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THE EFFECT OF NICOTINE CO-ADMINISTRATION ON ALCOHOL-INDUCED REACTIVE HIPPOCAMPAL CELL PROLIFERATION DURING ABSTINENCE IN AN ADOLESCENT MODEL OF AN ALCOHOL USE DISORDER

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THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Pharmacy
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By
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Lexington, KY

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2016

ABSTRACT OF THESIS

THE EFFECT OF NICOTINE CO-ADMINISTRATION ON ALCOHOL-INDUCED REACTIVE HIPPOCAMPAL CELL PROLIFERATION DURING ABSTINENCE IN AN ADOLESCENT MODEL OF AN ALCOHOL USE DISORDER

A significant consequence of alcohol use disorders (AUDs) is hippocampal neurodegeneration. The hippocampus is responsible for learning and memory, and neurodegeneration in this brain region has been shown to result in cognitive deficits. Interestingly, some alcoholics demonstrate improvements in hippocampus-dependent functions, potentially due the phenomenon termed adult neurogenesis. Adult neurogenesis, the process by which neural stem cells (NSCs) proliferate, differentiate into neurons, migrate into the granule cell layer, and survive, occurs in two brain regions; however, this study examines only neurogenesis occurring in the subgranular zone of the hippocampal dentate gyrus. Four-day binge ethanol exposure in an animal model causes a decrease in neurogenesis during intoxication; however, there is a reactive increase in cell proliferation on day seven of abstinence. The purpose of this study was to determine the timing of increased cell proliferation. Furthermore, most alcoholics also smoke tobacco, and nicotine, the addictive component of tobacco, has also been shown to affect hippocampal neurogenesis. As many people initiate alcohol and tobacco use during adolescence, the second experiment herein examined the effect of nicotine co-administration on alcohol-induced reactive hippocampal cell proliferation.

KEYWORDS: alcohol use disorder, nicotine, adult neurogenesis, adolescent alcohol use, hippocampal cell proliferation

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Chapter One: Introduction

Alcohol Use Disorder (AUD) Definitions and Epidemiology

The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) outlines the requirements for the diagnosis of an alcohol use disorder (AUD). In order to be diagnosed with an AUD, a person must meet two out of eleven criteria within the past 12 month period. These criteria include: (1) tolerance to the effects of alcohol, (2) withdrawal during abstinence, (3) persistent use despite knowledge of recurrent physical/psychological or (4) interpersonal problems resulting from alcohol use, (5) repeated use in physically dangerous situations, (6) sacrifice of social, occupational, or recreational activities because of alcohol, (7) use even when alcohol causes repeated failures at home, school, or work, (8) craving, (9) persistent desire for or inability to reduce the amount of alcohol consumed, (10) significant amount of time spent obtaining, using, or recovering from alcohol, (11) a greater amount of alcohol consumed or consumed for longer than intended (American Psychiatric Association, 2013). The severity of the AUD is then determined according to the number of criteria met: two to three symptoms indicate a mild AUD, four to five symptoms indicate a moderate AUD, and 6 or more symptoms indicate a severe AUD. Epidemiologic data show that according to DSM-5 criteria, the 12-month prevalence of an AUD from 2012-2013 was 13.9% and the lifetime prevalence of an AUD was 29.1% (Grant et al., 2015). This is a 5% increase from the 12-month prevalence in 2001-2002 of 8.5% that was based on DSM-IV criteria (Grant et al., 2004) and likely reflects changes to inclusion criteria for an AUD diagnosis in the DSM-5. Not only does excessive drinking affect the individuals with AUDs, but it also costs the U.S. economy approximately $223.5 billion each year (Bouchery et al., 2011). Because of the combined harm to the individual and the harms to society, the WHO and others have ranked alcohol as the greatest harm to society of all of the drugs of abuse (Nutt et al., 2010).

Binge drinking is a risky pattern of alcohol consumption in which a person rapidly attains a high blood ethanol concentration (BEC). The National Institute on Alcohol Abuse and Alcoholism defines binge drinking as drinking that results in a BEC of at least 0.08 mg/dL (2016). This BEC may be attained when men consume four drinks (or five drinks for women) within approximately two hours. In survey results from 2014, nearly 23% of adults over the age of 25 years old reported that they had engaged in binge alcohol drinking within the past month (Johnston et al., 2015). Furthermore, binge drinking is also thought to increase the risk of brain damage (Hunt, 1993). These definitions are important to keep in mind while studying any aspect of alcohol use disorders.

Adolescent Alcohol Consumption

Adolescence is defined by the WHO as the period between the ages of 10 and 19 years old (World Health Organization). Adolescents most commonly begin consuming alcohol between the ages of 12 and 14 years (Faden, 2006). Furthermore, 25% of 10th grade students reported in a 2014 survey that they had been drunk at some point, and 10.8% of 16 year olds reported that they had engaged in binge drinking (Miech et al., 2015). Although adolescents generally consume alcohol less frequently than adults (~6 days per month for 12-20 year olds versus ~9 days per month for adults over 25 years old), they tend to consume a greater number of drinks than adults when they do drink (~5 drinks per occasion for 12-20 year old versus ~3 drinks per occasion for adults over 25 years old (Johnston et al., 2007)). One possible explanation for the fact that adolescents tend to consume more alcohol at a time than adults is that they have been shown to be less sensitive to both the sedating effects of alcohol (Little et al., 1996, Silveri and Spear, 1998, White et al., 2002) and “hangover” effects (Varlinskaya and Spear, 2004), but are more sensitive to the rewarding properties of alcohol and social

facilitation (Varlinskaya and Spear, 2010). Not only does ethanol have an acute effect on adolescent drinkers, but it may also continue to affect them during adulthood. The younger a person is when they start consuming alcohol, the higher their risk for developing an AUD at some point during their life. For example, adolescents who start drinking when they are 13 years old or younger with a family history of alcoholism have a 57% chance of developing an AUD (Grant, 1998). Even these adolescents with no family history of alcoholism have a 26% chance of developing an AUD (Grant, 1998). This high risk of alcohol dependence is in stark contrast to the risk for those who do not initiate alcohol consumption until adulthood; adults who are at least 21 years old with a family history of an AUD have a 16% chance and those without a family history have only a 6.5% chance of developing an AUD (Grant, 1998). This epidemiological data demonstrates the importance of studying adolescent alcohol use.

Ethanol Pharmacology

Ethanol, commonly referred to as alcohol and used interchangeably herein, is a polar, water and lipid-soluble compound with the molecular formula CH_3CH_2OH. Once absorbed through the gastrointestinal tract, it is metabolized in the liver by alcohol dehydrogenase (ADH) to acetaldehyde and then by acetaldehyde dehydrogenase (ALDH) to acetic acid and water. A smaller portion of ethanol is also oxidatively metabolized via the cytochrome P450 liver enzyme (CYP) 2E1 to reactive oxygen species.

Alcohol is a promiscuous drug that has many different sites and mechanisms of action. It enhances the activity of 5-HT3, $GABA_A$, glycine, and nicotinic acetylcholine receptors (nAChRs) and inhibits the activity of NMDA receptors (Vengeliene et al., 2008). Furthermore, it inhibits dihydropyridine-sensitive L-type Ca^{2+} channels, opens G-protein-activated inwardly rectifying K^+ channels, and modulates dopaminergic, opioid,

peptide hormone, and endocannabinoid systems (Vengeliene et al., 2008). Alcohol's interactions with these various receptors and ion channels is dependent on the conformation of receptor and channel subunits and the concentration of alcohol present (Vengeliene et al., 2008); for example, ethanol inhibits α7 nAChRs but potentiates α4β2 nAChRs (Cardoso et al., 1999).

Nicotine Pharmacology

Tobacco is responsible for the deaths of 6 million people each year and roughly half of users eventually die from it (World Health Organization, 2015). Nicotine, which is widely considered to be the addictive component of tobacco, binds nicotinic acetylcholine receptors (nAChRs). These receptors are ligand-gated ion channels that bind endogenous acetylcholine. nAChRs have a pentameric structure made up of alpha, beta, gamma, delta, and epsilon subunits; however, the nAChRs in the central nervous system (CNS) consist of only alpha and beta subunits. The most common subtypes found within the brain are α4β2 and α7 and both of the subtypes exist in the hippocampus. When nAChRs are bound by an agonist, the channel opens and cations including sodium and calcium enter resulting in membrane depolarization. Voltage-dependent calcium channels then become activated, which increases intracellular calcium and activates cell signaling pathways. Nicotine binding to nAChRs on presynaptic neurons causes the release of various neurotransmitters into the synaptic cleft.

Agonism of the α4β2 subtype of nAChRs in the ventral tegmental area (VTA) by nicotine is thought to be the source of its addictive properties (Picciotto et al., 1998, Mansvelder and McGehee, 2002). Nicotine is a unique substrate in that when it binds to its receptor, it causes receptor upregulation. nAChRs also eventually become desensitized in the presence of nicotine and, essentially, are rendered inactive. For

example, α4β2 nAChRs become quickly desensitized in the presence of nicotine following a brief period of activation (Dani and Harris, 2005). Alpha7 nAChRs, however, have a lower affinity for nicotine and generally require higher nicotine concentrations to become desensitized by it (Fenster et al., 1997). Alpha7 receptors, which have a higher permeability to calcium than other nAChRs, are present on presynaptic glutamatergic neurons and excite DA neurons through activation of glutamatergic afferents (Pidoplichko et al., 1997, Albuquerque et al., 2009).

In general, nicotine is thought to have beneficial effects on cognitive function via its effects on the cholinergic system. Animal models have shown that both acute and chronic nicotine administration improves learning and memory as measured by performance in the Morris water maze, radial-arm maze, Lashley III maze, one-way active avoidance tasks, and object recognition tasks (reviewed in (Levin et al., 2006)). Furthermore, it has also been shown to increase attention in rats when measured by the visual signal detection test and five-choice serial reaction time test (Levin et al., 2006). Nicotine is thought to enhance cognitive function both through direct action on nAChRs and by causing the release of other neurotransmitters including glutamate, serotonin, dopamine, and GABA (Levin et al., 2006). Intriguingly, nicotine has been shown to reduce hippocampal neurogenesis, which will be reviewed in later sections of this document.

Alcohol and Nicotine Interactions

As 50-92% of people with an AUD also smoke, it is highly important to study these two drugs together in order to model the actual human condition (Miller and Gold, 1998, Falk et al., 2006, De Leon et al., 2007, Van Skike et al., 2016). Alcoholics are more likely to smoke than nonalcoholics and have more difficulty quitting smoking (DiFranza and Guerrera, 1990). Furthermore, nicotine and ethanol have been shown to

interact with each other in humans and animal models. In human subjects, ethanol potentiated the rewarding effects of nicotine, according to rates of satisfaction resulting from smoking a cigarette after consuming a dose of alcohol that resulted in a BEC of 0.03 g/dL (Rose et al., 2002). Nicotine has also been shown to promote compulsive alcohol drinking in nicotine-dependent rats (Leao et al., 2015), and humans given cigarettes with low nicotine content consumed less alcohol than those who smoked regular cigarettes (Dermody et al., 2016). Nicotine may also alleviate some of alcohol's withdrawal symptoms by decreasing the upregulation of $GABA_A$ that occurs during abstinence from alcohol (Staley et al., 2005). Moreover, as previously discussed, alcohol interacts directly with nAChRs. In fact, mecamylamine, a nicotinic receptor antagonist, has been shown to reduce ethanol-induced DA release into the nucleus accumbens and operant responding for alcohol by rats (Blomqvist et al., 1993, Blomqvist et al., 1996, Kuzmin et al., 2009). Furthermore, varenicline, a partial agonist of α4β2 nAChRs, has also been shown to decrease alcohol seeking and intake in rats (Steensland et al., 2007) and reduce alcohol intake in heavy-drinking people who smoke (McKee et al., 2009).

The Effect of Alcohol on the Hippocampus

Alcohol affects the structure and function of many regions of the brain, however one consequence of an AUD – and of particular importance to our studies – is hippocampal neurodegeneration. The hippocampus is a region of the brain known for its role in learning and memory. Several drugs of abuse have been shown to adversely affect hippocampal function, which may foster contextual conditioning that results in cue-induced drug craving (Koob and Volkow, 2010). Furthermore, alcoholics often display neurocognitive deficits that include impairments attributed to the hippocampus (Parsons, 1998). However, with abstinence, former alcoholics show significant improvements in hippocampus-dependent functions (Bartels et. al., 2007) and cognitive abilities (Fein et

al., 2006). For example, a meta-analysis showed that alcoholics demonstrate impaired spatial learning and memory during the first year of abstinence, but return to control levels during more prolonged abstinence (Stavro et al., 2013). Therefore, it is hypothesized that some mechanism of hippocampal recovery exists.

In addition to studies demonstrating the effect of alcohol on hippocampal function, other groups have found that alcohol also affects hippocampal structure. For example, one study measuring hippocampal volumes in humans found that chronic alcoholics had lower anterior hippocampal volume than social drinking controls (Sullivan et al., 1995). Several other groups have also shown hippocampal volume loss resulting from alcoholism (Agartz et al., 1999, Beresford et al., 2006, Mechtcheriakov et al., 2007); however, studies finding no effect of alcohol on hippocampal neuron loss exist as well (Harding et al., 1997). Additionally, adolescent drinkers appear to be particularly susceptible to alcohol-induced hippocampal damage (White and Swartzwelder, 2004). A study measuring hippocampal volume via magnetic resonance imaging found that people who developed AUDs during adolescence had lower hippocampal volumes than those who did not; furthermore, younger age at onset of AUD and longer duration were associated with lower hippocampal volumes (De Bellis et al., 2000).

Animal studies evaluating the neurodegenerative effects of alcohol on the hippocampus have also been performed. For example, an animal model has shown granule cell loss in the dentate gyrus and pyramidal cell loss in the CA regions following chronic ethanol consumption (Walker et al., 1980). Furthermore, rats that underwent four-day binge ethanol treatment had cell death in the DG as measured by silver staining and demonstrated impaired reversal learning in the Morris water maze (Obernier et al., 2002). Another studying employing the four-day binge ethanol model found that female rats had fewer DG granule cells and increased hippocampal cell death as measured by FluoroJade B (Leasure and Nixon, 2010). Finally, NDMAR-dependent hippocampal

cytotoxicity has been demonstrated in organotypic hippocampal slices following chronic intermittent ethanol exposure and ethanol withdrawal (Reynolds et al., 2015).

Adult Neurogenesis

Adult neurogenesis, the process by which neural stem cells (NSCs) proliferate, differentiate into neurons, migrate into the granule cell layer, and survive and integrate into the neural network (Kempermann et al., 2004b), occurs in two brain regions: the subventricular zone (SVZ) of the lateral ventricle (Alvarez-Buylla and Garcia-Verdugo, 2002) and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) in animals and humans (Altman and Das, 1965, Eriksson et al., 1998). In rodents, neurons generated in the SVZ migrate along the rostral migratory stream to the olfactory bulb (Alvarez-Buylla and Garcia-Verdugo, 2002). However, the significance of adult neurogenesis in the SVZ of humans is unclear (Inta et al., 2015); therefore, only hippocampal adult neurogenesis will be discussed herein. Greater than 9,000 neurons – 6% of the total population – are generated daily in the GCL of rats (Cameron and McKay, 2001). Hippocampal neurogenesis involves several steps: neural stem cells (NSCs) in the SGZ asymmetrically divide into amplifying progenitor cells, which differentiate into immature neurons and migrate into the GCL; if the cells survive to become mature neurons, they project dendrites into the molecular layer of the DG (von Bohlen und Halbach, 2011). NSCs, or Type 1 cells, have radial glial properties and are located in the SGZ; they express glial fibrillary acidic protein (GFAP), nestin (an intermediate filament), and SRY-related HMG-box gene2 – or Sox2 – a transcription factor (von Bohlen und Halbach, 2011). Type 2a and 2b cells are rapidly proliferating progenitors arising from Type 1 cells. Type 2b cells express doublecortin (DCX, a microtubule-associated protein that is a marker of immature neurons) and NeuroD (a transcripton factor), whereas Type 2a cells do not (Lee et al., 1995, Kempermann et al.,

2004a). Type 3 cells are immature neurons that begin to migrate into the granule cell layer. Immature neurons eventually become postmitotic mature neurons that express neuron-specific nuclear protein (NeuN). Immunohistochemical staining procedures are also used to identify other markers expressed by cells in various stages of neurogenesis.

Adult neurogenesis is hypothesized to be key to hippocampal function. Correlations provided the initial evidence that newborn neurons were related to DG structure and therefore function. For example, the number of granule cells in the hippocampal dentate gyrus doubled in rats that underwent hippocampus-dependent associative learning tasks (Gould et al., 1999). Furthermore, mice that had increased hippocampal neurogenesis via running or environmental enrichment showed enhanced learning (Nilsson et al., 1999, van Praag et al., 1999). Conversely, when neurogenesis was decreased, rats performed worse on hippocampus-dependent trace conditioning tasks (Shors et al., 2001). Causation was established by a study that showed that genetic ablation of newborn GCL neurons in mice resulted in contextual and spatial memory impairment (Imayoshi et al., 2008).

Alcohol has been shown to have different effects on hippocampal adult neurogenesis during intoxication and abstinence. Four-day binge ethanol exposure in an animal model causes a decrease in neurogenesis immediately following the last dose of ethanol (Nixon and Crews, 2002). The study by Nixon and Crews in 2002 found that both hippocampal neural stem cell proliferation and survival were negatively impacted by binge ethanol in rats. Decreased neural stem cell proliferation following one, two, and four weeks of chronic ethanol administration has also been reported in rats (He et al., 2005). Another study evaluating hippocampal neurogenesis in adult rats involving six weeks of alcohol administration showed decreased new cell survival contributing to impaired neurogenesis, but no effects on neural stem cell proliferation (Herrera et al., 2003). However, during abstinence from four-day binge ethanol exposure, adult

hippocampal neurogenesis has been shown to increase as a result of increased neural stem cell proliferation (Nixon and Crews, 2004). This particular study is discussed in greater depth in the introduction of Chapter 1.

Hippocampal adult neurogenesis has also been studied in adolescent animal models of AUDs. In rats, postnatal days (PND) 28-42 are generally recognized as the period of adolescence, while PND 63 and over is considered adulthood (Spear, 2000, Sengupta, 2013). The first study to show an effect of alcohol on hippocampal neurogenesis was conducted by Crews et al. in 2006. This study found that acute doses of ethanol administered intragastrically to male adolescent rats reduced BrdU and doublecortin immunoreactivity (Crews et al., 2006). Furthermore, our lab has shown that binge ethanol administration acutely decreases hippocampal neurogenesis through decreased neural stem cell proliferation and survival in adolescent rats (Morris et al., 2010a) and induces hippocampal neurogenesis on the seventh day of abstinence (McClain et al., 2014). The same study found an increased number of ectopic neuroblasts in the molecular layer and hilus of the hippocampal dentate gyrus at this same time point during abstinence in rats that underwent severe withdrawal, indicating that adolescents may have impaired neuronal incorporation into hippocampal circuitry (McClain et al., 2014). An adolescent intermittent ethanol exposure model showed decreases in dorsal and ventral hippocampal neurogenesis that persisted into adulthood and was associated with diminished cognitive function in the novel object recognition task (Vetreno and Crews, 2015). Another model using a different ethanol administration pattern during adolescence also showed persistent decreases in hippocampal neurogenesis that were hypothesized to be the result of increased immature neuronal cell death (Broadwater et al., 2014).

Nicotine has been shown to cause decreases in hippocampal adult neurogenesis in various animal models, which will be discussed in greater depth in Chapter 3. Briefly,

different doses and administration schedules have shown that nicotine decreases hippocampal neurogenesis when measured immediately following administration. Some studies have concluded that nicotine decreases neurogenesis by diminishing cell proliferation, while others have postulated that the decrease is a result of reduced cell survival. None of the studies have evaluated the effect of nicotine on neurogenesis during abstinence. Additionally, nicotine has been shown to induce hippocampal cell death in both adolescent and adult rats, which will also be thoroughly discussed in Chapter 3. However, this information is important to keep in mind when discussing the hypothesis.

Hypothesis

Alcoholics demonstrate impaired cognitive function and diminished ability to perform hippocampal-dependent tasks (Parsons, 1998). Moreover, alcohol has been shown to induce hippocampal neurodegeneration in humans and animal models of AUDs. One of its mechanisms of neurodegeneration is via the reduction of adult neurogenesis occurring in the hippocampal DG – a process that is essential for proper hippocampal function (Imayoshi et al., 2008) – during intoxication (Nixon and Crews, 2002). However, following four-day binge ethanol exposure, cell proliferation returns to normal by the third day of abstinence (Nixon and Crews, 2004, Nixon et al., 2008). On the seventh day of abstinence, it has been shown that a burst in cell proliferation occurs that results in increased neurogenesis in the DG (Nixon and Crews, 2004). In order to eventually reveal the mechanism by which this ethanol-induced reactive neurogenesis occurs, it is necessary to determine the timing of the initiation of cell proliferation. Therefore, the aim for Experiment 1 was to develop a timeline of cell proliferation in the SGZ of the hippocampal DG on days 4, 5, 6, and 7 of abstinence in order to determine on which day it begins. Then, Experiment 2 will build upon the results of Experiment 1 to

examine the effects of nicotine and alcohol co-administration on cell proliferation at the point of peak proliferation in adolescent rats.

Although nicotine is generally considered to have beneficial effects on cognition (Levin et al., 2006), it has been shown to have toxic effects on the hippocampus through multiple mechanisms. First, nicotine has been shown to induce hippocampal cell death in both adult and adolescent rats (Abrous et al., 2002, Jang et al., 2002, Abreu-Villaca et al., 2003, Demiralay et al., 2008). Additionally, nicotine has been shown to decrease adult hippocampal neurogenesis during exposure (Abrous et al., 2002, Jang et al., 2002, Shingo and Kito, 2005, Scerri et al., 2006, Wei et al., 2012). However, no studies have assessed nicotine's effect on hippocampal neurogenesis during abstinence, nor have they measured the effect of nicotine co-administration on alcohol-induced reactive neurogenesis. As most alcoholics smoke (Miller and Gold, 1998, Falk et al., 2006, De Leon et al., 2007, Van Skike et al., 2016), and many people begin smoking and drinking during adolescence, it is critical to evaluate the effect of these drugs when administered in combination during adolescence. Based on the fact that nicotine induces hippocampal neurotoxicity and acutely decreases hippocampal neurogenesis during administration – similar to the neurotoxicity and decreased neurogenesis seen during alcohol administration – the hypothesis of Experiment 2 is that nicotine increases reactive hippocampal cell proliferation during abstinence and has an additive effect on reactive cell proliferation during abstinence when co-administered with alcohol.

Chapter Two: Timeline of Reactive Alcohol-Induced Hippocampal Cell Proliferation

Introduction

Previous work has shown that there is a decrease in cell proliferation in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) immediately after the last dose of ethanol is administered after four days of binge ethanol exposure in a modified Majchrowicz model as depicted in Figure 2.1 (Nixon and Crews, 2002, 2004). However, cell proliferation returns to control levels on the first day of abstinence (T24) as shown in Figure 2.2 (Nixon et al., 2008). Then, on the second day of abstinence (T48), an increase in the number of proliferating cells marked by BrdU occurs; however, these cells were shown to be microglia and did not reflect changes in neurogenesis. Furthermore, these BrdU+ cells were located not only in the SGZ, but also throughout the hilus and molecular layer of the DG, the CA regions of the hippocampus, and cortical regions – areas where neurogenesis does not occur. Interestingly, on the seventh day of abstinence there is a 350% increase in the number of proliferating cells marked by BrdU in ethanol exposed animals compared to controls that does reflect increases in neurogenesis. This work showed that the increased cell proliferation at day 7 of abstinence resulted in increased neurogenesis by measuring doublecortin (DCX) on day 14 of abstinence. Doublecortin is a protein that is associated with microtubules and is expressed by migrating, newly differentiated neuroblasts within 4-7 days after cell birth (Gleeson et al., 1999, Brown et al., 2003). Furthermore, this study by Nixon and Crews showed that most of these cells survived 28 days after the burst in cell proliferation on day 7 of abstinence.

Figure 2.1: Timeline of cell proliferation

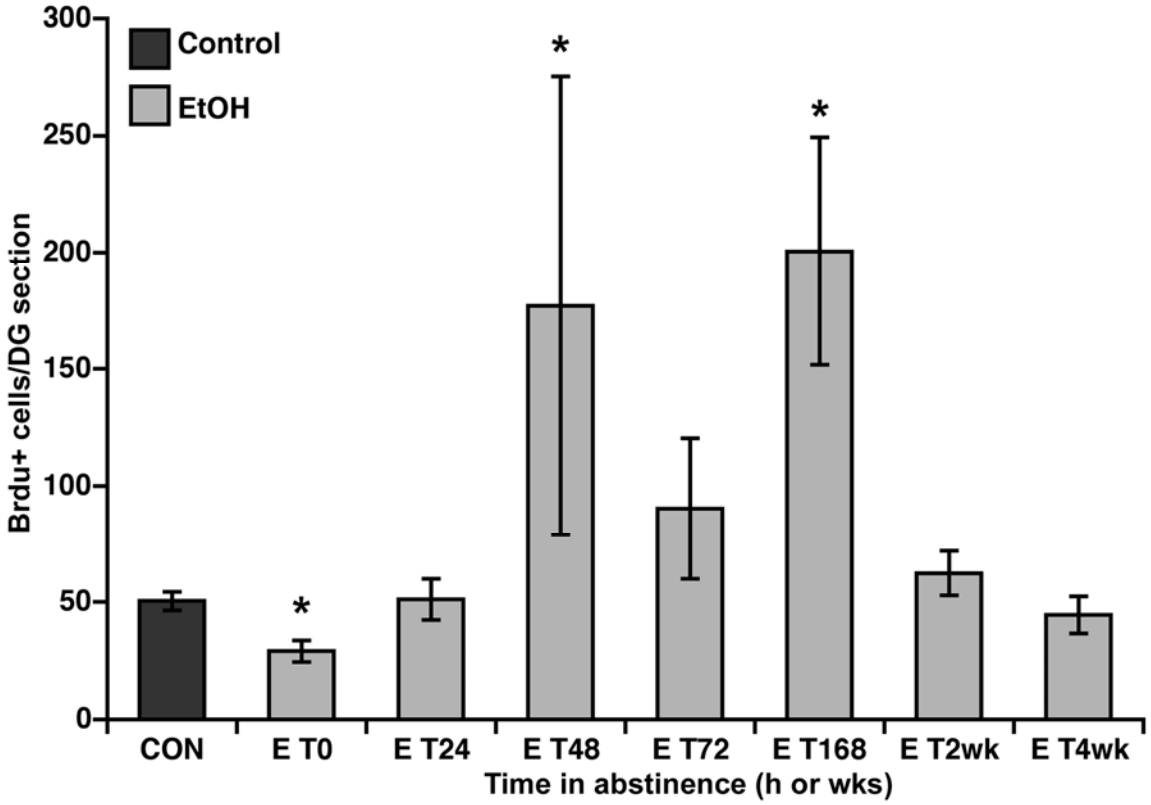


*Figure 2.1 Timeline of cell proliferation in the hippocampal dentate gyrus following four days of binge ethanol exposure in a modified Majchrowicz model. Cell proliferation was measured by BrdU incorporation into dividing cells. Cell proliferation on the second day of abstinence (T48) is reflective of microglia proliferation (Nixon et al., 2008), whereas cell proliferation on the seventh day of abstinence (T168 or T7) results in increased neurogenesis (Nixon and Crews, 2004). *p<0.05 versus control*

As previously discussed, neural stem cell proliferation is the first step in the process of adult neurogenesis. Therefore, the initial step in studying this phenomenon is to examine cell proliferation in the subgranular zone of the hippocampal dentate gyrus, a brain region where neurogenesis is known to occur. In order to better understand the timeline of reactive adult neurogenesis during abstinence from alcohol, Experiment 1

investigated the timeline of cell proliferation on days 4, 5, 6, and 7 to determine when the increase in adult neurogenesis begins.

Methods

Fifty-two adult male Sprague Dawley rats (~PND 70) with an initial weight of approximately 300 grams (Charles River Laboratories, Raleigh, NC) underwent four days of binge ethanol exposure as described below. There were a total of 32 rats in the ethanol treatment group and 20 rats in the control group (Table 2.1); however, not all of the BrdU injections successfully incorporated into the brain (cite Eisch et al., 2000 – in PNAS acknowledges this and has a method), so 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 control animals had to be excluded from analyses. At four, five, six, and seven days of abstinence following the last dose of alcohol (i.e. T4-T7), a group of ethanol and control animals were given BrdU and euthanized in the manner described below in order to develop a timeline of neural stem cell proliferation (Figure 2.2).

Table 2.1: Experiment 1 Design

Euthanasia time point	Ethanol	Control
T4	n=8	n=4
T5	n=8	n=4
T6	n=8	n=4
T7	n=8	n=8

Table 2.1 Original number of animals within each group. Twelve rats total were planned to be euthanized four, five, and six days after the last dose of ethanol (T4-T6) and 16 rats were planned to be euthanized on day seven of abstinence (T7).

Figure 2.2: Experiment 1 Timeline

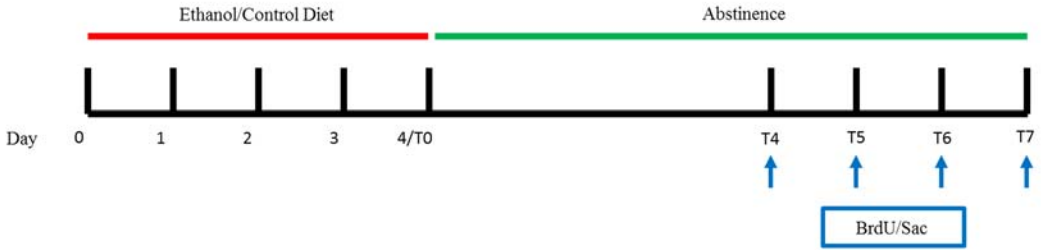


Figure 2.2 An experimental timeline showing the timing of ethanol administration and euthanasia during abstinence. Sac = sacrifice/euthanasia. BrdU = bromodeoxyuridine.

Animal Model of an Alcohol Use Disorder (AUD)

A binge ethanol exposure model modified from Majchrowicz (1975) was used throughout all experiments as previously described (Morris et al., 2010b). This model produces blood ethanol concentrations (BECs) of roughly 325 mg/dL, approximately four times the legal limit, to mimic the high BECs found in alcoholics who drink in a binge-bender pattern. The model has been shown in our lab and in others to cause neurodegeneration, decreased hippocampal neurogenesis during intoxication, and increased hippocampal neurogenesis during abstinence from alcohol (Collins et al., 1996, Nixon and Crews, 2002, 2004, Kelso et al., 2011). Additionally, the rats develop tolerance to alcohol and undergo withdrawal during abstinence.

All animals were group-housed with two to three rats per cage on a 12 hour light:dark cycle with free access to water and chow except during binge treatment when chow was removed. All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee and were consistent with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The animals were handled for three consecutive days prior to the beginning of ethanol administration in order to minimize stress. The rats were administered either ethanol (25% w/v in Vanilla Ensure Plus®) or control diet via intragastric gavage three times per day (at 7am,

3pm, and 11pm) for four days. The ethanol and control diets were calorie-matched. Rats were given an initial ethanol dose of 5 g/kg, and subsequent doses (ranging between 0 and 5 g/kg) were titrated according to their behavioral intoxication score (Table 2.2). This model maximizes BECs without affecting mortality (Majchrowicz, 1975). In order to ensure that there were no nutritional differences between ethanol and control groups, control animals were administered the average volume of diet received by ethanol animals. To prevent dehydration, any animal with an intoxication score of four or five was given two milliliters of water.

Table 2.2: Behavioral Intoxication Scale

Intoxication Score	Behavior	Dose of Ethanol
0	Normal animal	5 g/kg
1	Hypoactive, mildly ataxic	4 g/kg
2	Ataxic, elevated animal	3 g/kg
3	Ataxic, loss of abdominal elevation, delayed righting reflex	2 g/kg
4	Loss of righting reflex, retained eye blink reflex	1 g/kg
5	Loss of righting reflex, loss of eye blink reflex	0 g/kg

Table 2.2 Scale used to evaluate each rat's behavioral intoxication prior to ethanol administration and determine the dose of ethanol to give.

BECs were measured by collecting tail blood on the third day of the binge (after the 7th dose), 90 minutes after ethanol administration, the point at which rats reach peak BEC after oral gavage (Kelly et al., 1987, Livy et al., 2003). Blood was collected into eppendorfs that contained five microliters of heparin. Following blood collection, all samples were centrifuged at 1800g for five minutes, resulting in the separation of erythrocytes from plasma. Centrifuged samples were stored at -20°C. To measure the BEC of each animal, five microliters of blood plasma was placed into an AM1 Alcohol

Analyser (Analox, London, UK) and run in triplicate. The machine was calibrated using a 300 mg/dL standard.

Eight hours after the last dose of ethanol was administered, all animals were single-housed and given free access to standard rat chow. Ten hours after the last dose of ethanol was administered, monitoring of withdrawal behavior was initiated for all rats receiving ethanol. Monitoring occurred for 30 minutes of every hour for 18 consecutive hours and withdrawal was assessed according to a previously developed scale as shown in Table 2.3 (Penland et al., 2001, Morris et al., 2010b).

Table 2.3: Withdrawal Behavior Scale

Score	Withdrawal Behavior
1.0	Hyperactivity
1.4	Tail tremor
1.6	Tail spasm
2.0	Caudal tremor
2.2	Arched back, on tiptoes
2.4	Splayed limbs
2.6	General tremor
3.0	Head tremor
3.2	Induced running
3.4	Wet dog shakes
3.6	Chattering teeth
3.8	Spontaneous convulsions
4.0	Death

Table 2.3 Scale used to measure withdrawal severity based on behavior. This scale is a modification of Penland et al., 2001 which essentially assigned a score to each behavior rather than categorizing severity as in Majchrowicz's (1975) original paper.

Two hours prior to euthanasia, all rats were administered a saturating dose of 5-bromo-2'-deoxyuridine (BrdU, Roche Diagnostics, Mannheim, Germany; 300mg/kg) via intraperitoneal injection (Cameron and McKay, 2001). The 20mg/mL BrdU solution for injection was prepared by dissolving the BrdU crystals in 0.9% NaCl. Rats were

administered a lethal dose of pentobarbital sodium solution (Fatal-Plus®, Vortech Pharmaceuticals, Dearborn, MI) and underwent transcardial perfusion with 0.1M phosphate buffered saline (PBS, pH 7.5) followed by 4% paraformaldehyde. Rat brains were extracted shortly after euthanasia and placed into 4% paraformaldehyde for 24 hours of post-fixation before being transferred to and stored in 0.1M PBS at 4˚C until sectioning. Brains were sliced into 40 micrometer coronal sections in a 1:12 series using a vibrating blade microtome (Leica Biosystems, Buffalo Grove, IL) and stored in cryoprotectant at -20°C.

Bromodeoxyuridine (BrdU) Immunohistochemistry

Every sixth coronal brain section was processed for free-floating BrdU immunohistochemistry using published methods (Nixon and Crews, 2004). Tissue was washed in tris buffered saline (TBS) and endogenous peroxidases were quenched with 0.6% hydrogen peroxide. Antigen retrieval through DNA denaturation was then performed by placing the tissue into 50% formamide/2x saline sodium citrate for 2 hours at 65°C and 2N HCl for 1 hour at 37°C followed by pH neutralizing washes in 0.1M boric acid. The tissue was next placed into blocking solution to prevent nonspecific antibody binding (3% horse serum and 0.1% triton X in TBS). An additonal DNA denaturing step was then done by putting the tissue into a deoxyribonuclease I solution (100U DNase/1mL of 0.02% $MgCl_2$ in TBS) for 1 hour. The tissue was then incubated in a 1:4400 dilution (subjects from the T4 and T5 euthanasia time points) or 1:5000 dilution (subjects from the T6 and T7 euthanasia time points) of mouse anti-BrdU (Millipore MAB3424, Temecula, CA) overnight at 4°C. The next day, the tissue was rinsed in blocking solution and incubated in biotinylated horse anti-mouse, rat adsorbed secondary antibody (Vector Laboratories, Burlingame, CA) for 1 hour at 37°C. Following

this step, the tissue was placed into an avidin-biotinylated horseradish peroxidase complex solution (ABC Elite Kit; Vector Laboratories), which binds the biotin and amplifies the signal of the secondary antibody (Volpicelli-Daley and Levey, 2004). Finally, nickel-enhanced 3,3'-diaminobenzidine tetrahydrochloride (DAB; Polysciences, Warrington, PA), a chromagen that undergoes an enzymatic reaction with the horseradish peroxidase in ABC, was used to allow the BrdU+ cells to be visualized (black color). Following immunohistochemical staining, all tissue sections were mounted onto glass slides, dried, lightly counterstained with cresyl violet, and coverslipped with Cytoseal® (Stephens Scientific, Wayne, NJ).

All cells labeled with BrdU (BrdU+) in the subgranular zone of the hippocampal dorsal dentate gyrus (Bregma -2.64 to -5.28) were quantified via profile counting on an Olympus BX-41 light microscope at 1000x with an oil immersion lens (UPlanSApo 100x; numerical aperture 1.4, Olympus). During quantification, the experimenter was blinded to the treatment condition. Previous work has shown that the profile counting method results in percent change identical those produced by stereological methods of cell quantification and may be more appropriate for the non-homogenously distributed BrdU profiles (Crews et al., 2004, Noori and Fornal, 2011).

Statistical Analyses

Data were analyzed and graphed using Prism Version 6.05 (GraphPad Software, Inc. La Jolla, CA). Data are reported as mean ± standard error of the mean. Results were considered statistically significant if $p<0.05$. All animals that failed to incorporate BrdU into the brain were excluded from all analyses. Nonparametric, animal model subject data including intoxication scores and mean and peak withdrawal scores were analyzed with the Kruskal-Wallis test. Parametric, BECs and daily ethanol dose data were analyzed by one-way ANOVA. BrdU cell counts in rats receiving ethanol and

euthanized at all 4 time points were compared to a common control group, collapsed across time points via a one-way ANOVA with Dunnet's post hoc test. Correlations of parametric data, i.e. between BrdU+ cell counts and blood ethanol concentration or daily ethanol dose were analyzed by Pearson correlation; all other correlations of subject data with BrdU cell counts utilized the nonparametric Spearman correlation.

Results

Animal Model Subject Data

The mean weight of the rats on the first day of binge ethanol exposure was 329 ± 2.7 grams. Because diet restriction has been shown to promote adult neurogenesis, initial body weight and the change in body weight during the four day binge period were measured to identify potential differences between groups (Lee et al., 2002). One-way ANOVA showed no differences in initial body weights or percentage of body weight lost between any of the groups (Table 2.4).

Table 2.4: Experiment 1 Animal Weight Data

Group	Initial body weight (grams)	Change in body weight (%)
Control (n=11)	327 ± 6	-11.5 ± 0.3
T4 (n=4)	322 ± 5	-13.7 ± 1.3
T5 (n=6)	332 ± 6	-11.9 ± 1
T6 (n=6)	338 ± 4	-13.9 ± 0.7
T7 (n=4)	326 ± 8	-11.2 ± 2.9

Table 2.4 Initial body weight and change in body weight following 4 days of binge ethanol exposure were both statistically similar between groups.

One-way ANOVA of blood ethanol concentrations (grand mean = 358 ± 18.4 mg/dL) and daily dose of ethanol administered (grand mean = 9.9 ± 0.3 g/kg/day) revealed no significant differences between the four groups. The mean BEC and daily

ethanol dose are in accordance with the values routinely produced by this model (Nixon and Crews, 2004, Morris et al., 2010b, Kelso et al., 2011). Furthermore, Kruskal-Wallis tests of intoxication score, mean withdrawal score, and peak withdrawal score showed no significant differences between groups (Table 2.5).

Table 2.5: Experiment 1 Animal Model Subject Data

Group	Intoxication Score (0-5 scale)	Dose (g/kg/day)	BEC (mg/dL)	Mean Withdrawal (0-4 scale)	Peak Withdrawal (0-4 scale)
T4	1.8 ± 0.1	9.7 ± 0.3	324 ± 5	0.64 ± 0.1	3.4 ± 0.2
T5	1.6 ± 0.1	10.1 ± 0.4	330 ± 44	0.86 ± 0.2	3.1 ± 0.1
T6	1.9 ± 0.1	9.2 ± 0.3	412 ± 11	0.72 ± 0.2	3.4 ± 0.2
T7	1.5 ± 0.4	10.6 ± 1.1	356 ± 42	0.79 ± 0.1	3.5 ± 0.2

Table 2.5 No statistical differences between groups were detected in any of the behavioral parameters or BECs associated with the modified Majchrowicz model.

Time Course of Cell Proliferation

In order to investigate the timing of reactive cell proliferation during the period of abstinence following binge ethanol exposure, the number of BrdU+ cells in the subgranular zone of the hippocampal dentate gyrus were quantified 4, 5, 6, and 7 days post binge ethanol administration (T4-T7; Figure 2.3). A one-way ANOVA showed no difference in BrdU+ cell counts between control groups at all four time points. Therefore, all controls were collapsed into one group (n=11). A one-way ANOVA showed a significant effect of group on the number of BrdU+ cells ($F_{4,26}$=29.69; $p<0.0001$). Dunnett's post hoc test comparing each time point to control showed that there was no difference in the number of BrdU+ cells in the ethanol group versus the control group at four days of abstinence (T4; n=4); however, there was a 70% increase in the number of

BrdU+ cells at T5 ($p<0.05$; n=6), a 121% increase at T6 ($p<0.05$; n=6), and a 233% increase at T7 ($p<0.05$; n=4).

Figure 2.3: Time Course of Cell Proliferation in the Hippocampal Dentate Gyrus

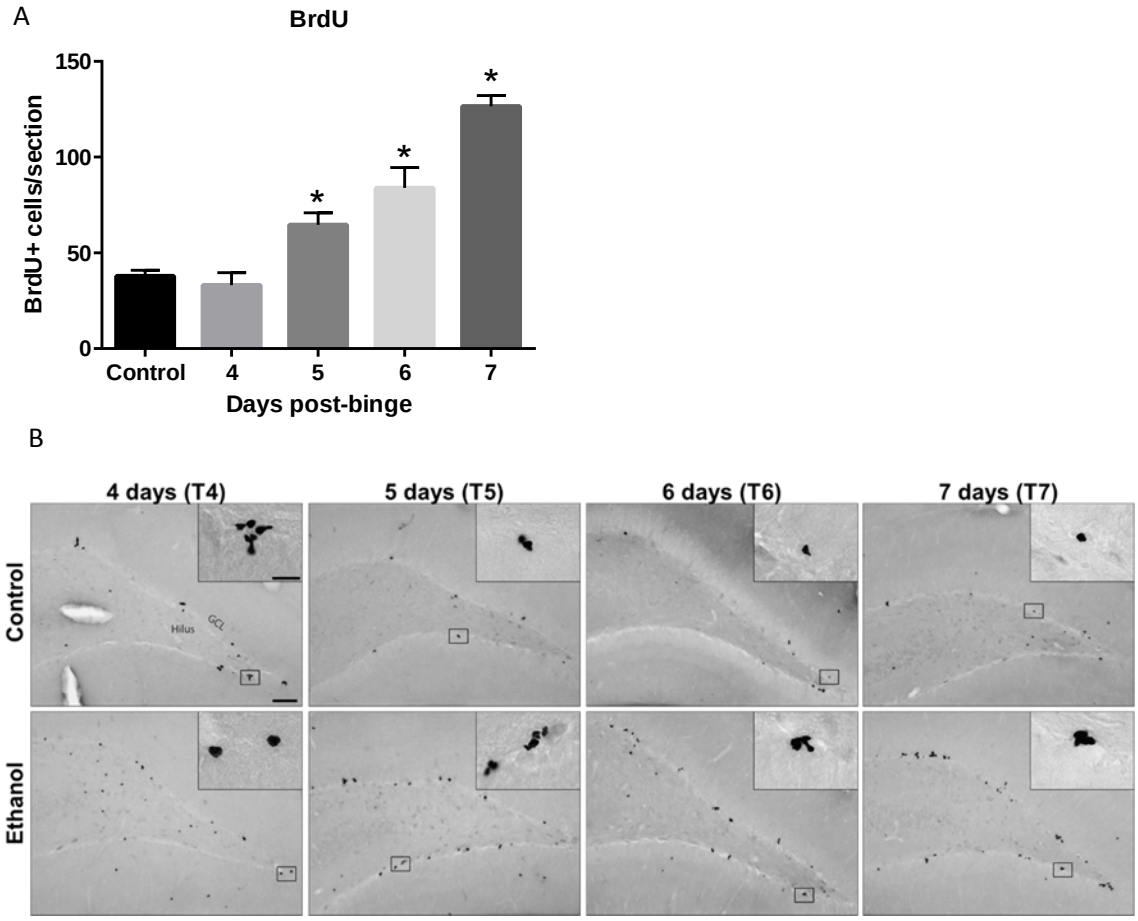


*Figure 2.3: The number of BrdU+ cells in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG), a measure of cell proliferation, increased beginning 5 days into abstinence (T5), graphed in panel A. Panel B shows representative images of BrdU+ cells in the DG for each group. BrdU+ cells located in the granule cell layer (GCL) or hilus were not quantified. * $p<0.05$ compared to the control group. Scale bar = 100µm, 20µm inset.*

Correlations were conducted to probe for a relationship between any of the subject data collected during binge ethanol exposure – including level of behavioral intoxication, BEC, and withdrawal severity – and the number of BrdU+ cells in the SGZ at T7, which appears to be the point of peak cell proliferation during abstinence (Table 2.6; Figure 2.4). Spearman nonparametric correlation of intoxication score, mean withdrawal, and peak withdrawal scores were not significantly correlated with BrdU+ cell count. Furthermore, Pearson correlation analysis showed that BEC and daily ethanol dose were not correlated with the number of BrdU+ cells.

Table 2.6: Correlations of Animal Model Subject Data with BrdU Cell Counts at T7

Animal model parameter	**Correlation coefficient (P value)**
Mean withdrawal	0.40 (0.75)
Peak withdrawal	-0.32 (0.50
Intoxication score	-0.20 (0.92)
Dose	0.23 (0.77)
BEC	0.02 (0.98)

Table 2.6 Correlations were used to examine whether any relationships exist between the measured animal model parameters and BrdU cell counts at day 7 of abstinence (T7). None of the animal model parameters showed a statistically significant correlation.

Figure 2.4: Correlations of Animal Model Subject Data with BrdU Cell Counts at T7

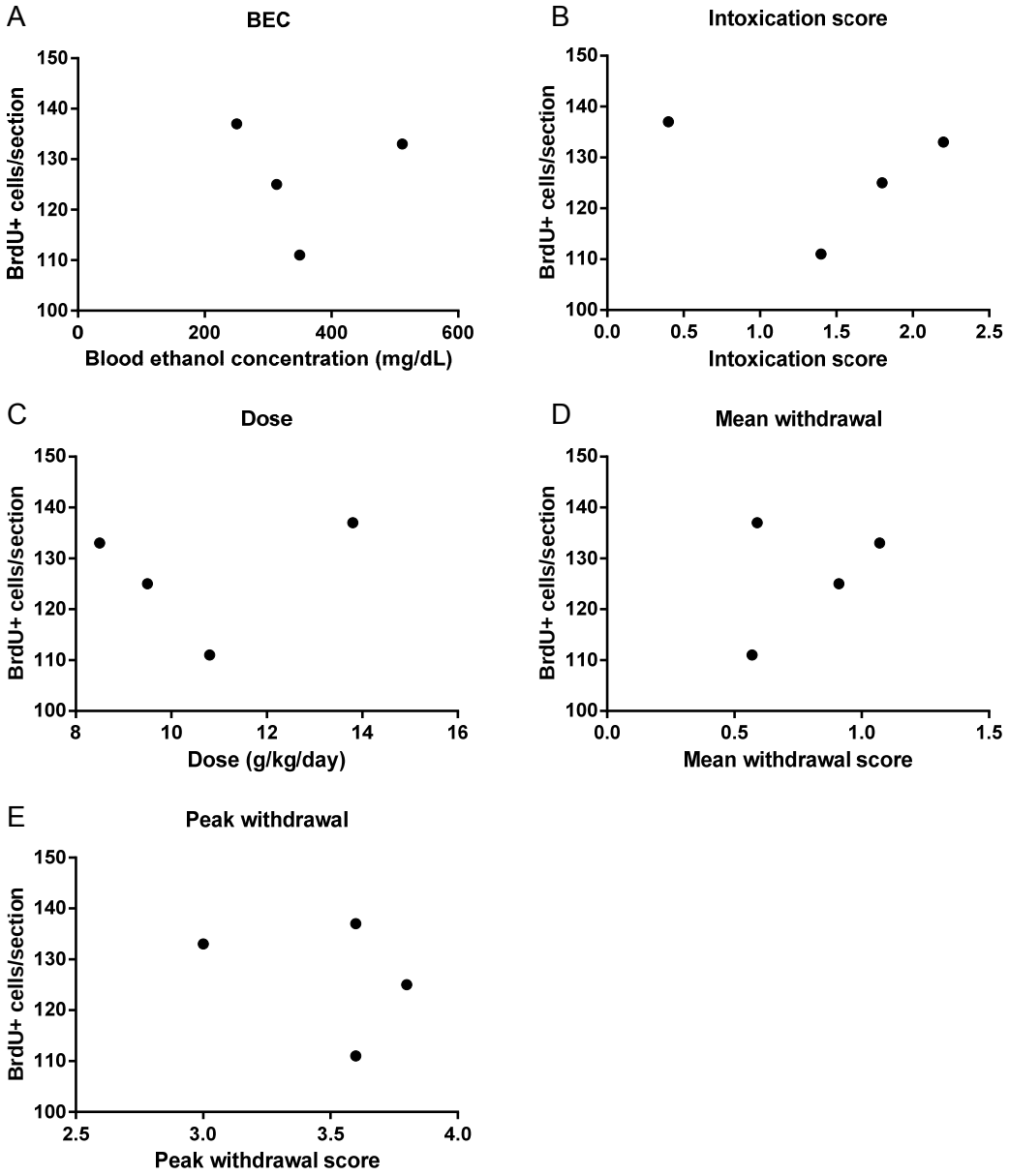


Figure 2.4. None of the animal model parameters showed a statistically significant correlation with BrdU cell count at T7.

Discussion

This experiment builds on the work of Nixon and Crews (2004) to determine the start of the reactive cell proliferation that has been shown to occur in rats during abstinence following four days of binge ethanol exposure. The results of this study show

that cell proliferation in the subgranular zone of the hippocampal dentate gyrus is increased in ethanol-exposed animals beginning on day 5 of abstinence (T5) and continues to increase on days 6 and 7. Furthermore, the T7 time point data replicated previous work though the fold change over control appears to be less. The BrdU analysis reported by Nixon and Crews (2004) included BrdU+ cells in both the granule cell layer and the subgranular zone. In this experiment, only BrdU+ cells in the SGZ were quantified, which may better predict increases in neurogenesis as this is the region where neural stem cells proliferate.

Studies evaluating reactive neurogenesis in response to insults other than alcohol have shown a similar timeline of cell proliferation. For example, one study of rats found that focal cerebral ischemia induced reactive cell proliferation in the SGZ one week after the insult; furthermore, cell proliferation returned to control levels two weeks after ischemia occurred (Jin et al., 2001). Another study examining focal ischemia in gerbils showed that cell proliferation increased beginning 6 days after the insult, peaked at day 11, and returned to control levels 3 weeks after the event (Liu et al., 1998). A model involving pilocarpine-induced status epilepticus in rats, which is notable because the Majchrowicz model produces withdrawal seizures in some rats, demonstrated increased cell proliferation in the SGZ 3, 6, and 13 days after seizure and resulted in increased neurogenesis (Parent et al., 1997). Thus, events inducing cell death including ischemia and seizures have been shown to result in compensatory neurogenesis along a somewhat similar timeline as alcohol. These data add to our understanding of the time course of cell proliferation events after an acutely damaging exposure to alcohol: cell proliferation increases gradually over days of abstinence until reaching a peak, at which point it returns to normal levels.

It is important to note that although it can be concluded that cell proliferation increases on day 5 of abstinence, it cannot be definitively concluded that *adult*

neurogenesis increases on day 5 of abstinence. BrdU labels all proliferating cells in the S phase of the cell cycle; thus, BrdU can label proliferating glial cells or neural stem cells that will eventually differentiate into glia. As previously discussed, increases in cell proliferation on day 2 of abstinence reflect microglial proliferation rather than NSC proliferation (Nixon et al., 2008). However, based on prior work showing that cell proliferation in this brain region on day 7 of abstinence (T7) results in increased neurogenesis, it can be inferred that increased cell proliferation on day 5 of abstinence also results in increased adult neurogenesis. In order to conclude that increased cell proliferation at T5 does in fact result in increased neurogenesis, future studies could involve quantifying doublecortin or NeuroD (markers of immature neurons) on day 12 of abstinence, which is one week after cell proliferation increases and by which time the cell fate of the proliferating NSCs would have been determined (Seki, 2002, Brown et al., 2003). Moreover, rats could be injected with BrdU on day 5 of abstinence and euthanized 28 days later (T35) in order to determine whether the proliferating cells survive, as cell survival is a crucial step in process of adult neurogenesis. Lastly, at T35, by which time the proliferating NSCs would have become mature neurons, fluorescent immunohistochemistry could be used to determine whether there is co-localization of BrdU and NeuN (a marker of mature neurons). Co-localization would indicate that the proliferating cells labeled with BrdU at T5 differentiated and survived to become mature neurons. Another issue to consider is that BrdU is exogenously administered and its bioavailability could influence the interpretation of these data. To obtain more conclusive results regarding cell proliferation, future work should involve quantifying the number of Ki-67+ cells in this same brain region on day 5 of abstinence. Ki-67 is an endogenous marker of cell proliferation that labels proliferating cells in all phases of the cell cycle except for the resting phase (Scholzen and Gerdes, 2000). Ki-67+ cells are increased in parallel to BrdU+ cells in all studies to date (Nixon and Crews, 2004, Nixon et al., 2008).

As discussed above, seizures have been shown to induce reactive hippocampal neurogenesis (Parent et al., 1997); therefore, it is necessary to rule out withdrawal seizure as the cause of alcohol-induced reactive neurogenesis. Although prior studies using the binge ethanol model have demonstrated that BrdU+ cell number positively correlates with withdrawal severity, a study using 10mg/kg diazepam to decrease withdrawal severity showed that animals exhibiting very few behavioral signs of withdrawal still had increased cell proliferation at T7 (Nixon and Crews, 2004). Moreover, although CNS hyperexcitability via glutamate transmission occurs during alcohol withdrawal (Rossetti and Carboni, 1995, Dahchour and De Witte, 1999), agonism of NMDA receptors by glutamate has actually been shown to result in decreased hippocampal neurogenesis via reduced cell proliferation (Cameron et al., 1995). Therefore, it is not thought that withdrawal contributes much to reactive adult neurogenesis during abstinence. Furthermore, in this experiment, none of the animal model subject data including BEC, daily ethanol dose, intoxication score, mean withdrawal score, or peak withdrawal score correlated with the number of BrdU+ cells in the SGZ. However, it is possible that the lack of any significant correlations is due to the small number of animals in the T7 ethanol group.

Chapter Three: Effect of Nicotine and Alcohol Co-administration on Reactive Cell Proliferation

Introduction

Nicotine has been shown to cause decreases in hippocampal adult neurogenesis in various animal models. One study found that when high doses of nicotine were intravenously self-administered daily for 42 days, hippocampal neurogenesis was decreased when measured during the last three days of self-administration (Abrous et al., 2002). Moreover, the decrease in neurogenesis was accompanied by an increase in cell death in the granule cell layer as measured by pyknotic cells. The authors hypothesize that these cells were a mix of mature neurons, newborn cells, and NSCs based on their location throughout the GCL but could not conclusively determine what cell phenotypes were dying. Another study showed that nicotine administered subcutaneously at a dose of 4 mg/kg/day through an osmotic mini pump for ten days – a dose that produces blood nicotine levels similar to that of heavy smoking and that results in desensitization of nAChRs – decreased cell proliferation in the DG and caused an impairment in spatial memory as measured by the Morris water maze (Scerri et al., 2006). A study conducted in male adolescent rats (PND 30) showed that three days of once daily 1 mg/kg intraperitoneal doses of nicotine decreased cell proliferation in the SGZ when measured by BrdU administration during all three days of nicotine exposure (Jang et al., 2002). Moreover, similarly to the study by Abrous et al., this study found increased cell death in the DG as measured by the number of TUNEL+ cells, pyknotic cells, and caspase-3 positive cells. Not only does nicotine impair cell proliferation, it has also been shown to reduce cell survival following three weeks of intravenous nicotine self-administration at a mean dose of 0.2 to 0.25 mg/kg (Wei et al., 2012). Finally, it has been shown that 0.1 mg/kg, 0.5 mg/kg, and 1 mg/kg daily intraperitoneal doses of

nicotine for 14 days causes decreased numbers of PSA-NCAM+ cells and NeuN+ cells in the DG of PND 28 rats (Shingo and Kito, 2005). PSA-NCAM – the polysialylated embryonic form of the neural cell adhesion molecule – is considered a marker of neurogenesis as it has been shown to be expressed in newborn granule cells (Seki and Arai, 1991, Seki, 2002, von Bohlen und Halbach, 2011). In summary, most of the studies evaluating nicotine's effect on neurogenesis have concluded that nicotine decreases hippocampal neurogenesis.

The mechanism by which nicotine effects adult hippocampal neurogenesis is unknown, but thought to be related to its effects on the cholinergic system (Nixon et al., 2011). The cholinergic system is well known to be associated with cognitive function and, as previously discussed, hippocampal neurogenesis is also thought to be necessary for hippocampal functions such as learning and memory (Eichenbaum, 2004, Imayoshi et al., 2008). Multiple approaches have been taken to determine the influence of the cholinergic system on adult neurogenesis. In one study, an immunotoxin was infused into the lateral ventricle of rats in order to lesion cholinergic neurons located in the cholinergic basal forebrain that send projections to the hippocampal DG (Cooper-Kuhn et al., 2004). Lesioning of these neurons resulted in decreased neurogenesis in the GCL and increased apoptotic cells in the SGZ; therefore, the authors of this study hypothesized that cholinergic input promotes cell survival. Moreover, when rats were treated with donepezil, an acetylcholinesterase inhibitor (AChEI) that increases levels of acetylcholine (ACh), neurogenesis in the DG was increased through increased cell survival (Kotani et al., 2006). Therefore, it can be hypothesized that nicotine acts to decrease hippocampal neurogenesis through its desensitization of nAChRs and the resulting decreases in cholinergic activity (Nixon et al., 2011).

Based on the finding from Experiment 1 that cell proliferation in the SGZ of the hippocampal DG peaks on day 7 of abstinence from alcohol (T7) and the knowledge that

most people start consuming alcohol and smoking tobacco during adolescence, the goal of Experiment 2 was to examine the effect that nicotine co-administration has on cell proliferation at this time point during abstinence from both alcohol and nicotine. As previously discussed, reactive neurogenesis has been shown to occur following events that induce cell death, including alcohol, ischemia, and seizure. Nicotine has also been shown to induce cell death in some animal models. The studies by Abrous et al. in 2002 and Jang et al. in 2002 that were just discussed showed that nicotine induced cell death in the DG at the same doses that affected neurogenesis. Furthermore, another study showed increased TUNEL-positive cells – a measure of apoptotic cell death – in the hippocampus of adult rats administered 0.6 mg/kg nicotine intraperitoneally daily for 21 days (Demiralay et al., 2008). A study conducted in adolescent rats that compared the effect of nicotine dose and route of administration on neurotoxicity showed that 0.3mg/kg doses of nicotine administered subcutaneously twice daily for one week beginning at PND 30 – a model similar to ours – showed neurotoxic effects in the hippocampus (Abreu-Villaca et al., 2003). The authors of this study evaluated neurotoxicity by assessing cell packing density, cell size, cell number, and neurite projections via fluorescent dye-binding and modified dot-immunobinding techniques. Finally, preliminary, unpublished data from our lab shows that our model of nicotine administration in adolescent rats potentiates ethanol-induced cell death in the entorhinal cortex, a brain region that projects to the hippocampus as measured by FluoroJade B. Although these studies demonstrate nicotine toxicity, many other studies support nicotine's role in neuroprotection. As neurogenesis has been shown to be acutely decreased by both alcohol and nicotine during use and reactively increased during abstinence from alcohol, we hypothesized that nicotine increases reactive hippocampal neurogenesis during abstinence and has an additive effect on reactive neurogenesis during abstinence when co-administered with alcohol.

Methods

In this experiment, a total of 46 adolescent male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) were used. Rats were group-housed and handled identically to rats in experiment 1 as described in Chapter 2. PND 29 rats were treated with ten days of either nicotine or saline with the modified Majchrowicz binge ethanol treatment overlapping with the last four days of nicotine treatment (see Figure 3.1). Binge ethanol administration and tail blood collection for BEC analysis were conducted as described in detail in Chapter 2, except for chow availability (discussed below). There were five treatment groups: ethanol diet plus nicotine injection (Ethanol/Nicotine), ethanol diet plus saline injection (Ethanol/Saline), control diet plus nicotine injection (Control/Nicotine), control diet plus saline injection (Control/Saline), and an *ad libitum* group (Table 3.1). Rats in the *ad libitum* group did not receive diet or injections; they had free access to water and standard rat chow throughout the whole experiment and were handled only while being weighed. All animals received BrdU injections (300mg/kg; i.p.) and were sacrificed on day seven of abstinence (T7) as described in Chapter 2. Two cohorts of animals were run for this experiment; Cohort 2 underwent the treatment model approximately nine months after Cohort 1. The experiment was run in two cohorts due to its size with the intention of combining them for analyses.

Figure 3.1: Experiment 2 Timeline

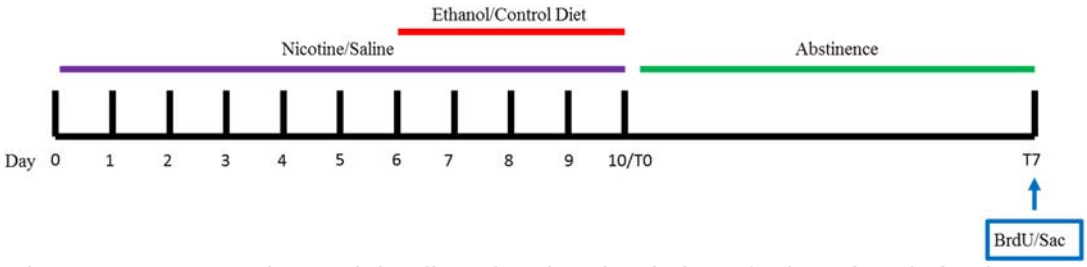


Figure 3.1 An experimental timeline showing the timing of ethanol and nicotine administration, BrdU labeling of cell proliferation and euthanasia during abstinence.

A sterile 0.3 mg/mL nicotine solution (pH=7.4) was prepared fresh, daily from (-)-nicotine liquid (Sigma-Aldrich, Saint Louis, Missouri) and 0.9% NaCl. Nicotine was injected subcutaneously as a 0.3 mg/kg dose and was given three times a day on the same schedule as ethanol administration beginning on postnatal day 29. An equal volume of 0.9% NaCl (normal saline; 1 mL/kg) was administered subcutaneously in control groups.

In Cohort 1, one rat from the ethanol/nicotine group died during the period of withdrawal monitoring; however, the death was most likely a delayed result of gavage error or severe ethanol intoxication as the animal did not show any behavioral signs of withdrawal. One rat from the control/nicotine group died during the four day binge period from gavage error.

Cohort 2 underwent the same nicotine/ethanol treatment paradigm as Cohort 1; however, Cohort 2 received rat chow during the four days of ethanol administration while Cohort 1 had no access to food during this period. A problem with excessive weight loss in adolescent rats during the 4 day binge period was noticed between the time that Cohort 1 and Cohort 2 were run. Therefore, Cohort 2 was given rat chow during the binge in order to combat the weight loss. On the first and second days of ethanol exposure, 10 grams of rat chow were placed into the bottom of the cage every day at the 3pm time point so that every rat in all groups (except the *ad libitum* group) received approximately 5 grams of food per day. All of the rat chow was eaten on both days, so on the third day of ethanol exposure 16 grams of rat chow was placed into the cage. All of the rat chow was again eaten, so on the fourth and final day of ethanol exposure, 20 grams of rat chow was placed into the cage. Additionally, 8 hours after the last dose of diet and injections were administered, all rats (except those in the *ad libitum* group), were given 20 mL/kg of control diet to prevent additional weight loss during the withdrawal period. No rats from Cohort 2 died.

Table 3.1: Experiment 2 – Experimental Design

Group	Ethanol/ Nicotine	Ethanol/ Saline	Control/ Nicotine	Control/ Saline	*Ad libitum*
Cohort 1	n=5	n=5	n=5	n=5	n=4
Cohort 2	n=5	n=5	n=4	n=4	n=4

Table 3.1 Original number of animals within each group in both cohorts.

BrdU immunohistochemistry was performed in the manner described in Chapter 2. The same primary antibody was used at a concentration of 1:4400. The only procedural difference in this experiment is that tissue sections were not counterstained with cresyl violet as DAB staining was dark enough to allow the GCL and other brain regions to be easily defined.

Statistical Analyses

Data were analyzed and graphed using Prism Version 6.05 (GraphPad Software, Inc. La Jolla, CA). Data are reported as mean ± standard error of the mean. Results were considered statistically significant if $p<0.05$. The Mann-Whitney U test was used to compare intoxication scores and mean and peak withdrawal scores between the ethanol/nicotine and ethanol/saline groups within each cohort; Mann-Whitney U tests were used in this experiment because only two groups were being compared, while Kruskal-Wallis tests were used in Experiment 1 because four groups were being compared. A student's t-test was used to compare BECs and daily ethanol doses between these groups. BrdU+ cell counts were analyzed by two-way ANOVA followed by a Tukey's post hoc test. BEC and daily ethanol dose correlations with BrdU+ cell counts were analyzed by Pearson correlation; all other correlations of subject data with BrdU+ cell counts were analyzed by Spearman nonparametric correlation.

Results

Animal Model Subject Data

Nicotine has been shown to cause weight loss through both decreased food intake and increased energy expenditure (Audrain-McGovern and Benowitz, 2011, Mineur et al., 2011). Furthermore, as previously discussed, caloric restriction can effect adult neurogenesis. Therefore, an analysis of initial body weight and the change in body weight over the course of the ten days of nicotine administration (and ethanol administration during the last four days of nicotine exposure) was conducted for both Cohorts 1 and 2 (excluding *ad libitum* animals; Table 3.2). Initial body weights of animals in the ad libitum group in Cohort 1 were not collected; therefore, no weight data from the ad libitum group is reflected in Table 3.2 and no statistical analyses could be conducted.

Table 3.2: Experiment 2 Animal Weight Data

Group	Initial body weight (grams)	Change in body weight (%)
Cohort 1	72.6 ± 1.8	37.4 ± 1.9
Cohort 2	83.6 ± 1.9	50.6 ± 2.5

In order to evaluate the appropriateness of combining Cohorts 1 and 2 for the BrdU cell count analysis, subject data from the animal model was analyzed to verify that the model was performed similarly between the two cohorts. Mann-Whitney U nonparametric tests showed that Cohort 2 had a significantly lower mean intoxication score ($p=0.0003$), mean withdrawal score ($p=0.0002$), and peak withdrawal score ($p=0.0014$) than Cohort 1. A t-test also showed that Cohort 2 received a significantly greater daily dose of ethanol ($t_{14}=5.546$; $p<0.0001$); however, the cohorts had a statistically similar BEC (Table 3.3). The mean BECs of both cohorts are lower than expected; moreover, the intoxication and withdrawal scores of Cohort 2 are lower than expected and the daily dose of ethanol administered is higher than previously reported (Morris et al., 2010a, McClain et al., 2011, McClain et al., 2014).

Table 3.3: Animal Model Subject Data – Cohort 1 vs. Cohort 2

Group	Intoxication Score (0-5 scale)*	Dose* (g/kg/day)	BEC (mg/dL)	Mean Withdrawal (0-4 scale)*	Peak Withdrawal (0-4 scale)*
Cohort 1 (n=7)	0.96 ± 0.1	12.1 ± 0.4	281 ± 34	1.7 ± 0.2	3.3 ± 0.2
Cohort 2 (n=9)	0.27 ± 0.05	14.3 ± 0.1	257 ± 31	0.12 ± 0.03	1.4 ± 0.3

*Table 3.3 Cohort 2 had significantly lower intoxication scores, mean withdrawal scores, and peak withdrawal scores than Cohort 1. Cohort 2 also received a significantly higher daily dose of ethanol than Cohort 1; however, the cohorts did not differ in BEC. *p<0.05.*

Although differences existed between the two cohorts, the primary parameter that is critical to whether ethanol has an effect – BEC – was statistically similar between groups. Therefore, cohorts were combined as the study was designed. When the ethanol/nicotine groups and the ethanol/saline groups from both cohorts were combined, there were no statistically significant differences between the ethanol/nicotine and ethanol/saline groups in any of the animal model parameters evaluated (Table 3.4). The overall animal model subject parameters are similar – although slightly lower with the exception of daily ethanol dose – to those previously reported (Morris et al., 2010a, Morris et al., 2010b, McClain et al., 2011, McClain et al., 2014).

Table 3.4: Animal Model Subject Data – Nicotine vs. Saline

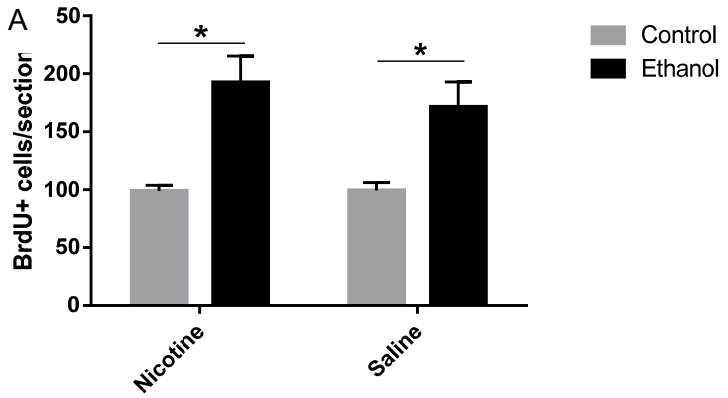
Treatment	Intoxication Score (0-5 scale)	Dose (g/kg/day)	BEC (mg/dL)	Mean Withdrawal (0-4 scale)	Peak Withdrawal (0-4 scale)
Nicotine (n=9)	0.53 ± 0.1	13.4 ± 0.4	297 ± 24	1 ± 0.3	2.2 ± 0.4
Saline (n=7)	0.61 ± 0.2	13.2 ± 0.5	230 ± 38	0.6 ± 0.2	2.3 ± 0.2

Table 3.4 When Cohorts 1 and 2 were combined, all measured animal model parameters were statistically similar between nicotine and saline groups.

Ethanol increases BrdU incorporation at T7

As discussed in Chapter 2, BrdU labels all proliferating cells in the S phase of the cell cycle; however, in order to more specifically identify proliferating NSCs, only BrdU+ cells in the SGZ were quantified. Control animals show a basal level of BrdU+ cells in the SGZ. A second analysis to determine whether it was appropriate to combine the two cohorts compared the number of BrdU+ cells in the various control groups between the two cohorts. A two-way ANOVA did not reveal any significant differences in BrdU+ cell counts in the control/saline group versus the ad libitum control group or between each cohort. Therefore, since the original intention of the experiment was to combine the two cohorts as long as there were no differences in BEC or control BrdU+ cell counts, and to combine the control/saline groups with the *ad libitum* controls, all controls were combined. All BrdU counts were then analyzed by two-way ANOVA which showed a significant main effect of ethanol on BrdU+ cell count ($F_{1,35}$=31.42; p<0.0001), but no effect of nicotine (Figure 3.2). Moreover, there was no interaction between ethanol and nicotine. Tukey's post hoc test showed that the ethanol/nicotine and ethanol/saline groups both had a greater number of BrdU+ cells in the SGZ than the control/nicotine and control/saline groups (p<0.05).

Figure 3.2: BrdU+ Cells in the SGZ of the Hippocampal DG at T7



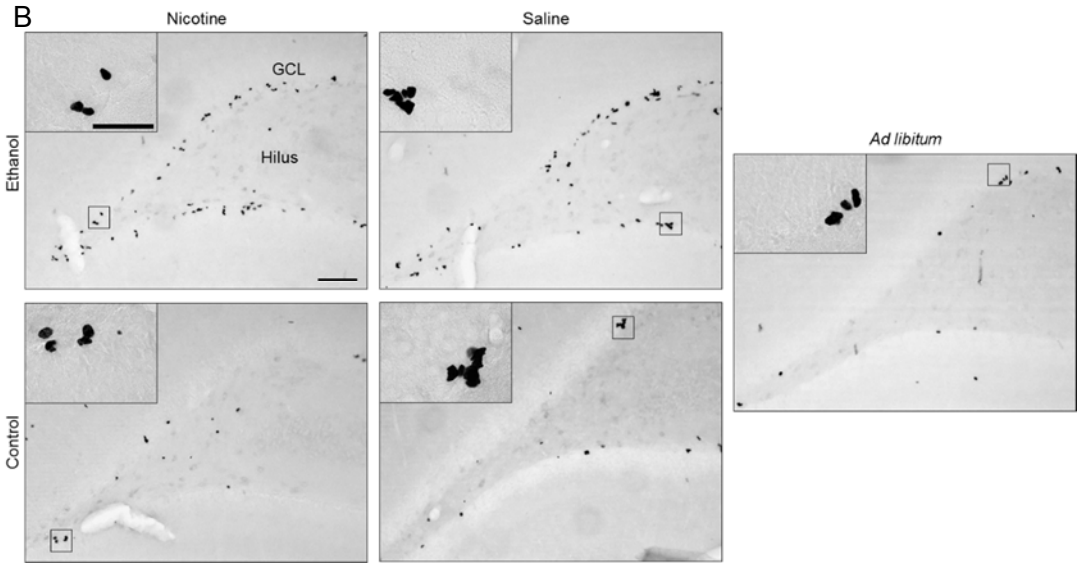


*Figure 3.2 Rats receiving ethanol showed greater numbers of BrdU+ cells in the subgranular zone of the hippocampal dentate gyrus at day 7 of abstinence than rats receiving control diet. Nicotine had no effect on BrdU+ cell counts in either the ethanol or control group, graphed in Panel A.*p<0.05 compared to each ethanol group's respective control group. Panel B shows representative images from each group in Cohort 1. Scale bar = 100µm, 40µm inset.*

Next, correlations were conducted to probe whether any of the factors involved in the adolescent nicotine/binge ethanol model, including level of behavioral intoxication, BEC, and withdrawal severity, were associated with the number of BrdU+ cells in the SGZ at T7 (Table 3.5; Figure 3.3). Spearman nonparametric correlation of intoxication score, mean withdrawal, and peak withdrawal revealed positive correlations of mean withdrawal score and intoxication score with BrdU+ cell count (Table 3.5). Pearson correlation analysis showed that neither daily ethanol dose nor BEC correlated with the number of BrdU+ cells.

Table 3.5: Animal Model Subject Data Correlations with BrdU Cell Counts at T7

Animal model parameter	Correlation coefficient (p value)
Mean withdrawal*	0.58 (0.02)
Peak withdrawal	0.36 (0.17)
Intoxication score*	0.68 (0.005)
Dose	-0.35 (0.35)
BEC	0.49 (0.055)

*Table 3.5 Correlations suggest a relationship between mean withdrawal score and mean intoxication score with the number of BrdU+ cells. *p<0.05.*

Figure 3.3: Correlations of Animal Model Subject Data with BrdU Cell Counts at T7

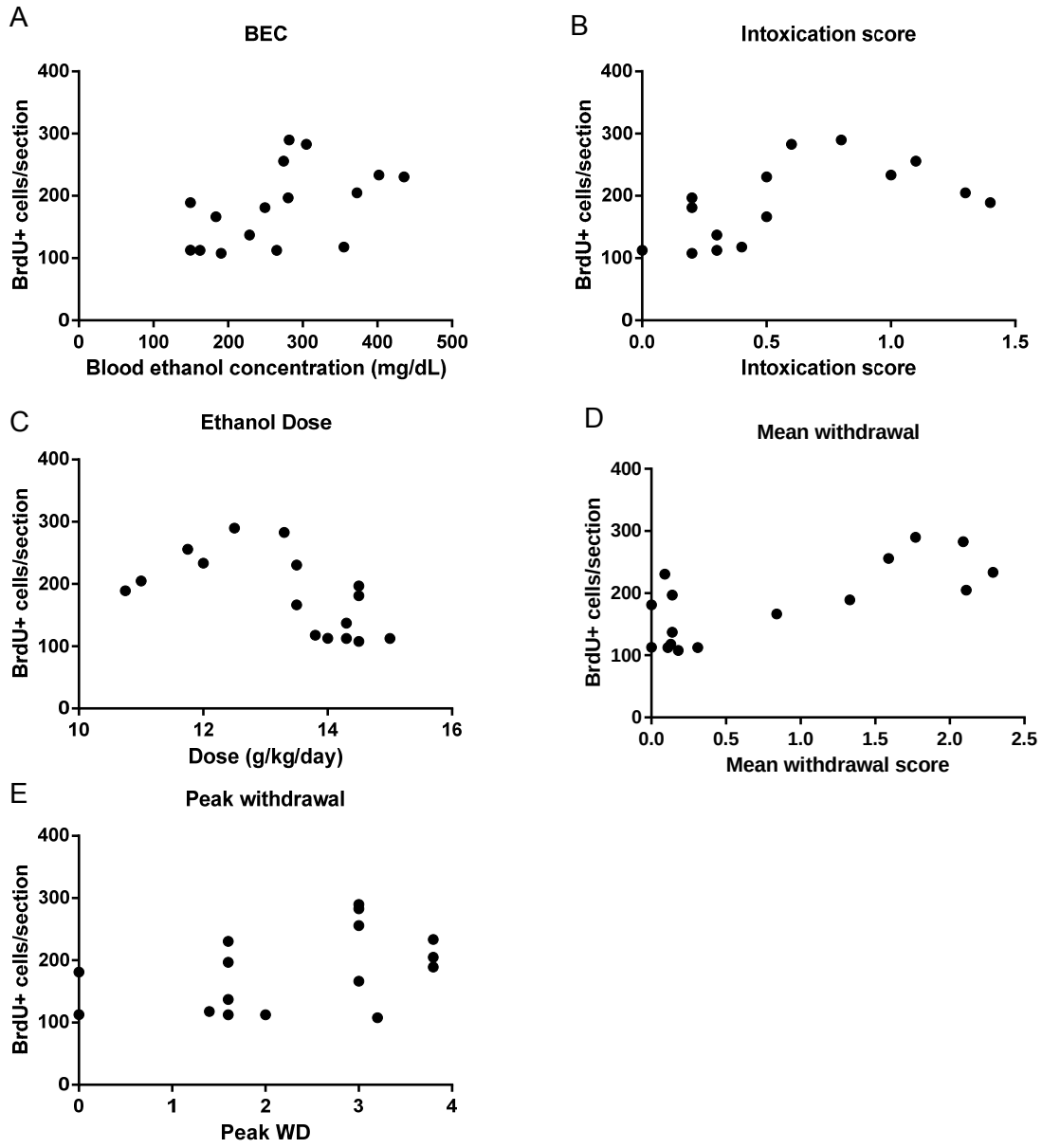


Figure 3.3 BrdU+ cell number positively correlates with mean withdrawal score and intoxication score. None of the other animal model parameters showed a statistically significant correlation with BrdU+ cell count.

Discussion

This study examined for the first time, the effect of nicotine on alcohol-induced reactive cell proliferation in a model of alcohol dependence. These data replicated the reactive cell proliferation that occurs after 4-day binge ethanol exposure (Nixon and Crews, 2004), however, nicotine exposure prior to and during the four-day binge had no effect on alcohol-induced reactive cell proliferation at T7 of abstinence. Even though previous studies reported an effect of nicotine on cell proliferation (Abrous et al., 2002, Jang et al., 2002, Scerri et al., 2006), a variety of factors may explain the lack of effect of nicotine on hippocampal cell proliferation in this study. First, this study was conducted following seven days of abstinence from nicotine, whereas all the other studies evaluated cell proliferation and neurogenesis immediately following the period of nicotine administration. From these results, it is possible to infer that acute nicotine-induced decreases in hippocampal neurogenesis do not persist to the seventh day of abstinence. Furthermore, nicotine-induced reactive cell proliferation during abstinence resulting from neurotoxicity to the developing adolescent brain may not occur on T7, the day at which it was evaluated in this study. In order to determine whether the timeline of nicotine-induced cell proliferation is different than that of ethanol-induced cell proliferation – or whether it occurs at all – an experimental design similar to that of Experiment 1 evaluating nicotine rather than ethanol could be performed. Second, the dose of nicotine chosen may not have been one that influences neurogenesis. In the seminal study where nicotine decreased neurogenesis, only the two highest doses of intravenous nicotine (0.04 mg/kg and 0.08 mg/kg) had an effect (Abrous et al., 2002). Moreover, the

study by Scerri et al. in 2006 showed that ten days of continuous nicotine exposure delivered subcutaneously by an osmotic mini pump at a dose of 4 mg/kg/day – but not 0.25 mg/kg/day – decreased cell proliferation in the DG. The authors of this study postulated that the lower dose of nicotine, which reflects the blood nicotine levels of light smokers, had no effect on neurogenesis because the low dose did not desensitize nAChRs, whereas the higher dose did. It is unknown whether the dose and administration schedule of nicotine in this study desensitized nAChRs. In order to evaluate whether our chosen dose and administration schedule acutely influences neurogenesis in the manner described by the other studies, animals could be injected with BrdU on the last day of nicotine administration and euthanized. This experimental design would make it possible to determine whether acute decreases in cell proliferation occur in the DG following ten days of thrice daily 0.3 mg/kg subcutaneous nicotine injections. However, preliminary, unpublished data from our lab shows that doublecortin, a marker of immature neurons, is decreased by nicotine immediately following our nicotine/ethanol administration model. Therefore, preliminary data with DCX shows that our nicotine administration model potentially acutely reduces neurogenesis during exposure. Furthermore, a different model of nicotine exposure may produce changes in neurogenesis not seen with our model. For example, although other models of nicotine exposure have resulted in decreased hippocampal neurogenesis, a model of extended access nicotine exposure with periodic deprivation produced an increase in immature hippocampal neurons (Cohen et al., 2015). In this model, adult male rats were provided with 0.03 mg/kg doses of nicotine to intravenously self-administer 21 hours per day for four days and then denied nicotine access for three days. The cycle was repeated for a total of 14 weeks. This model of nicotine administration was chosen by the authors because it has been shown to produce an increased motivation for nicotine self administration following deprivation as a result of nicotine dependence and withdrawal.

Therefore, it is possible to hypothesize that models of nicotine administration that induce nicotine dependence differentially affect hippocampal neurogenesis. Finally, our model of nicotine administration may not affect cell proliferation, but rather cell survival, which would result in effects on adult neurogenesis that could not be measured through our BrdU experiment that was designed to assay effects on cell proliferation. To evaluate this possibility, animals could be injected with BrdU at some point during abstinence and then euthanized four weeks later. BrdU cell counts could then be used to determine whether more proliferating cells survived for four weeks in animals receiving nicotine.

Importantly, animals in the *ad libitum* group showed the same level of basal cell proliferation at the time of euthanasia as animals receiving control diet via intragastric gavage and subcutaneous saline injections. This demonstrates that neither weight loss nor stress from injections and/or gavage have a long-term effect on cell proliferation in this region of the brain. This information is important as glucocorticoids, which are increased by stress, have been shown to reduce hippocampal neurogenesis in adult rats (Cameron and Gould, 1994).

Correlations revealed that mean withdrawal score and intoxication score were positively correlated with the number of BrdU+ cells in the SGZ. This indicates that the more intoxicated the rats behave, the greater the number of BrdU+ cells in the SGZ. Furthermore, as shown in Figure 3.3, the greater the withdrawal severity, the greater the number of BrdU+ cells. This correlation was not unexpected as prior studies using the binge ethanol model have demonstrated that BrdU+ cell number positively correlates with withdrawal severity in both adults and adolescents (Nixon and Crews, 2004, McClain et al., 2014). As discussed in Chapter 2, although seizures have been shown to induce reactive hippocampal neurogenesis (Parent et al., 1997), this group ruled out the role of alcohol-withdrawal seizures in reactive neurogenesis by using 10 mg/kg diazepam to decrease withdrawal severity. Again, they showed that adult rats exhibiting

very few behavioral signs of withdrawal still had increased cell proliferation at T7 (Nixon and Crews, 2004). Even though this study with diazepam was conducted in adult rats, it is expected to apply to adolescent rats as well based on the results of a study qualitatively and quantitatively comparing withdrawal from binge ethanol exposure in adult and adolescent rats that found no differences (Morris et al., 2010b).

Chapter Four: Overall Conclusions

Alcohol has been shown to be toxic to the hippocampus, a region of the brain involved in learning and memory. As evidence of this, alcoholics – or those with AUDs – demonstrate structural damage to the hippocampus and volume loss (Sullivan et al., 1995, Agartz et al., 1999, Beresford et al., 2006, Mechtcheriakov et al., 2007). Furthermore, alcohol has been shown in many animal models to induce cell death in the hippocampus and areas of the brain that project to the hippocampus (Walker et al., 1980, Obernier et al., 2002, Leasure and Nixon, 2010, Kelso et al., 2011). Although nicotine is generally considered to be neuroprotective, some groups have shown a toxic effect of nicotine on the hippocampus (Abreu-Villaca et al., 2003, Abrous et al., 2002, Demiralay et al., 2008, Jang et al., 2002). Additionally, several studies have evaluated the effects of co-abuse of nicotine and alcohol on neurodegeneration in humans through magnetic resonance imaging. Smoking worsened alcohol-induced gray matter loss in both heavy drinking alcoholics and alcoholics undergoing treatment who had been abstinent for one week (Gazdzinski et al., 2008). Moreover, both alcohol and nicotine have been shown to be particularly toxic in adolescents (Abreu-Villaca et al., 2003, White and Swartzwelder, 2004). Although alcohol may induce neurodegeneration through a variety of mechanisms (Crews and Nixon, 2009), our group is particularly interested in the contributions of adult hippocampal neurogenesis.

The impact of alcohol and nicotine on hippocampal function has not only been shown in animal models, but also in humans. The effect of nicotine on cognitive function has historically been thought to be beneficial (Levin et al., 2006). However, a study of the effect of nicotine on cognitive performance in adolescent smokers showed that adolescent smokers performed worse on tests of verbal memory than nonsmokers following 24 hours of abstinence, but performed the same as nonsmokers during periods

when they were allowed free access to cigarettes (Jacobsen et al., 2005). Furthermore, the effects of nicotine and alcohol co-administration on cognitive performance in humans have been studied. For example, one study that sampled people with AUDs involved in a clinical trial testing different behavioral therapies to treat their addiction found that chronic alcohol abuse was associated with reduced scores on neurocognitive tests. Furthermore, alcoholics who smoked performed even worse on these tests, indicating that nicotine has an additive effect on alcohol-induced cognitive impairments. However, the effect size of alcohol on cognitive impairment was very small – in fact, most alcoholics scored within normal limits – which conflicts with other studies that found significant reductions in performance on cognitive tests in alcoholics (Friend et al., 2005). Taken together, these studies and others indicate that nicotine and alcohol do in fact impact cognitive function in humans and that studies in animal models demonstrating cognitive impairment may be applicable to humans.

Mechanisms of hippocampal recovery have been suggested to exist. In one particular study, 60% of the alcoholics included initially demonstrated dysfunction in a battery of hippocampus-dependent tests that assessed verbal and nonverbal learning and visuospatial memory (Bartels et al., 2007). Following two years of abstinence, performance on these tasks was significantly improved and actually returned to normal. These data from Bartels et al. indicate that some mechanism of hippocampal recovery during abstinence must exist. The improvement in hippocampal functioning that abstinent alcoholics demonstrate could be related to reactive neurogenesis (Nixon and Crews, 2004), and as such it is very important to study this phenomenon in order to potentially develop therapies that target this process to aid cognitive recovery during abstinence. Adult neurogenesis – the process by which new neurons are born – in the hippocampus has been shown to be an important factor in hippocampal function (Gould et al., 1999, Nilsson et al., 1999, Shors et al., 2001, Imayoshi et al., 2008). Moreover, as

discussed herein, adult neurogenesis has been shown to be modulated by alcohol and nicotine. Both alcohol and nicotine have been shown to suppress hippocampal neurogenesis during use in adult and adolescent animal models (Abrous et al., 2002, Broadwater et al., 2014, Crews et al., 2006, Jang et al., 2002, Morris et al., 2010, Nixon and Crews, 2002, Scerri et al., 2006, Vetreno and Crews, 2015). However, neurogenesis has been shown to reactively increase during abstinence from alcohol (Nixon and Crews, 2004). No studies have examined the effect of nicotine on hippocampal neurogenesis during abstinence, nor have they evaluated the effect of nicotine co-administration on alcohol-induced reactive neurogenesis. Therefore, the goal of the experiments herein was to determine whether nicotine potentiates alcohol-induced reactive hippocampal cell proliferation on the day at which peak cell proliferation occurs during abstinence from alcohol.

In conclusion, this study showed that reactive cell proliferation increases on day 5 of abstinence and continues to increase through day 7 of abstinence. This discovery made it possible to examine the effect of nicotine on peak cell proliferation during abstinence from alcohol. Moreover, it is now possible to examine hippocampal brain slices at the time when reactive neurogenesis begins in order to determine the cellular mechanisms and signaling pathways responsible for reactive neurogenesis. Future work will aim to clarify that the increased cell proliferation at T5 does in fact result in increased neurogenesis. Furthermore, although the same time course of cell proliferation is hypothesized to occur in adolescent rats, future work could involve replicating this experiment in adolescent animals. Our study looking at the effect of nicotine on reactive adult hippocampal neurogenesis in adolescent rats at the point of peak cell proliferation showed that neither nicotine alone nor in combination with alcohol had an effect on cell proliferation. This result was not expected as we had anticipated that nicotine would potentiate alcohol-induced cell proliferation. Although the result was not expected, as

previously discussed, several possible explanations exist and it cannot be conclusively ruled that nicotine has no effect on hippocampal neurogenesis. Future work should involve demonstrating that our model of nicotine administration results in decreased cell proliferation during use or experimenting with different models of nicotine administration. Furthermore, coming studies could examine whether nicotine influences cell survival rather than cell proliferation during abstinence.

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Vita

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Education

Doctor of Pharmacy — May 2016
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Professional Experience

Pharmacy Intern — Feb. 2015-present
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Research Experience

Research Assistant — June 2013-present
Research Focus: Reactive adult neurogenesis in the hippocampus following exposure to nicotine and binge alcohol
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Bibliography

Hayes, D.M., Geil, C.R., Chen, K.Y., Heath, M.M., McClain, J.A., Deeny, M.A., & Nixon, K.
Reactive hippocampal neurogenesis following alcohol-induced neurodegeneration is due to activation of neural stem cells from quiescence. In revision

Abstracts

M. Heath, C. Geil, K. Nixon. Binge ethanol exposure increases hippocampal cell proliferation beginning 5 days into abstinence. — June 2014
Research Society on Alcoholism Annual Scientific Meeting; Bellevue, WA

C. Geil, **M. Heath,** K. Nixon. Binge alcohol administration does not inhibit alcohol-induced reactive proliferation. — Nov. 2013
Society for Neuroscience Annual Meeting; San Diego, CA

Honors and Awards

Merck Award — Apr. 2016
University of Kentucky College of Pharmacy

Gateway to Research Scholarship — May 2014-Aug. 2015
American Foundation for Pharmaceutical Education

Second Place Poster — Apr. 2014
Rho Chi Research Day
University of Kentucky College of Pharmacy

Student Merit Travel Award — Apr. 2014
Research Society on Alcoholism

Charles T. Lesshafft Jr. Scholarship — Apr. 2013-present
University of Kentucky College of Pharmacy

Dean's List — Dec. 2012-present
University of Kentucky College of Pharmacy

Dean's List — Dec. 2010-May 2012
University of Kentucky

Presidential Scholarship — Dec. 2010- May 2014
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