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ETHANOL DECREASES EXPRESSION OF DEVELOPING NEURONS, BUT NOT NEURONAL VIABILITY, IN A CULTURED RAT HIPPOCAMPUS

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THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
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By

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ABSTRACT OF THESIS

ETHANOL DECREASES EXPRESSION OF DEVELOPING NEURONS, BUT NOT NEURONAL VIABILITY, IN A CULTURED RAT HIPPOCAMPUS

As of 2019, alcohol use disorders (AUDs) affect roughly 14.1 million individuals over the age of 18 and cost up to $249 billion in economic burden in the United States. In addition to central nervous system (CNS), those affected by AUDs can present with cognitive and behavioral abnormalities. Specifically, those affected by AUDs experience deficits in memory consolidation and retrieval as well as executive functioning, which may be due to cellular changes within the hippocampus. There are at least two prominent and contended theories which explain the mechanism of functional impairment caused by alcohol: 1) alcohol induces excitotoxic neuronal death in the hippocampus and/or 2) alcohol diminishes neurogenesis, reducing production and proliferation of new neuronal cells. Determining the relative involvement of neuronal death and neuronal production in alcohol-induced hippocampal dysfunction is necessary to inform potential therapeutic targets aiming to restore cognitive deficits caused by alcohol exposure. The purpose of this experiment is to examine the effects of continuous alcohol exposure and explore the relative contributions of each of these processes in the conformational changes in the hippocampus after chronic alcohol exposure and establish organotypic hippocampal slice culture as a viable method to continue exploring these changes.

KEYWORDS: Chronic Alcohol Use, Organotypic Hippocampal Slice Culture, Neuronal Viability

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CHAPTER 1. INTRODUCTION

1.1 Alcohol Use Disorders

Alcohol Use Disorder (AUD) is a broad term used to describe alcohol misuse and uncontrolled alcohol drinking as characterized in the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition. AUD is a spectral disorder in which the severity is determined by the number of defined criteria of problematic drinking behavior met. In the United States alone, an estimated 14.5 million people over the age of 12 meet AUD criteria, according to the National Survey on Drug Use and Health (SAMHSA, 2019).

Alcohol misuse and disordered drinking contribute significantly to the disease burden in both the patient and those close to the patient. Each year, about 95,000 deaths are contributed to alcohol-related causes such as injuries, digestive diseases, cardiovascular diseases, infectious diseases, and cancers (SAMHSA, 2019; World Health Organization, 2014). Additionally, alcohol misuse ranked as the 7th leading cause of premature and preventable deaths and disabilities (Griswold et al., 2018). About 10.5% of children aged seventeen and younger live with a parent with an AUD, leading to consequences such as parental abuse or neglect and fewer household resources (Lipari and Van Horn, 2017). Economic burdens are also prevalent as alcohol misuse cost the United States $249 billion in 2010, 75% of which was due to binge drinking (Sacks et al. 2015).

1.1.1 Drinking Patterns

The definition of binge drinking has evolved and differs between societies, yet its definition remains contentious. One of the first classifications of binge drinking was defined by Jellinek (1952) as a chronic state of intoxication, in which intoxication or heavy drinking lasts for days or even weeks. However, the current classification of binge drinking

has changed quite a bit, partially due to the drinking habits exhibited by college students (Weschler and Nelson, 2001). Currently, binge drinking is defined by the National Institute of Alcohol Abuse and Alcoholism (NIAAA) as a pattern of drinking in which the blood alcohol concentration (BAC) of an individual reaches or exceeds .08 g/dL. This is about the equivalent of four drinks for women or five drinks for men in the span of about 2 hours (NIAAA).

Many different classifiers have been established to differentiate patterns of high consumption drinking such as high-risk drinking, heavy drinking, and high-intensity drinking. High risk drinking can be a small amount, but it interferes with the activities to be performed after the drinks are consumed (Weschler and Nelson, 2001). Heavy drinking is defined by SAMHSA (2019) as drinking four drinks in a day or 14 drinks per week for men and 3 drinks in a day or 7 drinks for women. High-intensity drinking is consuming two or more times the gender-specific quantities for binge-drinking (Hingson et al., 2017). These different classifications highlight different aspects of high quantity drinking that can lead to problematic behaviors. Binge drinking and these other classifications of large quantity consumption are important to understand, as these drinking patterns are a public health issue and a risk factor for developing AUD.

1.1.2 Animal Models of Alcohol Dependence

Animal models are widely used in alcohol research to gain experimental control that cannot be implemented in human research, such as genetic manipulations, elimination of the risks and ethical violations associated with giving humans a drug of abuse, and the ability to conduct biochemical and molecular investigations of the alcohol-exposed organs (Tabakoff and Hoffman, 2000). Voluntary drinking enables the animal *ad libitum* access

to alcohol. The two-bottle choice paradigm is a logical expansion of the voluntary drinking model in which the animal is presented with the choice of consumption of *ad libitum* alcohol or water (Samson & Czachowski, 2003). Operant chambers are also a method of voluntary drinking; however, they measure the rewarding aspects since the animal must perform a standardized task to obtain the substance (Samson & Czachowski, 2003). However, voluntary drinking animal models do not allow for control over alcohol consumption rate or quantity; therefore, rats generally do not drink enough to reach a BAC relevant to those seen in humans (McBride et al., 2014).

To control the rate and volume of consumption, involuntary drinking models are often required. One method of involuntary drinking is oral gavage, in which a needle goes down the throat to directly inject alcohol into the stomach to reach specific blood alcohol concentrations (Livy et al., 2003). Intraperitoneal injections of alcohol also maintain a specific blood alcohol concentration in rats (Livy et al., 2003). Placing rats in a chamber with ethanol introduced as a vapor is another method to ensure rats receive a certain concentration of ethanol (Le Bourhis, 1975). While involuntary drinking models enable precise control over experimentally desirable BACs, these paradigms are restricted by their inability to determine specific drinking patterns or investigate reinforcing aspects of alcohol use. (Jeanblanc et al., 2019).

1.1.3 Organotypic Hippocampal Slice Culture Model

To examine biochemical mechanisms of alcohol use, specifically in the hippocampus, the organotypic hippocampal slice culture model of neonatal Sprague-Dawley provides advantages in manipulating a deep brain structure. Stoppini et al. (1991) developed a widely used model for culturing hippocampal slices by demonstrating the

model's viability in maintaining morphological structure and the ability to monitor tissue changes for weeks. By creating an environment where tissue can maintain its natural morphology and continue developing outside of the organism, direct manipulations can be made to the tissue to remove interfering factors and thereby increase experimental control. This model permits unmatched experimental control which is important when standardizing the concentration of alcohol exposure which differs from some live-animal techniques that generate variability in blood alcohol levels. By eliminating a drinking paradigm and exposing the tissue directly to alcohol, much of the variability can be mitigated.

Immunohistochemical methods will be utilized to measure various markers of cellular integrity and expression. Propidium iodide (PI) is a commonly utilized marker to examine relative levels of neuronal death. PI is a small fluorescent molecule that binds to deoxyribonucleic acid (DNA). However, it is unable to pass through cellular membranes, so it can only bind if the cellular membrane has been disrupted, indicating the death of that cell (Crowley et al., 2016). Neuronal nuclear protein (NeuN) will also be utilized to measure relative expression of intact mature neurons. NeuN is a highly specific marker of neuronal nuclei in the brain, as it is only expressed once neurons have reached maturity (Sarnat et al., 1998). Bromodeoxyuridine (BrdU) and doublecortin (DCX) will be utilized as markers of newly dividing and developing cells, as discussed below.

1.2 Hippocampal Structure and Function

The hippocampus is a bilateral, deep brain structure that has many important functions. Within the hippocampus, there are specific regions called the dentate gyrus (DG), cornu ammonis 1 (CA1), cornu ammonis 3 (CA3), and subiculum (Ramon y Cajal,

1893; Lorente de No, 1933). Lorente de No (1934) also classified the region cornu ammonis 2 (CA2), but this region is not commonly imaged or referred to in the trisynaptic pathway. The pathway has been defined by the studies of Ramon y Cajal (1893) and Lorente de No (1933, 1934) as follows. Inputs from the frontal cortex project to the entorhinal cortex, which project to the DG. Projections, called mossy fibers, connect the granule cells of the dentate gyrus to CA3. Pyramidal cells in the CA3 connect to other neurons along the CA3 via 'auto-associative tracts', and to the CA1 via the Shaffer collaterals. The CA1 then connects to the subiculum, which sends projections back to the cortex (Lavenex & Amaral, 2000).

Each region of the hippocampus has a different neuronal makeup and function. The mossy fibers in the DG project mostly to gamma-aminobutyric acid (GABA) interneurons, which then project to the CA3 (Acsady et al., 1998). The DG is also a site for the creation and proliferation of new neurons (Altman & Das, 1965). The CA3 is largely connected with the rest of the hippocampus and makes excitatory projections to the surrounding excitatory and inhibitory neurons (Lorente de No, 1934). The CA1 contains a dense population of glutamatergic neurons, with a large quantity of N-methyl-D-aspartate (NMDA) receptors (Racca et al., 2000). This receptor makeup leads the CA1 to be particularly vulnerable to excitotoxicity (Bernal et al., 2000). The subiculum contains mainly pyramidal neurons, which are excitatory in nature (Harris et al., 2001).

1.2.1 Role in Learning, Memory, and Executive Function

The unique morphology and projections of the hippocampus make this structure critical for many important behaviors including learning, memory, and executive functioning. The hippocampus itself plays a critical role in memory, including the

acquisition and temporary storage of new long-term memories (Scoville & Milner, 1957). Memories are moved from the hippocampus into long-term storage in the cortex upon sleep, but the hippocampus is a critical short-term storage location (Preston & Eichenbaum, 2013). Studies of bilateral hippocampal damage have demonstrated that while there are deficits in the formation of new long-term memories, several other processes related to memory remain intact such as short-term memory, priming, and procedural learning (Rempel-Clower et al., 1996). The hippocampus also plays a key role in episodic memory, as it has been proven to play a crucial role in imagining new events, particularly the spatial element (Hassabis et al., 2007).

The hippocampus also plays a crucial role in learning, due to its importance in memory and its neuronal plasticity. Hippocampal removal experimentation has led to the conclusion that this structure is necessary for many aspects of learning behaviors. Zola-Morgan and Squires (1985) determined that monkeys with medial temporal lesions show deficits in concurrent object-discrimination learning. The hippocampus has also been implicated in the formation of configural associations being learned, meaning complex associations containing at least two elemental events (Sutherland and Rudy, 1989). Jarrard (1993) states that the role of the hippocampus in learning may be more related to processing and remembering spatial and contextual information than specific learning and memory processes. Whatever its' specific role may be, clearly, the hippocampus is intricately involved in various learning processes.

Due to its extensive projections throughout the brain and unique properties of neural plasticity, the hippocampus also plays an important role in executive functioning behaviors. The volume of both the hippocampus and the neocortex are significant

predictors of performance on various executive functioning tasks by various nonhuman primates, indicating the importance of these structures in executive functioning (Shultz & Dunbar, 2010). Hippocampal lesions in rats have been associated with impaired planning and initiation of social behaviors (Maaswinkel et al., 1997). The projections from the hippocampus to the prefrontal cortex are implicated in integrating spatial memory with executive functioning in rats (Seamans et al., 1998). Taken together, these findings indicate that the learning and memory processes in the hippocampus provide critical information for proper executive functioning in the prefrontal cortex.

1.2.2 Neurogenesis

The hippocampus is a unique structure in the brain, as it is one of the few areas in a mature brain in which new neurons are being continually produced (Eriksson et al., 1998). These newly developed neurons are critical for neural plasticity due to their enhanced synaptic plasticity once they have been differentiated (Ge et al., 2007). This means that new connections can be created and strengthened, which has behavioral implications in memory formation and cognition (Spalding et al., 2013; Clelland et al., 2009). Immature neurons in the hippocampus have been shown to have a crucial role in pattern separation in one's environment (Leutgeb et al., 2007). More recently, studies have shown that neurogenesis may have as much of a role in forgetting and memory clearance as it does in learning and forming memories (Akers et al, 2014). Since neurogenesis is so critical in all these behaviors, lack of neurogenesis has been associated with deficits in these behaviors (Winocur et al., 2006). Changes in neurogenesis have also been associated with many disorders such as Alzheimer's disease, Parkinson's disease, epilepsy, and mood disorders (Toda et al., 2019).

New neurons are produced via cellular division and become neural progenitor cells in between the hilus and the granule cell layer of the hippocampus (Kuhn et al., 1996; Altman and Das, 1965). Once the cells have differentiated into neurons, they either become excitatory neurons or dentate granule cells (Basu and Suh, 2020) and must integrate themselves into the DG and extend axonal projections where they form synaptic connections with neurons in the CA3 (Toda et al., 2019). These new synaptic connections are sensitive to environmental changes until these neurons have matured (Jessberger and Kempermann, 2003). The integration of new neurons is important for maintaining a balance of excitatory and inhibitory signals in the hippocampus (Basu and Suh, 2020),

There are several immunohistochemical markers that can be utilized to identify newly differentiated and immature neurons. One commonly used marker is BrdU as this marker incorporates a thymidine analog into the DNA of dividing cells (Yu et al., 2022). Through BrdU's specific incorporation into only newly dividing cells, the number of new cells created in a point in time can be quantified directly through measuring BrdU fluorescence via immunohistochemistry. This is not specific to neurons, however, as the thymidine analog will be incorporated by replicative machinery in any cell undergoing mitosis at that time. Therefore, BrdU is often used in conjunction with other markers to examine neurogenesis. A specific marker of immature neurons is doublecortin, which is a microtubule protein specific to neuronal precursors (Moores et al., 2004). Doublecortin is an endogenous protein involved in the stabilization of axonal microtubules specifically in immature neurons and is downregulated upon maturation as markers of mature neurons are upregulated (Brown et al., 2003). This marker alone is not necessarily sufficient to conclude neurogenesis changes as it is possible for doublecortin-expressing cells to be

generated other ways (Zhang et al., 2009). This is still a common marker of neurogenesis, but more definitive markers should be used in conjunction with it.

1.2.3 Alcohol's Physiological and Behavioral Influences

Chronic alcohol exposure to any region of the brain has been shown to cause structural changes, and the hippocampus is no exception. Alcohol exposure in the hippocampus leads to deficits such as alterations in neuronal density (Walker et al., 1980), patterns of dendritic branching (Cadete-Leite et al., 1989), and fluctuations in dendritic spine density in the CA1 (Tarelo-Acuña et al., 2000). The loss of hippocampal neurons upon chronic alcohol exposure can reach as high as 50%, and the remaining neurons may be unable to compensate for this loss (Bengochea and Gonzalo, 1990). Whether or not these are mature or immature neurons being lost is unclear, however. Further, studies have also shown that there is a loss of dorsal hippocampal grey matter (Mechtcheriakov et al., 2007).

Due to the morphological changes caused by alcohol, resulting behavioral changes may occur. In fact, chronic alcohol exposure leads to impairment in memory formation (Victor, 1994). Immediate memory, attention, acquisition, and working memory are also impaired in adults with AUD (Ozsoy et al., 2013). Since the hippocampus is a critical structure in memory tasks and cognitive function, these abilities are sensitive to the effects of alcohol (White et al., 2000). Evidence has also emerged implicating decreases in hippocampal volume with these cognitive and memory changes (Chanraud et al., 2007). These hippocampal changes do not appear to be associated with initiation of drinking behaviors, however (Nagel et al., 2005). While the exact relationship between the morphological changes in the hippocampus due to chronic alcohol exposure remains to be

defined, evidence suggests that alcohol does cause morphological changes as well as a range of behavioral deficits.

1.3 Alcohol's Interference with Hippocampal Neurogenesis

In addition to the morphological and structural changes discussed in the previous section, chronic alcohol exposure also interferes with hippocampal neurogenesis. Previously, it was believed that the deficits in hippocampal integrity were due to neurons dying (Bengochea and Gonzalo, 1990). Once hippocampal neurogenesis was established in adults, studies started to investigate how alcohol impacted this process. Studies have now shown that reductions in hippocampal neurogenesis are at least partially responsible for alcohol-induced hippocampal neurodegeneration (Morris et al., 2010). Additionally, another study showed that hippocampal integrity was impacted but there was a lack of cell death, indicating that the loss of hippocampal neuronal density may be due to lack of neurogenesis, not cell death (Leasure and Nixon, 2010). Alcohol affects neurogenesis differentially depending on whether the person is intoxicated or undergoing withdrawal, meaning they are abstaining from consuming alcohol. During intoxication, hippocampal neurogenesis is significantly decreased due to alterations in cell proliferation and cell survival (Crews and Nixon, 2009). During abstinence, there is increased neurogenesis, but usually to aberrantly high levels (Nixon and Crews, 2004). The mechanisms underlying these alcohol-induced alterations in hippocampal neurogenesis are still being investigated, but some of the direct effects of alcohol on neurogenesis include altered neurotransmission, cAMP-responsive element binding (CREB) protein and its downstream effectors, and functional changes in the sub-granular zone and its neurogenic niche (Geil et al., 2014).

1.3.1 Altered Neurotransmission

One way in which alcohol impacts neurogenesis is through its alteration of neurotransmission in the hippocampus. Alcohol is a unique drug that acts on virtually all the neurotransmitters in the central nervous system, including acetylcholine, endocannabinoids, neuropeptide Y (NPY), gamma-aminobutyric acid (GABA), glutamatergic NMDA receptors, and serotonin (Vengeliene et al., 2008). Most of these neurotransmitters also regulate hippocampal neurogenesis as well (Crews and Nixon, 2003).

GABA is one of the major targets of alcohol's action, and additionally, $GABA_A$ receptors are present on new neurons in the sub-granular zone, acting as an excitatory neurotransmitter for the beginning of its development (Espósito et al., 2005). Studies have shown that $GABA_A$ agonists reduce proliferation of neural progenitor cells in the sub-granular zone (Tozuka et al., 2005). Since alcohol is a modulator of $GABA_A$ receptors and acts as an agonist, this may be one mechanism by which alcohol is altering neurogenesis.

The endocannabinoid system may also be implicated in alcohol-induced alterations in hippocampal neurogenesis. Neural progenitor cells express both CB1 and CB2 receptors and have a functional endocannabinoid system (Aguado et al., 2005). Studies have shown that CB1 and CB2 knockout mice display deficits in neurogenesis (Jin et al., 2004). Additionally, activation of cannabinoid receptors promotes proliferation of neural progenitor cells (Aguado et al., 2005). While there is no direct evidence that the endocannabinoid system directly causes alcohol-induced neurogenesis deficits, the increase in NMDA receptor expression characterized in chronic alcohol use is dependent on the activity of CB1 receptors (Warnault et al., 2007). Since the endocannabinoid system

regulates neuroadaptations in the hippocampus due to chronic alcohol exposure, it may also influence the deficits in neurogenesis as well (Geil et al., 2014).

1.3.2 Cyclic Adenosine Monophosphate Downstream Effects

Cyclic adenosine monophosphate (cAMP) is a second messenger signaling cascade component and is a common intracellular mechanism of action of many neurotransmitter systems (McKnight, 1991). The phosphorylated form of CREB (pCREB), which is a transcription factor of cAMP, is present in the subgranular zone and is localized mostly in immature neurons (Mantamadiotis et al., 2012). Additionally, enhancement of CREB signaling led to increased dendritic branching, survival, and proliferation of neural progenitor cells (Nakagawa et al., 2012). Alcohol has been shown to modulate the second messenger pathway and its effectors such as NPY and brain-derived neurotrophic factor (BDNF), which may play a role in alcohol's modulation of neurogenesis (Moonat et al., 2010).

NPY has been shown to play a role in alcohol-related behavior (Thiele et al., 1998) and in the initiation of hippocampal neurogenesis (Malva et al., 2012). The GABAergic interneurons in the DG and the neural progenitor cells in the subgranular zone both express the NPY Y1 receptor (Sperk et al., 2007). NPY knockout mice show deficits in neuronal proliferation and administration of NPY stimulates the proliferation stage of neurogenesis and promotes differentiation into neurons (Decressac et al., 2010; Howell et al., 2007). NPY expression is also decreased after chronic exposure to alcohol (Bison and Crews, 2003), which is consistent with alcohol-induced decreases in proliferation of neural progenitor cells (He et al., 2005). These parallels suggest that NPY may be involved in the alcohol-induced changes in neuronal proliferation in the hippocampus.

BDNF also plays a role in both alcohol related behavior (Davis, 2008) and the initiation of hippocampal neurogenesis (Waterhouse et al., 2012). BDNF promotes hippocampal neurogenesis by enhancing cell birth, maturation, and survival (Lee et al., 2002). Alcohol-dependent patients have been shown to have decreased serum levels of BDNF, indicating that alcohol downregulates BDNF in the hippocampus (Joe et al., 2007). These alcohol-induced decreases are associated with depressive behaviors of rats during alcohol use, such as psychomotor retardation and behavioral despair (Hauser et al., 2011). Treatments with antidepressants to restore hippocampal BDNF shortly after alcohol exposure led to decreased depressive symptoms and mitigation of alcohol-induced neurogenesis alterations (Hauser et al., 2011). This relationship between alcohol exposure, BDNF, and neurogenesis suggests that it may play a role in alcohol's mechanism in altered neurogenesis.

1.3.3 Functional Changes in the Subgranular Zone

The unique properties of the subgranular zone make it one of the only locations in the adult brain where the production of new neurons can be facilitated. These properties include the specific organization of this zone which allows for maintenance of stem cell populations (Conover and Notti, 2008), the vasculature, (Palmer et al., 2000), the secreted factors (Alvarez-Buylla and Lim, 2004), and the presence of astrocytes (Seri et al., 2001).

Excessive consumption of alcohol is damaging to astrocytes as it either activates them or damages them entirely (Kelso et al., 2011; Korbo, 1999). Activation of astrocytes leads to astrogliosis, which consists of morphological changes that alter their functional role (Sofroniew, 2009). Since astrocytes are critical for providing metabolic support, taking up excessive neurotransmitters such as glutamate, buffering ions, and eliminating free

radicals, impaired astrocyte function will alter the environment in which new neurons are being produced and would no longer provide a proper environment for cell proliferation (Geil et al., 2014).

Microglia are also important in maintaining a proper environment for the neurogenic niche. Microglia are involved in neuroinflammatory processes and are critical for responding to insults to the brain (Baufeld et al., 2018). There are different phenotypes of microglia, including pro-inflammatory and anti-inflammatory microglia, which determines the effects of microglia on neurogenesis (Town et al., 2005). Only the pro-inflammatory phenotype of microglia appears to inhibit neurogenesis in the hippocampus (Russo et al., 2011). Studies show that administration of a proinflammatory cytokine IL-1β suppresses hippocampal neurogenesis (Koo and Duman, 2008), and blocking the alcohol-induced increase in IL-1β synthesis resolved this decrease in neuronal proliferation and cell survival (Zou and Crews, 2012). Since alcohol produces an inflammatory response in the brain, microglia are likely to become the pro-inflammatory phenotype, thereby reducing neurogenesis.

1.4 Purpose

Considering the presented information, these experiments will be a critical step in determining the specific mechanism of alcohol-induced decreases in neurogenesis. Four different measurements of neuronal status following alcohol exposure will be taken: 1) PI will be measured to compare the relative death of neurons after five days with or without alcohol exposure; 2) NeuN will be measured to compare the relative maturation of existing neurons after five days with or without alcohol exposure; 3) BrdU will be measured to compare initiation of cell division after five days with or without alcohol exposure; 4) DCX

will be measured to compare relative expression of developing neurons with or without alcohol exposure. Together, this study will provide insights to developing neurons, neuronal death, and neuronal maturation in the context of this alcohol paradigm. The hypothesis for each marker includes the following: 1) no difference will occur in neuronal death between alcohol and control tissue, 2) no difference will occur in neuronal maturation between alcohol and control tissue, 3) BrdU expression will be significantly decreased in ethanol compared to control tissue, and 4) DCX expression will be significantly decreased in ethanol compared to control tissue. The goal of these experiments is to establish viability of this model to further investigate results of alcohol exposure on neurogenesis. The use of these markers will confirm viability and allow for continual exploration of other markers of neurogenesis to clarify the specific alterations in hippocampal neurogenesis. The combination of the findings from these four markers will provide insight into the specific mechanism of alcohol-induced hippocampal morphological changes and will validate that this model can be utilized to further explore markers of neurogenesis in the hippocampus.

CHAPTER 2. METHODS

2.1 Organotypic Hippocampal Slice Culture

Neonatal male and female Sprague-Dawley rat pups (Envigo-Harlan Laboratories, Indianapolis, IN) were euthanized to obtain hippocampal tissue. Once bilateral hippocampal tissue was removed and isolated, the tissue was sliced into 200 micrometer thick slices using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, Surrey, UK). Slices were then selected for experimental use if they contained all 3 regions of interest (CA1, CA3, and dentate gyrus) and there were no visible holes or tears. Selected slices were randomized and plated on .4 micrometer Millicell-CM biopore membranes (Millipore Sigma, Marlborough, MA), with 4 sex-matched slices per well. The inserts were placed in 35-mm 6 well culture plates, each plate containing 3 wells of male tissue and 3 wells of female tissue. Each well contained 1 mL of culture medium containing minimum essential medium, dextrose anhydrous, HEPES, Sodium Hydroxide, Hank's balanced salt solution, heat-inactivated horse serum, penicillin/streptomycin, and amphotericin B solution. Cultures were kept in an incubator maintaining a temperature of 37 degrees Celsius and an air composition of 5% CO2/95% air and 95% humidity. This procedure was followed for each experiment, using tissue from a different litter with each replication.

2.2 Ethanol Exposure

After 5 days in the incubator to recover from the procedure and adhere to the membrane, slices were transferred to new plates with culture medium with or without 50 mM of EtOH included. The plates were incubated for 5 days at 37 degrees Celsius and 5%

CO2 in plastic tubs containing 50 mL of double-distilled H_2O or 50 mL of double-distilled water containing 50mM of 100% ethanol. These tubs were placed in gallon polypropylene bags and filled with a gas mixture to mimic the air conditions of the incubator, then placed in the incubator for the 5-day exposure. Previous studies within this laboratory have shown that merely adding ethanol to the cultures leads to evaporation across the 5-day exposure period, which isolating tissue within bagged environments seems to resolve (Prendergast et al., 2004).

2.3 Experimental Design

In these studies, three different cellular markers were measured in 3 specific regions of the hippocampal slices after the 5 days of experimental manipulation. Each plate was exposed to one specific marker and had one experimental manipulation. Each slice in each well provided a data point, as optical density, for each region of interest. This led to 12 male slices and 12 female slices for each marker and experimental manipulation per litter. There was a plate of control for comparison to each manipulation and marker considered. Measurements were taken at the same time point for each group within a marker. The slices from the control plate for a given marker and the slices for the experimental manipulation for that marker were compared through statistical analysis. The means of the optical density were obtained for each group and marker combination, then converted to percentages of the mean of the control group for that marker. This allowed for the experimental groups within a marker to be compared to the mean of the control via "% control", as indicated in the results.

2.4 Immunohistochemistry Procedures

On the fourth day of experimental exposure (24 hours before the end of exposure), plates were exposed to markers that needed to be integrated into live tissue. Some plates were exposed to culture media containing 100uM of BrdU (Millipore Sigma) for 2 hours at 37 degrees Celsius. After the two hours of exposure, inserts were placed into fresh culture medium for the remaining 22 hours, until time of fixation in formalin, to dilute the BrdU. At the same time, other slices were placed in culture medium containing 2.5ug/mL of PI for 24 hours. These slices were then imaged using fluorescence microscopy, as detailed below. Slices that were to be analyzed with NeuN or DCX were placed into fresh culture medium for this time, until fixation in formalin 24 hours later. For slices in the EtOH experimental group, 50mM of ethanol was also added to the media for these 24 hours in addition to the marker for the 5^{th} day of ethanol exposure.

Slices prepared for BrdU, NeuN, and DCX measurement were fixed in 10% formalin for 30 minutes at room temperature. All plates were then washed twice with phosphate-buffered saline (PBS). BrdU slices were placed into wells of 1mL of PBS and 1 mL of 2N HCl was added on top to incubate for 45 minutes. After this step, BrdU, NeuN, and DCX tissue was placed in a permeabilization and blocking buffer (PBB: Triton-X100, bovine serum albumin, and PBS) on the top and bottom of the wells for 45 minutes. Once the incubation was complete, NeuN, BrdU, and DCX tissue was placed into plates containing 1mL of PBS. Plates for NeuN were incubated with anti-NeuN antibody (1:200, Millipore Sigma) diluted with PBB and were placed in the refrigerator for 24 hours. Plates for BrdU were incubated with anti-BrdU antibody (1:200, Millipore Sigma) diluted with PBB and were placed in the fridge for 24 hours. Plates for DCX were incubated with anti-DCX antibody (1:200, Thermo Fisher). After the 24-hour incubation, NeuN, BrdU, and

DCX slices were rinsed twice with PBS, then placed into wells containing 1mL of PBS. NeuN, BrdU, and DCX slices were incubated with Fluorescein isothiocyanate (FITC) (1:200, Millipore Sigma) and incubated in the fridge for 24 hours. After the final incubation, all slices were rinsed twice with PBS then transferred to plates with 1mL of fresh PBS on the bottom for fluorescence imaging.

SPOT Advanced version 4.0.2 software for Windows (W. Nuhsbaum Inc., McHenry Illinois) was used to measure fluorescent emission from PI, NeuN, BrdU, and DCX tissue. A Leica DMIRB microscope (W. Nuhsbaum Inc.) fitted with a mercury-arc lamp for fluorescent detection and a x5 objective lens was connected to a personal computer through a SPOT 7.2 color mosaic camera (W. Nuhsbaum Inc.) to capture the fluorescent images. PI tissue was exposed to a band-pass filter at 515 to 560 nm, while BrdU, DCX, and NeuN tissue was exposed to a band-pass filter at 495 nm. After the images were captured, ImageJ software (NIH) was used to capture optical intensity of each hippocampal region of interest.

2.5 Statistical Analysis

Due to litter variation and anatomical differences between slices themselves due to the shape of the hippocampus, steps were taken to mitigate the difference in optical density not caused by the experimental manipulation of interest. One such measure was to take a background optical density measurement of each slice and subtract this value from the fluorescence measurements from each region of this slice. This removes the variability in background fluorescence, caused by nonspecific binding, that may change from slice to slice. This left “background removed” optical density values for analysis in each region of a slice. The % control optical density within each marker was analyzed for the effect of

treatment and sex in each region of the hippocampus individually via a univariate analysis of variance (ANOVA) in SPSS. Slices in the same experimental group and marker were combined for analysis and graphing purposes to illustrate universal effect of each marker in this paradigm.

CHAPTER 3. RESULTS

3.1 PI Immunofluorescence

In the CA1 region, there was no significant effect of treatment, sex, or the interaction of the two, $F(1, 92) = .161$, $p = .689$, $F(1, 92) = 1.348$, $p = .249$, $F(1, 92) = 1.347$, $p = .249$ respectively (Figure 3.1.1). In the CA3 region, there was no significant effect of treatment, sex, or the interaction of the two, $F(1, 92) = .274$, $p = .602$, $F(1, 92) = .393$, $p = .532$, $F(1, 92) = .393$, $p = .532$ respectively (Figure 3.1.2). In the DG region, there was no significant effect of treatment, sex, or the interaction of the two, $F(1, 92) = 1.136$, $p = .289$, $F(1, 92) = 1.318$, $p = .254$, $F(1, 92) = 1.318$, $p = .254$ respectively (Figure 3.1.3).

3.2 NeuN Immunofluorescence

In the CA1 region, there was no significant effect of treatment, sex, or the interaction of the two, $F(1, 90) = .066$, $p = .798$, $F(1, 90) = .757$, $p = .387$, $F(1, 90) = .757$, $p = .387$ respectively (Figure 3.2.1). In the CA3 region, there was no significant effect of treatment, sex, or the interaction of the two, $F(1, 90) = .010$, $p = .922$, $F(1, 90) = 3.026$, $p = .086$, $F(1, 90) = 3.026$, $p = .086$ respectively (Figure 3.2.2). In the DG region, there was no significant effect of treatment, sex, or the interaction of the two, $F(1, 90) = .087$, $p = .769$, $F(1, 90) = 3.719$, $p = .057$, $F(1, 90) = 3.719$, $p = .057$ respectively (Figure 3.2.3).

3.3 BrdU Immunofluorescence

In the CA1 region, there was no significant effect of sex nor an interaction of treatment*sex, $F(1, 45) = .044$, $p = .835$, $F(1, 45) = .044$, $p = .835$ respectively. There was a significant main effect of treatment, $F(1, 45) = 14.115$, $p < .001$ with the ethanol-treated slices having significantly lower BrdU fluorescence than the control slices ($M = 84.58$, SE

= 4.87 and M = 100.00, SE = 3.26 respectively) (Figure 3.3.1). In the CA3 region, there was no significant effect of sex nor an interaction of treatment*sex, $F(1, 45) = .635$, $p = .430$, $F(1, 45) = .635$, $p = .430$ respectively. There was a significant main effect of treatment, $F(1, 45) = 9.132$, $p = .004$ with the ethanol-treated slices having significantly lower BrdU fluorescence than the control slices ($M = 82.77$, $SE = 5.22$ and $M = 100.00$ and $SE = 5.70$ respectively) (Figure 3.3.2). In the DG there was no significant effect of sex nor an interaction of treatment*sex, $F(1, 45) = .903$, $p = .348$, $F(1, 45) = .903$, $p = .348$ respectively. There was a significant main effect of treatment, $F(1, 45) = 4.297$, $p = .045$ with the ethanol-treated slices having significantly lower BrdU fluorescence than the control slices ($M = 88.70$, $SE = 4.97$ and $M = 100.00$, $SE = 5.85$ respectively) (Figure 3.3.3).

3.4 DCX Fluorescence

In the CA1 region, there was no significant effect of treatment, sex, or the interaction of the two, $F(1, 135) = 2.232$, $p = .138$, $F(1, 135) = .264$, $p = .608$, $F(1, 135) = .264$, $p = .608$ respectively (Figure 3.4.1). In the CA3 region, the was no significant effect of sex or interaction of treatment*sex, $F(1, 135) = 3.207$, $p = .076$, $F(1, 135) = 3.207$, $p = .076$ respectively. There was a significant main effect of treatment, $F(1, 135) = 5.932$, $p = .016$ with the ethanol-treated slices having significantly lower DCX immunofluorescence than control slices ($M = 90.49$, $SE = 4.12$ and $M = 100.00$, $SE = 3.71$ respectively) (Figure 3.4.2). In the DG region, there was no significant effect of treatment, sex, or the interaction of the two, $F(1, 135) = 2.389$, $p = .125$, $F(1, 135) = .974$, $p = .325$, $F(1, 135) = .974$, $p = .325$ respectively (Figure 3.4.3).

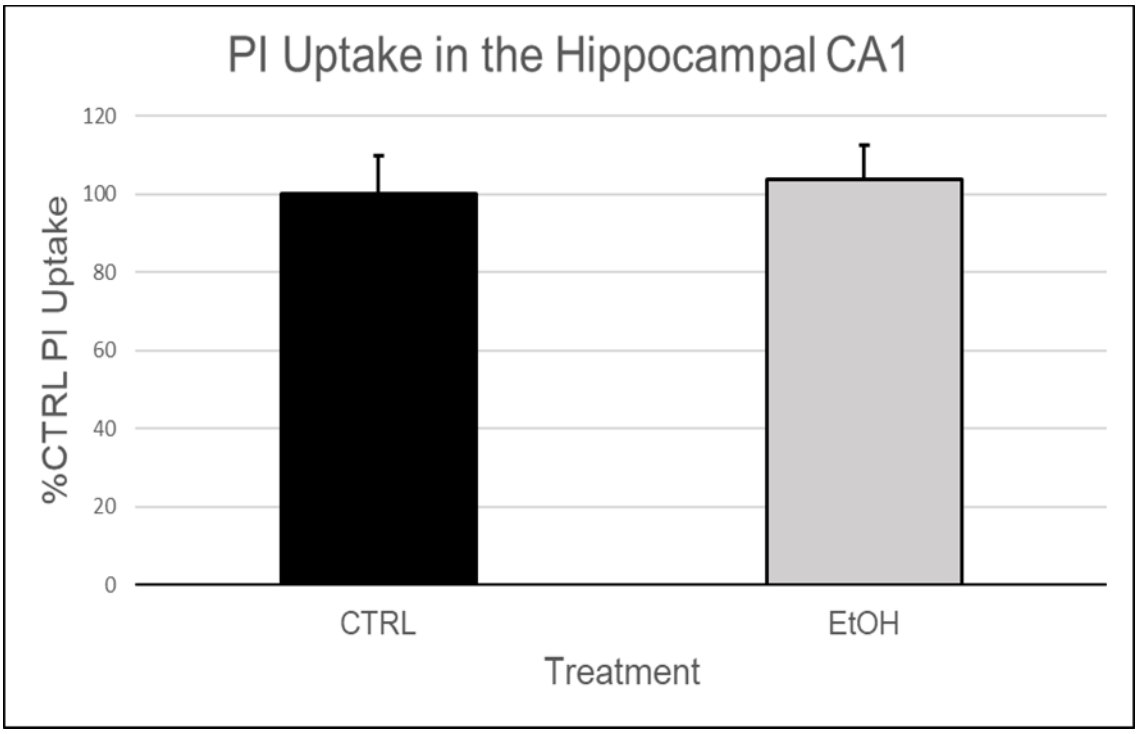

Figure 3.1.1: Effect of ethanol treatment on PI uptake in the CA1 region of hippocampal slices. There was no effect of treatment, $p = .689$ or an effect of sex, $p = .249$. Error bars represent standard error of the mean.

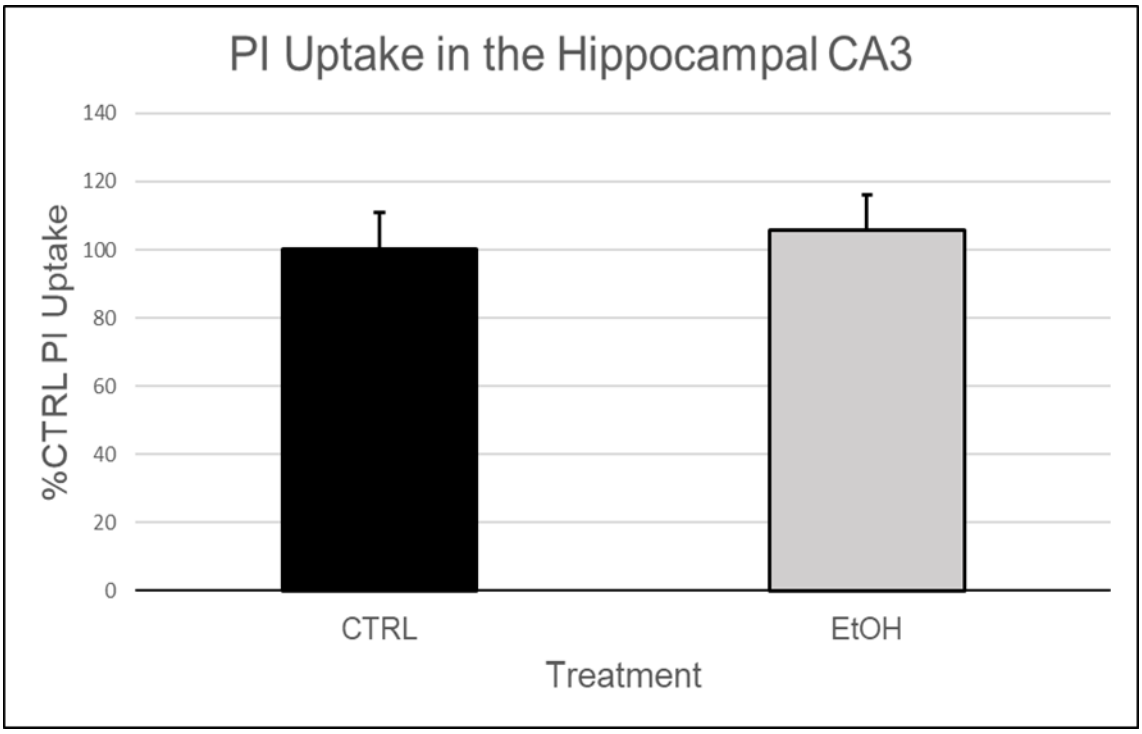

Figure 3.1.2: Effect of ethanol treatment on PI uptake in the CA3 region of hippocampal slices. There was no effect of treatment, $p = .602$ or an effect of sex, $p = .393$. Error bars represent standard error of the mean.

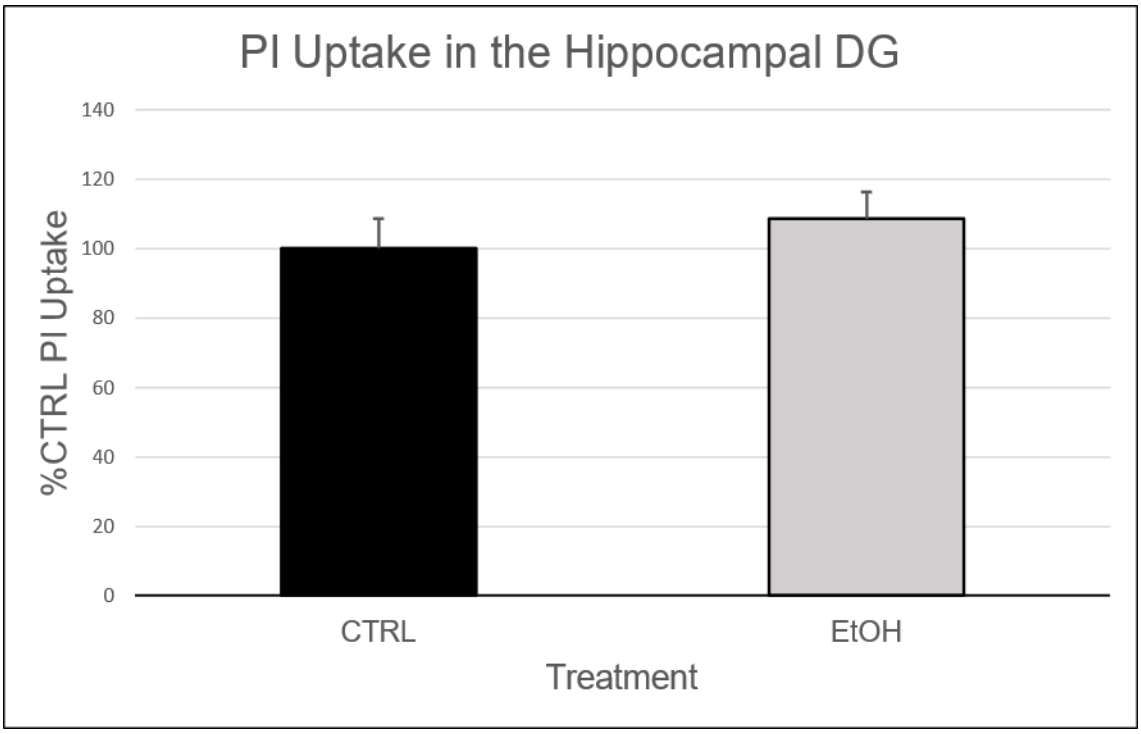

Figure 3.1.3: Effect of ethanol treatment on PI uptake in the DG region of hippocampal slices. There was no effect of treatment, $p = .289$ or an effect of sex, $p = .254$. Error bars represent standard error of the mean.

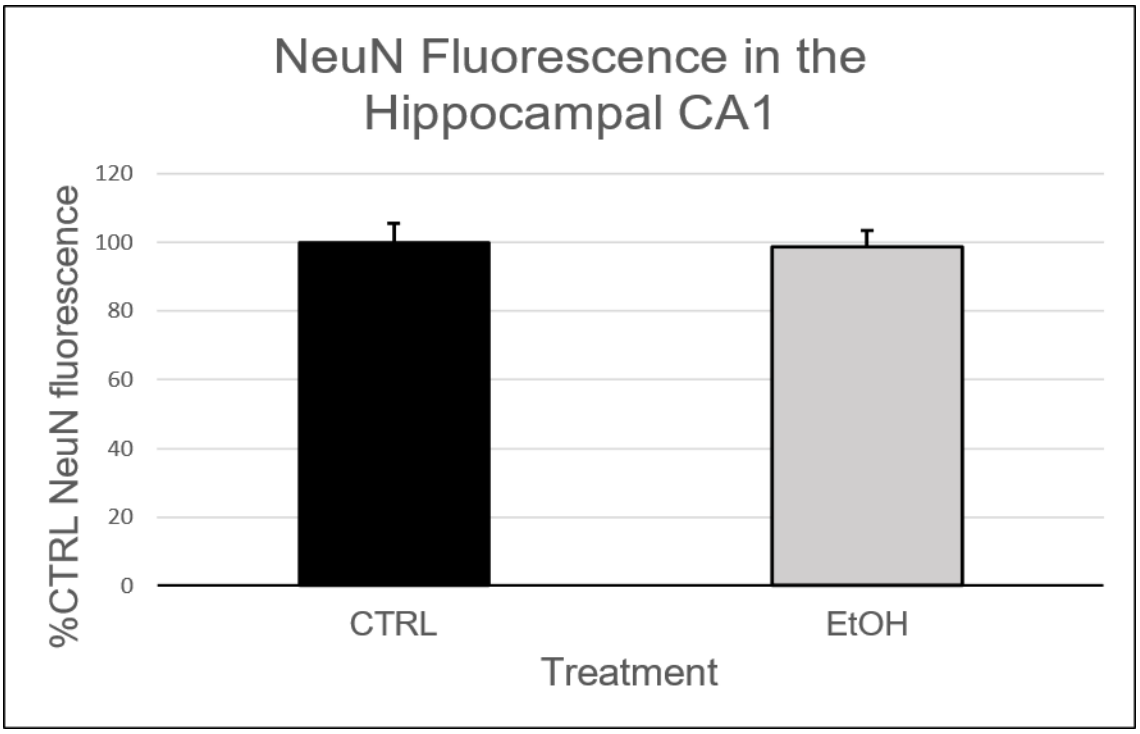

Figure 3.2.1: Effect of ethanol treatment on NeuN immunofluorescence in the CA1 region of hippocampal slices. There was no effect of treatment, $p = .798$ or an effect of sex, $p = .387$. Error bars represent standard error of the mean.

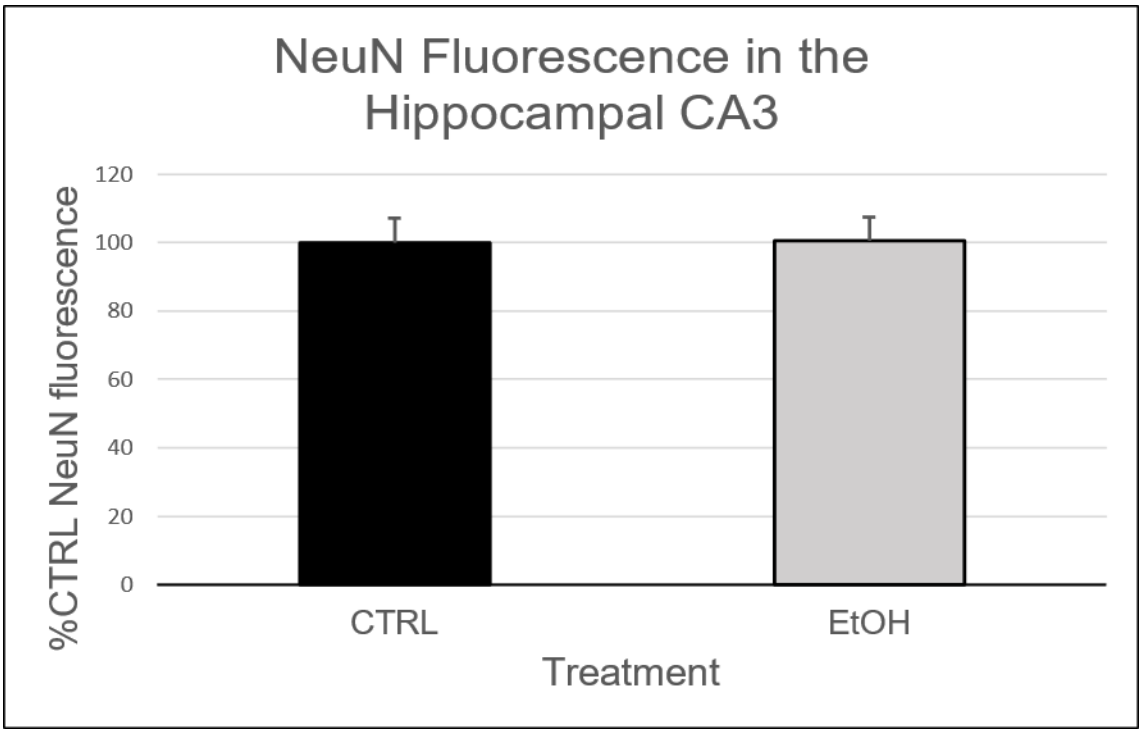

Figure 3.2.2: Effect of ethanol treatment on NeuN Immunofluorescence in the CA3 region of hippocampal slices. There was no effect of treatment, $p = .922$ or an effect of sex, $p = .086$. Error bars represent standard error of the mean.

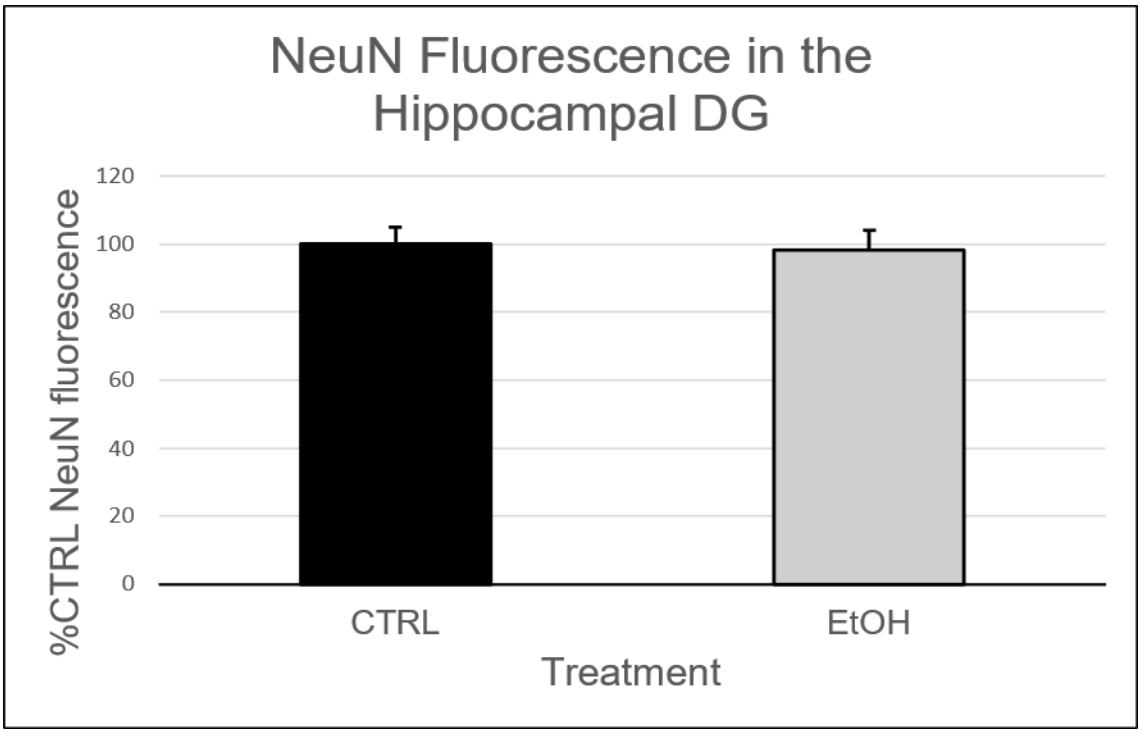

Figure 3.2.3: Effect of ethanol treatment on NeuN immunofluorescence in the DG region of hippocampal slices. There was no effect of treatment, $p = .769$ or an effect of sex, $p = .057$. Error bars represent standard error of the mean.

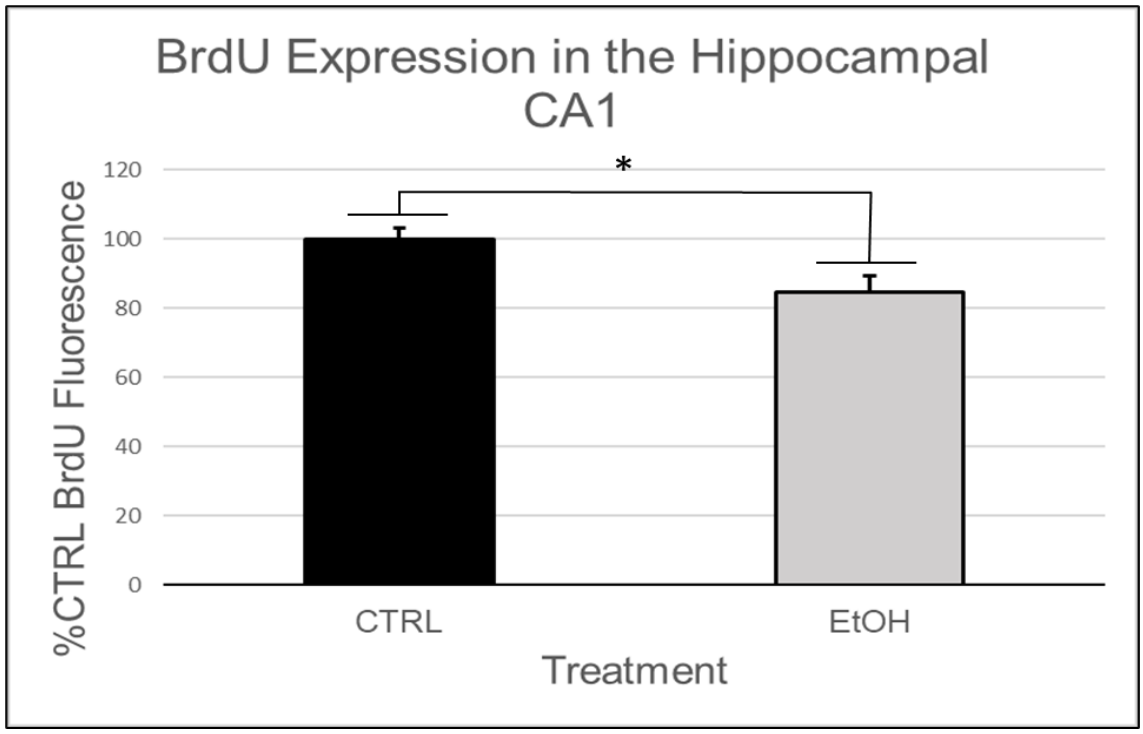

Figure 3.3.1: Effects of ethanol treatment on BrdU immunofluorescence in the CA1 region of hippocampal slices. There was no effect of sex, $p = .835$. There was a significant decrease in BrdU uptake in ethanol-exposed tissue compared to control tissue, $p < .001$. Error bars represent standard error of the mean.

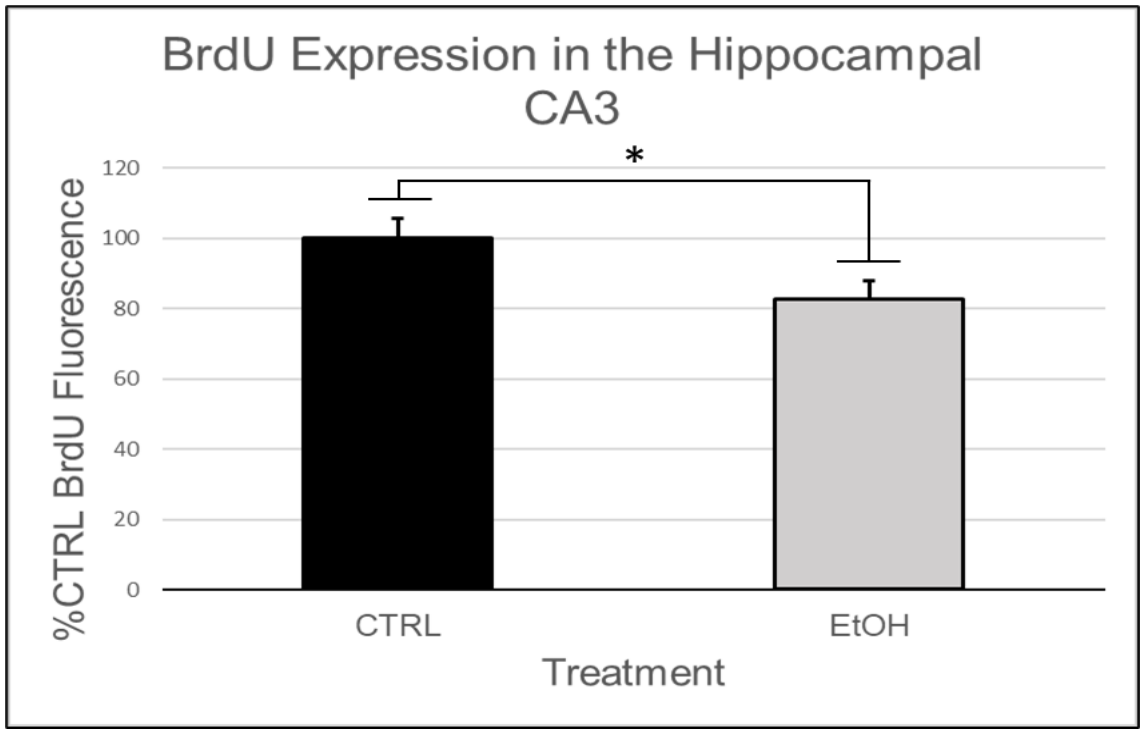

Figure 3.3.2: Effects of ethanol treatment on BrdU immunofluorescence in the CA3 region of hippocampal slices. There was no effect of sex, $p = .635$. There was a significant decrease in BrdU uptake in ethanol-exposed tissue compared to control tissue, $p = .004$. Error bars represent standard error of the mean.

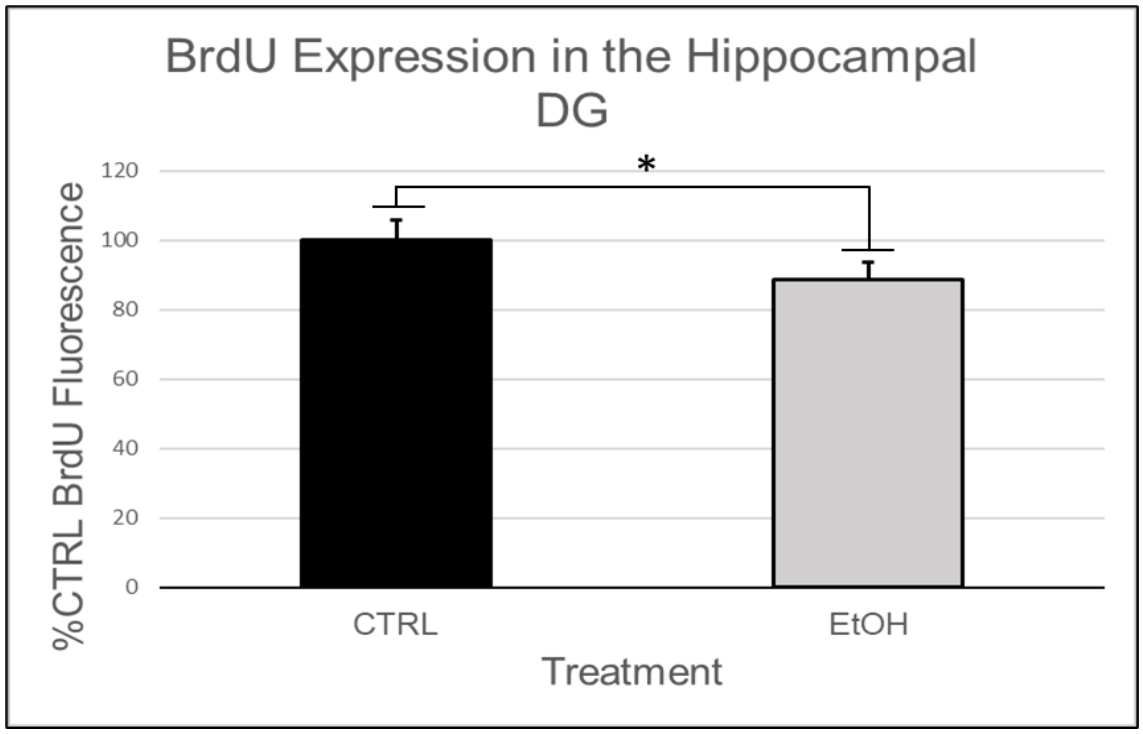

Figure 3.3.3: Effects of ethanol treatment on BrdU immunofluorescence in the DG region of hippocampal slices. There was no effect of sex, $p = .903$. There was a significant decrease in BrdU uptake in ethanol-exposed tissue compared to control tissue, $p = .045$. Error bars represent standard error of the mean.

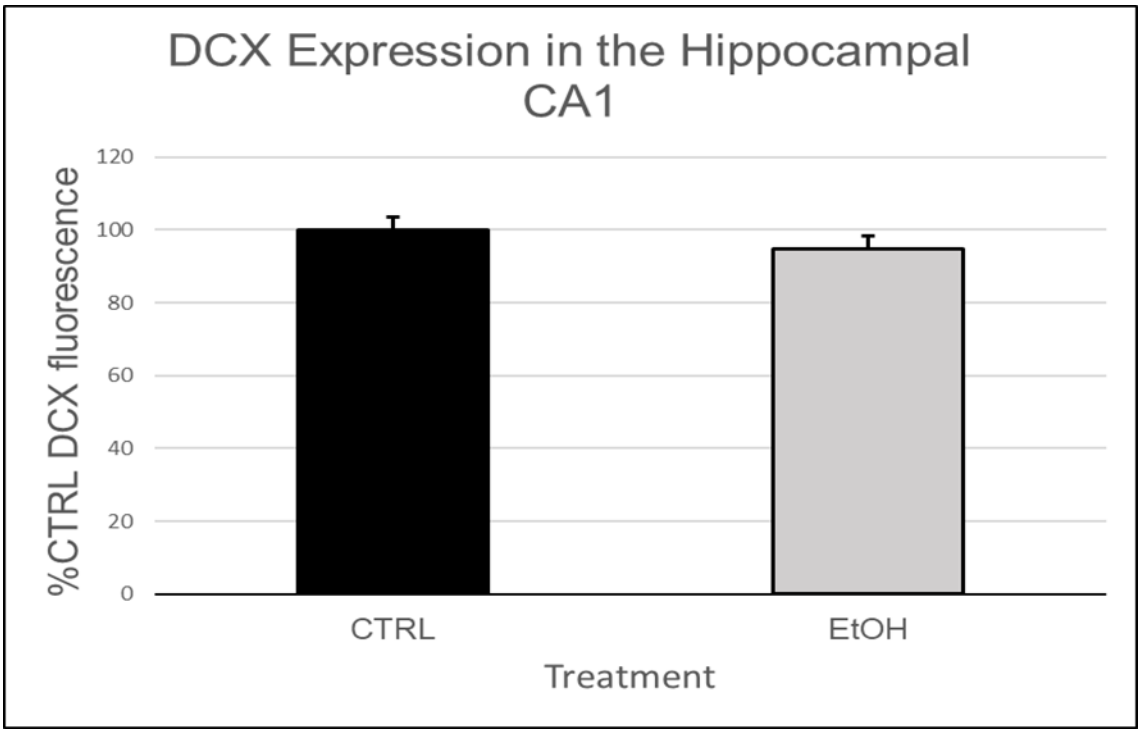

Figure 3.4.1: Effect of ethanol treatment on DCX immunofluorescence in the CA1 region of hippocampal slices. There was no effect of treatment, $p = .138$ or an effect of sex, $p = .608$. Error bars represent standard error of the mean.

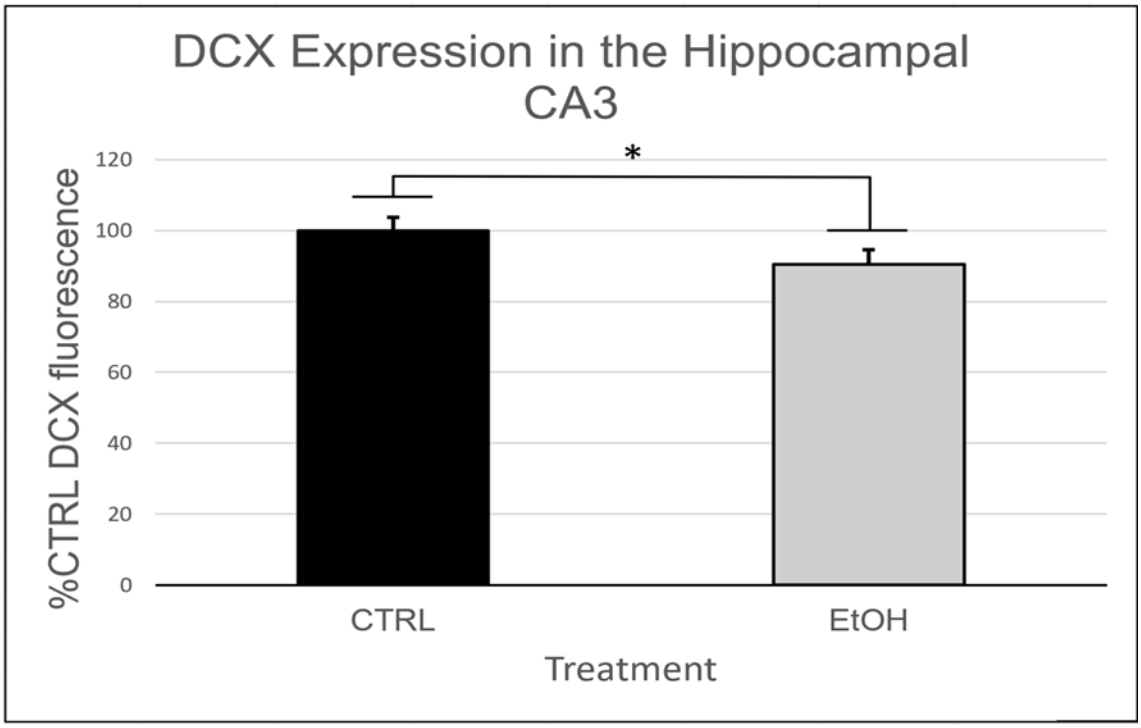

Figure 3.4.2: Effects of ethanol treatment on DCX immunofluorescence in the CA3 region of hippocampal slices. There was no effect of sex, $p = .076$. There was a significant decrease in DCX uptake in ethanol-exposed tissue compared to control tissue, $p = .016$. Error bars represent standard error of the mean.

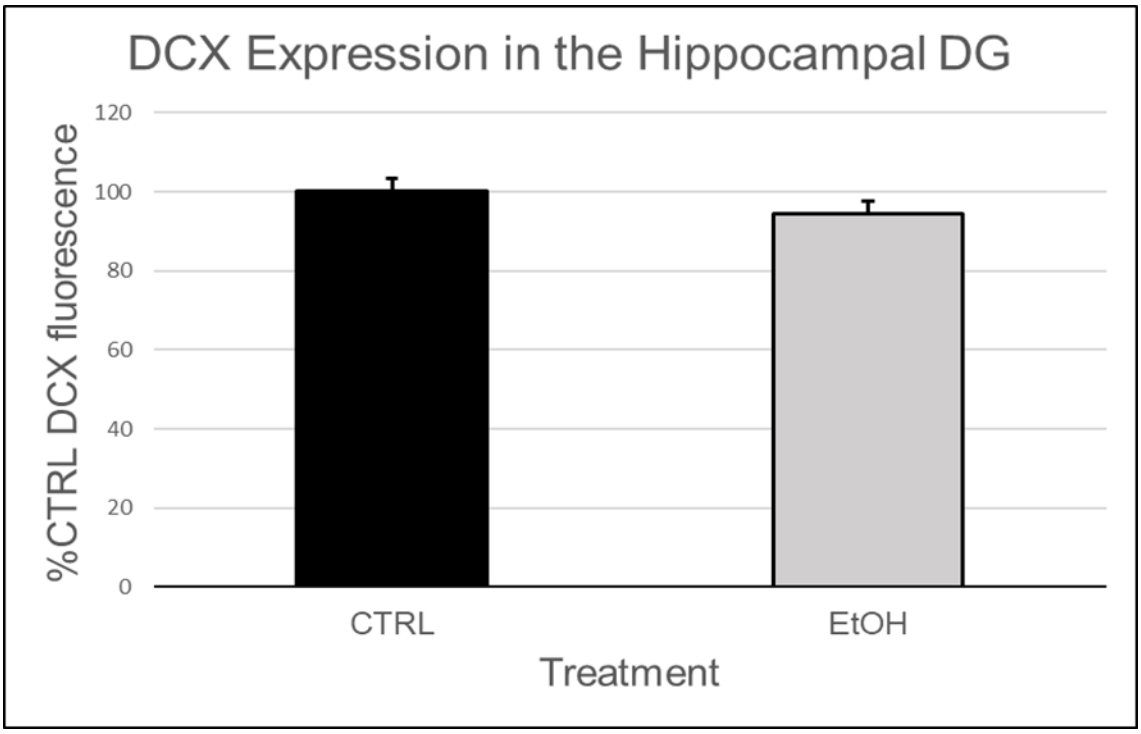

Figure 3.4.3: Effect of ethanol treatment on DCX immunofluorescence in the DG region of hippocampal slices. There was no effect of treatment, $p = .125$ or an effect of sex, $p = .325$. Error bars represent standard error of the mean.

CHAPTER 4. DISCUSSION

4.1 Implications

Due to the many deficits in memory and cognition seen in those who chronically abuse alcohol (Ozsoy et al., 2013), examining alcohol's influence on mechanisms of neural plasticity is critical. Development of new neurons in the hippocampus is a major contributor to neuronal plasticity, thus understanding alcohol's influence on these mechanisms can aid in the understanding of these alcohol-related deficits. By measuring neuronal death, neuronal viability, cellular division, and mature neurons, the specific effects of alcohol in the hippocampus can be further elucidated, allowing for more specific interventions.

4.1.1 Sustained PI Uptake Regardless of Ethanol Exposure

In these experiments, there was no difference between PI uptake in control tissue compared to ethanol-exposed tissue, regardless of sex or region. This is an important finding that contributes to the understanding of ethanol-induced hippocampal dysfunction after prolonged exposure. If there is no difference in the number of neurons dying between control tissue and ethanol tissue, this indicates that this is not the mechanism by which hippocampal dysfunction is occurring. Since alcohol is a molecule that is permeable to the neuronal lipid bilayer membrane, it is reasonable to assume that this teratogen can cause cellular damage within the cell (Li et al., 1994). However, this study shows that alcohol itself may not be sufficient to cause cellular damage alone to result in neuronal death, consistent with previous findings (Harding et al., 1998). Studies have shown ethanol withdrawal in the hippocampus leads to a significant increase in neuronal cell death in tissue undergoing withdrawal compared to control tissue (Prendergast et al., 2000).

Possible causative mechanisms include increased NMDA receptor expression and hyperexcitable state of the central nervous system in ethanol's absence (Prendergast et al., 2004). This hyperexcitable state of glutamatergic excitotoxicity is not present while alcohol is in the tissue, which may explain why there was no difference in cellular death during continuous alcohol exposure. The present results indicate that a mechanism beyond mere alcohol exposure may be the cause of hippocampal damage and behavioral dysfunction experienced with chronic alcohol use. By ruling out this mechanism, conclusions about other measures are strengthened.

4.1.2 Sustained NeuN Expression Regardless of Ethanol Exposure

In these experiments, there was no difference in NeuN fluorescence between control and ethanol-exposed tissue, regardless of sex or region. This means that there is no difference in the amount of mature hippocampal neurons in control tissue and ethanol tissue. This finding is important because it further solidifies the idea that alcohol is not causing excitotoxic cell death because there is a similar expression of mature neurons in control tissue compared to ethanol tissue. This finding is also important to differentiate between neurons that are fully matured and integrated versus neurons that are currently differentiating and proliferating. By having a specific measure for healthy mature neurons, it distinguishes the effects of alcohol on already developed neurons versus those that are still developing. Without evidence regarding ethanol's effects on mature neurons, it cannot be concluded that the effects of alcohol are specific to the newly developing neurons. The present data indicates that these mature neurons do not seem to be changed by alcohol exposure. Studies have shown that as PI uptake increases, NeuN fluorescence decreases (Berry et al., 2016), suggesting that as more neurons die, there will be less mature neurons

expressed. The findings are consistent with the relationship of these markers as there is no change in the amount of cell death nor the expression of mature neurons.

4.1.3 Regionally Universal Decreases in BrdU Expression

The present results show a decrease in BrdU expression in all investigated regions of the hippocampus, regardless of sex. Therefore, a noted decrease in cellular division in each of these regions is likely. BrdU is substituted for thymidine during cell division, thus a reduction in the expression of BrdU means that there is less cell division occurring in these hippocampal regions. Clearly, less cellular division indicates a corresponding decrease in daughter cell production. This indicates that ethanol is interfering in the production of new cells in a manner yet to be defined. This is important because the creation and integration of new cells is a critical component of neural plasticity in the hippocampus, which is important for learning and memory (Spalding et al., 2013; Clelland et al., 2009). Ethanol's interference with the creation of new cells in the hippocampus may be implicated in the learning and memory deficits seen in people who chronically abuse alcohol. While this is a promising finding, it is limited by the fact that this marker indicates any newly dividing cell and is not specific to neurons (Matatall et al., 2017). The neuronal projections in the hippocampus are a major component in the neuronal plasticity involved in learning and memory. The lack of specificity of cell types marked by this measure limits the conclusions that can be drawn about this neural circuitry, but it still illuminates a promising piece of information.

4.1.4 Regional Decreases in DCX Expression

These experiments showed a decrease in DCX expression in the CA3 region, regardless of sex, indicating significantly lower expression of developing neurons as

compared to control tissue. This marker is specific to developing neurons as doublecortin is expressed for the first ten days of a neuron's life when NeuN is in turn upregulated, so this marker differentiates developing neurons from mature neurons (Brown et al., 2003). The difference in expression of newly developing neurons upon exposure to ethanol compared to no change in expression of mature neurons indicates that ethanol's impact on hippocampal neurons may be specific to newly developing neurons. It makes sense that the CA3 alone would be affected because axonal projections from developing neurons located in the DG go through the CA3 region. DCX is a structural protein, so expression in the axons would be most likely, therefore explaining why the change in expression was seen in the CA3. This supports the BrdU findings because if cell division is decreased there will in turn be a decrease in the expression of immature neurons. While DCX is specific to neurons, unlike BrdU, further confirmation of impaired neuronal development using additional methods is required to describe ethanol's apparent interference in neural production.

4.1.5 Overall Implications

Taken together, these findings suggest that chronic ethanol exposure in the hippocampus may be interfering with production of new neurons, as evidenced by a decrease in both BrdU and DCX expression in ethanol-exposed tissue compared to controls. These findings are consistent with previous studies that suggest chronic alcohol exposure impairs hippocampal neurogenesis (Morris et al., 2010). These findings also suggest that cellular death is not a mechanism of ethanol-induced hippocampal dysfunction as displayed by no change in PI or NeuN expression of ethanol-exposed tissue compared to controls.

4.2 Limitations

Slice culture methodology was chosen to isolate and manipulate brain tissue that cannot be executed in a live organism. By adding ethanol directly to the tissue with no other influences, the direct effects of ethanol can be more confidently concluded. However, by isolating a slice of the brain, the connections to other brain structures and organic operation of a live brain cannot be observed. Severing connections to other brain structures may also change the operation of that specific slice of tissue in its cultured environment. This limits the translatability to a living organism, but it still provides important insights about how the hippocampus specifically is impacted by ethanol exposure.

The timing of antibody administration is also a potential limitation of this study. These experiments capture the expression of these markers at a designated time point in the exposure. It is possible that the inclusion of additional timepoints may have revealed different effects of alcohol. The time point was chosen strategically to see the state of this structure after several days of exposure to alcohol as those were the effects of interest. However, it is possible there was cellular death in the early days that the timing of this marker did not capture. But, including NeuN as an additional marker would capture the early loss of cells by showing a decrease in the expression of mature neurons, which was not the case in the present study. An examination of the effects of a longer exposure to ethanol should also be examined as this time frame may be too early to catch some of the effects. These findings are still valuable and informative, but the temporal nature of the findings must be considered when translating or applying these results.

Ideally, each of these markers would be examined within a single slice of tissue to know the expression of each marker in that slice. However, due to limited wavelengths on the microscope for imaging, it was not possible to have four different wavelengths of

excitation to differentiate each marker. To avoid the spectral overlap and false inflation of fluorescence due to bleed through of another marker, each slice was analyzed for only one marker. It is reasonable to believe that there would not be significant enough differences between slices to cause different results in one slice of the hippocampus from a singular pup and its littermates. To control for individual slice defects, there were at least 12 slices in each manipulation for each marker, and this was replicated in another litter as well to ensure the effects were not slice or litter specific.

AUD is a complex disorder, so creating a model that accounts for every influence and aspect of this disorder while isolating the variables of interest is not yet possible. Specifically, the present study modeled a high-dose binge across several days but does not incorporate other aspects of AUD including withdrawal. This model also removes the behavioral aspects of binge drinking as ethanol is given directly to the tissue across time instead of voluntary consumption in a human. While there are differences in the pharmacokinetics of ethanol reaching the brain, the concentration of ethanol given to the slices is representative of a BAC of ~0.23. While the BAC threshold to be considered a binge episode is ~0.08 (Lange and Voas, 2001), 0.23 is within the range of human consumption and significantly under the lethal dose of 0.45 (Minion et al., 2008). Additionally, Corbin et al. (2014) questioned the utility of this BAC as a marker of binge drinking, as it does not capture the higher BACs that lead to the negative behavioral consequences associated with binge drinking. While the method of administration may differ, this model still offers valuable insights into the effects of binge-level alcohol concentrations in the brain that cannot be examined in vivo.

4.3 Applications

These findings that alcohol is interfering with the development of hippocampal neurons is critical information that can inform interventions for those with an AUD. Complete abstinence is often a challenging or undesirable goal for those with AUD due to the negative withdrawal symptoms (McKeon et al., 2008) and the habitual nature of their drinking behaviors (Barker and Taylor, 2014). Due to the challenge of getting people to completely abstain from drinking alcohol, a common approach in treatment of substance use disorders is harm reduction. In this treatment strategy, the goal is to allow continued use of the substance while mitigating some of the consequences of the drug (Muckle et al., 2012). In the case of alcohol abuse, learning and memory impairments are something that can be targeted for mitigation if alcohol use is going to be continued. By understanding the mechanisms underlying these behavioral deficits under chronic alcohol abuse, interventions can be appropriately targeted. These studies reveal a potential mechanism to target interventions for these behavioral deficits.

4.4 Future Directions

These findings are a building block upon which to continue understanding the mechanisms of alcohol-induced neural alterations implicated in behavioral changes from continued alcohol abuse. As indicated by this study, the neural consequences of prolonged alcohol exposure seem to influence the creation of new neurons. This is a broad finding, and the specific mechanisms by which alcohol is inducing these changes need to be further investigated. Additionally, these results alone are not enough to conclude that neurogenesis is impaired. Other validated markers of neurogenesis should be utilized to confirm this is the affected process influencing reduced expression of developing neurons.

There are several explanations for the decreases in DCX expression that should be further investigated. One possibility is adding a substance to initiate neurogenesis to see if this overcomes alcohol's blunting effects. This would suggest that decreased DCX is due to a failure to initiate the creation of new neurons. A pilot study investigating this was started to see if NPY would initiate neurogenesis in ethanol-exposed tissue since it has been shown to induce production of new neurons (Decressac et al., 2010). In the pilot study, the concentration of NPY used appeared to be toxic, so the concentration needs to be optimized to continue this possibility. Other methods to promote neurogenesis in ethanol-exposed tissue would also provide valuable mechanistic information.

Another avenue that should be explored is the axonal and dendritic integrity of newly developed neurons. Since the reduction in DCX expression was specific to the CA3, these results suggest the axonal projections of the newly developed neurons may be influenced by alcohol. Studies should investigate the integrity of these axons and if there are structural differences in these ethanol-exposed axons compared to controls and the axons of mature neurons that have also been exposed to alcohol. Studies have also shown that continued alcohol exposure impacts dendritic integrity (Lindsley and Clarke, 2004), so dendritic integrity in developing neurons should be examined as well to examine hippocampal neuronal circuitry.

Migration of these newly developed neurons should also be investigated as a potential explanation for the decrease in DCX expression upon ethanol exposure. The migration and integration of the newly developed hippocampal neurons is critical for them to be functional in the hippocampal circuitry. If ethanol is impairing newly developing neurons from migrating and being integrated into the pathway, they will be unable to

survive and will most likely undergo phagocytosis or apoptosis, as consistent with studies showing reduction in survival of new cells upon alcohol exposure (Morris et al, 2010). This possible mechanism of alcohol-induced reductions in DCX expression should be investigated as a potential explanation as well.

4.5 Conclusions

These studies show that continual ethanol exposure is interfering with proper hippocampal functioning. Reduced DCX and BrdU expression suggest that ethanol's effects may be differentially impacting developing neurons as compared to mature neurons. This aligns with behavioral deficits of impairment in learning and memory displayed with chronic alcohol abuse. Development and integration of new neurons is critical for neural plasticity and creating new memories, so it would make sense that these neurons are impaired. Understanding how ethanol interferes with the development of neurons would greatly aid in interventions and treatments for the cognitive symptoms of AUD.

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