The Effects of Dual Ethanol and Nicotine Exposure on Hippocampal Neurogenesis

By

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Abstract

Binge alcohol consumption is one of the most prevalent, yet problematic, patterns of substance abuse behavior in this country. Further, individuals who binge drink account for about half of all tobacco consumption in the United States. Both ethanol and nicotine have been shown to impair adult hippocampal integrity; however, little is known about the combined influence this may have over hippocampal neurogenesis.

The aim of the current study was to confirm the impairing effects nicotine and ethanol have been shown to have on hippocampal cell proliferation in adolescent and adult rats, and to further extend previous findings by identifying the interactive effects nicotine and ethanol may have on proliferation within the dentate gyrus. Adolescent (N = 42) and adult (N = 37) male Sprague-Dawley rats were administered nicotine (0.3 mg/kg in 0.9% saline, subcutaneously) or saline alone every 8 hours for 10 days. On the last 4 days of nicotine exposure, an ethanol (25% w/v in Vanilla Ensure® Plus, adult: 9.25 and adolescent: 11.87 g/kg/day, oral gavage) or isocaloric dextrose diet was administered following a modified Majchrowicz procedure. Animals were euthanized following the last dose in the treatment schedule. Brains were extracted, and coronal tissue sections were obtained. Ki67 immunohistochemistry was used to quantify cell proliferation in the sub-granular zone of the dentate gyrus within the hippocampus.

As hypothesized, individual exposure to chronic nicotine and binge levels of ethanol produced deficits in cell proliferation for both adolescent and adult treatment groups. Further, an additive effect was observed in the adult subjects following combined alcohol and nicotine treatment. However, this effect was not observed in adolescent subjects.

When comparing adolescents to adults and adults exposed to ethanol or nicotine alone, adolescent proliferation appears more impaired than in their adult counterparts. Lastly, non-injected controls provided evidence to suggest that the method of administration of ethanol may also impair cell proliferation.

In conclusion, there is sufficient evidence to suggest that nicotine and ethanol both produce impairments in hippocampal proliferation. Future research should focus on eliminating a potential confound produced by the route of administration. Once addressing this issue, further focus can be employed to understand better the relationship between ethanol- and nicotine-induced impairments in cell proliferation and how these affects may promote memory alterations as shown in the Morris water maze and radial arm maze paradigms.

Dedication

To both Galen and Brenda Hartless, without whom nothing in my life would have shown the shiny side of the coin. To you for planting the seed for happiness, fostering its growth within my being, and allowing it to flourish naturally. Thank you for instilling my desire to fight for the elements making me who I am. Thank you for encouraging the self-exploration of my curiosities, both recreational and intellectual, while supporting me during my failures and successes. There is no greater gift than that of knowledge and happiness. You have given me the ability to find both and there is nothing more I could have asked for. I love you for these things and will always cherish the gifts you have given.

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Introduction

Alcohol and tobacco are two of the most commonly co-abused substances in the United States. In 2011, about 75% of U.S. citizens reported consuming at least one alcoholic beverage within a month (NSDUH, 2012). Among these consumers, 58.3 million reported binge-drinking behaviors, which is defined as five or more drinks for men (four or more for women) per occasion (SAMHSA, 2012). Furthermore, binge drinkers are responsible for nearly half of the tobacco consumed in the United States (SAMHSA, 2012). Concern regarding the co-abuse of these substances is this: the National Institute on Alcohol Abuse and Alcoholism has reported increases in morbidity from the co-abuse of alcohol and nicotine in varying forms of terminal peripheral disease and neuro-cognitive dysfunction (NIAAA, 2012). Little is known about these cognitive abnormalities resulting from exposure to nicotine and alcohol, nor about their effects on adolescent development.

Co-Abuse Determinants of Nicotine and Alcohol

The abuse of nicotine with alcohol is not surprising, given that both substances are legally and commercially available to individuals over 18 and 21, respectively. Many reports distinguish the initial use of alcohol as a depressant, producing inhibitory effects on self-control while having a general inhibitive effect on central nervous system (CNS) activity (Valenzuela, 1997). More specifically, binge drinking has been shown to be highly damaging to the hippocampus, a region within the brain primarily responsible for spatial and temporal aspects associated with memory functioning (Chin, Skike, Berry, Kirk, Diaz-Granados, & Matthews, 2011; Garcia-Moreno & Cimadevilla, 2012). Such neuroanatomical changes may be responsible for the behavioral/cognitive deficits seen in

human and animal models alike. Regardless, alcohol exposure has been observed in humans to produce subjective rewarding effects and facilitate dependent behaviors and chronic patterns of abuse (Durazzo, Tosun, Buckley, Gazdzinski, Mon, Fryer, & Meyerhoff, 2011).

Evidence regarding the effects of nicotine on cognition seems more complex. Acute administration of nicotine has been shown to improve working memory, learning, and attention on tasks such as the radial arm maze and Morris water maze. This would indicate some improvement in the ability to cognitively function in adult and aged subjects (Levin, McClernon, & Rezvani, 2006; Rezvani & Levin, 2001; Fujii & Sumikawa, 2001). However, evidence regarding chronic exposure suggests nicotine to produce impairing effects on motor learning, short-term memory and long-term potentiation throughout perinatal, adolescent, and adult stages of life (Trauth, Seidler, McCook, & Slotkin, 1999; Han, An, Yang, Si, & Zhang, 2014; Gonzalez, Gharbawie, Whishaw, & Kolb, 2005). These differences may be related to nicotine's cholinergic modulation of localized function, such as hippocampal and cerebellar dependent functioning; however, the current investigation targets structural differences expressed only within the hippocampal formation. These differences in behavior may also result from adaptive changes in neuronal circuitry associated with the onset of dependence, or differences in methodological technique, such as the dose concentration, schedule, and duration of exposure. Regardless, neurological evidence supports nicotine's impairing effect on hippocampal functioning and its boosting effect on hippocampal cell toxicity (Abrous, Adriani, Montaron, Aurousseau, Rougon, Moal, & Piazza, 2002; Berger, Gage, & Vijayaraghavan, 1998).

Alcohol and tobacco are commonly co-abused, with nicotine consumption more prevalent in those who abuse alcohol than in those who abstain (Dani & Harris, 2005; Le, Funk, Shram, Li, & Shaham, 2006). Nicotine and alcohol possess shared neuronal circuitry and genetic ties, both of which heavily contribute to the observed patterns in behavior characteristics of addictive pathologies, dependence, and substance-related mental disorders (Li, Volkow, Baler, & Egli, 2007; Dani & Harris, 2005; Schlaepfer, Hoft, & Ehringer, 2008).

Nicotine and alcohol are functionally linked to shaping the neurological reactions and behavioral responses associated with chemical dependence. Acetylcholine, gamma-aminobutyric acid (GABA), glutamate (NMDA), and dopamine (DA) activity are directly involved in addictive psychopathologies, states of dependence, and comorbidity of substance abuse (Valenzuala, 1997; Picciotto, 2003; Jang, Shin, Jung, Lee, Bahn, Kwon, Kim, & Kim, 2002; Larsson, Edstrom, Svensson, Soderpalm, & Engel 2005). Likewise, alcohol significantly increases nicotine's affinity for nicotinic receptors, potentiating the subjective rewarding effects and other physiological effects associated with nicotine use (Yoshida, Engel, & Liljequist, 1982; Dohrman and Reiter, 2003; Rose, Brauer, Behm, Cramblett, Calkins, & Lawhon, 2002). This relationship is inversely existent, with nicotine use increasing self-administration of alcohol, alcohol's rewarding effects, and subsequent abuse potential (Barrett, Tichauer, Leyton, & Pihl, 2006; Doyon, Dong, Ostroumov, Thomas, Zhang, & Dani, 2013).

Evidence also supports nicotine's facilitation of drinking behaviors, while alcohol has been shown to increase smoking behaviors (Burns & Proctor, 2013; Olausson, Jentsch, & Taylor, 2003; Epstein, Sher, Young, & King, 2007; Sayette, Martin, Wertz,

Perrott, & Peters, 2005). Research addressing genetic ties has identified specific chromosomes involved in nicotine and alcohol addiction, functioning to influence the expression of the subjective rewarding effects and behavioral tendencies characterized by dependent and addictive profiles (Bierut, Rice, Goate, Hinrichs, Saccone, Foroud, Edenberg, Cloninger, Begleiter, Conneally, Crowe, Hesselbrock, Li, Nurnberger, Porjesz, Schuckit, & Reich, 2004; Schlaepfer, Hoft, & Ehringer, 2008). Such potential drug interactions could be the synergistic product of the overlap in genetic expression, neuronal circuitry, environmental factors, and behavioral mechanisms underlying addictive pathologies (Li, Volkow, Baler, & Egli, 2007).

Hippocampal Neurogenesis and the Influences of Adolescent Development

Neurogenesis is the process by which new neurons form throughout the lifespan (Gage, Kempermann, & Song, 2008). Currently, adult neurogenesis is known to occur in two regions of the CNS. These areas are the sub-ventricular zone (SVZ), responsible for the sustained sense of smell, and the sub-granular zone (SGZ) of the dentate gyrus (DG) within the hippocampus (Alveraz-Buylla & Garcia-Verdugo, 2002; Cameron & McKay, 2001; Snyder, Kee, & Wojtowicz, 2001). The functionality of hippocampal neurogenesis is not fully understood; however, evidence suggests that the neurogenic process increases the complexity of the hippocampal neuronal network, sustaining a healthy, and overall more developed, network dedicated to the continued memory formation of new experiences throughout the lifespan (Kempermann, 2002). Adult hippocampal neurogenesis also seems to provide an overall strengthening of memory functioning, building associations, and modulating dependent states, stress responses, and mental

disorders (Vollmayr, Mahlstedt, & Henn, 2007; Snyder, Soumier, Brewer, Pickel, & Cameron, 2011; Balu, & Lucki, 2009).

During the process of hippocampal neurogenesis, radial and non-radial precursors within the SGZ give rise to neural progenitor cells (NPC), as well as glia subtypes (Ming & Song, 2005; Cameron, Woolley, McEwen, & Gould, 1993). NPCs function as self-renewing and multi-potent, meaning they are able to differentiate into different cell types, which serve a multitude of purposes for long and continuous periods of time (Mackowiak, Chocyk, Markowicz-Kula, & Wedzony, 2004; Breedlove, Watson, & Rosenzweig, 2010; Ming & Song, 2011). Proliferating cells form dense clusters and efficiently produce either glia or granule cells that mature and migrate through the CA1 and CA3 region of the hippocampal structure, known for its involvement in complex and higher-order learning and memory processes (Deng, Aimone, & Gage, 2010; Cameron, Woolley, McEwen, & Gould, 1993; Gage, Kempermann, & Song, 2008). After proliferated cells reach their destination via cell adhesion molecules, gene expression results in the differentiation of neuronal functioning (Breedlove, Watson, & Rosenzweig, 2010). Neurogenesis produces cells that migrate throughout the hippocampus, supporting the integrity of the neuronal network (Deng, Aimone, & Gage, 2010; Kempermann, 2002). Migratory pathways extend from the SGZ of the DG to the CA1 and CA3 regions of the hippocampus, where concentrations of interconnected dopaminergic, serotonergic, acetylcholinergic, and GABAergic neuronal systems exist (Gage, Kempermann, & Song, 2008). It is through these systems that neurogenesis is influenced by alcohol and nicotine (Gage, Kempermann, & Song, 2008).

The adolescent period, or the developmental transition into adulthood, is characterized by dramatic synaptic pruning, myelination of cortical matter throughout the brain, and a wide fluctuation in neuronal activity (Sisk & Foster, 2004; Spear, 2014; Tamnes, Ostby, Fjell, Westlye, Due-Tonnessen, & Walhovd, 2010). The refinement of synapses, increases in functional connectivity, and development of a premature neuronal system promotes reward seeking, dependent behaviors, and addictive pathologies (Borsari & Carey, 2001; Tamnes, Ostby, Fjell, Westlye, Due-Tonnessen, & Walhovd, 2010; Spear, 2010). This may in part be due to the increases in impulsivity, risky decisions, stress response, peer-influenced interactions, exploratory use of psychoactive substances, and sensation and reward-seeking behaviors characteristic of this developmental period (Spear, 2010; Leijenhorst, Zanolie, Meel, Westenberg, Rombouts, & Crone, 2010). Evidence strongly suggests such behavioral alterations and physiological responses are mediated by the developmental changes in neurotransmission and hormonal fluctuations (Sisk & Zehr, 2005; Buchanan, Eccles, & Becker, 1992; Forbes & Dahl, 2010). The changes in GABA, NMDA, and dopaminergic systems during adolescence are directly related to the increases in reward response, sensation seeking, and dependent behaviors (Spear, 2010; Wahlstrom, White, & Luciana, 2010; Brenhouse & Anderson, 2011).

The Effects of Alcohol on Cognitive Functioning

Shortly after consumption, alcohol is dispersed throughout the CNS, acting upon DA, GABA, NMDA, and cholinergic receptors, some of which are implicated in cell degeneration and interference with the neurogenic process (Crews, Morrow, Crinswell, & Breese, 1996; Crews & Nixon, 2009; Gage, Kempermann, & Song, 2008). Dense

concentrations of these receptors exist in the hippocampus, specifically throughout the SGZ of the DG and the CA3 regions of the hippocampus, where proliferation occurs (Gage, Kempermann, & Song, 2008).

Alcohol has been reported to increase GABA and decrease NMDA activity, leading to the impaired neuronal excitability and overall CNS functioning (Valenzuela, 1997). In response to chronic abuse, the CNS attempts to compensate for such continuous GABA and NMDA dysregulation. A decrease in GABA and an increase in NMDA sensitivity occur, which have been implicated in addictive pathologies and states of dependence (Valenzuela, 1997; Koob, Roberts, Schulteis, Parsons, Heyser, Hyytia, Merlo-Pich, & Weiss, 1998). This reversal in neuronal circuitry, specifically within the hippocampal formation, may be associated with changes in learning and memory processes involved in dependence and addiction, neurotoxic effects, and increases in cognitive deficits (Nelson & Gruoi, 2005; Crews, Collins, Dlugos, Littleton, Wilkins, Neafsey, Pentney, Snell, Tabatoff, Zou, & Noronha, 2003; Valenzuela, 1997).

Long-term alcohol exposure also produces brain damage, decreases in neuronal matter, and increases in ventricular volume (Sullivan, Harris, & Pfefferbaum, 2010; Bonthius & West, 2006). Chronic, high level alcohol consumption also produces neurodegeneration, which further disrupts hippocampal integrity (Collins, Zou, & Neafsey 1998; Crews, Collins, Dlugos, Littleton, Wilkins, Neafsey, Pentney, Snell, Tabakoff, Zou, & Noronha, 2004). Neurodegeneration is an apoptotic decrease in the number of cells within the CNS, leading to prolonged impairments in memory functioning (Obernier, Bouldin, & Crews, 2006).

The memory impairment observed in chronic alcoholics is similar to that of more traditional, endogenous neurodegenerative illness, such as neurocognitive disorder, dementia, and Alzheimer's disease (Kopelman & Corn, 1987). To further this concern, periodic use, such as a single binge episode, has been shown to produce inflammation and cell death along hippocampal system pathways (Pascual, Blanco, Cauli, Minarro, & Guerri, 2007; Cippitelli, Zook, Bell, Damadzic, Eskay, Schwandt, & Helig, 2010). Alcohol-induced neurodegeneration is prominent in a number of regions throughout the brain, with particular severity in the DG of the hippocampus (Obernier, Bouldin, & Crews, 2006; Crews, Collins, Dlugos, Littleton, Wilkins, Neafsey, Pentney, Snell, Tabatoff, Zou, & Noronha, 2003; Gage, Kempermann, & Song, 2008). Research has identified the subsequent cognitive impairment to be far reaching, with working memory deficits and significant disruption of learning, memory retrieval, and long-term potentiation processes (Cippitelli, Zook, Bell, Damadzic, Eskay, Schwandt, & Helig, 2010; Valenzuela, 1997; White, Matthews, & Best, 2000).

In addition to the neurotoxic effects on hippocampal functioning, alcohol has been shown to disrupt neurogenic functioning, impairing cell proliferation and neuronal maturation (Nixon & Crews 2004; Morris et al, 2010; Nixon & Crews, 2002; Crews, He, & Hodge, 2006). Further evidence suggests that the primary source of such overall neurogenic dysfunction may stem from alcohol's impairment on cell proliferation (Khun, Dickinson-Anson, & Gage, 1996; Crews, Miller, Ma, Nixon, Zawada, & Zakhari, 2003; Goritz & Frisen, 2012). Taffe, Kotzebue, Crean, Crawford, & Edwards (2009) showed that binge levels of alcohol significantly decreased multiple types of proliferating cells. Even when exposed to moderate doses of ethanol, progenitor cell production within the

DG was significantly decreased by 40% one week into the withdrawal period (Anderson, Nokia, Govindaraju, & Shors, 2012).

The Effects of Nicotine on Cognitive Functioning

The primary psychoactive and reinforcing constituent in tobacco is nicotine (Bergen & Caporaso; 1999). Nicotine is an agonist of the nicotinic acetylcholinergic receptors, producing both inhibitory and excitatory neurotransmission through various receptor subtypes (Akk & Auerbach, 1999; Picciotto, 2003), with adolescence resulting in an increase in cholinergic sensitivity (Shingo & Kito, 2005). After nicotine is administered, primary stimulation occurs at the nicotinic acetylcholine receptors (nAChR). However, activation of NMDA and GABA receptor sites are also involved, which may partly be responsible for the increases in attention, alertness, learning, and memory performance observed following acute nicotine exposure (Balfour, 2006; Warburton, 1992; Pomerleau & Arbor, 1992; Rangani, Upadhya, Nakhate, Kokare, & Subhedar, 2012). Dopamine is yet another key neurotransmitter, eliciting a major influence on the rewarding properties that motivate dependent patterns and addictive pathologies (Balfour, 1994). The cognitive benefits, such as stimulation of learning and memory functioning, diminish over chronic use as nAChR becomes desensitized (Ernst, Heishman, Spurgeon, & London, 2001; Picciotto, 2003). Depending upon dose and duration of use, nicotine has been demonstrated to produce varying effects on cognition (Picciotto, 2003; Small, Shah, Davenport, Geier, Yavarovich, Yamada, Sabarinath, Derendorf, Pauly, Gold, & Bruijnzeel, 2010; Ernst, Heishman, Spurgeon, & London, 2001).

At moderate doses, nicotine has been shown to significantly impair neurogenesis within the DG (Abrous, Adriani, Montaron, Aurousseau, Rougon, Moal, & Piazza, 2002). Evidence also suggests that nicotine disrupts the maturation of new neurons, while still providing some neuroprotective effects in adult neuronal circuitry (Shingo & Kito, 2005). Even with this taken into account, evidence strongly suggests that nicotine exposure increases cell death, disrupts developing neurons, and impairs cell proliferation and maturation tracts within and around the DG (Jang, Shin, Jung, Lee, Bahn, Kwon, Kim, & Kim, 2002; Silva, Manhaes, Rodrigues, Filgueiras, & Abreu-Villaca, 2010). Such neurotoxic effects have been demonstrated to, at least in part, result from a disruption of calcium's production and/or distribution throughout the cellular system (Berger, Gage, & Vijayaraghavan, 1998; Sharma, 2013; Satriotomo, Miki, Itoh, Ameno, Ijiri, & Takeuchi, 2000; Mulholland, Harris, Wilkins, Self, Blanchard, Holley, Littleton, & Prendergast, 2003).

Effects of Nicotine and Alcohol Co-Abuse on Neurogenesis

Abuse of either alcohol or nicotine produces both neurodegeneration and dependent/addictive behaviors. The need for understanding their effects on memory function throughout the lifespan and its influences over dependent states is imperative, as healthy cognitive functioning rests upon the ability to integrate information and experience. Research has identified the effects of nicotine and ethanol in isolation; however, dual administration to identify the specific neurobiological and behavioral effects of co-abuse remains fairly elusive.

In adults and adolescents, binge episodes of alcohol consumption are known to produce neurodegeneration; however, nicotine has been shown to produce both cognitive

improvements in the short-term and neurodegenerative effects following repeated exposure (McClain, Hayes, Morris, & Nixon, 2011; He, Nixon, Shetty, & Crews, 2005; Abrous, Adriani, Montaron, Aurousseau, Rougon, Moal, & Piazza, 2002; Bruijnzeel, Bauzo, Munikoti, Rodrick, Yamada, Fornal, Ormerod, & Jacobs, 2011). Research suggests that alcohol and nicotine's combined effects may lead to dysregulation in the neurogenic process, with as much as a two-fold deficit over either substance enacting its effects alone (Jang, Shin, Jung, Lee, Bahn, Kwon, Kim, & Kim, 2002). Applying similar curiosities with significant design and methodological improvements is warranted to gain a better understanding of ethanol and nicotine and their interactions with neurogenesis. Furthermore, the current investigation's control comparisons have been implemented and will be a novel account, comparing baseline levels of cell proliferation, with minimal influences of the experimental manipulation.

Current Investigation: Hypotheses

The proposed research seeks to identify the potential effects of alcohol and nicotine dual exposure on hippocampal cell proliferation in adolescent and adult models of abuse. A modified Majchrowicz (1975) binge ethanol has been combined with a nicotine administration paradigm. Following nicotine and binge ethanol administration, subjects were sacrificed and brain tissue was processed and analyzed for hippocampal cell proliferation in the SGZ of the DG.

It is hypothesized that ethanol and nicotine alone in adolescent and adult subjects will produce deficits in cell proliferation when compared to experimental/injected (EI) controls. Dual exposure to nicotine and ethanol is expected to produce even further deficits in hippocampal cell proliferation than either nicotine or alcohol alone. The non-

injected (NI) control proliferation was not subsequently affected by oral gavage procedure, and therefore should have a higher proliferating cell count than all orally gavaged subjects. Lastly, adolescent subjects are expected to have more proliferating cells than adults, however adolescent sensitivity to the effects of ethanol and nicotine on proliferation is expected to be more responsive.

Methodology

Subjects

Adolescent (PD 28; N=42) and adult (PD 70; N=37) male Sprague-Dawley rats were obtained from Charles River Laboratories (Portage, MI). Upon arrival, subjects were paired in standard tubs (44 cm x 22 cm x 20.5 cm) with bedding. Subjects were maintained on a 12:12 light cycle in a temperature and humidity controlled vivarium and allowed *ad libitum* access to food and water. After a 5-day acclimation period to the laboratory environment with three days of animal handling for habituation, drug exposure began. Standard rat food was removed during the binge treatment while animals received oral gavage of a nutritionally complete liquid diet containing ethanol or an isocaloric-equivalent dextrose control solution. Experimental procedures and animal care were enacted in accordance with the NIH Guide for Animal Care and Use of Laboratory Animals (NCR, 2011). Experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky. Table 1 represents the number of subjects used in the current investigation. The small number of animals in the non-injected (NI) control condition was included for a preliminary understanding regarding the routes of administration and their effects on cell proliferation. Teasing apart these impairments is essential for observing a more accurate relationship between nicotine, ethanol, and hippocampal neurogenesis.

Table 1. Number of Subjects in Each Experimental Condition (Total N=79)

	NI Controls	EI Controls	Nic. and Control	EtOH and Saline	EtOH and Nic
Adult	4	9	9	8	7
Adolescent	4	10	10	8	10

Design

The 86 subjects utilized in this study were divided into five conditions as depicted in the table above. Prior to ethanol treatment, all group sizes were equivalent (N=10), not including the additional 8 Non-Injected (NI) controls divided between adolescent and adult conditions. The reduction of subject sample size is the result of attrition due to outliers of the intoxication parameters (i.e. death). Experimental/injected (EI) controls received vehicle solutions administration via a subcutaneous (s.c.) injection of saline and an oral gavage of a dextrose sustaining diet. NI controls differed from EI controls in that they received no injections and remained undisturbed, handled only to retrieve weight data. Adolescent and adult subjects were exposed to a 10-day model of nicotine with 4 days of binge ethanol, beginning on the 6^{th} consecutive day of nicotine treatment. Nicotine was administered prior to its co-administration with ethanol as tobacco use typically precedes the initiation of alcohol exposure (John, Meyer, Rumpf, & Hapke, 2003). Administration of nicotine and ethanol was discontinued after the tenth day, at which point subjects were immediately euthanized for brain extraction. After the removal of tissue, coronal sections were obtained along the entire rostral to caudal extent of the limbic cortices and the brain tissue was preserved at -20 degrees Celsius until immunohistochemistry. Ki67 antibodies combined with location in the brain allow for the detection of neural progenitor cells. A standard light emission microscope (Model BX-43, Olympus America Inc.) was used in order to quantify cell proliferation after the 10-day binge co-administration model of nicotine and alcohol. A visual representation of procedural events via timeline is provided in Figure 1.

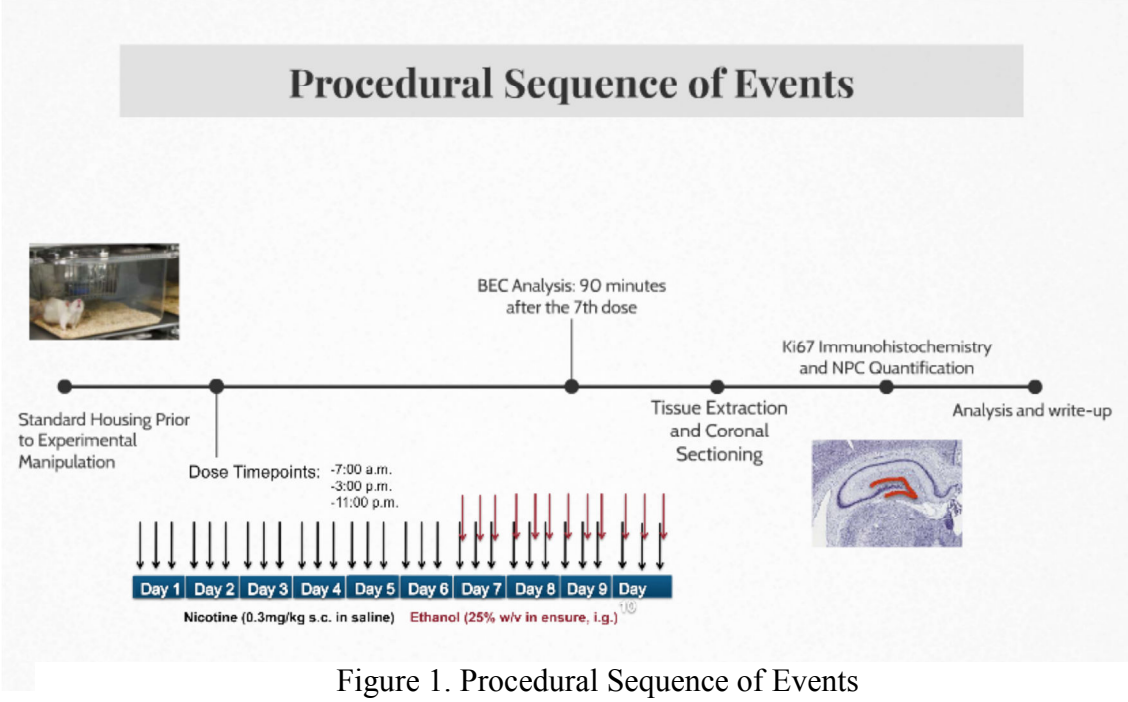

Figure 1. Procedural Sequence of Events

Ethanol and Nicotine Preparation and Administration

Nicotine was administered to adolescent (Postnatal day (PD) 28) and adult subjects (PD 70) via subcutaneous (s.c.) injections. Nicotine (100%, Sigma-Aldrich) was diluted in 0.9% saline and delivered at 0.3 mg/kg three times a day (7:00 a.m., 3:00 p.m., and 11:00 p.m.). Administered doses of nicotine were equivalent to a pharmacologically moderate dose in humans (Smith & Stolerman, 2009). For adolescents, injections began on PD 28, an age corresponding with the developmental patterns observed during early-adolescence in humans (Spear, 2000).

Adolescent and adult subjects received intragastric (oral-gavage) administration of either a mixture of ethanol (25% w/v, Pharmco-AAPER, Brookfield, CT) and Vanilla Ensure® Plus (Abbott Laboratories, Abbott Park, IL) or control diet of equivalent isocaloric value containing dextrose. Ethanol was introduced after the administration of

nicotine to better represent the patterns of co-abuse observed in those who consume alcohol (Torabi, Bailey, & Majd-Jabbari, 2009).

Blood ethanol levels were detected via tail blood, extracted 90 minutes after the 7^{th} dose of ethanol (8^{th} day of nicotine exposure). Blood was centrifuged (1800xg for 5 minutes) and stored at -20°C. Plasma blood ethanol concentrations (BEC) were determined using an AM1 Alcohol Analyzer (Analox, Lunenberg, MA).

Measures of Behavioral Intoxication

Subjects exposed to ethanol were assessed for behavioral impairment. An initial dose of 5g/kg was administered, followed by titrated doses that were dependent upon the level of behavioral intoxication shown by the subjects prior to each dose. This maintenance of dose administered ensured subjects' BECs remained relatively stable (~.3-.4 g/dl) without producing ethanol-induced mortality. The behavioral intoxication scale ranges from 0-5. For example, if a subject were to elicit a behavioral intoxication score of 2, then the subject would be administered a dose of 4g/kg. Table 2 identifies the behaviors elicited from ethanol administration in the rat. Each subject's observed behavioral intoxication, in respect to severity, is indicated in a score of 1-5. This number corresponds with the appropriate dose, administered at each exposure time-point.

Table 2. Observable Intoxication Parameters and Equivalent Ethanol Doses

Behavioral Intoxication	Observable Behavior	Subsequent EtOH Dose
0	Standard behavior	5 g/kg
1	Hypoactive, mild ataxia	4 g/kg
2	Ataxic w/ elevated abdomen	3 g/kg
3	Ataxic w/ loss of elevated abdomen and delayed righting reflex	2 g/kg
4	Loss of righting reflex w/ retained eye-blink reflex	1 g/kg

5	Loss of righting reflex w/ loss of eye-blink reflex	0 g/kg

Tissue Preparation

Following the final dose of nicotine and ethanol, subjects were exposed to an overdose of sodium pentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI). After sedation, subjects were transcardially perfused with 0.1M of phosphate buffered saline (PBS; pH 7.4). Following perfusions, brains were retrieved and soaked in paraformaldehyde (4%) for 24 hours. Next, brains were washed and stored in PBS at 4°C to await sectioning. Coronal sections, spanning the entire rostral to caudal extent of the limbic cortices, were obtained via a vibrating microtome (40□l; Leica Microsystems, Wetzlar, Germany) and stored in cryoprotectant, a lipid/protein enriched solution that prevents crystallization of the brain tissue while stored in the freezer.

Immunohistochemistry: Ki67

Free floating tissue was removed from cryoprotectant and washed in Tris Buffered Saline (TBS; 20 mM Tris, 500 mM NaCl, pH 7.5; BioRad Laboratories, Inc., Hercules, CA), followed by exposure to H_2O_2 (0.6%) to ensure the elimination of endogenous peroxidase activity. After washes, tissue was incubated in standard sodium citrate (heat bath: 65°C for 1 hour) for antigen retrieval, followed by a 30-minute incubation in blocking solution (TBS/0.1% Triton-X/ 3% Horse Serum). Anti-mouse Ki67 primary antibody (neural proliferation; 1:200, Vector Laboratories, Burlingame, CA) was used during a 4-day incubation period at room temperature.

Ki67 was used as a marker of cell proliferation, producing specific binding to actively proliferating cells (resting cells unaffected) throughout all stages of the cell cycle

(Schlozen & Gerbes, 2000). As shown in Figure 2, when compared to BrdU (Bromodeoxyuridine) and pHisH3 (Phosphoinositide-3), Ki67 appears more exhaustive when accounting for cell proliferation. BrdU is incorporated during the S phase of the cell cycle and pHisH3 is a marker of the G2 and M phases. Ki67, however, is exogenous and is present throughout the entire cell cycle, allowing for the identification of all actively dividing cells (Scholzen & Gerdes, 2000; Kee, Sivalingam, Boonstra, & Wojtowicz, 2002).

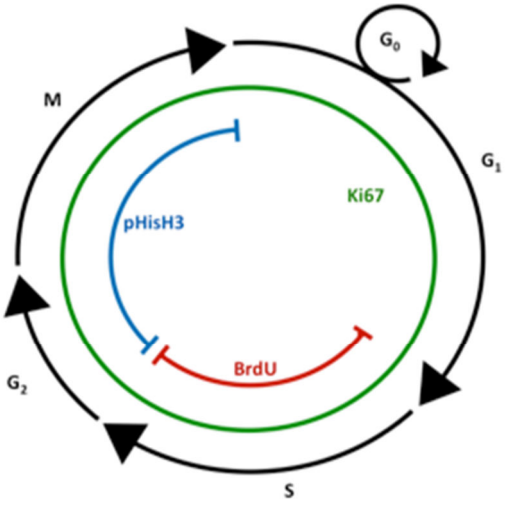

Figure 2. Ki67: Cell Cycle Integration

After primary antibody exposure, tissue was washed in species-specific blocking solution, followed by exposure to the biotinylated secondary antibody for 1 hour (horse anti-mouse-rat absorbed, Vector Laboratories, Burlingame, CA; 1:200). After another series of TBS washes, tissue was incubated in avidin-biotin peroxidase complex (ABC; ABC elite-kit, Vector Laboratories) for 1 hour. ABC was enhanced with Nickel-enriched diaminobenzidine tetrahydrochloride (DAB; Polysciences, Warrington, PA) and H_2O_2 (0.0006%). Tissue was then counterstained in cresyl violet, mounted, and cover-slipped with Cytoseal (Richard Allen Scientific, Kalamazoo, MI).

Quantification

Cell proliferation within the hippocampus was determined using a standard profile cell counting method (Crews, Mdzinarishvili, Kim, He, and Nixon, 2006; Hayes, Deeny,

Shaner, and Nixon, 2013) via BX43 Olympus Microscope (Olympus, Center Valley, PA). Profile cell counting methods were chosen as Ki67 localizes to distinct, non-homogenous cell clusters (Noori & Fornal, 2011; Hayes, Deeny, Shaner, & Nixon, 2013). Cell counts focused on the SGZ of the DG of the hippocampus in every 12^{th} coronal slice. NPCs were quantified at 1000x using a 100x oil-immersion objective and presented as Ki67+ cells/section +/- SEM.

SPSS version 21 was used to analyze the data from the current investigation. Ki67+ cells were analyzed by a 2 (age) x 4 (treatment) Analysis of Variance, followed by detailed comparisons addressing adolescent differences, adult differences, and the differences between adult and adolescent developmental time points.

Results

Blood Ethanol Concentrations

Adult and adolescent subjects were administered binge levels of ethanol via oral gavage three times a day for 4 days. Tail blood samples were taken 90 minutes following the 7th administration of ethanol (Day 3) in order to assess peak BECs. Descriptive statistics for both adolescent and adult rat ethanol exposure are provided in Table 3. Subjects' sample sizes are also provided (decreases indicate attrition due to mortality via researcher manipulation during the administration of ethanol).

Firstly, a nonparametric point-biserial analysis was used to show that adolescent subjects (M = 3.89, SE = .06) received higher doses of ethanol than adult subjects (M = 3.16, SE = .10), r = .523, n = 33, p < .001. An independent samples t-test indicated that adolescents (M = 435.64, SE = 6.08) maintained higher BECs on average than adult subjects (M = 280.95, SE = 10.29), $t(64) = 13.65$, p < .001. An additional point-biserial analysis revealed that adolescent subjects (M = 1.11, SE = 0.06) are more resilient to the behavioral effects of ethanol compared to adult subjects (M = 1.84, SE = 0.10), r = -.246, n = 33, p < .001. This accounts for the increased levels in adolescent subjects over adult subjects. Figure 3 represents the administered dose and intoxication levels for ethanol-exposed subjects, patterns that are documented as typical with an equivalent ethanol-binge paradigm (Nixon & Crews, 2002; Morris, Eaves, Smith, Nixon, 2011; Morris, Kelso, Liput, Marshall, and Nixon, 2010). Table 3 displays subjects' pattern of observed behavioral intoxication throughout ethanol administration.

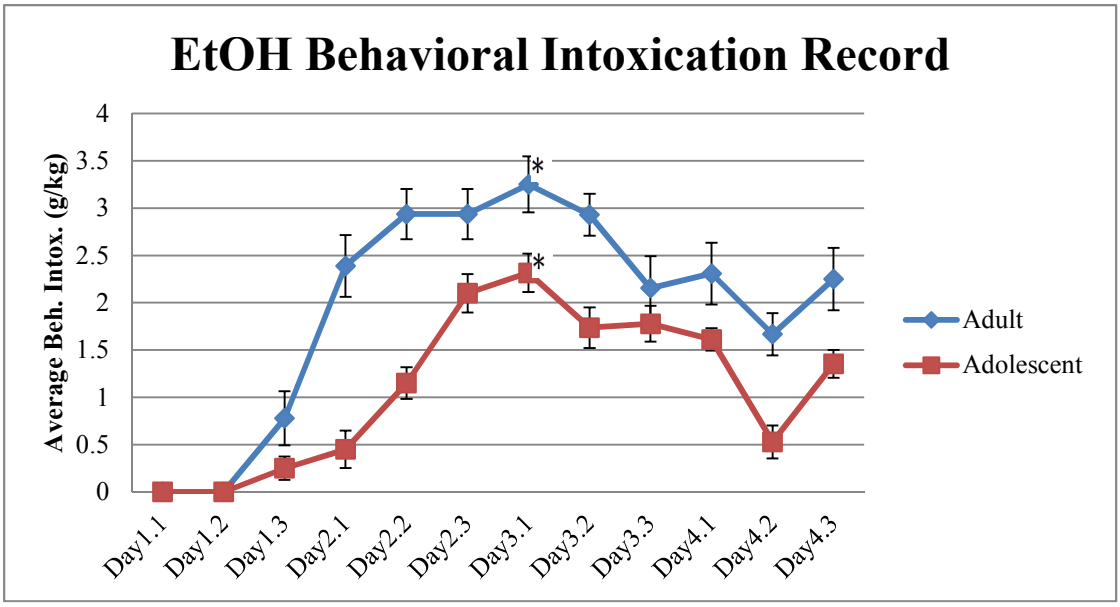

Figure 3. Record of Behavioral Intoxication Throughout the Administration Paradigm

Table 3. Summary of Average Behavioral Intoxication and Associated Administered Doses

		Treatment	Admin. Dose (g/kg)	Sum of Admin. (g/kg/Day)	Behavioral Intoxication
Adult	Day 1	EtOH	4.76 ± 0.14	14.29 ± 0.42	0.24 ± 0.14
		EtOH/Nic	4.79 ± 0.11	14.38 ± 0.32	0.21 ± 0.11
	Day 2	EtOH	2.33 ± 0.29	7.00 ± 0.87	2.67 ± 0.29
		EtOH/Nic	2.25 ± 0.35	6.75 ± 1.05	2.75 ± 0.35
	Day 3	EtOH	2.19 ± 0.22	6.57 ± 0.65	2.81 ± 0.22
		EtOH/Nic	2.44 ± 0.19	7.33 ± 0.56	2.56 ± 0.19
	Day 4	EtOH	3.09 ± 0.23	9.29 ± 0.84	1.91 ± 0.23
		EtOH/Nic	4.76 ± 0.14	8.40 ± 0.75	0.24 ± 0.14
Adolescent	Day 1	EtOH	4.89 ± 0.06	14.67 ± 0.17	0.11 ± 0.06
		EtOH/Nic	5 ± 0.00	15.00 ± 0.00	0 ± 0.00
	Day 2	EtOH	3.70 ± 0.20	11.11 ± 0.59	1.30 ± 0.20
		EtOH/Nic	4.04 ± 0.18	12.13 ± 0.55	0.96 ± 0.18
	Day 3	EtOH	2.89 ± 0.16	8.87 ± 0.47	2.11 ± 0.16
		EtOH/Nic	3.38 ± 0.19	10.13 ± 0.58	1.62 ± 0.19
	Day 4	EtOH	3.78 ± 0.11	11.33 ± 0.33	1.22 ± 0.11
		EtOH/Nic	3.92 ± 0.14	11.75 ± 0.41	1.08 ± 0.14

Effect of Nicotine on BEC in Adult and Adolescent Subjects

Underlying metabolic factors of nicotine and ethanol are similar; therefore, it is important to determine whether nicotine had any impact on BEC levels. Adult subjects' BECs were affected by the presence of nicotine, $t(13) = 3.616$, $p = .003$, $d = 2.28$. Adolescent subjects' BECs were not significantly affected by the administration of nicotine, $t(17) = .649$, $p = .525$. Average BECs between ethanol and nicotine vs. ethanol for both adult and adolescent subjects are displayed in Figure 4.

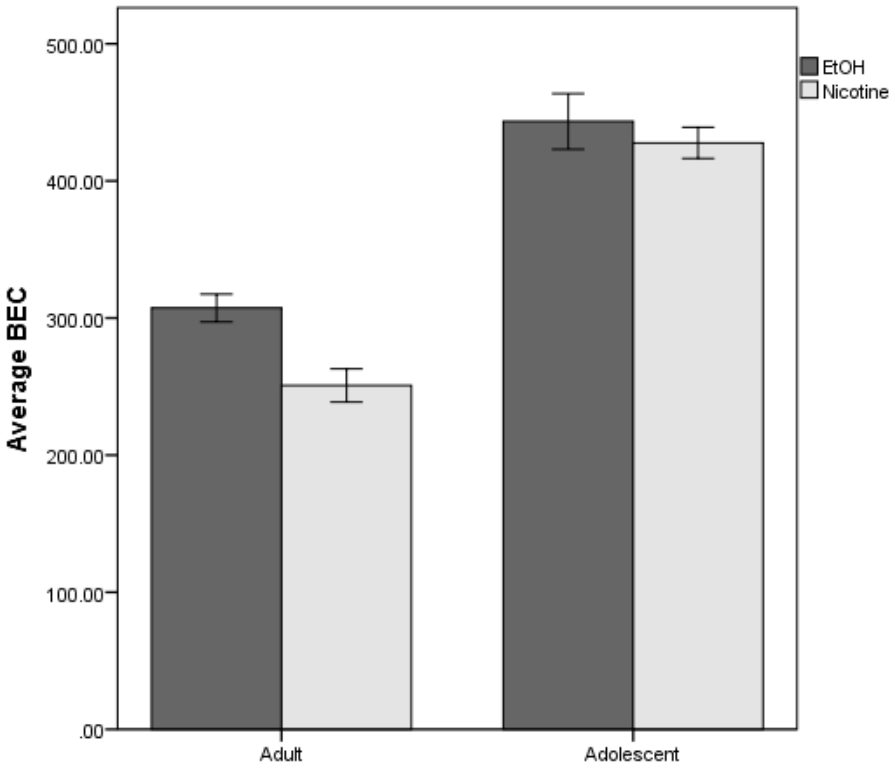

Figure 4. Average Blood Ethanol Concentrations

Further analysis addresses the metabolic factors of nicotine's influence over BECs. Nonparametric analyses using point-biserial coefficients help to determine

whether significant differences existed between the doses administered at each time point throughout the ethanol-binge paradigm. Analyses for both adult and adolescent subjects revealed that those given nicotine and ethanol received comparable amounts of ethanol as did subjects exposed to ethanol and saline, (adult) $r = .477$, $n = 17$, $p = .053$; (adolescent) $r = -.082$, $n = 15$, $p = .722$. Nicotine did not appear to significantly affect the metabolism of ethanol in adolescents; however, this is not the case for adult subjects. Given the trend in the relationship between ethanol and nicotine, in addition to the significant effect nicotine has in decreasing BECs, it is clear that nicotine might affect the metabolic reaction to ethanol in adult subjects.

The Effects of Ethanol and Nicotine on Adult Weight

Weight was used as an indicator of overall health and functioned to discern potential stress and methodological confounds. A mixed model analysis of variance was used to evaluate the weights of all five conditions across the four-day ethanol binge. Mauchly's test indicated a violation of sphericity, $x^2(5) = 29.10$, $p < .001$, therefore SPSS corrected the degrees of freedom using Greenhouse-Geisser estimates of sphericity ($\varepsilon = .63$).

Within subjects analysis revealed both a main effect for the day of exposure and an interaction between experimental condition and day throughout the ethanol binge. Firstly, all animals' weights, regardless of treatment, were significantly different by the end of the binge compared to when they began, $F(1.89, 56.78) = 383.20$, $p < .001$, *partial* $\eta^2 = .927$. In addition, the Non-Injected (NI) control subjects' pattern of weight gain was significantly increased throughout the ethanol exposure period when compared to experimentally manipulated conditions, $F(7.57, 56.78) = 34.53$, $p < .001$, *partial* $\eta^2 =$

.822. Between subject analyses revealed a significant effect in that group weights were different as a result of the binge-ethanol exposure, $F(4, 30) = 8.44$, $p < .001$, *partial* $\eta^2 = .529$. As shown in Figure 5, unhandled subjects ($M = 483.37$, $SE = 9.87$) showed a pattern of weight gain, whereas ethanol treated subjects displayed decreasing patterns in weight change: ethanol and nicotine ($M = 420.39$, $SE = 7.46$), $p < .001$, ethanol and saline ($M = 419.89$, $SE = 8.06$), $p < .001$, nicotine and control ($M = 428.53$, $SE = 6.58$), $p < .001$, and control and saline ($M = 437.86$, $SE = 6.58$), $p = .004$. Tukey post-hoc analyses revealed no further differences in weight between those groups exposed to oral gavage and subcutaneous administration techniques.

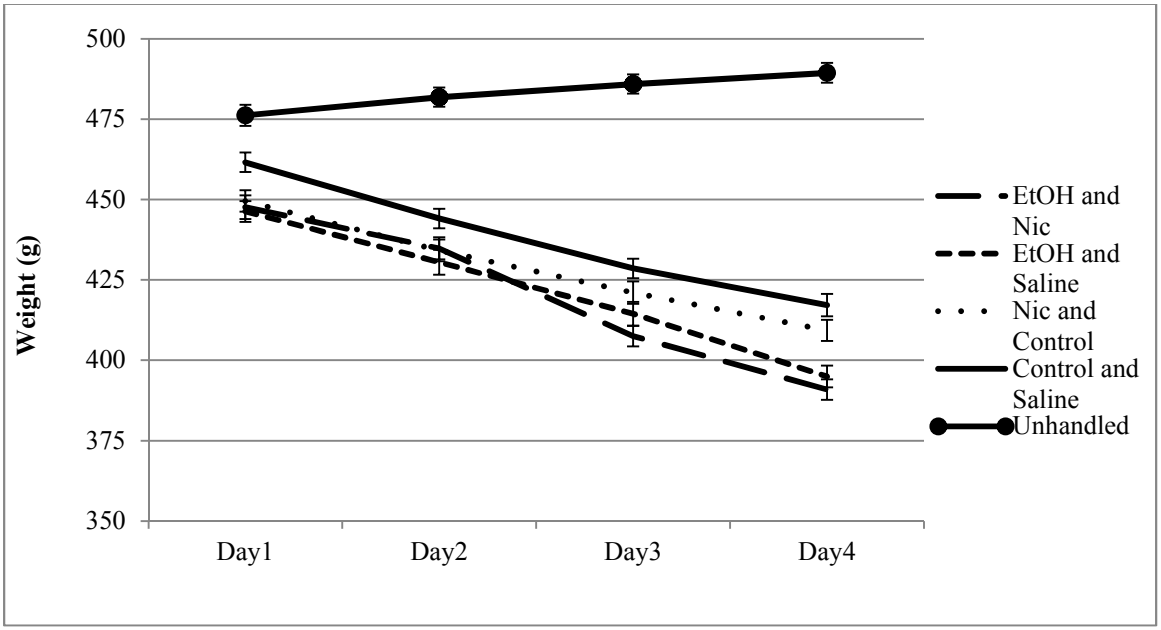

Figure 5. Weight Change in Adult Subjects

Nicotine, prior to the ethanol administration, produced similar differences in weight change throughout the exposure period. Again, Mauchly's test indicated a violation of sphericity, $x^2(14) = 191.46$, $p < .001$, therefore SPSS corrected the degrees of freedom using Greenhouse-Geisser estimates of sphericity ($\varepsilon = .29$). A mixed-model

ANOVA revealed a significant main effect in that body weights significantly increased from the initial injection of nicotine to the dose prior to ethanol administration (6 days), $F(1.45, 50.84) = 64.84$, $p < .001$, *partial* $\eta^2 = .660$. A significant interaction also existed, indicating that the patterns in weight change for each experimental condition were different throughout the nicotine administration period, $F(5.81, 50.84) = .44$, $p = .019$, *partial* $\eta^2 = .242$. Though weights fluctuated independently of each other, subject weights were not significantly different throughout nicotine administration, $F(4, 35) = .1.86$, $p = .139$.

The Effects of Ethanol and Nicotine on Adolescent Weight

A mixed model analysis of variance was used to assess the weights of all adolescent subjects throughout the administration period. Mauchly's test provided evidence in support of sphericity, $x^2(5) = 42.54$, $p < .001$, however SPSS corrected the degrees of freedom in using Greenhouse-Geisser estimates ($\varepsilon = .67$).

Similar to that of the adult subjects, analyses revealed both a main effect and an interaction between experimental conditions and weights throughout the ethanol binge. Firstly, all animals' weights, regardless of condition, changed over the injection period $F(2.01, 74.21) = 387.24$, $p < .001$, *partial* $\eta^2 = .913$. A significant interaction also existed, supporting that unhandled subjects' weight change throughout the injection period was different from that of experimentally handled subjects, $F(8.02, 74.21) = 125.77$, $p < .001$, *partial* $\eta^2 = .931$.

Between subjects analyses revealed a significant effect regardless of day, in that conditions weighed different from each other regardless of the day during the binge, $F(4, 37) = 9.82$, $p < .001$, *partial* $\eta^2 = .515$. As shown in Figure 6, Tukey's statistics identified

unhandled subjects (M = 142.58, SE = 5.03) showed a pattern of weight gain, whereas those experimentally handled produced patterns of decreasing weights: ethanol and nicotine (M = 107.20, SE = 3.35), $p < .001$, ethanol and saline (M = 109.33, SE = 3.35), $p < .001$, nicotine and control (M = 114.80, SE = 3.18), $p < .001$, and control and saline (M = 113.62, SE = 3.18), $p < .001$. Subjects experimentally manipulated were not significantly different from each other.

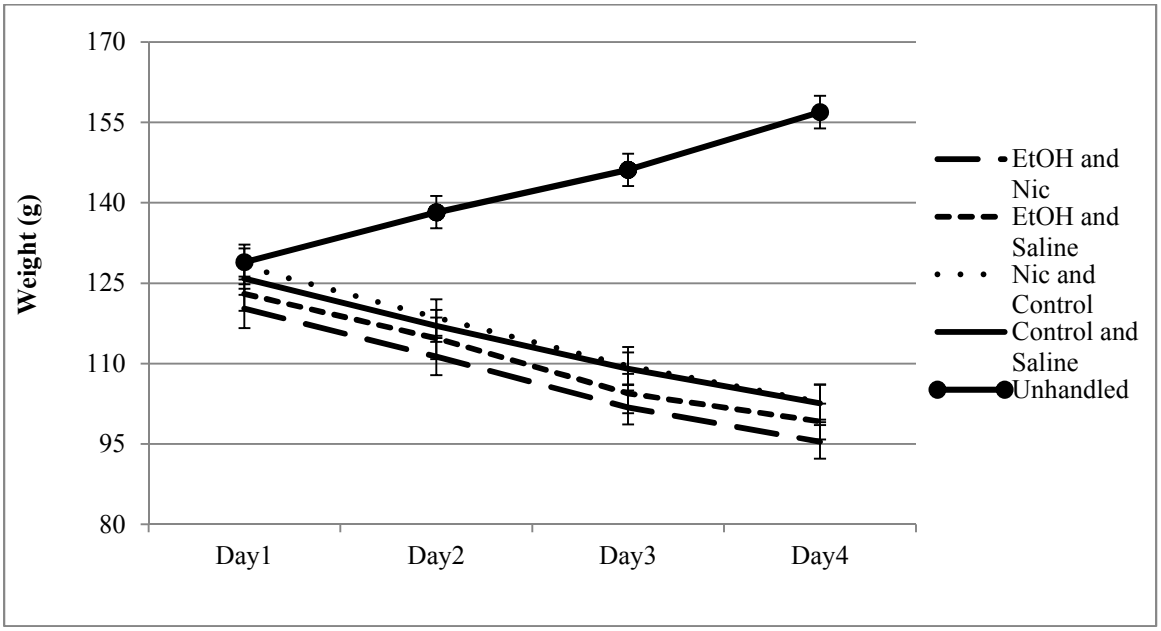

Figure 6. Weight Change in Adolescent Subjects

Nicotine's effects on adolescent weight prior to the addition of ethanol administration produced no differences in weights. Mauchly's test provided evidence in support of sphericity, $x^2(14) = 128.45$, $p < .001$, however SPSS corrected the degrees of freedom in using Greenhouse-Geisser estimates ($\varepsilon = .45$). In regards to weight gain throughout the injection period, all subjects significantly increased in weight more across the nicotine administration period, $F(2.23, 86.77) = 2419.44$, $p < 001$, *partial* $\eta^2 = .984$. There was no significant interaction, in that the pattern of subjects' weight did not

significantly differ, $F(8.90, 86.77) = 1.32$, $p = .239$, nor was there evidence to suggest that conditions were significantly different, regardless of day, $F(4, 39) = 1.22$, $p = .317$.

Ki67 Proliferation Analysis

A 2x4 ANOVA was used to determine the differences in the observed Ki67+ cells within the DG of the hippocampus in both adult and adolescent subjects. Analyses revealed, as hypothesized, main effects for both treatment and developmental time course. Overall, adolescent subjects ($M = 42.57$, $SE = 4.52$) had significantly more Ki67+ cells than adult subjects ($M = 24.90$, $SE = 3.40$), $F(1, 66) = 29.84$, $p < .001$, *partial* $\eta^2 = .311$. There was also a main effect for treatment conditions, $F(4, 66) = 25.48$, $p < .001$, *partial* $\eta^2 = .607$. There was no significant interaction when considering baseline Ki67+ levels for treatment and developmental time course, $F(4, 66) = 1.52$, $p = .220$.

In order to compare treatment conditions within adolescent and within adult subjects, two 1 x 4 ANOVAs were conducted addressing the effects of treatment on Ki67+ proliferating cells. Adolescent analysis revealed a significant effect, $F(3, 34) = 21.78$, $p < .001$. Comparison analyses using Least Squares Differences (LSD) was used for adolescent comparisons, revealing injected control condition ($M = 60.36$, $SE = 3.99$) as having significantly more Ki67+ cells than nicotine ($M = 40.19$, $SE = 5.02$), ethanol ($M = 25.69$, $SE = 3.65$), and nicotine/ethanol ($M = 19.59$, $SE = 4.27$) exposed subjects, all $p < .001$. Also ethanol ($p = .019$) and nicotine/ethanol ($p < .001$) exposed subjects showed fewer Ki67+ cells than nicotine exposed subjects. Nicotine/ethanol exposed subjects were not shown to significantly differ from those exposed to only ethanol, $p = .128$, indicating no further impairment in cell proliferation resulting from dual exposure

to ethanol and nicotine. The differences between adolescent treatment conditions are further represented in Figure 7.

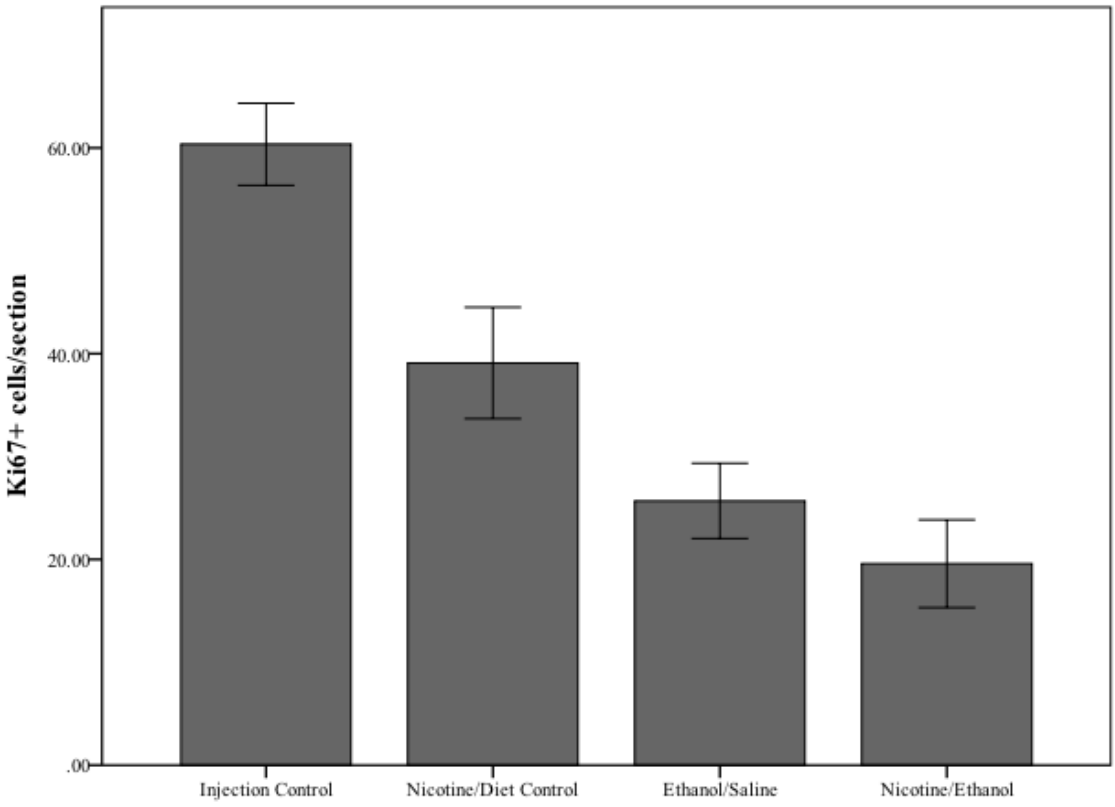

Figure 7. Adolescent comparisons of Ki67+ cells per section

The second 1 x 4 ANOVA for the adult model also produced a significant effect with adults showing a fairly similar pattern to adolescent subjects, $F(3, 26) = 6.54$, $p = .002$. LSD comparisons revealed the injected control condition ($M = 33.96$, $SE = 5.38$) as having significantly more Ki67+ cells than nicotine ($p = .029$; $M = 23.18$, $SE = 5.23$), ethanol ($p = .025$; $M = 20.26$, $SE = 5.29$), and nicotine/ethanol ($p < .001$; $M = 4.12$, $SE = 1.39$) exposed subjects. Furthermore, nicotine/ethanol exposed subjects produced significantly fewer Ki67+ cells than either nicotine ($p = .006$) or ethanol ($p = .037$) exposed subjects, indicating that adult subjects were more potently affected by the

combined effects of ethanol and nicotine than those exposed to only either ethanol or nicotine. Ethanol exposed subjects were not significantly different from nicotine exposed subjects, $p = .679$. The differences between adult treatment conditions are further represented in Figure 8.

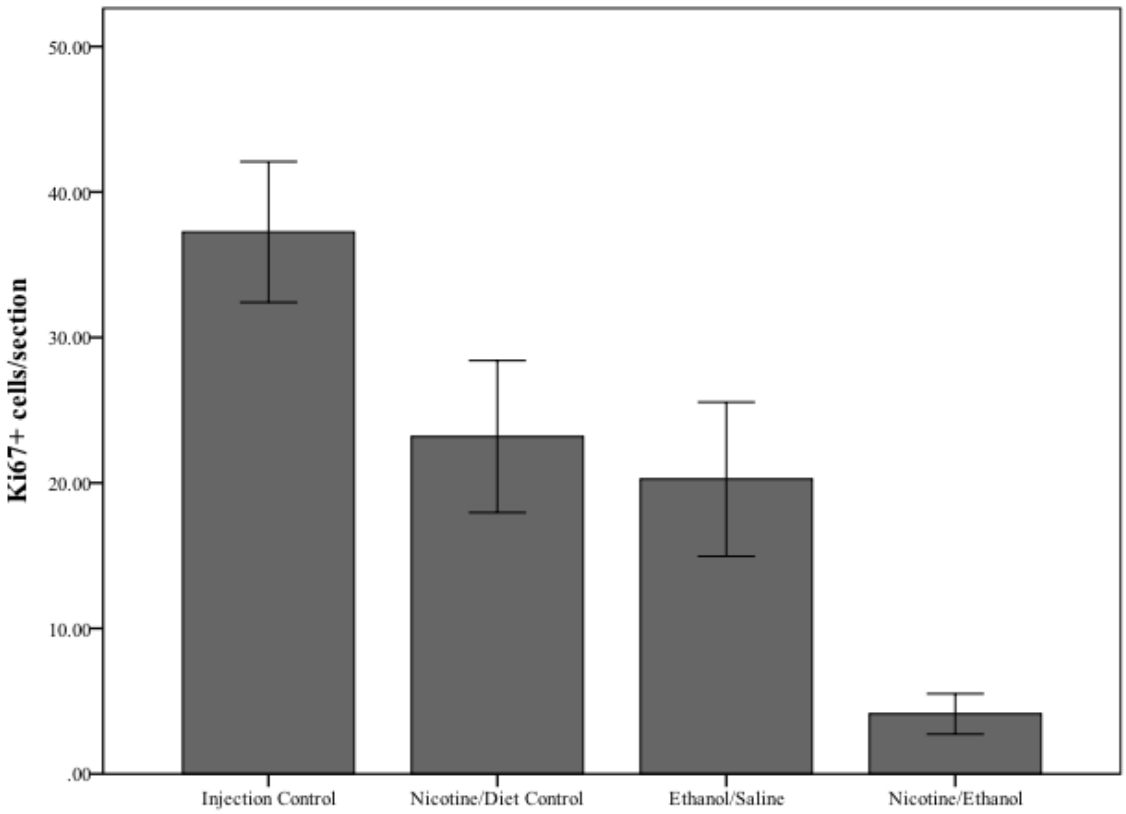

Figure 8. Adult comparisons of Ki67+ cells per section

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In order to assess the impact of the route of administration's role on hippocampal proliferation, a one-tail t-test was conducted for both adolescent and adult models. This was done in order to compare control-injected subjects to those not experiencing i.g. and s.c. injections, with the understanding that i.g. administration may produce levels of stress that would impair proliferation compared to their control counterparts. Non-

injected adolescent controls (M = 98.10, SE = 21.38) were observed to have significantly more Ki67+ cells than those exposed to s.c. and i.g. administration (M = 60.36, SE = 3.99), $t(12) = 2.66$, $p = .011$, $d = 1.41$ The same pattern, however not quite significant, was observed between adult subjects exposed to i.g. and s.c. administration (M = 33.96, SE = 5.38) and those who were only handled (M = 50.53, SE = 12.05), $t(11) = 1.48$, $p = .084$, $d = 0.62$. Given this trending pattern and the number of non-injected controls (N = 4), additional non-injected subjects would be expected to culture significant results. Given these findings, some interest should be directed towards the additional factor accounting for some of the variability observed in Ki67+ cell counts. A visual representation of these results is detailed in Figure 9.

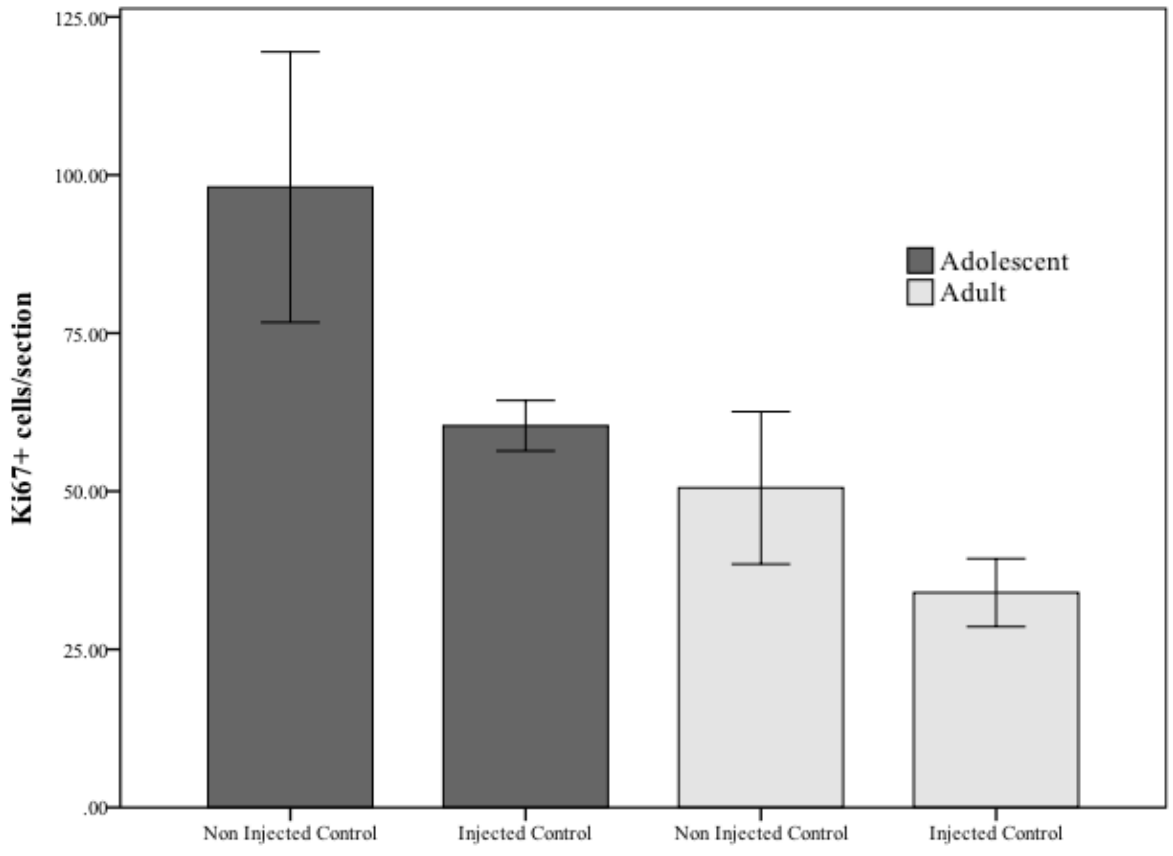

Figure 9. Comparisons of non-injected and injected controls

To address the differences between adolescent and adult conditions, an analysis of covariance was first used to determine the influence of age over treatment conditions. Analysis revealed that regardless of age, subjects differed significantly as a result of treatment conditions, $F(4, 70) = 22.73$, $p < .001$, *partial* $\eta^2 = .51$. Following this, an independent samples t-test was conducted to determine the differences between adult and adolescent NI controls. As expected, adolescent NI controls (M = 98.10, SE = 21.38) were observed to have significantly more Ki67+ cells than adult NI controls (M = 50.53, SE = 12.05), $t(6) = 1.94$, $p = .05$, $d = 1.77$. Adolescent NI subjects were observed to have significantly higher levels of proliferation compared to adult NI subjects. For this reason, proliferation counts were adjusted for appropriate comparisons between adult and adolescent treatment conditions. In order to best compare adult and adolescent impairments resulting from experimental manipulation, the differences in relation to NI controls was established. This was to provide an understanding of the severity in impairment produced by routes of administration, nicotine exposure, ethanol exposure, and co-exposure to nicotine and ethanol. In regard to EI controls, adolescent subjects (M = 37.74, SE = 3.99) showed a greater reduction of Ki67+ cells than adult subjects (M = 16.57, SE = 5.38), $t(17) = 3.20$, $p < .001$, $d = 1.46$. Adolescent NI controls had a difference of 37.74 while adult NI controls differed by 16.57, further suggesting adolescent subjects may be more affected by the routes of administration than adult subjects.

The impairment of Ki67+ cells in adolescent subjects (M = 57.91, SE = 5.02) exposed to nicotine was also significantly different than in their adult counterparts (M = 27.35, SE = 5.23), $t(18) = 4.19$, $p < .001$, $d = 1.89$. Compared to NI controls, adolescent

EI subjects had a difference of 57.91 while adult injection controls differed by 27.35 to that of NI subjects, indicating the potential increase in sensitivity that adolescents may experience to nicotine compared to adult rats. In regard to ethanol exposure, adolescent subjects (M = 72.41, SE = 3.65) were different from adult subjects (M = 30.27, SE = 5.29), $t(11) = 6.78$, $p < .001$, $d = 3.79$. It is clear that ethanol produced a harsher effect on Ki67+ cells, with adolescent differences of 72.41 and adult differences of 30.27. Lastly, adolescent subjects (M = 82.03, SE = 2.70) exposed to both nicotine and ethanol were significantly different from their adult counterparts (M = 46.41, SE = 1.39), $t(15) = 10.73$, $p < .001$, $d = 5.66$. Again, when examining the differences from that of the NI control conditions, there was a greater impairment in the number of Ki67+ cells, with adolescent differences of 82.03 and adult differences of 46.41. Figure 10 details the differences in the severity of impairment on Ki67+ cell counts, compared between adult and adolescent models regarding the current model of ethanol and nicotine exposure.

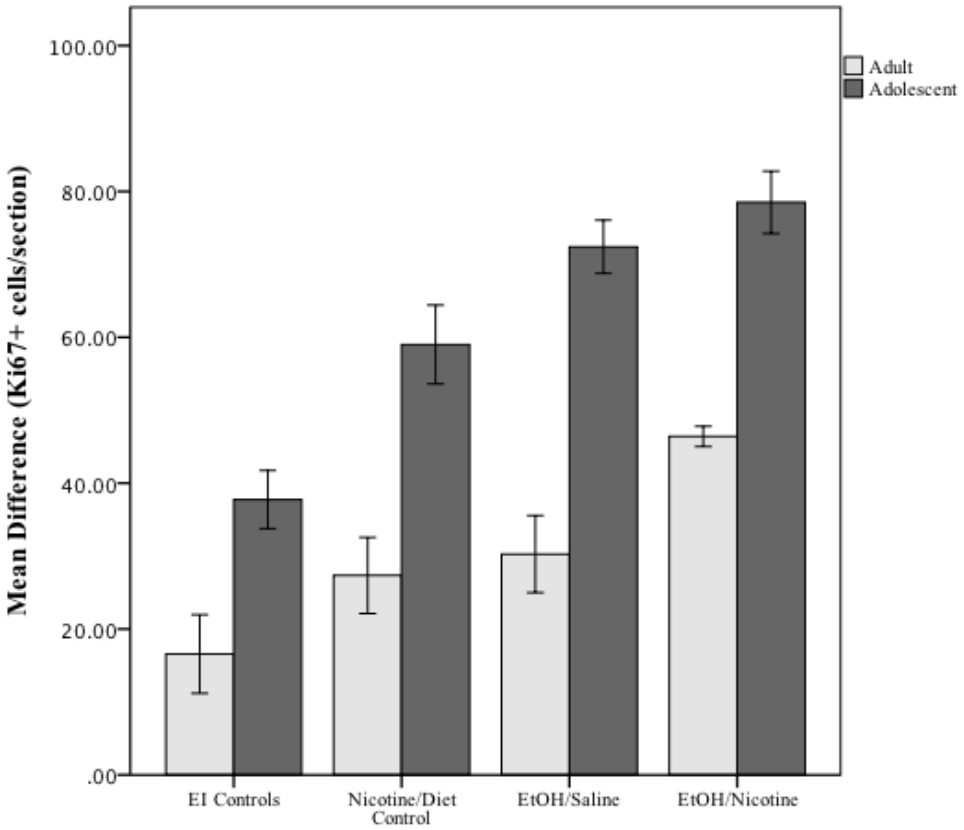

Figure 10. Comparison of Ki67+ cells in adolescent and adult subjects: Levels of impairment resulting from experimental manipulation.

Discussion

Statistical inference provided supporting evidence for the hypotheses of both nicotine and ethanol's unique contributions to the impairment of cell proliferation in adult and adolescent models. In addition, the combined effects of nicotine and ethanol in adult subjects produced significant impairments much greater than subjects exposed to either nicotine or ethanol. Co-administration of nicotine and ethanol in adolescence did not further affect cell proliferation when compared to ethanol treated adolescents. Furthermore, evidence suggests adolescent subjects had more proliferating cells than their adult counterparts and responded with greater sensitivity as a result of each drug treatment condition.

Evidence in both adults and adolescents also suggests the need for identifying the nature of the administration technique as a contributing factor to the impairment of cell proliferation. In the control comparisons it was apparent that adolescent subjects produced more proliferating cells than adult subjects.

After averaging nicotine and ethanol BECs for each condition, adolescent subjects had higher BECs than adult subjects. This may largely be a result of a hardy behavioral resilience, resulting in ethanol exposed adolescent subjects experiencing an inflated administration dose. Such levels of behavioral sensitivity have been noted in previous literature (Morris, Kelso, Liput, Marshall, & Nixon, 2010). In the current study, this increase in behavioral resilience and subsequent increased the ethanol administration may contribute to the magnitudes of impairments observed in adolescent animals when compared to adult subjects.

The impairment of cell proliferation resulting from ethanol is supported by previous findings. Nixon and Crews (2002), using a similar binge paradigm, also showed adult rats to have significantly reduced proliferation when exposed to binge levels of ethanol. Anderson and colleagues exposed subjects to a moderate dose of ethanol and observed similar deficits in progenitor cells within the hippocampus (Anderson, Nokia, Govindaraju, & Shors, 2012). Similar claims have been made regarding ethanol's effects during adolescent development. Crews, Mdzinarishvili, Kim, He, & Nixon (2006) identified via BrdU, in adolescent subjects exposed to both 2.5g/kg and 5g/kg of ethanol, a 29% - 78% proliferation reduction, which in turn drastically affected developing and maturing neurons. Furthermore, research using the same ethanol administration paradigm in an adolescent model concluded that there is significant impairment in hippocampal proliferation within the DG when compared to controls (Morris, Eaves, Smith, & Nixon, 2011; Morris, Kelso, Liput, Marshall, and Nixon, 2010).

Research addressing nicotine's effects on adult hippocampal plasticity is also supported by the current findings. Adolescent models of nicotinic exposure have come to analogous conclusions, with moderate levels producing apoptosis and neurotoxicity (Silva, Manhaes, Rodrigues, Filgueiras, & Abreu-Villaca, 2010; Dwyer, McQuown, & Leslie, 2009). Abrous, Adriani, Montaron, Aurousseau, Rougon, Moal, and Piazza (2002) also showed decreases in adult hippocampal proliferation with similar doses of nicotine. Furthermore, low dose infusion via subcutaneous osmotic pumps have also been shown to produce deficits in hippocampal proliferation, in addition to subsequent behavioral deficits observed in the Morris water maze (Scerri, Stewart, Breen, & Balfour, 2006).

The dual administration of nicotine and ethanol in adult subjects produced a further decrease in adult cell proliferation, providing some support for the additive impairment produced by ethanol and nicotine. Research regarding this relationship is minimal; however suggests that the dual administration of nicotine and ethanol will produce greater deficits in proliferation. Evidence has indicated that the co-contaminant use of nicotine and ethanol produces cell death and decreased neuronal densities, which appears to be more severe than in nicotine exposed subjects (Silva, Manhaes, Rodrigues, Filgueiras, & Abreu-Villaca, 2010). Nicotine and ethanol could disrupt the production and development of neural stem cells, potentially through their shared influences over cholinergic systems, as well as down-regulation of the mechanisms involved in healthy development and function, (Dwyer, McQuown, & Leslie, 2009; Ribeiro-Carvalho, Lima, Filgueiras, Manhaes, & Abreu-Villaca, 2008; Costa, Giordana, & Guizzetti, 2013). Furthermore, such deficits may be modulated by ethanol and nicotine's effects on microRNA production, expression, and regulatory networks, as evidence from fetal models suggesting that the development of neural stem cells are disrupted by the mutually teratogenic effects (Balaraman, Ursala, Winzer-Serhan, Miranda, 2012).

Adolescent subjects exposed to both nicotine and ethanol did not show a further impairment in cell proliferation than those receiving ethanol or nicotine alone. There are several factors that may account for this difference from adult subjects.

All conditions receiving s.c. and i.g. routes of administration produced fewer proliferating cells than that of unhandled subjects, in both adult and adolescent groups. Intragastric administration is known as a stressful procedure for the subject, being shown to increase corticosterone levels, as well as induce physical discomfort (Brown, Dinger,

& Levine, 2000; Murphy, Smith, Shaivitz, Rossberg, & Hurn, 2001; Alban, Dahl, Hansen, Hejgaard, Jensen, Kragh, Thomsen, & Steensgaard, 2001). Such stress and distress is not readily habituated to and has been shown to last for prolonged periods after the initial administration procedure (Walker, Boberg, Walsh, Wolf, Trujillo, Duke, Palme, & Felton, 2012; Atcha, Rourke, Neo, Goh, Lim, Aw, Browne, & Pemberton, 2010; Bonnichsen, Dragsted, & Hansen, 2005). This would indicate that a repeated exposure pattern to a stressful route of administration may in part be responsible for the impairments on hippocampal proliferation.

Such extraneous influences were further evident in the substantial and consistent weight decreases observed in all subjects exposed to s.c. and i.g. administration. Throughout the administration process, unhandled subjects steadily gained weight, as is expected during adolescent development; however, weights decreased in those exposed to nicotine via s.c. and ethanol via i.g. These decreases in weight are understood to partly result from a stress response to i.g. administration, as stress has been shown to cause similar changes in weight (Diane, Victoriano, Fromentin, Tome, & Larue-Achagiotis, 2008; Jeong, Lee, Kang, 2013).

These patterns of weight loss may also be partially due to the administration of a liquid diet over standard rat chow, as recent findings have suggested that such liquid diets commonly substituted for standard lab rat chow produce significant decreases in weight compared to control conditions (Patten, Moller, Graham, Gil-Mohapel, & Christie, 2013). These techniques, though efficient and the best technique for administering ethanol in this attenuated paradigm, should be carefully considered when interpreting results of the

current, and similar investigations (Mirescu & Gould, 2006; McEwen, 1999; Gould, Tanapat, Rydel, & Hastings, 2000, Gould & Tanapat, 1999).

Despite these limitations, the current examination provides statistically significant treatment differences in how nicotine and ethanol affects adult and adolescent cell proliferation. Adolescent resilience to the co-exposure of nicotine and ethanol still may result from the developmental changes in neuronal circuitry, involving cholinergic, glutaminergic, and GABAergic functioning (Zanardi, Leo, Biagini, & Zoli, 2002; Slotkin, 2004; Sharma, 2013; Abreu-Villaca, Seidler, Tate, & Slotkin, 2003; Levin, Lawrence, Petro, Horton, Rezvani, Seidler, & Slotkin, 2007). Furthermore, such changes may be associated with a resilience factor in response to the interactive effects (Balaraman, Winzer-Serhan, Miranda, 2012; Dwyer, McQuown, & Leslie, 2009; Doura, Gold, Keller, & Perry, 2009; Abreu-Villaca, Seidler, Tate, & Slotkin, 2003; Levin, Lawrence, Petro, Horton, Rezvani, Seidler, & Slotkin, 2007). Though more than one mechanism may be involved in the neurotoxic impairments of cell proliferation that occur within the hippocampus, resolving the additional influences created by the research methodology is essential before determining the nature of nicotine and ethanol's co-effect on adolescent cell proliferation and the underlying neuronal mechanisms involved.

As previously stated, both adult and adolescent unhandled controls showed drastically more proliferating cells compared to all conditions. Additionally, all subjects except unhandled controls showed decreases in weight gain across the exposure period. Research has suggested that adolescents may be more susceptible to the effects of ethanol and stress than adults (Somerville, Jones, & Casey, 2010; McCormick & Mathews, 2007; Morris, Kelso, Liput, Marshall, & Nixon, 2010). This increased sensitivity to stress is an

important concern when considering the current findings. When examining cell proliferation, it is evident that ethanol and nicotine reduced proliferating cells, however co-exposure produced no greater impairment than either substance had done alone. This could be due to the fact that during adolescent development, neurogenesis is so rapidly occurring that the observed levels are impaired by ethanol and nicotine, however still maintained at some baseline level (Khun, Dickinson-Anson, & Gage, 1996; He & Crews, 2007).

The current investigation provides some understanding of the effects of nicotine and ethanol on hippocampal cell proliferation; however, there are a few things to consider prior to future endeavors. Comparisons were conducted in order to provide some preliminary understanding of the differences between adolescent and adult subjects. Adolescent groups compared to adult proliferation counts appear to produce more proliferating cells than adult subjects, with adolescent subjects more sensitive to the structural impairments produced by ethanol and nicotine (Abrous, Koehl, & Moal, 2005; Nixon, Morris, Liput, & Kelso, 2010).

Also, the administration of ethanol via intragastric gavage is a stressful and discomforting experience, as observed by the high Ki67+ cells in unhandled controls compared to all other experimentally manipulated conditions. Such influences may have produced the observed decreases in weight. With stress known to affect neurogenesis, it is imperative to identify the severity of change and resolve its confounding influence. By using bidirectional selection for high and low ethanol preference, an oral consumption model of self-administration would be more appropriate in regard to face validity (Grahame, Li, & Lumeng, 1999; Oberlin, Best, Matson, Henderson, & Grahame, 2011).

Alcohol drinking can be further attenuated by utilizing a drinking in the dark (DID) procedure. Consumption may naturally compare with that of moderate to heavy binge-drinkers, producing pharmacologically relevant BECs (Thiele, Crabbe, & Boehm, 2014). The BEC levels here would also be expected to range closer to that of the human population. With this approach, the stress from intragastric administration can be eliminated.

Other influences could have impacted hippocampal proliferation, such as the introduction of a nutritionally enriched liquid supplement (used in addition with ethanol exposure). Research suggests that such diets alone decrease the number of mature neurons, as well as the proliferation phase of neurogenesis; however, they may not impact the overall number of neurons produced by neurogenesis, suggesting the new glia to be a primary target of ethanol and nicotine mediated effects on hippocampal plasticity (Patten, Moller, Graham, Gil-Mohapel, & Christie, 2013; Okihara, Ito, Kokai, Ishida, Hiranuma, Kato, Yabushita, Ishida, Ono, & Michikawa, 2014). Further investigation is required to identify glia's role in ethanol and nicotine induced neurodegeneration and toxicity.

Determining the theorized impairments in proliferation was the primary goal of this research. The nature and health of this first neurogenic process is important because if impaired, a myriad of downstream mechanisms may be affected, especially with involved in the process of neurogenesis (i.e. maturation, differentiation/migration, and integration). Ki67 is a marker of proliferation; however, it does not distinguish between which of these neurons will mature into glia cells and which of these will develop into neurons. Research has identified impairments in subtypes of glia as well as neurons,

making this distinction vital to tweezing apart ethanol and nicotine's effects (Hayes, Deeny, Shaner, & Nixon, 2013; Vemuri & Chetty, 2005; Nixon & Crews, 2004). Incorporating fluorescent neuronal nuclei (NeuN) immunohistochemistry techniques in addition to the Ki67 would identify proliferating neurons, at which point investigation into the specific impairments throughout all of neurogenesis can result.

The current research has extended evidence for the impairment of neurogenesis resulting from either nicotine or ethanol exposure in both adolescent and adult subjects. Evidence further indicates an additive impairment of neurogenesis in adult subjects exposed to both ethanol and nicotine; however, this relationship was not apparent in adolescent subjects.

These findings are important, as a high percentage of the population consumes binge levels of alcohol while also partaking in nicotine use. Hippocampal cell proliferation, especially involving that of neurons, is a dominating component in the continued integration of experience as meaningful learned associations and memories. Evidence suggests that binge ethanol and exposure to moderate amounts of nicotine exposure produce impairments in hippocampal proliferation. Furthermore, the combined effects of these substances produce additional impairments in adult models; however, more research needs to be conducted in order to determine its effects in adolescents.

In conclusion, both nicotine and ethanol create addictive pathologies, furthering abuse of each drug, respectively. Furthermore, these substances produce impairments in hippocampal functioning, and in combination may contribute to a further deficit than either nicotine or ethanol could produce on its own. This may be one underlying factor

contributing to the increased reports of addictive pathologies and cognitive and overall mental health concerns related to alcohol/nicotine use.

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