.

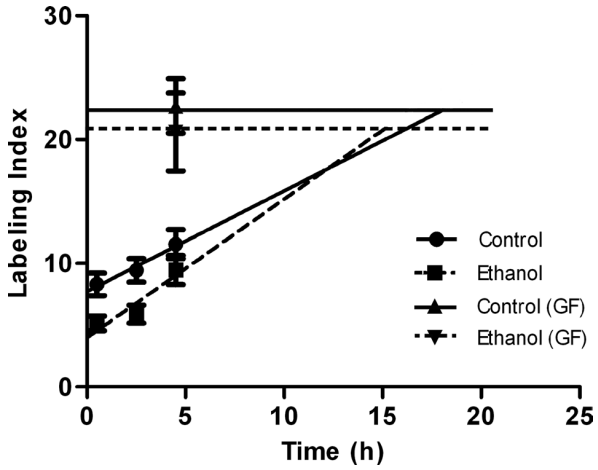


Figure 4: Example of the BrdU labeling index versus the growth fraction. Over time, as the number of BrdU injections increases, the BrdU labeling index (BrdU+ cells / Sox-2+ cells) approaches the value of the growth fraction (Ki67+ cells / Sox-2+ cells). These two values can be used to calculate total cell cycle length. In this instance, adolescent binge-like alcohol exposure significantly decreased the BrdU labeling index, indicating a shortened S phase. Image obtained from McClain et al., 2011.

2.5 Immunohistochemistry

On PD10, animals were transcardially perfused with heparinized .1M phosphate buffer (PBS) and 4% paraformaldehyde in PBS (pH 7.2). Following overnight storage in paraformaldehyde, brains were transferred to a solution of 30% sucrose in paraformaldehyde. Brains were sectioned using a cryostat at 20 μm and then stored in cryoprotectant at -20C. Sections containing the dorsal hippocampus were identified, and a random systematic fraction of these sections (1:12) was selected for analysis and mounted onto subbed slides. Slides were placed in a desiccator for at least 48 hours to ensure that the sections adhered to the slide.

2.5.1 BrdU

To visualize BrdU+ cells by DAB immunocytochemistry, slides were rinsed in 1X TBS and then incubated in .6% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. Slides were washed again in TBS, followed by incubation in a solution of 50% formamide in 2X concentrated saline-sodium citrate buffer (SSC) for 2 hours at 65C. After being rinsed in 2X SSC, sections were incubated in 2N HCl for 30 min at 37C. Sections were then neutralized in 0.1M boric acid and washed in TBS. Following 1-hour exposure to blocking solution (3% normal goat serum and .05% Triton X in TBS), the sections were incubated with primary antibody (rat anti-BrdU, 1:1000; OBT0030 [Accurate Chemical, Westbury, NY]). A PAP pen was used to draw a hydrophobic circle around the sections, and then drops of the antibody in blocking solution were placed on the sections. Slides were kept in a humid environment for 72 hours at 4 degrees Celsius. To stop antigen-antibody conjugation, the slides were rinsed in 1X TBS. To increase the signal, sections were then incubated in secondary antibody (goat anti-rat; 1:500; biotinylated antibody,

Vector) for two hours. Following two 1X TBS washes and a rinse in washing solution (3% goat serum in TBS), avidin–biotin-peroxidase complex (made in washing solution) was applied to the slides. One hour later, slides were rinsed again in 1X TBS. Localization of the binding sites was visualized with nickel-enhanced diaminobenzidine (DAB). Slides were counterstained with Pyronin Y and coverslipped with DPX mounting solution the following day.

2.5.2 Sox-2

Sox-2 is a transcription factor that helps maintain pluripotency; thus, it labels multipotent neural progenitor cells (36). For Sox-2 detection, slides were similarly washed in TBS and incubated in .6% hydrogen peroxide for 30 minutes. Following three additional 1X TBS washes, tissue was blocked in a solution of 3% normal goat serum and .1% triton X in TBS for 30 minutes. A PAP pen was used to draw a hydrophobic circle around the sections, and then drops of primary antibody (rabbit anti-Sox-2; 1:500; AB5603 [Millipore]) in blocking solution were placed on the sections. Slides were kept in a humid environment for 48 hours. Following a TBS rinse, sections were incubated in secondary antibody (goat anti-rabbit; 1:500; biotinylated antibody, Vector) for one hour. Then, the slides were rinsed with TBS followed by washing solution (3% goat serum in TBS). Similar to BrdU, avidin–biotin-peroxidase complex (made in washing solution) was applied to the slides, allowed to incubate for one hour at room temperature, and then rinsed with 1X TBS. Localization of the binding sites was visualized with nickel-enhanced DAB. The following day, sections were dehydrated in ethanol and Histoclear and then coverslipped with DPX.

2.5.3 Ki67

Ki67 is an intrinsic marker that labels cells in all active phases of the cell cycle (i.e. G1, S, G2, and M). The first day of staining is similar to that described for BrdU above. Using a PAP pen, drops of the primary antibody in blocking solution (3% normal goat serum and .5% Triton X in TBS) were placed on the sections (mouse anti-Ki67; 1:200; NCL-Ki67-MM1 [Novacastra Laboratories]). Slides were incubated for 48 hours. On the second day of staining, sections were washed twice with TBS and then with blocking solution. Then, slides were incubated with the secondary antibody (goat anti-mouse; 1:1000; biotinylated antibody, Vector) for 1 hour. Following rinses with TBS and washing solution, avidin–biotin-peroxidase complex (made in washing solution) was applied to the slides, allowed to incubate for one hour at room temperature, and then rinsed with 1X TBS. As previously described for BrdU and Sox-2, localization of the binding sites was visualized with nickel-enhanced diaminobenzidine (DAB). Slides were counterstained with Pyronin Y and coverslipped with DPX mounting solution the following day.

2.6 Stereological Counting

Light microscopy was used to identify BrdU+ cells. Slides were coded so that the experimenter was blind to the animal's condition. The optical fractionator probe of the Stereoinvestigator program was used to perform unbiased stereology on sections containing dorsal hippocampus. Previous work upon which this study is based has focused on changes in the dorsal hippocampus (Helfer et al., 2007; McClain et al., 2010). Within the dorsal hippocampus, 5 sequential sections were analyzed per animal. If a section in the series had a poor stain, a torn DG, or was missing, then the missing section was accounted for. Only one missing section was allowed for an

animal, and if more than one section had to be excluded, the animal could not be included in the analysis. The DG for each section was traced at 100x and analyzed at 400x. Sections were analyzed with 100 x 100µm counting frames. The grid size was set for the same dimensions. 2µm guard zones were implemented, and the dissector volume was set to 7µm. Section thickness was recorded at every site where a count was made.

2.7 Data Analysis

Using SPSS Statistics, the influence of postnatal treatment on body weights was determined using a repeated-measures ANOVA with postnatal condition (AE, SI, SC) as the between subjects factor and postnatal day as a within subjects factor, followed by post hoc analysis (Tukey's test). 69 male rats were used in this study (17 AE, 27 SI, and 25 SC). 11 animals did not survive the intubation procedure. For the preliminary data presented for BrdU immunolabeling, one to two animals per postnatal condition were analyzed for each of the three BrdU injection time points: i.e. animals that were sacrificed after receiving one, two, or three BrdU injections.

Chapter 3

RESULTS

3.1 Body Weights and Blood Alcohol Concentration (BAC)

Animal weights were recorded every day during alcohol administration (PD4 and 9) and at the time of sacrifice (PD10). A repeated measures ANOVA comparing PD4 and PD9 weights revealed a day x postnatal treatment interaction [$F_{2,69}$=21.616, $p < .001$] and a main effect of day [$F_{1,69}$=1503.503, $p < .001$]. A univariate ANOVA of PD4 weights determined that there were no significant differences between groups ($p = .116$). The same type of analysis on PD9 weights demonstrated a significant main effect of postnatal treatment [$F_{2,69}$=12.476, $p < .001$]. Post hoc tests demonstrated that AE animals weighed significantly less than both SI ($p < .001$) and SC ($p < .001$), suggesting a powerful effect of alcohol. On the day of sacrifice (PD10), a main effect of alcohol was similarly found [$F_{2,69}$=12.476, $p < .001$], such that AE pups weighed significantly less than SI ($p = .001$) and SC ($p < .001$) animals (Table 2).

Blood alcohol Concentration (BAC) was measured 1.5 hours after the second alcohol dose on PD4, the first day of alcohol exposure, by blood taken from the tail vein. The average BAC for AE animals was 388.37 mg/dl.

Table 1: Average body weights +/-SEM in grams. ** Indicates significance ($p < .001$)

	PD4	PD9	PD10
AE	10.835 ±.154	17.253 ±.463**	18.876 ±.593**
SI	10.977 ±.169	20.737 ±.414	22.607 ±.479
SC	11.56 ±.349	21.092 ±.687	23.216 ±.699

3.2 Cumulative BrdU Immunolabeling

As previously described, pups from all three postnatal treatment groups were sacrificed on PD10 after having received one, two, or three injections with BrdU administered two hours apart. This was done in order to assess the length of the S-phase of the cell cycle. Preliminary data (n = 2 per group) suggests that the number of BrdU+ cells increased over time as the animals received a greater number of injections, an outcome that was expected and is crucial for future calculation of cell cycle length (Figure 5, 6, and 7). However, there did not appear to be any differences between postnatal treatment groups at any of the time points. This suggests that alcohol does not impact the rate of proliferation shortly following repeated exposure to alcohol (Figures 5 and 6). During counting, measured volume of the DG was calculated for each animal that was analyzed. Comparison of the average measured volume for each of the three treatment groups (including animals from all three injection time points) indicates that AE animals exhibited decreased volume of the DG (Figure 8).

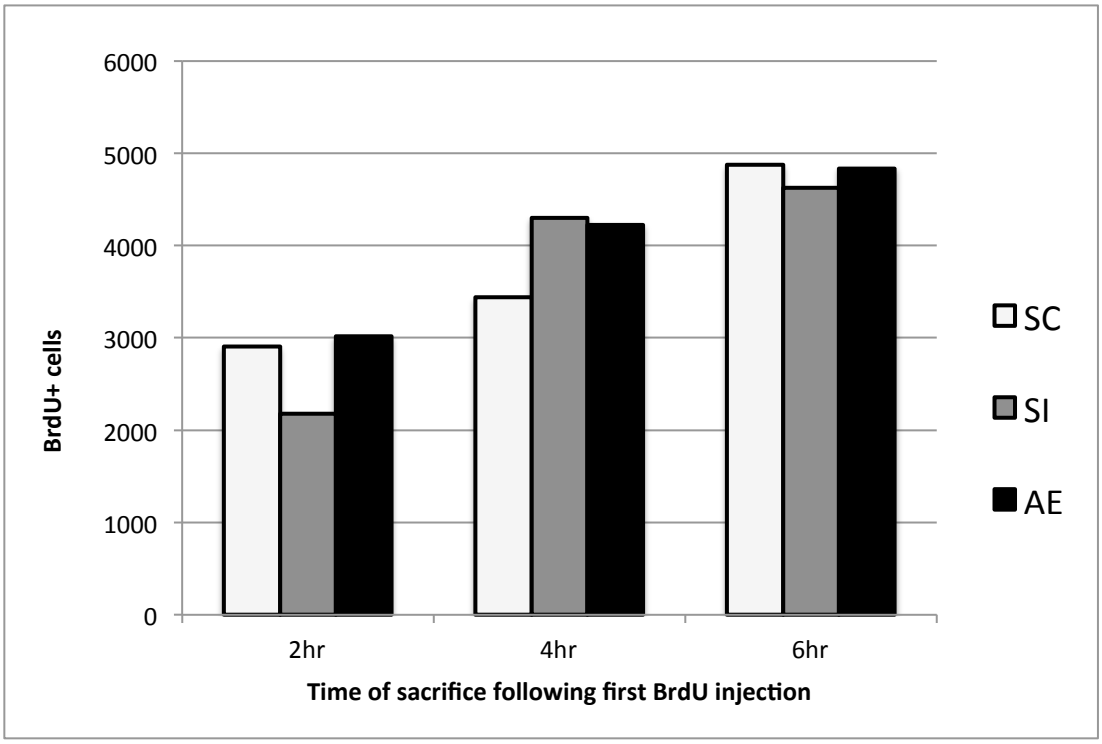


Figure 5: Cumulative immunolabeling with BrdU on PD10. While the number of cells increases over time, there appear to be no differences as a result of postnatal treatment.

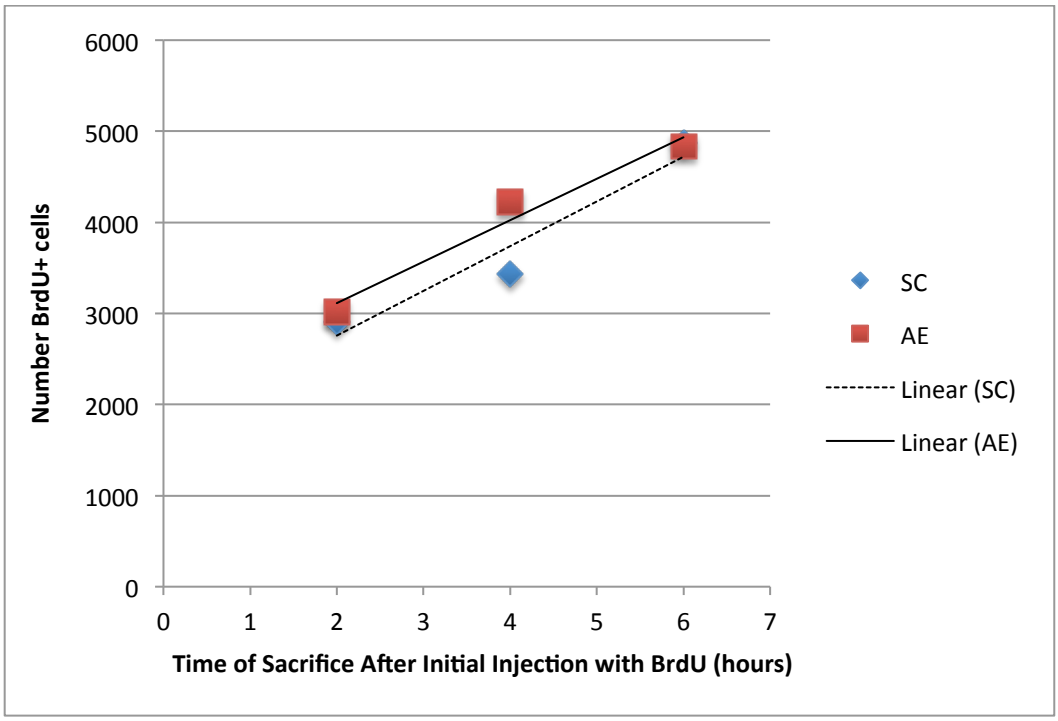
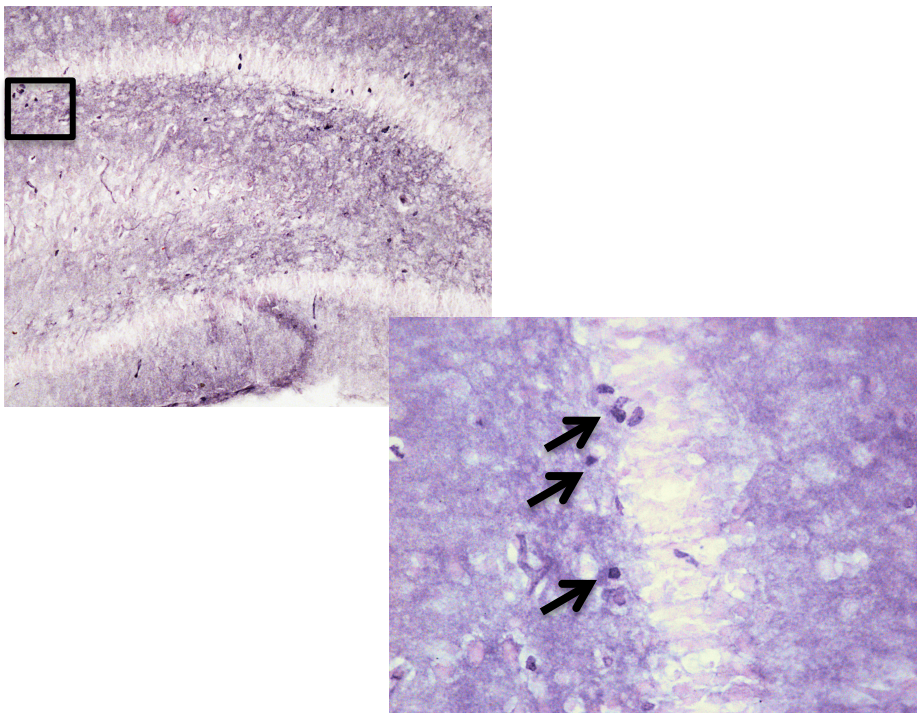
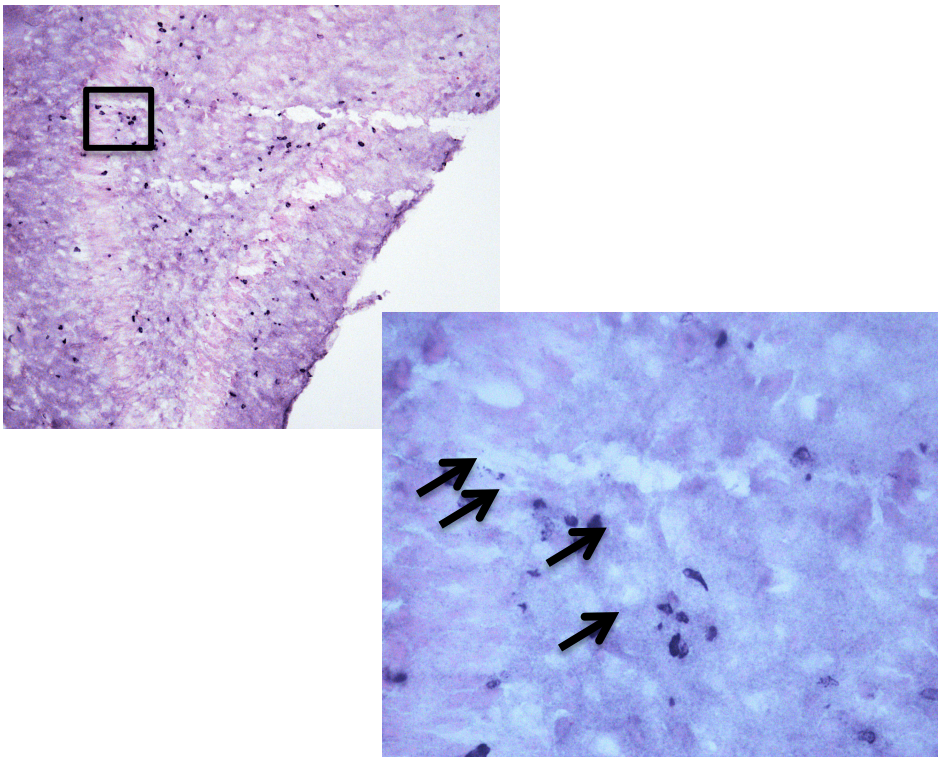


Figure 6: Comparison of trendlines for number of BrdU+ cells in SC versus AE animals.

A



B



C

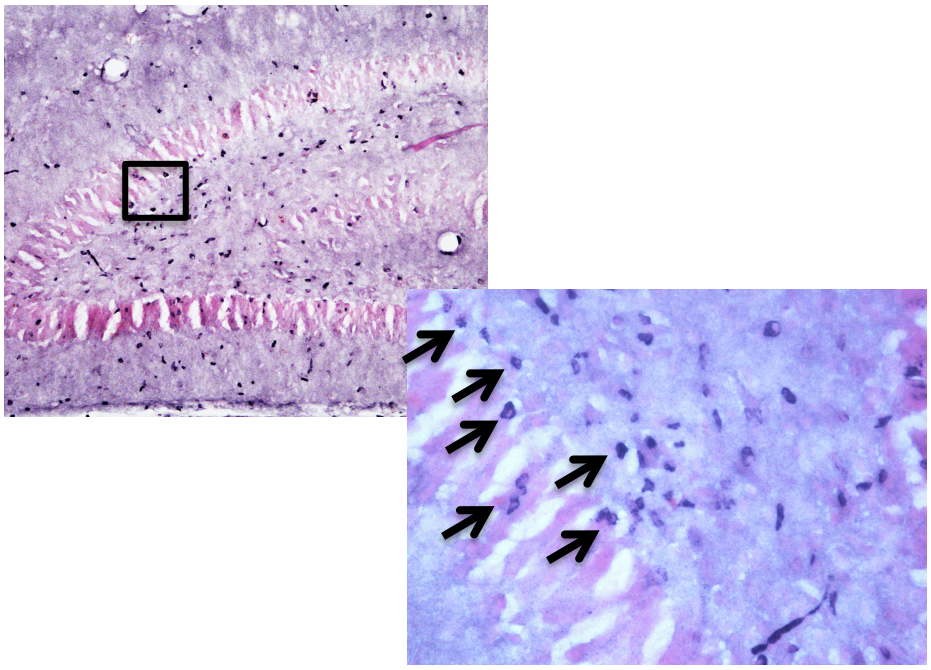


Figure 7: Comparison of BrdU labeling following one (A), two (B), and three (C) injections. It is evident that more cells are labeled as the number of injections increases. All images obtained from AE animals with 10x (left) and 40x (right) objectives. Arrows indicate examples of BrdU+ cells that were counted.

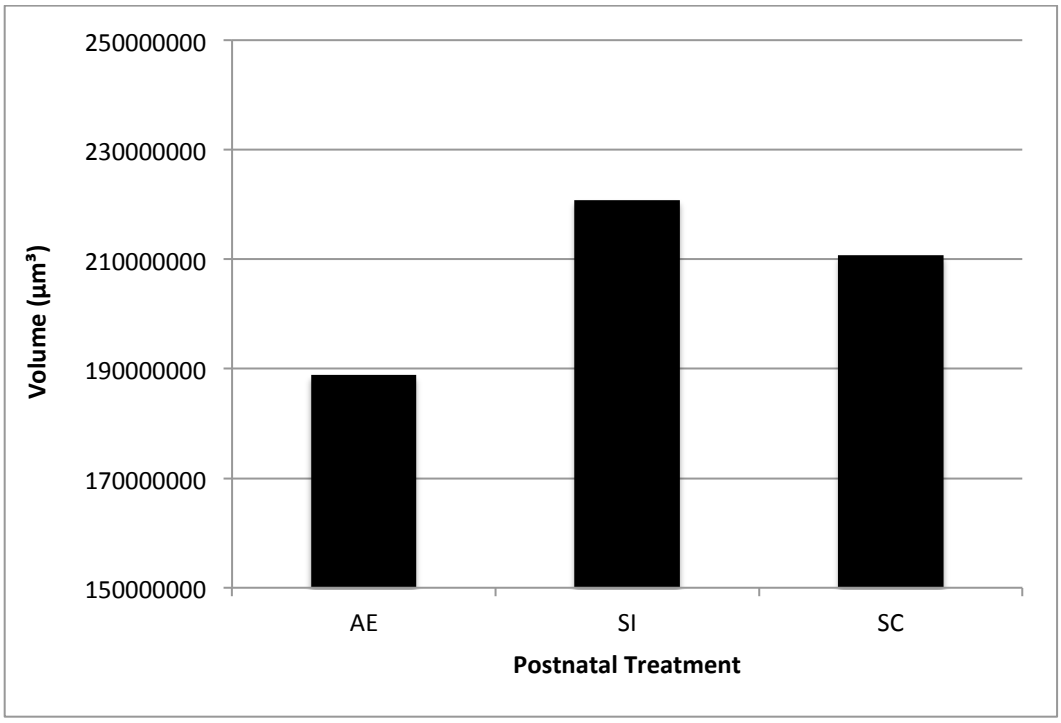


Figure 8: Average measured volume of DG for analyzed sections. AE animals appear to have decreased hippocampal volume relative to SI and SC animals. Averages include animals from all three injection time points.

3.3 Sox-2 Immunolabeling

Immunolabeling for Sox-2 is in progress (Figure 9). Labeling this cell population is essential for calculation of the growth fraction (Ki67+ cells / Sox-2+ cells) and BrdU labeling index (BrdU+ cells / Sox-2+ cells), both of which can be used to assess the total cell cycle length.

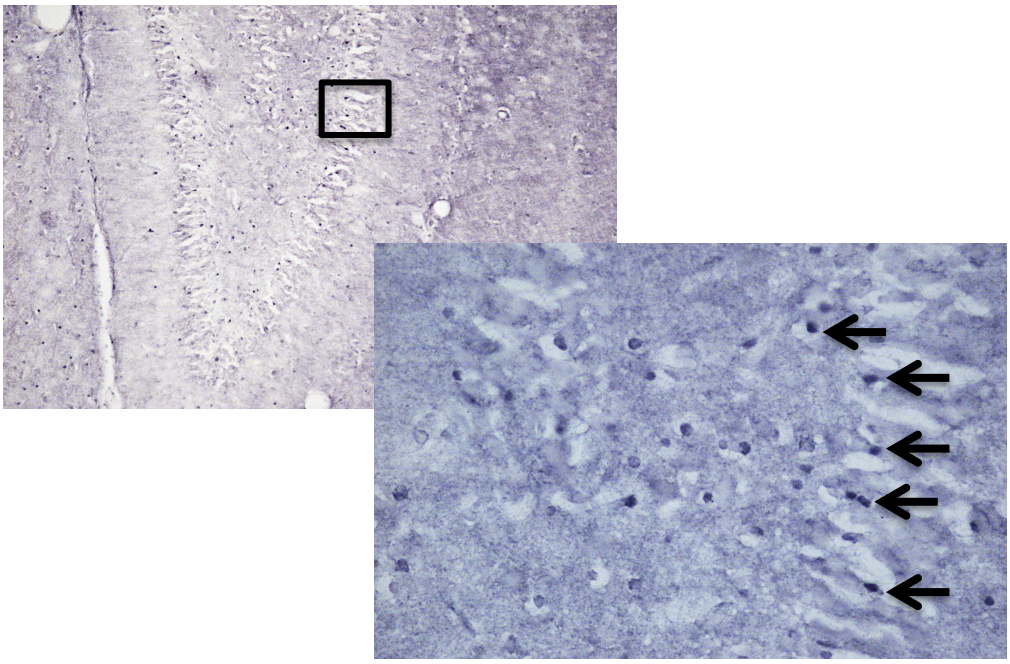


Figure 9: Sox-2 immunolabeling. Images obtained with 10x and 40x objectives. Arrows indicate examples of Sox-2+ cells.

Chapter 4

DISCUSSION

The purpose of this thesis was to assess the rate of proliferation of postnatally generated granule cells in the DG of the hippocampus shortly following binge-like exposure to alcohol on PD4-9. This work will serve as a part of a larger study that will examine the effect of developmental alcohol exposure on cell cycle kinetics in this unique cell population. We hypothesize that following a binge-like exposure to alcohol on PD4-9, the duration of NPC cell cycle will be lengthened when examined at PD10, likely due to a change in the G1 phase.

4.1 Present Findings

Our preliminary data indicate that a greater number of cells are labeled with BrdU with the passage of time as the number of injections increases. This outcome was expected for the cumulative BrdU labeling method and is essential for future calculations of cell cycle and S phase length. The BrdU labeling index should steadily increase and ultimately intersect graphically with the growth fraction, as this point of intersection determines total cell cycle length (Figure 4). Binge-like alcohol exposure on PD4-9 did not impact the generation of BrdU+ cells. An alteration in the number of BrdU+ cells would have suggested that the S phase of the cell cycle was being affected by alcohol exposure, since this endogenous marker labels cells that are specifically in the S-phase. Thus, the observed outcome supports the hypothesis that the G1 phase will be most greatly impacted by neonatal alcohol exposure. Future staining with Sox-2 and Ki67 as well as the addition of more animals to the current study will enable the assessment of total cell cycle and S phase length.

4.2 Qualitative Observations

The observed increase in the number of labeled cells across the time points is evident even at low magnification (see Figure 7), and this high level of proliferation is much greater than that seen in adult animals (see Figure 10A and C). PD10 marks the end of a highly active period in hippocampal development: the second dentate migration. During PD5-10, a conspicuous increase in the depth of the granule cell layer occurs, especially in the later developing external limb, due to proliferation in the tertiary (intrinsic) dentate matrix (Figure 11; 22). As previously mentioned, the cell cycle is shorter early in development and lengthens over time, which also contributes to the higher proliferation rates observed (reviewed in 32). Furthermore, BrdU+ cells at this earlier time point looked much different than those labeled in adulthood. Chromatin aggregation and spindles were evident in these cells (Figure 10D), while adult cells displayed a more characteristic round shape and even impregnation with BrdU (10B). Due to the higher proliferation rates in the DG early in development, there is a greater likelihood that "mitotic figures," cells that are undergoing division, will be labeled and visualized during analysis. The observed differences in immunolabeling required us to modify our criteria for defining what constituted a BrdU+ cell prior to analysis, as previous work from our lab primarily examined adult tissue. This effect was exacerbated by slight differences in strength of immunolabeling that can occur in different staining batches.

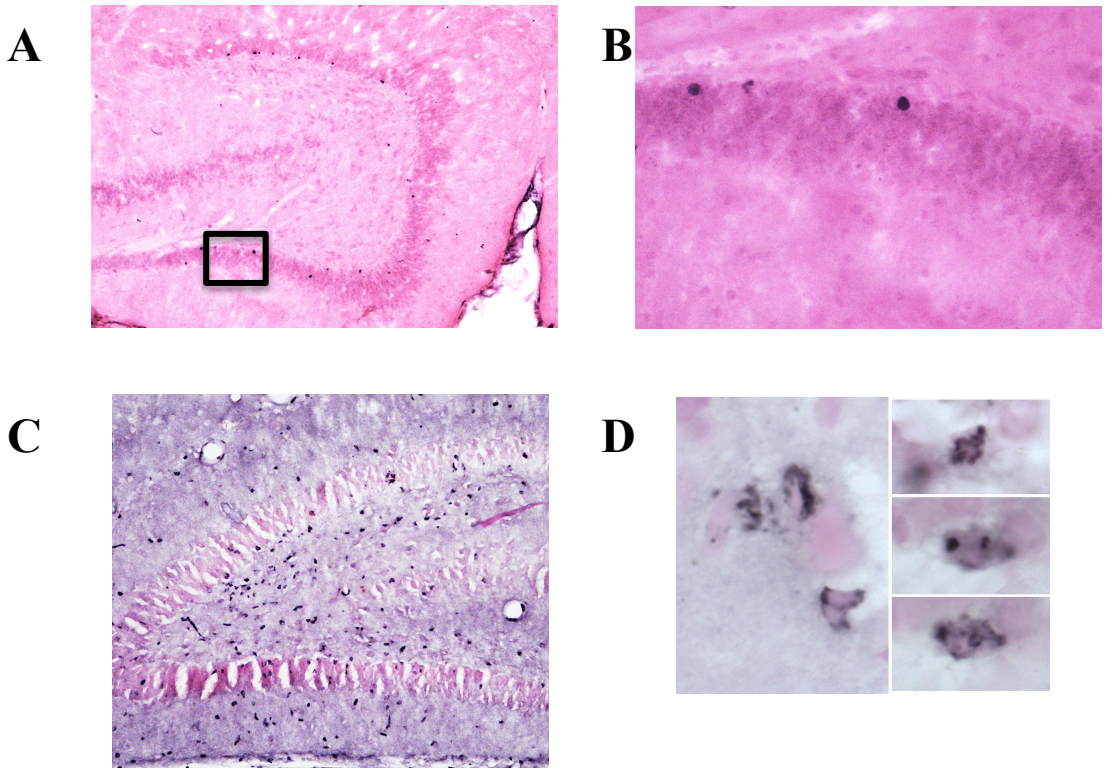


Figure 10: BrdU immunolabeling in the adult brain (A and B) versus PD10 (C and D). A) Horizontal section of the DG obtained from an animal that received a single, saturating dose of BrdU on PD42 and was sacrificed on PD72. Image obtained with a 10x objective. B) Image taken with a 40x objective. C) Coronal section of the DG obtained from an animal that received three injections and was sacrificed on PD10. Image obtained with a 10x objective. D) Montage of BrdU+ cells visualized with a 100x objective. Images taken by Dr. Anna Klintsova.

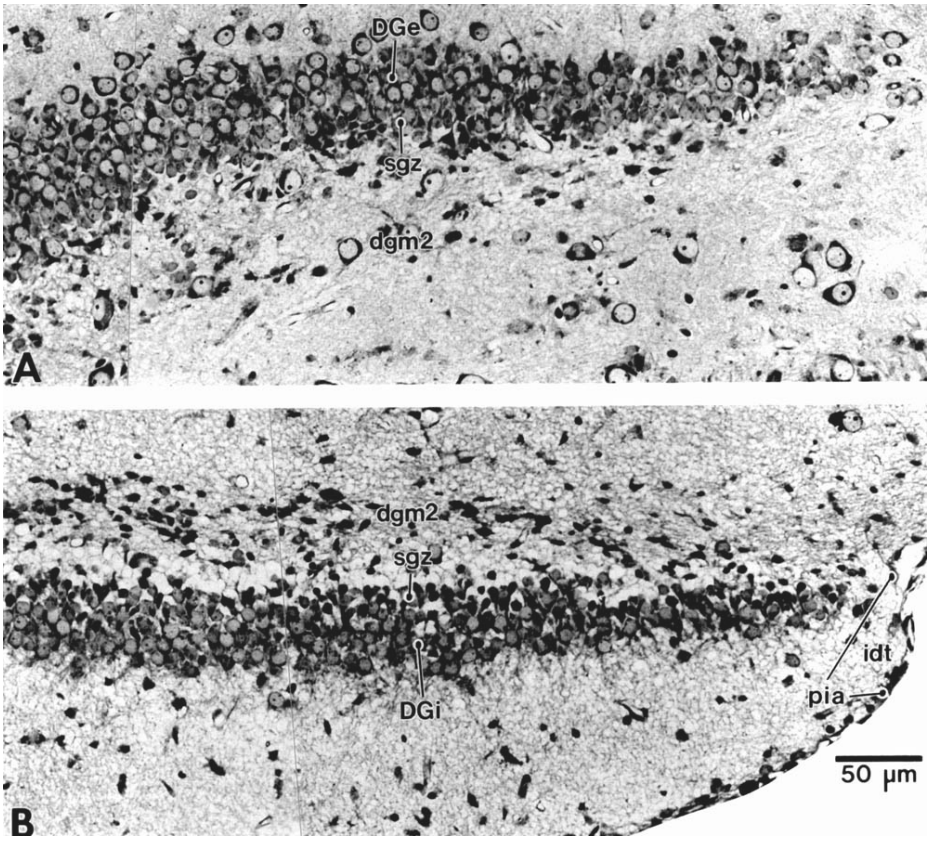


Figure 11: Sagittal section of the external and internal limbs (A and B, respectively) of the DG from PD10 rat that received a subcutaneous injected with [H3] thymidine. Darkly-stained, smaller cells represent "primitive" cells, which differentiate into the more characteristic round, lighter staining, larger granule cells. Image obtained from Altman and Bayer, 1990.

4.3 Present Findings in the Context of Current Literature

To the author's knowledge, the ongoing project will be the first to examine the impact of developmental alcohol exposure on cell cycle kinetics of hippocampal progenitor cells in the early postnatal rodent brain. However, evidence from studies examining different brain regions as well as *in vitro* work suggests that alcohol impacts the cell cycle. For example, prenatal administration of alcohol resulted in a lengthening of the cell cycle of proliferating cells in the ventricular zone due to an

alteration of the G1 phase (5). Cultures of granule precursor cells obtained from the cerebellum of PD3 rats exposed to ethanol exhibited a 135% increase in the length of the cell cycle due to a lengthening of the S phase (41). Moreover, relatively few studies have examined the impact of developmental alcohol exposure on proliferation in the short term as assessed by BrdU immunolabeling. In a study of female rats, Uban and colleagues (48) used a model of prenatal alcohol exposure to examine the density of BrdU+ labeled cells in the DG 24 hours (proliferation) and 3 weeks (survival and phenotyping) after BrdU injection on PD10. While proliferation and survival remained unchanged, the proportion of new neurons and glial cells was decreased, which the authors attributed to delayed differentiation of newly generated cells (48). The findings of the aforementioned study not only supports the preliminary findings of this thesis, which was that postnatal alcohol exposure did not impact proliferation in the short term; but it also supports our hypothesis that an alteration in the cell cycle due to alcohol exposure could persist and, by fostering neurogenesis over expansion of the proliferative pool, generate postmitotic cells that either have impaired migration or differentiation.

4.4 Conclusions

Overall, it was found that alcohol exposure on PD4-9 did not impact the generation of BrdU+ NPCs in the DG of the hippocampus on PD10. This data as well as that from upcoming stains of Sox-2 and Ki67 will allow for the assessment of the short-term impact of neonatal alcohol exposure on the cell cycle kinetics of these NPCs. It is hoped that this project will provide insight as to why neuronal progenitors in the DG are impaired in their ability to mature and survive when assessed during adulthood, a finding that we have observed previously (3). Future work should

determine whether an alteration of the cell cycle in this cell population persists into adolescence and adulthood, and should uncover the mechanism by which alcohol alters the speed of cell cycle progression.

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Appendix

IACUC APPROVED ANIMAL PROTOCOL

University of Delaware
Institutional Animal Care and Use Committee
Annual Review

(Please complete below using Arial, size 12 Font.)

Title of Protocol: Therapeutic Motor Training and Fetal Alcohol Effects	
AUP Number: 1134-2013-2	← **(4 digits only)**
Principal Investigator: Dr. Anna Y. Klintsova	
Common Name: Rat, Long-Evans **Genus Species:** Rattus norvegiccus	

Pain Category: ***(please mark one)***

USDA PAIN CATEGORY: ***(Note change of categories from previous form)***

Category	Description
☐ B	Breeding or holding where NO research is conducted
☐ C	Procedure involving momentary or no pain or distress
X D	Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)
☐ E	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

Official Use Only

IACUC Approval Signature: _____

Date of Approval: 7/1/2012

Principal Investigator Assurance

1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.
2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).
3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.
4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist listed on this AUP. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.
5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.
6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.
7. I assure that any modifications to the protocol will be submitted to the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.
8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.
9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.
10. I assure that the experimental design has been developed with **consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.**
11. I assure that the proposed research does not unnecessarily duplicate previous experiments. *(Teaching Protocols Exempt)*
12. I understand that by signing, I agree to these assurances. Signature of Principal Investigator — Date: May 23, 2012

SIGNATURE(S) OF ALL PERSONS LISTED ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only the procedures that have been approved by the IACUC.

Name	Signature
1. Anna Klintsova	A Klintsova
2. Gillian Hamilton	Gill F Hamilton
3. Karen Boschen	Karen Boschen
4. Kerry Criss	Kerry J Criss
5. Elizabeth Hetterly Will sign in August as soon as she is back	
6. James Delorme will sign in August as soon as ~~he~~ she is back	
7. Mia Castiglione	Mia Castiglione
8. Shannon Houlihan will sign in August as soon as she is back	
9. Samuel Modin will sign in August as soon as he is back	
10. Brielle Gerry will sign in August as soon as she is back	
11.	
12.	
13.	
14.	
15.	

IACUC approval of animal protocols must be renewed on an annual basis.

1. **Previous Approval Date:** July 11, 2011

   Is Funding Source the same as on original, approved AUP?

   X Yes ☐ **No**

   If no, please state Funding Source and Award Number:

2. **Record of Animal Use:**

Common Name	Genus Species	Total Number Previously Approved	Number Used To Date
1. Rat (Long Evans)	Rattus norvegicus	360	292
2.			
3.			
4.			
5.			

3. **Protocol Status:** *(Please indicate by check mark the status of project.)*

   Request for Protocol Continuance:

   X A. Active: Project ongoing

   ☐ B. Currently inactive: Project was initiated but is presently inactive

   ☐ C. Inactive: Project never initiated but anticipated starting date is:

   Request for Protocol Termination:

   ☐ D. Inactive: Project never initiated

   ☐ E. Completed: No further activities with animals will be done.

4. **Project Personnel:** Have there been any personnel changes since the last IACUC approval? X Yes ☐ No

   If Yes, fill out the Amendment to Add/Delete Personnel form to "Add" Personnel.

Project Personnel Deletions:

Name	Effective Date
1. Alejandro Morales	June 1, 2012
2.	

5. **Progress Report:** If the status of this project is 3.A or 3.B, please provide a brief update on the progress made in achieving the aims of the protocol. We have nearly completed animal generation for the two year ARRA-funded project. The data collection (tissue immunostaining and quantification) is in its final stage to be completed, three papers were published, one more is in preparation. However we experienced some problems with tissue processing after perfusions that led to partically unsuccessful immunohistochemical staining. Thus we need to add more animals to experimental groups: we need to generate 8 more litters, 8 pups in each litter (64 total animals). These pups will be divided between 3 postnatal treatments as described in the initial protocol: alcohol-exposed between PD4-9, sham-intubated and suckle controls. At PD30 rats will be assigned either to wheel-running or social cage environment. After 12 days of exercise rats will be transferred to environmental enrichment for 30 days, then anesthetized and perfused.

   The project is in the final stage of preparation for a competitive renewal grant submission.

6. **Problems or Adverse Effects:** If the status of this project is 3.A or 3.B, please describe any unanticipated adverse events, morbidity, or mortality, the cause if known, and how these problems were resolved. If there were none, this should be indicated.

   No unanticipated adverse events in terms of animal welfare happened.