Ethanol but not Nicotine Administration during Adolescence Leads to Alterations in Neurogenesis in the Hippocampal Dentate Gyrus During Protracted Abstinence

By

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Abstract

During adolescence increased risk-taking behavior can lead to experimentation with drugs and alcohol. The two most commonly co-abused drugs are ethanol and nicotine. Adolescents are particularly susceptible to a host of alterations following drug use, such as decreases in neurogenesis. Neurogenesis is the production of new neurons and consists of four stages: proliferation, differentiation, maturation, and survival. One area of the brain where neurogenesis occurs is the hippocampus, which plays an essential role in spatial memory. To examine the separate and combined effects of ethanol and nicotine on proliferation, adolescent male Sprague-Dawley rats were administered nicotine (0.3 mg/kg; s.c.) or saline every 8 hours for 10 days. Animals were also given ethanol (25% in nutritionally complete diet; oral gavage) or a control diet containing dextrose on the final 4 days of injections. An unhandled control condition was added to examine potential influences of administration procedures. Animals went through a withdrawal period of 17 days. During withdrawal, animals performed a behavioral test of spatial memory, the Morris water maze. Animals were perfused transcardially and brains were extracted. Brain tissue slices were stained using Ki67, and newly born neurons along the subgranular zone (SGZ) of the dentate gyrus were quantified. During extended withdrawal, ethanol administration lead to decreased proliferation in the SGZ. An effect of nicotine or an interaction between ethanol and nicotine was not observed on proliferation. This study provides some insight into the effects of binge type ethanol consumption and nicotine use like that of a light smoker.

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Introduction

Throughout life many mammals, humans included, will continuously produce new neurons via a process known as neurogenesis (Zhao, Deng, & Gage, 2008). Adolescence is associated with a higher rate of neurogenesis compared to later stages of life (Nixon et al., 2010). Adolescence is also a period of exploration, with an increased likelihood of experimentation with drugs and alcohol (Nixon et al., 2010). Unfortunately, adolescents are also more vulnerable to the neurotoxic effects of drug use, such as decreases in neurogenesis (Nixon et al., 2010). Two of the most commonly used drugs among teens are alcohol and nicotine (NSDUH, 2013). Substance Abuse and Mental Health Services Administration (SAMHSA) has found that about 2.9 million adolescents in America admit to current alcohol use, while more than 1.6 million adolescents admit to cigarette use in the past month (NSDUH, 2013). Furthermore, people who consume alcohol have an increased likelihood of nicotine use, with alcoholics being close to three times more likely than the general population to be smokers (Lê et al., 2014). However, many of the neurobiological effects of combined alcohol and nicotine use have yet to be elucidated.

Neurogenesis

Neurogenesis is a broad term that incorporates several processes including cell proliferation, differentiation, maturation, and integration into existing neural circuitry (Zhao, et al., 2008). Postnatal neurogenesis was initially observed in various species during the 1960s (Altman & Gopal, 1965). Until recently, it was believed that neurogenesis did not occur in the adult human brain. Eriksson et al. (1998) were the first to demonstrate neurogenesis in the hippocampus of adult humans by examining postmortem brains of cancer patients who received bromodeoxyuridine (BrdU) injections as part of their treatment. BrdU is a thymidine analog with

a half-life of 30 minutes. As a thymidine analog, it replaces thymine during DNA replication and is incorporated into newly created DNA of cells undergoing cell division (Nixon et al., 2010). Since Eriksson's work in 1998, neurogenesis in the adult human brain has been repeatedly verified with its existence no longer in doubt (Aberg et al., 2005; Laplagne et al., 2007; Nixon et al., 2008).

While disagreement surrounds whether neurogenesis occurs throughout the entire adult brain, there are two areas of the brain that clearly demonstrate neurogenesis: the subgranular zone (SGZ) of the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Aberg et al., 2005). Neural stem cells (NSC) are cells in the brain that are capable of replication in order to create new daughter cells with the potential to become either neurons or glial cells (Zhao et al., 2008). While NSCs are located throughout the brain, it is likely that there are microenvironments created within the SGZ and the SVZ that permit neurogenesis. Researchers point to glial cells, specifically astrocytes, as the primary creator of the microenvironment (Morrison & Spradling, 2008). Though it clearly occurs in both regions, the processes of neurogenesis in the SGZ and the SVZ differ from each other in a number of ways, such as types of NSCs, migration distance, and ultimate function (Zhao et al., 2008).

Neurogenic Regions

Cells born in the SVZ originate from the lateral ventricles and migrate large distances through the rostral migratory stream before being incorporated into the olfactory bulb circuitry (Zhao et al., 2008). The new neurons in the SVZ will become similar to mature olfactory bulb neurons in structure and function at close to 4 weeks of age (Zhao et al., 2008). These neurons will first develop functional gamma-aminobutyric acid (GABA) receptors, then the cells will begin to develop dendrites and glutamate receptors, with all of these events occurring within the

first 2 weeks of their birth (Zhao et al., 2008). According to research, a greater variety of olfactory experiences positively impacts cell survival and integration in the olfactory bulb, while an environment that is deprived of many different odors decreases cell survival (Alonso et al., 2006). Moreover, exposure to a large variety of odors alone is not enough to increase neurogenesis. Olfactory learning, such as an odor discrimination task, is a critical component that enhances cell survival (Alonso et al., 2006) and dendritic arborization (Zhao et al., 2008).

Neurons born in the SGZ move a much shorter distance compared to the SVZ. In this area, cells are born in the SGZ of the dentate gyrus and migrate inward into the granule cell layer of the hippocampus (Zhao et al., 2008). The maturation of new neurons can be determined by examining how the neurons behave when exposed to various neurotransmitters or stimuli, as compared to pre-existing neurons. Newly born neurons of the SGZ generally resemble mature neurons after 2-4 weeks (Zhao et al., 2008). New neurons of the SGZ at first show a depolarizing response when in contact with GABA, but after several weeks, begin to behave more like mature neurons and hyperpolarize in response to GABA (Zhao et al., 2008). At 4 weeks of age, neurons of the SGZ are similar to existing neurons of the granule layer, but continue to develop and can still become differentially activated by experience related influences (Kee et al., 2007). While neurons can take more than 6 weeks to fully mature, survival and integration into the existing neural circuitry is generally determined within the first 3 or 4 weeks (Zhao et al., 2008). Kee and colleagues (2007) demonstrated that as neurons mature they become more activated in response to hippocampal-dependent learning, such as spatial memory, compared to pre-existing neurons. They used a marker of neuronal activity, c-fos, in conjunction with BrdU to determine that at 6 to 8 weeks of age, these neurons become much more likely than mature neurons to become incorporated into spatial memory networks.

Hippocampal dependent learning tasks

The hippocampus plays a critical role in the encoding of memories, and as such investigations were conducted into the function of hippocampal neurogenesis (Bruel-Jungerman, Rampon, & Laroche, 2007). Neurogenesis appears to influence many tasks that rely on the hippocampus, such as contextual fear conditioning, the Morris water maze (MWM), and trace eye-blink conditioning (Balu & Lucki, 2009). It is possible that neurogenesis may play a role in most hippocampal dependent functions (Zhao et al., 2008). Furthermore, factors such as environmental enrichment and exercise, which improve performance on these tasks, also increase neurogenesis in the hippocampus (Balu & Lucki, 2009). In addition, factors that decrease neurogenesis also appear to decrease performance on these hippocampal-dependent learning tasks (Balu & Lucki, 2009). Positive correlations between basal levels of neurogenesis and MWM performance provide additional support that neurogenesis plays an important role in hippocampal functioning (Bruel-Jungerman et al., 2007). This indicates that neurogenesis as a whole affects these memory tasks. Many of the underlying mechanisms of neurogenesis remain unknown (Bruel-Jungerman et al., 2007). A variety of experimental methods have been used to alter neurogenesis to determine how neurogenesis influences hippocampal-dependent learning (Bruel-Jungerman et al., 2007). One such protocol is the use of irradiation, where x-ray irradiation is focused at the hippocampus. While this procedure reduces neurogenesis, the effects may be more widespread, leading to changes in performances from damage to surrounding areas (Zhao et al., 2008). Other protocols have selectively destroyed the NSCs through non-invasive genetic approaches. The genetic modification method appears to impair contextual fear conditioning, but have no effect on spatial memory (Balu & Lucki, 2009). In a third model, which involved the ablation of nestin positive neural precursors, led to a deficit in spatial

learning and memory (Balu & Lucki, 2009). It is likely that each method of interference of neurogenesis alters neurogenesis differently, leading to the differences in impairment (Bruel-Jungerman et al., 2007).

Ethanol

Alcohol has many acute and chronic behavioral effects. Acute effects of alcohol include disturbances of motor control, poor decision-making, and even unconsciousness in larger administered doses (Van Skike et al., 2010). Chronic effects include damage to almost every body system and organ, with potential for developing an alcohol use disorder (Novier et al., 2013). An alcohol use disorder as defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM) 5 is “a problematic pattern of alcohol use leading to clinically significant impairment or dis-tress” (p. 490). Many of the behaviors outlined in the DSM 5 are related to inability to stop alcohol use, consuming large quantities of alcohol, and general negative effects on overall life quality. Long-term effects of ethanol also include various cognitive deficits, many of which are functions of the hippocampus, such as decreases in spatial memory, declarative memory, and impulsivity (Nixon, Pauly, & Hayes, 2011).

Pharmacokinetics and pharmacodynamics

In general, ethanol is consumed by drinking alcoholic beverages. Once consumed, the stomach and upper intestines rapidly absorb ethanol. Ethanol crosses membranes in the body quite readily, entering the brain as well as most other tissues (Mumenthaler, Taylor, O’Hara, & Yesavage, 1999). Ethanol is known to have overall inhibitory effects on the brain (Criswell et al., 2003). Ethanol exerts its influence on the brain through changes in the functioning of various types of neurotransmitters and receptors. Researchers have implicated glutamate and GABA as the major neurotransmitters that are responsible for the cognitive and behavioral effects of

ethanol. However, ethanol is also able to influence dopamine, serotonin, and other various neurotransmitters (Cosgrove et. al., 2014; Hosp, Nolan, & Luft, 2015; Roberto et al., 2004). GABA is believed to be the primary cause of the neural inhibition (Cosgrove, et. al., 2014). The inhibition produced by ethanol may be restricted to only some brain regions, such as the limbic system (Roberto et al., 2004). It is believed that this neural inhibition of the various structures in the limbic system results in effects such as reduction in motor control, sedation, and impaired cognition (Novier et al., 2013). Furthermore, the neurotransmitters affected by ethanol play important roles in neurogenesis such as cell maturation (Zhao et al., 2008).

A variety of ethanol administration protocols have been developed to model various forms of ethanol intake such as social, light, and heavy use. When examining effects of high ethanol intake, animals will generally not voluntarily consume enough ethanol to reach blood ethanol levels equivalent to those often seen in human drinkers (Meinhardt & Sommer, 2015). Therefore, some researchers began using a forced administration binge model, which is able to reliably reach desired levels of blood ethanol. One major difference between humans and rats is metabolic rate. In general, rats have a faster metabolism than humans (Meinhardt & Sommer, 2015). To reach clinically relevant levels of blood alcohol that are representative of those in humans, rats must be given higher amounts of alcohol per unit of body weight, which in general is around 9 to 10 g/kg/day ethanol (25% w/v) (Crews, Braun, Hoplight, Switzer, & Knapp, 2000).

Alcohol and neurogenesis

Studies that have employed a binge model of ethanol use similar to Majchrowicz (1975) generally find consistent results in relation to neurogenesis. Specifically, it has been found that the number of cells going through apoptosis, as evidenced by amino cupric silver stain, peaks

during the final day of a 4-day binge procedure (Crews & Nixon, 2009). Next, the number of cells going through apoptosis decreases over the course of approximately a week (Crews & Nixon, 2009). Generally, once committed to cell death, it can take from 1 to 3 days for the cell to fully die (Switzer III, 2000). However, during the first week of abstinence, there are two major bursts in neurogenesis. The first burst of neurogenesis occurs on the second day and is thought to be an increase in proliferation of microglia (Nixon et al., 2008). The second burst occurs at approximately the seventh day post ethanol binge. The second burst of neurogenesis consists of an increase of proliferation of neurons within the SGZ (Nixon et al., 2008). This burst of neurogenesis occurs as glia remove the materials left from binge-associated cell death (Crews & Nixon, 2009). As stated above, glial cells may be responsible for the promotion of adult neurogenesis by creating microenvironments (Zhao et al., 2008). Thus, it is possible that ethanol disrupts the microenvironments. During abstinence, the glial cells recreate the microenvironments, which leads to the later burst in neuron production (Crews & Nixon, 2009).

GABA has been proposed to be one of the major neurotransmitters that is influenced by ethanol to produce changes in neurogenesis (Nixon et al., 2011). GABA is believed to have a direct influence on differentiation of certain NSCs within the hippocampus. Research conducted by Tozuka and colleagues (2005) demonstrated that GABA can cause some NSCs to produce more NeuroD, a neuronal differentiation factor. In their study, they conducted several *in vivo* manipulations of the two major types of NSCs contained in the SGZ. These NSCs are called type one and type two. Type two NSCs were influenced by the presence of GABA to depolarize and lead to an action potential. The cells involved did not respond in a similar manner to other neurotransmitters, implicating changes in GABA functioning as one of the mechanisms by which ethanol influences neurogenesis (Tozuka et al., 2005).

Research conducted by Roberto et al. (2004) suggests that ethanol tends to inhibit the activity of glutamate, an excitatory neurotransmitter at both N-methyl-D-aspartate (NMDA) and non-NMDA receptors. Research has yielded mixed results on whether the inhibition of glutamate exists in specific brain regions like parts of the limbic system and basal ganglia, or whether this inhibition occurs throughout the brain (Criswell at al., 2003). While it is uncertain how widespread the effects of ethanol are, it is certain that proliferation within the hippocampus is affected. The role of glutamate in neurogenesis appears to be more indirect. For example, neurogenesis can occur even when NCSs do not have functional NMDA receptors; however, overall levels of NMDA in the brain appear to be inversely correlated with proliferation in the SGZ (Jang et al., 2008). The exact mechanisms that mediate the role of NMDA in proliferation have yet to be determined.

Differences between adults and adolescents

Crews and colleagues (2000) assert that ethanol can impact adults and adolescents differently. There have been inconsistent results as to the extent of damage to the brain of adults who are diagnosed with alcohol use disorder; however, research on adolescents with an alcohol use disorder has shown more consistently that ethanol use leads to hippocampal degeneration (Nixon et al., 2010). There is evidence that adolescents are particularly susceptible to changes in neurogenesis, which may spill over into decreases in hippocampal-dependent learning and memory tasks (Nixon et al., 2010).

Furthermore, ethanol dose-dependently decreases proliferation of neural stem cells (NSCs) in adolescent animals (Crews et al., 2009). According to Hargraves et al. (2009), adolescents are susceptible to decreases in protein production from ethanol use, but this effect is generally absent in adults. Some of the proteins affected are involved in the cell cycle as well as

synaptic functioning, and decreases in these proteins may lead to neurodegeneration. There are also differences in behavioral reactions to ethanol consumption. For example, while adolescents are less susceptible to reductions in motor control than adults, they are more susceptible to decreases in body temperature and hippocampal-related synaptic activity (Rezvani & Levin, 2004).

Nicotine

Pharmacokinetics and pharmacodynamics

Nicotine is the primary psychoactive substance in tobacco (O'Dell et al., 2013). Nicotine is easily absorbed into the body through a number of ways: skin, inhalation, gastrointestinal tract, and mucus membranes (Yildiz, 2004). While there are many other ways to consume nicotine, such as gum, dermal patches, and electronic cigarettes, nicotine is overwhelmingly consumed through the use of tobacco products, with cigarettes and cigars being the two most common (NSDUH, 2013). Chronic use of tobacco products has been associated with various health problems including cardiovascular disease, ulcers, and some types of cancer (Hurley, Taylor, & Tizabi, 2012).

Almost all the psychoactive properties of nicotine are carried out on a specific type of receptor. The nicotinic family of receptors are subtypes of acetylcholine receptors (O'Dell et al., 2013). These types of receptors are found in both the central nervous system (CNS) and the peripheral nervous system (PNS) (Gotti et al., 2009). Nicotinic receptors exert considerable influence over other neurotransmitter systems within the brain via direct interactions (Hurley et al., 2012). Nicotinic receptors have the potential to facilitate the release of several different neurotransmitters, such as dopamine, glutamate, acetylcholine, and serotonin. Of importance to the proposed research is the α7 subtype of nicotinic receptor. This receptor subtype is highly

distributed throughout the hippocampus, and believed to be important for neuronal growth, survival, and certain cognitive processes such as attention (Hurley et al., 2012). A key feature of nicotine dependence is an upregulation of nicotinic receptors; therefore, nicotine is believed to have an influence on hippocampal neurogenesis. Changes in nicotinic binding can even be seen after about a month of abstinence in adolescent animals (Slotkin, 2002). The activation of the α7 subtype of receptor may explain results observed by Abrous et al. (2002), where hippocampal neurogenesis was decreased, but neurogenesis of the SVZ was unaffected following an intermittent nicotine self-administration protocol. In their study, it appeared that the newborn neurons were unable to establish essential connections, leading to their death.

Furthermore, nicotine exerts several different effects on cognitive function (Hurley et al., 2012). A recent study by Grundey et al. (2015) has suggested that a potential acute effect of nicotine includes some cognitive enhancement; however, according to their results this enhancement is generally limited to people who are considered smokers. In addition, the smokers used in the study showed cognitive impairments from nicotine abstinence. In animal studies, it appears that the cognitive enhancement from nicotine is task-dependent, improving performance on some measures while decreasing task performance on others (Slotkin, 2002). Additionally, Abrous and collegues (2002) have stated that regular smokers who go through withdrawal have a high level of cognitive impairment. Nicotine has been reported to improve cognitive function in some cases (Grundey et al., 2015) and produced impairments in other reports (Ijomone & Nwoha, 2015). In some instances researchers have even stated that some nicotinic receptor subtypes are able to serve as a neuroprotectant when activated (Hurley et al., 2012). Two potential reasons for conflicting results may be due to differences in dosages and routes of administration. An experiment by Scerri, Stewart, Breen, and Balfour (2006) found that

differences in dosages of nicotine had different effects on spatial memory. In their study, researchers assessed performance on the MWM and the number of proliferating cells of the SGZ was also measured. Animals that received higher doses of nicotine (4 mg/kg/day) had reduced neurogenesis and longer trial times, when compared to a low dose group (0.25 mg/kg/day). A study by Ijomone and Nwoha (2015) found that similar doses of nicotine produced decreases in neurogenesis. It should be noted that in the first study animals were administered nicotine continuously during testing via a mini-osmotic pump, while animals in the second study were given subcutaneous injections.

Research by Slotkin (2002) also suggested that nicotine may impact regions of the brain differently. For example, nicotine reduces norepinephrine activity in the hippocampus, and increases catecholaminergic activity in the midbrain. Nicotine may even act as a teratogen (Slotkin, 2002). Many researchers have concluded that nicotine is damaging to growing neurons and it has the potential to influence cell differentiation, leading to long-term changes to developing neurons. According to Slotkin (2002), nicotine is able to negatively impact brain development in adolescent animals.

Differences between adults and adolescents

Often adult nicotine users who smoke cigarettes began as adolescents (Vieira-Brock et al., 2013). Researchers have concluded that adolescents are more susceptible to the development of nicotine addiction (Kandel & Chen, 2000). Some of the key features of nicotine dependence, observed in both adults and adolescents, include increased motivation to consume nicotine and negative emotional states during withdrawal and abstinence, (Cohen et al., 2015). It is believed that adolescents are more resistant to some of the negative effects associated with nicotine use and withdrawal. Some of these effects include decreases in brain reward functioning and fewer

somatic signs of nicotine withdrawal (O'Dell et al., 2006). Research has also suggested that adolescent animals metabolize nicotine more quickly than adults, and it is believed that development of nicotine dependence is positively correlated with speed of nicotine metabolism (Vieira-Brock et al., 2013).

The decreased sensitivity to negative effects associated with use and withdrawal likely contributes to increased response of adolescent animals observed in place conditioning and taste aversion studies when compared to adult animals (Torres et al., 2008). The place conditioning task, as done by Torres and colleagues (2008), consists of a 2-day procedure that is repeated several times. After receiving drug the animal is placed into one of the chambers for a period of time. Then, on the next day the animal is not given drug and is placed into a different chamber for a period of time. After repeating this several times the animals form an association between being receiving drug and one of the chambers. Then, the two chambers are placed beside each other with an opening in between. The animals are placed into the middle of the two chambers and then the amount of time each animal spends in each chamber is measured. The longer an animal spends in the chamber associated with drug use, the more rewarding the drug is believed to be for that animal. Taste aversion studies also rely on associations between drug use and the environment. In this procedure, an animal is given drug and then immediately given access to a desirable solution, such as one that contains glucose. This pairing of drug and solution is performed repeatedly. The amount of solution consumed when measuring is the dependent variable, usually performed when not given drug. It is believed that the less solution that is consumed, the more aversive the drug is to the animal (Sanchis-Segura & Spanagel, 2006).

Interaction between ethanol and nicotine

Often both ethanol and nicotine are consumed together, with 53% of people 12 or older who are heavy drinkers also smoking in the past month, compared to the general population with only 15% smoking in the past month (SAMHSA, 2013). Additionally, 65% of current cigarette smokers reported alcohol use in the past month, compared to 48% who did not use cigarettes in the past month (SAMHSA, 2013). The interactions between ethanol and nicotine are poorly understood (Ceballos, 2006). Interactions between ethanol and nicotine may lead to increased use of both substances (Oliveira-da-Silva et al., 2010). When ethanol and nicotine are taken together, the two drugs may synergistically influence each other, increasing their potency (Clark & Little, 2004). It is likely that this effect is due to each drug altering the metabolism of the other (Hurley et al., 2012). It has also been proposed that these drugs may interact in a way that suppresses the adverse effects of either drug alone (Ceballos, 2006). Additionally, nicotine has been reported to have neuroprotective properties (Hurley et al., 2012). The neuroprotective properties of nicotine may also play a role in how these two drugs interact (Hurley et al., 2012). Work by Gomez et al. (2015) has reported that animals that received a co-administration of alcohol and tobacco smoke, twice a day for 29 days, had decreases in neurogenesis. This study also found that when administered together, animals had a higher level of cotinine, a metabolite of nicotine in their blood plasma. It has also been implied that differences between short-term and long-term administration paradigms may lead to either cross-tolerance or sensitization (Hurley et al., 2012). A study conducted by Jang and colleagues (2002) found that when ethanol and nicotine were taken together, the number of BrdU-positive cells in the dentate gyrus of the hippocampus was decreased compared to control animals. The decrease in neurogenesis was found to be greater in magnitude than the decreases in neurogenesis that were observed in

animals that received either drug alone. This finding lends support to the conclusion that when ethanol and nicotine are taken together, they lead to a greater decrease in neurogenesis overall.

At the time of writing this article, there are only a handful of published research papers that attempt to examine the interaction between ethanol and nicotine in relation to their impact on neurogenesis. Additionally, the interactions mentioned above may only represent a portion of all the interactions between the two drugs. This study will attempt to help shed some more light on the issue.

Current Study

In the current study, neural cell proliferation was assessed in adolescent Sprague-Dawley rats. The animals used in this study were subjected to a dual administration procedure of ethanol and nicotine. The animals then went through a period of protracted abstinence to investigate potential long-term changes in proliferation in the subgranular zone of the hippocampal dentate gyrus. Finally, proliferation in the subgranular zone was assessed from slices of brain tissue, using an immunohistochemical staining procedure for Ki67. Ki67 is a protein found in cells that are going through active phases of the cell cycle, including G1, S, G2, and M phases (Scholzen, & Gerdes, 2000; Gerdes et al., 1984). The number of Ki67 positives cells was measured using cell profile counting with data being expressed as Ki67+ cells/section +/- SD.

One of the previous experiments in our lab has found evidence that adolescents who received either nicotine or ethanol had suppressed proliferation compared to control animals immediately following drug administration (Hayes lab, unpublished results). While the animals used in the previous study had a trend towards an additive interaction between ethanol and nicotine on proliferation suppression, the results failed to reach statistical significance. A more recent analysis of the MWM data has revealed substantial behavioral implications for dual drug

administration. These animals demonstrated that co-administration of both ethanol and nicotine interacted to have an additive effect, which impaired their ability to perform a test of spatial memory to a greater degree than either drug alone. Performance on the MWM is a hippocampal-dependent learning task and is heavily influenced by changes in hippocampal neurogenesis (Balu & Lucki, 2009). This analysis also revealed that ethanol alone produced decreases in acquisition performance on the MWM task, as expected. Thus, the behavioral results suggest that the combination of ethanol and nicotine is leading to a greater degree of suppression of proliferation within the dentate gyrus of the hippocampus.

Design

The research design was a 2 (ethanol or dextrose) x 2 (nicotine or saline) between-subjects design, with an additional control group, designed to address potential changes in cell proliferation due directly to the stress associated with the administration of a liquid diet as well as injections. Adolescent animals (post-natal day 38) were randomly assigned to 1 of 5 conditions: ethanol and nicotine, ethanol and saline injection, dextrose gavage and nicotine, dextrose gavage and saline injection, or to an unhandled control group. Animals in the unhandled control group were yoked to the dextrose gavage. The unhandled animals received an equivalent volume of dextrose containing diet; however, these animals had free access to the diet in a water bottle at their home cage. After drug administration animals went through a withdrawal period, during which time the animals were exposed to the MWM. Following behavioral testing, tissue samples were collected and cell proliferation was measured.

Method

Animals

Male, adolescent (post-natal day 38), Sprague-Dawley rats were used in this study (*N* = 26). Animals weighed an average of 105.47g at the beginning of the study. All animals were acquired from Charles River laboratories (Raleigh, NC) and either bred in-house or used after becoming acclimated to the lab. Rats were kept in individual hanging cages in a humidity and temperature controlled environment at approximately 23 °C, with a 12-hour light dark cycle (lights on at 7 a.m.). Except during ethanol administration and during behavioral testing sessions, animals had ad libitum access to food and water, and body weights were monitored. All procedures were approved by Radford Institutional Animal care and Use Committee (IACUC) and conducted in accordance with National Institute of Health (NIH) guidelines. Animals were assigned to groups randomly; however, littermates were not assigned to the same experimental groups. Animals were weighed daily except for 2 days immediately following the end of drug administration, 2 days right before behavioral testing, and after completion of behavioral testing. A timeline of events can be seen in Figure 1.

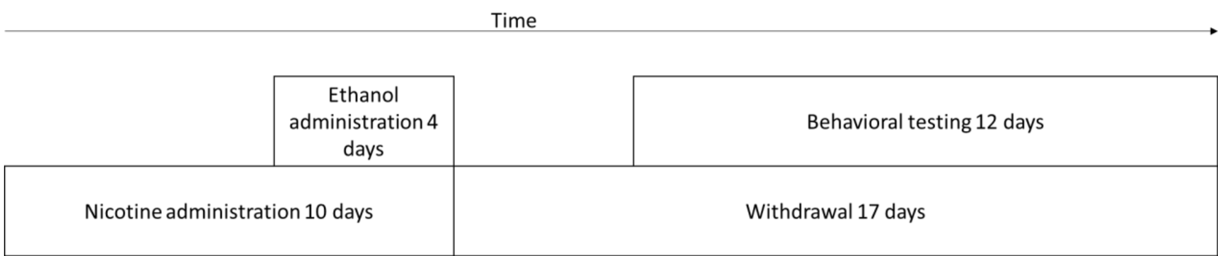


Figure 1. Timeline of study events. Nicotine or saline was administered (s.c.) for the first 10 days of the experiment. Ethanol or control diet was administered on the final 4 days of nicotine administration. After 5 days, behavioral testing (MWM) began and lasted 12 days. Tissue was collected from animals on the day following the end of behavioral testing.

Drug Administration and Behavioral Testing

Nicotine administration

Animals were given subcutaneous injections of nicotine (0.3 mg/kg) every 8 hours for 10 days (3 p.m., 11 p.m., and 7 a.m.). Administration of this dose reflects the levels observed during light smoking (Scerri et al., 2006). Control animals received equal volumes of 0.9% saline solution.

Ethanol administration

On the final 4 days of nicotine injections, ethanol (25% w/v in nutritionally complete diet or isocaloric dextrose diet) was administered via oral gavage every 8 hours starting at 3 p.m. The oral gavage procedure was performed with an oral intubation needle with a bulbous end. This is a modified procedure from an original model, which is used to emulate high levels of ethanol consumption (Majchrowicz, 1975; Crews & Nixon, 2009). The first dose is 5 g/kg and then adjusting the dose of ethanol administered depending on the animal's level of intoxication (Crews & Nixon, 2004). Behavioral scoring was used to determine level of intoxication and subsequent doses were administered in order to titrate a high BAC (Nixon & Crews, 2004). Animals were randomly assigned to either ethanol (25% w/v in nutritionally complete diet) or isocaloric dextrose diet.

Withdrawal

At the end of day 10, drug administration ceased and the animals experienced withdrawal from both of drugs simultaneously. The withdrawal from nicotine was not anticipated to be severe due to the dose selected. However, the withdrawal from ethanol warranted observation beginning 10 hours after the final dose and continuing for 18 hours. Independent observers monitored animals for a range of behaviors associated with withdrawal, such as tail twitches,

spasms, general tremors, and convulsion. The animals were observed for the first 30 minutes of each hour. The scale used ranged from 0 being still intoxicated or no withdrawal behaviors to 4 being death from withdrawal.

Memory assessment

The MWM is a test of spatial memory, working memory, and reversal learning. Animals began the MWM 5 days after the end of the drug administration procedure. A plastic pool was filled with water and contained non-toxic white paint, which made the water opaque. There was a platform placed in the water in one of four quadrants depending on task. Visual cues hung from each wall, which were held constant throughout the experiment. Spatial memory was assessed during three various phases in the MWM: acquisition, reversal learning, and spatial working memory.

The first test the animals performed is the acquisition task. This analizes the animal’s overall spatial memory. The platform was located in the south west quadrant for the duration of the task. Animals were tested for 5 consecutive days. The animals were placed in different starting quadrants that had been predetermined for the day and were measured for distance and time to reach the platform. Reversal learning was assessed on the day after the completion of the acquisition task. In this test the platform was moved to the north east corner of the pool. Each animal performed four trials to determine how quickly animals change strategies to find the platform. Finally, spatial working memory was tested via two trials per day for 5 days. During this task animals first completed a sample trial and then a test trial. The difference in time between reaching the platform in the first trial and the second trial was measured in this task. The platform was located in different quadrants for each day during this task.

Analysis of Cell Proliferation

Tissue preparation

Following completion of behavioral tasks, tissue samples were prepared for collection via transcardial perfusion. The procedure was performed on animals deeply anesthetized with sodium pentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI; 117 ml/kg). Perfusions were performed using a gravity flow perfusion system. The animals were first perfused with 0.1M phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (PFA; pH 7.4). The PBS and the PFA were prepared the day before and were refrigerated overnight at 4°C. Following blood removal and initial tissue fixation, brains were extracted and post-fixed in 4% PFA for 24 hours before being transferred to PBS for storage at 4°C until slicing. Slices consisted of coronal sections of tissue 50 microns in width. The sections spanned the entire rostral-caudal distance of limbic and associated cortices starting at approximately 0.8 mm anterior bregma to 7.6 mm posterior bregma (Paxinos & Watson, 2012) and were sectioned using a vibrating microtome (Electron Microscopy Sciences, Hatfield, PA). The samples were submerged in cryoprotectant (ethylene glycol and glycerol), which prevents crystallization, and stored in a freezer (-20 °C) until mounting and immunohistochemical staining.

Immunohistochemistry

Tissue samples were first washed in tris buffered saline (TBS) to remove any residual cryoprotectant followed by a 0.6% peroxide bath for 30 minutes to block endogenous peroxidases. After the peroxide incubation, the samples were once again washed with TBS three times, and then subjected to antigen retrieval procedures using 1x sodium citrate incubation at 65°C for 1 hour. Following another wash in TBS, the samples were incubated in a blocking solution containing 10% Triton-X 100 and normal horse serum in TBS for 30 minutes. The

samples were submerged in 1:200 mouse anti-Ki67 antibodies (Dako North America, Inc., Carpinteria, CA) at room temperature for 96 hours. After 3 rinses in the blocking solution, the tissue was incubated in horse anti-mouse secondary antibody solution (Vector Labs, Burlingame, CA; in TBS with 3% normal horse serum) for an hour. Three washes in TBS occurred and were followed by samples being incubated in avidin-biotin complex (ABC) solution. Then peroxidase detection was conducted using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a chromogen with a wash step in between the incubation in ABC and the peroxidase detection. After three more rinses in TBS, the samples were stored before final processing, which included being mounted on slides, 24 hours of time to dry at room temperature, and counterstaining to visualize neuroanatomic landmarks using a typical cresyl violet staining procedure.

Counterstaining began by submerging slides in progressively weaker solutions of ethanol for 30 seconds each: 100%, 95%, 70%, and 50%. Then the slides were submerged in distilled water for 30 seconds, followed by 3 minutes in cresyl violet solution. Next, slides were submerged in progressively stronger solutions of ethanol for 30 seconds each: 50%, 95%, and 100%. Finally, the slides were placed into xylenes for 5 minutes, before being cover slipped.

Quantification

Overall estimates of Ki67 labeled cells were calculated using a cell profile counting procedure outlined by Noori and Casimir (2011). Profile counting methodology was conducted using a BX43 Microscope (Olympus, Center Valley, PA) under a 100x oil immersion objective. All slides were masked for quantification so that the researcher was blind to experimental condition during counting. Intact coronal slices were examined throughout the entire rostral to caudal extent of the dorsal hippocampus. Specifically, every eighth coronal slice of the hippocampus was examined for Ki67+ cells and cell clusters. The number of cells expressing

Ki67 was quantified in each slice (approximately 6-8 per brain) and averaged to yield overall Ki-67+ cells/section per hippocampus. Ki67 is produced by cells that are in all active cell cycle phases (Scholzen, & Gerdes, 2000). Ki67 has advantages over other staining methods such as proliferating cell nuclear antigen (PCNA). Because proliferating cell nuclear antigen can be sensitive to fixation procedures, cells in resting phase sometimes test positive (Scholzen & Gerdes, 2000). Additionally, Ki67 staining may be applied to tissue post fixation, whereas Bromo-deoxy-Uridine (BrdU) must be injected or consumed orally before termination of the animal. Ki67 is also seen in the G1, S, G2, and M phases, whereas BrdU is only observed in cells that are currently dividing in S phase (Kee et al., 2002). Ki67 also has support from the clinical setting where it is often used as a prognostic tool for cancer (Borre et al., 1989).

Results

Body Weights

As stated by Zainuddin and Thuret (2012), nutrition directly influences hippocampal neurogenesis. Thus, body weights were taken throughout the experiment to determine if any differences in proliferation at the end could be attributed to differences in nutrition. A series of 2 (ethanol or dextrose) x 2 (nicotine or saline) analysis of variances (ANOVAs) were conducted. Body weight information is presented in Figure 2. Differences in body weights began to appear by the end of drug administration due to ethanol administration. Throughout the experiment no differences in body weight were detected between the placebo control group and the unhandled control group. The weights of animals that received ethanol were significantly lower than animals that received dextrose control diet, $F(1, 18) = 7.02$, $p = .016$. This trend continued 2 days post binge with ethanol receiving animals weighing significantly less than animals that received dextrose control diet, $F(1, 18) = 11.88$, $p = .003$. Differences in body weights were still apparent at the beginning of behavioral testing, where animals that received ethanol weighed significantly less than animals that received dextrose control diet, $F(1, 18) = 8.23$, $p = .01$. Weight differences disappeared by the end of behavioral testing such that the weight of ethanol receiving animals was not significantly different from animals that received dextrose diet, $F(1, 18) = 2.80$, $p = .11$.

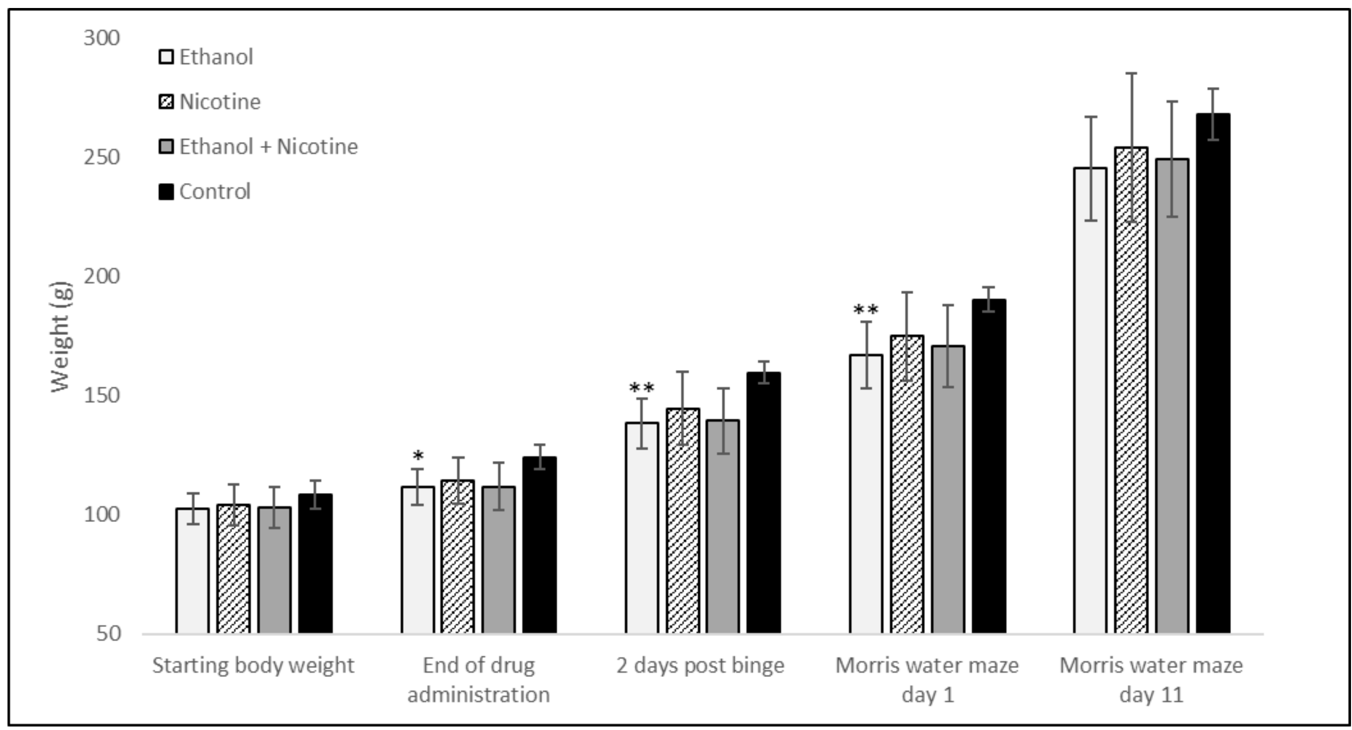


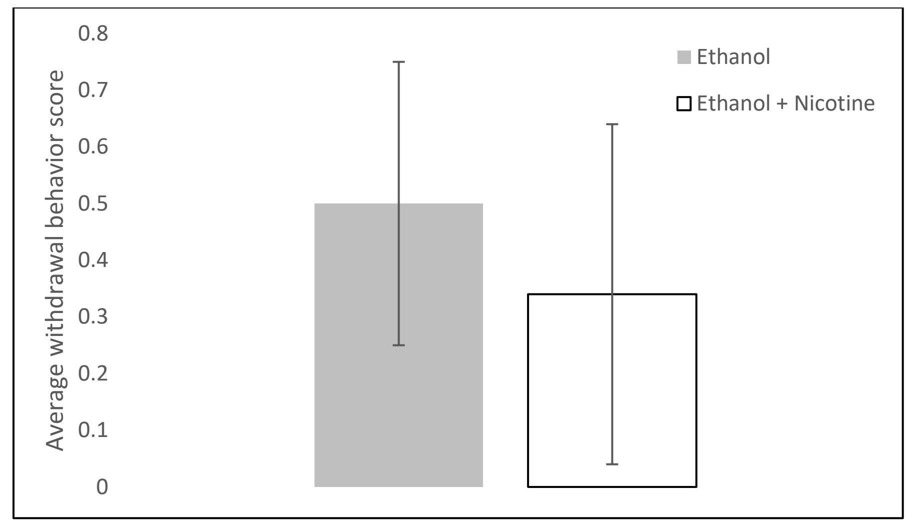
Figure 2. A series of 2 (ethanol or dextrose) x 2 (nicotine or saline) ANOVAs were conducted to determine if there were differences in body weight (in grams) between the groups of animals at various timepoints of interest during the study (±S.D.). Differences in body weights due to ethanol began to appear by the end of drug administration; however, differences in body weight disappeared by the end of behavioral testing. $*p < 0.05$. $**p < 0.01$.

Ethanol dose

High levels of ethanol exposure can produce decreases in cell proliferation (Crews et al., 2009). Therefore, an independent samples t-test was conducted to determine if there were differences in doses of ethanol between the ethanol/nicotine and ethanol/saline groups. The analysis revealed that there were no differences in the amount of ethanol received by animals receiving nicotine ($M = 12.17$, $SD = 0.34$) and those receiving saline ($M = 12.13$, $SD = 0.61$), $t(10) = .15$, $p = .89$.

Withdrawal

Independent samples t-tests were conducted to determine if there were differences in withdrawal severity between animals that received ethanol and nicotine versus those receiving ethanol alone. Higher withdrawal scores were associated with more severe withdrawal behaviors. There were similar levels in the average severity of withdrawal for dual drug exposed animals ($M = 0.34$, $SD = 0.30$) and those administered ethanol alone ($M = 0.50$, $SD = 0.25$), $t(10) = -0.99$, $p = .35$. However, animals that received ethanol alone ($M = 2.70$, $SD = 0.70$) had significantly worse peak withdrawal behaviors when compared to animals that received ethanol and nicotine ($M = 1.90$, $SD = 0.49$), $t(10) = -2.30$, $p = .045$. See Figure 3 for means and standard deviations of withdrawal behavior scores of peak and average withdrawal scores.



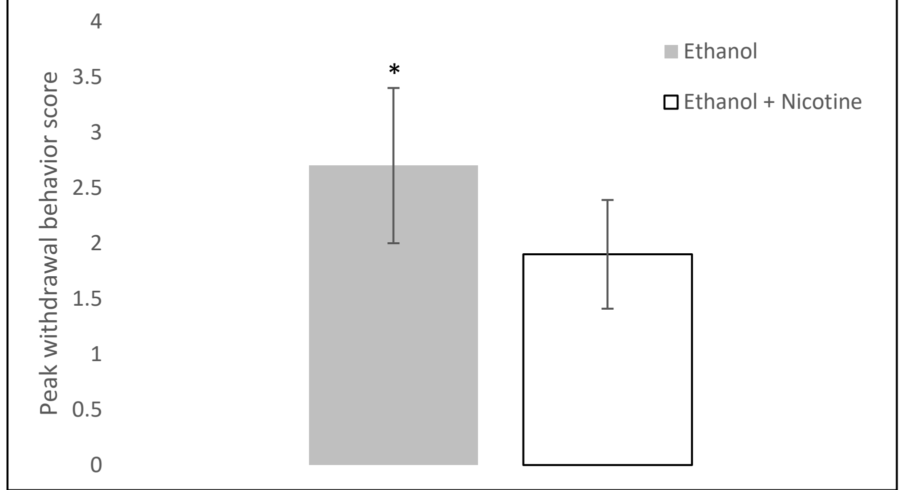


Figure 3. Withdrawal behaviors. Independent samples t-tests revealed a similar average withdrawal behavior score for animals that received ethanol only and animals receiving ethanol and nicotine (±S.D.). Higher withdrawal scores were associated with more severe withdrawal behaviors. The average withdrawal scores in the current study were quite low; scores between 1 and 2 included behaviors such as hyperactivity, tail tremors, tail spasms, and caudal tremors. Another independent samples t-test found that animals only receiving ethanol have more severe peak withdrawal behavior scores than animals receiving ethanol and nicotine. Peak withdrawal behaviors between 2.6 and 3.2 include general tremors, head tremors, and induced running. *$p < 0.05$.

Due to differences emerging in average peak withdrawal, a series of ANOVAs were run to further explore differences in peak withdrawal behavior between animals receiving ethanol only and animals receiving ethanol and nicotine. Individual timepoints were examined. There was a significant difference between the groups at 12 a.m. where animals receiving ethanol only showed greater peak withdrawal scores (*M* = 1.29, *SD* = 0.95) compared to animals receiving ethanol and nicotine (*M* = 1.90, *SD* = 0.49), $F(1, 13) = 5.08$, $p = .042$; partial $\eta^2 = .28$. No other timepoint reached statistical significance, but several timepoints had moderate effect sizes: 8 p.m. (partial $\eta^2 = .09$), 10 p.m. (partial $\eta^2 = .11$), 1 a.m. (partial $\eta^2 = .10$), 5 a.m. (partial $\eta^2 = .08$), 6 a.m. (partial $\eta^2 = .08$), 7 a.m. (partial $\eta^2 = .08$) See Figure 4 for the peak withdrawal scores over time. Means and standard deviations for withdrawal scores at various timepoints are reported in Table 1.

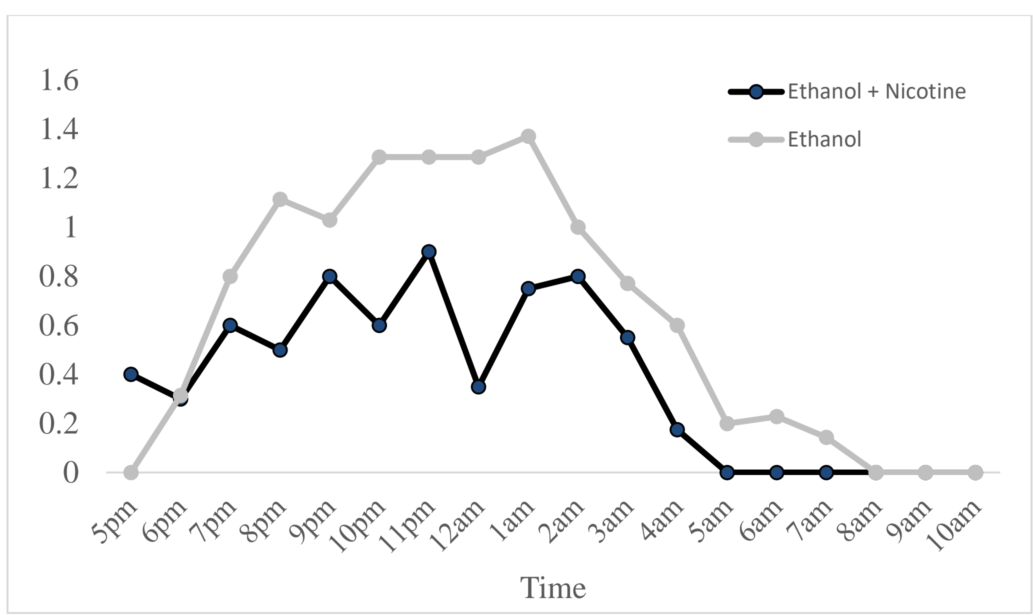


Figure 4. Withdrawal behaviors over the course of withdrawal. When additional analyses were run, the 12 a.m. timepoint was the only timepoint to reach statistical significance. However, 8 p.m., 10 p.m., 1 a.m., 5 a.m., 6 a.m., and 7 a.m. had moderate effect sizes.

Table 1. Means and standard deviations of withdrawal scores for animals in the ethanol group and animals in the ethanol and nicotine group at withdrawal timepoints.

	Ethanol + Nicotine		Ethanol	
	M	*SD*	*M*	*SD*
5 p.m.	0.40	0.74	0.00	0.00
6 p.m.	0.30	0.85	0.31	0.83
7 p.m.	0.60	1.11	0.80	1.03
8 p.m.	0.50	0.95	1.11	1.11
9 p.m.	0.80	1.13	1.03	0.81
10 p.m.	0.60	1.11	1.29	1.01
11 p.m.	0.90	1.24	1.29	1.30
12 a.m.	0.35	0.65	1.29	0.95
1 a.m.	0.75	0.81	1.37	1.16
2 a.m.	0.80	0.89	1.00	1.38
3 a.m.	0.55	0.76	0.77	0.74
4 a.m.	0.18	0.50	0.60	0.75
5 a.m.	0.00	0.00	0.20	0.53
6 a.m.	0.00	0.00	0.23	0.60
7 a.m.	0.00	0.00	0.14	0.38
8 a.m.	0.00	0.00	0.00	0.00
9 a.m.	0.00	0.00	0.00	0.00
10 a.m.	0.00	0.00	0.00	0.00

Behavioral effects

During the acquisition phase of the MWM, a main effect of ethanol was observed where animals that received ethanol took significantly longer ($M = 22.67$, $SE = 1.39$) to reach the hidden platform per trial than animals that did not receive ethanol ($M = 17.29$, $SE = 1.53$), $F(1, 18) = 6.80$, $p = .018$. There was no main effect of nicotine on time taken to reach the hidden platform, $F(1, 18) = 1.90$, $p = .18$). Additionally, an interaction occurred where animals that received both ethanol and nicotine ($M = 26.69$, $SE = 1.97$) took longer to find the hidden platform than animals that only received ethanol ($M = 18.66$, $SD = 1.97$), $F(1, 18) = 6.28$, $p = .022$. A similar pattern of results was observed for the distance the animals swam to find the hidden platform.

Ki67+ cells/section

A 2 (ethanol or dextrose) x 2 (nicotine or saline) ANOVA was conducted using the average number of Ki67+ cells/section as the dependent variable to assess cell proliferation (see Figure 5). The analysis revealed a main effect of ethanol, $F(1, 18) = 13.98$, $p = .002$, where animals that received ethanol had a significantly fewer Ki67+ cells/section ($M = 8.91$, $SD = 5.19$) than animals that received dextrose containing control diet ($M = 25.33$, $SD = 13.25$). Nicotine did not significantly affect the average number of Ki67+ cells/section, $F(1, 18) = 0.01$, $p = .92$. Additionally, the interaction between ethanol and nicotine did not significantly influence the average number of Ki67+ cells/section, $F(1, 18) = 0.51$, $p = .49$.

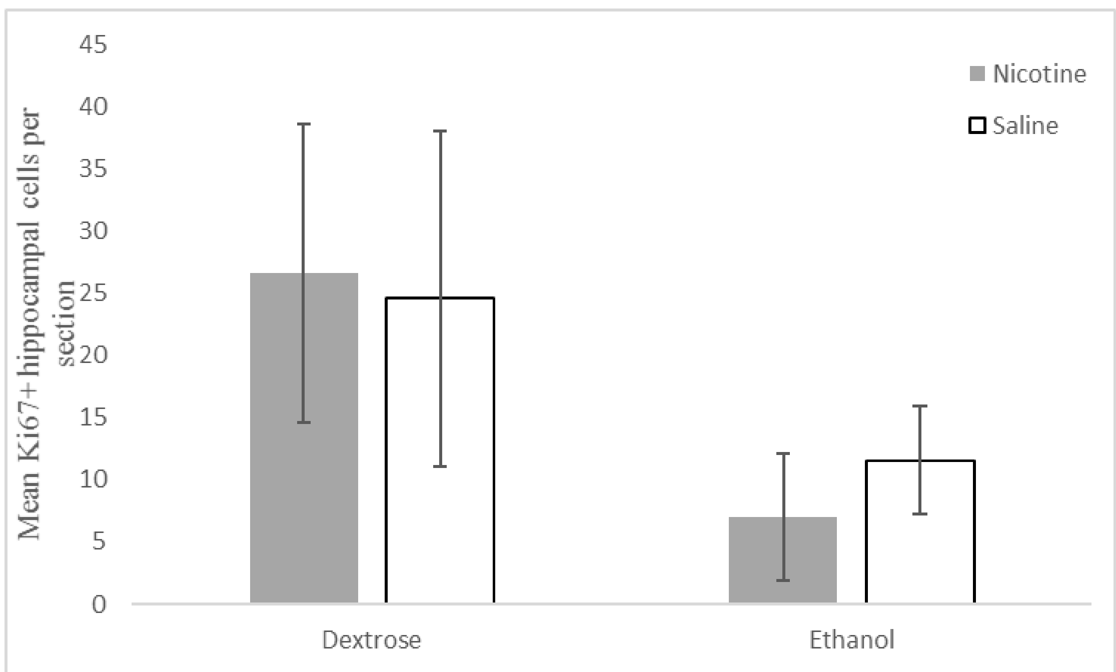


Figure 5. Mean number of Ki67+ hippocampal cells/section (±S.D.). Animals that received ethanol had significantly fewer Ki67+ cells/section on average than animals that received control dextrose containing diet. Nicotine did not significantly affect mean Ki67+ cells/section and there was no significant interaction effect.

A separate analysis was conducted to determine if stress caused by subcutaneous injections and oral gavage procedures lead to decreases in cell proliferation. Stress is known to cause decreases in neural cell proliferation (Mirescu & Gould, 2006). It is also known that the oral gavage procedure may cause anxiety in rats (Boschen et al., 2015). To determine if stress from administration procedures could have influenced proliferation, an independent samples t-test was performed between animals that received injections of saline and control dextrose-containing diet and unhandled animals, who were given free access to an equivalent volume of the same dextrose diet as the control animals. The analysis indicated that animals that received the dextrose gavage and saline injections (M = 24.08, SD = 15.72) did not differ from unhandled animals (M = 25.37, SD = 11.87), $t(6) = -0.12$ $p = .93$. The means and standard deviations for the two groups are shown in Figure 6.

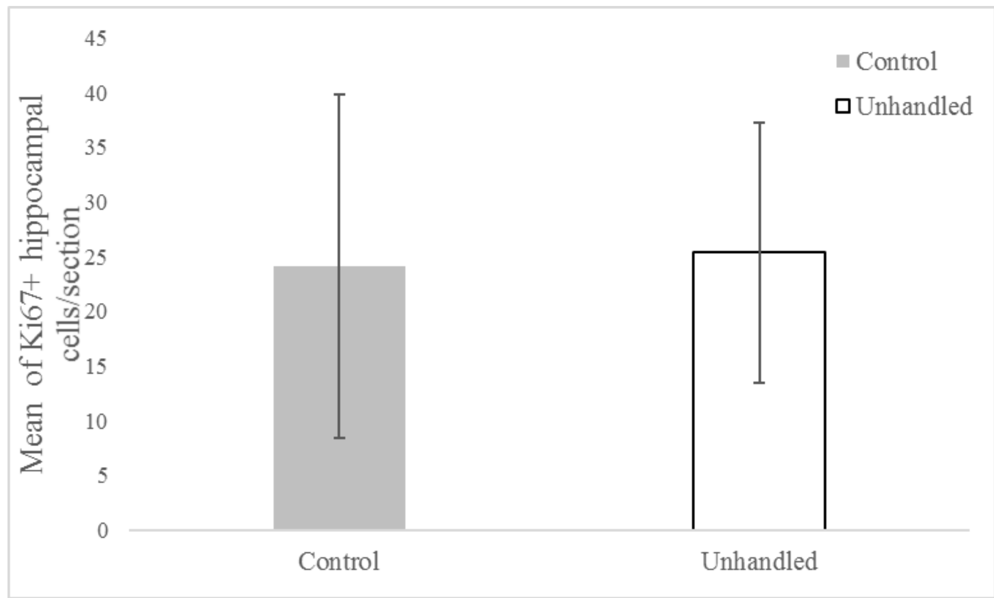


Figure 6. Mean number of K67+ hippocampal cells/section (±S.D.) control vs unhandled animals. Control animals received dextrose diet gavage and saline injections and were compared to unhandled animals that had access to equivalent volumes of dextrose diet in a bottle at their home cage. An independent samples t-test revealed no significant differences in mean number of Ki67+ hippocampal cells/section between the two groups, $t(6) = -0.12$ $p = .93$.

Discussion

The main finding of this experiment is that ethanol decreased the proliferation of neuronal cells as measured by average number of Ki67+ cells per section of hippocampal tissue following extended withdrawal. However, neither nicotine alone nor the interaction between ethanol and nicotine influenced cell proliferation during extended withdrawal. Furthermore, ethanol decreased the body weights of the animals for part of the study. Body weight was decreased from ethanol at the end of the binge through the beginning of behavioral testing, but returned to normal by the end of behavioral testing. Nicotine alone and the dual administration of nicotine and ethanol did not seem to influence body weights. Additionally, there were no differences in doses of ethanol administered to animals that received ethanol and nicotine versus receiving ethanol and saline. During the withdrawal period, previous ethanol exposure decreased performance in the MWM. Animals that had received ethanol took longer to find the hidden platform. Nicotine did not influence the time it took for animals to reach the platform. Finally, it appeared that ethanol and nicotine interacted to cause a greater deficit in MWM performance than the effects of either drug alone. The interaction between the two drugs led to animals taking a longer time to reach the platform.

Proliferation

The current study demonstrated that after a period of extended withdrawal, proliferation of progenitor cells in the SGZ of the hippocampal dentate gyrus was suppressed. Most of the neuronal damage resulting from ethanol occurs during intoxication due to inflammation, and from interruptions in neurogenesis (Crews & Nixon, 2009). It is possible that the specific model of ethanol exposure may be an important component in determining the extent of neurological damage observed following ethanol exposure. A binge model of forced ethanol administration

was employed in the current study. Previous research using this model has concluded that cell death caused by ethanol intoxication peaks on the final day of drug administration and then begins to subside during abstinence (Crew & Nixon, 2009). Neurogenesis is suppressed for approximately two days following abstinence before increasing beyond control levels (Cews & Nixon, 2009). Morris et al. (2010a) used a binge model of ethanol exposure to show that proliferation is increased 7 days after exposure compared to control animals, via BrdU labeling. In their study, there were no significant differences at 14 days after exposure when comparing ethanol exposed animals to control animals. However, another study found that 35 days after an ethanol binge procedure, differences in the cell survival and differentiation compared to control animals were observable (McClain et al., 2014). Longer exposure periods than what was done within the current study, such as an 11-month chronic exposure procedure, have produced changes in neurogenesis lasting up to 2.5 months (Taffe et al., 2010). Various models of ethanol exposure may determine how long alterations to proliferation within the SGZ of the hippocampus are observable.

One study examined ethanol dependent adult rats, using an extended vapor exposure paradigm. Animals in the study were exposed to ethanol vapor for 17 hours per day for 8 weeks. These animals had decreased proliferation within the SGZ at the end of exposure, but proliferation returned to control values after 7 days of abstinence (Hansson et al., 2010). During ethanol administration, blood ethanol levels ranged from 150 to 300 mg/dl, which is slightly lower than the levels expected from the binge exposure paradigm. Specifically, proliferation decreased immediately after ethanol administration, and was greater than control levels at 3 days of abstinence, before returning to control levels at 7 days of abstinence. However, a trend towards decreased proliferation was observed during extended withdrawal at their next timepoint

of 21 days post-administration. In the current study, reductions in cell proliferation were also observed during protracted withdrawal at 17 days after completion of drug administration.

Another study found decreases in proliferation after a similar withdrawal period as the current study. Stevenson et al. (2009) conducted a study in which adult mice had free choice between ethanol (10% v/v) and water in drinking bottles, and showed decreased cell proliferation within the SGZ at 14 days of abstinence compared to control animals. Animals had access to ethanol for a period of 4 weeks and consumed approximately 17.8 g/kg/day of ethanol, leading to average blood ethanol concentrations of 112.8 mg/dl, expectedly much lower than in a binge model. Then the animals went through a period of abstinence of 0, 2, or 14 days. While no differences in hippocampal proliferation were observed between the control animals and those with 0 or 2 days of abstinence, proliferation was significantly decreased at day 14 of abstinence. The results of this study provide supporting evidence to what was observed in the current study; ethanol can influence proliferation during extended withdrawal, even if proliferation returns to or above control levels during shorter withdrawal periods.

Nicotine did not have an influence on neurogenesis in the present study. It was predicted that nicotine would reduce proliferation. However, the observation that nicotine did not influence proliferation is not entirely surprising, due to quite a few studies reporting differing results (Ijomone, & Nwoha, 2015; Scerri et al., 2006). The dose of nicotine selected in the current study was approximately that of light smoking in humans. In various studies, nicotine dose played a large role in the effects of nicotine on neuronal processes. While lower doses tend to produce fewer negative consequences, as the dose of nicotine increases there is a greater likelihood for adverse effects, such as altered proliferation within the subgranular zone of the hippocampus. Ijomone and Nwoha (2015) reported that there were no observable reductions in proliferation in

animals that received daily subcutaneous injection of nicotine for 28 days of 0.25 mg/kg; however, doses of both 2 mg/kg and 4 mg/kg were both able to produce decreases in hippocampal proliferation, without the inclusion of a withdrawal period. In the current study, animals received injections of 0.9 mg/kg/day for 10 days. Likely the inclusion of a withdrawal period in the current study led to a lack of differences between animals that received nicotine and those that did not. Previous research within our lab using similar doses and administration found that nicotine decreased proliferation in the SGZ. Tissue was collected at the end of drug administration without the inclusion of a withdrawal period (unpublished Hayes lab results). In the current study, animals had a withdrawal period of 17 days. Trauth et al. (2000) suggest that nicotine may influence the binding of cholinergic receptors after 12 days of withdrawal, but after 27 days of withdrawal, the binding of cholinergic receptors had returned to levels comparable to control animals. The timepoints selected in their study were based on findings in fetal studies. Studies involving fetal exposure models have demonstrated a similar time course of neural effects, although of a greater magnitude than adolescent animals (Slotkin, 2002). This indicates that adolescence may be either a separate period of vulnerability or a continuation of vulnerability to psychoactive drugs seen during the fetal period (Slotkin, 2002). The withdrawal period used in the current study is slightly longer, but comparable to the 12-day withdrawal period used by Trauth et al. (2000). As observed in the current study, nicotine may not lead to changes at the receptor level that last much longer than 12 days (Trauth et al., 2000).

Similarly, Slotkin (2002) reported an increase in the overall number of nicotinic receptors after 13 days of nicotine abstinence within the hippocampus of female animals. While this upregulation did not reach significance for male animals, a similar trend was observed. Furthermore, the trend in males completely vanished by after 27 days of abstinence; however,

female animals still had an increased number of receptors at the 27-day timepoint. While changes in neuronal function from nicotine withdrawal could be present at day 12 or 13, receptor functioning may not return to normal until between week 2 and week 3 of nicotine cessation. It is possible the effects of nicotine on proliferation may occur at similar timepoints as changes to receptor functioning, occurring shortly after cessation and not lasting much longer than 2 weeks.

Activity of the cholinergic system, according to research, seems to be necessary for proliferation within the subgranular zone of the hippocampus. Cholinergic activity is also quite important for learning and memory (Veena, Rao, & Srikmuar, 2011). Destruction of cholinergic inputs to the dentate gyrus of the hippocampus from the forebrain lead to a decrease in both short-term survival and cell proliferation in the SGZ (Mohapel et al., 2005). The researchers also discovered that administration of a cholinergic agonist, physostigmine, could increase proliferation within the SGZ to levels greater than controls. In addition, the decreases in survival and proliferation were accompanied by decreases in performance during the MWM task (Veena et al., 2011). In contrast to findings of Veena et al. (2011), nicotine did not have an influence on either proliferation or the MWM, possibly due to differences in administration protocols.

In the current study, there was not a significant interaction between ethanol and nicotine on cell proliferation within the subgranular zone of the hippocampus. In a study by Jang and colleagues (2002), animals that received both ethanol and nicotine had complex interactions on neurogenesis within the dentate gyrus. Animals that received both drugs had a decrease in proliferation that was significantly greater than the decreases observed by either drug alone. This effect did not seem to extend to cell survival. The authors also assessed apoptosis by measuring the number of transferase-mediated dUTP nick-end labeling (TUNEL) positive cells. The number of TUNEL+ cells were elevated for groups that received nicotine, ethanol, or both,

indicating an increase in cell death due to drug administration. However, there were no significant differences in TUNEL+ cells between treatment groups 3 days post-administration (Jang et al., 2002). It is possible that the interactions between the two drugs may lead to short-term changes in neurogenesis. The changes may be specific to cell proliferation, and may not lead to lasting changes, similar to what was observed in the current study.

A more recent study found that when ethanol was combined with tobacco smoke, proliferation within SGZ was decreased in the group receiving ethanol alone and the group receiving both ethanol and tobacco smoke. Tobacco smoke alone did not seem to decrease proliferation compared to the control group (Gomez et al., 2015). Research by Oliveira-da-Silva et al. (2010) examined the effects of combined exposure of ethanol and nicotine in several different regions of the hippocampus at 2 days and 5 days of abstinence. In their study, they found an increase in TUNEL+ cells in animals receiving ethanol or nicotine, but not both, after 2 days of withdrawal. At 5 days post-administration, all drug receiving animals had fewer TUNEL+ cells than control animals. This could indicate that ethanol and nicotine are more likely to interact in a way that produces behavioral effects from the inhibition of proliferation rather than from cell death. However, an inhibition of proliferation from combined use was not observed in the current study, suggesting the interaction between the two drugs does not last 17 days after cessation.

Nicotinic antagonists have been reported to decrease ethanol intake (Lajtha & Sershen, 2010). It is possible for cross tolerance to develop between these two drugs and it is unlikely that the tolerance is completely dependent on cholinergic receptors (Lajtha & Sershen, 2010). More likely, the two drugs alter the metabolism of the other (Hurley et al., 2012). As reported by Gomez et al. (2015), animals that received both ethanol and tobacco smoke had higher blood

levels of cotinine, the primary metabolite of nicotine, compared to animals that only received tobacco smoke. It is possible that when the animals in the current study were exposed to both ethanol and nicotine, the drugs led to an increased metabolic tolerance to nicotine. Similar to results observed by Gomez et al. (2015).

In adolescence, there is an increased vulnerability to many of the negative effects of drug use (Slotkin, 2002). The decrease in proliferation from ethanol exposure seen in our study could have been due to the increased vulnerability of this stage of life. One of the most negatively impacted brain regions in adolescents is the hippocampus (Nixonet al., 2011). Human research involving ethanol use and abuse has shown that adolescents tend to have greater impairments in hippocampal-dependent memory tasks compared to adults (Nixon et al., 2010). Research on alcohol use disorders in adolescents tends to reveal more consistent decreases in spatial memory, verbal memory, and attention, when compared to similar studies in adults (Nixon et al., 2010). One study compared teens, between the ages of 15 to 17, who had been diagnosed with an alcohol abuse or dependence disorder, to healthy cohorts. MRI scans revealed that the teens with alcohol abuse or dependence disorder had decreased hippocampal volumes (Nagel et al., 2005).

Adolescent animals also seem to be more vulnerable to certain long-term changes in protein expression due to ethanol administration, with some of the proteins involved in neurogenic processes (Hargraves et al., 2009; Zhao et al., 2008). In the current study, decreased proliferation within the SGZ was observed at 17 days of abstinence. Changes in proliferation in adolescents have been observed after longer periods of abstinence. One study found that differences between animals receiving ethanol and control animals were still detectable via BrdU labeling after 35 days of abstinence (Morris et al., 2010a).

The reduction in proliferation could have potential negative consequences for adolescents. Learning that relies on the hippocampus, such as spatial memory, is correlated with neurogenesis (Balu & Lucki, 2009). Increased neurogenesis has been shown to be positively correlated with performance on tasks such as contextual fear conditioning, the MWM, and trace eye-blink conditioning (Balu & Lucki, 2009). Furthermore, factors that decrease neurogenesis typically also decrease performance on hippocampal-dependent learning tasks (Zhao et al., 2008). Neurogenesis has been implicated in almost every functional aspect of the hippocampus (Nixon et al., 2010). Neurogenesis could even play a role in disorders related to alterations in hippocampal functioning including depression, schizophrenia, and epilepsy (Balu & Lucki, 2009). However, further research is needed to determine the role of neurogenesis in these disorders.

Morris water maze

During the experiment, animals underwent behavioral testing. All groups were exposed to the MWM for 12 days during the withdrawal period. The MWM was performed to determine if expected changes in proliferation were accompanied by decreases in spatial memory. As observed in the current study, animals that received ethanol had decreased performance in the acquisition phase of the MWM. The decrease in performance was expected as decreases in hippocampal proliferation have been linked with decreased performance on the MWM (Balu & Lucki, 2009). Supporting the finding of decreased spatial learning in the current study, a previous study indicated that concurrent exposure to tobacco and ethanol also lead to decreases in proliferation, albeit without the inclusion of a withdrawal and lower ethanol doses (4 g/kg/day) (Gomez et al., 2015).

It is possible that the use of this task itself could have influenced neurogenesis. Researchers have reported both that the MWM task experience has increased neurogenesis (Gould et al., 1999), and that it had no influence on neurogenesis (Van Praag et al., 1999). Performance on the MWM task relies on spatial memory, which is thought to be directly influenced by hippocampal neurogenesis. Hippocampal neurogenesis, in turn, influences overall hippocampal functioning (Balu & Lucki, 2009; Ehninger & Kempermann, 2006). Currently, there appears to still be debate as to whether the learning that occurs during the MWM task causes changes in proliferation or cell survival in the hippocampus (Ehninger & Kempermann, 2006). The MWM task has several components that have been shown to influence proliferation, such as physical activity, stress, and spatial learning (Ehninger & Kempermann, 2006).

First, the MWM involves physical activity. Voluntary physical exercise has been reported to increase proliferation (Balu & Lucki, 2009). However, this effect is unlikely to have led to alterations in neurogenesis between groups present in the current study since all animals in all groups participated. Additionally, as reported by Van Praag et al. (1999), animals that had access to a running wheel displayed an increase in hippocampal proliferation, but animals that were subjected to the MWM or swim-time-yoked control animals did not reveal any increase in proliferation. Another component of the MWM is stress. The MWM task is believed to be a significant source of stress. Researchers such as Ehninger and Kempermann (2006) have shown that pre-exposure to conditions similar to the MWM before MWM testing have led to significant decreases in stress when compared to animals that did not have pre-exposure. Stress is believed to lead to decreases in proliferation (Kirby, 2013). Stress associated with MWM testing is also unlikely to have led to differences between groups due to stress, as all animals in the current study participated in the MWM. Finally, spatial learning itself could have an influence on

proliferation (Balu & Lucki, 2009). A review by Leuner, Gould, and Shors (2006) states that much evidence exists both for and against the hypothesis that spatial learning may influence proliferation within the SGZ. More research is needed to reach a definitive conclusion. It is possible that spatial learning is more likely to affect other aspects of neurogenesis such as cell survival and maturation (Shors, Anderson, Curlik, & Nokia, 2012).

Withdrawal

In the current study, nicotine did not influence the average withdrawal behavior observed in ethanol receiving animals compared to animals that received ethanol and saline. Adolescents tend to be more resistant to the negative effects associated with nicotine use and withdrawal (O'Dell et al., 2006). As demonstrated by O'Dell et al. (2006), adolescent animals (receiving nicotine doses of 1.6, 3.2, or 4.7 mg/kg/day) did not have a significantly different number of withdrawal behaviors, such as eye-blinks, yawns, or foot licks, compared to control animals. However, adult animals receiving 2.1 or 3.2 mg/kg/day, but not 1 mg/kg/day, performed a greater number of withdrawal behaviors compared to control animals. Animals in the current study received slightly less nicotine than the groups that did not show withdrawal symptoms.

Withdrawal scores in the current study were quite low, with average withdrawal scores being less than 1 for animals receiving ethanol and those receiving ethanol and nicotine. This indicates that most animals during observational timepoints did not show withdrawal symptoms. Morris and colleagues (2010b) used a binge ethanol protocol similar the one currently employed, as well as a similar withdrawal behavior measure with the only difference being 17 hours of observation rather than 18. In their study, adolescent and adult animals had comparable withdrawal scores, except for one timepoint at 11 hours of abstinence where adults showed more severe withdrawal behaviors than adolescents. The average withdrawal score for adolescents was

1.5, indicating behaviors such as hyperactivity, tail tremors, and tail spasms. Animals in the current study showed fewer withdrawal behaviors on average, 0.5 for animals receiving ethanol only and 0.34 for animals receiving both ethanol and nicotine. Additionally, a high degree of variability in withdrawal scores was observed in the current study. Variability in withdrawal behaviors observed in the Morris and colleagues (2010b) study was less than the current study.

A difference in peak withdrawal behaviors emerged between ethanol exposure groups such that animals receiving saline had more severe withdrawal behaviors than those receiving nicotine. It is possible that animals in the current study metabolized nicotine more quickly, giving them more time to recover from the effects of the drug. Vieira-Brock and colleagues (2013) reported differences in metabolism between post-natal day (PND) 40 rats and PND 90 rats following a single administration of nicotine. PND 90 animals had significantly higher blood concentrations of nicotine compared to PND 40 animals for the first 2 hours. Also, for the first 2 hours following administration, PND 90 animals had lower concentrations of cotinine in their blood when compared to PND 40 animals.

Furthermore, the differences in peak withdrawal behaviors could have been due to an interaction between the nicotine and ethanol. To further explore this possibility, statistical analyses were conducted on all the observed withdrawal timepoints. While the only timepoint where the groups had statistically different peak withdrawal scores was at midnight, several timepoints had moderate effect sizes. Animals that received ethanol and nicotine had reduced withdrawal scores compared to animals that received ethanol and saline at most timepoints. One study that examined both ethanol and tobacco found a significantly greater amount of cotinine levels in their blood compared to animals exposed to tobacco only (Gomez et al., 2015). This indicates that when animals are exposed to both drugs concurrently, nicotine seems to become

metabolized more rapidly than when used alone. This is possible as animals receiving both ethanol and nicotine stopped showing signs of withdrawal at 5 a.m., but animals in the ethanol only group still showed minor signs of withdrawal through the 7 a.m. timepoint. Hurley and colleagues (2012) have also suggested that the co-use of these drugs leads to a combination of the analgesic effects of the drugs, as well as negation of adverse effects associated with either drug. These effects could have persisted during the period of withdrawal behavior monitoring, preventing animals from experiencing the more severe effects of ethanol withdrawal.

Drug Administration

In the current study, cell proliferation within the SGZ was decreased approximately two weeks after binge administration of ethanol. Research has found that ethanol impacts neurogenesis in a dose-dependent manner, where larger doses of ethanol produced more pronounced decreases in proliferation (Crews et al., 2000). In a study that employed the binge model of administration, adolescent animals received doses of ethanol averaging 12.1 g/kg/day (McClain et al., 2011). In their study, blood ethanol concentrations were approximately 353.5 mg/dl during the second day of administration. In the current study, the animals received an average of 12.15 g/kg/day ethanol (25% w/v). Animals in the current study received very similar levels of ethanol administration as previous studies that employed similar methods of ethanol administration in adolescent animals (McClain et al., 2011). Often ethanol doses received by adults using this procedure are lower because adults are more susceptible to reductions in motor ability. Nixon and Crews (2004) administered an average of 8.9 g/kg/day to adults using similar procedures. With doses is this model being determined based on behavioral measures of intoxication, this is to be expected (Nixon & Crews, 2004). When comparing adults to adolescent animals in amount of ethanol received from a drug administration, Morris et al. (2010b) found

that adolescents received significantly more ethanol, 12.3 g/kg/day compared to adults that received 9.2 g/kg/day during the binge procedure.

In a study by Abrous et al. (2002), animals that were given access to higher concentrations of nicotine over the course of 42 days had decreased proliferation in the SGZ. Animals who received doses of nicotine of either 0.04 mg/kg/infusion or 0.08 mg/kg/infusion had decreased proliferation when compared to control animals. These animals self-administered approximately 0.2 mg/kg/day and 0.3 mg/kg/day respectively. This effect was not seen in animals receiving doses of 0.02 mg/kg/infusion, receiving an average of 0.1 mg/kg/day. The withdrawal period used in their study was 2 days. Some studies have even reported negative effects of nicotine on various proteins involved in processes that are directly related to neurogenesis, such as synaptic plasticity, cell-to-cell interactions, and regeneration (Shingo & Kito, 2005). In Shingo and Kito's (2005) study, animals that received injections of nicotine for 2 weeks at doses of 0.1 mg/kg, 0.5 mg/kg, and 1 mg/kg all decreased the production of proteins involved in cell adhesion, while other proteins remained unaffected. Doses of nicotine used in the current study were likely to have elicited physiological changes in the animals that received them. This is because an interaction between ethanol and nicotine on MWM performance was observed. However, the effect likely did not last until the end of the withdrawal period, as there was no main effect of nicotine or an interaction between ethanol and nicotine on cell proliferation.

Nutrition

The animals in the current were weighed at various points throughout the study. Body weight group averages for the animals in the study were comparable to each other at the beginning and end of experimentation, but ethanol-exposed animals reduced body weight

compared to other animals at select points. Importantly, alterations in nutritional components are believed to influence neurogenesis. Park and Lee (2011) state that dietary restriction or excess has been shown to influence neurogenesis. Specifically, dietary restriction is believed to increase survival of newly born cells without influencing proliferation, and dietary excess is believed to decrease survival of newly born neurons. Other diet-related factors are also believed to influence hippocampal functioning, including proportion of diet that is sugar or fat, and texture (Zainuddin & Thuret, 2012). A study by Ross et al. (2007) compared animals on a high fructose diet versus animals receiving standard laboratory rat chow. While acquisition of the MWM task was unaffected, retention was decreased in the fructose diet group. Retention was measured as amount of time spent in the quadrant where the hidden platform was located, and time to travel to platform location. Texture of food has also been shown to influence neurogenesis. Research by Yamamoto et al. (2009) found that animals placed on a powdered diet compared to standard rat chow containing the same ingredients had a reduction in proliferation in the SGZ as evidenced by a reduction in the number of BrdU+ cells. As all groups in the current study received similar diets, the influence of food content was uniform and unlikely to lead to differences in neurogenesis between groups. Furthermore, by the time the study ended, there were no differences between groups based on body weight. Even if animals receiving ethanol began to consume more food by the end of the study, changes in the number of calories consumed influences cell survival rather than proliferation, as reported by Park and Lee (2011).

Future considerations

Two of the most common drugs of abuse among adolescents are alcohol and nicotine (NSDUH, 2013). Close to three million adolescents in America admitted to current alcohol use and about half that number admit to cigarette use in the past month (NSDUH, 2013). Users of

one of these drugs are also more likely to use the other (Lê et al., 2014). Adolescents seem to be at an increased risk of developing use and addiction to either drug, possibly due to experimentation at this age and differences in physiological effects compared to adults (Vieira-Brock et al., 2013; Kandel & Chen, 2000; Nixon et al., 2010). While this experiment was able to shed some light on extended withdrawal from ethanol and nicotine within adolescent populations, additional questions still need to be answered. Testing the effect of various doses of nicotine combined with ethanol should be one of the next steps. The current study examined the effects of ethanol dependence and nicotine doses that approximated light smoking. The results of the current study support the finding that lower doses of nicotine may not have lasting effects on proliferation within the SGZ of the hippocampus. However, larger doses of nicotine, similar to smoking at a rate that is closer to that of a heavy smoker, have negative neural consequences and may reveal an interaction between these two drugs.

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