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Probing the Functional Relevance of Reactive Hippocampal Neurogenesis in a Model of an Alcohol Use Disorder

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PROBING THE FUNCTIONAL RELEVANCE OF REACTIVE HIPPOCAMPAL NEUROGENESIS IN A MODEL OF AN ALCOHOL USE DISORDER

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Pharmacy
at the University of Kentucky

By
Chelsea Rhea Geil Nickell

Lexington, Kentucky

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2016

ABSTRACT OF DISSERTATION

PROBING THE FUNCTIONAL RELEVANCE OF REACTIVE HIPPOCAMPAL NEUROGENESIS IN A MODEL OF AN ALCOHOL USE DISORDER

Alcoholism, or alcohol use disorders (AUDs), represent a major public health concern both locally and globally. Critically, excessive alcohol consumption results in neurodegeneration in brain regions such as the hippocampus which is known for its role in learning and memory. Recovery of hippocampal volume loss has been observed after prolonged abstinence, but the mechanisms underlying this process are not well understood. Adult neurogenesis is thought to contribute to this recovery since after alcohol exposure a reactive increase in adult neurogenesis is observed. This reactive neurogenesis (the process by which brain insult results in a compensatory increase in neurogenesis) may represent a beneficial endogenous mechanism of brain recovery. However, the role of alcohol-induced reactive neurogenesis in hippocampal recovery is not known. We hypothesize that this reactive neurogenesis promotes the functional recovery of the hippocampus following a 4-day binge model of an AUD. To that end, the experimental studies herein were developed to decrease reactive proliferation to study its impact on hippocampal function (learning and memory). The first set of experiments attempted to decrease reactive proliferation by administering alcohol during the period of increased proliferation. Although these experiments were unsuccessful, they triggered an informative investigation into the timing of alcohol-induced reactive neurogenesis. The second set of experiments established an in-depth time course and revealed that increased proliferation occurred 5-7 days after binge alcohol exposure. With the new time course of proliferation in mind, the third set of experiments used an anti-proliferative drug that successfully decreased alcohol-induced reactive proliferation/neurogenesis but revealed no differences between groups in a hippocampal-dependent task. The fourth set of experiments revealed that granule cells born during reactive neurogenesis were capable of activation. In conclusion, the finding that reactive neurogenesis resulted in neurons capable of activation indicated that reactive neurogenesis may be involved in promoting the recovery of hippocampal function. These studies furthered our understanding of the role of alcohol-induced reactive neurogenesis in recovery

from AUDs. Furthermore, these studies provided valuable insight into modulation of the NPC pool as a potential therapeutic target for the treatment of AUDs.

KEYWORDS: Alcohol use disorder, neurogenesis, reactive proliferation, reactive neurogenesis, binge ethanol exposure.

Chelsea Rhea Geil Nickell

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10 – 17 – 2016

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Dedicated to my loving parents, Philip and Mary

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CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

This chapter contains excerpts from a previously published manuscript (Geil et al., 2014) which have been reproduced with permission from Elsevier and in accordance with guidelines provided by the University of Kentucky Graduate School.

Adult neurogenesis is now widely accepted as an important contributor to hippocampal integrity and function but also dysfunction when adult neurogenesis is affected in neuropsychiatric diseases such as alcohol use disorders (AUDs). Excessive alcohol consumption, the defining characteristic of AUDs, results in a variety of cognitive and behavioral impairments related in part to the structural and functional integrity of the hippocampus. Recent preclinical work has shown that adult neurogenesis may be one route by which alcohol produces hippocampal neuropathology. Alcohol is a pharmacologically promiscuous drug capable of interfering with adult neurogenesis through multiple mechanisms. This introduction discusses AUDs and the primary mechanisms underlying alcohol induced changes in adult hippocampal neurogenesis including alcohol's effects on neurotransmitters and hippocampal integrity.

1.2 ALCOHOL AND ALCOHOL USE DISORDERS

1.2.1 Alcohol use disorders

According to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) AUDs, more commonly called alcoholism, are classified based on disease severity (mild, moderate, severe); which corresponds

to the number of diagnostic criteria an individual possesses (American Psychiatric Association et al., 2013). A sampling of the 11 criteria include 1) drinking more or for longer than intended, 2) unsuccessfully trying to reduce or stop drinking, or 3) continuing to drink despite negative consequences (American Psychiatric Association et al., 2013). Individuals are considered to have an AUD if they meet a minimum of 2 criteria and 6+ criteria is indicative of a severe AUD.

AUDs represent major social and economic problems as well. Nearly 13.9% of the US population met the diagnostic criteria (in the last 12 months) for an AUD (Grant et al., 2015). Additionally, the economic burden was estimated to be $223.5 billion dollars annually (Bouchery et al., 2011). Further, excessive alcohol consumption (including binge drinking) is the third leading cause of preventable death in the United States (Mokdad et al., 2004). Alcohol is responsible for approximately 88,000 deaths per year (Centers for Disease Control and Prevention, 2006). More specifically, excessive alcohol consumption was responsible for 1 in 10 deaths of working aged individuals in the US (Stahre et al., 2014).

Although there are many theories about the development of AUDs, all involve the fact that repeated bouts of excessive alcohol intake change the brain in a way that drives a loss of control over consumption (Koob and Le Moal, 1997). This loss of control may be driven by hijacked learning processes, impairments in behavioral control, and/or impaired decision making (Crews, 1999; Noel et al., 2013). All of these processes involve, at least partially, the contribution of an intact hippocampus, and a wide variety of studies have shown

that excessive alcohol intake impacts the structure and function of hippocampal circuitry.

1.2.2 Alcohol

Alcohol (ethanol) is a small, highly lipid soluble and pharmacologically promiscuous drug capable of penetrating virtually every organ system including the brain. Alcohol, more specifically ethanol, may have stimulant-like effects at small concentrations but, unsurprisingly for those who have imbibed, it is in fact a CNS depressant. Alcohol can have variable effects on individuals based on their body size, gender, and race. Individuals of Asian descent are more prone to facial flushing. Females are generally smaller than males and have less body water, this results in less fluid for alcohol to be diluted in and therefore, women become intoxicated with lower doses of alcohol. As such, charts used to determine blood ethanol concentration (BEC) are based on weight and sex.

Alcohol consumption decreases blood flow to the cerebellum which could account for deficits in hand-eye coordination and delays in reaction time which makes driving very dangerous (Volkow et al., 1988). Excessive alcohol consumption can also affect memory storage and retrieval; in extreme cases it is called a blackout and can be categorized as a partial (greyout) or complete blackout (McKim, 2007). During a partial blackout (greyout) individuals remember bits and pieces of events that occurred while they were intoxicated and can be reminded of things and will remember them. On the other hand, if an individual had a complete blackout, the memories were never put into long-term storage, which results in total memory loss (White, 2003). Individuals are conscious and

appear to have intact recall of past events but long-term memories were not created. This is likely caused by alcohol's effect on the hippocampus, a brain region needed to form long-term memories (McKim, 2007; White, 2003).

Excessive alcohol consumption has widespread deleterious effects on many organ systems, but central nervous system injury is a critical consequence as 50 to 75% of alcohol-dependent adults show permanent cognitive impairment (Eckardt and Martin, 1986). These impairments are thought to be due to both structural and functional changes resulting from excessive alcohol consumption (Crews, 1999; Sullivan and Pfefferbaum, 2005). Alcohol appears to target some brain regions more than others with a critical cluster of alcohol-induced impairments in behavioral control, learning, memory, mood, and decision-making attributed, at least in part, to the integrity of the hippocampus (Mechtcheriakov et al., 2007). Because ethanol is so pharmacologically promiscuous, there are many reported and potential mechanisms of alcohol-induced effects on the hippocampus. The focus here is on the emerging role of adult hippocampal neurogenesis in alcoholic neuropathology and the various potential mechanisms involved in alcohol's effects on neural progenitor cells (NPCs).

1.2.3 Alcohol and neurodegeneration

Excessive alcohol consumption results in neurodegeneration in several brain regions. Alcoholism in humans results in volume loss in cerebral cortex and subcortical brain regions, reducing the number of both neurons (grey) and glial cells (white matter tracts; reviewed in Crews and Nixon, 2009). These cortical and subcortical regions include the limbic system, which is important for

emotional processing, essential brain communication, and memory, among other things (Oscar-Berman and Marinković, 2007). Interestingly, alcohol does not uniformly affect the brain; the prefrontal cortex, hippocampus, cerebellum, and white matter tracts and glial cells are the most affected (Alfonso-Loeches and Guerri, 2011; Sullivan and Pfefferbaum, 2005). Human studies show decreased brain size in postmortem and *in vivo* imaging studies (Oscar-Berman and Marinković, 2007). Brain weight and volume loss in chronic alcoholics is primarily due to white matter loss in the frontal lobe; however, several human and animals studies have also shown neuronal cell loss (Chanraud et al., 2007; Pfefferbaum et al., 1997; Sullivan and Pfefferbaum, 2005; Walker et al., 1980).

Glutamate's upregulation during alcohol withdrawal results in glutamate excitotoxicity (Chandler et al., 1993a, 1993b, Prendergast et al., 2000, 2004). *In vitro* studies suggested that excitotoxicity may contribute to neurodegeneration following alcohol exposure (Chandler et al., 1993a, 1993b). However, NMDA glutamate receptor antagonists have not been successful in reducing neurodegeneration caused by excessive alcohol exposure (Collins et al., 1998; Corso et al., 1998; Crews et al., 2004a; Hamelink et al., 2005). Additionally, silver stain (which is used to identify cell death) is observed during intoxication as early as day two in a four-day binge model of an AUD (Collins et al., 1996; Obernier et al., 2002a). In this four-day binge model of an AUD, peak cell death occurs on the fourth and final day of the binge exposure (Crews and Nixon, 2009). As such, it is unlikely that the mechanism of alcohol-induced neurodegeneration (*in vivo*) is glutamate excitotoxicity. An alternative mechanism of alcohol-induced

neurodegeneration is oxidative stress resulting from pro-inflammatory cell signaling pathways (Crews and Nixon, 2009). cAMP responsive element-binding protein (CREB) is a transcription factor that's involved in neuroprotection against oxidative stress (Lee et al., 2009) and during alcohol intoxication there is a reduction in DNA binding of CREB (Crews and Nixon, 2009; Zou and Crews, 2005). Thus, oxidative stress and CREB-related signaling cascades represent a potential mechanism of alcohol-induced neurodegeneration (because cell death occurs primarily during intoxication and intoxication is linked to decreased action of CREB and its downstream targets).

1.2.4 Alcohol and the hippocampus

Excessive alcohol consumption results in extensive deficits in neuropsychological functions, many of which involve the hippocampus (Chanraud et al., 2007; Ozsoy et al., 2013; Parsons, 1993). As has been reviewed extensively elsewhere (Belujon and Grace, 2011; Eichenbaum, 2001; Gilbert and Kesner, 2006; Johnson et al., 2007), the hippocampus is especially critical for aspects of learning and memory and as such is implicated in the acquisition, consolidation, and expression of memories associated with drug use (Hyman et al., 2006; Nixon et al., 2011). Many now suggest a role for the hippocampus in relapse and drug seeking as well (Belujon and Grace, 2011; Vorel et al., 2001). This expanded role has emerged from data indicating the hippocampus's broad role in cognitive functions, specifically through its interconnections with frontal cortices and reward systems. For example, glutamatergic efferents projecting from the hippocampus and terminating in the

prefrontal cortex (PFC) have been implicated in the proper processing of executive functions, working memory, and contextual information (Godsil et al., 2013). Therefore, disruptions in the structural integrity of the hippocampus may be an underlying substrate for impairments in these functions. Certainly, long-lasting deficits in executive function and working memory are observed following excessive alcohol consumption (O'Daly et al., 2012; Stavro et al., 2013; Stephens and Duka, 2008) and it is hypothesized that compromised hippocampal integrity in alcoholics may contribute to these impairments. In support of this hypothesis, a correlation between deficits in executive function and hippocampal gray matter volume have been reported (Chanraud et al., 2007). Although correlation does not imply causation, new evidence is mounting to support that hippocampal integrity may influence a more broad range of cognitive functions than classically considered. With new and improved technology, more consistent reports of hippocampal pathology have emerged in the last several years (Beresford et al., 2006; Ozsoy et al., 2013) including grey matter loss (Mechtcheriakov et al., 2007). Debate is evident in the human literature over whether the hippocampus is impacted by excessive alcohol; whether the left, right, or both hippocampi are affected, whether degeneration is due to effects in white or gray matter, and/or the magnitude of this effect (Agartz et al., 1999; Laakso et al., 2000; Sullivan et al., 1995). Agartz et al. (1999) found the left hippocampus to be larger than the right in men and women, but saw no hippocampal volume difference between alcoholics and healthy subjects. Although Agartz et al. (1999) did account for age and sex in their study, the more

recent study by Beresford et al. (2006) used more stringent inclusion criteria (excluding individuals with mental health issues like polysubstance abuse, schizophrenia, depression, and post-traumatic stress disorder) and an MRI capable of generating twice the magnetic field (Agartz et al., 1999; Beresford et al., 2006). Beresford et al. (2006) revealed a decrease in hippocampal volume in alcoholics compared to controls. The strict inclusion criteria and improved MRI resolution provided by new instruments are likely responsible for the divergent results from these studies. This study indicated that accounting for polysubstance drug abuse, and mental health with appropriate controls is likely important for accurately detecting differences in hippocampal volume. However, age should also be considered since alcohol is particularly toxic to the hippocampus of the adolescent brain (Ozsoy et al., 2013). Alcoholics that begin drinking during adolescence show greater hippocampal volume deficits than those that began drinking as adults (Ozsoy et al., 2013). However, greater anterior volume loss was reported in the hippocampi of older alcoholics (Sullivan et al., 1995). Regardless, the additive effect that alcohol damage has on the brain may be responsible for some age-related differences and for that reason a measure of lifetime alcohol consumption may be a better variable to control for than age of disease onset. Earlier studies did not always properly account for confounding variables such as sex differences, polysubstance drug use, mental health, and age differences. Importantly, when these variables are accounted for and groups are appropriately separated, as they are in more recent studies, the picture

becomes clearer and the debate declines in favor of new evidence which specifically shows hippocampal volume reductions in alcoholics.

Current imaging studies lack sufficient resolution to identify specific sub-regions of the hippocampus or cellular populations. Therefore, post-mortem studies in humans and experimental evidence in animal models have been necessary to offer insight into dentate gyrus specific effects and/or alcohol-induced neuronal loss. One study observed significant reductions in neuron number in all hippocampal subfields, including the dentate gyrus, in alcoholics less than 45 years old (Bengochea and Gonzalo, 1990). However, a more rigorous stereological estimation failed to observe neuronal loss in any hippocampal subfield, though these subjects were older, averaging 55 years of age (Harding et al., 1997). Although Harding et al. (1997) associated hippocampal volume deficits in alcoholic cases with white matter loss, others have proposed that astroglial loss underlies hippocampal neurodegeneration (Korbo, 1999). Nevertheless, animal models of chronic alcohol exposure have shown consistently that alcohol is toxic to hippocampal neurons, including the dentate gyrus granule cells (Cadete-Leite et al., 1988; Lukoyanov et al., 2000; Walker et al., 1980). Furthermore, animal models allow researchers to examine the effect of dose, duration and/or pattern of exposure which led to the discovery that subtle evidence of damage in the hippocampus is apparent after as little as 24-48 hours of high dose, binge-like ethanol exposure (Hayes et al., 2013; Obernier et al., 2002b). In the 4-day binge model of alcohol dependence, neurons are lost throughout the corticolimbic pathway with degenerating cells

particularly evident in the entorhinal cortex and ventral dentate gyrus (Collins et al., 1996; Crews et al., 2000; Kelso et al., 2011; Obernier et al., 2002a, 2002b). Importantly, these binge models mimic the high blood ethanol concentrations (BECs) experienced by binge drinking alcoholics, which is estimated to be 60% or more of the alcoholic population (Robin et al., 1998; Zeigler et al., 2005). Additionally, alcoholics who drink in a binge pattern are much more likely to have neurodegeneration (Hunt, 1993).

1.2.5 Recovery in abstinence

Human studies have shown that some portion of alcohol-induced neurodegeneration and impairments in cognitive function can recover with abstinence from alcohol (Bartels et al., 2007; Carlen et al., 1978; Gazdzinski et al., 2005; Mann et al., 1999; Pfefferbaum et al., 1995). Research has shown that hippocampal function returns to normal in alcoholics after two years of abstinence for about half of the study participants (Bartels et al., 2007). Another study noted that after four years of sobriety there were significant improvements in overall memory and short-term memory (Rosenbloom et al., 2004). In addition, hippocampal volumes increase and neuronal markers normalize one month into abstinence (Gazdzinski et al., 2008). For the hippocampus, a host of plastic changes were originally thought to underlie this recovery in rats, such as, dendritic expansion (Cadete-Leite et al., 1988, 1989a, 1989b) and spine density recovery (King, 1988); however, alcohol withdrawal may compromise some of these effects (Durand et al., 1989). Although it has long been hypothesized that this plasticity underlies recovery in hippocampal volume and/or function observed

in animals (Lukoyanov et al., 2000) and humans (Bartels et al., 2007), the contribution of these mechanisms seems insufficient to overcome the hippocampal volume loss (16% decrease in total hippocampal volume) observed in alcoholics compared to controls (Beresford et al., 2006). These theories, however, did not take into consideration the newly accepted phenomena of adult neurogenesis in the dentate gyrus (Armstrong and Barker, 2001; Nixon, 2006).

1.3 ADULT NEUROGENESIS – A CRITICAL COMPONENT OF HIPPOCAMPAL INTEGRITY

Adult neurogenesis, the process by which new neurons are created in the postnatal brain, occurs constitutively in two brain regions, the subventricular zone (SVZ) of the walls of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Altman and Das, 1965; Doetsch et al., 1999). Newly generated SVZ neurons migrate via the rostral migratory stream to the olfactory bulb which is primarily responsible for olfactory sensation. However, recent discoveries about the lack of newborn neurons in the adult *human* olfactory bulb (Bergmann et al., 2012) suggest that adult neurogenesis may only occur in the hippocampus of adult humans. Altman and Das's (1965) pivotal discovery of adult neurogenesis was not widely accepted until the late-1990s (Eriksson et al., 1998; Gross, 2000; Kaplan and Hinds, 1977; Palmer et al., 1997). Since then there has been a large increase in published work on adult neural stem cells (NSCs) and as such adult neurogenesis has fundamentally changed how we think about the hippocampus; specifically, the mechanism of ongoing neuron

generation and how this potential for regeneration impacts its function and dysfunction in neuropsychiatric disorders.

The hippocampal formation is part of the brain's limbic system and is involved in spatial navigation and short-term to long-term memory consolidation. Within the hippocampal formation lies the dentate gyrus, a structure unique in formation due to unilateral connectivity (Amaral et al., 2007; Jonas and Lisman, 2015; Treves et al., 2008). The dentate gyrus and the CA3 receive significant input from the entorhinal cortex. The entorhinal cortex projects to the dentate gyrus via a pathway commonly referred to as the perforant pathway which is thought to be the first phase in the production of episodic memory. Following the perforant pathway, the dentate granule cells project to the CA3, termed the mossy fiber pathway. All dentate granule cells project to the CA3 (Amaral et al., 2007). Interestingly, only 2-5% of granule cells are active while exploring a novel environment, indicating a low activity among the mossy fiber pathway's granule cell population (Snyder and Cameron, 2012). Additionally, a single granule cell only synapses with 11-15 CA3 pyramidal cells (Acsády et al., 1998) and multiple granule cells synapse onto one CA3 pyramidal cell (Amaral et al., 2007). Importantly, Snyder and Cameron indicated that an individual granule cell can "trigger firing in downstream CA3 targets" (Snyder and Cameron, 2012) and since CA3 pyramidal cells form widespread connections with one another it becomes easy to see that a single granule cell can have a sizeable impact. The next stage in the hippocampal connectivity pathway (following the mossy fiber pathway), is the Schaffer collateral pathway, where cells from CA3 project to the

CA1. Thus the entire hippocampal network involves the entorhinal cortex → dentate gyrus → CA3 → CA1 → back to the entorhinal cortex or to the subiculum (Jonas and Lisman, 2015).

1.3.1 Components of neurogenesis

In the hippocampus, it is now well accepted that new neurons are generated constitutively throughout life in a multi-step process that originates from radial glia-like stem cells that reside along the SGZ of the dentate gyrus (Zhao et al., 2008). Adult hippocampal neurogenesis consists of multiple stages that cells progress or mature through. Stem-like radial glia act as the NSCs which can proliferate and generate NPCs which have limited self-renewal and a more restricted cell fate. NSCs are capable of producing both glial and neuronal NPCs to generate neurons, glial, or endothelial cells (Bonaguidi et al., 2011, 2012; Dranovsky et al., 2011; Encinas et al., 2011; Gage, 2000). The NPC term is used throughout this document when it is not clear which cell type, the NSC or one of the progenitors, has been examined or if the distinction was not made.

The process of adult neurogenesis begins with Type 1 radial glia-like NSCs (Figure 1.1). Type 1 cells are located in the SGZ and have vascular end feet (characteristic of astrocytes), they display morphological and electrophysiological astrocytic properties, and they express cellular marker combinations unique to NSCs (Filippov et al., 2003; Kempermann et al., 2015). Type 1 NSCs are capable of proliferation but tend to divide infrequently and asymmetrically to generate Type 2 progenitor cells (Figure 1.1 B). Type 2 NPCs are primarily responsible for the expansion of NPCs because they are highly proliferative

Figure 1.1 - Drawing of the hippocampal dentate gyrus

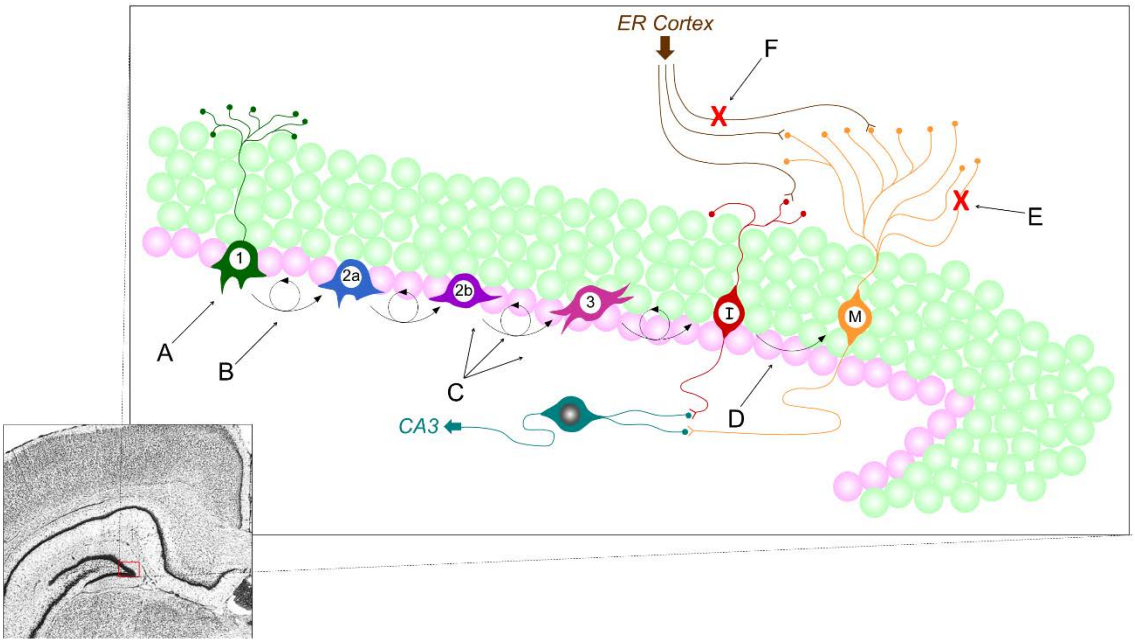


Figure 1.1 - Schematic representation of adult neurogenesis and known ways in which alcohol affects this process. Type 1, Type 2, and Type 3 neural stem/progenitor cells can be seen along the SGZ located at the intersection of the GCL and the hilus. Circular arrows indicate that the cell is capable of replicating. I and M indicate immature neurons and mature neurons, respectively (Gage, 2000; Zhao et al., 2008). Figure reproduced from Geil et al., 2014.

<u>Alcohol impacts these processes at multiple stages:</u>

(A) Alcohol dependence may kill progenitor cells with long duration, chronic exposure (Richardson et al., 2009; Taffe et al., 2010).

(B) Alcohol intoxication reduces NPC proliferation whereas reactive increases in neurogenesis occur one week into abstinence (Nixon and Crews, 2002, 2004). Alcohol also alters the cell cycle of NPCs (McClain et al., 2011).

(Figure 1.1 continued)

(C) Multiple ways in which alcohol may directly affect the neurogenic niche, notably alcohol produces reactive astrocytes and reactive microglia (He et al., 2005; Kelso et al., 2011; Marshall et al., 2013, 2013).

(D) High peak blood ethanol concentrations are likely required to impact survival of new born cells (Herrera et al., 2003; Nixon and Crews, 2002).

(E) Chronic alcohol exposure blunted dendritic arborization and impaired dendritic spine density in newborn cells (Golub et al., 2015; He et al., 2005; Staples et al., 2015), an effect that would have functional implications for new cells incorporation into hippocampal circuitry.

(F) Binge alcohol exposure kills entorhinal cortex cells that project to the hippocampal dentate gyrus (Collins et al., 1996; Kelso et al., 2011).

(Figure 1.1 C) (Kempermann et al., 2015). Additionally, Type 2 cells are further differentiated (Type 2a and Type 2b) since these two cell types possess different cell fates. Type 2 NPCs do not have the radial morphology seen in Type 1 NSCs but still express the glial/stem cell marker Sox2 (Filippov et al., 2003; Fukuda et al., 2003; Kempermann et al., 2015). Type 2a NPCs can give rise to Type 2b NPCs which express neuronal specific markers (ex. NeuroD, DCX, and Prox1), indicating a neuronal fate (Steiner et al., 2006). Previous research has shown that the transcription factor Eoms (tbr2) is capable of suppressing Sox2 which is thought to be important in the transition of NSCs to NPCs with a neuronal fate (Hodge et al., 2012; Kempermann et al., 2015). As the Type 2b early neuronal progenitor cells mature they give rise to Type 3 NPCs which continue to express early neuronal cell markers (ex. NeuroD) and although Type 3 cells are still fully capable of replicating, it occurs less frequently than it does in the rapidly multiplying Type 2 cells (Kempermann et al., 2015). NeuroD is a transcription factor expressed in Type 2b, Type 3, and (early) immature neurons. NPCs exit the cell cycle becoming postmitotic as they transition from Type 3 cells and become immature neurons (neuroblasts). Neuroblasts extend axons and dendrites as they migrate and integrate into the granule cell layer (Sun et al., 2013). Immature neurons begin to develop dendritic spines when they are around 16 days old (Zhao et al., 2006). Excitatory synapses are first seen when cells are 21 days old (Kelsch et al., 2008; Kempermann et al., 2015). Unsurprisingly, as the neurons mature (into mature granule cells) the dendritic spines become progressively longer, thicker, and more intricate until neurons are

around 8-10 weeks old, at which point they plateau (Llorens-Martín et al., 2015a; van Praag et al., 2002). Interestingly, neurons are considered mature when they are 28 days old. However, young neurons (~6-8 weeks old), although mature, have increased synaptic plasticity compared to older neurons (Ge et al., 2007). When compared to older mature neurons, young neurons require lower thresholds to induce long-term potentiation (Schmidt-Hieber et al., 2004; Wang et al., 2000). Furthermore, young mature neurons (6-8 weeks old) can be preferentially incorporated into hippocampal networks (Kee et al., 2007). Once the neuronal maturation process is complete (8-10 weeks) the new granule cell neurons are considered fully integrated into hippocampal circuits and these neurons can no longer be distinguished from older neurons; they are electrophysiologically and morphologically identical (Llorens-Martín et al., 2015a, 2015b; Zhao et al., 2006).

1.3.2 Adult neurogenesis and hippocampal function

Adult neurogenesis generates new neurons throughout life. Approximately 6% of the GCL is generated each month in the adult rat (Cameron and McKay, 2001). Although, not every cell that divides survives through incorporation into the hippocampus. Baseline rates of proliferation, survival, and incorporation vary across species, strain, age, and sex (Kempermann, 2012; Kempermann and Gage, 2002a; Zhao et al., 2008). While this phenomenon of adult neurogenesis is now accepted as a contributor to the structural integrity of the hippocampus (Imayoshi et al., 2008), the functional role of newborn neurons remains unclear. Early work relied upon correlation to implicate adult neurogenesis in many of the

same functions of the hippocampus (van Praag et al., 1999). However, conditional and inducible transgenic animals have provided the most compelling evidence for the role of adult neurogenesis. These animals have allowed for the discovery that adult hippocampal neurogenesis is critical for a computational process termed pattern separation associated with hippocampal dentate gyrus (Deng et al., 2010). Pattern separation is a process that acts to enhance differences between similar experiences so they can be distinguished from one another (Clelland et al., 2009; Nakashiba et al., 2012). For example, an individual may park their car in the same garage every day for work but the exact position of the car is likely to change from day to day. The hippocampus uses pattern separation to distinguish between today's parking spot and yesterday's parking spot by highlighting subtle differences between the two events. In addition to transgenic and knock-out animals providing evidence for hippocampal pattern separation, knock-out animal models have also helped resolve contradictory results across the hallmark of hippocampal function in rodents, spatial learning and memory. Well-controlled, genetic manipulations by inducible knockouts or lentiviral transgene delivery are able to decrease neurogenesis then show, fairly consistently, impairments in spatial memory (Marin-Burgin and Schinder, 2012). More importantly, of those that did not detect deficits with the classic test of spatial memory, the Morris water maze (MWM), several did report impairments in other hippocampus-dependent behavioral tasks such as contextual fear conditioning (Saxe et al., 2006), trace fear conditioning (Shors et al., 2002), or place recognition (Madsen et al., 2003). Therefore, although there are some

contradictory results, new technologies are helping to resolve these issues to strengthen the conclusion that decreases in adult neurogenesis result in some kind of deficit in hippocampal-dependent behavior (Marin-Burgin and Schinder, 2012; Zhao et al., 2008).

1.4 ALCOHOL AND HIPPOCAMPAL NEUROGENESIS

For over 30 years, scientists have speculated that a direct relationship exists between alcohol intake and disruptions in hippocampal integrity (Walker et al., 1980). Preliminary conjecture coupled with extensive literature revealing overt cell death following alcohol exposure *in vivo* led to the original assumption that cell loss in the hippocampus was the result of dying neurons (Bengochea and Gonzalo, 1990; Collins et al., 1996; Walker et al., 1980). However, this theory failed to incorporate the then controversial idea that adult neurogenesis was an essential component of hippocampal structure and function (Zhao et al., 2008). In fact, more recent data utilizing adult and adolescent alcohol exposure models strongly suggest that alcoholic neuropathology is at least *partially* due to an attenuation of adult neurogenesis (Crews et al., 2006b; Morris et al., 2010a; Nixon and Crews, 2002). In this same 4-day binge model of an AUD, hippocampal integrity is impacted, resulting in an 16% loss of granule cells despite a lack of equivalent levels of cell death (Leasure and Nixon, 2010). Therefore, alcohol likely impairs hippocampal integrity, in part, through its effects on adult neurogenesis (Morris et al., 2010a). As described above, adult neurogenesis is a process comprised of multiple components: cell proliferation, differentiation, migration, and survival/integration. Alcohol exposure can affect

neurogenesis at any one of these individual phases or through a combination of effects at multiple stages (Figure 1.1). Indeed, the various phases are differentially affected by alcohol intoxication, alcohol dependence, and other sequelae that result from chronic alcohol exposure such as seizures and/or neurodegeneration. Therefore, the effects of alcohol intoxication versus alcohol withdrawal and abstinence are discussed separately. The majority of *in vivo* studies have shown that alcohol intoxication leads to an overall decrease in neurogenesis through alcohol's effects on cell proliferation and cell survival (Anderson et al., 2012; Crews and Nixon, 2009; Golub et al., 2015; Herrera et al., 2003; Jang et al., 2002; Nixon and Crews, 2002; Pawlak et al., 2002; Richardson et al., 2009; Taffe et al., 2010), while increased neurogenesis has been observed in abstinence following alcohol dependence (He et al., 2009; Nixon and Crews, 2004). Although a few studies showed no effect *or* an increase in proliferation resulting from 10 days (Rice et al., 2004), 2 weeks (Aberg et al., 2005), 6 weeks (Herrera et al., 2003), or 10 weeks (Aberg et al., 2005) of chronic alcohol exposure, these differences could result from the use of different animal models, the animal's BEC at time of sacrifice/analysis, the timing of analysis with respect to alcohol exposure, and methods used to label proliferating cells (Nixon, 2006). Despite all of these explanations, an interesting trend can be observed from these reports: ethanol intoxication appears to dose-dependently inhibit NSC proliferation. This dose-dependency is noted since low BECs appear to increase NSC proliferation (Aberg et al., 2005), while higher doses decrease it (Nixon, 2006). This dose-dependent effect has been shown *in vivo* with acute doses in

adolescent rats (Crews et al., 2006b) and can be extrapolated from a similar correlation in mice (Contet et al., 2014). Determining or estimating the BEC at the time of sacrifice and labeling of the proliferating population continues to support the view that some level of significant intoxication, perhaps above 80 mg/dl, is necessary to inhibit proliferation (Aberg et al., 2005; Crews et al., 2006b; Nixon, 2006). Recent publications continue to support this point. For example, rats that were fed an ethanol containing liquid diet had an average BEC of 86.4 mg/dl (0.08%) and showed a 40% decrease in proliferation (Anderson et al., 2012). It is important to note that these BECs were obtained from animals at sacrifice, which is typically done during lights on, at the start of the sleeping period for rodents, and thus 12+ hours past when they would likely have had a drinking bout and BECs would have peaked. Therefore, alcohol may have reduced proliferation in some of these divergent studies, but the sacrifice time point missed the time of peak intoxication and therefore peak inhibition of cell proliferation. Alternatively, it is of note that the two studies that have observed an increase in proliferation have been in mice (Aberg et al., 2005; Pawlak et al., 2002). Interestingly, Snyder and colleagues showed that when compared to mice, rats generally had more adult born neurons that matured quicker and were more involved in behavior (Snyder et al., 2009). Mice also catabolize ethanol much faster than rats (Livy et al., 2003), meaning withdrawal events will occur relatively earlier.

A few studies have observed persistent decreases in proliferation despite animals being days to weeks past their last exposure and therefore no longer significantly intoxicated. The number of bromodeoxyuridine- and Ki76-positive

cells (exogenous and endogenous proliferation markers, respectively) remains decreased compared to controls in these reports, but it is worth noting that the majority of these reports were from long-term chronic exposure models with weeks or months of ethanol exposure (Broadwater et al., 2014; Contet et al., 2014; Hansson et al., 2010; Richardson et al., 2009; Taffe et al., 2010). Therefore, the duration (i.e. number of days, whether repeated binge-like or chronic) may impact the number of proliferating progenitors. Alcohol also potentially kills NSCs and/or NPCs according to long-term alcohol dependence models, again highlighting that dose and duration of exposure may be critical factors in understanding the mechanism of alcohol's effects on NSCs, NPCs, and adult neurogenesis (Contet et al., 2014; Hansson et al., 2010; Nixon and Crews, 2002; Richardson et al., 2009; Taffe et al., 2010).

Although hippocampal NPC proliferation is decreased during alcohol intoxication, the percentage of newly born cells that differentiate into neurons or glia appears unchanged. However, no studies have examined differentiation specifically. Several intriguing reports on alcohol induced effects in human NSCs within the context of Fetal Alcohol Spectrum Disorders (Vangipuram and Lyman, 2010; Zhou et al., 2011) suggest that differentiation might be a potential area for discovery in AUD models. NPC/neuroblast survival is generally decreased in alcohol exposure models, but again, this effect is heavily dose dependent, i.e., higher doses of alcohol produce greater decreases in new cell survival (Nixon and Crews, 2002; Richardson et al., 2009). This dose and duration dependency is not surprising as alcohol-induced neuronal cell death, in general, is also

hypothesized to be dose and duration dependent. Several groups, including ours, speculate that sustained BECs greater than 200 mg/dl (0.20%) are necessary to produce neurodegeneration (Collins et al., 1996). In support of this idea, a single 5 g/kg dose of alcohol, which only transiently produces >0.20% BEC, does not inhibit survival of hippocampal NPCs (Nixon and Crews, 2002).

In addition to alcohol affecting proliferation, it also affects the developing dendritic arbors of immature post-mitotic neurons (Figure 1.1 E). Chronic alcohol consumption decreases the number, size, and thickness of dendrites in new neurons, effectively blunting dendritic arborization (Golub et al., 2015; He et al., 2005). This dendritic blunting likely has functional implications as young neurons become incorporated into the hippocampal circuitry since the number of dendrites (connections) are reduced. Reduced neural connectivity can result in impaired neuronal processing ability.

1.5 ALCOHOL AND REACTIVE NEUROGENESIS

Here the process of neurogenesis is described using the general term neurogenesis (which encompasses all stages of neurogenesis: proliferation, differentiation, migration, and maturation), or using the more specific term, proliferation (which refers only to the proliferation stage of the neurogenesis process). Although chronic alcohol intoxication inhibits multiple aspects of adult neurogenesis, a different set of effects on adult neurogenesis are observed in abstinence after intoxication. The most distinct change involves a large, transient (reactive) increase in NPC proliferation seen well after the cessation of alcohol exposure in both binge and chronic alcohol models. Specifically, in a 4-day binge

model of alcohol dependence, a burst in NPC proliferation (called reactive proliferation) occurs after one week of abstinence, leading to an increased number of newly formed mature neurons four weeks later (Nixon and Crews, 2004). Notably, proliferation returns to control levels by at least two weeks following the last ethanol dose, which emphasizes the short-lived nature of the reactive neurogenesis response (Nixon et al., 2008). Similar increases in NPC proliferation and neurogenesis in abstinence have been described in alcohol-preferring rats subjected to a seven-week two bottle choice paradigm and in rats exposed chronically to ethanol via vapor chambers (Hansson et al., 2010; He et al., 2009). In contrast, Taffe and colleagues reported a decrease in NPC proliferation and neurogenesis after two months of abstinence in adolescent rhesus macaques given alcohol chronically for 11 months (Broadwater et al., 2014; Taffe et al., 2010).This discrepancy may involve several factors related to the vastly different models: species of the animals, the time in abstinence that was analyzed, and possibly even the age of the animals. In addition, given the transient nature of reactive neurogenesis observed early in abstinence in rodent models of alcohol exposure, it is possible that a similar transient change occurred but was missed due to the timing of analysis (which was months into abstinence). Importantly, if a short-lived increase in neurogenesis was missed, the subsequent return to an overall decrease in neurogenesis represents a potential barrier to full recovery of hippocampal structure and function. For this reason, understanding the mechanisms responsible for reactive neurogenesis may be

important for designing strategies aimed at sustaining neurogenesis at normal levels after long-term abstinence (Mandyam and Koob, 2012).

The specific components and the degree to which each stage of adult neurogenesis is affected by alcohol depend upon the dose, duration, and pattern of alcohol exposure as well as the age of the organism (Nixon, 2006). The mechanisms of alcohol induced effects on neurogenesis have yet to be described and remain a critical gap in understanding the role of alcohol-induced brain damage in AUDs. In the following section, select plausible interactions are discussed through which alcohol may exert its effects on adult neurogenesis.

1.6 POTENTIAL MECHANISMS OF ALCOHOL'S EFFECTS ON ADULT NEUROGENESIS

Excessive consumption of alcohol results in behavioral and neurochemical changes that are difficult to isolate due to the promiscuous pharmacology of ethanol on the brain. Ethanol's effects range from direct modulation of many neurotransmitter systems to indirect outcomes that are caused by or are comorbid with alcohol dependence. Alcohol's effects also vary depending on dose and duration of exposure, especially noting that a variety of neuroadaptations occur with the development of dependence (Vengeliene et al., 2008). Here, the direct effects of ethanol on neurotransmission are summarized. However, it is likely that neurotransmission, cell signaling, and ethanol’s cellular effects on the neurogenic niche all interact with each other as well as the host of indirect effects/comorbidities to generate the net effect alcohol has on the components of hippocampal adult neurogenesis.

Ethanol affects virtually every neurotransmitter system in the CNS (Vengeliene et al., 2008). For example, concentrations of alcohol that reflect those seen in human consumption may directly interact with a host of neurotransmitters, receptors and ion channels including acetylcholine, endocannabinoids, neuropeptide Y (NPY), gamma-aminobutyric acid (GABA) receptors, glutamate receptors, dopamine receptors, and serotonin receptors along with G-protein-activated inwardly rectifying K+ channels (Arnone et al., 1997; Fadda and Rossetti, 1998; Grobin et al., 2001; Lewohl et al., 1999; Lovinger, 1999; Lovinger et al., 1989; Vengeliene et al., 2008). This list notably overlaps with the neurotransmitter systems that regulate adult hippocampal neurogenesis, including acetylcholine, endocannabinoids, GABA, glutamate, norepinephrine, NPY, and serotonin to name a few (Aguado et al., 2005; Bruel-Jungerman et al., 2011; Cameron et al., 1998; Conover and Notti, 2008; Gray, 2008). As many of these have been reviewed previously (Crews and Nixon, 2003; Nixon et al., 2010, 2011), only the primary targets GABA and glutamate are discussed below.

The process of adult neurogenesis and maturation of newly born neurons involves two neurotransmitter systems that are also considered the primary pharmacodynamic targets of ethanol, GABA and glutamate. $GABA_A$ receptors are present on newly born neurons in the SGZ and GABA acts as an excitatory neurotransmitter during the first two - four weeks of new neuron development (Esposito et al., 2005; Ge et al., 2006; Overstreet Wadiche et al., 2005). Around this time, the neuron begins to express NMDA receptors and undergoes a switch

from GABA being excitatory to inhibitory and NMDA being excitatory, recapitulating that seen in development (Nacher et al., 2007; Zhao et al., 2008). Previous studies show that treatment with a $GABA_A$ agonist results in decreased SGZ NPC proliferation while antagonist treatment yielded the opposite effect (Tozuka et al., 2005). However, treatment with a $GABA_A$ agonist was shown to enhance activity-dependent neuronal differentiation (Tozuka et al., 2005). Since ethanol is a positive allosteric modulator of the $GABA_A$ receptor it is possible that this receptor could be one avenue through which alcohol acts to alter neurogenesis. These findings also suggest that alcohol could have very specific effects on NPCs and developing neuroblasts depending on their state of ontogeny, especially when GABA is initially excitatory. However, no one has explored this potential relationship or whether alcohol affects the maturation of neuroblasts in the dentate gyrus.

The role of NMDA receptors in adult neurogenesis is complex. Several studies have shown that NMDA receptor activation inhibits adult hippocampal neurogenesis (Cameron et al., 1995; Maekawa et al., 2009; Nacher et al., 2001; Pava and Woodward, 2012); whereas NMDA receptor antagonism increases adult neurogenesis (Cameron et al., 1995; Petrus et al., 2009). Acutely, ethanol intoxication *inhibits* NMDA receptors (Lovinger et al., 1989) which indicates that NMDA receptors are not likely the mechanism by which ethanol intoxication decreases adult neurogenesis (Nixon and Crews, 2002). Prolonged ethanol exposure and the development of dependence, however, augments NMDA function and enhances glutamate release, both of which contribute to neuronal

hyperexcitability associated with the ethanol withdrawal syndrome (Hoffman, 2003). Increased neurogenesis is observed after four days of ethanol inhibition of NMDA receptors, which is consistent with an NMDA antagonist similarly increasing neurogenesis (Cameron et al., 1995). Furthermore, ethanol withdrawal-induced hyperexcitability increases activation of granule cell neurons as measured by c-Fos immunoreactivity (Knapp et al., 1998; Matsumoto et al., 1993). Although hyperexcitability associated with ethanol withdrawal typically subsides within 24 hours (Matsumoto et al., 1993), previous studies demonstrate that the effects of granule cell excitation on neurogenesis can be delayed by several days (Bruel-Jungerman et al., 2006; Stone et al., 2011a). Additional studies are needed, but the delayed neurogenic effects associated with activity-dependent neurogenesis fit the temporal profile for reactive neurogenesis that occurs after a 4-day binge ethanol exposure (Nixon and Crews, 2004).

Seizures have been shown to result in increased levels of neurogenesis (Parent et al., 1997). Seizures can also occur during alcohol withdrawal and therefore represent a potential confound. However, this issue has been addressed in studies utilizing a 4-day binge model of an AUD. Using diazepam, researchers were able to significantly reduce the number of rats that experienced alcohol withdrawal-induced seizures as indicated by decreased withdrawal severity scores (Nixon and Crews, 2004). Proliferation levels were assessed seven days post-binge, when an increase is normally seen in this model, and even when withdrawal severity was reduced, there was still a significant increase

in proliferation over controls (Nixon and Crews, 2004). Therefore, withdrawal-induced seizure is not a confounding variable.

1.7 CONCLUSION

Numerous studies have illustrated that AUDs result in damage to the CNS with the hippocampus being one of the targets of its neurotoxic effects. Recent preclinical studies have highlighted that alcohol's effects on the various components of adult neurogenesis are one way in which excessive alcohol intake produces hippocampal pathology. By impacting the structure and function of the dentate gyrus through inhibition of adult neurogenesis, alcohol has downstream effects on hippocampal circuitry and subsequently the host of behaviors controlled or influenced by the hippocampus. Importantly, research has shown that some recovery is possible when alcoholics abstain from alcohol use (Bartsch et al., 2007; Carlen et al., 1978; Pfefferbaum et al., 1995; Shear et al., 1994). Although there are a host of plastic changes that occur with abstinence, one way that the hippocampus may recover in abstinence is through the repopulation of the dentate gyrus by adult neurogenesis. Although it has been reported that a compensatory increase in neurogenesis occurs in abstinence, it is still not known whether the new cells are correctly integrated or the extent to which this process influences cognitive recovery. Despite observation of this potential mechanism of recovery in animal models, detectable cognitive impairments remain in over 50% of AUD cases (Eckardt and Martin, 1986; Parsons, 1993). Therefore, important future questions should be aimed at understanding 1) alcohol-induced decreases

in pathology, 2) reactive neurogenesis following alcohol exposure in recovery, and 3) hippocampal integrity and function in AUDs.

1.8 DISSERTATION OBJECTIVES

As described above, reactive neurogenesis is the process by which central nervous system (CNS) injury yields an increase in neurogenesis, and is a potentially beneficial endogenous mechanism of brain recovery. At this time, the role of alcohol-induced reactive neurogenesis in hippocampal recovery is not known. However, since alcohol intoxication reduces proliferation and then cessation of alcohol results in an increase in proliferation/neurogenesis, we hypothesize that this reactive neurogenesis promotes the functional recovery of the dentate gyrus following alcohol exposure. To investigate the functional contribution that reactive neurogenesis makes to hippocampal recovery it was necessary to reduce reactive neurogenesis to basal neurogenesis levels (which are seen in control animals, throughout life). This study was designed to a) expose rats to a binge model of an AUD that results in reactive neurogenesis, b) inhibit reactive neurogenesis (with alcohol or drug), and then c) determine how a lack of reactive neurogenesis affects hippocampal recovery (measured with the MWM). This study was designed with the overall goal of assessing the behavioral effect of essentially eliminating reactive neurogenesis. To that end, Chapter 2 detailed the results of inhibiting reactive neurogenesis using a secondary alcohol exposure since acute high doses of alcohol are anti-proliferative (Nixon and Crews, 2002). Remarkably, this secondary alcohol exposure did not inhibit reactive proliferation. In trying to understand these negative results it was

postulated that perhaps reactive neurogenesis begins prior to when the secondary dose of alcohol was administered and that this may underlie why reactive neurogenesis was so robust and resistant to suppression. Regardless, these negative results emphasized the lack of knowledge regarding the timing of alcohol-induced reactive neurogenesis, which necessitated a thorough investigation into when, exactly, the increase in reactive proliferation began. Chapter 3 addressed this issue by investigating the time course of alcohol-induced reactive proliferation. The goal of these experiments was to pinpoint the day proliferation began to increase (in ethanol rats compared to controls) so that future experiments could target it to reduce reactive proliferation to basal proliferation levels. Based on this newly established time-course, Chapter 4 determined the optimal dose of the antimitotic temozolomide needed to attenuate alcohol-induced reactive proliferation/neurogenesis in the dentate gyrus. Additionally, the functional implications of inhibiting reactive neurogenesis were examined by investigating changes in MWM performance. The last chapter, Chapter 5 investigated activation of young neurons. Following training on the hippocampal-dependent task, functional incorporation was assessed via activation of granule cells born during alcohol-induced reactive neurogenesis. The proposed studies are critical in evaluating the role of alcohol-induced reactive neurogenesis in recovery from AUDs. Furthermore, these studies provided valuable insight into modulation of the NPC pool as a potential therapeutic target for the treatment of AUDs.

CHAPTER 2: INHIBITION OF REACTIVE PROLIFERATION WITH ETHANOL

2.1 INTRODUCTION

Excessive alcohol consumption, characteristic of an AUD, results in neurodegeneration in brain regions such as the hippocampus (Beresford et al., 2006; Wilhelm et al., 2008). With abstinence from alcohol use there is some recovery seen in human brain volume and function (Bartels et al., 2007; Pfefferbaum et al., 1995). However, the mechanisms involved in recovery are poorly understood (Nixon, 2006). Adult hippocampal neurogenesis is the process by which neural progenitor cells (NPCs) proliferate in the SGZ, differentiate, migrate, and integrate as granule cells into the GCL of the hippocampal dentate gyrus (Ming and Song, 2011). The functional significance of adult neurogenesis remains a subject of debate. Many, however, think it is critical for normal hippocampal function (Braun and Jessberger, 2014). Ethanol intoxication inhibits adult neurogenesis, specifically, the proliferation of NPCs and cell survival, depending on the extent of exposure, which is thought to contribute to behavioral impairments in hippocampal-dependent tasks such as the MWM following a binge model of an AUD (Nixon and Crews, 2002; Obernier et al., 2002b). Conversely, after seven days of abstinence (T7) from a binge model of an AUD there is a reactive increase in NPC proliferation which may contribute to recovery of hippocampal function (Nixon, 2006; Nixon and Crews, 2004).

The functional significance of alcohol-induced reactive neurogenesis has not been investigated (Geil et al., 2014; Richardson et al., 2009). Currently, it is not known if this reactive neurogenesis contributes to improvements in

hippocampal-dependent tasks seen during abstinence. To that end, this experiment was designed to decrease alcohol-induced reactive neurogenesis so that hippocampal-dependent behavioral testing could help determine if alcohol-induced reactive neurogenesis is required for recovery of hippocampal function.

Previously, it has been shown that ethanol exposure affects a variety of organ systems (Manzo-Avalos and Saavedra-Molina, 2010). This includes the nervous system in general and the neurogenic region of the hippocampus (Jang et al., 2002; Nixon and Crews, 2002; Richardson et al., 2009). Specifically, a single high dose of ethanol (5 g/kg) administered via gavage significantly reduced proliferation in the SGZ of ethanol-naïve rats (Nixon and Crews, 2002). Therefore, in this study, two doses of ethanol (5 g/kg) were administered to decrease alcohol-induced reactive proliferation. This study tests the hypothesis that alcohol (administered six days after the end of a 4-day binge alcohol exposure) would decrease alcohol-induced reactive proliferation.

2.2 METHODS

2.2.1 Binge model of an alcohol use disorder

Twenty-eight adult male Sprague-Dawley rats (Charles River Laboratory; approximately PND 70) weighing 275-300 g on arrival were used in the following experiment. A total of six rats were excluded from analysis because they failed to incorporate BrdU; three rats were removed from the C+C (control + control) group, one from the E+C (ethanol + control) group, and three from the E+E (ethanol + ethanol) group. Groups described in detail on page 40. This experiments primary outcome measure was to assess proliferation with BrdU+

staining which is not possible when rats to not take up the injected BrdU. Reported results are based on the 22 rats that successfully incorporated BrdU.

All rats were allowed to acclimate to the vivarium for five days before alcohol exposure began. All experimental procedures were completed in accordance with the University of Kentucky Institutional Animal Care and Use Committee and followed the Guidelines for the Care and Use of Laboratory Animals (NRC, 1996). The vivarium was temperature and humidity controlled and allowed 12 hours of lights-on at 6:00 AM, and 12 hours lights-off at 6 PM. Upon arrival, animals were housed 2/cage and allowed *ad libitum* access to rat chow and water at all times except during the four days of binge alcohol exposure when rat chow was removed. While rats became acclimated to their new environment they were individually handled for three minutes a day for three days to familiarize the rats with being held. On their sixth day in the animal vivarium, rats were subjected to a 4-day rodent binge model of an AUD that achieves high blood alcohol concentrations (BACs) and produces tolerance, physical dependence, and corticolimbic neurodegeneration with low mortality (Collins et al., 1996; Crews et al., 2000; Majchrowicz, 1975; Nixon and Crews, 2004). This binge model of alcohol consumption was chosen because up to 60% of human alcoholics consume alcohol in a binge pattern (Robin et al., 1998). Furthermore, this model produces high BACs similar to those observed in human alcoholics (Cartlidge and Redmond, 1990; van Hoof et al., 2011; Lindblad and Olsson, 1976). This model maintains high blood alcohol levels and induces alcohol-induced reactive neurogenesis (Nixon and Crews, 2004).

This model of binge alcohol exposure has been described previously and in detail (Majchrowicz, 1975; Nixon and Crews, 2004). Briefly, rats were randomly divided into three groups with similar starting weights and were gavaged with either 25% (w/v) ethanol in Vanilla Ensure Plus® (Abbott Laboratories, Columbus, OH) or an isocaloric control diet (made calorically equivalent with dextrose) in Vanilla Ensure Plus®. Ethanol or control diet was administered via gavage every eight hours for four days. Ethanol animals were initially given a 5 g/kg ethanol dose and all subsequent doses were titrated based on the animal's behavioral intoxication score according to the scale in Table 2.1 (Majchrowicz, 1975; Nixon and Crews, 2004). The desired clinical effect is alcohol intoxication resulting in consistent blood alcohol concentrations with minimal mortality (Collins et al., 1996; Knapp and Crews, 1999). Essentially, dosages are adjusted to achieve a desired clinical effect. According to the dosing scale, less intoxicated rats (e.g. hypoactive and mildly ataxic) receive more alcohol than rats showing stronger behavioral signs of intoxication (e.g. loss of righting reflex while maintaining the eye blink reflex). Each rat that receives alcohol is individually scored for behavioral intoxication and then the corresponding dose of ethanol is administered via gavage. The average volume of diet given to the ethanol animals was determined and that volume of isocaloric control diet was given to all control animals. This ensured that control and ethanol rats receive approximately the same number of calories. Food restriction has been shown to be associated with changes in neurogenesis in some models (Barbarich-Marsteller et al., 2013; Park and Lee, 2011). However, previous

Table 2.1 - Behavioral intoxication and dosing scale

Intoxication score	Behavioral features	Ethanol dose (g/kg)
0	Normal animal	5
1	Hypoactive, mildly ataxic	4
2	Ataxic, elevated abdomen	3
3	Ataxic, no abdominal elevation, delayed righting reflex	2
4	Loss of righting reflex, maintain eye blink reflex	1
5	Loss of righting reflex, loss of eye blink reflex	0

*Table 2.1 - Animals were assessed based on their behavioral features and intoxication scores were used to calculate the appropriate dose of ethanol for administration. This modified Majchrowicz scale indicates proper ethanol dose based on behavioral intoxicatio*n *(Majchrowicz, 1975; Morris et al., 2010a; Nixon and Crews, 2004).*

studies have shown that free-feeding rats (un-handled, *ad libitum* controls) and control diet rats (food restricted to match ethanol rat's caloric intake) showed no differences in neurogenesis (Nixon and Crews, 2002). All animals were weighed daily during alcohol administration since dosing calculations are based on animal weight.

The ethanol groups underwent monitored withdrawal for 17 hours beginning 10 hours after the last dose of ethanol (Morris et al., 2010a). This time frame was selected because it is associated with peak alcohol withdrawal symptoms (Faingold, 2008; Majchrowicz, 1975; Nixon and Crews, 2004; Penland et al., 2001). Rats were observed for 30 minutes every hour for 17 hours and withdrawal behavior was scored using a Majchrowicz behavioral withdrawal scale (Table 2.2) modified by Penland et al. 2001 (Morris et al., 2010a). Behaviors were assigned a numerical score according to severity, where higher scores indicated increased withdrawal severity. The highest rated behavioral feature was determined for each 30 minute interval. For example, if from 10:00-10:30 PM an animal had splayed limbs (score of 2.4) and a head tremor (score of 3.0), then the highest score (a 3.0, head tremor) was recorded for that animal at that time point. Peak withdrawal score was also analyzed for each rat and consists of the highest withdrawal score observed at any time point across the entire 17 hour withdrawal period. Mean and Peak withdrawal scores are calculated across all animals and are expressed as mean withdrawal score and peak withdrawal score ± standard error of the mean.

Table 2.2 - Behavioral withdrawal scale

Withdrawal score	Behavioral feature
1.0	Hyperactivity
1.4	Tail Tremor
1.6	Tail Spasm
2.0	Caudal Tremor
2.2	Tip-Toe Arch
2.4	Splayed Limbs
2.6	General Tremor
3.0	Head Tremor
3.2	Induced Running
3.4	Wet Dog Shakes
3.6	Teeth Chattering
3.8	Spontaneous Convulsions
4.0	Death

Table 2.2 - Withdrawal scores are an index of withdrawal severity and were quantified using this scale (Morris et al., 2010a).

2.2.2 Blood ethanol concentration

Ninety minutes following the seventh dose of ethanol, tail bloods were collected to determine peak blood ethanol concentration (BEC; Morris et al., 2010b). Blood samples were centrifuged at 1,800 x g for five minutes to separate blood plasma from red blood cells and then stored at -20º C until processing. To quantify BECs, 5 µl supernatant blood serum was inserted into an AM1 Alcohol Analyzer (Analox Instruments, Lunenberg, MA). All blood serum samples were run in triplicate with frequent calibration to a 300 mg/dl ethanol standard. Data are presented as mean blood ethanol concentration ± standard error of the mean.

2.2.3 Ethanol-induced inhibition of reactive proliferation

Previous studies showed that a single dose of 5 g/kg ethanol reduced proliferation in ethanol-naïve rats (Nixon and Crews, 2002). Since the rats in the present study were not ethanol-naïve, they were given an additional 5 g/kg dose of ethanol (for a total of 2 doses). This second dose was added because we hypothesized that *alcohol-induced* reactive proliferation would be more difficult to inhibit than *basal* proliferation in ethanol-naïve rats. Furthermore, if a single dose of ethanol did not decrease reactive proliferation then it would have likely required an additional experiment with multiple doses of ethanol. Consequently, in an effort to reduce the number of animals used to inhibit alcohol-induced reactive proliferation, rats were administered two 5 g/kg doses of ethanol beginning on the sixth day of abstinence (T6), following the 4-day binge (Figure 2.1). The first additional dose of ethanol was administered at the end of T6 and

the second dose was given eight hours later at the beginning of T7.

Rats underwent either (a) standard 4-day binge with control diet (dextrose in Vanilla Ensure Plus®) + two doses of control diet on T6/7 (Control + Control, C+C), or (b) standard 4-day binge with ethanol diet + two doses of control diet on T6/7 (Ethanol + Control, E+C), or (c) standard 4-day binge with ethanol diet + two doses of 5 g/kg ethanol (25% w/v) in Vanilla Ensure Plus® (Ethanol + Ethanol, E+E). Groups are outlined in Table 2.3.

2.2.4 Tissue preparation

Seven days after the 4-day binge, animals were injected (*i.p.*) with a saturating dose (Cameron and McKay, 2001) of bromodeoxyuridine (300 mg/kg BrdU; Roche, Mannheim, Germany) in 0.9% saline. The proposed dose of BrdU has been shown not to result in toxicity or to label DNA repair via IHC methods (Cameron and McKay, 2001; Palmer et al., 2000). Two hours after BrdU injections, all rats were administered a lethal dose of sodium pentobarbital (*i.p.*; Fatal-Plus®, Vortech Pharmaceuticals, Dearborn, MI) and transcardially perfused using 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were removed and post-fixed for 24 hours in 4% PFA. Brains were then cut into 40 µm thick coronal sections on a vibrating microtome (Leica VT1000S, Wetzlar, Germany) and collected in a 1:12 series. Tissue sections were stored in 24-well plates with cryoprotectant at -20° C until processing.

2.2.5 Bromodeoxyuridine immunohistochemistry

IHC was performed on free floating sections in a 1:6 series as previously described (McClain et al., 2011; Nixon and Crews, 2002). Sections were rinsed in

Table 2.3 - Groups for additional ethanol administration experiment

Treatment	4-Day binge	Additional	n
C + C	Control	Control	8
E + C	Ethanol	Control	6
E + E	Ethanol	Ethanol	8

Table 2.3 - Rats received four days of ethanol diet or isocaloric control diet followed by two additional doses of either 5 g/kg ethanol diet or control diet.

Figure 2.1 - Ethanol administration to reduce reactive proliferation

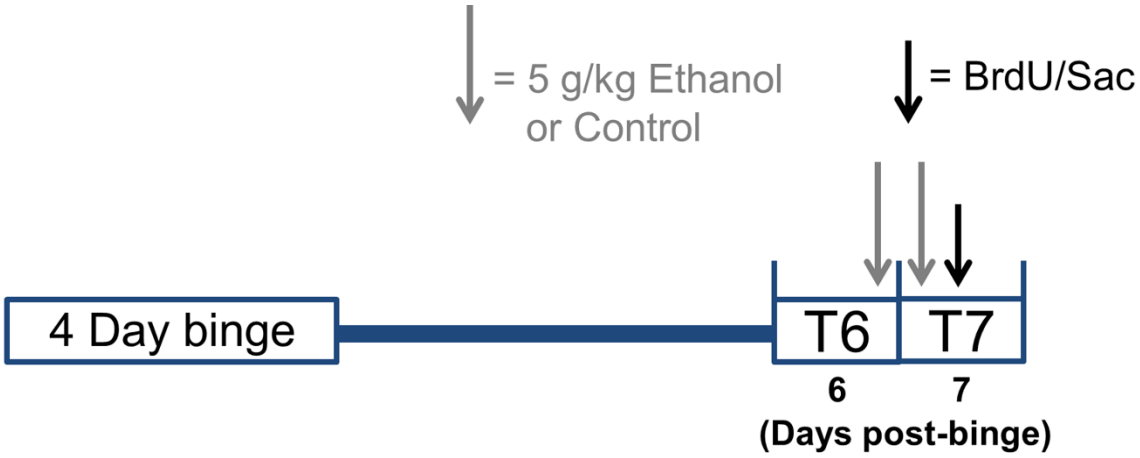


Figure 2.1 - Experimental timeline. Additional doses of 5 g/kg ethanol (or isocaloric control) were given to decrease alcohol-induced reactive cell proliferation.

tris-buffered saline (TBS) between each incubation step (day one, 2 x 5 minutes and day two, 3 x 10 minutes, unless otherwise noted). Sections were incubated in 0.6% hydrogen peroxide for 30 minutes, rinsed 3 x 5 minutes in TBS, followed by incubation in antigen retrieval with standard sodium citrate (SSC) and 50% formamide for 2 hours at 65° C. Then tissue was rinsed in 2X SSC for 2 x 5 minutes, incubated in 2N HCl for 1 hour at 37° C to denature DNA, and was neutralized in 0.1 M boric acid, pH 8.5 for 2 x 5 minutes. Tissue was rinsed for 3 x 3 minutes in TBS and was incubated for 30 minutes in blocking buffer (3% serum/0.1% Triton X-100/TBS) with normal horse serum (Vector Laboratories, Burlingame, CA). Tissue was rinsed in reaction buffer (0.1 g MgCl in 500 ml TBS), incubated for 1 hour in DNAse 100U/1 ml (in reaction buffer), rinsed/incubated in blocking buffer 2 x 5 minutes, and incubated in primary antibody overnight (1:5000 mouse anti-BrdU, MAB 3424 Millipore, Billerica, MA). The following day tissue was rinsed in blocking buffer 3 x 10 minutes, followed by incubations in biotinylated horse anti-mouse secondary antibody for 1 hour at room temperature (Vector Laboratories, Burlingame, CA), avidin-biotin-complex for 1 hour at room temperature (ABC Elite Kit, Vector Laboratories, Burlingame, CA), and processed with nickel-enhanced 3,3'-Diaminobenzidine for visualization (DAB, Polysciences, Warrington, PA). Tissue was then washed in TBS (to quench the reaction) for 3 x 5 minutes. All sections were mounted onto glass slides and allowed to dry overnight. Sections were then lightly counterstained with cresyl violet (Acros Organics, Morris Plains, New Jersey): mounted tissue was rehydrated through decreasing concentrations of ethanol followed by

distilled water (dH_2O) and cresyl violet (0.1% in Walpole buffer) for 3 minutes. After differentiation in dH_2O, tissue was then dehydrated in increasing concentrations of ethanol followed by clearing in xylenes (Leasure and Nixon, 2010; Nixon and Crews, 2002). Glass coverslips and Cytoseal® (Stephens Scientific, Wayne, NJ) were used to seal the slides.

2.2.6 Quantification

All slides were coded to ensure the experimenter was blind to the treatment conditions. BrdU+ cells were counted between Bregma -2.28 mm and -5.52 mm as determined by Paxinos and Watson (2009). Prior to quantification, all sections were inspected to determine which hemisphere was most intact and would therefore be best to count cells in. Cells were then counted along the SGZ, a band of cells approximately three cell widths that runs between the GCL and the hilus of the hippocampal dentate gyrus. Cells were counted using a 100x objective lens hooked up to an Olympus BX41 microscope (Olympus, Center Valley, PA). On the anterior portion of the hippocampus the dentate gyrus is a single blade, to account for this, the blades were quantified separately then the averages were totaled across all sections. Data are expressed as average cells/section ± standard error of the mean. Since proliferating cells expressing BrdU at this time point are located in clusters scattered unevenly throughout the SGZ, cell profiles were counted. Cell profile counts were more appropriate here since unbiased stereology requires evenly distributed cells in excess of 200 cells/section and those conditions are not met with BrdU proliferation studies so it was more appropriate to use profile cell counts (Crews et al., 2004b; Noori and

Fornal, 2011). Additionally, an identical percent change is obtained by this profile counting method when compared to stereology (Crews et al., 2004b).

2.2.7 Statistics

All data were analyzed with GraphPad Prism® (version 7.0, GraphPad software, La Jolla, CA) using a one-way ANOVA followed by Tukey's multiple comparison tests. Intoxication data was analyzed by comparing treatment groups using the student's t-test (ethanol dose and BEC) or the nonparametric, Mann-Whitney test (intoxication scores and withdrawal scores). P-values were accepted as significant when $p < 0.05$ (two-tailed).

2.3 RESULTS

2.3.1 Animal model data

Data were collected to assess intoxication between treatment groups and are detailed in Table 2.4. Animals treated with ethanol had an average intoxication score of 1.7 ± 0.1 on the 6-point intoxication scale (Table 2.4), which is expressed behaviorally as ataxia with an elevated abdomen. Intoxication scores resulted in animals receiving a total of 10 ± 0.3 g/kg/d ethanol, and corresponded to an average BEC of 366 ± 20 mg/dl. These values are similar to those previously reported (Morris et al., 2010). Student's t-tests revealed no differences between the treatment groups in terms of dose and BEC. The Mann-Whitney test also revealed no differences between the treatment groups when comparing intoxication scores. Intoxication was assessed only for the 4-day binge. The 4-day binge doses were titrated based on intoxication behavior, while the ethanol doses to decrease reactive proliferation (post-binge) were kept

Table 2.4 - Four-day binge intoxication data

Treatment group	Intoxication score	Dose (g/kg/day)	BEC (mg/dl)	n
Ethanol + Control	1.6 ± 0.2	10.3 ± 0.6	354 ± 24	6
Ethanol + Ethanol	1.7 ± 0.1	9.8 ± 0.4	375 ± 31	8

Table 2.4 - Intoxication parameters show average intoxication scores, ethanol dose, and BEC for ethanol animals during a 4-day binge treatment; all treatment groups were statistically similar.

constant at 5 g/kg to maintain consistent effects on proliferation. As such, treatment groups in Table 2.4 and 2.5 represent intoxication behavior during the 4-day binge treatment only.

2.3.2 Intoxication withdrawal data

Withdrawal scores were assigned based on observations of behavioral signs of ethanol withdrawal as described above (Table 2.2; Morris et al., 2010; Penland et al., 2001). Mann-Whitney test revealed no significant difference between treatment groups for mean or peak withdrawal scores (Table 2.5). The mean withdrawal (WD) score (i.e. mean WD) across the entire 17-hour period of monitored WD corresponded approximately with hyperactivity on the behavioral scale (0.8 ± 0.1). Additionally, the mean of the peak WD score achieved (peak WD) across the entire monitored WD period corresponded approximately with head tremor (3.1 ± 0.3).

2.3.3 Effect of additional doses of ethanol on reactive proliferation

Immunohistochemistry was used to label BrdU positive (+) cells. Figure 2.2 shows BrdU+ cells in clusters lining the SGZ for each treatment group. BrdU+ cells were profile counted and then analyzed by one-way ANOVA, revealing a significant effect of 4-day ethanol treatment [$F_{(2,19)}=23.78$; $p < 0.0001$]. Tukey's multiple comparisons test revealed that rats treated with 4-days of ethanol + additional doses of control diet (E+C) or 4-days of ethanol + additional doses of ethanol diet (E+E) had increased BrdU+ cell counts ($p < 0.05$) compared to 4-days of control diet + additional doses of control diet (C+C). However, there was no significant difference between the two ethanol treatment groups (Figure 2.2).

Table 2.5 - Mean and peak withdrawal (WD) scores separated by treatment

Treatment groups	Mean WD	Peak WD	n
Ethanol + Control	0.6 ± 0.2	2.8 ± 0.6	6
Ethanol + Ethanol	0.8 ± 0.2	3.4 ± 0.2	8

Table 2.5 - There were no statistical differences between treatment groups for mean WD or peak WD.

Figure 2.2 - Representative images and BrdU+ cell counts

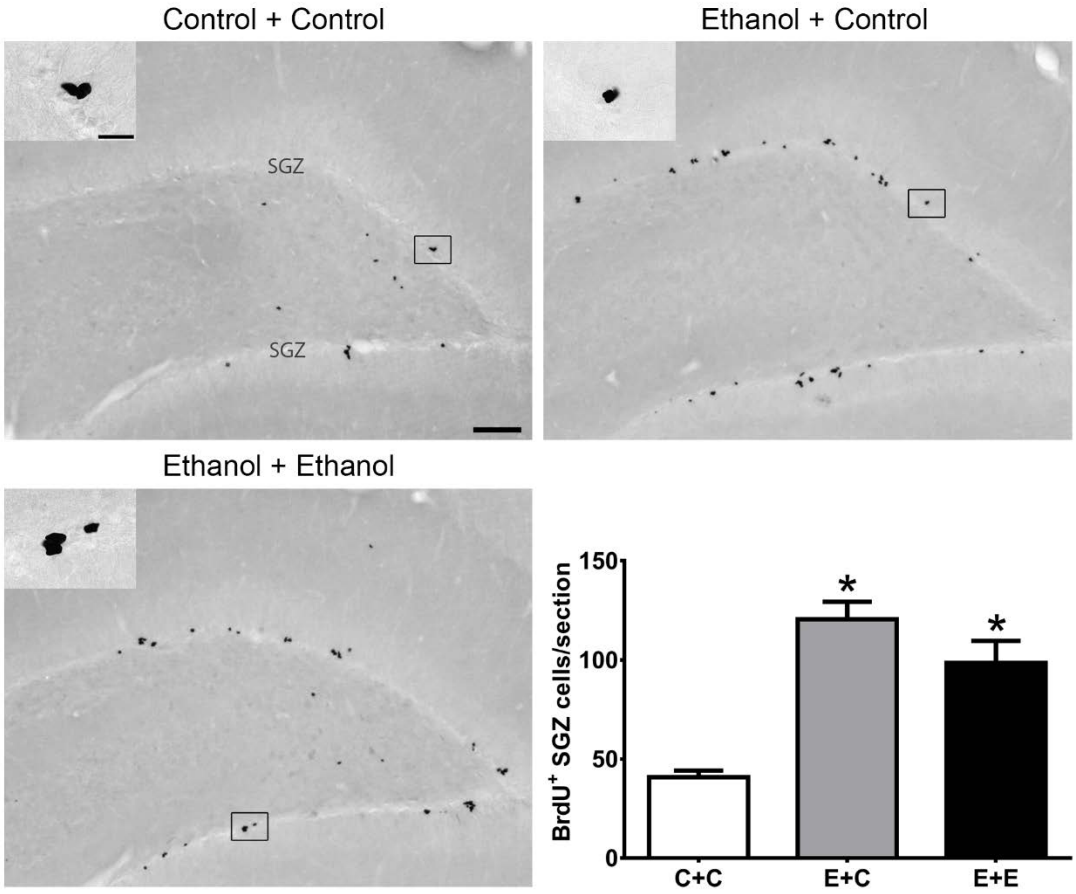


*Figure 2.2 - Representative photomicrographs of BrdU+ cells seven days post-binge ethanol exposure. As expected, BrdU+ cells were located in clusters throughout the SGZ. Ethanol administered during reactive proliferation does not significantly decrease BrdU+ cell counts. Tukey's multiple comparison test revealed an increase between ethanol treated groups (E+C and E+E) and controls (C+C), but no difference between the two ethanol groups. *p < 0.05 ; n = 6-8; scale bar = 100 μm; insets = 20 μm; GCL = granule cell layer.*

These results indicated that the two additional doses of 5 g/kg ethanol administered during reactive proliferation did not reduce BrdU+ cell counts at T7 (seven days post-binge).

2.4 DISCUSSION

Decreases in hippocampal cell proliferation are seen during alcohol intoxication across several animal models (Anderson et al., 2012; Jang et al., 2002; Nixon and Crews, 2002; Taffe et al., 2010; Tesone-Coelho et al., 2013). Indeed, a single high dose of ethanol significantly decreased proliferation in the SGZ of the hippocampal dentate gyrus of ethanol-naïve rats (Nixon and Crews, 2002). Additionally, alcohol has been shown to dose dependently reduce cell proliferation in adolescent rats (Crews et al., 2006b). However, Nixon and Crews (2004) revealed that seven days after a 4-day binge model of an AUD there was a reactive increase in cell proliferation in the SGZ and this effect was replicated in the present study. The current experiment was designed to test the hypothesis that two doses of 5 g/kg ethanol, given six to seven days following alcohol exposure, would decrease alcohol-induced reactive proliferation. Surprisingly, alcohol-withdrawal induced reactive proliferation in the dentate gyrus at T7 was robust and resistant to inhibition by ethanol. However, ethanol did slightly decrease BrdU+ cell counts (18% decrease) in animals that received additional doses of ethanol; however, this decrease was not significant. Since additional doses of ethanol did not decrease BrdU+ cell counts, there was no need to test for additional proliferation markers (i.e. Ki67).

Binge-exposed, ethanol animals show a large increase in NPC

proliferation compared to controls and therefore decreasing reactive proliferation could require lengthier or earlier ethanol administration (Hayes et al., in preparation). A recent study administered a second 4-day binge ethanol exposure beginning seven days post-binge (a measure that would most likely decrease reactive proliferation). However this study resulted in cell counts with a wide variability following the second binge (Maynard and Leasure, 2013). Therefore, alcohol is likely not the best choice for decreasing alcohol-induced reactive proliferation. Timing of drug administration should also be considered. Alcohol-induced reactive proliferation is reported at seven days after binge alcohol exposure with 80% of surviving cells becoming neurons 28 days later (Nixon and Crews, 2004), i.e. they express the mature neuronal marker NeuN (Mullen et al., 1992). However, it is not known if this increase in reactive proliferation begins prior to T7 (seven days post-binge). But since reactive proliferation/neurogenesis occurs over multiple days following traumatic brain injury, seizure, and transient global ischemia in rodents it is possible that alcohol-induced reactive neurogenesis could follow a similar multi-day proliferation increase (Dash et al., 2001; Liu et al., 1998; Parent et al., 1997). If alcohol-induced reactive proliferation also had multiple days when proliferation was increased then the drug chosen to decrease proliferation should be administered ideally across multiple days when the increase in proliferation is seen. For example, if there was an increase five days after binge alcohol exposure then (anti-proliferative) drug administration could begin four days after alcohol exposure and last through six days post-binge (since animals are killed only

hours into T7). In this way, one could prevent proliferation across the period when cell proliferation was greatly increased. Therefore, the timeline of reactive proliferation needs to be investigated.

Alcohol is a pharmacologically promiscuous drug that that affects virtually every bodily organ system and brain neurotransmitter system (reviewed in Geil et al., 2014). Initially it was thought that alcohol would be a relevant way to decrease proliferation since individuals with alcohol abuse problems tend to repeatedly undergo alcohol binges (Hunt, 1993; McMahon et al., 1991). However, this process of inhibiting reactive neurogenesis was not necessarily meant to mimic human behavior. The goal of this experiment was to prevent reactive proliferation from occurring and unfortunately, alcohol may not be the best choice since it is not a selective inhibitor of proliferation/neurogenesis. Temozolomide (TMZ) on the other hand is a selective anti-proliferative drug and will be used in future experiments. In addition to addressing issue of timing of drug administration, future chapters will discuss TMZ, a drug that can specifically target rapidly proliferating cells, such as those undergoing alcohol-induced reactive proliferation.

CHAPTER 3: DETERMINE TIMELINE OF REACTIVE NEUROGENESIS

3.1 INTRODUCTION

Previous research has shown that seven days after binge alcohol exposure (T7) there is a reactive increase in neurogenesis (reactive neurogenesis), which may contribute to neuronal regeneration following alcohol-induced hippocampal damage (Nixon and Crews, 2004). However, a more in-depth time course for reactive neurogenesis, including pinpointing when it begins, has not been determined. Classically, reactive neurogenesis has been seen following brain insults including stroke, traumatic brain injury (TBI), and seizure (Dash et al., 2001; Parent et al., 1997). Rodent models of TBI and stroke have been shown to produce increases in neurogenesis (NPC proliferation), three to seven days post-injury (Dash et al., 2001; Jin et al., 2001). It is likely that binge alcohol consumption produces a similarly timed reactive increase in neurogenesis (Nixon and Crews, 2004). Although reactive proliferation following a 4-day binge model of an AUD was assessed in previous experiments at several time points (e.g. 0, 2, 4, 7, 10, 14, 28, and 35 days post-binge), the days immediately prior to the increase in proliferation seen 7 days post-binge (i.e. T5 and T6) had not been previously investigated (Nixon and Crews, 2004; Nixon et al., 2008). Furthermore, previous experimental time points (listed above) counted cells across the entire GCL (not just the SGZ) which contributed to high variability in cell counts. Therefore, the present experiments only counted BrdU+ cells in the SGZ in effort to more accurately assess hippocampal cell counts at previous quantified time points (T4 and T7) as well as at new time points (T5 and T6).

Perhaps one reason why the previous attempt to decrease reactive neurogenesis was unsuccessful (Chapter 2) was due to incomplete information about the time course of reactive proliferation four to seven days following binge alcohol exposure. Essentially, reactive proliferation may begin earlier than was previously suggested. Additionally, here we investigated a time course for neurogenic differentiation factor 1 (NeuroD) expression following binge alcohol exposure. This was done so future experiments could confirm neurogenesis with NeuroD+ cell counts. NeuroD is one of the earliest markers that a cell has adopted a neuronal fate and represents an excellent neurogenesis marker (von Bohlen und Halbach, 2011).

3.2 METHODS

3.2.1 Binge model of an alcohol use disorder

Eighty-three male Sprague-Dawley rats (Charles River Laboratory; approximately PND 70), each weighing 275-300 grams on arrival, were used for studies performed with three cohorts. There were two studies in this chapter: one examined the time course of proliferation and the other investigated neurogenesis (Table 3.1, Figure 3.1). The time course study included 52 rats, 32 received ethanol diet and 20 received control diet; however, not all rats took-up BrdU (Eisch et al., 2000). A total of 21 rats were removed and excluded from the analyses due to failure to take up BrdU: four ethanol rats from the T4 group, two ethanol rats from the T5 group, two ethanol rats from the T6 group, four ethanol rats from the T7 group, and nine rats from the control groups. The neurogenesis study included 31 rats, 17 received ethanol diet and 14 received control diet.

Table 3.1 - Time course and neurogenesis study: experimental design

Group	Study	Ethanol	Control
T4	Time course	4	2
T5	Time course	6	2
T6	Time course	6	3
T7	Time course	4	4
T7	Neurogenesis	8	6
T14	Neurogenesis	9	8

Table 3.1 - Animal numbers separated by diet and study.

Figure 3.1 - Experimental timeline for proliferation study

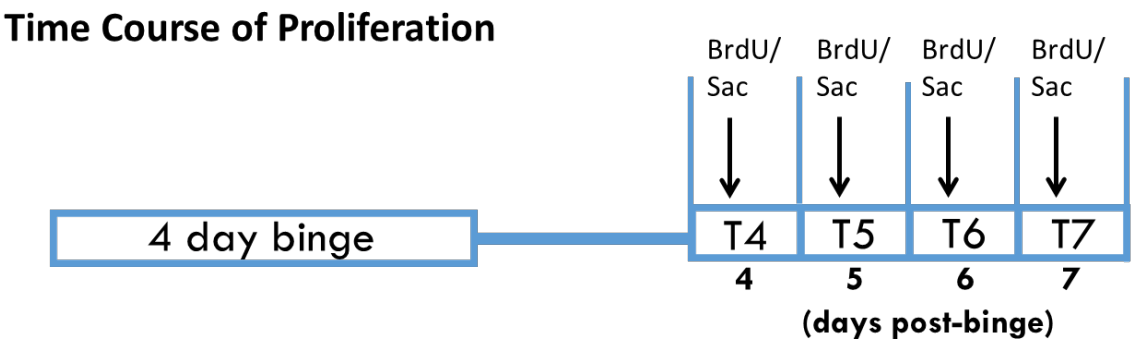


Figure 3.1 - All animals underwent a 4-day binge model of an AUD, were injected with BrdU four to seven days after the binge and sacrificed two hours later.

All rats in the neurogenesis study were included in the analyses because NeuroD, an endogenous cellular marker was quantified (no BrdU injections were needed and therefore no animals were excluded due to their inability to take up BrdU).

Upon arrival, rats were double housed and allowed to acclimate to the University of Kentucky AAALAC-accredited vivarium for five days before experimental procedures began. Animals were kept on a 12-hour light/dark cycle with *ad libitum* access to food and water except when noted. All rats were handled for three days during the acclimation period. All experimental procedures were completed in accordance with the University of Kentucky Institutional Animal Care and Use Committee and followed the Guidelines for the Care and Use of Laboratory Animals (NRC, 1996). On the sixth day in the animal vivarium, a 4-day binge model of an AUD was initiated (Majchrowicz, 1975; Morris et al., 2010a).

Animals were randomly divided into two groups with statistically similar weights, their food was removed and they were administered either 25% (w/v) ethanol in Vanilla Ensure Plus® (Abbott Laboratories, Columbus, OH) or an isocaloric control diet (calorically equivalent quantities of dextrose) in Vanilla Ensure Plus®. Ethanol or control diet was administered every eight hours for four consecutive days. Ethanol animals received a 5 g/kg ethanol dose initially and the following doses were titrated based on the animal's behavioral intoxication score as described in Chapter 2 (Majchrowicz, 1975; Morris et al., 2010a). All animals receiving ethanol were scored individually for behavioral intoxication and

the appropriate dose of ethanol was calculated and administered. After all ethanol animals had been dosed, the average volume of diet administered to the ethanol animals was calculated and that volume of control diet was given to all control animals (Knapp and Crews, 1999). All animals were weighed daily during alcohol administration to ensure accurate dosing and to monitor animal health.

All ethanol animals underwent monitored withdrawal for 17 hours beginning 10 hours after the last dose of ethanol (previously described in section 2.2.1). Rats were observed for 30 minutes of every hour for 17 hours (previously described in Table 2.2). Data are presented as mean withdrawal ± standard error of the mean and peak withdrawal ± standard error of the mean.

3.2.2 Blood ethanol concentration

Ninety minutes following the seventh dose of ethanol, tail bloods were collected to determine BEC (as described in section 2.2.2). Briefly, blood samples were centrifuged at 1,800 x g for five minutes and stored at -20º C until processing. BECs were determined by an AM1 Alcohol Analyzer (Analox, Lunenberg, MA) in triplicate with frequent calibration to a 300 mg/dl ethanol standard. Data are presented as BEC (mg/dl) ± standard error of the mean.

3.2.3 Tissue preparation

Rats in the proliferation time course group were injected with BrdU (300 mg/kg, Roche, Mannheim, Germany) in 0.9% saline 4, 5, 6, or 7 days following the 4-day binge. Rats in the neurogenesis groups did not receive BrdU injections and were sacrificed 7 or 14 days following the 4-day binge. Two hours after BrdU injections (or at a similar time when BrdU injections were not given) rats were

overdosed with sodium pentobarbital (Fatal-Plus®) and transcardially perfused using 0.1 M PBS followed by 4% PFA. Brains were removed and post-fixed for 24 hours in 4% PFA. Brains were then cut into 40 µm thick coronal sections and collected in a 1:12 series on a vibrating microtome (Leica VT1000S; Wetzlar, Germany). Sections were stored in a cryoprotectant at -20º C until use. Brains were coded to ensure the experimenter was blind to the treatment conditions.

3.2.4 Immunohistochemistry

3.2.4.1 Bromodeoxyuridine

Immunohistochemistry was performed on every sixth section (free floating tissue) as previously described in section 2.2.5 (McClain et al., 2011; Nixon and Crews, 2002). Briefly, sections were rinsed in TBS between each incubation step. Sections were incubated in 0.6% hydrogen peroxide, followed by antigen retrieval and DNA denaturing steps using standard sodium citrate (SSC) and formamide. Tissue was rinsed in SSC, incubated in 2N HCl, and then neutralized in boric acid (pH 8.5). Tissue was incubated in blocking buffer with normal horse serum (Vector Laboratories; Burlingame, CA), rinsed in reaction buffer, incubated in DNAse, rinsed in blocking buffer (3% normal horse serum/0.1% Triton X-100/TBS), and incubated in primary antibody overnight (1:5000 mouse anti-BrdU MAB3424, Millipore). Sections were rinsed in blocking buffer, followed by incubations in secondary antibody (1:200, biotinylated anti-mouse IgG made in horse), avidin-biotin-complex (ABC Elite Kit, Vector Laboratories, Burlingame, CA), and nickel-enhanced 3,3'-Diaminobenzidine (DAB; Polysciences, Warrington, PA) for visualization. All sections were mounted onto slides and

allowed to dry overnight. Sections were then counterstained lightly with Cresyl Violet (as described in Chapter 2). Then, glass coverslips and Cytoseal® (Richard Allen Scientific, Kalamazoo, MI) were used to seal the slides. Slides were coded to ensure experimenter was blind to the treatment conditions.

3.2.4.2 Neurogenic differentiation factor 1

NeuroD is a basic helix-loop-helix transcription factor that is essential for proper pancreatic and neuronal development. Interestingly, NeuroD knockout mice have dramatically reduced insulin levels and do not survive past postnatal day five due to hyperglycemia (Chae et al., 2004; Naya et al., 1997). Since knock-out mice die shortly after birth, researchers used a transgenic technique to specifically rescue the pancreatic NeuroD phenotype while leaving the neuronal NeuroD phenotype knocked-out (Chae et al., 2004; Miyata et al., 1999). Amazingly, these mice survived over a year but completely lacked a hippocampal dentate gyrus (Miyata et al., 1999). As such, NeuroD is very strongly linked to neurogenesis. Additionally, NeuroD expression is considered one of the earliest indicators that a NPC has adopted a neuronal fate (i.e. will become a neuron). NeuroD has an expression profile very similar to the microtubule-associated protein doublecortin (DCX), the *former* 'gold standard' marker of cells undergoing neurogenesis (Brown et al., 2003; Nicola et al., 2015; Pataskar et al., 2016). NeuroD offers multiple advantages over DCX: the first, NeuroD is more specific than DCX. Recent studies show that DCX is not specific to neurons (Diaz et al., 2013). Second, NeuroD is a transcription factor and therefore results in a nuclear stain that is easier to quantify with profile cell counts (Gao et al., 2009; Lee et al.,

1995). DCX stains the entire cell body and processes which can require analysis with densitometry, a non-preferred method of quantification that counts pixels of color rather than the actual number of cells. Although NeuroD and DCX have similar expression profiles (von Bohlen und Halbach, 2011), NeuroD has not been investigated following a 4-day binge model of an AUD. To that end, we investigated a time course for NeuroD expression following binge alcohol exposure so future experiments could confirm neurogenesis with NeuroD+ cell counts instead of having to use DCX.

Immunohistochemistry was performed on every 12th section (free floating tissue). Tissue was rinsed in TBS between incubation steps. Tissue was incubated in 0.6% hydrogen peroxide for 30 minutes, Antigen Retrieval Citra Solution (BioGenex, Fremont, CA) for 1 hour at 65º C, and blocking buffer [3% normal rabbit serum (Vector Laboratories, Burlingame, CA), 0.1% triton-X, and TBS] for 30 minutes. Tissue was incubated in primary antibody (1:1000 goat anti-NeuroD SC-1084, Santa Cruz Biotechnologies, Dallas, TX) in blocking buffer for ~42 hours at 4º C. Tissue was rinsed in blocking buffer, incubated in secondary antibody (1:200, biotinylated anti-goat IgG made in rabbit) for 1 hour, avidin-biotin-complex (ABC Elite Kit, Vector Laboratories, Burlingame, CA) for 1 hour, and nickel-enhanced 3,3'-Diaminobenzidine for 70 seconds (DAB; Polysciences, Warrington, PA) for visualization. All sections were mounted onto slides and allowed to dry overnight then coverslipped with Cytoseal® (Richard Allen Scientific, Kalamazoo, MI). Slides were coded to ensure that the experimenter was blind to the treatment conditions.

3.2.5 Quantification

3.2.5.1 Bromodeoxyuridine

BrdU+ cell profiles were counted in the SGZ of the dorsal dentate gyrus using a 100x objective lens attached to an Olympus BX41 microscope (as previously described). Briefly, BrdU+ cells were quantified on a single hemisphere between Bregma -2.28 mm and -5.52 mm as determined by an atlas (Paxinos and Watson, 2009). For each brain, all sections were inspected and the side without tears to the SGZ was selected for obtaining cell counts. Data are presented as average SGZ cells/section ± standard error of the mean.

3.2.5.2 Neurogenic differentiation factor 1

Unbiased stereology utilizing the optical fractionator method (West, 1991) was used to estimate the number of NeuroD+ cells in the SGZ of the dorsal hippocampal dentate gyrus (McClain et al., 2011). An Olympus BX51 microscope coupled to a ProScan II motorized stage, microcator, DP70 digital camera (Olympus, Center Valley, PA), and a Dell Precision 380 computer with newCAST Stereology System software was utilized (Visiopharm version 3.6.4.0, Hoersholm, Denmark). For each hippocampus section, the SGZ or GCL was traced at 100x magnification and an average section thickness was obtained from three measurements at different locations at 600x magnification (60x oil objective, PlanApo N, Olympus). Immunohistochemical tissue processing yielded a mean section thickness of ~18 μm and consequently, a dissector height of 14 μm with 2 μm guard zones was used. Random sampling was set up using a 20 μm X 20 μm counting frame and an 80 μm x,y step length for NeuroD

(parameters established with assistance from Dr. Justin McClain). Cells were counted at 1200x magnification from live images projected onto the computer screen with the overlaying counting frame. The total number of NeuroD+ cells in the SGZ and the total number of granule cells in the GCL was estimated using the following formula (West, 1991):

$$N = \sum Q \times \frac{1}{asf} \times \frac{1}{tsf} \times \frac{1}{ssf}$$

Where N is the estimated total number of cells, Q is the number of immunopositive cell profiles that were counted, asf is the area sampling fraction (the counting frame: x,y step ratio), tsf is the thickness sampling fraction (height of dissector: section thickness ratio), and ssf is the section sampling fraction (the fraction of sections examined). One brain was removed because it was missing dorsal hippocampus sections. Data are presented as total NeuroD+ cells/SGZ ± standard error of the mean.

3.2.6 Statistics

All data were analyzed with GraphPad Prism (version 7.0, GraphPad software, La Jolla, CA). Intoxication data were analyzed by comparing ethanol groups using a one-way ANOVA (ethanol dose and BEC) and the nonparametric Kruskal-Wallis test (intoxication and withdrawal scores). Time course data (BrdU) were designed to be assessed with a one-way ANOVA. Therefore, controls were first compared via one-way ANOVA and collapsed into a common control group when not statistically different. Then, time course data was assessed by one-way

ANOVA followed by Dunnett's multiple comparisons test. Neurogenesis data (T7 and T14) were assessed using a two-way ANOVA (diet x day) followed by Bonferroni's multiple comparisons test. For neurogenesis groups, intoxication data were analyzed by comparing ethanol groups using a two-tailed student's t-test (ethanol dose and BEC) and the nonparametric Mann-Whitney test (intoxication and withdrawal scores). P-values were accepted as significant when $p < 0.05$.

3.3 RESULTS

3.3.1 Time course of alcohol-induced reactive proliferation

3.3.1.1 Animal model and withdrawal data for time course study

During the 4-day binge model of an AUD, data were collected to assess similarities in intoxication between time points and are detailed in Table 3.2. Increased intoxication can correlate with increased levels of proliferation. Therefore, intoxication parameters were analyzed to determine if significant differences were present among the different time points. One-way ANOVA revealed no significant difference in dose or BEC across the different time points. The Kruskal-Wallis test showed no significant difference in intoxication or withdrawal scores across the different time points. Therefore, ethanol animals had a mean intoxication score of 1.7 ± 0.1 on the 6-point intoxication scale which is represented behaviorally by ataxia with an elevated abdomen (Majchrowicz, 1975). Intoxication scores resulted in animals receiving a mean of 9.9 ± 0.3 g/kg/d ethanol, and corresponded to a mean BEC of 359 ± 18 mg/dl. These values are similar to those previously reported (Morris et al., 2010a).

Table 3.2 - Animal model data: time course study

Days post-binge	Intoxication score	Dose (g/kg/day)	BEC (mg/dl)	Mean WD	Peak WD	n
4	1.8 ± 0.1	9.7 ± 0.3	324 ± 5	0.6 ± 0.1	3.4 ± 0.2	4
5	1.6 ± 0.1	10.1 ± 0.4	330 ± 44	0.9 ± 0.2	3.1 ± 0.1	6
6	1.9 ± 0.1	9.2 ± 0.3	412 ± 11	0.7 ± 0.2	3.4 ± 0.2	6
7	1.5 ± 0.4	10.6 ± 1.1	356 ± 42	0.8 ± 0.1	3.5 ± 0.2	4

Table 3.2 - All time points had statistically similar intoxication scores, daily ethanol doses, BEC's, and withdrawal scores. Thus, exposures are equivalent across the different time points.

Withdrawal scores were assigned based on observations of behavioral signs of ethanol withdrawal (Penland et al., 2001). The Kruskal-Wallis test revealed no significant differences among time points for mean or peak withdrawal scores (Table 3.2). Here, the mean withdrawal score was 0.8 ± 0.1 which corresponded to hyperactivity on the behavioral withdrawal scale (Table 2.2). Additionally, the peak withdrawal score was 3.3 ± 0.1 which corresponded to induced running.

3.3.1.2 Time course of proliferation following binge alcohol exposure

Proliferation was measured using IHC for cells that have incorporated BrdU during DNA synthesis. BrdU with DAB-IHC was performed on rat brain tissue obtained either 4, 5, 6, or 7 days post-alcohol exposure. Representative images illustrate BrdU+ cells along the SGZ in ethanol and control animals for each time point (Figure 3.2). One-way ANOVA revealed that control BrdU+ cell counts at each time point were statistically similar [$F_{(3,7)}$=3.1; p=0.10] and therefore controls were combined into one common control group. One-way ANOVA compared BrdU+ cell counts at each time point against controls and revealed an effect of diet [$F_{(4,26)}$=29.69; $p < 0.0001$]. Dunnett's multiple comparisons test revealed a significant increase in BrdU+ cell counts in ethanol groups on T5 ($p < 0.005$), T6 ($p < 0.0005$), and T7 ($p < 0.0005$) when compared to the combined control group (Figure 3.3).

Figure 3.2 - Representative images for time course of reactive proliferation

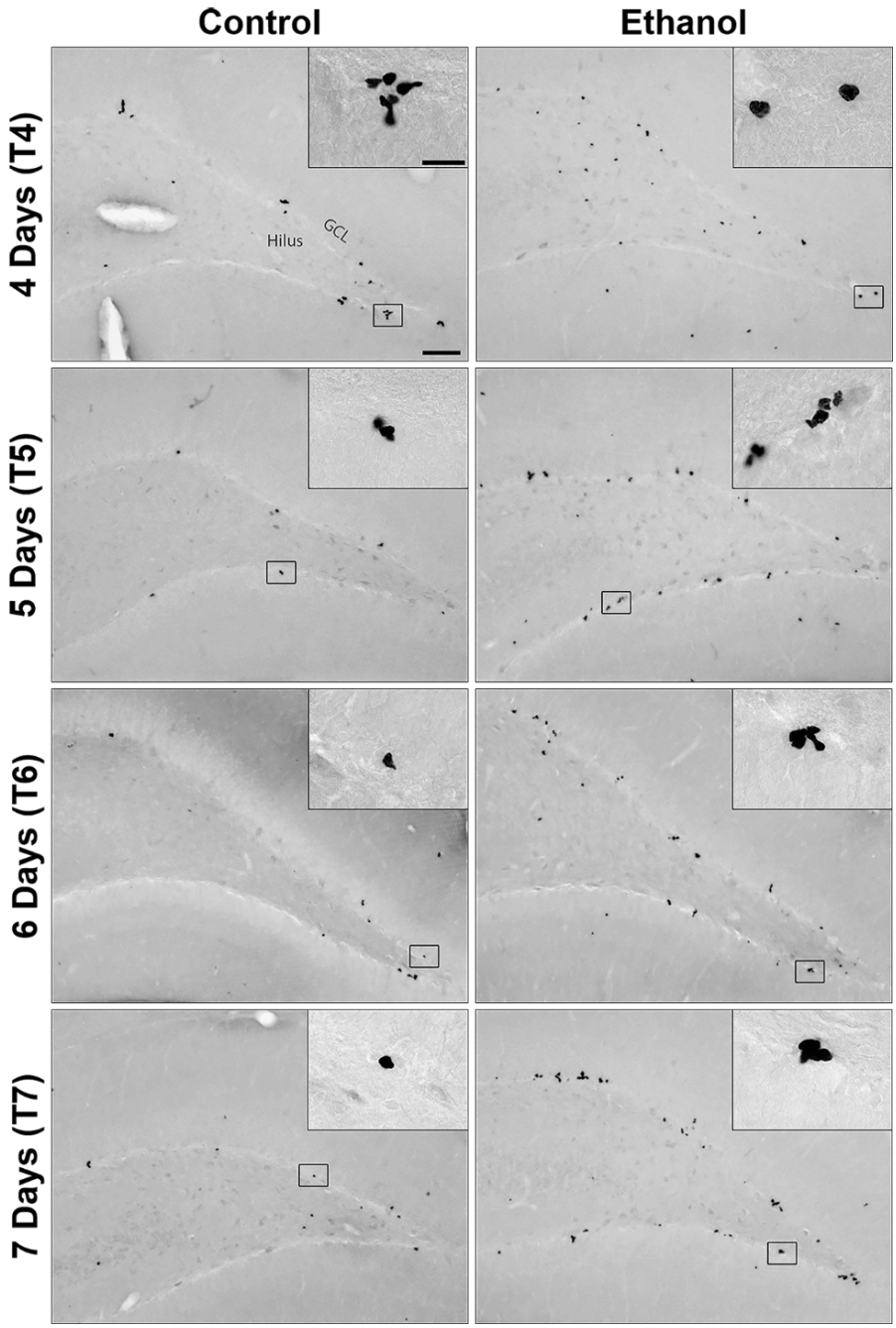


Figure 3.2 - Representative images are shown for each time point for both control and binge ethanol groups. Only cells that resided within the SGZ were quantified. Images were taken at 80x magnification, boxed inset images were taken at 1200x magnification. Scale bar = 100 μm; 20 μm inset.

Figure 3.3 - Time course of alcohol-induced reactive proliferation

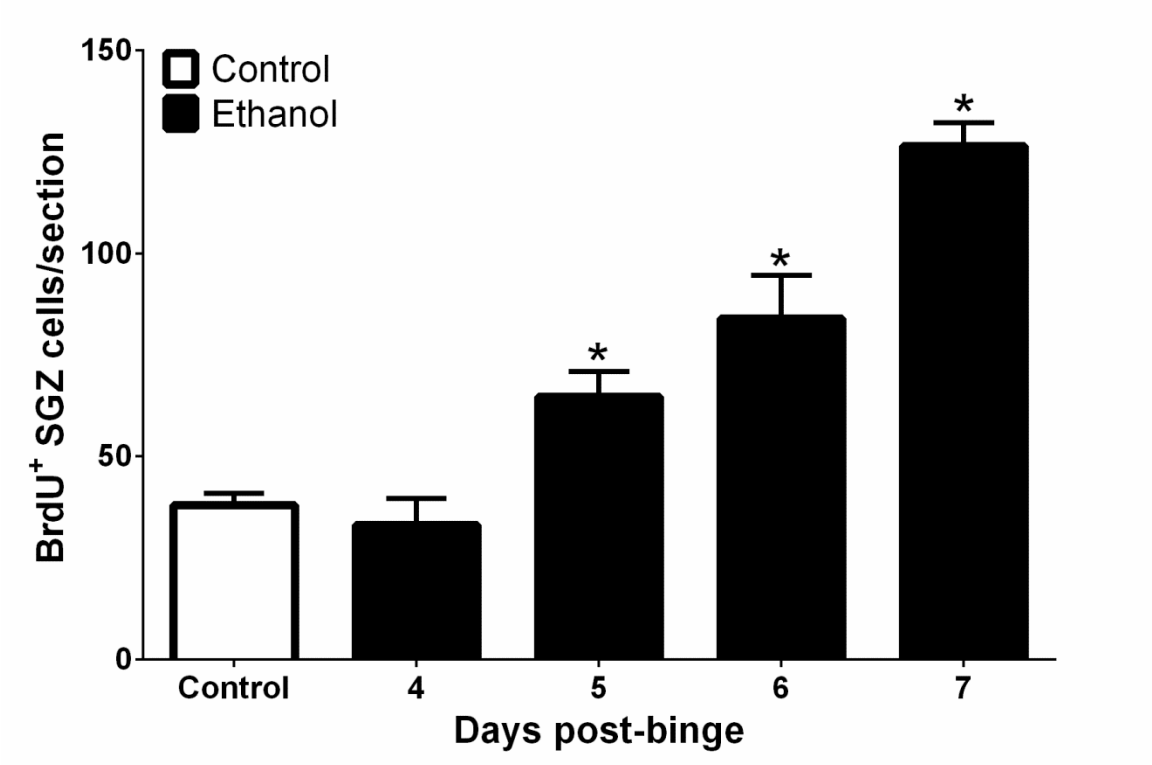


*Figure 3.3 - Increased alcohol-induced reactive proliferation begins five days post-binge and continues through 7 days post-binge. *p < 0.005 indicates a significant increase from control.*

3.3.2 Confirmation of reactive neurogenesis

3.3.2.1 Animal model and withdrawal data

To confirm reactive neurogenesis following a 4-day binge model of an AUD, rats were sacrificed either 7 (T7) or 14 (T14) days post-binge. These time points were selected because the increase in proliferation seen at T7 results in an increase in neurogenesis at T14 (Brown et al., 2003; Nixon and Crews, 2004). Table 3.3 summarizes animal model data from the two different groups of animals given ethanol (T7 and T14). Student's t-test's compared T7 and T14 ethanol groups and showed no significant difference in daily ethanol dose, but revealed a significant difference in BEC's [$t_{(15)}$=2.55, p=0.022]. Nonparametric data was analyzed with Mann-Whitney tests and showed no significant difference in intoxication score, but revealed a significant difference in both mean [U=5, p=0.001] and peak [U=8, p=0.001] withdrawal scores between T7 and T14 ethanol groups. Although there were differences between T7 and T14 groups in terms of BEC and withdrawal, all animals had similar intoxication scores and received similar doses of ethanol.

3.3.2.2 Alcohol-induced reactive neurogenesis seen with NeuroD

To further characterize the time course of reactive neurogenesis following binge ethanol exposure, NeuroD immunoreactivity was examined at both one and two week's post-ethanol administration. As shown in Figure 2.4 A-D, the SGZ of the hippocampal dentate gyrus displayed ample NeuroD+ labeling in all groups. A two-way ANOVA (diet x time point) revealed significant main effects of both diet [$F_{(1,27)}$=6.48, p<0.05] and time point [$F_{(1,27)}$=22.31, p<0.001], as well as a

Table 3.3 - Animal model parameters for the neurogenesis study

Days Post-binge	Intox. score	Dose (g/kg/day)	BEC (mg/dl)	Mean WD	Peak WD	n
7	1.8 ± 0.1	9.8 ± 0.4	307 ± 25*	1.0 ± 0.1*	3.0 ± 0.0*	8
14	1.9 ± 0.1	9.3 ± 0.3	377 ± 13	1.8 ± 0.2	3.6 ± 0.1	9

*Table 3.3 - Intoxication scores and daily ethanol doses were statistically similar between T7 and T14 time points; however, there were differences between BEC's and withdrawal (WD) scores between T7 and T14. *=p < 0.05*

Figure 3.4 - Representative images and NeuroD+ cell counts for T7 and T14

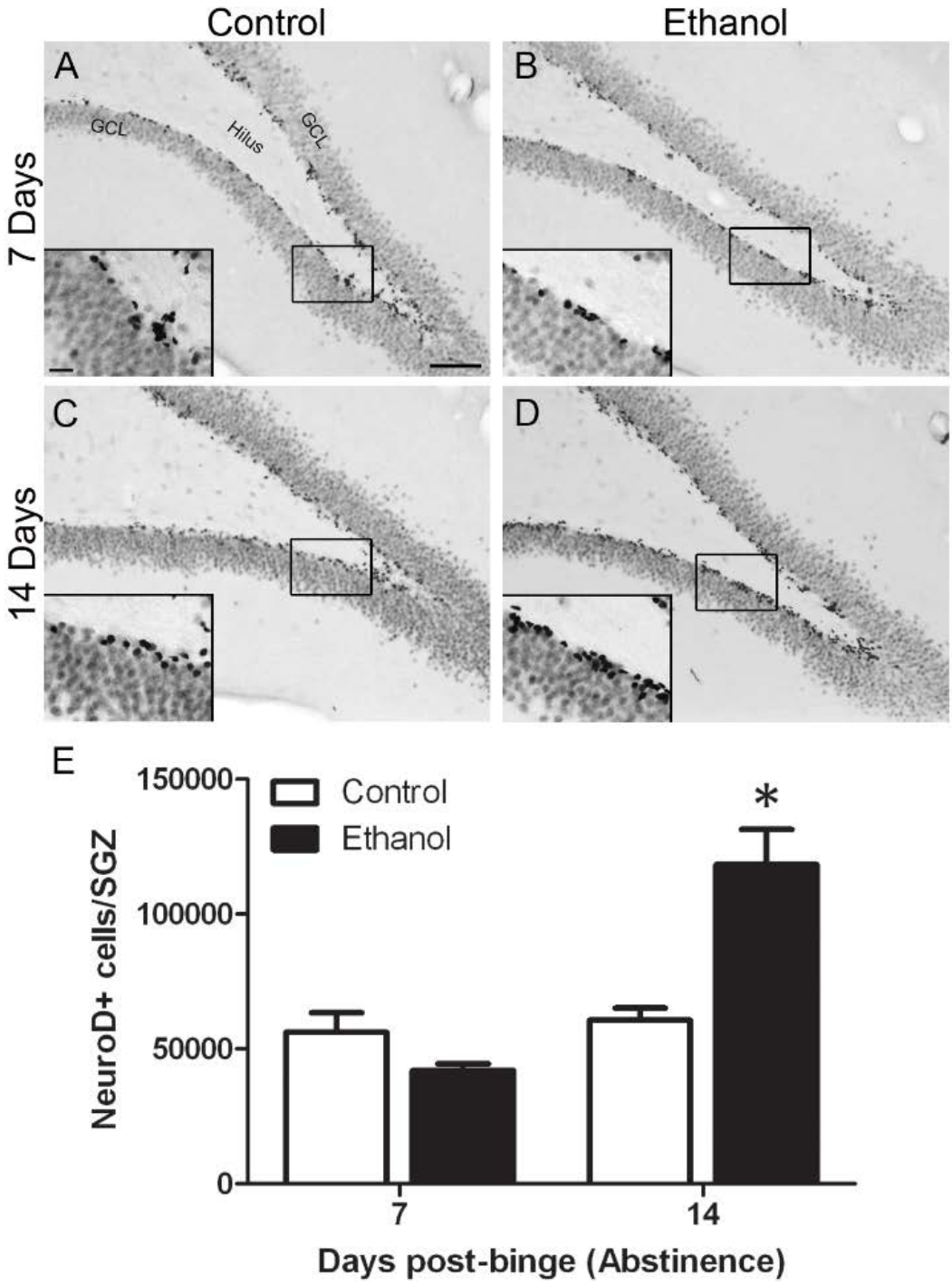


Figure 3.4 - The total number of NeuroD+ cells are increased following 14, but not 7, days of abstinence in ethanol-treated rats. Representative images of the hippocampal dentate gyrus taken at 100x are shown for control (A, C) and

Figure 3.4 (continued)

*ethanol-exposed animals (B, D) at 7 days (A, B) and 14 days (C, D) post-ethanol exposure. Insets for all images were taken at 600x. The number of NeuroD+ cells in the SGZ of the hippocampal dentate gyrus was determined using unbiased stereology as shown in panel E. GCL, granule cell layer; scale bar = 100 μm; inset scale bar = 20 μm; *p < 0.05 = a significant increase in NeuroD+ cells in the ethanol group compared to controls at T14.*

significant interaction [$F_{(1,27)}$=17.68, p<0.001]. Bonferroni multiple comparisons test revealed a significant increase in the total number of NeuroD+ cells in the SGZ of ethanol rats compared to controls at the 14-day time point (p<0.001), but not at the 7-day time point. Because NeuroD is a marker of cells committed to a neuronal fate (Gao et al., 2009), the increase in NeuroD+ cells at T14 may reflect an increase in the number of new neurons originating from reactive neurogenesis (including the cells undergoing reactive proliferation on day 7 post-binge).

3.4 DISCUSSION

Here we show, for the first time, when the increase in alcohol-induced reactive proliferation is first detectable. This finding indicates that binge alcohol exposure resulted in an increase in BrdU+ cell counts 5-7 days post-binge (T5, T6, T7). BrdU was used as a marker of proliferating cells and, as such, an increase in BrdU+ cells represents an increase in the number of proliferating cells. Because this proliferation occurred following binge alcohol exposure, it is further classified as reactive proliferation. BrdU+ cell counts revealed no difference between ethanol and control rats 4 days (T4) after the end of binge alcohol exposure but showed increased proliferation 5, 6, and 7 days after the end of binge alcohol exposure. Although previous reports investigated proliferation at multiple time points following binge alcohol exposure, proliferation had not been examined 5 and 6 days post-binge (Nixon and Crews, 2004; Nixon et al., 2008). Here, a more extensive time course was necessary to ensure a thorough understanding of the timing of alcohol-induced reactive proliferation; a

necessary step to establish a novel protocol to decrease alcohol-induced reactive proliferation.

The increase in cell proliferation (BrdU+ cell counts) spanning multiple days (T5-T7) is consistent with that reported in animal models of ischemia and seizure (other models that result in neurodegeneration) which show a reactive increase in cell proliferation one week later (Liu et al., 1998; Parent et al., 1997; Smith et al., 2005). Research involving the 4-day binge model used here has shown increased proliferation at two time points post-binge (keep in mind BrdU is not specific to neurons – it labels all cells replicating at the time of injection). We have already thoroughly discussed the increase in proliferation occurring on T7 that results in ~80% mature neurons 28 days later (Nixon and Crews, 2004). In addition to T7 neurogenesis, there is also an increase in BrdU+ cells (proliferation) two days post-binge (T2). T2 studies used a BrdU injection on T2 followed by a 28 day waiting period (to allow cells to mature) and then triple-label IHC revealed that cells proliferating on T2 (assuming survival) primarily matured into microglia, not neurons (Nixon et al., 2008). There are no studies that birthdate proliferating cells at T5 (with BrdU) and examine them 28 days later, when they are considered mature neurons. As such, we cannot definitively say that T5 proliferation resulted in an increase in mature neurons; for that additional studies are needed. It is possible that T5 proliferation may generate microglia instead of neurons however, that is unlikely for a couple of reasons. Recently, we have used quadruple-label IHC and revealed that, at T5, ethanol rats show a 2-fold increase in the number of cells expressing an early neuronal marker

compared to controls (Hayes et al., in preparation). This recent study by Hayes et al. revealed some important points: one, it confirmed that there was an increase in proliferation with Ki67 at T5 in ethanol rats, and two, it was able to show that the increased proliferation seen at T5 was generating cells expressing neuronal markers (NeuroD). This means that the increased proliferation seen at T5 is neuronal. Furthermore, previous studies have shown that proliferation increases at T2 (yielding microglia; BrdU+/Iba1+/NeuN- cells), which normalizes by T4 and is followed by a separate increase in proliferation at T7 which yields neurons (Nixon and Crews, 2004; Nixon et al., 2008). The present study did not observe a difference in the number of SGZ BrdU+ cells between ethanol and controls at the T4 time point which lends support to separation between the two proliferation increases (seen at T2 and T7). Additionally, the T4 time point was investigated in Nixon and Crews (2004) and showed similar results to the present study in that there was no significant difference between ethanol and control groups at T4. Although, the dentate gyrus regions that were quantified were not identical between Nixon and Crews (2004) and the present study. The entire GCL was counted in the 2004 study (while only the SGZ was quantified in the present study) which resulted in increased variability compared to the present study. Additional clues to indicate the cell type come from examining the tissue sections at the various time points. For example, T2 hippocampal sections show a random, yet even, distribution of BrdU+ cells across the dentate gyrus including numerous cells in the hilus region (Nixon et al., 2008). That same, hilar, even, random pattern (and hilar region staining) of BrdU+ cell expression is seen in the

present experiments but *only* in the T4 ethanol brain (Figure 3.2). In summary, it is likely that there is some separation between the two events (T2 and T7) and that the proliferation occurring at T5-7 likely results in neurogenesis. However, an additional experiment involving triple-label IHC would be needed to determine if the cells proliferating at T5 and T6 go on to primarily become *mature* neurons. The present studies can only conclusively state that there is an increase in proliferation from T5-T7 and that there is an increase in neurogenesis at T14 in ethanol animals compared to controls.

Original studies investigating the time course of reactive neurogenesis following 4-day binge ethanol exposure showed that an increase in cell proliferation at T7 is followed by an increase in doublecortin (DCX)-immunoreactivity one week later (on T14) suggesting that the newly born neurons are capable of differentiation into immature neurons (Nixon and Crews, 2004). However, recent research reports indicate that DCX should be cautiously interpreted because DCX+ cells are not neuron-specific; DCX+ cells can co-express the oligodendrocyte precursor marker, Olig2 (Diaz et al., 2013). Therefore, we utilized NeuroD, a transcription factor expressed by progenitor cells committed to a neuronal fate and known to overlap with DCX expression to confirm the original findings of reactive neurogenesis (Lee et al., 1995; Steiner et al., 2006). As anticipated, we showed a significant increase in $NeuroD^{+}$ cells at T14, but not at T7, thereby mirroring the DCX timeline originally reported by Nixon and Crews in 2004.

Differences in the animal model data could represent a study limitation. Analysis of animal model data revealed an increase in BEC's, and mean and peak withdrawal in T14 animals when compared to T7 animals. An increase in intoxication level could potentially result in a larger increase in reactive neurogenesis. However, BEC is only examining blood alcohol levels at a single time point and as such is not necessarily indicative of intoxication throughout the entire 4-day binge. A more accurate representation of intoxication throughout the duration of the binge comes from the behavioral intoxication scores which are taken every eight hours for the entire 4-day binge and these were not significantly different between T7 & T14. The mean dose of alcohol given was also not significantly different between time points. This information coupled with similar studies showing increases in DCX, a marker with a similar expression pattern to NeuroD, indicates that the increased NeuroD expression seen in ethanol animals at T14 is likely due to increased neurogenesis and not a result of differences in animal model data (McClain et al., 2014). However, since two measures of intoxication suggest that the model, overall, may be different between groups, model differences cannot be ruled out.

Overall this research represents a significant contribution to the reactive neurogenesis field. Previously, alcohol-induced increases in neuronal proliferation were only reported on T7 (McClain et al., 2014; Nixon and Crews, 2004). Here we show that this increased proliferation starts earlier than realized, occurs over multiple days, and is followed by an increase in reactive neurogenesis at T14. The research reported in this study indicated that (for future

studies detailed in the following chapter) inhibition of reactive proliferation must start beginning on T4, just prior to the increase seen at T5-7, and administration should continue through at least T6. This information will assist in establishing a method to reduce reactive proliferation/neurogenesis, thereby allowing us to investigate reactive neurogenesis's contribution to neuronal recovery following a binge model of an AUD.

CHAPTER 4: INHIBITION OF REACTIVE PROLIFERATION AND NEUROGENESIS USING TEMOZOLOMIDE

4.1 INTRODUCTION

Excessive alcohol consumption contributes to widespread neurodegeneration (discussed in detail in section 1.2.3). Alcohol can damage both white matter tracts comprised of glial cells (microglia, astrocytes, and oligodendrocytes) and grey matter tracts composed of neurons (reviewed in Alfonso-Loeches and Guerri, 2011). In humans, cognitive deficits are associated with alcohol-induced cellular loss (Beresford et al., 2006; Parada et al., 2011; Pfefferbaum et al., 1992). Animal models of alcohol use disorders also show deficits in learning and memory (Cippitelli et al., 2010; Kuzmin et al., 2012; Obernier et al., 2002b). A 4-day binge model of an AUD resulted in a learning deficit in alcohol exposed rats (compared to controls) one week post-binge (see Appendix, Supplemental Figure 1). However, with cessation of alcohol intake comes recovery and studies show that a certain amount of brain regeneration (in terms of grey and white matter volume) can occur (van Eijk et al., 2013; O'Neill et al., 2001; Pfefferbaum et al., 1995).

Adult neurogenesis may contribute to neuronal recovery. When high concentrations of alcohol are administered (like during binge exposure), adult neurogenesis is substantially reduced (Nixon and Crews, 2002) and then increases in proliferation are seen 5-7 days post-binge (Hayes et al., in preparation; Nixon and Crews, 2004). This increase is thought to be the brain's way of compensating for the cells it was unable to produce during intoxication

(Crews and Nixon, 2009; Crews et al., 2006a). Therefore, the brain's reaction to alcohol-induced reduced neurogenesis is to temporarily increase neurogenesis to make-up for lost time (or, lost neurons in this case). The process has been termed alcohol-induced reactive neurogenesis and its function is unknown.

An important question that has not been address is whether reactive neurogenesis is beneficial for recovery from AUDs. Since the hippocampus is involved in learning and memory, studies have investigated alcohol's effects on hippocampal-dependent tasks. In fact, research in the Nixon lab has shown that adult rats tested in the MWM 7-10 days after binge alcohol treatment swam longer distances before locating the hidden platform, which is indicative of a learning deficit (see Appendix, Supplementary Figure 1). However, these deficits normalized by 3 weeks post-binge (Obernier et al., 2002b). Since hippocampal-dependent learning and memory returns to normal after reactive neurogenesis, it is possible that reactive neurogenesis may contribute to the functional recovery of the hippocampal dentate gyrus. Although the previous statement is purely correlative evidence, additional experiments must be done to test causation.

To assess the contribution that reactive neurogenesis makes to regaining hippocampal function we administered Temozolomide (TMZ), a DNA alkylating agent that can penetrate the blood brain barrier and kill rapidly proliferating cells (Chowdhury et al., 1999; Omar and Mason, 2009). Since Type 2 NPCs are rapidly proliferating cells produced during neurogenesis, it is possible that TMZ could reduce alcohol-induced reactive neurogenesis. However, the effect of TMZ on alcohol-induced reactive neurogenesis has never been investigated.

Furthermore, there is no way to know which cells are reactive or which cells are contributing to constitutive neurogenesis since there is not a way to tease apart these populations. Thus, there is no way to target only the reactive population since we cannot differentiate this population from the cells undergoing normal neurogenesis. Therefore, the best approach was to reduce proliferation in general with TMZ and then test rats in a hippocampal dependent task (MWM). Using this approach it was possible to investigate the role of reactive neurogenesis in recovery of hippocampal function (learning and memory). If endogenous reactive neurogenesis is involved in hippocampal recovery then it would represent a novel target for pharmaceutical intervention.

The purpose of this study was to develop an approach to reduce alcohol-induced reactive neurogenesis to basal neurogenesis levels seen in control animals. First, an appropriate dose of TMZ was determined. Second, the appropriate dose of TMZ was tested to ensure that it could decrease proliferation and the associated neurogenesis; care was taken to ensure that TMZ did not simply delay reactive proliferation. Third, we assessed how inhibiting reactive neurogenesis (with TMZ) would affect hippocampal learning and memory. The main purpose of these experiments was to address this third point.

Determining a dose of TMZ that was able to decrease reactive proliferation was the first goal. Previous studies used 25-50 mg/kg TMZ to decrease basal neurogenesis by cyclical administration, three days on/four days off for multiple weeks (Garthe et al., 2009; Niibori et al., 2012; Nokia et al., 2012). Since alcohol-induced reactive proliferation occurs over a short period (T5-T7),

the present study only allowed for a single three-day cycle to effectively target alcohol-induced reactive proliferation (Figure 3.1). Also, data from Chapter 3 indicated that the optimal time for TMZ administration would be four to seven days following alcohol administration. Although there is no increase in proliferation at T4, there is an increase at T5, and we hypothesized that to effectively decrease proliferation at T5 the drug needs to be administered prior to the increase. A dose response curve determined the appropriate concentration of TMZ to administer and the following experiments used that regimen that reduce reactive NPC proliferation to control levels.

Once a dose of TMZ that effectively decreased reactive proliferation was determined, we confirmed the results by examining a marker of neurogenesis. Because the proliferation marker BrdU is not neuron-specific, it was necessary to use a neurogenesis-specific marker to confirm neurogenesis (NeuroD). Previous studies revealed that reactive proliferation is seen at T7 and reactive neurogenesis is seen at T14 (Nixon and Crews, 2002; 2004). Neurogenesis was investigated 14 days after the last dose of ethanol ensure that reactive proliferation was not delayed.

The ultimate goal of these experiments was to determine if alcohol-induced reactive neurogenesis contributed to recovering the deficit seen 7-10 days post-binge in the MWM in rats (Supplemental Figure 1). Therefore, after the normalization of reactive proliferation and neurogenesis were confirmed, a hippocampal-dependent learning and memory task (MWM) was administered to assess the potential function of alcohol-induced reactive proliferation.

4.2 METHODS

4.2.1 Binge model of an alcohol use disorder

Three separate experiments used a total of 114 male Sprague-Dawley rats weighing 275-300 grams on arrival (Table 4.1; Charles River Laboratory; PND 70). One rat (a T14 saline + ethanol rat) was excluded from analysis because of failure to incorporate BrdU. Rats were double housed and allowed to acclimate to the University of Kentucky AAALAC-accredited vivarium for five days before experimental procedures began. Rats were kept on a 12-hour light/dark cycle with *ad libitum* access to food and water except when noted. All rats were handled for three days before the experiments began. All experimental procedures were completed in accordance with the University of Kentucky Institutional Animal Care and Use Committee and followed the Guidelines for the Care and Use of Laboratory Animals (NRC, 1996).

For each of the three experiments detailed below, the rats were divided into two groups with statistically similar weights and administered either 25% (w/v) ethanol in Vanilla Ensure Plus® (Abbott Laboratories, Columbus, OH) or an isocaloric control diet (calorically equivalent quantities of dextrose) in Vanilla Ensure Plus®. Ethanol or control diet was administered every eight hours for four consecutive days. Ethanol animals received 5 g/kg ethanol diet initially and the subsequent doses were titrated based on the animal's behavioral intoxication score according to the scale in Chapter 2 (Table 2.1; Majchrowicz, 1975; Morris et al., 2010).

Table 4.1 - Temozolomide studies: experimental design

Time point	TMZ dose	Study	Ethanol	Control
T7	Saline	4.3.1	8	6
T7	25 TMZ	4.3.1	9	6
T7	50 TMZ	4.3.1	8	6
		Total	**43**	
T14	Saline	4.3.2	7	8
T14	50 TMZ	4.3.2	8	8
		Total	**31**	
T35	Saline	4.3.3	9	10
T35	50 TMZ	4.3.3	11	10
		Total	**40**	

Table 4.1 - Animal numbers separated by time point, TMZ dose (mg/kg/day), study, and diet.

All ethanol animals underwent monitored withdrawal for 17 hours beginning 10 hours after the last dose of ethanol (previously described in Chapter 2). Rats were observed for 30 minutes of every hour for 17 hours and withdrawal behavioral features (listed in Table 2.2) were recorded (Majchrowicz, 1975; Morris et al., 2010a; Penland et al., 2001). Results for each experimental time point (T7, T14, and T38) were analyzed separately because each is considered a separate experiment. Data are presented as mean withdrawal ± standard error of the mean and peak withdrawal ± standard error of the mean.

4.2.1.1 Blood ethanol concentration

Ninety minutes following the seventh dose of ethanol, tail bloods were collected to determine BEC (previously described in Chapter 2). Blood samples were centrifuged at 1,800 x g for five minutes to separate blood plasma from blood cells and then stored at -20º C until processing. Supernatant blood serum was processed in triplicate using an AM1 Alcohol Analyzer (Analox, London, UK). Data are presented as BEC (mg/dl) ± standard error of the mean.

4.2.2 Temozolomide

Following the 4-day binge model of an AUD, rats were administered TMZ, a DNA alkylating agent used to decrease cellular proliferation (Garthe et al., 2009, 2016; Nokia et al., 2012). Animals were weighed daily just prior to TMZ administration and TMZ (Cayman Chemicals, Ann Arbor, MI) was made fresh prior to each injection (5 mg/ml in 0.9% normal saline). TMZ was sonicated for 30 minutes at 35º C in a water bath and then the solution was then transferred to a cell culture hood and filter sterilized. A vehicle solution of 0.9% normal saline was

injected as a control into all rats not receiving a TMZ injection. All rats in these experiments (listed here and below) received the same number of i.p. (intraperitoneal) injections at approximately the same times to ensure similarity between groups.

4.2.2.1 Temozolomide optimization study: T7

To establish a dose of TMZ capable of decreasing alcohol-induced reactive proliferation, 43 rats (Table 4.1) were given one injection every 12 hours for 3.5 days starting 3.5 days after the last dose of ethanol (Figure 4.1). Rats received either (A) one saline injection every 12 hours, (B) alternating injections of 25 mg/kg TMZ and saline (to balance the number of injections), or (C) one 25 mg/kg TMZ injection given every 12 hours – resulting in a total daily dose of 50 mg/kg/d TMZ. Animals were injected with BrdU (300 mg/kg, Roche, Mannheim, Germany) in 0.9% saline seven days following binge alcohol exposure (T7, ~168 hours after their last dose of ethanol). Two hours later rats were sacrificed by pentobarbital overdose (Fatal Plus®) followed by intracardial perfusion and brain extraction (as previously described in Chapter 2). Animals were sacrificed on T7 to determine if TMZ was able to decrease the reactive increase in proliferation on T7. TMZ was not given on T7 due to timing and consistency issues. Animals are injected with BrdU ~168 hours (7 days) after their last alcohol exposure and sacrificed two hours later. It is not known if BrdU and TMZ interact and therefore administering these drugs within a couple hours of one another is not ideal (i.e. giving TMZ at 7 AM and then giving BrdU at 9 AM).

Figure 4.1 - Experimental design diagram for TMZ studies

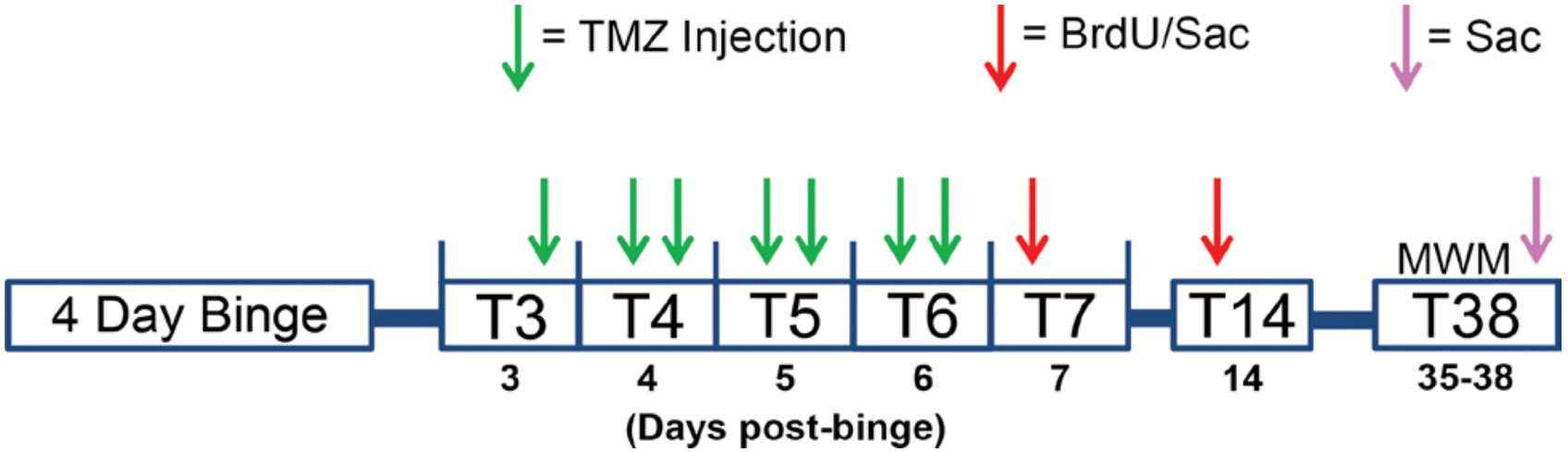


Figure 4.1 - Timeline of Temozolomide (TMZ) administration. Rats underwent four days of binge alcohol exposure and were then given TMZ (or saline) injections every 12 hours for 3.5 days. Rats were then injected with BrdU and sacrificed (Sac) either seven or 14 days post-binge. T38 rats were sacrificed 38 days post-binge after the MWM.

Also, BrdU's solubility is such that large volumes must be i.p. injected and closely timed BrdU and TMZ injections would exceed max injection volumes if animals were injected with BrdU and TMZ within a couple hours of each other. Additionally, in order to maintain consistency with previous studies within our laboratory the rats needed to be sacrificed early on T7 (which begins at 7 AM) and so it was not ideal to push back the sacrifice time. Therefore, a 7 AM dose of TMZ was not included so BrdU could be administered instead.

4.2.2.2 Proliferation and neurogenesis study: T14

Based on the success of the dosing regimens listed above the rats in this experiment (n=32) were given one 25 mg/kg injection every 12 hours, resulting in a total daily dose of 50 mg/kg/d TMZ (as described immediately above). Injections began 3.5 days after the last dose of ethanol and continued for 3.5 days (Figure 4.1). Rats were injected with BrdU (300 mg/kg, Roche, Mannheim, Germany) in 0.9% saline, 14 days following binge alcohol exposure (T14). Animals were sacrificed two hours later by pentobarbital overdose (Fatal Plus®) followed by transcardial perfusion and brain extraction (previously described in Chapter 2). This T14 time point was selected because reactive proliferation occurring on T7 results in a reactive increase in neurogenesis and can be seen with an early marker of neurogenesis, one week following the T7 proliferation increase on T14. Furthermore, the addition of an anti-proliferative drug during reactive proliferation could simply delay the reactive increase that occurs on T7, so proliferation was examined one week later.

4.2.2.3 T38 MWM study: determine the effect of TMZ on MWM behavior

Based on the success of the dosing regimens listed above, rats in this experiment (n=40) were given one 25 mg/kg injection every 12 hours, resulting in a total daily dose of 50 mg/kg/d TMZ (as described in section immediately above). Injections began 3.5 days after the last dose of ethanol and continued for 3.5 days (Figure 4.1). Animals were sacrificed 38 days following binge alcohol exposure (T38) by pentobarbital overdose (Fatal Plus®) followed by intracardial perfusion and brain extraction, previously described in Chapter 2. The T38 time point was selected since reactive neurogenesis occurs 7 days after binge exposure (Nixon and Crews, 2004) and because Jessberger and Kempermann (2003) indicated that newborn neurons require 28 days to express mature neuronal markers (7 days + 28 days + 4 days of MWM = 38 days). Additional studies show that cells that are a minimum of 28 days old are recruited for use in the MWM, and young neurons that are six to eight weeks old are more than twice as likely to be utilized for the MWM task (Kee et al., 2007). T38 animals were not given BrdU because this experimental time point was focused on behavioral outcome measures. However, these brains were harvested in the event that further analysis was needed.

The MWM was used to assess spatial learning and memory following binge alcohol exposure and TMZ administration. Experiments were conducted similar to previous studies (Verbois et al., 2003) with equipment in collaboration with the laboratory of Dr. James Pauly. Briefly, five weeks post-binge alcohol exposure (four weeks post-TMZ/saline injections), rats were trained in the MWM

for four days. Each day consisted of a block of four 60-second trials where the rat searched for a submerged platform, 13 cm in diameter, hidden in a 180 cm diameter pool of water dyed black with non-toxic paint. Once the rat located the platform, he was allowed to remain on the platform for 15 seconds before being placed back in his home cage to begin a four minute inter-trial interval. The maze starting points (N, S, E, W) were the same for all animals in a given day, but each day the sequence of starting points was different (counterbalanced in a Latin square design). For example, day 1 was organized so that the entry points for the four trials were N, S, E, W while entry points on day 2 were W, E, S, N. The platform remained in the same position for all four days. On the final day, approximately one hour after their last trial, the platform was removed and the rat was placed back in the pool at a novel entry point (SW) for a probe trial. The settings were fixed so that if a rat spent 5 seconds in the target zone (12.7 cm zone around old platform location), the trial would end. If the rat did not spend 5 seconds in the target zone then the rat had 60 seconds to try and complete this task. Animals were organized into blocks for behavioral testing so that each trial contained a different treatment group, ensuring that no treatment group was run consecutively. A video camera and a motion analyzer (EthoVision XT 10, version 10.1, Noldus, Wageningen, The Netherlands) connected to a Dell Precision T3610 computer was used to measure distance to reach the platform (cm), time to reach the platform (seconds), and velocity (cm/second). Daily blocks (four trials) were averaged for each rat then averaged across treatment groups to obtain daily means for distance, time, and speed ± standard error of the mean.

4.2.3 Immunohistochemistry and quantification

Tissue was stained for NeuroD using previously described IHC methods (Chapter 3). Previous studies have shown that NeuroD is increased 14 days after binge alcohol exposure (Hayes et al., in prep) and therefore an increase at T14 likely represents T7 NPCs that have differentiated into a neuronal phenotype (Gao et al., 2009). Quantification of NeuroD was performed differently for experiments in this chapter. In Chapter 3, unbiased stereology was used to count NeuroD+ cells and since NeuroD is expressed uniformly, with cell counts over 150 cells/section, West (1991) showed that this was an appropriate way to accurately quantify the SGZ (West, 1991). However, profile cell counts represent a logical alternative to stereology and provide a way to quantify the SGZ allowing the experimenter to count all the cells in the region of interest (Crews et al., 2004b). Importantly, counting all cells should theoretically be as accurate, if not more accurate than only counting a sample of cells, even if it is randomly generated. In this experiment, NeuroD+ cells were counted using profile cell counts since the number of NeuroD+ cells was easily quantifiable, accuracy was similar to stereology, and profile counting saved experimenter time.

BrdU+ (240 µm between sections) and NeuroD+ (480 µm between sections) cells were quantified on a single hemisphere between Bregma -2.28 mm and -5.52 mm (as determined by Paxinos and Watson, 2009). For each rat, all tissue sections were inspected and the side without tears to the SGZ was selected for quantification. BrdU+ and NeuroD+ cells were then counted along the SGZ, a band of cells approximately three cell widths that runs between the

GCL and the hilus of the hippocampal dentate gyrus. Cells profiles were counted manually at 1000x magnification with a 100x objective lens coupled to an Olympus BX41 microscope. Brains were coded to blind the experimenter to the treatment conditions during quantification. Data are presented as the average number of immunopositive SGZ cells/section ± standard error of the mean.

4.2.4 Statistics

All data were analyzed with GraphPad Prism® (version 7.0, GraphPad software, La Jolla, CA) or SPSS Statistics (version 22, IBM, Armonk, NY). Intoxication parameters were analyzed by comparing ethanol groups using a one-way ANOVA (ethanol dose and BEC) or the non-parametric Kruskal-Wallis test (intoxication and withdrawal scores). BrdU+ (T7 and T14) and NeuroD+ (T14) cell counts were analyzed by two-way ANOVA (diet x drug) followed by planned t-tests. It was expected that there would not be a significant interaction between diet and drug because TMZ would likely decrease proliferation across all conditions and therefore *a priori* t-tests were used to analyze the critical comparisons (the most critical being between the ethanol + TMZ group and the control + saline group). MWM data was analyzed by three-way repeated measures ANOVA (diet x drug x day) and posthoc Bonferroni's multiple comparisons test, when appropriate. The MWM probe trial data was analyzed by two-way ANOVA (diet x drug). *P*-values were accepted as significant when $p < 0.05$.

4.3 RESULTS

4.3.1 TMZ normalizes alcohol-induced reactive proliferation at T7

Animals were administered 0, 25, or 50 mg/kg/d TMZ following binge alcohol (or control) exposure to decrease alcohol-induced reactive proliferation (Table 4.1 and Figure 4.1). Intoxication parameters were compared between treatment groups to rule out potential confounding effects of model-differences on TMZ's effect on neurogenesis (Figure 4.2). One-way ANOVA of BEC and alcohol dose as well as Kruskal-Wallis tests of intoxication score and withdrawal score revealed no differences between groups. Therefore, T7 rats displayed a mean intoxication score of 1.8 ± 0.06 on the 6-point intoxication scale (Figure 4.2) which is represented behaviorally by ataxia and an elevated abdomen. T7 rats received a mean daily ethanol dose of 9.7 ± 0.2 g/kg/d and displayed average BECs of 354 ± 8 mg/dl. These values are similar to those previously reported (Morris et al., 2010a).

Clusters of BrdU+ cells were located along the SGZ and were quantified seven days after binge alcohol administration and 12 hours after the last administration of TMZ to assess the effect of TMZ on alcohol-induced reactive proliferation. A two-way ANOVA revealed a main effect of diet [$F_{(1,37)}$=30.86; P < 0.0001] and a main effect of drug [$F_{(2,37)}$=6.098; P=0.0051]. Planned t-tests revealed no differences between 0 and 25 mg/kg/d TMZ for either ethanol or control groups. However, there was a difference between 0 and 50 mg/kg/d TMZ for both ethanol [$t_{(14)}$=4.708; p=0.0003] and control [$t_{(10)}$=5.316; p=0.0003] groups such that the number of BrdU+ cells/section was significantly reduced in TMZ

Figure 4.2 - T7 animal model data

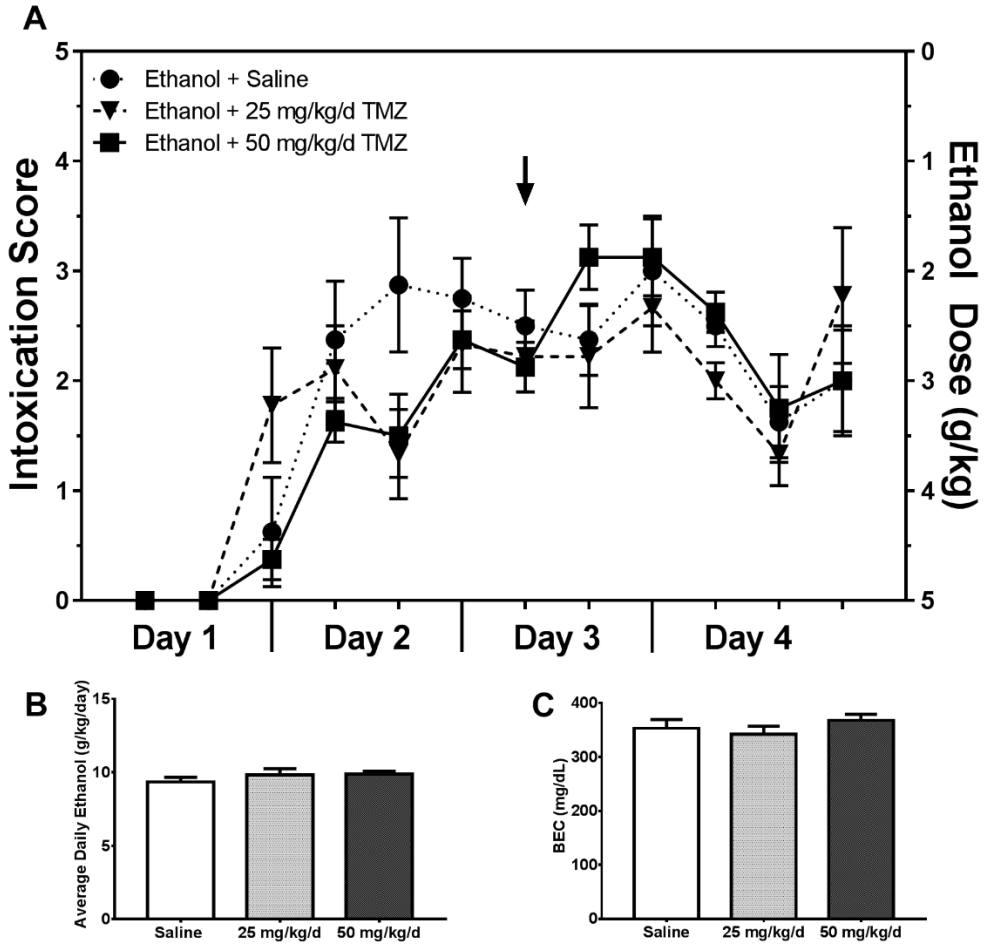


Figure 4.2 - Animals were treated with four days of binge alcohol administration followed by TMZ administration (0, 25, or 50 mg/kg/day). (A) Behavioral intoxication scores (left axis) and corresponding ethanol dose (right axis) were similar between groups. (B, C) Average daily ethanol dose and BEC did not differ between groups. Arrow = when bloods were taken for BEC; n = 8-9 for ethanol groups.

Figure 4.3 - Representative images and BrdU+ cell counts for T7 optimization

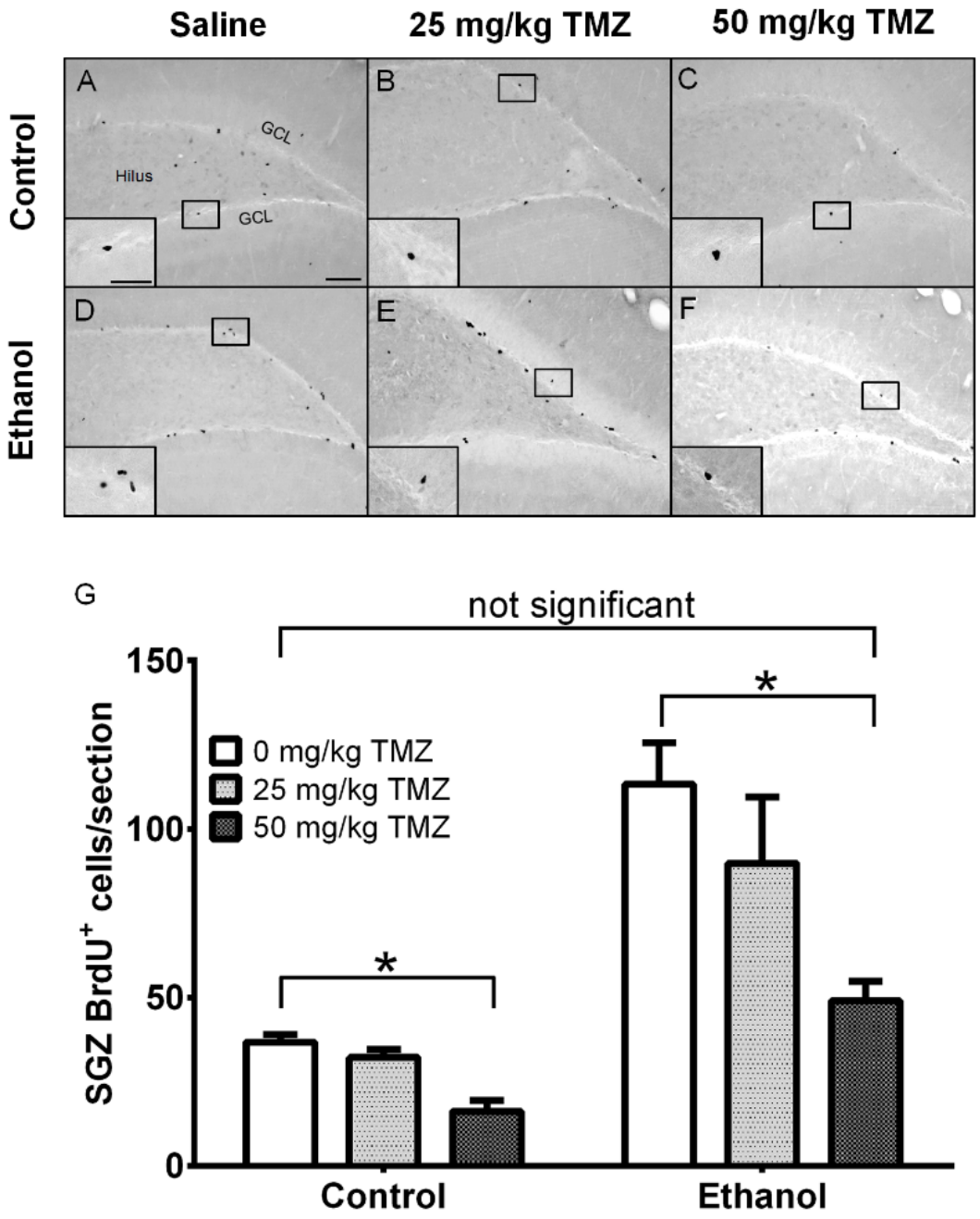


Figure 4.3 - Representative photomicrographs of BrdU+ cells for control (A-C) and ethanol (D-F) animals at indicated TMZ doses seven days post-alcohol exposure. Large and inset images were taken with a 10x and 100x objective, respectively. (G) The number of BrdU+ cells was significantly decreased

(Figure 4.3 continued)

*following 50 mg/kg/d TMZ. Note the lack of significance between ethanol + 50 mg/kg/d TMZ and control + saline; it suggests that proliferation returned to levels seen in controls. Scale bar = 100 μm; inset 40 μm. GCL = granule cell layer. *p = < 0.05; n = 8-9 for ethanol groups and n = 6 for control groups.*

versus saline animals. Additionally, a planned t-test revealed no significant difference in BrdU+ cell counts between 50 mg/kg/d TMZ ethanol rats and saline control rats (Figure 4.3), which indicates that TMZ decreased proliferation to levels seen in control animals.

4.3.2 TMZ normalizes alcohol-induced reactive neurogenesis at T14

In order to verify the results from the previous experiment, rats were administered 0 or 50 mg/kg/d TMZ to decrease alcohol-induced reactive proliferation/neurogenesis. Intoxication parameters were again compared between treatment groups to rule out potential confounding effects of model differences on TMZ's effect on cell proliferation and neurogenesis (Figure 4.4). Student's t-test of BEC and daily ethanol dose revealed no differences between TMZ and Saline groups. Non-parametric (Mann-Whitney) tests of intoxication scores and withdrawal scores also revealed no differences between TMZ and saline groups. As such, data were collapsed across groups resulting in a mean intoxication score of 1.7 ± 0.1 on the 6-point intoxication scale (Figure 4.4) which is represented behaviorally by ataxia and an elevated abdomen. The T14 mean daily ethanol dose was 9.8 ± 0.3 g/kg/d ethanol, and the corresponding mean BEC was 376 ± 17 mg/dl. These values are similar to those previously reported (Morris et al., 2010a) and those reported in the previous experiment (T7).

NeuroD is expressed in newborn neurons and is used as an early marker of neurogenesis (Gao et al., 2009; Pataskar et al., 2016). NeuroD+ cells were stained by immunohistochemistry 14 days after ethanol exposure.

Figure 4.4 - T14 animal model data

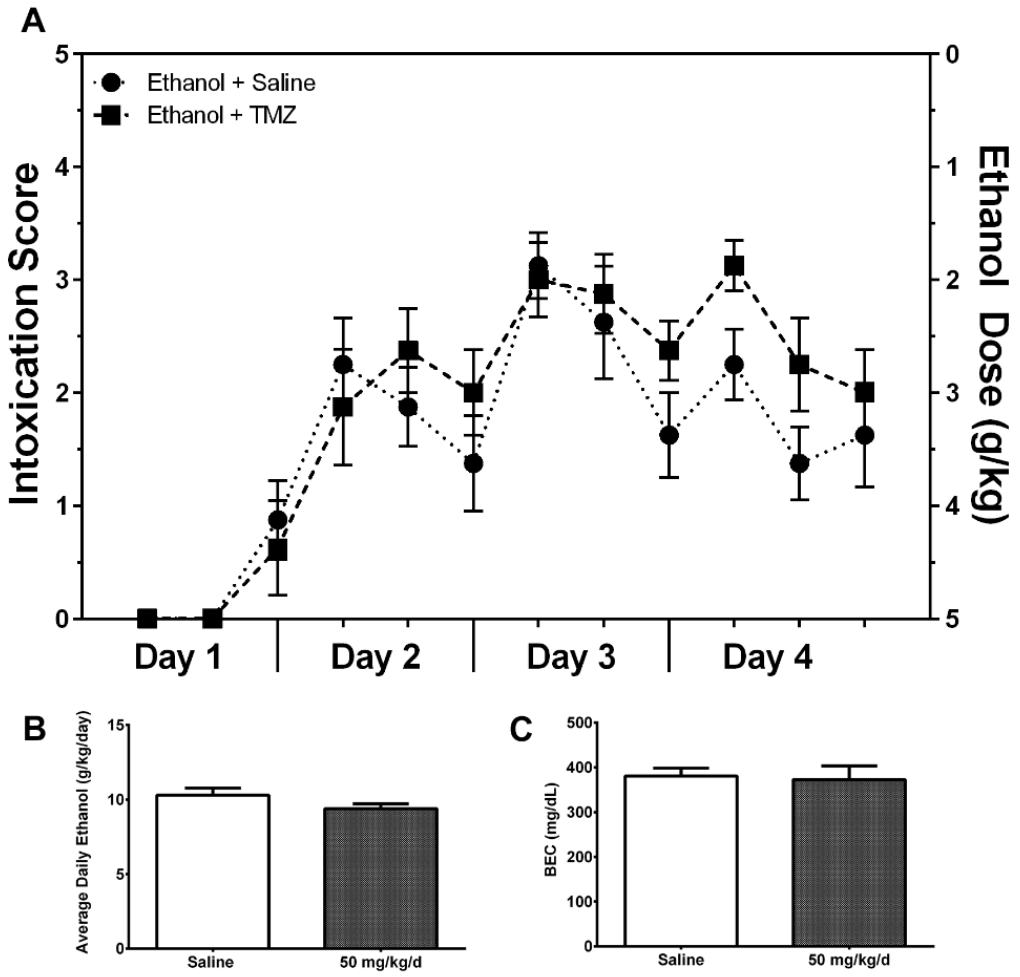


Figure 4.4 - Rats were treated with binge alcohol or control diet for four days followed by saline or 50 mg/kg/day TMZ. (A) Behavioral intoxication scores (left axis) and corresponding ethanol dose (right axis) were similar between groups. (B, C) Average daily ethanol dose and BEC did not differ between groups. n = 8/group.

NeuroD+ cells were seen along the SGZ and the inner GCL and were consistent with markers of neuroblasts or immature neurons. NeuroD+ cells were quantified by profile cell counts which revealed decreased NeuroD+ cell counts in rats administered TMZ *vs* saline (Figure 4.5). Representative images show NeuroD+ nuclear staining throughout the SGZ in control diet (Figure 4.5 A, C) or ethanol diet (Figure 4.5 B, D) followed by saline or TMZ. Two-way ANOVA (diet x drug) revealed a main effect of diet [$F_{(1,28)}$=12.70; p=0.0013] and a main effect of drug [$F_{(1,28)}$=20.41; p=0.0001]. A planned t-test revealed that administration of ethanol + TMZ resulted in NeuroD+ cell counts similar to control + saline [$t_{(14)}$=1.19; p=0.25], indicating that neurogenesis levels were similar to those seen in control animals. Additional planned t-tests revealed that administration of TMZ decreased NeuroD+ cell counts for both control [$t_{(14)}$=2.79; p=0.014] and ethanol [$t_{(14)}$=3.64; p=0.0027] groups. This means that regardless of diet (ethanol or control), the rats injected with TMZ had decreased NeuroD+ cell counts when compared to rats injected with saline.

The number of BrdU+ cells were counted 14 days post-binge. BrdU+ cells were again observed all along the SGZ (Figure 4.6 A-D). There was a significant difference between saline and TMZ, but only in ethanol-exposed rats (Figure 4.6). Two-way ANOVA (diet x drug) revealed a main effect of diet [$F_{(1,27)}$=4.28; p=0.048] and a main effect of drug [$F_{(1,27)}$=17.69; p=0.0003]. A planned t-test revealed that administration of ethanol + TMZ resulted in cell counts not significantly different from control + saline [$t_{(14)}$=1.76; p=0.1], indicating that proliferation levels returned to those observed in controls. Additional planned

Figure 4.5 - Representative images and NeuroD+ cell counts at T14

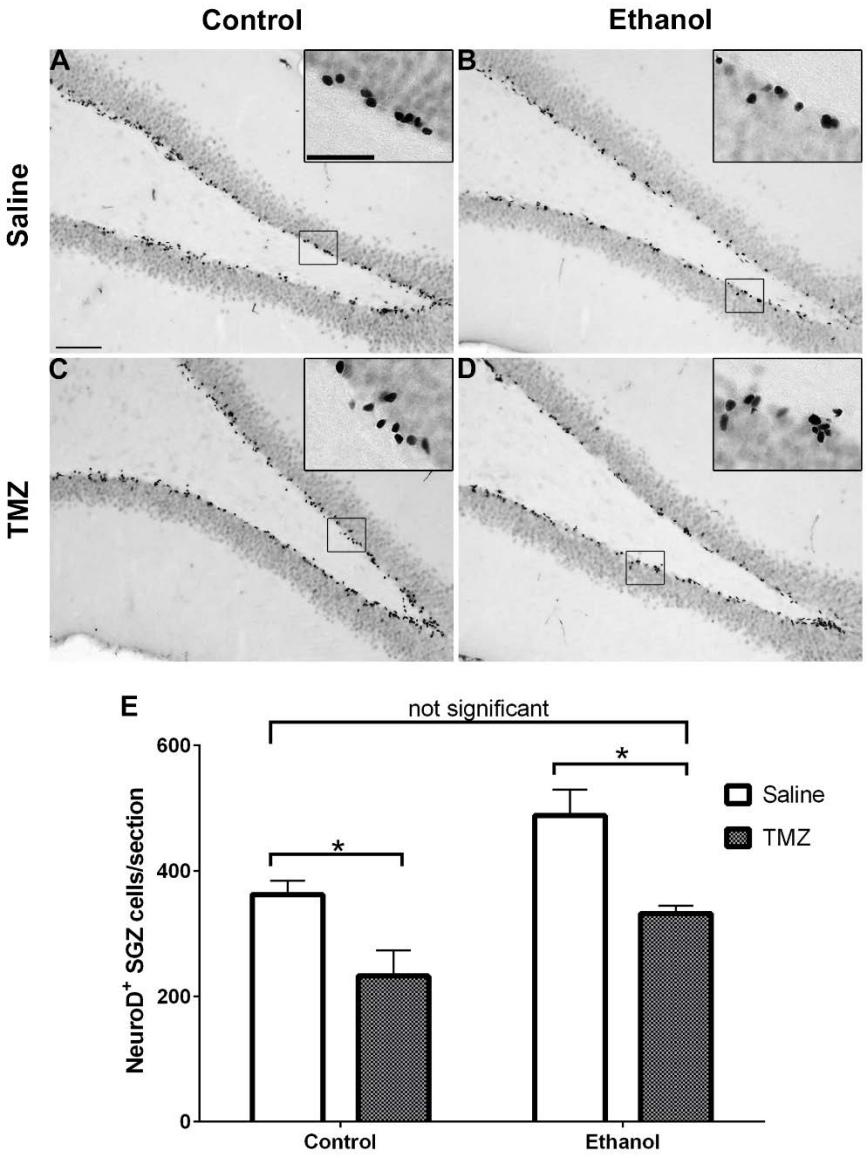


Figure 4.5 - Rats were treated with binge alcohol or control diet for four days followed by saline or TMZ injections and were sacrificed 14 days post-binge (T14). Representative images show NeuroD+ nuclear staining throughout the SGZ in control diet (A, C) or ethanol diet (B, D) followed by saline or TMZ. (E) TMZ decreased NeuroD+ cell counts in control and ethanol groups. Additionally, TMZ decreased NeuroD+ cell counts in ethanol-exposed rats to levels seen in

(Figure 4.5 continued)

*control + saline rats. Scale bar = 100 μm; inset = 40 μm. *p < 0.05; n = 8/group.*

Figure 4.6 - Representative images and BrdU+ cell counts at T14

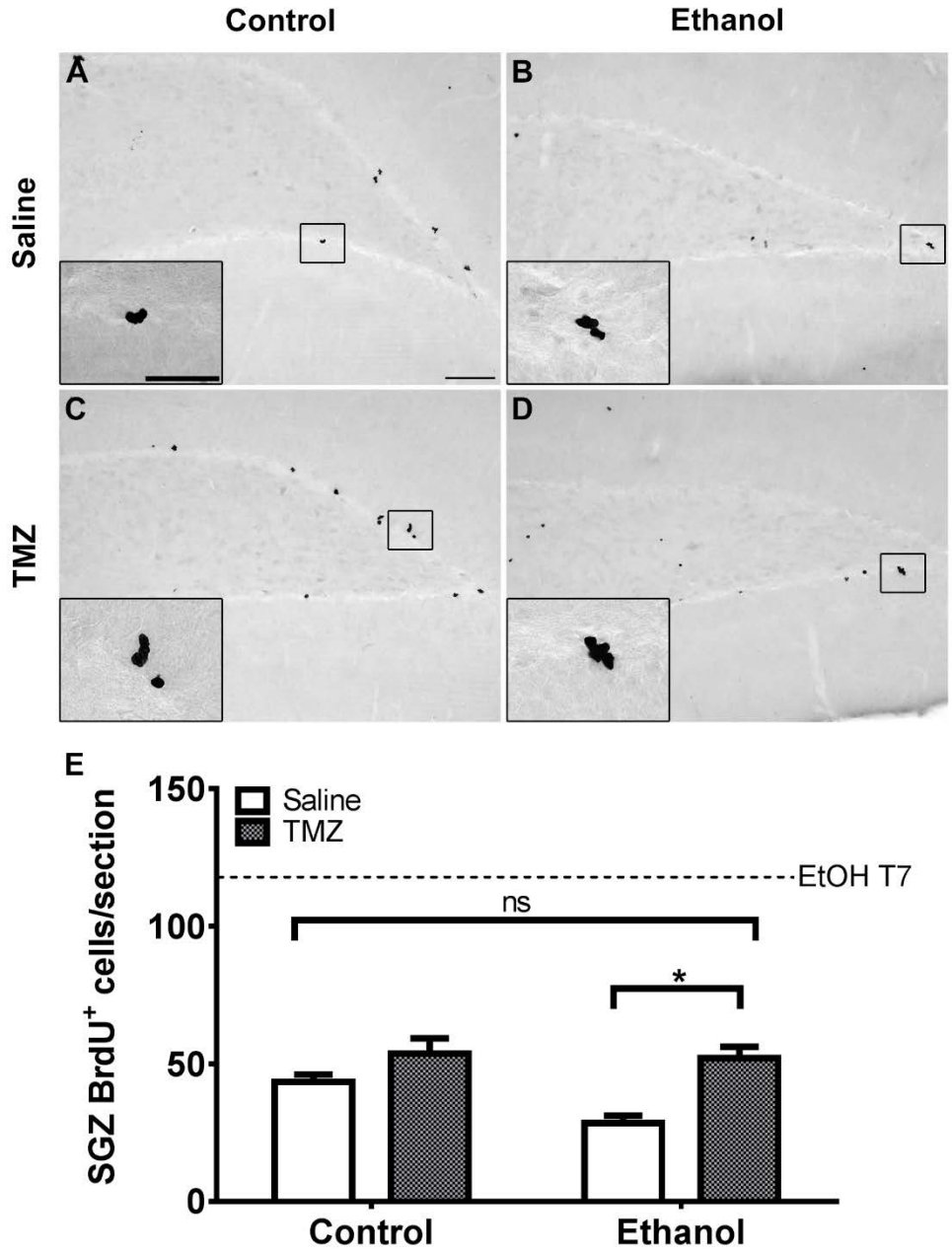


*Figure 4.6 - Representative images show BrdU+ cells 14 days after four-day binge treatment with control (A, C) or ethanol diet (B, D) followed by saline or TMZ injections. (E) Quantification of BrdU+ cell counts. Dashed line indicates T7 data to illustrate magnitude of T7 increase compared to T14. Scale bar = 100 μm; inset = 40 μm. *p < 0.05; n = 7-8/group.*

t-tests revealed that administration of TMZ significantly decreased BrdU+ cell counts for ethanol [$t_{(13)}$=4.642; p=0.0005] but not control groups. Figure 4.6 E includes a dashed line that represents the large increase in BrdU+ cells seen in the ethanol + saline group on T7. This dashed line indicates BrdU+ cell counts that would be expected if reactive proliferation was delayed one week.

4.3.3 TMZ does not affect Morris water maze behavior at T38

In order to determine if inhibiting reactive neurogenesis would impact hippocampal-dependent learning and memory, rats were set aside for four weeks after TMZ administration to allow newborn neurons time to mature. Rats were then tested on a hippocampal-dependent version of the MWM to assess whether inhibiting reactive neurogenesis prevented functional recovery. To that end, intoxication parameters were compared between treatment groups to rule out potential confounding effects of model differences on TMZ treatment and behavior (Figure 4.7). Student's t-test of BEC and daily alcohol dose revealed no differences between TMZ and saline groups. Non-parametric (Mann-Whitney) tests of intoxication score and mean withdrawal score also revealed no differences between TMZ and saline groups. There was a small but significant decrease in peak withdrawal score for the TMZ group compared to saline controls [U=19.5; p=0.028; Figure 4.7 E]. To that end, data resulted in a mean intoxication behavioral score of 1.87 ± 0.09 on the 6-point intoxication scale (Figure 4.7), which is represented behaviorally by an elevated abdomen and ataxia. The mean daily ethanol dose was 9.4 ± 0.3 g/kg/d ethanol, and corresponded to a mean BEC of 429 ± 11 mg/dl. The intoxication scores and

Figure 4.7 - T38 animal model data

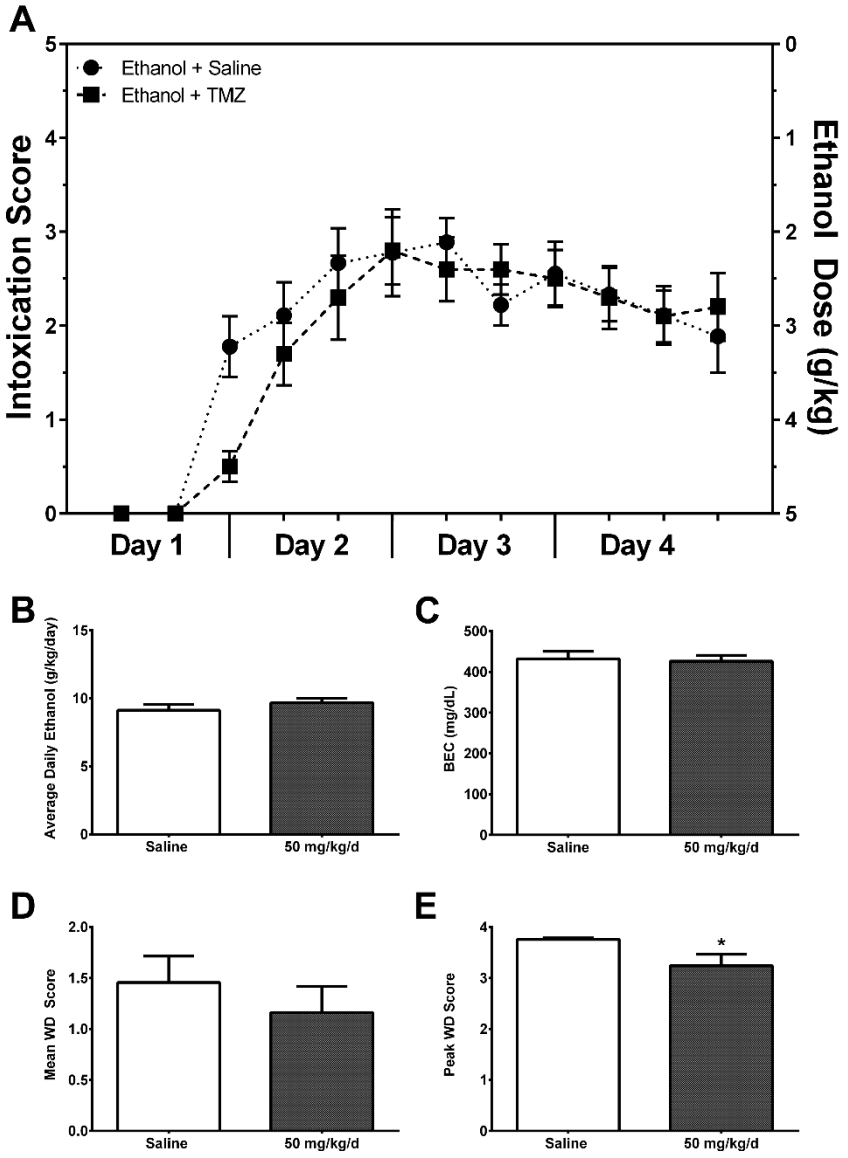


*Figure 4.7 – Similarity of ethanol groups shown. Behavioral intoxication scores (A, left axis) and corresponding ethanol dose (right axis) were similar between groups. Average daily ethanol dose, BEC, and mean withdrawal score did not differ between groups (B-D). Peak withdrawal scores were slightly, but significantly lower in animals that received TMZ (E). *p < 0.05; n = 9-11/group.*

daily ethanol dose are similar to previous reports (Morris et al., 2010a) and previous experiments with the exception that the BECs appeared slightly higher. The mean withdrawal score was 1.3 ± 0.2 on the behavioral withdrawal scale (Table 2.2). Peak withdrawal scores for ethanol + TMZ and ethanol + saline cohorts were 3.75 ± 0.03 and 3.24 ± 0.2, respectively.

Following four days of binge alcohol exposure, temozolomide (or saline vehicle) was administered to decrease/inhibit reactive neurogenesis. Thirty-five days post-binge ethanol/control exposure the rats underwent training and testing in the MWM (Figure 4.8). Thirty-five days post-binge was chosen as the end-point since newborn neurons require approximately 28 days to become mature neurons (Jessberger and Kempermann, 2003) and young neurons become activated in response to the MWM as early as four weeks of age (Kee et al., 2007). Three-way repeated measures ANOVA (day x diet x drug) revealed a main effect of day (within-subjects) for distance to goal [$F_{(3,108)}$=81.3; $p<0.001$], time to goal [$F_{(3,108)}$=93.8; $p<0.001$], and velocity [$F_{(3,108)}$=48.7; $p<0.001$]. There was no main effect of diet or drug (between-subjects) for distance to goal, time to goal, or velocity. Essentially, distance, time, and velocity all showed changes across the days of testing, but didn't show differences between treatment conditions (i.e. ethanol/control and/or TMZ/saline). To clarify, this means that during the MWM at T35, rats given ethanol and TMZ injections did not differ from those given control diet and saline injections. Probe trial data was analyzed by two-way ANOVA (diet x drug) and revealed no differences between treatment groups in terms of time spent in goal zone (5-inch area where platform was

Figure 4.8 - T38 Morris water maze data

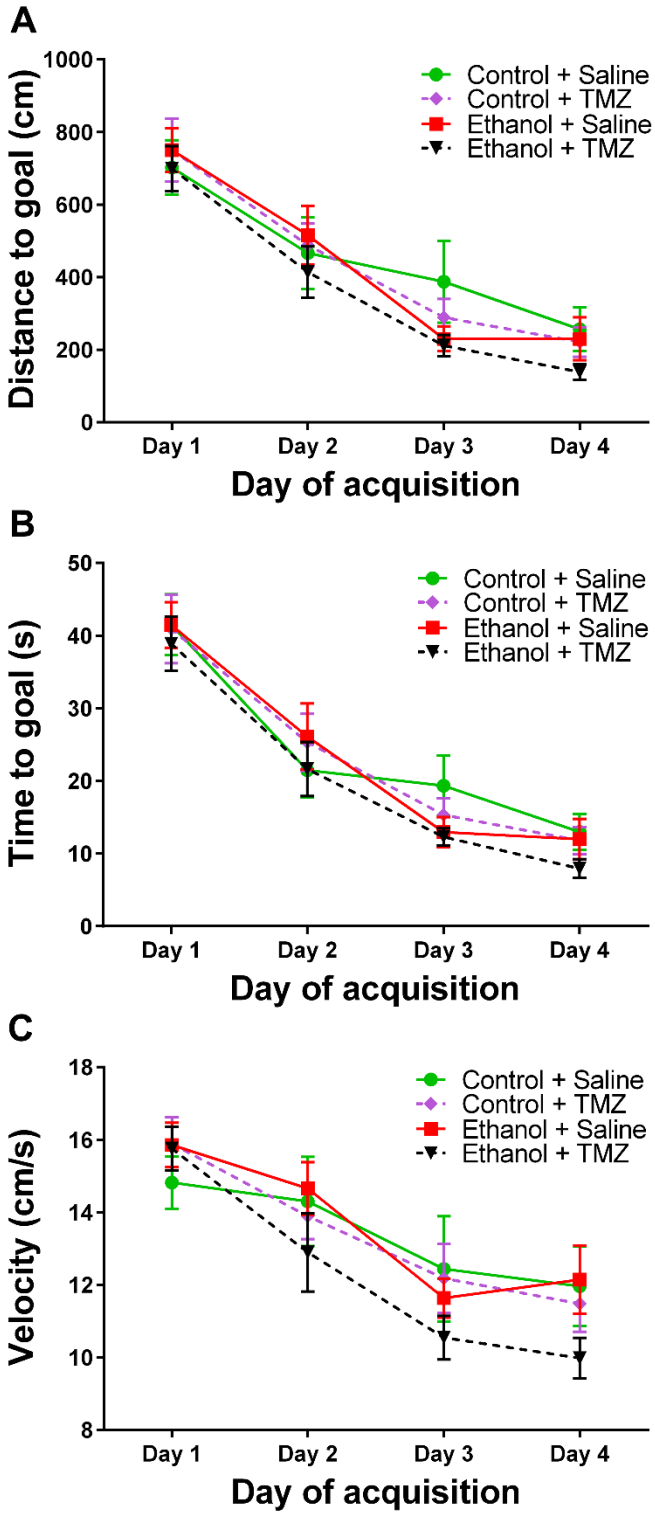


Figure 4.8 - Effects of binge ethanol and TMZ on Morris water maze behavior five

(Figure 4.8 continued)

weeks post-binge. Three-way repeated measures ANOVA (day x diet x drug) revealed a main effect of day (within-subjects) for (A) distance to goal, (B) time to goal, or (C) velocity, indicating that all groups successfully learned to locate the hidden platform. There was no main effect of diet or drug (between-subjects) for distance to goal, time to goal, or velocity. n = 9-11/group.

previously located), latency to reach the goal zone, or swim velocity. In conclusion, ethanol rats did not differ from controls and although TMZ successfully normalized (reduced) reactive neurogenesis, it did not prevent normalization/recovery of MWM behavior (i.e. MWM behavior recovered without reactive neurogenesis).

4.4 DISCUSSION

The key finding of this series of experiments is that TMZ decreased alcohol-induced reactive neurogenesis but that did not alter MWM behavior. This study indicates that TMZ decreased alcohol-induced reactive proliferation (BrdU+ cell counts) and corresponding neurogenesis (NeuroD+ cell counts) to levels seen in controls. These findings are consistent with previous reports showing that TMZ is able to decrease neurogenesis (Garthe et al., 2009, 2016; Niibori et al., 2012; Nokia et al., 2012). However, previous studies showed that TMZ decreased basal neurogenesis, while the present studies demonstrate decreases in reactive neurogenesis. In fact, this experiment represents the first time that TMZ was used in combination with alcohol exposure to study reactive neurogenesis. Aside from the fact that alcohol was administered during the present experiments, the results from both studies demonstrate that TMZ yields a general decrease in proliferation/neurogenesis. Currently, there is no way to identify and therefore selectively inhibit proliferation of cells undergoing reactive proliferation. Therefore, TMZ was used to inhibit proliferation/neurogenesis overall and, consequently, decreased proliferation was expected in all animals given TMZ (including control diet groups). TMZ's non-selective inhibition of

proliferation is a limitation of the study; however, it was the best option available. Nevertheless, it is noteworthy that TMZ was able to bring reactive proliferation down to control levels, especially since extra doses of ethanol were not able to achieve this task. Now, TMZ represents a tool to inhibit reactive neurogenesis and can be used for studies wanting to investigate the mechanistic basis of alcohol-induced reactive proliferation.

An additional concern was that TMZ would merely delay reactive proliferation. Therefore, since alcohol-induced reactive proliferation occurs seven days post-binge, we assessed proliferation by counting BrdU+ cells seven days after TMZ administration, on T14. We observed a small but significant decrease in proliferation in the ethanol + TMZ group compared to the ethanol + saline group on T14. Although statistically significant, this ~10 cell/section difference at T14 is nowhere near the ~70 cell/section increase in proliferation observed at T7, plus it is in the opposite direction. Additionally, previous studies have examined this same time point (T14) and have not seen any differences in proliferation between ethanol and control rats (McClain et al., 2014; Nixon and Crews, 2004; Nixon et al., 2008), so this could represent an incongruity where proliferation in these animals simply happened to be on the low side. Alternatively, the decrease in proliferation could be the result of the pool of proliferating cells becoming exhausted since a week earlier there was a large increase in proliferation (Kempermann, 2008). Although, one would have expected to observe this proliferation at T14 previously, and that has not been the case.

Proliferation levels (BrdU) at T7 following alcohol/control diet and TMZ/saline administration are mirrored at T14 with the neurogenesis marker, NeuroD. Previous studies have shown that NeuroD is increased at T14 (Chapter 3 and Hayes et al., in prep) and therefore an increase at T14 likely represents the increase in proliferating NPCs at T7 that have differentiated into immature neurons (McClain et al., 2014; Nixon and Crews, 2004). NeuroD at T14 demonstrated that TMZ decreased reactive neurogenesis (Figure 4.5). Importantly, ethanol rats given TMZ showed no difference in NeuroD+ cell counts from control + saline rats indicating that administration of TMZ through this protocol effectively inhibits the increase in alcohol-induced reactive neurogenesis seen at T14. Taken together with the T7 BrdU data this likely indicates that TMZ decreases alcohol-induced reactive neurogenesis to levels seen in control-saline rats. Not surprisingly, and in agreement with other TMZ-neurogenesis reports (Garthe et al., 2009, 2016; Martinez-Canabal et al., 2013; Niibori et al., 2012; Nokia et al., 2012), a decrease in neurogenesis (NeuroD+ cells at T14) was also seen in control animals (non-ethanol) receiving TMZ. Although unavoidable, some may view this as a methodological weakness since TMZ administration could result in behavioral deficits in-and-of-itself, which would complicate interpretation. Indeed, if TMZ alone caused behavioral deficits at the doses administered then the drug could not be used since one would not be able to determine if behavioral deficits were caused by TMZ or by inhibition of reactive neurogenesis. However, we did not observe behavioral differences between any groups in the MWM, indicating that it is unlikely that TMZ alone causes

behavioral deficits. Surprisingly, MWM training and testing 35-38 days post-binge did not reveal a deficit in animals where reactive neurogenesis was decreased to control levels (Figure 4.8). Here we showed that a single round of TMZ did not appear to affect learning and memory in a hippocampal-dependent version of the water maze task. This finding is supported by a study that used eyeblink classical conditioning, specifically, trace and very long delay conditioning, both hippocampal-dependent tasks, to study the effects of TMZ on learning and memory (Nokia et al., 2012). They showed that administration of a *single* 3-day cycle of TMZ was not sufficient to induce deficits, however, *multiple* cycles of TMZ (over several weeks) did, in fact, disrupt learning (Nokia et al., 2012). This study supports our findings that the one cycle of TMZ used in our study should not be detrimental to learning and memory.

Interestingly, despite TMZ's ability to decrease reactive neurogenesis, there were no differences between any experimental groups in MWM training or testing. MWM is the 'gold-standard' measure of hippocampal-dependent learning and memory (Nunez, 2008). These data, combined with previous research from our lab which show a MWM deficit 7-10 days post-binge (Supplemental Figure 1), indicates that alcohol-exposed rats recover hippocampal function, at least in the MWM, by T35. Indeed, previous research has shown that hippocampal function recovers in humans (Gazdzinski et al., 2008) and in animal models of AUDs (Cadete-Leite et al., 1988, 1989a, 1989b; King, 1988).

Binge alcohol treatment was not considered a confounding variable since intoxication data between treatment groups was similar for the vast majority of

measures (all except peak withdrawal score). Only a slight difference was seen during peak withdrawal between the two ethanol groups (T35 saline and T35 TMZ) and no other differences in intoxication measures were observed, including mean withdrawal. Therefore, it is likely that the two T35 ethanol groups showed a difference in peak withdrawal score due to one groups unusually consistent peak withdrawal scores and/or random group assignments. Indeed, all ethanol + saline animals achieved the exact same peak withdrawal score, resulting in no error and therefore significance between saline and TMZ groups. TMZ was given after withdrawal and rats were randomly assigned to a TMZ or saline group. All groups learned similarly, whether they received alcohol followed by TMZ injections or control diet followed by saline injections. Slight differences in peak withdrawal score, although significant, do not explain the MWM data since no differences were observed between groups. However, a thorough analysis of TMZ's effects on basal neurogenesis and learning literature indicated that even with multiple weeks of TMZ administration, differences between treatments (TMZ vs. saline) were primarily seen when the platform location was changed in the MWM during a reversal task (Garthe et al., 2009, 2016). This indicates that the standard water maze test may not have been challenging enough to flush out differences between the treatment groups and a harder version of the water maze may be necessary to tease apart subtle deficits in learning and memory (Garthe et al., 2009, 2016). Therefore, the hypothesis that reactive neurogenesis is necessary for normalization of learning and memory involved in the MWM may still be valid and reactive neurogenesis could be required for proper hippocampal function. In

addition, since reversal learning was not tested in this study, it is possible that a single cycle of TMZ could still induce behavioral deficits. However, since TMZ was only administered for a single 3.5 day cycle in this study, as opposed to several weeks, it seems unlikely that TMZ alone would induce behavioral deficits in a reversal learning task (Nokia et al., 2012). Indeed, Nokia et al., 2012 demonstrated that the detrimental effects of TMZ were only seen after several weeks of treatment. Despite that information, at this time we cannot rule out the possibility that TMZ could induce behavioral deficits since TMZ has not been studied following a binge model of an AUD. As such, any future experiments should include a more challenging reversal water maze task.

In conclusion this novel TMZ administration paradigm was able to decrease alcohol-induced reactive neurogenesis. Additionally, there was not a rebound in reactive proliferation one week following TMZ administration. Interestingly, treatment with TMZ had no detectable effect on MWM behavior 35 days after alcohol exposure. Future experiments will increase the difficulty of the water maze by adding a reversal component, since binge alcohol exposure could cause subtle deficits in hippocampal function that may be difficult to detect without the appropriately challenging test.

CHAPTER 5: MWM-INDUCED NEURONAL ACTIVATION FOLLOWING ALCOHOL-INDUCED REACTIVE NEUROGENESIS

5.1 INTRODUCTION

Alcohol-induced neurodegeneration is characterized by decreased MRI brain volumes, decreased hippocampal granule cell counts, and deficits in learning and memory (Pfefferbaum et al., 1992; Walker et al., 1980; Zahr et al., 2015). Neurogenesis is one of the ways that alcohol leads to neurodegeneration since alcohol intoxication results in decreased levels of adult neurogenesis. But, following a 4-day binge model of an AUD, when alcohol is removed there is a reactive increase in neurogenesis and its role in repair is unknown. This alcohol-induced reactive increase in neurogenesis occurs seven days post-binge (T7) and results in new, mature neurons 28 days later (Nixon and Crews, 2004). However, it is also not known if neurons born during alcohol-induced reactive neurogenesis are functionally incorporated. This lack of knowledge regarding the function of neurogenesis is due to the fact that, in general, it has been technically difficult to study, and as such the function of adult-born neurons is still up for debate (as discussed in Chapter 2). Nevertheless, most agree that adult neurogenesis has been evolutionarily conserved for good reason and that it serves multiple functions (Kempermann, 2008).

Under basal adult neurogenesis conditions (i.e. when alcohol is not involved) newborn neurons integrate into the hippocampal circuitry. At around three weeks post-birth they sprout axons that project along the mossy fiber tract towards the CA3 region, and they also become capable of receiving excitatory

input from the perforant pathway (Hastings and Gould, 1999; Kaplan and Hinds, 1977; Kee et al., 2007; van Praag et al., 2002). For over two decades, researchers have used double-label IHC technology along with immediate early genes (IEGs), like c-Fos, as markers of neuronal activity (Cruz et al., 2013; Guzowski et al., 2005). IEGs are genes that are activated quickly and briefly by a variety of cellular stimuli; many are transcription factors or other kinds of DNA binding proteins (Guzowski et al., 2005). The expression of IEGs like c-Fos and Zif268 are correlated with neuronal excitation and can be used to indicate neuronal activation (Guzowski, 2002; Kee et al., 2007; Labiner et al., 1993). C-Fos is involved in learning and memory and as such has been used as a neuronal activity marker (Guzowski et al., 2005). Under baseline conditions, IEG expression levels are generally undetectable immunohistochemically. However, increases in IEG expression occur quickly if the brain is in an attentive state and/or if synaptic plasticity is induced (Cole et al., 1989; Guzowski et al., 1999). Since IEGs are highly conserved evolutionarily, they have been used to study neural circuits in many animal species (Guzowski et al., 2005).

We investigated whether new neurons produced during alcohol-induced reactive neurogenesis are incorporated functionally following a hippocampal-dependent task of spatial learning and memory, the MWM. Here we used c-Fos as an indicator of neuronal activity since it is selectively upregulated in granule cells of the dentate gyrus following the MWM (Geibig et al., 2012; Kee et al., 2007). Studies have also shown that 6-8 week old granule cells are preferentially recruited and activated following the MWM task in mice (Kee et al., 2007).

Therefore, we used the MWM to induce (activate) c-Fos expression and then determined if the neurons born during reactive neurogenesis are capable of being activated in response to the MWM. To induce activation we selected the MWM since there is a clear increase in granule cell activation following this task (Kee et al., 2007). Then, we identified these activated cells by triple-label IHC which results in a precise population of neurons being labeled (Geibig et al., 2012). We used BrdU to label cells proliferating on T7 during reactive neurogenesis. These young neurons, born and labeled on T7, were then allowed to mature/integrate into hippocampal networks for six weeks, an amount of time that has been shown to be sufficient for preferential incorporation for the MWM (Kee et al., 2007). After the neurons had time to integrate, the rats were tested in the MWM. Only newborn cells that were labeled with BrdU during reactive neurogenesis (on T7), survive six weeks, express a mature neuronal marker (NeuN), and were activated in response to a hippocampal-dependent task (MWM) were triple labeled. Therefore, we examined the contribution of reactive neurogenesis to learning the MWM task, after neurons had 6 weeks to mature. Using the IEG c-Fos, we determined if neurons born during reactive neurogenesis were incorporated into hippocampal networks and if the number and percentage of neurons activated was different from controls. We hypothesized that reactive neurogenesis produced neurons capable of hippocampal integration (activation) following the MWM task.

5.2 METHODS

5.2.1 Binge model of an Alcohol Use Disorder

Twenty-one male Sprague-Dawley rats (Charles River Laboratory) weighing 275-300 grams on arrival, were double-housed and allowed to acclimate to the University of Kentucky AAALAC-accredited vivarium for five days before experimental procedures began. All experimental procedures were completed in accordance with the University of Kentucky Institutional Animal Care and Use Committee and followed the Guidelines for the Care and Use of Laboratory Animals (NRC, 1996). Animals were divided into two groups with statistically similar weights and administered either 25% (w/v) ethanol in Vanilla Ensure Plus® (Abbott Laboratories, Columbus, OH) or an isocaloric control diet (calorically equivalent quantities of dextrose) in Vanilla Ensure Plus®. Ethanol (n=10) or control diet (n=11) was administered every eight hours for four consecutive days (previously described). Ninety minutes following the seventh dose of ethanol, tail bloods were collected to determine BEC (as previously described in Chapter 2). An average of the triplicate samples are presented as BEC ± standard error of the mean.

All ethanol animals underwent monitored withdrawal for 17 hours beginning 10 hours after the last dose of ethanol (previously described in Chapter 2). Briefly, rats were observed for 30 minutes every hour for 17 hours and withdrawal behavior (listed in Table 2.2) was scored. Data are reported as mean or peak withdrawal score ± standard error of the mean.

5.2.2 Bromodeoxyuridine administration

The proposed dose of BrdU has been shown not to result in toxicity or to label DNA repair using IHC methods (Cameron and McKay, 2001; Palmer et al., 2000). Beginning exactly 168 hours (7 days) after the last dose of alcohol, all animals received the first of three total *i.p.* BrdU injections (100 mg/kg BrdU in 0.9% saline), scheduled injections were eight hours apart at 7AM, 3 PM, and 11PM (Figure 4.1). BrdU gets incorporated into the DNA in place of Thymidine when the cell is in the synthesis phase (S-phase) of the cell cycle. Typical duration of the S-phase in adult rats is eight hours (Cameron and McKay, 2001) and therefore BrdU injections were administered every eight hours to label a large pool of cells proliferating on T7 (seven days post-binge alcohol exposure). Administering saturating doses of BrdU (200-300 mg/kg), i.e. doses known to label **all** proliferating cells, was not possible due to BrdU's poor solubility (max 20 mg/ml) which would necessitate large volumes being repeatedly injected into the *i.p*. cavity. However, several studies administered much lower concentrations of BrdU and achieved measurable results (Jessberger and Kempermann, 2003; Kee et al., 2002; Weig et al., 2016). Thus, multiple BrdU injections of 100 mg/kg should label a large pool of cells that could potentially be recruited for use in the MWM.

5.2.3 Morris water maze

The MWM was used to activate neurons generated during alcohol-induced reactive neurogenesis (Figure 5.1). The MWM was performed as described in Chapter 4. Briefly, seven weeks post-binge alcohol exposure (when newborn

Figure 5.1 - Experimental design for functional neurogenesis study

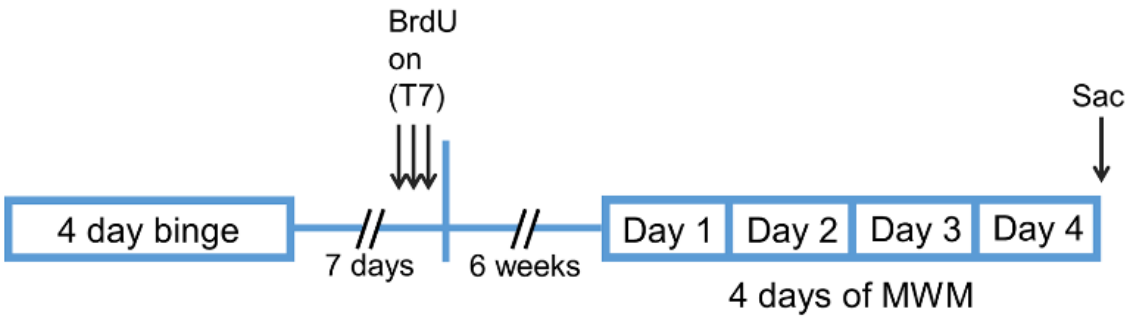


Figure 5.1 - Four days of binge alcohol exposure were followed by three BrdU injections seven days post-binge (T7) during reactive proliferation. Proliferating cells were allowed to mature for six weeks and then animals were all trained and tested in the Morris water maze for four days. One hour after their last trial on the fourth day, the platform was removed and all rats were tested in a probe trial. All animals were sacrificed (sac) approximately 90 minutes after their last MWM trial (not including the probe trial) on the fourth day.

neurons are six weeks old) the rats were trained and tested in the MWM for four days. Daily blocks (four trials) were averaged for each animal then averaged across treatment groups (control and ethanol diet) to obtain daily means for latency (s), distance (cm), and swim speed (cm/s) ± standard error of the mean. On the final day, approximately one hour after their last trial, the platform was removed and the rats were placed back in the pool at a novel entry point (SW) for a probe trial. The settings were fixed so that if a rat spent 5 seconds in the target zone (12.7 cm zone around old platform location), the trial would end. If the rat did not spend 5 seconds in the target zone then the rat had 60 seconds to try and complete this task.

5.2.4 Tissue preparation

Fifty-two days following their last dose of alcohol and ~90 minutes after their last trial in the MWM (not including the probe trial), rats were sacrificed by pentobarbital overdose (Fatal Plus®) followed by intracardial perfusion (previously described). Ninety minutes was selected based on previous research that demonstrated its appropriateness for examining c-Fos expression in hippocampal neurons (Snyder et al., 2009). Brains were then extracted, post-fixed, and sliced in 40 µm sections on a vibrating microtome as previously described in Chapter 2.

5.2.5 Immunohistochemistry

A subset of three ethanol rats and three control rats were selected for triple-label IHC based on their pre-binge weights being closest to the average. Care was also taken to ensure that the three selected ethanol rats had

intoxication and withdrawal scores that were close to the averages. Tissue was stained using triple label fluorescent IHC for BrdU, c-Fos, and NeuN, similar to Geibig et al. (2012). Individual dilution curves were run for each primary antibody (BrdU, c-Fos, and NeuN) to determine the best working concentration. The foundation of the triple label protocol was the BrdU antibody protocol since it was the most rigorous. Free-floating tissue sections were rinsed in phosphate buffered saline (PBS) pH 7.4 (Gibco, Life Technologies, Grand Island, NY) + 1% Triton®X-100 (Acros Organics, New Jersey, US), PBST. Tissue was then incubated for 30 minutes in 1 N HCl at 37º C (to denature DNA so the anti-BrdU antibody can bind) and neutralized with 0.1 M Boric acid pH 8.5. Tissue was then rinsed in PBST and incubated in primary antibody for three hours at room temperature followed by 42 hours at 4º C. The primary antibodies: rabbit anti-c-Fos (1:200, SC7202, Santa Cruz Biotechnology Inc., Dallas, TX), rat anti-BrdU (1:400, H7786, Accurate Chemical and Scientific Co., Westbury, NY), and mouse anti-Neuronal Nuclei (1:10,000, MAB377, Millipore, Temememecula, CA), were diluted in 15% normal goat serum (Vector Laboratories, Burlingame, CA) in PBST. Tissue was rinsed in PBST and incubated in a light-proof box at room temperature for 90 minutes in secondary antibody. From this point on all light exposure was limited. Secondary antibody solution consisted of 15% normal goat serum in PBST and 1:200 of the following Alexa Fluor's® (Invitrogen™ Molecular Probes®, Eugene, OR): goat anti-rabbit IgG 488, goat anti-rat IgG 546, and goat anti-mouse IgG 633. Tissue was rinsed in PBST, mounted onto glass slides,

allowed to dry, coverslipped with ProLong® Gold antifade reagent (Molecular Probes® by Life Technologies, Eugene, OR), and left to cure for two nights.

5.2.6 Quantification

Brains were sliced at 40 µm thickness and collected in a 1:12 series resulting in approximately 6-8 hippocampal sections per brain. All sections per brain were inspected and the side without tears to the SGZ was selected for quantification. All cells were quantified using a Leica TCS SP5 inverted laser scanning confocal microscope (Wetzlar, Germany). Z-plane optical stacks were collected at 1 µm thickness using a 20x objective. Multiple z-stacks were taken across each section to capture the entire GCL (2-5 z-stacks per section, 6-8 sections per brain). Immediately, after each z-stack the images were visually inspected to determine if any triple labeling occurred. This was accomplished using Leica software by hiding the NeuN (blue) channel and looking for overlap of BrdU+ (red) and c-Fos+ (green) cells = yellow. Yellow was much easier to detect than the white color made by blue/red/green and was just as accurate for detecting a cell that needed further investigation. If it was suspected that a cell may be triple labeled, then the suspect cell underwent another z-stack at a higher magnification (63x lens). All cells were quantified on a single hemisphere (480 µm between sections), between Bregma -2.28 mm and -5.52 mm as determined by Paxinos (Paxinos and Watson, 2009).

All Z-stack images were viewed and manually quantified using Image-Pro Plus software (version 3.6 windows, Image-Pro Plus, Media Cybernetics, Rockville, MD). Although Image-Pro Plus software can be used to generate a 3D

render of collected z-stack images, this type of analysis was not feasible because of the very high concentration of NeuN+ cells. The GCL consists of densely packed granule cell neurons and since these are what NeuN labels, the signal takes over the 3D render making it very difficult to assess with 3D rendering software. Nevertheless, Image-Pro Plus software was still used because it makes viewing z-stack images easier. This software allowed viewing all three cell marker images and the overlay simultaneously; overlay images help determine if a single cell expressed more than one marker (co-labeling). Essentially, each marker BrdU, c-Fos, and NeuN was a different color image. For each z-stack, the series of images were compiled such that each color/marker (including the overlay) could be visualized and used to count all BrdU+ and c-Fos+ cells in the GCL (see Figure 5.3 for examples of each color channel/image and corresponding overlay and how this method can be used to count cells). NeuN cells were not counted because NeuN immunoreactivity was only used to confirm that the cell was a mature neuron. Each BrdU+ cell was assessed for its expression of c-Fos and NeuN. Similarly, each c-Fos+ cell was assessed for its expression of BrdU and NeuN. All BrdU+ and c-Fos+ cells were counted and then each cell was determined to be either BrdU+/NeuN+, BrdU only, c-Fos+/NeuN+, c-Fos only, or c-Fos+/BrdU+/NeuN+ (similar to Geibig et al., 2012). Cell counts were then totaled for each animal (2-5 z-stacks per brain section, 6-8 sections per animal) and averages for control and ethanol animals were calculated ± standard error of the mean.

5.2.7 Statistics

All data were analyzed with GraphPad Prism (version 7, GraphPad Software, La Jolla, CA) and reported as mean ± standard error of the mean. Histological data were analyzed by student's t-test. MWM data were analyzed by two-way repeated measures ANOVA (diet x day) and when appropriate, followed by Bonferroni's multiple comparisons test. MWM probe trial data were analyzed by one-way ANOVA (diet). P-values were accepted as significant when $p < 0.05$ (two-tailed).

5.3 RESULTS

5.3.1 Animal model data

Adult male Sprague-Dawley rats were fed an ethanol or control diet for four days with the ethanol dose titrated based on the rat's intoxication score. Ethanol-fed rats had a mean intoxication score of 1.8 ± 0.07, which resulted in an average ethanol dose of 9.7 ± 0.2 g/kg/d, and average BECs of 401 ± 14 mg/dl. Mean withdrawal scores were 1.4 ± 0.2 and peak withdrawal scores were 3.6 ± 0.1 on the behavioral withdrawal scale (Table 2.2). With the exception of BECs being slightly higher than usual, these values are similar to previous reports (Morris et al., 2010a).

5.3.2 Morris water maze at T52

MWM was used to activate neurons born during alcohol-induced reactive neurogenesis. Animals were trained and tested in the MWM for four days beginning seven weeks post-binge (six weeks post-BrdU injections). Two-way repeated measures ANOVA revealed a main effect of day for time to goal

(latency) [$F_{(3,57)}$=64.2, p<0.0001], distance to goal [$F_{(3,57)}$=63.8, p<0.0001], and swim speed [$F_{(3,57)}$=42.4, p<0.0001]. There was a significant diet x day interaction for time to goal (latency) [$F_{(3,57)}$=4.7, p<0.05], distance to goal [$F_{(3,57)}$=4.7, p<0.05], and swim speed [$F_{(3,57)}$=3.8, p<0.05]. The Bonferroni post-hoc multiple comparisons test revealed that ethanol-fed animals had decreased time to goal compared to controls on day 1 and 2 (p<0.05, Figure 5.2 A), which indicated that during the first two days of MWM, rats exposed to four days of ethanol (seven weeks earlier) took less time to locate the platform than rats fed a control diet. Additionally, a decrease was seen in distance to goal and velocity in alcohol-fed animals compared to controls on day 2 (p<0.05, Figure 5.2 B, C), indicating that ethanol animals did not locate the platform more quickly simply because they were swimming faster. If anything the ethanol animals appeared to swim slower and swam shorter distances. Probe trial one-way ANOVA did not reveal any differences between ethanol and control.

Figure 5.2 - T52 Morris water maze results

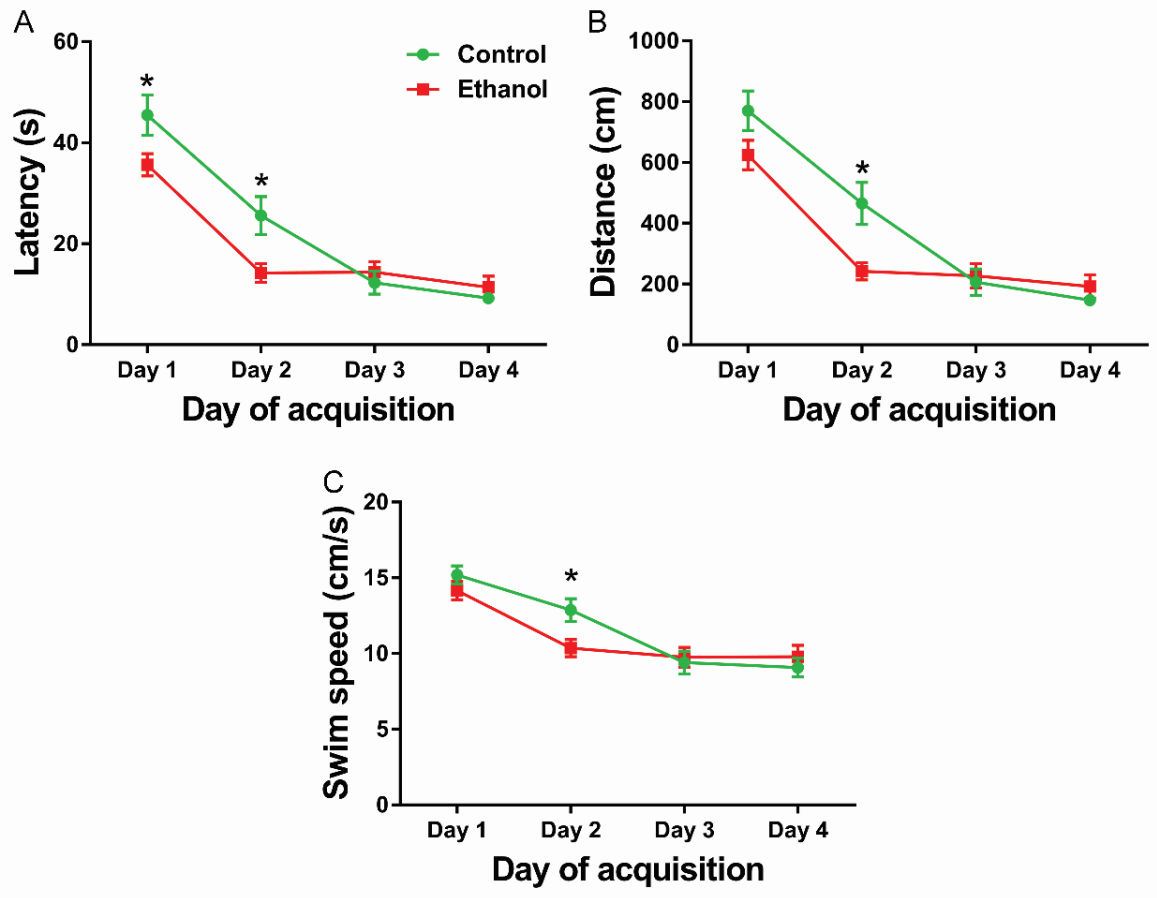


*Figure 5.2 - The MWM was used as a tool to activate hippocampal neurons, still, MWM data was collected and is reported here. Rat performance on the MWM was assessed by three measures: (A) time to goal (latency), (B) distance to goal, and (C) swim speed. *p < 0.05, n = 10-11/group.*

5.3.3 Fluorescent triple label (BrdU/c-Fos/NeuN)

Representative photomicrographs are detailed in Figure 5.3 and include (A) a BrdU+/NeuN+ cell, (B) c-Fos+/NeuN+ cells, and (C) a BrdU+/c-Fos+/NeuN+ cell. The total number of BrdU+ cells was counted across the GCL and each BrdU+ cell was assessed for the presence of NeuN, a marker of mature neurons. Ethanol rats showed an increase [$t_{(4)}$=3.34; p=0.029] in the number of BrdU+ cells compared to controls at T52, six weeks after reactive neurogenesis (Figure 5.4 A). Additionally, ethanol animals revealed an increase [$t_{(4)}$=5.347; p=0.0059] in the number of BrdU+/NeuN+ cells compared to controls (Figure 5.4 B). BrdU indicated which cells were proliferating on T7 and then survived to T52, while the combination of BrdU+/NeuN+ cells indicated that the BrdU+ cells became mature neurons. The total number of BrdU+ cells and the total number of c-Fos+ cells was also counted across the GCL. C-Fos+ cells and c-Fos+/NeuN+ cell counts (Figure 5.4 C, D) revealed no differences between ethanol and control animals, suggesting that ethanol consumption did not alter the number of cells activated by the MWM. There was no significant difference [$t_{(4)}$=0.591; p=0.5861] in the number of triple labeled cells (BrdU+/c-Fos+/NeuN+) between ethanol and controls (Figure 5.4 E). Additionally, the percentage of BrdU+ cells that also expressed c-Fos and NeuN (i.e. cells that were triple labeled) was not different between ethanol and control groups (Figure 5.4 F). Overall, very few cells were triple labeled, which is consistent with previous studies (Geibig et al., 2012).

Figure 5.3 - Representative images for BrdU/c-Fos/NeuN at T52

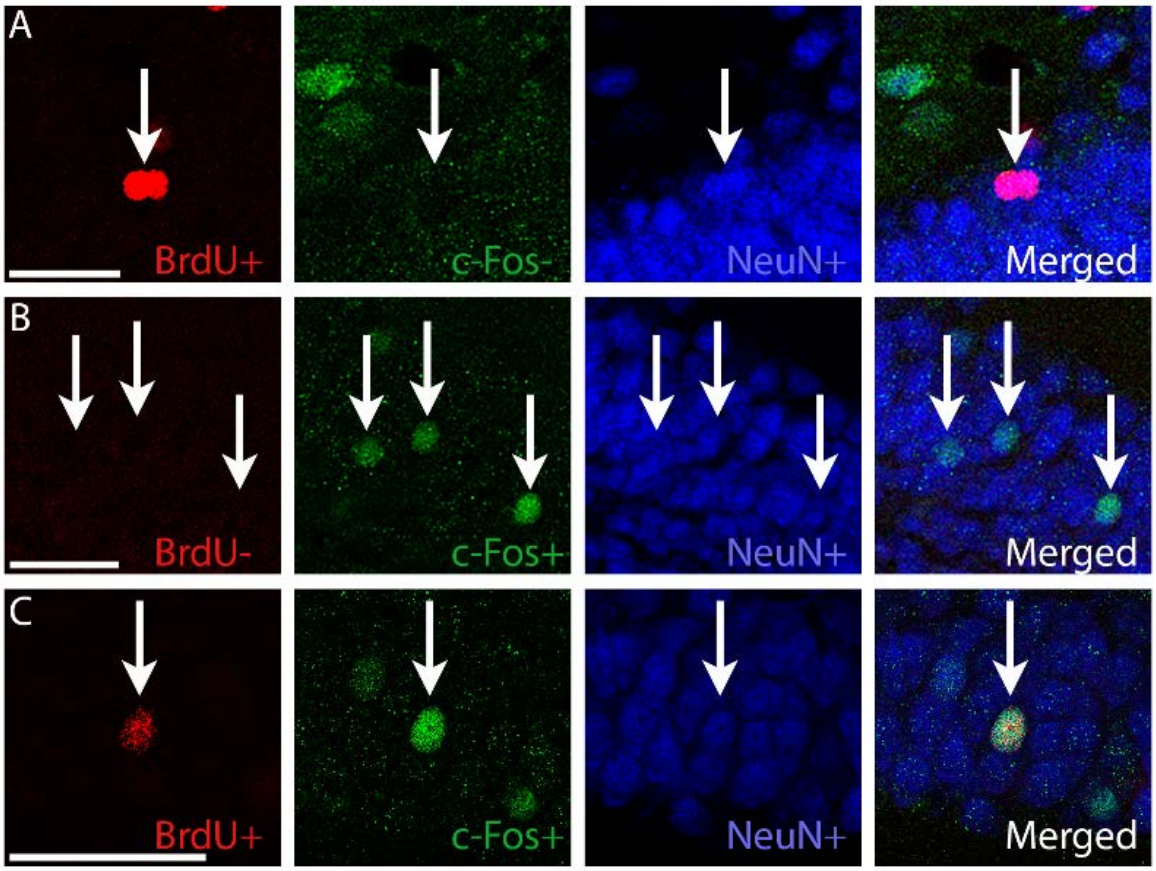


Figure 5.3 - Representative photomicrographs of the GCL at T52 for individual fluorochromes illustrating BrdU+ cells (red), c-Fos+ cells (green), NeuN+ cells (blue), and merged. (A) BrdU+/c-Fos-/NeuN+ cells, (B) BrdU-/c-Fos+/NeuN+ cells, and (C) BrdU+/c-Fos+/NeuN+ cells. Scale bars = 40 μm.

Figure 5.4 - Triple label cell counts for BrdU/c-Fos/NeuN at T52

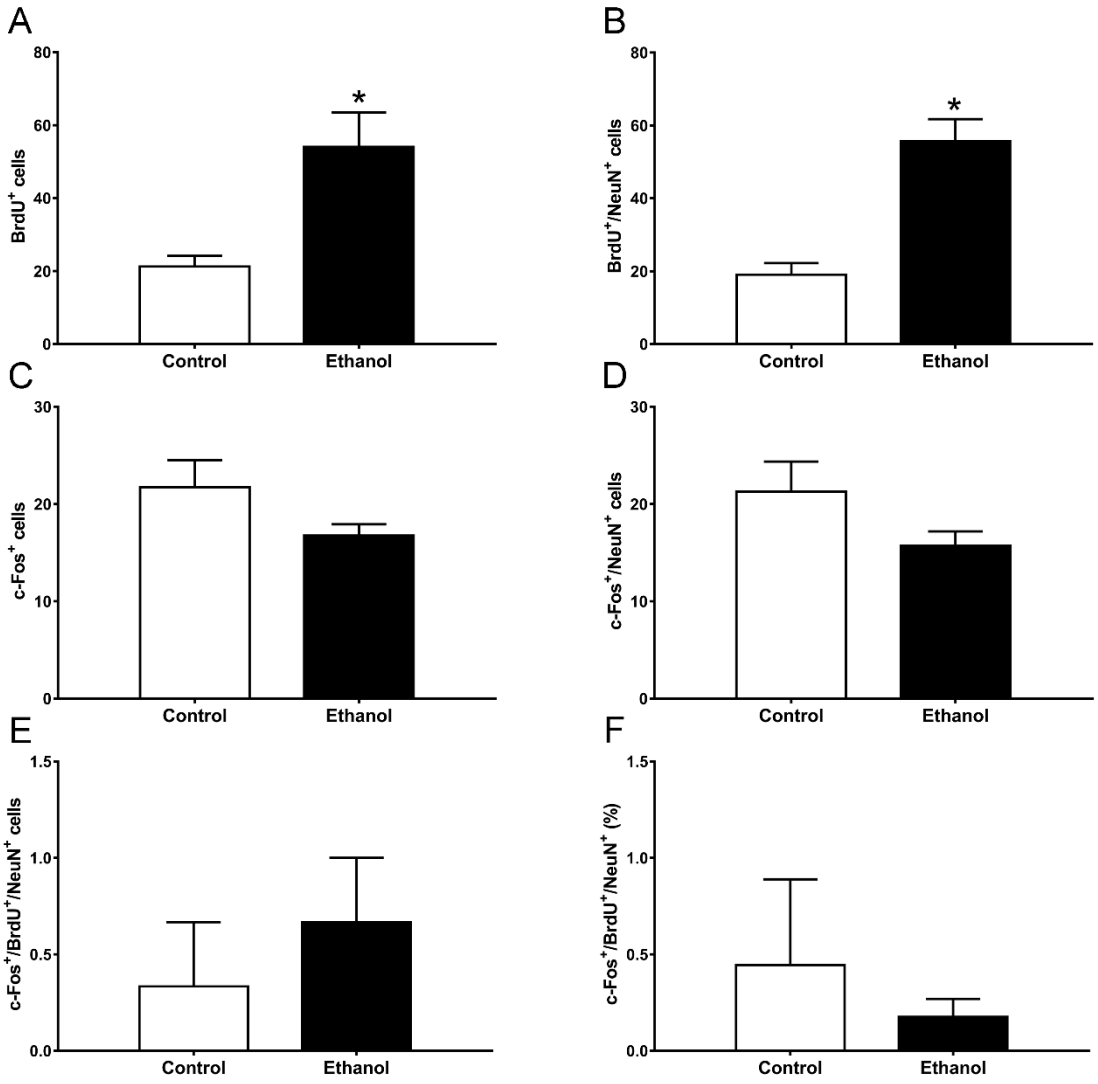


*Figure 5.4 - Total cell counts are included in the following graphs for: (A) BrdU+ cells, (B) BrdU+/NeuN+ cells, (C) c-Fos+ cells, (D) c-Fos+/NeuN+ cells, and (E) c-Fos+/BrdU+/NeuN+ cells. Note the percentage of BrdU+ cells that also expressed c-Fos and NeuN (i.e. cells that were triple labeled) are included in the last graph (F). *p < 0.05; n = 3/group.*

5.4 DISCUSSION

Ethanol rats displayed increased neurogenesis, indicated by BrdU+/NeuN+ cell counts seven weeks-post binge, confirming that reported previously (Nixon and Crews, 2004). Indeed, more than 90% of BrdU+ cells co-expressed NeuN, which means that 90% of the cells labeled on T7 that survived to T52 (in the SGZ) became mature neurons. This is consistent with previous studies that show increases in neurogenesis following alcohol exposure (Hansson et al., 2010; He et al., 2005; Maynard and Leasure, 2013; Nixon and Crews, 2004). Although there was an increase in neurogenesis in ethanol rats, there was no difference between ethanol and control rats in terms of the number of cells that were activated in response to the MWM (measured with c-Fos; Figure 5.4). Studies have shown that c-Fos expression is similar between newborn neurons and older granule cells following MWM in untreated (normal) animals (Jessberger and Kempermann, 2003). However, assessing the number of c-Fos+ cells was not the goal here, the goal was to determine if more of the newborn neurons (labeled on T7) were activated by MWM. Interestingly, despite the increase in neurogenesis (BrdU+/NeuN+) there was no increase in the number of triple labeled neurons, i.e. activated neurons. In summary, there were significantly more six-week old neurons in ethanol rats and those neurons were the optimal age for preferential recruitment in the MWM (Kee et al., 2007). However, preferential recruitment was not observed since there was no difference between the number of triple labeled cells in ethanol and control rats. This was consistent with Geibig et al. (2012) who noted an increase in

neurogenesis (in post-stroke mice) but an equal number of activated young neurons (triple labeled neurons) compared to controls. These unexpected results, however, may be the result of the very low number of triple labeled cells. Although, low c-Fos expression was expected since only 1-2% of all dentate granule cells express c-Fos in response to the present stimuli (Geibig et al., 2012). These low cell counts are comparable (Geibig et al., 2012) or slightly lower than previous studies (Kee et al., 2007). These results are counterintuitive and difficult to explain.

Originally, the specificity of the triple label was viewed as a methodological advantage since it allowed for highly precise labeling. Although in this instance the specificity could be seen as a disadvantage since so few cells are labeled which results in difficulty assessing the meaningfulness of such a small number of cells. Increasing the number of triple labeled cells would be possible if the rats were given BrdU injections over a longer period of time so that a larger population could be captured. It is possible that a larger pool of cells to quantify would reveal more triple labeled cells. However, it seems unlikely that this would change the results since the ethanol animals already had a significant increase in the number of neurons (BrdU/NeuN) and did not show a similar increase in the number of triple labeled cells (and hundreds of cells were quantified for each animal across all sections). Essentially, the percentage of cells that were triple labeled did not change despite the large increase in the total number of BrdU+ cells in ethanol animals compared to controls. This lack of change was unexpected since cells that are 6 weeks old have been shown to be preferentially

incorporated (c-Fos+) following the MWM (Kee et al., 2007). Since there were more newborn neurons in ethanol animals, we hypothesized that more of those neurons would be available for recruitment in the MWM and that was not the case. There were some potential differences that could shed light on the lack of a difference in recruitment despite the increase in neurogenesis in ethanol animals. The research done by Kee and colleagues in 2007 illustrated that there was a preferential age when neurons are recruited for the MWM task, was done in mice. Species differences in neurogenesis are known to exist between rats and mice and could account for differences in the timing of when cells are considered mature. Indeed, researchers have reported that in adult rats, young neurons display mature neuronal markers and IEG expression 1-2 weeks before mice (Snyder et al., 2009). Therefore, it is possible that looking at c-Fos expression 1-2 weeks earlier may have yielded different results. Although, the preferential recruitment of newborn neurons in mice occurred when young neurons were 6-8 weeks old (Kee et al., 2007). Thus, investigating 4 week old neurons would likely yield the same results seen in the 6 week old neurons.

We hypothesized that the increase in neurogenesis would also generate an increased number of triple labeled cells that were activated following the MWM; however, that was not the case. There was no difference between ethanol and control rats in terms of the number of triple labeled cells, or the percentage of BrdU+ cells that also expressed c-Fos/NeuN. However, MWM testing revealed that ethanol animals located the platform more quickly than controls (typically interpreted as an improvement in learning). These results are interesting since

improved MWM performance typically correlates with increased neurogenesis (Garthe et al., 2016; Geibig et al., 2012; van Praag et al., 2002), but the more direct assay of cellular functional incorporation, the triple label, does not. Future studies will focus on investigating a more in-depth time course of MWM testing to determine when/if improvements in learning and memory normalize. Additionally, future studies should investigate additional IEGs such as zif268 and Arc (Clark et al., 2011; Stone et al., 2011b) in place of c-Fos in the triple-label because each IEG has a slightly different expression profile, while still being indicative of activation in response to a hippocampal-dependent task. For example, Zif268 expression is roughly four times higher than c-Fos and has been used to confirm findings related to c-Fos expression (Geibig et al., 2012).

The goal of this project was to determine if neurons generated during reactive neurogenesis were capable of activation in response to the MWM. While there was an increase in the number of young neurons born during alcohol-induced reactive neurogenesis, there were no differences in the number of activated newborn neurons between ethanol and control animals. However, there were triple labeled cells in both ethanol and control animals. Therefore, we concluded that the neurons generated during reactive neurogenesis were capable of activation in response to MWM. However, this conclusion required proof that c-Fos was activated as a direct result of the MWM. Indeed, previous research has shown that rats not exposed to MWM expressed no detectable levels of c-Fos with IHC (Jessberger and Kempermann, 2003). To confirm that c-Fos expression was undetectable in animals not exposed to the water maze,

tissue sections from multiple animals (from a similar 4-day binge study) were stained for c-Fos alongside a positive control (MWM rats) and we observed no c-Fos+ staining in animals that did not undergo the MWM. Therefore, although we recognize the importance of this group, the lack of staining seen here allowed us to conclude that a non-MWM group was not necessary for this study.

In conclusion, seven weeks post-binge alcohol exposure (six weeks after the reactive increase in neurogenesis) animals exposed to alcohol performed better on the MWM, an effect that likely is due to increases in neurogenesis, which were confirmed with BrdU+/NeuN+ cell counts. The number of activated neurons (c-Fos+/NeuN+) did not differ between ethanol and control animals. Lastly, the number of triple labeled cells did not differ between ethanol and control animals indicating that activation is similar between cells generated during basal neurogenesis and cells generated during reactive neurogenesis. Regardless of the treatment administered (and the fact that ethanol animals had increased BrdU+/NeuN+ cell counts) a similar number of cells were activated in ethanol and control rats. These data support the conclusion that neurons generated during alcohol-induced reactive neurogenesis are capable of becoming incorporated into hippocampal networks. Additionally, since there was no difference between ethanol and controls in terms of the number of activated neurons (c-Fos+/NeuN+), it indicates that activation may not be affected by binge alcohol exposure at this time point (T52). We recognize that a triple-labeled cell may not necessarily be functionally normal and this should be tested in future studies. Although the number of activated neurons is very small, there is

important research that indicates that the contribution of a small number of new neurons could contribute substantially to the hippocampal network (Geibig et al., 2012; Kempermann, 2008; Wiskott et al., 2006). In addition, activation in response to MWM represents only one facet of functional integration (Geibig et al., 2012). A complete analysis will require additional studies to investigate connectivity and electrophysiological properties to ensure that neurons born during reactive neurogenesis are properly functionally integrated.

This is the first study to investigate the function of alcohol-induced reactive neurogenesis. The idea of triple labeling cells to determine if they are able to functionally incorporate into the hippocampal circuits came from similar research investigating reactive neurogenesis following an experimental model of a stroke which revealed that reactive neurons were activated in response to MWM (Geibig et al., 2012). Reactive neurogenesis occurring in response to alcohol-induced neurodegeneration is potentially similar to other models of neurodegenerative disease including stroke and TBI (Geil et al., 2014). Any neurodegenerative disease that affects the hippocampus can result in deficits in hippocampal-dependent learning and memory. Since ethanol decreases neurogenesis, it has been postulated that this likely contributes to its neurodegenerative effects (Armstrong and Barker, 2001; Nixon, 2006). Thankfully, research has shown that some hippocampal functions can normalize with time and here we show that it is possible that neurons generated during reactive neurogenesis could contribute to this recovery.

CHAPTER 6: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Excessive alcohol consumption can result in the development of an AUD, commonly referred to as alcoholism. In the United States an estimated 30% of the population will meet the criteria for an AUD at some point during their lifetime (Hasin DS et al., 2007). Chronic alcoholics display cognitive deficits including impairments in executive functioning, learning, and memory (Sullivan et al., 2000). Indeed, human alcoholics and animal models of alcoholism show hippocampal degeneration (Bengochea and Gonzalo, 1990; Taffe et al., 2010; Walker et al., 1980; Zahr et al., 2010). Rodent models have been able to show the relationship between excessive alcohol ingestion, cell death, and corticolimbic neurodegeneration (Collins et al., 1996; Crews et al., 2006a; Walker et al., 1980). Research has also shown that adult neurogenesis is essential for maintaining hippocampal integrity (Gould et al., 1999; Imayoshi et al., 2008; Shors et al., 2002). Additionally, rodent models have revealed a temporally-specific increase in adult neurogenesis seen during abstinence from binge alcohol exposure (Nixon and Crews, 2004). Reactive neurogenesis can be observed following multiple forms of brain injury including TBI where research has indicated a potential role for newborn neurons in hippocampal recovery (Blaiss et al., 2011). Investigators utilized transgenic mice to show that neurons generated during TBI-induced reactive neurogenesis were able to stably incorporate into the hippocampus (Blaiss et al., 2011). Similarly, previous research has shown that stroke-induced reactive neurogenesis also yielded

newborn neurons capable of integration into hippocampal circuits (Geibig et al., 2012).

The studies described in this dissertation aim to define the contribution of reactive neurogenesis in hippocampal recovery following a binge model of an AUD in rats. To address this novel aim the first experiments focused on elucidating a methodological approach capable of successfully inhibiting alcohol-induced reactive neurogenesis. The next step was to examine how inhibiting reactive neurogenesis affected learning and memory in a hippocampal-dependent task (MWM). In order to normalize (inhibit or decrease) the alcohol-induced reactive increase in proliferation seen at T7, rats were exposed to four days of binge alcohol followed by two additional doses of alcohol which were given on T6/T7. Although high doses of alcohol had been previously successful in decreasing ***basal*** levels of hippocampal proliferation (Nixon and Crews, 2002), surprisingly, the additional doses of alcohol did not prevent the reactive increase in proliferation. The fact that high doses of alcohol inhibit basal proliferation, but not reactive proliferation, indicate that reactive proliferation is robust, possibly due to its potential role in hippocampal recovery following an AUD. These results prompted a more thorough investigation into the time frame surrounding alcohol-induced reactive proliferation (and subsequent reactive neurogenesis).

A timeline study investigated proliferation levels between four and seven days post-binge. Results revealed that reactive proliferation begins on T5 and lasts through T7. Discovering that proliferation was increased as early as T5 was important for elucidating a method to normalize alcohol-induced reactive

proliferation/neurogenesis. Without this timeline it would not have been clear that reactive proliferation needed to be inhibited across multiple days.

Because our previous attempt to use alcohol to decrease reactive proliferation was unsuccessful, we incorporated the use of TMZ to inhibit neurogenesis. TMZ has been used for studies of basal neurogenesis and is considered to be a well-tolerated drug among existing anti-proliferative agents (Niibori et al., 2012; Nokia et al., 2012). Although new information regarding the timeline of reactive proliferation may have provided the information needed to properly time alcohol administration to decrease proliferation (ethanol could have been given from T4-T6), we hypothesized that using a drug known to target rapidly proliferating cells would allow for the greatest chance of success in inhibiting rapidly proliferating NPCs. Therefore, TMZ was administered to overlap with the reactive increase in proliferation seen on T5-T7. Importantly, TMZ administration began before T5 to ensure that cells that were in the process of replicating were inhibited. Essentially, cells may already be in the process of proliferating by/on T4 and those T4 cells generate the increase seen on T5; for this reason TMZ was administered prior to T5. Results revealed that TMZ normalized the reactive increase in proliferation seen on T7, and the respective increase in neurogenesis seen on T14. These experiments indicated that TMZ was able to decrease alcohol-induced reactive proliferation and neurogenesis to levels seen in controls without simply delaying reactive proliferation. As a result, these experiments supported the use of TMZ as an effective method of inhibiting alcohol-induced reactive neurogenesis and therefore these methods can be used

in future experiments to study the function of inhibiting alcohol-induced reactive neurogenesis.

One main objective of this dissertation was to determine the role of alcohol-induced reactive neurogenesis in hippocampal recovery following a binge model of an AUD. With TMZ successfully inhibiting reactive neurogenesis, an important question could be asked: if reactive neurogenesis is inhibited, does it prevent recovery of hippocampal function (assessed with MWM). Previously, our lab has shown deficits in the MWM 7-10 days post-binge (Supplemental Figure 1). In the present studies rats were tested in the MWM when neurons produced during reactive neurogenesis were four weeks old (T38). Interestingly, TMZ did not appear to affect MWM behavior at this time point as there were no differences between the different drug exposure groups (i.e. the ethanol-TMZ rats preformed just as well as the control-saline group). These data indicated that inhibiting reactive neurogenesis did not affect functional hippocampal recovery measured by the MWM. While these results were unexpected (the original hypothesis predicted that inhibiting reactive neurogenesis would prevent MWM normalization), they are not totally unexpected. Previous research has shown hippocampal recovery following alcoholism in humans (Bartels et al., 2007) and animal models of AUDs (Lukoyanov et al., 2000) and as such, the present findings support the conclusion that regeneration is possible. However, to be thorough, future experiments should increase the complexity of the MWM test by incorporating a reversal learning component which could result in a greater challenge to the injured hippocampus, thereby revealing more subtle deficits not

seen with standard MWM testing procedures (Garthe et al., 2009). Regardless of the final conclusion of this line of research (i.e. whether reactive neurogenesis is beneficial or inconsequential), it will provide a valuable addition to the field of regeneration and hippocampal recovery following AUD.

The next part of this study investigated the functional incorporation of neurons born during alcohol-induced reactive neurogenesis. Importantly, this study did not use TMZ because the goal here was to investigate if reactive neurons were capable of activation. Neurons born during alcohol-induced reactive neurogenesis were allowed time to mature and incorporate into the hippocampal circuitry. Here, results revealed that cells born during alcohol-induced reactive neurogenesis were capable of activation following the MWM (although they may be less prone to activate). This finding is supported by previous studies in other models of neurodegeneration, including stroke and TBI, which have observed neuronal activation in response to reactive neurogenesis (Blaiss et al., 2011; Geibig et al., 2012).

Here the MWM was used as a means to activate neurons generated during reactive neurogenesis seven weeks post-binge alcohol exposure (Geibig et al., 2012; Snyder et al., 2012). However, when all of the available post-binge MWM data was considered it showed a time course with a deficit in MWM ~one week post-binge that normalized by five weeks post-binge and then resulted in an improvement in MWM in ethanol rats at seven weeks post-binge (compared to controls). Importantly, the MWM timeline (one, five, and seven weeks post-binge) in rats appears consistent with previous research that demonstrates an

improvement in cortical grey matter volumes within four weeks of alcohol abstinence in humans (O'Neill et al., 2001; Pfefferbaum et al., 1995). Additionally, increased neurogenesis observed four to eight weeks post-insult in animals correlated with improved performance on hippocampal-dependent tests (Garthe and Kempermann, 2013; Kempermann and Gage, 2002b; van Praag et al., 1999). Previous research has also shown that it can take four to eight weeks for young neurons to mature and integrate into hippocampal networks (Deng et al., 2010; Kee et al., 2007). Therefore, four week old neurons may not be fully integrated by T38 and therefore may require at least six weeks to incorporate (T52), particularly for the MWM (Kee et al., 2007). This timeline of maturation could also explain why there was no change when neurons were four weeks old (T38) but showed improvements seven-weeks post-binge (T52) in ethanol rats with increased neurogenesis levels. To confirm the results that indicate that alcohol rats outperform controls, the T52 MWM time point should be repeated and include a reversal learning test and another hippocampal-dependent behavioral test, such as contextual fear conditioning (Daumas et al., 2005; Phillips and LeDoux, 1992). The combination of the experiments listed above would allow for an in-depth investigation into the functional contribution of reactive neurogenesis.

The function of newborn neurons is still being debated but most adult neurogenesis studies agree that the constant generation of new neurons is important for hippocampal function (including pattern separation). Young granule neurons display different electrophysiological properties than fully mature granule

neurons which result in young neurons being highly active in the weeks following functional incorporation (Nakashiba et al., 2012). Previous research has used several methods to inhibit neurogenesis in an attempt to study how new neurons contribute to hippocampal function with the newest methods involving the use of transgenic rats to fully inhibit adult neurogenesis (Snyder et al., 2016).

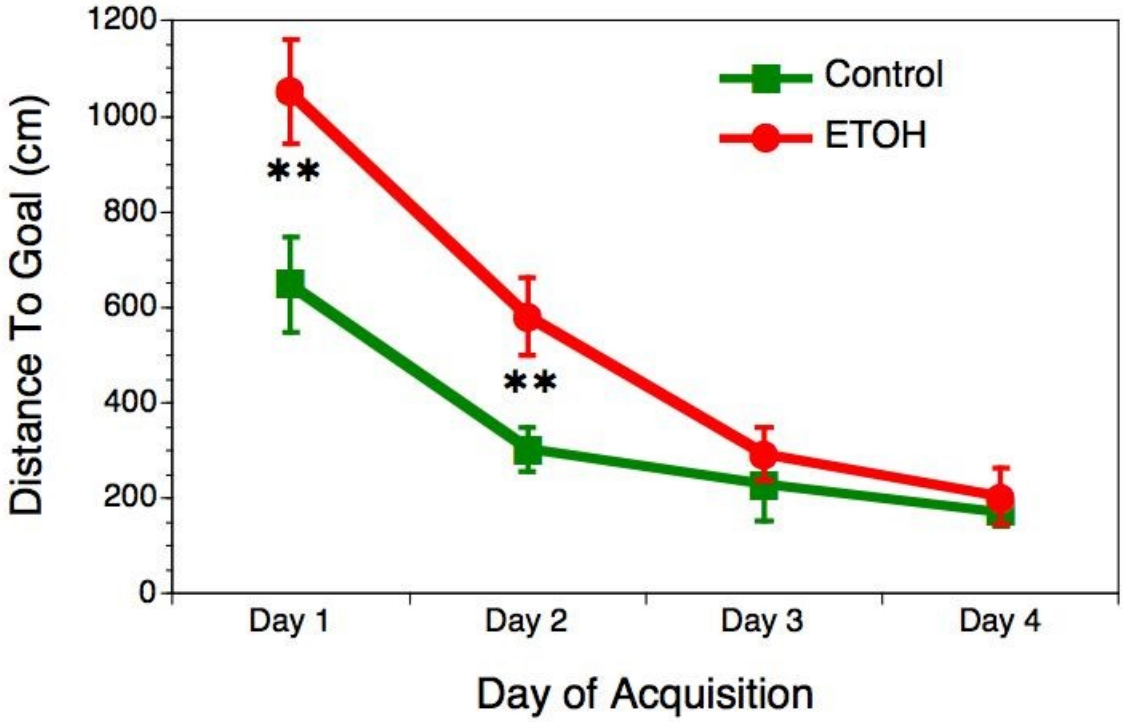
Here we use TMZ to inhibit reactive neurogenesis following alcohol exposure. Previous studies propose a role for adult neurogenesis as a potential mechanism contributing to functional and structural repair of the hippocampus following binge alcohol exposure (Nixon, 2006). This mechanism is supported by a robust increase in neurogenesis (i.e. reactive neurogenesis) that occurs after the cessation of binge alcohol consumption in rats. Furthermore, the correlation between alcohol-induced increases in neurogenesis and improvements in hippocampal-specific behavior are striking. Additionally, the young neurons generated during reactive neurogenesis are activated when induced with a hippocampus-specific task, which supports that they are functional. If neurons are indeed functionally incorporated then they represent a potential mechanism by which the brain can help repair itself. Additionally, it implies that neurogenesis represents a target for therapeutic development in the treatment of AUDs. Advances towards understanding reactive neurogenesis could contribute to the development of interventions capable of improving abstinence success rates or speeding up the process of neural recovery. There is high demand for therapeutic-based treatment options for AUDs since currently, only three

medications are FDA approved: aversive compounds, opioid receptor antagonists, and glutamate-based interventions.

It should be noted that not all studies show a role for adult neurogenesis or reactive neurogenesis in hippocampal recovery. One study ablated adult neurogenesis only to reveal that the brain was able to compensate and recover normal LTP function despite neurogenesis being completely inhibited (Singer et al., 2011). Another study showed that although chronic alcohol exposure resulted in significant neuronal loss in multiple hippocampal regions, the rodent brain was able to reorganize (potentially at the synaptic level), such that functional deficits were not observed (Lukoyanov et al., 2000). As such, we must consider the possibility that the brain is capable of compensating for a lack of adult neurogenesis, which may complicate an assessment of the function of alcohol-induced reactive neurogenesis. However, the present research indicates that hippocampal deficits are recoverable and neurons born during reactive neurogenesis are not only functionally incorporated, but potentially lead to improvements in hippocampal function. Therefore, further investigation should remain a priority, especially with the long-term prospect of revealing novel targets to treat neurodegenerative disorders including AUDs.

APPENDIX

SUPPLEMENTAL FIGURE 1



Supplemental Figure - EtOH (ethanol) rats swim longer distances before locating the hidden platform which is indicative of a learning deficit. Binge alcohol disrupts spatial learning in the MWM 1 week (7-10 days) after a 4-day binge alcohol treatment (n=7). **p<0.05. Figure and data reproduced with permission from Dr. Nixon and Dr. Pauly.

ABBREVIATIONS

AAALAC, Association for assessment and accreditation of laboratory animal care
ANOVA, analysis of variance
AUD, alcohol use disorder
BEC, blood ethanol concentration
BrdU, bromodeoxyuridine
CA, cornu amonis
CREB, cAMP response element binding protein
CNS, central nervous system
DAB, 3,3'-diaminobenzidine tetrahydrochloride
DCX, doublecortin
DG, dentate gyrus
dH_2O, distilled water
DNA, deoxyribonucleic acid
DSM, diagnostic and statistical manual of mental disorders
FASD, fetal alcohol spectrum disorders
GABA, gamma-amino-butyric acid
GCL, granule cell layer
GFAP, glial fibrillary acidic protein
Iba1, ionized calcium-binding adapter molecule 1
IEG, immediate early gene
IgG, immunoglobulin G
IHC, immunohistochemistry
i.p., intraperitoneal
LTP, long-term potentiation
MRI, magnetic resonance imaging
MWM, Morris water maze
nAChR, nicotinic acetylcholine receptor
NeuN, neuronal nuclei
NeuroD, neurogenic differentiation factor 1
NIAAA, National Institute on Alcohol Abuse and Alcoholism
NIH, National Institute of Health
NMDA, N-methyl-D-aspartate
NPC, neural progenitor cell
PBS, phosphate buffered saline
PBST, phosphate buffered saline + Triton-x
PFA, paraformaldehyde
PND, postnatal day
SGZ, subgranular zone
S-phase, synthesis phase of cell cycle
SSC, standard sodium citrate
TBI, traumatic brain injury
TBS, tris buffered saline
TMZ, temozolomide
WD, withdrawal

REFERENCES

Aberg, E., Hofstetter, C.P., Olson, L., and Brene, S. (2005). Moderate ethanol consumption increases hippocampal cell proliferation and neurogenesis in the adult mouse. The International Journal of Neuropsychopharmacology / Official Scientific Journal of the Collegium Internationale Neuropsychopharmacologicum *8*, 557–567.

Acsády, L., Kamondi, A., Sík, A., Freund, T., and Buzsáki, G. (1998). GABAergic Cells Are the Major Postsynaptic Targets of Mossy Fibers in the Rat Hippocampus. The Journal of Neuroscience : The Official Journal of the Society for Neuroscience *18*, 3386–3403.

Agartz, I., Momenan, R., Rawlings, R.R., Kerich, M.J., and Hommer, D.W. (1999). Hippocampal volume in patients with alcohol dependence. Archives of General Psychiatry *56*, 356–363.

Aguado, T., Monory, K., Palazuelos, J., Stella, N., Cravatt, B., Lutz, B., Marsicano, G., Kokaia, Z., Guzman, M., and Galve-Roperh, I. (2005). The endocannabinoid system drives neural progenitor proliferation. FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology *19*, 1704–1706.

Alfonso-Loeches, S., and Guerri, C. (2011). Molecular and behavioral aspects of the actions of alcohol on the adult and developing brain. Crit Rev Clin Lab Sci *48*, 19–47.

Altman, J., and Das, G.D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol *124*, 319–335.

Amaral, D.G., Scharfman, H.E., and Lavenex, P. (2007). The dentate gyrus: fundamental neuroanatomical organization (dentate gyrus for dummies). Prog Brain Res *163*, 3–22.

American Psychiatric Association, American Psychiatric Association, and DSM-5 Task Force (2013). Diagnostic and statistical manual of mental disorders: DSM-5.

Anderson, M.L., Nokia, M.S., Govindaraju, K.P., and Shors, T.J. (2012). Moderate drinking? Alcohol consumption significantly decreases neurogenesis in the adult hippocampus. Neuroscience *224*, 202–209.

Armstrong, R.J., and Barker, R.A. (2001). Neurodegeneration: a failure of neuroregeneration? Lancet *358*, 1174–1176.

Arnone, M., Maruani, J., Chaperon, F., Thiebot, M.H., Poncelet, M., Soubrie, P., and Le Fur, G. (1997). Selective inhibition of sucrose and ethanol intake by SR

141716, an antagonist of central cannabinoid (CB1) receptors. Psychopharmacology *132*, 104–106.

Barbarich-Marsteller, N.C., Fornal, C.A., Takase, L.F., Bocarsly, M.E., Arner, C., Walsh, B.T., Hoebel, B.G., and Jacobs, B.L. (2013). Activity-based anorexia is associated with reduced hippocampal cell proliferation in adolescent female rats. Behav Brain Res *236*, 251–257.

Bartels, C., Kunert, H.J., Stawicki, S., Kroner-Herwig, B., Ehrenreich, H., and Krampe, H. (2007). Recovery of hippocampus-related functions in chronic alcoholics during monitored long-term abstinence. Alcohol and Alcoholism (Oxford, Oxfordshire) *42*, 92–102.

Bartsch, A.J., Homola, G., Biller, A., Smith, S.M., Weijers, H.-G., Wiesbeck, G.A., Jenkinson, M., De Stefano, N., Solymosi, L., and Bendszus, M. (2007). Manifestations of early brain recovery associated with abstinence from alcoholism. Brain *130*, 36–47.

Belujon, P., and Grace, A.A. (2011). Hippocampus, amygdala, and stress: interacting systems that affect susceptibility to addiction. Annals of the New York Academy of Sciences *1216*, 114–121.

Bengochea, O., and Gonzalo, L.M. (1990). Effect of chronic alcoholism on the human hippocampus. Histol Histopathol *5*, 349–357.

Beresford, T.P., Arciniegas, D.B., Alfers, J., Clapp, L., Martin, B., Du, Y., Liu, D., Shen, D., and Davatzikos, C. (2006). Hippocampus volume loss due to chronic heavy drinking. Alcohol. Clin. Exp. Res. *30*, 1866–1870.

Bergmann, O., Liebl, J., Bernard, S., Alkass, K., Yeung, M.S., Steier, P., Kutschera, W., Johnson, L., Landen, M., Druid, H., et al. (2012). The age of olfactory bulb neurons in humans. Neuron *74*, 634–639.

Blaiss, C.A., Yu, T.S., Zhang, G., Chen, J., Dimchev, G., Parada, L.F., Powell, C.M., and Kernie, S.G. (2011). Temporally specified genetic ablation of neurogenesis impairs cognitive recovery after traumatic brain injury. The Journal of Neuroscience : The Official Journal of the Society for Neuroscience *31*, 4906–4916.

von Bohlen und Halbach, O. (2011). Immunohistological markers for proliferative events, gliogenesis, and neurogenesis within the adult hippocampus. Cell Tissue Res *345*, 1–19.

Bonaguidi, M.A., Wheeler, M.A., Shapiro, J.S., Stadel, R.P., Sun, G.J., Ming, G.L., and Song, H. (2011). In vivo clonal analysis reveals self-renewing and multipotent adult neural stem cell characteristics. Cell *145*, 1142–1155.

Bonaguidi, M.A., Song, J., Ming, G.L., and Song, H. (2012). A unifying hypothesis on mammalian neural stem cell properties in the adult hippocampus. Current Opinion in Neurobiology *22*, 754–761.

Bouchery, E.E., Harwood, H.J., Sacks, J.J., Simon, C.J., and Brewer, R.D. (2011). Economic costs of excessive alcohol consumption in the U.S., 2006. Am J Prev Med *41*, 516–524.

Braun, S.M.G., and Jessberger, S. (2014). Adult neurogenesis: mechanisms and functional significance. Development *141*, 1983–1986.

Broadwater, M.A., Liu, W., Crews, F.T., and Spear, L.P. (2014). Persistent loss of hippocampal neurogenesis and increased cell death following adolescent, but not adult, chronic ethanol exposure. Developmental Neuroscience *36*, 297–305.

Brown, J.P., Couillard-Després, S., Cooper-Kuhn, C.M., Winkler, J., Aigner, L., and Kuhn, H.G. (2003). Transient expression of doublecortin during adult neurogenesis. J Comp Neurol *467*, 1–10.

Bruel-Jungerman, E., Davis, S., Rampon, C., and Laroche, S. (2006). Long-term potentiation enhances neurogenesis in the adult dentate gyrus. The Journal of Neuroscience : The Official Journal of the Society for Neuroscience *26*, 5888–5893.

Bruel-Jungerman, E., Lucassen, P.J., and Francis, F. (2011). Cholinergic influences on cortical development and adult neurogenesis. Behav Brain Res

Cadete-Leite, A., Tavares, M.A., Uylings, H.B.M., and Paula-Barbosa, M.M. (1988). Granule cell loss and dendritic regrowth in the hippocampal dentate gyrus of the rat after chronic alcohol consumption. Brain Research *473*, 1–14.

Cadete-Leite, A., Tavares, M.A., Alves, M.C., Uylings, H.B., and Paula-Barbosa, M.M. (1989a). Metric analysis of hippocampal granule cell dendritic trees after alcohol withdrawal in rats. Alcoholism, Clinical and Experimental Research *13*, 837–840.

Cadete-Leite, A., Tavares, M.A., Pacheco, M.M., Volk, B., and Paula-Barbosa, M.M. (1989b). Hippocampal mossy fiber-CA3 synapses after chronic alcohol consumption and withdrawal. Alcohol *6*, 303–310.

Cameron, H.A., and McKay, R.D. (2001). Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. J Comp Neurol *435*, 406–417.

Cameron, H.A., McEwen, B.S., and Gould, E. (1995). Regulation of adult neurogenesis by excitatory input and NMDA receptor activation in the dentate gyrus. J Neurosci *15*, 4687–4692.

Cameron, H.A., Hazel, T.G., and McKay, R.D. (1998). Regulation of neurogenesis by growth factors and neurotransmitters. J Neurobiol *36*, 287–306.

Carlen, P.L., Wortzman, G., Holgate, R.C., Wilkinson, D.A., and Rankin, J.C. (1978). Reversible cerebral atrophy in recently abstinent chronic alcoholics measured by computed tomography scans. Science *200*, 1076–1078.

Cartlidge, D., and Redmond, A.D. (1990). Alcohol and conscious level. Biomed. Pharmacother *44*, 205–208.

Centers for Disease Control and Prevention (2006). Alcohol and public health: Alcohol-related disease impact (ARDI).

Chae, J.H., Stein, G.H., and Lee, J.E. (2004). NeuroD: the predicted and the surprising. Mol Cells *18*, 271–288.

Chandler, L.J., Newsom, H., Sumners, C., and Crews, F. (1993a). Chronic ethanol exposure potentiates NMDA excitotoxicity in cerebral cortical neurons. Journal of Neurochemistry *60*, 1578–1581.

Chandler, L.J., Sumners, C., and Crews, F.T. (1993b). Ethanol inhibits NMDA receptor-mediated excitotoxicity in rat primary neuronal cultures. Alcohol Clin Exp Res *17*, 54–60.

Chanraud, S., Martelli, C., Delain, F., Kostogianni, N., Douaud, G., Aubin, H.J., Reynaud, M., and Martinot, J.L. (2007). Brain morphometry and cognitive performance in detoxified alcohol-dependents with preserved psychosocial functioning. Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology *32*, 429–438.

Chowdhury, S., Vaughan, M.M., and Gore, M.E. (1999). New approaches to the systemic treatment of melanoma. Cancer Treat Rev *25*, 259–270.

Cippitelli, A., Zook, M., Bell, L., Damadzic, R., Eskay, R.L., Schwandt, M., and Heilig, M. (2010). Reversibility of object recognition but not spatial memory impairment following binge-like alcohol exposure in rats. Neurobiol Learn Mem *94*, 538–546.

Clark, P.J., Bhattacharya, T.K., Miller, D.S., and Rhodes, J.S. (2011). Induction of c-Fos, Zif268, and Arc from acute bouts of voluntary wheel running in new and pre-existing adult mouse hippocampal granule neurons. Neuroscience *184*, 16–27.

Clelland, C.D., Choi, M., Romberg, C., Clemenson, G.D., Jr., Fragniere, A., Tyers, P., Jessberger, S., Saksida, L.M., Barker, R.A., Gage, F.H., et al. (2009). A functional role for adult hippocampal neurogenesis in spatial pattern separation. Science *325*, 210–213.

Cole, A.J., Saffen, D.W., Baraban, J.M., and Worley, P.F. (1989). Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. Nature *340*, 474–476.

Collins, M.A., Corso, T.D., and Neafsey, E.J. (1996). Neuronal degeneration in rat cerebrocortical and olfactory regions during subchronic “binge” intoxication with ethanol: possible explanation for olfactory deficits in alcoholics. Alcohol Clin Exp Res *20*, 284–292.

Collins, M.A., Zou, J.Y., and Neafsey, E.J. (1998). Brain damage due to episodic alcohol exposure in vivo and in vitro: furosemide neuroprotection implicates edema-based mechanism. FASEB *12*, 221–230.

Conover, J.C., and Notti, R.Q. (2008). The neural stem cell niche. Cell and Tissue Research *331*, 211–224.

Contet, C., Kim, A., Le, D., Iyengar, S., Kotzebue, R.W., Yuan, C.J., Kieffer, B.L., and Mandyam, C.D. (2014). Mu Opioid Receptors Mediate the Effects of Chronic Ethanol Binge Drinking on the Hippocampal Neurogenic Niche. Addict Biol *19*, 770–780.

Corso, T.D., Mostafa, H.M., Collins, M.A., and Neafsey, E.J. (1998). Brain neuronal degeneration caused by episodic alcohol intoxication in rats: effects of nimodipine, 6,7-dinitro-quinoxaline-2,3-dione, and MK- 801. Alcohol Clin Exp Res *22*, 217–224.

Crews, F.T. (1999). Alcohol and neurodegeneration. CNS Drug Reviews *5*, 379–394.

Crews, F.T., and Nixon, K. (2003). Alcohol, neural stem cells, and adult neurogenesis. Alcohol Res Health *27*, 197–204.

Crews, F.T., and Nixon, K. (2009). Mechanisms of Neurodegeneration and Regeneration in Alcoholism. Alcohol Alcohol *44*, 115–127.

Crews, F., Nixon, K., Kim, D., Joseph, J., Shukitt-Hale, B., Qin, L., and Zou, J. (2006a). BHT blocks NF-kappaB activation and ethanol-induced brain damage. Alcohol Clin Exp Res *30*, 1938–1949.

Crews, F.T., Braun, C.J., Hoplight, B., Switzer, R.C., and Knapp, D.J. (2000). Binge ethanol consumption causes differential brain damage in young adolescent rats compared with adult rats. Alcohol Clin Exp Res *24*, 1712–1723.

Crews, F.T., Collins, M.A., Dlugos, C., Littleton, J., Wilkins, L., Neafsey, E.J., Pentney, R., Snell, L.D., Tabakoff, B., Zou, J., et al. (2004a). Alcohol-induced neurodegeneration: when, where and why? Alcohol Clin Exp Res *28*, 350–364.

Crews, F.T., Nixon, K., and Wilkie, M.E. (2004b). Exercise reverses ethanol inhibition of neural stem cell proliferation. Alcohol *33*, 63–71.

Crews, F.T., Mdzinarishvili, A., Kim, D., He, J., and Nixon, K. (2006b). Neurogenesis in adolescent brain is potently inhibited by ethanol. Neuroscience *137*, 437–445.

Cruz, F.C., Koya, E., Guez-Barber, D.H., Bossert, J.M., Lupica, C.R., Shaham, Y., and Hope, B.T. (2013). New technologies for examining the role of neuronal ensembles in drug addiction and fear. Nat Rev Neurosci *14*, 743–754.

Dash, P.K., Mach, S.A., and Moore, A.N. (2001). Enhanced neurogenesis in the rodent hippocampus following traumatic brain injury. J Neurosci Res *63*, 313–319.

Daumas, S., Halley, H., Francés, B., and Lassalle, J.-M. (2005). Encoding, consolidation, and retrieval of contextual memory: differential involvement of dorsal CA3 and CA1 hippocampal subregions. Learn Mem *12*, 375–382.

Deng, W., Aimone, J.B., and Gage, F.H. (2010). New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nat Rev Neurosci *11*, 339–350.

Diaz, F., McKeehan, N., Kang, W., and Hébert, J.M. (2013). Apoptosis of glutamatergic neurons fails to trigger a neurogenic response in the adult neocortex. J Neurosci *33*, 6278–6284.

Doetsch, F., Caille, I., Lim, D.A., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell *97*, 703–716.

Dranovsky, A., Picchini, A.M., Moadel, T., Sisti, A.C., Yamada, A., Kimura, S., Leonardo, E.D., and Hen, R. (2011). Experience dictates stem cell fate in the adult hippocampus. Neuron *70*, 908–923.

Durand, D., Saint-Cyr, J.A., Gurevich, N., and Carlen, P.L. (1989). Ethanol-induced dendritic alterations in hippocampal granule cells. Brain Research *477*, 373–377.

Eckardt, M.J., and Martin, P.R. (1986). Clinical assessment of cognition in alcoholism. Alcohol Clin Exp Res *10*, 123–127.

Eichenbaum, H. (2001). The hippocampus and declarative memory: cognitive mechanisms and neural codes. Behav Brain Res *127*, 199–207.

van Eijk, J., Demirakca, T., Frischknecht, U., Hermann, D., Mann, K., and Ende, G. (2013). Rapid partial regeneration of brain volume during the first 14 days of abstinence from alcohol. Alcohol Clin Exp Res *37*, 67–74.

Eisch, A.J., Barrot, M., Schad, C.A., Self, D.W., and Nestler, E.J. (2000). Opiates inhibit neurogenesis in the adult rat hippocampus. Proc Natl Acad Sci U S A *97*, 7579–7584.

Encinas, J.M., Michurina, T.V., Peunova, N., Park, J.H., Tordo, J., Peterson, D.A., Fishell, G., Koulakov, A., and Enikolopov, G. (2011). Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult hippocampus. Cell Stem Cell *8*, 566–579.

Eriksson, P.S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A.M., Nordborg, C., Peterson, D.A., and Gage, F.H. (1998). Neurogenesis in the adult human hippocampus. Nature Medicine *4*, 1313–1317.

Esposito, M.S., Piatti, V.C., Laplagne, D.A., Morgenstern, N.A., Ferrari, C.C., Pitossi, F.J., and Schinder, A.F. (2005). Neuronal differentiation in the adult hippocampus recapitulates embryonic development. J Neurosci *25*, 10074–10086.

Fadda, F., and Rossetti, Z. (1998). Chronic ethanol consumption: From neuroadaptation to neurodegeneration. Progress in Neurobiology *56*, 385–431.

Faingold, C.L. (2008). The Majchrowicz binge alcohol protocol: an intubation technique to study alcohol dependence in rats. Curr Protoc Neurosci *Chapter 9*, Unit 9.28.

Filippov, V., Kronenberg, G., Pivneva, T., Reuter, K., Steiner, B., Wang, L.P., Yamaguchi, M., Kettenmann, H., and Kempermann, G. (2003). Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrophysiological and morphological characteristics of astrocytes. Mol Cell Neurosci *23*, 373–382.

Fukuda, S., Kato, F., Tozuka, Y., Yamaguchi, M., Miyamoto, Y., and Hisatsune, T. (2003). Two distinct subpopulations of nestin-positive cells in adult mouse dentate gyrus. J Neurosci *23*, 9357–9366.

Gage, F.H. (2000). Mammalian neural stem cells. Science *287*, 1433–1438.

Gao, Z., Ure, K., Ables, J.L., Lagace, D.C., Nave, K.-A., Goebbels, S., Eisch, A.J., and Hsieh, J. (2009). Neurod1 is essential for the survival and maturation of adult-born neurons. Nat Neurosci *12*, 1090–1092.

Garthe, A., and Kempermann, G. (2013). An old test for new neurons: refining the Morris water maze to study the functional relevance of adult hippocampal neurogenesis. Front Neurosci *7*, 63.

Garthe, A., Behr, J., and Kempermann, G. (2009). Adult-generated hippocampal neurons allow the flexible use of spatially precise learning strategies. PLoS ONE *4*, e5464.

Garthe, A., Roeder, I., and Kempermann, G. (2016). Mice in an enriched environment learn more flexibly because of adult hippocampal neurogenesis. Hippocampus *26*, 261–271.

Gazdzinski, S., Durazzo, T.C., and Meyerhoff, D.J. (2005). Temporal dynamics and determinants of whole brain tissue volume changes during recovery from alcohol dependence. Drug and Alcohol Dependence *78*, 263–273.

Gazdzinski, S., Durazzo, T.C., Yeh, P.H., Hardin, D., Banys, P., and Meyerhoff, D.J. (2008). Chronic cigarette smoking modulates injury and short-term recovery of the medial temporal lobe in alcoholics. Psychiatry Research *162*, 133–145.

Ge, S., Goh, E.L., Sailor, K.A., Kitabatake, Y., Ming, G.L., and Song, H. (2006). GABA regulates synaptic integration of newly generated neurons in the adult brain. Nature *439*, 589–593.

Ge, S., Yang, C.H., Hsu, K.S., Ming, G.L., and Song, H. (2007). A critical period for enhanced synaptic plasticity in newly generated neurons of the adult brain. Neuron *54*, 559–566.

Geibig, C.S., Keiner, S., and Redecker, C. (2012). Functional recruitment of newborn hippocampal neurons after experimental stroke. Neurobiol Dis *46*, 431–439.

Geil, C.R., Hayes, D.M., McClain, J.A., Liput, D.J., Marshall, S.A., Chen, K.Y., and Nixon, K. (2014). Alcohol and adult hippocampal neurogenesis: Promiscuous drug, wanton effects. Prog Neuropsychopharmacol Biol Psychiatry *54*, 103–113.

Gilbert, P.E., and Kesner, R.P. (2006). The role of the dorsal CA3 hippocampal subregion in spatial working memory and pattern separation. Behavioural Brain Research *169*, 142–149.

Godsil, B.P., Kiss, J.P., Spedding, M., and Jay, T.M. (2013). The hippocampal-prefrontal pathway: the weak link in psychiatric disorders? European Neuropsychopharmacology : The Journal of the European College of Neuropsychopharmacology *23*, 1165–1181.

Golub, H.M., Zhou, Q.-G., Zucker, H., McMullen, M.R., Kokiko-Cochran, O.N., Ro, E.J., Nagy, L.E., and Suh, H. (2015). Chronic Alcohol Exposure is Associated with Decreased Neurogenesis, Aberrant Integration of Newborn Neurons, and Cognitive Dysfunction in Female Mice. Alcohol Clin Exp Res *39*, 1967–1977.

Gould, E., Beylin, A., Tanapat, P., Reeves, A., and Shors, T.J. (1999). Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci *2*, 260–265.

Grant, B.F., Goldstein, R.B., Saha, T.D., Chou, S.P., Jung, J., Zhang, H., Pickering, R.P., Ruan, W.J., Smith, S.M., Huang, B., et al. (2015). Epidemiology of DSM-5 Alcohol Use Disorder: Results From the National Epidemiologic Survey on Alcohol and Related Conditions III. JAMA Psychiatry *72*, 757–766.

Gray, W.P. (2008). Neuropeptide Y signalling on hippocampal stem cells in health and disease. Mol Cell Endocrinol *288*, 52–62.

Grobin, A.C., Matthews, D.B., Montoya, D., Wilson, W.A., Morrow, A.L., and Swartzwelder, H.S. (2001). Age-related differences in neurosteroid potentiation of muscimol-stimulated 36Cl(-) flux following chronic ethanol treatment. Neuroscience *105*, 547–552.

Gross, C.G. (2000). Neurogenesis in the adult brain: death of a dogma. Nature Reviews Neuroscience *1*, 67–73.

Guzowski, J.F. (2002). Insights into immediate-early gene function in hippocampal memory consolidation using antisense oligonucleotide and fluorescent imaging approaches. Hippocampus *12*, 86–104.

Guzowski, J.F., McNaughton, B.L., Barnes, C.A., and Worley, P.F. (1999). Environment-specific expression of the immediate-early gene Arc in hippocampal neuronal ensembles. Nat Neurosci *2*, 1120–1124.

Guzowski, J.F., Timlin, J.A., Roysam, B., McNaughton, B.L., Worley, P.F., and Barnes, C.A. (2005). Mapping behaviorally relevant neural circuits with immediate-early gene expression. Curr Opin Neurobiol *15*, 599–606.

Hamelink, C., Hampson, A., Wink, D.A., Eiden, L.E., and Eskay, R.L. (2005). Comparison of cannabidiol, antioxidants, and diuretics in reversing binge ethanol-induced neurotoxicity. J Pharmacol Exp Ther *314*, 780–788.

Hansson, A.C., Nixon, K., Rimondini, R., Damadzic, R., Sommer, W.H., Eskay, R., Crews, F.T., and Heilig, M. (2010). Long-term suppression of forebrain neurogenesis and loss of neuronal progenitor cells following prolonged alcohol dependence in rats. Int J Neuropsychopharmacol *13*, 583–593.

Harding, A.J., Wong, A., Svoboda, M., Kril, J.J., and Halliday, G.M. (1997). Chronic alcohol consumption does not cause hippocampal neuron loss in humans. Hippocampus *7*, 78–87.

Hasin DS, Stinson FS, Ogburn E, and Grant BF (2007). Prevalence, correlates, disability, and comorbidity of dsm-iv alcohol abuse and dependence in the united states: Results from the national epidemiologic survey on alcohol and related conditions. Arch Gen Psychiatry *64*, 830–842.

Hastings, N.B., and Gould, E. (1999). Rapid extension of axons into the CA3 region by adult-generated granule cells. J. Comp. Neurol. *413*, 146–154.

Hayes, D.M., Geil C.R., and Chen, K.Y. (in preparation). Alcohol-induced reactive hippocampal neurogenesis is due to activation of neural stem cells.

Hayes, D.M., Deeny, M.A., Shaner, C.A., and Nixon, K. (2013). Determining the Threshold for Alcohol-Induced Brain Damage: New Evidence with Gliosis Markers. Alcoholism, Clinical and Experimental Research.

He, J., Nixon, K., Shetty, A.K., and Crews, F.T. (2005). Chronic alcohol exposure reduces hippocampal neurogenesis and dendritic growth of newborn neurons. Eur. J. Neurosci. *21*, 2711–2720.

He, J., Overstreet, D.H., and Crews, F.T. (2009). Abstinence from moderate alcohol self-administration alters progenitor cell proliferation and differentiation in multiple brain regions of male and female P rats. Alcohol Clin Exp Res *33*, 129–138.

Herrera, D.G., Yague, A.G., Johnsen-Soriano, S., Bosch-Morell, F., Collado-Morente, L., Muriach, M., Romero, F.J., and Garcia-Verdugo, J.M. (2003). Selective impairment of hippocampal neurogenesis by chronic alcoholism: protective effects of an antioxidant. Proc Natl Acad Sci U S A *100*, 7919–7924.

Hodge, R.D., Nelson, B.R., Kahoud, R.J., Yang, R., Mussar, K.E., Reiner, S.L., and Hevner, R.F. (2012). Tbr2 Is Essential for Hippocampal Lineage Progression from Neural Stem Cells to Intermediate Progenitors and Neurons. J. Neurosci. *32*, 6275–6287.

Hoffman, P.L. (2003). NMDA receptors in alcoholism. Int. Rev. Neurobiol. *56*, 35–82.

van Hoof, J.J., Van Der Lely, N., Bouthoorn, S.H., Van Dalen, W.E., and Pereira, R.R. (2011). Adolescent alcohol intoxication in the dutch hospital departments of pediatrics: a 2-year comparison study. J Adolesc Health *48*, 212–214.

Hunt, W.A. (1993). Are binge drinkers more at risk of developing brain damage? Alcohol *10*, 559–561.

Hyman, S.E., Malenka, R.C., and Nestler, E.J. (2006). Neural mechanisms of addiction: the role of reward-related learning and memory. Annual Review of Neuroscience *29*, 565–598.

Imayoshi, I., Sakamoto, M., Ohtsuka, T., Takao, K., Miyakawa, T., Yamaguchi, M., Mori, K., Ikeda, T., Itohara, S., and Kageyama, R. (2008). Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. Nat. Neurosci. *11*, 1153–1161.

Jang, M.-H., Shin, M.-C., Kim, E.-H., and Kim, C.-J. (2002). Acute alcohol intoxication decreases cell proliferation and nitric oxide synthase expression in dentate gyrus of rats. Toxicol. Lett. *133*, 255–262.

Jessberger, S., and Kempermann, G. (2003). Adult-born hippocampal neurons mature into activity-dependent responsiveness. Eur. J. Neurosci. *18*, 2707–2712.

Jin, K., Minami, M., Lan, J.Q., Mao, X.O., Batteur, S., Simon, R.P., and Greenberg, D.A. (2001). Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. Proc. Natl. Acad. Sci. U.S.A. *98*, 4710–4715.

Johnson, A., van der Meer, M.A., and Redish, A.D. (2007). Integrating hippocampus and striatum in decision-making. Curr Opin Neurobiol *17*, 692–697.

Jonas, P., and Lisman, J. (2015). Structure, function, and plasticity of hippocampal dentate gyrus microcircuits (Frontiers Media SA).

Kaplan, M.S., and Hinds, J.W. (1977). Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science *197*, 1092–1094.

Kee, N., Sivalingam, S., Boonstra, R., and Wojtowicz, J.M. (2002). The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. J. Neurosci. Methods *115*, 97–105.

Kee, N., Teixeira, C.M., Wang, A.H., and Frankland, P.W. (2007). Preferential incorporation of adult-generated granule cells into spatial memory networks in the dentate gyrus. Nat. Neurosci. *10*, 355–362.

Kelsch, W., Lin, C.-W., and Lois, C. (2008). Sequential development of synapses in dendritic domains during adult neurogenesis. Proc. Natl. Acad. Sci. U.S.A. *105*, 16803–16808.

Kelso, M.L., Liput, D.J., Eaves, D.W., and Nixon, K. (2011). Upregulated vimentin suggests new areas of neurodegeneration in a model of an alcohol use disorder. Neuroscience *197*, 381–393.

Kempermann, G. (2008). The neurogenic reserve hypothesis: what is adult hippocampal neurogenesis good for? Trends in Neurosciences *31*, 163–169.

Kempermann, G. (2012). New neurons for “survival of the fittest.” Nature Reviews. Neuroscience *13*, 727–736.

Kempermann, G., and Gage, F.H. (2002a). Genetic influence on phenotypic differentiation in adult hippocampal neurogenesis. Brain Res Dev Brain Res *134*, 1–12.

Kempermann, G., and Gage, F.H. (2002b). Genetic determinants of adult hippocampal neurogenesis correlate with acquisition, but not probe trial performance, in the water maze task. Eur. J. Neurosci. *16*, 129–136.

Kempermann, G., Song, H., and Gage, F.H. (2015). Neurogenesis in the Adult Hippocampus. Cold Spring Harb Perspect Biol *7*, a018812.

King, M.A. (1988). Alterations and recovery of dendritic spine density in rat hippocampus following long-term ethanol ingestion. Brain Research *459*, 381–385.

Knapp, D.J., and Crews, F.T. (1999). Induction of cyclooxygenase-2 in brain during acute and chronic ethanol treatment and ethanol withdrawal. Alcohol Clin Exp Res *23*, 633–643.

Knapp, D.J., Duncan, G.E., Crews, F.T., and Breese, G.R. (1998). Induction of Fos-like proteins and ultrasonic vocalizations during ethanol withdrawal: further evidence for withdrawal-induced anxiety. Alcohol Clin Exp Res *22*, 481–493.

Koob, G.F., and Le Moal, M. (1997). Drug abuse: hedonic homeostatic dysregulation. Science *278*, 52–58.

Kuzmin, A., Liljequist, S., Meis, J., Chefer, V., Shippenberg, T., and Bakalkin, G. (2012). Repeated moderate-dose ethanol bouts impair cognitive function in Wistar rats. Addict Biol *17*, 132–140.

Laakso, M.P., Vaurio, O., Savolainen, L., Repo, E., Soininen, H., Aronen, H.J., and Tiihonen, J. (2000). A volumetric MRI study of the hippocampus in type 1 and 2 alcoholism. Behavioural Brain Research *109*, 177–186.

Labiner, D.M., Butler, L.S., Cao, Z., Hosford, D.A., Shin, C., and McNamara, J.O. (1993). Induction of c-fos mRNA by kindled seizures: complex relationship with neuronal burst firing. J. Neurosci. *13*, 744–751.

Leasure, J.L., and Nixon, K. (2010). Exercise Neuroprotection in a Rat Model of Binge Alcohol Consumption. Alcohol Clin Exp Res *34*, 404–414.

Lee, B., Cao, R., Choi, Y.-S., Cho, H.-Y., Rhee, A.D., Hah, C.K., Hoyt, K.R., and Obrietan, K. (2009). The CREB/CRE transcriptional pathway: protection against oxidative stress-mediated neuronal cell death. J. Neurochem. *108*, 1251–1265.

Lee, J.E., Hollenberg, S.M., Snider, L., Turner, D.L., Lipnick, N., and Weintraub, H. (1995). Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Science *268*, 836–844.

Lewohl, J.M., Wilson, W.R., Mayfield, R.D., Brozowski, S.J., Morrisett, R.A., and Harris, R.A. (1999). G-protein-coupled inwardly rectifying potassium channels are targets of alcohol action. Nature Neuroscience *2*, 1084–1090.

Lindblad, B., and Olsson, R. (1976). Unusually high levels of blood alcohol? JAMA *236*, 1600–1602.

Liu, J., Solway, K., Messing, R.O., and Sharp, F.R. (1998). Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. J. Neurosci *18*, 7768–7778.

Livy, D.J., Parnell, S.E., and West, J.R. (2003). Blood ethanol concentration profiles: a comparison between rats and mice. Alcohol *29*, 165–171.

Llorens-Martín, M., Rábano, A., and Ávila, J. (2015a). The Ever-Changing Morphology of Hippocampal Granule Neurons in Physiology and Pathology. Front Neurosci *9*, 526.

Llorens-Martín, M., Jurado-Arjona, J., Avila, J., and Hernández, F. (2015b). Novel connection between newborn granule neurons and the hippocampal CA2 field. Exp. Neurol. *263*, 285–292.

Lovinger, D.M. (1999). 5-HT3 receptors and the neural actions of alcohols: an increasingly exciting topic. Neurochem Int *35*, 125–130.

Lovinger, D.M., White, G., and Weight, F.F. (1989). Ethanol inhibits NMDA-activated ion current in hippocampal neurons. Science *243*, 1721–1724.

Lukoyanov, N.V., Brandao, F., Cadete-Leite, A., Madeira, M.D., and Paula-Barbosa, M.M. (2000). Synaptic reorganization in the hippocampal formation of alcohol-fed rats may compensate for functional deficits related to neuronal loss. Alcohol *20*, 139–148.

Madsen, T.M., Kristjansen, P.E., Bolwig, T.G., and Wortwein, G. (2003). Arrested neuronal proliferation and impaired hippocampal function following fractionated brain irradiation in the adult rat. Neuroscience *119*, 635–642.

Maekawa, M., Namba, T., Suzuki, E., Yuasa, S., Kohsaka, S., and Uchino, S. (2009). NMDA receptor antagonist memantine promotes cell proliferation and production of mature granule neurons in the adult hippocampus. Neurosci. Res. *63*, 259–266.

Majchrowicz, E. (1975). Induction of physical dependence upon ethanol and the associated behavioral changes in rats. Psychopharmacologia *43*, 245–254.

Mandyam, C.D., and Koob, G.F. (2012). The addicted brain craves new neurons: putative role for adult-born progenitors in promoting recovery. Trends Neurosci *35*, 250–260.

Mann, K., Gunther, A., Stetter, F., and Ackermann, K. (1999). Rapid recovery from cognitive deficits in abstinent alcoholics: a controlled test-retest study. Alcohol Alcohol *34*, 567–574.

Manzo-Avalos, S., and Saavedra-Molina, A. (2010). Cellular and Mitochondrial Effects of Alcohol Consumption. Int J Environ Res Public Health *7*, 4281–4304.

Marin-Burgin, A., and Schinder, A.F. (2012). Requirement of adult-born neurons for hippocampus-dependent learning. Behavioural Brain Research *227*, 391–399.

Marshall, S.A., McClain, J.A., Kelso, M.L., Hopkins, D.M., Pauly, J.R., and Nixon, K. (2013). Microglial activation is not equivalent to neuroinflammation in alcohol-induced neurodegeneration: The importance of microglia phenotype. Neurobiol. Dis. *54*, 239–251.

Martinez-Canabal, A., Akers, K.G., Josselyn, S.A., and Frankland, P.W. (2013). Age-dependent effects of hippocampal neurogenesis suppression on spatial learning. Hippocampus *23*, 66–74.

Matsumoto, I., Leah, J., Shanley, B., and Wilce, P. (1993). Immediate early gene expression in the rat brain during ethanol withdrawal. Mol Cell Neurosci *4*, 485–491.

Maynard, M.E., and Leasure, J.L. (2013). Exercise enhances hippocampal recovery following binge ethanol exposure. PLoS ONE *8*, e76644.

McClain, J.A., Hayes, D.M., Morris, S.A., and Nixon, K. (2011). Adolescent binge alcohol exposure alters hippocampal progenitor cell proliferation in rats: effects on cell cycle kinetics. J. Comp. Neurol. *519*, 2697–2710.

McClain, J.A., Morris, S.A., Marshall, S.A., and Nixon, K. (2014). Ectopic hippocampal neurogenesis in adolescent male rats following alcohol dependence. Addict Biol *19*, 687–699.

McKim (2007). Drugs and behavior: an introduction to behavioral pharmacology (Pearson Prentice Hall).

McMahon, R.C., Davidson, R.S., Gersh, D., and Flynn, P. (1991). A comparison of continuous and episodic drinkers using the MCMI, MMPI, and ALCEVAL-R. J Clin Psychol *47*, 148–159.

Mechtcheriakov, S., Brenneis, C., Egger, K., Koppelstaetter, F., Schocke, M., and Marksteiner, J. (2007). A widespread distinct pattern of cerebral atrophy in patients with alcohol addiction revealed by voxel-based morphometry. Journal of Neurology, Neurosurgery, and Psychiatry *78*, 610–614.

Ming, G.-L., and Song, H. (2011). Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron *70*, 687–702.

Miyata, T., Maeda, T., and Lee, J.E. (1999). NeuroD is required for differentiation of the granule cells in the cerebellum and hippocampus. Genes Dev. *13*, 1647–1652.

Mokdad, A.H., Marks, J.S., Stroup, D.F., and Gerberding, J.L. (2004). Actual causes of death in the United States, 2000. JAMA *291*, 1238–1245.

Morris, S.A., Kelso, M.L., Liput, D.J., Marshall, S.A., and Nixon, K. (2010a). Similar withdrawal severity in adolescents and adults in a rat model of alcohol dependence. Alcohol *44*, 89–98.

Morris, S.A., Eaves, D.W., Smith, A.R., and Nixon, K. (2010b). Alcohol inhibition of neurogenesis: a mechanism of hippocampal neurodegeneration in an adolescent alcohol abuse model. Hippocampus *20*, 596–607.

Mullen, R.J., Buck, C.R., and Smith, A.M. (1992). NeuN, a neuronal specific nuclear protein in vertebrates. Development *116*, 201–211.

Nacher, J., Rosell, D.R., Alonso-Llosa, G., and McEwen, B.S. (2001). NMDA receptor antagonist treatment induces a long-lasting increase in the number of proliferating cells, PSA-NCAM-immunoreactive granule neurons and radial glia in the adult rat dentate gyrus. Eur. J. Neurosci. *13*, 512–520.

Nacher, J., Varea, E., Miguel Blasco-Ibanez, J., Gomez-Climent, M.A., Castillo-Gomez, E., Crespo, C., Martinez-Guijarro, F.J., and McEwen, B.S. (2007). N-methyl-d-aspartate receptor expression during adult neurogenesis in the rat dentate gyrus. Neuroscience *144*, 855–864.

Nakashiba, T., Cushman, J.D., Pelkey, K.A., Renaudineau, S., Buhl, D.L., McHugh, T.J., Rodriguez Barrera, V., Chittajallu, R., Iwamoto, K.S., McBain, C.J., et al. (2012). Young dentate granule cells mediate pattern separation, whereas old granule cells facilitate pattern completion. Cell *149*, 188–201.

Naya, F.J., Huang, H.P., Qiu, Y., Mutoh, H., DeMayo, F.J., Leiter, A.B., and Tsai, M.J. (1997). Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. Genes Dev. *11*, 2323–2334.

Nicola, Z., Fabel, K., and Kempermann, G. (2015). Development of the adult neurogenic niche in the hippocampus of mice. Front Neuroanat *9*, 53.

Niibori, Y., Yu, T.-S., Epp, J.R., Akers, K.G., Josselyn, S.A., and Frankland, P.W. (2012). Suppression of adult neurogenesis impairs population coding of similar contexts in hippocampal CA3 region. Nat Commun *3*, 1253.

Nixon, K. (2006). Alcohol and adult neurogenesis: roles in neurodegeneration and recovery in chronic alcoholism. Hippocampus *16*, 287–295.

Nixon, K., and Crews, F.T. (2002). Binge ethanol exposure decreases neurogenesis in adult rat hippocampus. J. Neurochem. *83*, 1087–1093.

Nixon, K., and Crews, F.T. (2004). Temporally specific burst in cell proliferation increases hippocampal neurogenesis in protracted abstinence from alcohol. J. Neurosci. *24*, 9714–9722.

Nixon, K., Kim, D.H., Potts, E.N., He, J., and Crews, F.T. (2008). Distinct cell proliferation events during abstinence after alcohol dependence: microglia proliferation precedes neurogenesis. Neurobiol. Dis. *31*, 218–229.

Nixon, K., Morris, S.A., Liput, D.J., and Kelso, M.L. (2010). Roles of neural stem cells and adult neurogenesis in adolescent alcohol use disorders. Alcohol *44*, 39–56.

Nixon, K., Pauly, J.R., and Hayes, D.M. (2011). Alcohol, nicotine, and adult neurogenesis. In Drug Addiction and Adult Neurogenesis, M.F. Olive, ed. (Kerala, India: Research Signpost), pp. 95–119.

Noel, X., Brevers, D., and Bechara, A. (2013). A neurocognitive approach to understanding the neurobiology of addiction. Current Opinion in Neurobiology.

Nokia, M.S., Anderson, M.L., and Shors, T.J. (2012). Chemotherapy disrupts learning, neurogenesis and theta activity in the adult brain. Eur. J. Neurosci. *36*, 3521–3530.

Noori, H.R., and Fornal, C.A. (2011). The appropriateness of unbiased optical fractionators to assess cell proliferation in the adult hippocampus. Frontiers in Neuroscience *5*, 140.

Nunez, J. (2008). Morris Water Maze Experiment. J Vis Exp.

Obernier, J.A., Bouldin, T.W., and Crews, F.T. (2002a). Binge ethanol exposure in adult rats causes necrotic cell death. Alcohol Clin Exp Res *26*, 547–557.

Obernier, J.A., White, A.M., Swartzwelder, H.S., and Crews, F.T. (2002b). Cognitive deficits and CNS damage after a 4-day binge ethanol exposure in rats. Pharmacol. Biochem. Behav. *72*, 521–532.

O'Daly, O.G., Trick, L., Scaife, J., Marshall, J., Ball, D., Phillips, M.L., Williams, S.S., Stephens, D.N., and Duka, T. (2012). Withdrawal-Associated Increases and Decreases in Functional Neural Connectivity Associated with Altered Emotional Regulation in Alcoholism. Neuropsychopharmacology *37*, 2267–2276.

Omar, A.I., and Mason, W.P. (2009). Temozolomide: The evidence for its therapeutic efficacy in malignant astrocytomas. Core Evid *4*, 93–111.

O'Neill, J., Cardenas, V.A., and Meyerhoff, D.J. (2001). Effects of abstinence on the brain: quantitative magnetic resonance imaging and magnetic resonance spectroscopic imaging in chronic alcohol abuse. Alcohol. Clin. Exp. Res. *25*, 1673–1682.

Oscar-Berman, M., and Marinković, K. (2007). Alcohol: Effects on Neurobehavioral Functions and the Brain. Neuropsychol Rev *17*, 239–257.

Overstreet Wadiche, L., Bromberg, D.A., Bensen, A.L., and Westbrook, G.L. (2005). GABAergic signaling to newborn neurons in dentate gyrus. J Neurophysiol *94*, 4528–4532.

Ozsoy, S., Durak, A.C., and Esel, E. (2013). Hippocampal volumes and cognitive functions in adult alcoholic patients with adolescent-onset. Alcohol *47*, 9–14.

Palmer, T.D., Takahashi, J., and Gage, F.H. (1997). The adult rat hippocampus contains primordial neural stem cells. Mol Cell Neurosci *8*, 389–404.

Palmer, T.D., Willhoite, A.R., and Gage, F.H. (2000). Vascular niche for adult hippocampal neurogenesis. Journal of Comparative Neurology *425*, 479–494.

Parada, M., Corral, M., Caamaño-Isorna, F., Mota, N., Crego, A., Holguín, S.R., and Cadaveira, F. (2011). Binge drinking and declarative memory in university students. Alcohol. Clin. Exp. Res. *35*, 1475–1484.

Parent, J.M., Yu, T.W., Leibowitz, R.T., Geschwind, D.H., Sloviter, R.S., and Lowenstein, D.H. (1997). Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. J. Neurosci. *17*, 3727–3738.

Park, H.R., and Lee, J. (2011). Neurogenic contributions made by dietary regulation to hippocampal neurogenesis. Ann. N. Y. Acad. Sci. *1229*, 23–28.

Parsons, O.A. (1993). Impaired neuropsychological cognitive functioning in sober alcoholics. In Alcohol-Induced Brain Damage (NIAAA Research Monograph No. 22), W.A. Hunt, and S.J. Nixon, eds. (Rockville, MD: National Institutes of Health), pp. 173–194.

Pataskar, A., Jung, J., Smialowski, P., Noack, F., Calegari, F., Straub, T., and Tiwari, V.K. (2016). NeuroD1 reprograms chromatin and transcription factor landscapes to induce the neuronal program. The EMBO Journal *35*, 24–45.

Pava, M.J., and Woodward, J.J. (2012). A review of the interactions between alcohol and the endocannabinoid system: implications for alcohol dependence and future directions for research. Alcohol *46*, 185–204.

Pawlak, R., Skrzypiec, A., Sulkowski, S., and Buczko, W. (2002). Ethanol-induced neurotoxicity is counterbalanced by increased cell proliferation in mouse dentate gyrus. Neurosci Lett *327*, 83–86.

Paxinos, G., and Watson, C. (2009). The Rat Brain in Stereotaxic Coordinates (Academic).

Penland, S., Hoplight, B., Obernier, J., and Crews, F.T. (2001). Effects of nicotine on ethanol dependence and brain damage. Alcohol *24*, 45–54.

Petrus, D.S., Fabel, K., Kronenberg, G., Winter, C., Steiner, B., and Kempermann, G. (2009). NMDA and benzodiazepine receptors have synergistic and antagonistic effects on precursor cells in adult hippocampal neurogenesis. Eur J Neurosci *29*, 244–252.

Pfefferbaum, A., Lim, K.O., Zipursky, R.B., Mathalon, D.H., Rosenbloom, M.J., Lane, B., Ha, C.N., and Sullivan, E.V. (1992). Brain gray and white matter volume loss accelerates with aging in chronic alcoholics: a quantitative MRI study. Alcohol. Clin. Exp. Res. *16*, 1078–1089.

Pfefferbaum, A., Sullivan, E.V., Mathalon, D.H., Shear, P.K., Rosenbloom, M.J., and Lim, K.O. (1995). Longitudinal changes in magnetic resonance imaging brain volumes in abstinent and relapsed alcoholics. Alcohol. Clin. Exp. Res. *19*, 1177–1191.

Pfefferbaum, A., Sullivan, E.V., Mathalon, D.H., and Lim, K.O. (1997). Frontal lobe volume loss observed with magnetic resonance imaging in older chronic alcoholics. Alcohol Clin Exp Res *21*, 521–529.

Phillips, R.G., and LeDoux, J.E. (1992). Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behav. Neurosci. *106*, 274–285.

van Praag, H., Christie, B.R., Sejnowski, T.J., and Gage, F.H. (1999). Running enhances neurogenesis, learning, and long-term potentiation in mice. Proc Natl Acad Sci U S A *96*, 13427–13431.

van Praag, H., Schinder, A.F., Christie, B.R., Toni, N., Palmer, T.D., and Gage, F.H. (2002). Functional neurogenesis in the adult hippocampus. Nature *415*, 1030–1034.

Prendergast, M.A., Harris, B.R., Blanchard, J.A., 2nd, Mayer, S., Gibson, D.A., and Littleton, J.M. (2000). In vitro effects of ethanol withdrawal and spermidine on viability of hippocampus from male and female rat. Alcohol Clin Exp Res *24*, 1855–1861.

Prendergast, M.A., Harris, B.R., Mullholland, P.J., Blanchard, J.A., 2nd, Gibson, D.A., Holley, R.C., and Littleton, J.M. (2004). Hippocampal CA1 region neurodegeneration produced by ethanol withdrawal requires activation of intrinsic polysynaptic hippocampal pathways and function of N-methyl-D-aspartate receptors. Neuroscience *124*, 869–877.

Rice, A.C., Bullock, M.R., and Shelton, K.L. (2004). Chronic ethanol consumption transiently reduces adult neural progenitor cell proliferation. Brain Res *1011*, 94–98.

Richardson, H.N., Chan, S.H., Crawford, E.F., Lee, Y.K., Funk, C.K., Koob, G.F., and Mandyam, C.D. (2009). Permanent impairment of birth and survival of

cortical and hippocampal proliferating cells following excessive drinking during alcohol dependence. Neurobiol Dis *36*, 1–10.

Robin, R.W., Long, J.C., Rasmussen, J.K., Albaugh, B., and Goldman, D. (1998). Relationship of binge drinking to alcohol dependence, other psychiatric disorders, and behavioral problems in an American Indian tribe. Alcohol. Clin. Exp. Res. *22*, 518–523.

Rosenbloom, M.J., Pfefferbaum, A., and Sullivan, E.V. (2004). Recovery of short-term memory and psychomotor speed but not postural stability with long-term sobriety in alcoholic women. Neuropsychology *18*, 589–597.

Saxe, M.D., Battaglia, F., Wang, J.W., Malleret, G., David, D.J., Monckton, J.E., Garcia, A.D., Sofroniew, M.V., Kandel, E.R., Santarelli, L., et al. (2006). Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. Proceedings of the National Academy of Sciences of the United States of America *103*, 17501–17506.

Schmidt-Hieber, C., Jonas, P., and Bischofberger, J. (2004). Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus. Nature *429*, 184–187.

Shear, P.K., Jernigan, T.L., and Butters, N. (1994). Volumetric magnetic resonance imaging quantification of longitudinal brain changes in abstinent alcoholics. Alcohol Clin Exp Res *18*, 172–176.

Shors, T.J., Townsend, D.A., Zhao, M., Kozorovitskiy, Y., and Gould, E. (2002). Neurogenesis may relate to some but not all types of hippocampal-dependent learning. Hippocampus *12*, 578–584.

Singer, B.H., Gamelli, A.E., Fuller, C.L., Temme, S.J., Parent, J.M., and Murphy, G.G. (2011). Compensatory network changes in the dentate gyrus restore long-term potentiation following ablation of neurogenesis in young-adult mice. Proc. Natl. Acad. Sci. U.S.A. *108*, 5437–5442.

Smith, P.D., McLean, K.J., Murphy, M.A., Turnley, A.M., and Cook, M.J. (2005). Seizures, not hippocampal neuronal death, provoke neurogenesis in a mouse rapid electrical amygdala kindling model of seizures. Neuroscience *136*, 405–415.

Snyder, J.S., and Cameron, H.A. (2012). Could adult hippocampal neurogenesis be relevant for human behavior? Behav Brain Res *227*, 384–390.

Snyder, J.S., Choe, J.S., Clifford, M.A., Jeurling, S.I., Hurley, P., Brown, A., Kamhi, J.F., and Cameron, H.A. (2009). Adult-born hippocampal neurons are more numerous, faster-maturing and more involved in behavior in rats than in mice. J Neurosci *29*, 14484–14495.

Snyder, J.S., Clifford, M.A., Jeurling, S.I., and Cameron, H.A. (2012). Complementary activation of hippocampal-cortical subregions and immature neurons following chronic training in single and multiple context versions of the water maze. Behav. Brain Res. *227*, 330–339.

Snyder, J.S., Grigereit, L., Russo, A., Seib, D.R., Brewer, M., Pickel, J., and Cameron, H.A. (2016). A Transgenic Rat for Specifically Inhibiting Adult Neurogenesis. eNeuro *3*.

Stahre, M., Roeber, J., Kanny, D., Brewer, R.D., and Zhang, X. (2014). Contribution of excessive alcohol consumption to deaths and years of potential life lost in the United States. Prev Chronic Dis *11*, E109.

Staples, M.C., Kim, A., and Mandyam, C.D. (2015). Dendritic remodeling of hippocampal neurons is associated with altered NMDA receptor expression in alcohol dependent rats. Mol. Cell. Neurosci. *65*, 153–162.

Stavro, K., Pelletier, J., and Potvin, S. (2013). Widespread and sustained cognitive deficits in alcoholism: a meta-analysis. Addict Biol *18*, 203–213.

Steiner, B., Klempin, F., Wang, L., Kott, M., Kettenmann, H., and Kempermann, G. (2006). Type-2 cells as link between glial and neuronal lineage in adult hippocampal neurogenesis. Glia *54*, 805–814.

Stephens, D.N., and Duka, T. (2008). Review. Cognitive and emotional consequences of binge drinking: role of amygdala and prefrontal cortex. Philosophical Transactions of the Royal Society of London *363*, 3169–3179.

Stone, S.S., Teixeira, C.M., Devito, L.M., Zaslavsky, K., Josselyn, S.A., Lozano, A.M., and Frankland, P.W. (2011a). Stimulation of entorhinal cortex promotes adult neurogenesis and facilitates spatial memory. The Journal of Neuroscience : The Official Journal of the Society for Neuroscience *31*, 13469–13484.

Stone, S.S., Teixeira, C.M., Zaslavsky, K., Wheeler, A.L., Martinez-Canabal, A., Wang, A.H., Sakaguchi, M., Lozano, A.M., and Frankland, P.W. (2011b). Functional convergence of developmentally and adult-generated granule cells in dentate gyrus circuits supporting hippocampus-dependent memory. Hippocampus *21*, 1348–1362.

Sullivan, E.V., and Pfefferbaum, A. (2005). Neurocircuitry in alcoholism: a substrate of disruption and repair. Psychopharmacology *180*, 583–594.

Sullivan, E.V., Marsh, L., Mathalon, D.H., Lim, K.O., and Pfefferbaum, A. (1995). Anterior hippocampal volume deficits in nonamnesic, aging chronic alcoholics. Alcohol Clin Exp Res *19*, 110–122.

Sullivan, E.V., Rosenbloom, M.J., and Pfefferbaum, A. (2000). Pattern of motor and cognitive deficits in detoxified alcoholic men. Alcohol. Clin. Exp. Res. *24*, 611–621.

Sun, G.J., Sailor, K.A., Mahmood, Q.A., Chavali, N., Christian, K.M., Song, H., and Ming, G. (2013). Seamless reconstruction of intact adult-born neurons by serial end-block imaging reveals complex axonal guidance and development in the adult hippocampus. J. Neurosci. *33*, 11400–11411.

Taffe, M.A., Kotzebue, R.W., Crean, R.D., Crawford, E.F., Edwards, S., and Mandyam, C.D. (2010). Long-lasting reduction in hippocampal neurogenesis by alcohol consumption in adolescent nonhuman primates. Proc Natl Acad Sci U S A *107*, 11104–11109.

Tesone-Coelho, C., Varela, P., Escosteguy-Neto, J.C., Cavarsan, C.F., Mello, L.E., and Santos-Junior, J.G. (2013). Effects of ethanol on hippocampal neurogenesis depend on the conditioned appetitive response. Addict Biol *18*, 774–785.

Tozuka, Y., Fukuda, S., Namba, T., Seki, T., and Hisatsune, T. (2005). GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. Neuron *47*, 803–815.

Treves, A., Tashiro, A., Witter, M.P., and Moser, E.I. (2008). What is the mammalian dentate gyrus good for? Neuroscience *154*, 1155–1172.

Vangipuram, S.D., and Lyman, W.D. (2010). Ethanol alters cell fate of fetal human brain-derived stem and progenitor cells. Alcoholism, Clinical and Experimental Research *34*, 1574–1583.

Vengeliene, V., Bilbao, A., Molander, A., and Spanagel, R. (2008). Neuropharmacology of alcohol addiction. Br J Pharmacol *154*, 299–315.

Verbois, S.L., Hopkins, D.M., Scheff, S.W., and Pauly, J.R. (2003). Chronic intermittent nicotine administration attenuates traumatic brain injury-induced cognitive dysfunction. Neuroscience *119*, 1199–1208.

Volkow, N.D., Mullani, N., Gould, L., Adler, S.S., Guynn, R.W., Overall, J.E., and Dewey, S. (1988). Effects of acute alcohol intoxication on cerebral blood flow measured with PET. Psychiatry Res *24*, 201–209.

Vorel, S.R., Liu, X., Hayes, R.J., Spector, J.A., and Gardner, E.L. (2001). Relapse to cocaine-seeking after hippocampal theta burst stimulation. Science *292*, 1175–1178.

Walker, D.W., Barnes, D.E., Zornetzer, S.F., Hunter, B.E., and Kubanis, P. (1980). Neuronal loss in hippocampus induced by prolonged ethanol consumption in rats. Science *209*, 711–713.

Wang, S., Scott, B.W., and Wojtowicz, J.M. (2000). Heterogenous properties of dentate granule neurons in the adult rat. Journal of Neurobiology *42*, 248–257.

Weig, B.C., Richardson, J.R., Lowndes, H.E., and Reuhl, K.R. (2016). Trimethyltin intoxication induces the migration of ventricular/subventricular zone cells to the injured murine hippocampus. Neurotoxicology *54*, 72–80.

West, M.J. (1991). Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator. Anatomical Record *231*, 482–497.

White, A.M. (2003). What happened? Alcohol, memory blackouts, and the brain. Alcohol Res Health *27*, 186–196.

Wilhelm, J., Frieling, H., Hillemacher, T., Degner, D., Kornhuber, J., and Bleich, S. (2008). Hippocampal volume loss in patients with alcoholism is influenced by the consumed type of alcoholic beverage. Alcohol Alcohol. *43*, 296–299.

Wiskott, L., Rasch, M.J., and Kempermann, G. (2006). A functional hypothesis for adult hippocampal neurogenesis: avoidance of catastrophic interference in the dentate gyrus. Hippocampus *16*, 329–343.

Zahr, N.M., Mayer, D., Rohlfing, T., Hasak, M.P., Hsu, O., Vinco, S., Orduna, J., Luong, R., Sullivan, E.V., and Pfefferbaum, A. (2010). Brain injury and recovery following binge ethanol: evidence from in vivo magnetic resonance spectroscopy. Biol. Psychiatry *67*, 846–854.

Zahr, N.M., Rohlfing, T., Mayer, D., Luong, R., Sullivan, E.V., and Pfefferbaum, A. (2015). Transient CNS responses to repeated binge ethanol treatment. Addict Biol.

Zeigler, D.W., Wang, C.C., Yoast, R.A., Dickinson, B.D., McCaffree, M.A., Robinowitz, C.B., and Sterling, M.L. (2005). The neurocognitive effects of alcohol on adolescents and college students. Prev Med *40*, 23–32.

Zhao, C., Teng, E.M., Summers, R.G., Jr., Ming, G.L., and Gage, F.H. (2006). Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. J Neurosci *26*, 3–11.

Zhao, C., Deng, W., and Gage, F.H. (2008). Mechanisms and Functional Implications of Adult Neurogenesis. Cell *132*, 645–660.

Zhou, F.C., Balaraman, Y., Teng, M., Liu, Y., Singh, R.P., and Nephew, K.P. (2011). Alcohol alters DNA methylation patterns and inhibits neural stem cell differentiation. Alcoholism, Clinical and Experimental Research *35*, 735–746.

Zou, J.Y., and Crews, F.T. (2005). TNF alpha potentiates glutamate neurotoxicity by inhibiting glutamate uptake in organotypic brain slice cultures: neuroprotection by NF kappa B inhibition. Brain Res *1034*, 11–24.

VITA

CHELSEA RHEA GEIL NICKELL

EDUCATION

Degree	Doctor of Philosophy in Pharmaceutical Sciences
2010-Current	College of Pharmacy, University of Kentucky, Lexington, Kentucky Mentor: Kimberly Nixon, Ph.D. Dissertation: Probing the functional relevance of hippocampal reactive neurogenesis in a model of an alcohol use disorder
Degree Awarded	Bachelors of Science in Biology, College of Natural Sciences
May 2007	University of Texas, Austin, Texas

PROFESSIONAL EXPERIENCE

2010-Present	Graduate Student, Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky Mentor: Kimberly Nixon, Ph.D.
2007-2010	Research Assistant, Waggoner Center for Alcohol and Addiction Research, College of Natural Sciences, University of Texas, Austin, Texas Mentor: Dr. Yuri Blednov, Ph.D.

RESEARCH SUPPORT

2014-2016	F31 - Ruth L. Kirschstein National Research Service Award (NRSA) Individual Predoctoral Grant, F31AA023459, Awarded by the National Institute on Alcohol Abuse and Alcoholism
2013-2014	T32 - Ruth L. Kirschstein National Research Service Award (NRSA) Institutional Training Grant, T32DA016176, Awarded by the National Institute on Drug Abuse

2006-2007 University of Texas Undergraduate Research Fellowship, Awarded by the University of Texas at Austin

HONORS & AWARDS

2016 Bluegrass Society for Neuroscience Poster Presentation Award, Graduate student category

2011-2016 Student merit award, Research Society on Alcoholism

PRESENTATIONS

2014 Investigation of the functional contribution of alcohol-induced reactive proliferation. National Institute on Drug Abuse T32 Research Retreat, University of Kentucky

2013 Reactive neurogenesis following excessive alcohol consumption. Pharmaceutical Sciences Departmental Seminar, University of Kentucky

2012 Temporal activation of neurogenesis following binge ethanol exposure in adolescent rats. Pharmaceutical Sciences, Drug Discovery Seminar, University of Kentucky

PUBLICATIONS

Marshall SA, **Geil CR**, Nixon K. (2016). Prior binge ethanol exposure potentiates the microglial response in an alcohol-induced neurodegeneration model. Brain Sciences 26:6(2).

Wagner JL, Barton EA, **Geil CR**, Leasure JL, Nixon K (2015). The role of neural stem cells in alcohol use disorders. In A. K. Shetty (Ed.) Neural Stem Cells in Health and Disease.

Geil CR, Hayes DM, McClain JA, Liput DJ, Marshall SA, Chen KY, Nixon K (2014). Alcohol and adult hippocampal neurogenesis: Promiscuous drug, wanton effects. Progress in Neuro-Psychopharmacology and Biological Psychiatry. 54:103-13.

Blednov YA, Ponomarev I, **Geil C**, Bergeson S, Koob GF, Harris RA (2011). Neuroimmune regulation of alcohol consumption: behavioral validation of genes obtained from genomic studies. Addiction Biology. 17(1):108-20.

Blednov YA, Benavidez JM, **Geil C**, Perra S, Morikawa H, Harris RA (2011). Activation of inflammatory signaling by lipopolysaccharide produces a prolonged increase of voluntary alcohol intake in mice. Brain Behavior and Immunity. 25 Suppl 1:S92-S105.

Blednov YA, Borghese CM, McCracken ML, Benavidez JM, **Geil CR**, Osterndorff-Kahanek E, Werner DF, Iyer S, Swihard A, Harrison NL, Homanics GE, Harris RA (2011). Loss of ethanol conditioned taste aversion and motor stimulation in knockin mice with ethanol-insensitive α2-containing GABA(A) receptors. Journal of Pharmacology and Experimental Therapeutics. 336(1):145-54.

MANUSCRIPTS IN PREPARATION

Hayes DM, **Geil CR**, Chen KY, McClain JA, Nixon K. Alcohol-induced reactive hippocampal neurogenesis is due to activation of neural stem cells. In preparation for submission to Biological Psychiatry.

Geil CR, Nixon K. Temozolomide inhibits alcohol-induced reactive neurogenesis in the granule cell layer of the dentate gyrus. In preparation for submission to Alcoholism: Clinical and Experimental Research.

Geil CR, McClain JA, Hayes DM, Nixon K. Reactive neurogenesis is due to recruitment of additional progenitors or changes in the cell cycle. In preparation for submission to Alcohol.

ABSTRACTS

Geil CR and Nixon K. Temozolomide decreases alcohol-induced reactive neurogenesis in the dentate gyrus. 39th Research Society on Alcoholism Scientific Meeting, New Orleans, LA, June 2016.

Geil CR and Nixon K. Temozolomide decreases reactive cell proliferation in the subgranular zone of the hippocampal dentate gyrus following 4-day binge ethanol exposure. 38th Annual Scientific Meeting of the Research Society on Alcoholism, San Antonio, TX, June 2015; 31st Annual Bluegrass Society for Neuroscience Conference, Lexington, KY, April, 2016.

Geil CR and Nixon K. Adolescent rats show increased Sox2+/BrdU+ co-labeled cells indicating an increase in neural stem/progenitor cells 7 days after binge ethanol exposure. 37th Annual Scientific Meeting of the Research Society on Alcoholism, Bellevue, WA, June 2014.

Heath MM, **Geil CR**, Nixon, K. Binge ethanol exposure increases hippocampal cell proliferation beginning 5 days into abstinence. 37th Annual Scientific

Meeting of the Research Society on Alcoholism, Bellevue, WA, June 2014.

Geil CR, Heath M, Nixon K. Binge alcohol administration does not inhibit alcohol-induced reactive proliferation. 2013 Society for Neuroscience Scientific Meeting, San Diego, CA, November 2013; 29th Annual Bluegrass Society for Neuroscience Conference, Lexington, KY, April, 2014.

Geil CR, Hayes DM, Nixon K. Increased NeuroD in adult rat hippocampal dentate gyrus following binge ethanol exposure confirms increased neurogenesis. 36th Annual Scientific Meeting of the Research Society on Alcoholism, Orlando, FL, June 2013; 2nd Annual Symposium on Drug Discovery and Development, Lexington, KY, October 2014.

Geil CR, McClain JA, Deeny MA, Nixon K. Increase in Doublecortin and NeuroD indicates an increase in progenitor cells and neurogenesis after binge ethanol exposure in adolescent rats. 35th Annual Scientific Meeting of the Research Society on Alcoholism, San Francisco, CA, June 2012; 28th Annual Bluegrass Society for Neuroscience Conference, Lexington, KY, April, 2013; 1st Annual Symposium on Drug Discovery and Development, Lexington, KY, October 2013.

Geil CR, Margoleskee RF, Harris RA, Blednov YA. Voluntary alcohol consumption is blocked in mice lacking TRPM5, the transducer of sweet taste. 33rd Annual Scientific Meeting of the Research Society on Alcoholism, San Antonio, TX, June 2010.

TEACHING EXPERIENCE

2010-2011	University of Kentucky, College of Pharmacy - teaching assistant for neuropharmacology and exam proctor coordinator

PROFESSIONAL SOCIETIES

2011-Present	Research Society on Alcoholism
2010-Present	Bluegrass Society for Neuroscience
2010-2011	American Association of Pharmaceutical Scientists
2013-2016	Society for Neuroscience