Alterations in Spatial Learning and Memory Following the Co-Abuse of Alcohol and Nicotine:

An Adult Model

by

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Thesis submitted to the faculty of Radford University

in partial fulfillment of the requirements for the degree of

Master of Arts in the Department of Psychology

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May 2015

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Abstract

Alcohol and nicotine are two of the most commonly used psychoactive substances, and are typically consumed in levels of excess causing a variety of neurobiological and behavioral alterations that range from slight abnormalities to profound neurodegeneration and cognitive impairment. However, research on the concomitant use of alcohol and nicotine has been relatively lacking. As self-identified practitioners of the binge type pattern of alcohol use are generally more likely to also engage in a regular smoking habit, it is imperative to evaluate the interaction of these substances. For this reason, researchers in the present study were interested in whether combined exposure to a binge model of ethanol consumption, and a chronic model of nicotine administration would differentially affect spatial learning and memory, as compared to the independent use of either substance. To that end, adult male Sprague-Dawley rats were administered a nicotine solution (0.3 mg/kg; s.c.) or saline, three times a day at 8 hr intervals for 10 days. During the final four days of nicotine exposure, ethanol (25% w/v in Vanilla Ensure Plus ®) or a dextrose containing complete nutritional diet was administered via intragastric intubation. Following either a 5 day or 19 day abstinence period, animals were assessed on a variety of spatial learning and memory tasks (reference memory, cognitive flexibility, and working memory) in the Morris water maze (Morris, 1984). It was found that prior exposure to nicotine dramatically impaired learning during the reference memory assessment, and the task of cognitive flexibility. Ethanol, independently, induced differential search strategies commonly associated with hippocampal damage but this did not contribute to increased difficulty completing learning assessments. However, when administered simultaneously, ethanol appeared to ameliorate the nicotine-induced learning impairment potentially acting as a neuroprotective agent. Further, prior nicotine administration prompted the development of anxiety related

behaviors, which were also attenuated by simultaneous ethanol exposure. These results provide additional concern for potential adverse health outcomes as a result of nicotine use, and posit a role for ethanol administration as a possible inhibitor of nicotine-induced damage.

Keywords: Morris water maze, ethanol, binge, nicotine, chronic, spatial learning and memory

Dedication

To my mother,

who was always there.

And to my father,

who would have been.

Acknowledgements

I would like to take this opportunity to express my gratitude to Dr. Dayna Hayes. I am grateful for the time she spent sharing her knowledge, and the guidance and encouragement continually extended to my academic pursuits. I would also thank my committee members, Dr. Jackson and Dr. Davis, for all their support.

Further acknowledgement must be made to Analise Roccaforte, Emily Tenshaw, Stasi Formica, and Justin Asbee for their continued support and countless hours spent working on the project, without their help this project would not have happened.

Table of Contents

Table of Figures

List of Tables

Chapter 1: Purpose of Study

The present study further elaborates on the neurobiological and behavioral effects resultant from binge level alcohol intoxication in addition to the chronic use of nicotine. In particular, researchers were interested in the combined effects of dual drug exposure on spatial learning and memory. A variety of studies have examined the biological and behavioral correlates of singular drug exposure and identified numerous deficits following either binge alcohol or chronic nicotine abuse. Of particular importance, extended alcohol abuse or chronic levels of nicotine abuse dramatically impair numerous phases of the neurogenic process (McClain, Hayes, Morris, & Nixon, 2011; Morris et al., 2009; Nixon & Crews, 2002; Nixon et al., 2008; Obernier, Bouldin, & Crews, 2002; Richardson et al., 2009; Saito et al., 2005; Shingo & Kito, 2005). Furthermore, significant cognitive deficits in spatial learning and memory have been evidenced following independent drug exposure; possibly as a result of deficits in neurogenesis (Obernier, White, Swartzwelder, & Crews, 2002; Garcia-Moreno & Cimadevilla, 2012; Heffernan, 2008). However, relatively few studies have focused on the potential additive effects of concurrent alcohol/nicotine abuse. Thus, the aim of the current study is to extend this line of research to more fully characterize the effects of dual drug exposure on cognitive functioning (e.g. spatial learning and memory). In line with previous observations on ethanol and nicotine exposure, it was expected that cognitive deficits would be evident in singular drug administration. However, researchers expected dual ethanol and nicotine administration to exacerbate the impairments observed during independent exposure.

Chapter 2: Overview of Previous Research

Excessive alcohol consumption and the abuse of nicotine are persistent concerns throughout the United States, especially among young people who are commonly engaged the use of such substances. Overall, approximately half (51.8%), or 126.8 million American individuals (aged 12+) report a current alcohol consumptive behavior (SAMHSA, 2010). Among these individuals, young adults, otherwise identified as approximately aged 18 - 25 yrs old, account for upwards of 45.9% of the alcohol consumed (SAMHSA, 2010). Additionally, the primary mode of alcohol consumption during young adulthood is in the form of binge drinking behavior (at least 4 or 5 drinks per session) (U.S. Department of Justice, 2002). Further, while binge drinking behavior does indeed decrease among individuals aged beyond young adulthood (>26yr), rates are consistently above 18.9% until late adulthood (>55yr) (SAMSHA, 2010). As such, a significant portion of the population may be susceptible to the development of alcohol use disorders (AUDs) and/or the widespread consequences of excessive alcohol consumption (Crews, Braun, Hoplight, Switzer III, & Knapp, 2000; White & Swartzwelder, 2003; Ziegler et al., 2005).

The consumptive pattern of an alcohol use disorder is generally typified by maladaptive and/or excessive consumption of alcohol that is identified under the nomenclature alcohol abuse, with continued patterns of this behavior potentially resultant in alcohol dependence (Hasin et al., 2007). Broadly, alcohol abuse and dependence are associated with numerous societal, cognitive/behavioral, and/or adverse health outcomes. These include problems such as traumatic injury resultant from an increased prevalence of automobile accidents or domestic violence in addition to increased reports of cardiovascular disease, cirrhosis of the liver, neuropsychological

deficits and cancer, as well as an elevated economic cost to society stemming from lost productivity (Bouchery et al., 2011; Hasin et al., 2007; SAMHSA, 2010).

Importantly, reports have consistently highlighted the comorbidity of associated drug abuse, particularly that of nicotine, with alcohol abuse (Hasin et al., 2007; SAMHSA, 2010). Surprisingly, despite the demonstrated adverse health outcomes associated with tobacco use, nicotine is among the most commonly used legal substances (NIH, 2006; SAMHSA, 2010). Furthermore, while reports have indicated slight decreases over recent years in the overall pattern of smoking behavior among adult populations, the use of nicotine is more prevalent among alcohol users than among those who do not use alcohol regularly (Dani & Harris, 2005; NIH, 2006; SAMHSA, 2010). This pattern of consumption is evidenced in that approximately half of the tobacco products produced in the United States are procured in order to maintain the tobacco use habits of individuals who consistently report binge alcohol consumption (SAMSHA, 2010).

As such a large section of the population is engaged in some form of regular drinking behavior while simultaneously maintaining a consistent cigarette smoking habit, it would appear necessary to more fully evaluate the combined effects of alcohol and nicotine on neurological and associated cognitive functioning in this population.

Overview of Drug Effects

Ethanol is a two-carbon molecule that is generally diluted from its pure form to concentrations of anywhere from approximately five (5) percent in beers to 50 percent in hard liquors, and is characterized as a central nervous system (CNS) depressant (Li, Baler, Egli, 2007). Importantly, alcohol is both water and lipid soluble, meaning it readily diffuses across the blood brain barrier (BBB), whereby some of its major pharmacodynamic actions may be observed (Li, Baler, Egli, 2007). In particular, ethanol exerts agonistic activation of gamma-

aminobutyric acid (GABA) receptors and an inhibition of glutamatergic processes, as well as differentially affecting numerous other neurotransmitter systems (Durazzo, Tosun, Buckley, Gazdzinski, Mon, Fryer, Meyerhoff, 2011). Specifically, alcohol will disrupt excitatory glutamatergic functioning by inhibiting activation of N-methyl-D-aspartate (NMDA) receptor sites (Garcia-Moreno & Cimadevilla, 2012; Durazzo, Tosun, Buckley, Gazdzinski, Mon, Fryer, Meyerhoff, 2011). This suppression of glutamatergic activity results in an up-regulation of NMDA receptors, and has been identified as an important mechanistic determinant by which neuronal damage and loss is incurred, particularly within the hippocampal formation (Soderpalm, Lof, & Ericson, 2009). Additionally, ethanol exerts pharmacodynamic actions through its activation of inhibitory GABAergic processes resulting in the most commonly observed behavioral effects of alcohol: sedation, muscle relaxation, and impaired motor and cognitive functioning (Soderpalm, Lof, & Ericson, 2009). Stimulation of such GABAergic processes also results in subsequent activation of endogenous opioid receptors which modulate dopaminergic functioning within mesolimbic structures; particularly the ventral tegmental area (VTA) (Buccafusco, Letchworth, Bencherif, & Lippiello, 2006; Soderpalm, Lof, & Ericson, 2009). Ultimately, this agonistic action on GABAergic processes results in the augmentation of dopamine functionality within the VTA and may be implicated in addictive pathologies and/or the rewarding effects of ethanol (Soderpalm, Lof & Ericson, 2009). Particularly important, the inhibition of glutamatergic, and stimulation of GABAergic processes following ethanol administration have been implicated in the disruption of long term potentiation, discussed in later sections as an important factor in memory formation (Kandel, 2000).

Likewise, nicotine is effectively distributed throughout the body, exerting its effects on numerous physiological structures. Of particular importance, nicotine also easily diffuses across

the BBB leading to various neurological effects (Soderpalm, Lof, Ericson, 2009). Specifically, nicotine administration is associated with the activation of nicotinic acetylcholine receptors (nAChRs), particularly that of the $\alpha_4\beta_2$ and α_7 subtypes (Buccafusco, Letchworth, Bencherif, & Lippiello, 2006; Soderpalm, Lof, & Ericson, 2009). Acetylcholine receptors (AChR) are found throughout the CNS and PNS, with relatively large concentrations of nAChRs found within the CA3 region and dentate granule cells of the hippocampus (Buccafusco, Letchworth, Bencherif, & Lippiello, 2006; Felix & Levin, 1997). It has been demonstrated that the innervation of such nicotinic receptors within the hippocampus is implicated in hippocampal synaptic activity, thereby possibly facilitating long-term potentiation (Rezvani & Levin, 2001). However, such findings appear more indicative of acute nicotine administration (Rezvani & Levin, 2001). Chronic levels of nicotine administration are associated with prolonged nAChR upregulation and dysfunctional cholinergic activity, which is implicated in impaired synaptic functionality (Trauth et al., 2001). Furthermore, nicotine supports addictive pathologies by exerting an indirect agonistic action of dopamine neurotransmitter systems within mesolimbic structures similar to that observed in ethanol administration (Buccafusco, Letchworth, Bencherif, & Lippiello, 2006; Felix & Levin, 1997; Soderpalm, Lof, & Ericson, 2009). Such agonistic action within mesolimbic structures appears to be the mechanism by which the stimulatory and reinforcing effects of the drug develop. As such, the synergistic potentiation of alcohol use by nicotine, and vice versa is evident, as both drugs activate similar pathways. Therefore, the dual effects of the drugs may be more dramatic than either drug produces independently.

Process of Neurogenesis

Neurogenesis is a four-stage process (proliferation, differentiation, maturation, and survival/integration) involving "stem" and/or "progenitor" cells (Abrous, Koehl & Le Moal,

2005; Balu & Lucki, 2009, Hsieh & Eisch, 2010). Ongoing developments in hippocampal plasticity have focused on the "progenitor" distinction of cell type, primarily due to the relative ambiguity associated with such cells. Progenitor cells are distinguished from "stem" cells by their inability to maintain indefinite self-renewal (Abrous, Koehl & Le Moal, 2005; Balu & Lucki, 2009, Hsieh & Eisch, 2010). In the adult mammalian brain, two regions have been identified as possessing the ability to generate such progenitor cells (i.e neurogenesis); the subgranular zone (SGZ) of the dentate gyrus and the subventricular zone (SVZ) of the lateral ventricles. Importantly, progenitor cells within the subgranular zone (SGZ) of the dentate gyrus are located within the hippocampal formation (HF), an area implicated in ongoing plastic developments associated with spatial learning and memory (Nixon & Crews, 2004).

Progenitor cells that have differentiated into neurons are incorporated into the granule cell layer of the dentate gyrus post mitosis (Balu, & Luckin, 2009; Emsley et al., 2005, Abrous et al., 2005). These newborn cells are incorporated in the existing GCL 4-10 days following genesis, whereby dendritic and axonal extensions begin projecting to the CA3 region of the hippocampal formation (Balu, & Luckin, 2009; Emsley et al., 2005, Abrous et al., 2005). Four to eight weeks following generation, neurons within the DG will exhibit synaptic integration within the pre-existing circuitry (Abrous et al., 2005; Balu, & Luckin, 2009; Emsley et al., 2005). Subsequent cholinergic, serotonergic, dopaminergic, glutamatergic, in addition to GABAergic processes may then be observed projecting into cortical regions (Abrous et al., 2005; Balu, & Luckin, 2009; Emsley et al., 2005). Importantly, Tashiro et al. (2006) identified NMDA receptor (a subtype receptor of glutamatergic processes) regulatory specificity in facilitating the survival of the newly born cells. Thus, NMDA receptor activity during early periods of neuronal development may be an important factor toward the survival and subsequent integration of

neurons into existing hippocampal circuitry. Additionally, these newly born neurons display long-term potentiation (LTP) induction at a lower threshold of synaptic strengthening than observed in the existing neural framework, discussed in later sections as indicative of functional relevance toward learning and memory (Balu & Luckin, 2009).

Function of Neurogenesis within the Hippocampal Formation

The hippocampus is generally thought of as the region most associated with memory (Kemperman, 2002). Essentially, the hippocampus acts as a relay station, whereby short-term memories are consolidated into the existing neural circuitry of cortical regions (Kemperman, 2002; Moscovitch, 2005). Axonal tracts project from the dentate gyrus to pyramidal cells in the CA3 region (Balu, & Luckin, 2009; Emsley et al., 2005, Abrous et al., 2005; Kemperman, 2002; Moscovitch, 2005) and will then further progress to the CA1 region and subsequently to associated cortices (Balu, & Luckin, 2009; Emsley et al., 2005, Abrous et al., 2005; Kemperman, 2002; Moscovitch, 2005). Importantly, hippocampal connections from the CA1 region through the subiculum to extended hippocampal regions, such as the mammillary bodies and anterior thalamic nuclei have been identified as integral in the spatial context of memories (Moscovitch, 2005). As such, the maintenance of hippocampal connectivity would appear integral to learning and memory formation. Indeed, Kandel (2000) identified that the process of learning results in the synaptic strengthening of hippocampal connections to adjacent areas (i.e. long-term potentiation). Snyder, Kee and Wojtowicz (2000) extended this development suggesting that newly born neurons may be more influential in the learning process than mature neurons, as the threshold for long-term potentiation is relatively lower among younger neurons. Subsequently, it is this progression of connectivity and strengthening of synaptic processes that would appear integral toward the consolidation of memories within cortical regions (Kemperman, 2002;

Snyder, Kee, & Wojtowicz, 2000). Therefore, dysfunctional neurogenic processes within the dentate gyrus may limit the consolidation of information into cortical regions by disrupting the connectivity necessary for such integration, in particular that of the functional necessity of plastic rearrangements and synaptic strengthening attributed to newly born neurons (Gage, 2002; Kandel, 2000; Snyder, Kee, & Wojtowicz, 2000). The importance of such neurogenic processes toward memory formation may be evidenced, in part, by an organism's ability to form trace memories, a form of classical conditioning requiring an intact hippocampus wherein the conditioned and unconditioned stimuli presentation is partitioned by an inter-stimulus interval (Abrous et al., 2006; Bangasser, Waxler, Santollo & Shors, 2006). Methylazoxymethanol, a DNA methylating agent is observed disrupting such trace conditioning by actively decreasing the number of newly born neurons (Abrous et al., 2006). Following cessation of methylazoxymethanol treatment, animals are observed acquiring the trace conditioned response, suggestive of a return to normal levels of immature neurons (Abrous et al., 2006). Therefore, as such cognitive impairments are associated with decreased levels of neuroproliferative action, it is reasonable to assume the relative importance of such functionality toward performance on cognitive tasks.

Ethanol-Induced Neurodegeneration

Widespread neural damage occurs following ethanol intoxication; this damage is generally associated with significant losses of cortical volume, including gray and white matter reductions, as well as neuronal loss among the frontal lobes (Crews, Braun, Hoplight, Switzer III, & Knapp, 2000). These areas are associated with the regulation of more complex cognitive abilities, such as memory formation, learning, attention, mood, and many others (Crews et al., 2000; Crews & Nixon, 2008). Indeed, binge ethanol exposure will result in neurodegenerative

patterns within corticolimbic areas producing deficits to spatial learning and memory (Obernier, White, Swartzwelder, & Crews, 2002). Of particular importance is the damage following binge exposure apparent in hippocampal and adjacent (entorhinal) regions, evidenced by the emergence of actively degenerating neurons (Obernier et al., 2002).

Extensive work has been devoted to the understanding of the effects of ethanol intoxication on the hippocampus. As such, it has been identified that the hippocampal formation is particularly susceptible to the effects of ethanol intoxication, and while this damage may be dependent on the method of administration (i.e. chronic and binge), the impairments are profound (Crews & Nixon, 2008). For example, chronically high levels of ethanol consumption will result in decreased numbers of CA1 and CA3 pyramidal neurons, as well as decreased cell numbers in the granule cell layer of the dentate gyrus (Fadda & Rosseti, 1998). Furthermore, binge level ethanol consumption has been shown to result in deficits to hippocampal neurogenesis, evidenced by reductions in proliferation and survival of new neurons (Nixon & Crews, 2004; Morris, Eaves, Smith, & Nixon, 2010; Shingo & Kito, 2005). Additionally, increased levels of necrotic and apoptotic forms of cell death in hippocampal and adjacent areas (piriform, entorhinal, perirhinal cortices) are observed following binge level ethanol consumption (Crews et al., 2000; Crews & Nixon, 2009; Crews et al., 2004; Nixon & Crews, 2004).

It has also previously been established that dramatic cognitive impairments will result from binge level ethanol intoxication. For example, spatial learning and memory has been examined using the Morris water maze following binge level ethanol intoxication (Obernier et al., 2002). Initial completion of the task among ethanol administered experimental subjects is similar to that observed in drug naïve animals (Obernier et al., 2002). However, subsequent trials

wherein the animal is required to relearn the task (i.e., platform is moved to another quadrant) in a manner opposite or different from the initial learning prove difficult (Crews, & Nixon, 2009; Nixon, & Crews, 2002; Obernier et al., 2002). This inability to relearn the task is evidenced, in part, by a perseverative behavior toward the initial requirements of the task. These deficits are generally consistent with those observed in patients with dysfunctional frontal lobe activity (Crews, & Nixon, 2009; Nixon, & Crews, 2002). As such, dysfunctional neurogenic processes may limit the ability of the hippocampus to process this change of information by impairing functionally relevant rearrangements necessary to the learning process, subsequently inhibiting consolidation into the existing neural framework (Crews et al., 2000; Gage, 2002; Kandel, 2000; Kemperman, 2002; Moscovitch, 2005; Snyder, Kee, & Wojtowicz, 2000).

Effects of Nicotine on Cognitive Functioning

Smoking behavior has extensive effects on neural functioning, however, these effects are complex and difficult to characterize. Divergent reports have elaborated on both the deleterious effects and the beneficial uses of nicotine in cognitive functioning. As discussed previously, nicotinic acetylcholine receptors (nAChr), the agonistic site of action for nicotine, are found throughout the CNS and PNS (Buccafusco, Letchworth, Bencherif, & Lippiello, 2006; Felix & Levin, 1997). Of particular importance are the relatively high concentrations of nAChr found within the CA3 region and dentate gyrus granule neurons of the hippocampus. Thus, as identified previously, high concentrations of nAChr in the dentate granule cells may be suggestive of the functional importance to memory processing (Buccafusco, Letchworth, Bencherif, & Lippiello, 2006; Felix, & Levin, 1997). Indeed, reductions in nicotinic receptor density are found throughout the brains of individuals with Alzheimer's disease, particularly in the frontal lobe regions (Buccafusco, Letchworth, Bencherif, & Lippiello, 2006; Felix, & Levin, 1997). This

reduction of nACH receptor density has been posited as a direct correlate of the cognitive issues facing Alzheimer's disease patients, namely dysfunctional memory systems.

Furthermore, administration of acute levels of nicotine has been shown to improve memory functioning among laboratory animals (Buccafusco, Letchworth, Bencherif, & Lippiello, 2006). This improvement has also been observed throughout a variety of nicotinic agonists acting on various subtypes of ACh receptors (i.e. ABT-418 and GTS-21), suggesting the functional diversity of nAChRs in relation to memory functioning (Buccafusco, Letchworth, Bencherif, & Lippiello, 2006; Felix & Levin, 1997). Furthermore, nicotinic antagonists have provided additional confirmatory determinations toward the influence of nAChrs, namely impairments to working memory dependent tasks following antagonistic action (Felix & Levin, 1997). For example, administration of the nicotinic antagonist Mecamylamine has been shown to impair memory performance in a radial arm maze examination (Felix, & Levin, 1997). Thus, the beneficial effects of nicotine exposure by way of nACh receptor activation are evident.

It has also been demonstrated that nACh receptor agonists provide for similar strengthening of synaptic processes that are identified in learning and memory. For example, numerical increases of neurons as well as functionally more efficient synaptic connectivity (Buccafusco, Letchworth, Bencherif, & Lippiello, 2006; Felix & Levin, 1997; Kandel, 2000; Snyder, Kee, & Wojtowicz, 2000). Specifically, two subtypes of nACh receptor sites (α7 and β2) associated with highly permeable Ca^{2+} dependent ligand-gated channels have been identified as important determinants of long term potentiation (LTP), previously defined as increases in synaptic strength (Buccafusco et al., 2006; Kandel, 2000). As such, the activation of nACh receptor sites results in cellular signaling directly correlated with the structural changes identified as important determinants of enhanced cognitive capacities (i.e., synaptic strengthening)

(Buccafusco et al., 2006; Kandel, 2000). However, these enhancements appear to be diminished in examinations of the chronic use of nicotine; potentially as a result of nAChR desensitization (Ernst et al., 2001). Thus, this appears to suggest that the beneficial uses of nicotine are associated with relative drug naiveté rather than regular use (Ernst et al., 2001).

Indeed, increased levels of degenerating cells, evidenced in part by the number of pyknotic cells throughout the hippocampal complex, are seen following chronic nicotine administration (Abrous et al., 2002). Furthermore, recent examinations of the effects of chronic nicotine use on neurogenesis have shown impaired processes (Abrous et al., 2002). Specifically, chronic nicotine administration will reduce the number of proliferating cells within the SGZ of the dentate gyrus (Abrous et al., 2002). As such, memory systems may be adversely impacted, as the integration of newly born cells appears integral to the synaptic strengthening associated with learning and memory. Interestingly, this reduction in proliferative cells has been suggested as primarily dependent on the nicotinic induction of calcium-mediated apoptosis (Berger, Gage & Vijayaraghavan, 1998). As previously discussed, several nAChR subtypes are highly permeable to Ca2+, excitatory action at which will increase levels of intracellular free calcium (Berger, Gage & Vijayaraghavan, 1998). This pathway is implicated in the cognitive enhancements associated with nAChR activity, particularly that of LTP, as an abundance of nAChR sites are located on hippocampal cells (Buccafusco et al., 2006). However, it has been demonstrated that undifferentiated proliferative cells lack a major calcium buffer that is not incorporated into the cellular structure until neuronal or glial fate has been determined (Berger, Gage & Vijayaraghavan, 1998). Therefore, excessive innervation of nAChRs by chronic levels of nicotine results in elevated levels of intracellular free calcium in undifferentiated progenitor cells that are incapable of buffering against calcium cytotoxicity.

Furthermore, nicotine promotes the release of the neurotransmitter glutamate, possibly through cholinergic innervation interacting with glutamatergic transmission (Radcliffe, Fisher, Gray, Dani, 1999). This process would appear particularly important when coupled with ethanol, as ethanol intoxication depresses aspects of glutamatergic activity, which results in a persistent up-regulation of NMDA receptor subtypes (Chefer et al., 2011; Julien, 2011; Bruijnzeel et al., 2011). Provided this is the case, excessive glutamate release in addition to NMDA receptor up-regulation will result in glutamatergic excitotoxicity and may be particularly implicated in the neurotoxic effects of combined exposure (Chefer et al., 2011).

Interestingly, Shingo and Kito (2005) also reported decreases in neuronal nuclei positive cells, markers of mature neurons, following chronic nicotine exposure; indicating detrimental effects of chronic nicotine use in multiple stages of neurogenesis. This would appear to suggest additional neurodegenerative mechanisms throughout the neurogenic cycle other than just the calcium cytotoxicity observed in undifferentiated proliferative cells (Berger et al., 1998; Shingo & Kito, 2005).

This inhibition and/or disruption of neurogenic processes in the DG may provide the mechanism by which working memory is impaired. Indeed, a comparison of performance on an N-Back memory task, a reflection of working memory capacities in which the subject is required to remember a series of letters that is continually updated, indicated faster reaction times with more accurate responses among drug naïve subjects than evidenced in individuals categorized as regular smokers (Ernst et al., 2001). This examination suggests potential impairments to working memory following prolonged use of nicotine (i.e., chronic use), as retrieval of the memory items in the N-Back task among regular smokers was deficient when compared to naïve drug controls (Ernst et al., 2001). Therefore, the chronic use of nicotine reduces the ability of working memory

systems to function as effectively as is apparent in individuals who do not engage in a regular smoking habit.

Combined Nicotine and Ethanol-Related Neurodegeneration

As it is apparent that both alcohol and nicotine exhibit complex neural actions (and these drugs are commonly co-abused) an understanding of how these substances interact when administered simultaneously is critical to future endeavors. Presently, binge alcohol consumptive behavior has been demonstrated to result in the reduced capacity to perform neurogenic functions integral to hippocampal integrity, subsequently increasing the prevalence of cognitive deficits. Furthermore, an active degeneration of neuronal and glial structures is evident following ethanol exposure (Crews & Nixon, 2009; He et al., 2005; McClain et al., 2011; Morris et al., 2010; Obernier et al., 2002).

Additionally, while acute nicotine exposure has been shown to enhance certain cognitive capabilities, chronic models of nicotine administration evidence similar effects to that of binge alcohol exposure (Abrous et al., 2005). As discussed previously, chronic nicotine use has been demonstrated increasing the number of actively degenerating cells, as well as inhibiting aspects of the neurogenic process (Abrous et al., 2005). Due to the similarities following ethanol and nicotine exposure in neural damage, as well as the mechanisms by which this damage is incurred, the potential for additive and/or synergistic effects is evident. However, research toward an understanding of such concomitance has been relatively lacking.

Therefore, due to the complexity of such effects, it was necessary to elaborate on any potential cognitive deficits. To that end, an examination of the Morris water maze (MWM), a hippocampal dependent task of spatial learning and memory (Obernier et al., 2002), was explored following dual nicotine/alcohol exposure. It was expected that the combined exposure

of alcohol and nicotine would result in additive deficits to the capacity to perform cognitive tasks associated with such spatial learning and memory.

Chapter 3: Methodology

Subjects

Adult (N = 46) male Sprague-Dawley rats, bred in-house, were used. Animals were approximately 192 days post birthing. Animals were maintained on a 12:12 light cycle in a temperature and humidity-controlled vivarium. Prior to experimentation, animals were allowed *ad libitum* access to regular rat chow and water. Vanilla Ensure Plus®, a nutritionally complete diet containing dextrose, replaced regular food chow for unhandled control subjects upon commencement of binge ethanol exposure. Experimental animals were housed in individual stainless steel hanging cages (10" x 8" X 8"). In addition, the NIH Guide for Animal Care and Use of Laboratory animals was utilized in order to maintain appropriate experimental procedures and ethical/humane care of laboratory animals. Furthermore, all experimental procedures and associated protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Radford University.

Design and Specific Aims

The present study utilized an experimental design focusing on drug exposure modeled after binge alcohol and chronic nicotine use in a population of Sprague-Dawley rats. Spatial memory and learning was examined using a hippocampal-dependent learning task (Morris Water Maze) in a laboratory setting (Obernier et al., 2002). A 2 (short term vs protracted withdrawal) X 2 (ethanol vs. dextrose ensure) X 2 (nicotine vs. saline) repeated measures factorial design was expected to show main effects for drug conditions. The trials and/or days for MWM testing was the within-subjects variable, and the drug and period of abstinence conditions were the between subjects variables. Two periods of withdrawal/abstinence before learning assessment {Short term (day 5-17), Protracted (day 19 – 31)} were implemented to assess the influence of an extended

abstinence period following drug administration. However, no effect of withdrawal was expected. A significant main effect for ethanol diet was expected, such that animals in the ethanol diet experimental condition were expected to show significantly increased impairments in the MWM than those animals in the dextrose diet group. A significant main effect for the nicotine solution was also expected. As such, animals receiving nicotine injections were expected to show significantly elevated levels of impairment in the MWM when compared to saline injections. A significant ethanol X nicotine interaction was expected; animals receiving the combined drug exposure paradigm were expected to require significantly longer periods of time/distance to complete the MWM than either nicotine or ethanol produced independently.

Furthermore, as recent developments have suggested potential complications involving the effect of stress resultant from the method of administration used to model binge alcohol consumption, the present study utilized an additional experimental design feature focusing on methodological comparisons. A comparison of an unhandled control (receiving isocaloric dextrose containing supplement in water bottle form) and the existing experimental control (receiving IG and SQ injections of dextrose and saline, respectively) was completed. It was expected that the experimental control would perform significantly worse on the MWM than unhandled controls due to heightened stress responses.

Procedures: Unhandled Control

A non-injected control group, referred to as "unhandled," were housed in stainless steel hanging cages (10" x 8" X 8"). and given an equivalent volume to that received by the injected control of a dextrose containing liquid Vanilla Ensure Plus® diet in a water bottle during the binge ethanol paradigm. This provided access to the same form of diet (liquid) as experimental

conditions. Animals in this condition did not receive regular food chow during the binge ethanol exposure.

Chronic Nicotine Treatment

Adult male Sprague-Dawley rats in the nicotine experimental groups (nicotine*dextrose, & ethanol*nicotine) were administered an injection of nicotine solution subcutaneously three times daily (7am, 3pm, 11pm) for 10 days. Nicotine (100%) was administered at a dosage of 0.3 mg/kg, prepared by dilution in isotonic saline (0.9%) in order to reach final concentration. Control rats received a subcutaneous injection of saline on the same timeframe as experimental subjects.

Binge Ethanol Treatment

Adult male Sprague-Dawley rats in the ethanol-receiving experimental groups were administered ethanol (25% w/v in Vanilla Ensure Plus ®), following a modified model of binge exposure (Majchrowicz, 1975), via intragastric intubation. Ethanol administration began on the sixth day of nicotine exposure and continued for the remaining four days of the drug exposure paradigm. As such, ethanol (25% w/v in Vanilla Ensure Plus®) was administered along the same timeline as nicotine: 3 times a day (7am, 3pm, 11pm). Rats received an initial 5.0 g/kg priming dose of ethanol to induce intoxication, with subsequent doses determined by the behavioral intoxication of the rat (see Appendix A). Experimental control rats received an isocaloric diet of Vanilla Ensure Plus® containing a caloric control, administered along the previously discussed timeframe via intragastric intubation. Refer to Figure 1 for a representation of the procedures timeline.

Blood Ethanol Concentrations

Ninety minutes following the 7th dose of ethanol, subject blood was extracted via tail snip to determine blood ethanol levels. Blood fractionation then followed via centrifuge (1800xg for 5 minutes) in order to separate plasma. An Analox (AM1) analyzer was then used to obtain plasma blood ethanol concentrations.

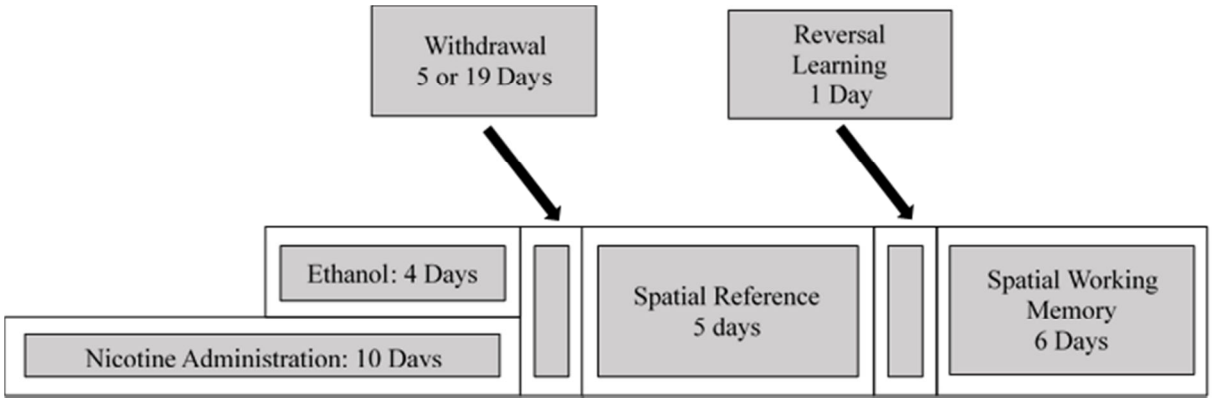

Figure 1. Timeline

Withdrawal Monitoring

At the beginning of every hour for 18hrs, and for a total duration of 30 min. following the final doses of alcohol and nicotine, withdrawal behaviors were monitored. Animals were transferred to standard housing tubs (44cm x 22 cm x 20.5 cm) with commercial bedding for the monitoring period. See Appendix B for complete descriptions of withdrawal behaviors (Majchrowicz, 1975).

Morris Water Maze

Following elimination of withdrawal behaviors (see Appendix B), animals were randomly separated into either a five day (short term abstinence) or a nineteen day (protracted abstinence) interval period before examination on spatial acquisition, reversal learning, and working memory using the Morris Water Maze (MWM) (Obernier et al., 2002; Vorhees & Williams, 2000). During the abstinence period animals were housed in stainless steel hanging cages and returned to *ad libitum* access to regular rat chow. The MWM is an open circular,

plastic pool with a moveable platform that is placed in one of the four directional quadrants, depending on the particular task. The pool is divided into four directional quadrants (NE, NW, SE, SW) by perpendicular bisecting lines not visible to the rats. Each line marks the four cardinal directions (N, S, E, W) with south facing the experimenter. The pool was housed within a square room with distal cues hung from each wall. Distal cues remained constant throughout the experimental phase. The pool was filled with water (~22°C ± 2°C) mixed with a non-toxic white paint, to mask the platform. The animal's movement during behavioral tasks was measured by DVR video camera and analyzed using HVS imaging software (Mountain View, CA). Upon completion of each individual trial, the water in the tank was stirred in order to account for odor trails (Obernier et al., 2002; Vorhees & Williams, 2010).

Spatial Memory – Acquisition Task

Procedures were based on the protocol outlined by Vorhees and Williams (2010). Reference memory assessment was conducted across five days with four trials per day, during which the submerged platform was placed in the SW quadrant and remained for each day of the acquisition task (Morris, 1984). At each trial the animal is placed systematically in a different starting quadrant facing the wall of the pool (See Table 2 for starting positions). Each animal was allotted 90 seconds per trial to locate the platform. Upon successful completion of the task, the animal was allowed to remain on the platform for 15 seconds. If the animal was unable to locate the platform within the 90-second period, the experimenter guided the animal to the platform where the animal remained for 15 seconds. Upon completion of each trial, the animal was placed in a separate housing unit for 30 seconds before the next trial began.

Spatial Memory – Reversal Learning/Probe Trial

An additional day following spatial acquisition was devoted to a probe trial and reversal learning. For the probe trial, the platform was removed from the pool and each animal was administered a 90-second trial, starting from the NW quadrant, with no potential for escape. Immediately following the probe trial, each animal was assessed on reversal learning. During this period the platform was moved to the opposite quadrant (NE) of the pool relative to its original position during acquisition learning (SW; Morris, 1984). See Table 3 for starting positions. Each animal again underwent four trials with an allotment of 90 seconds per trial to locate the platform. Following successful attempts, the animal was given 15 seconds to remain on the platform. Again, if the animal failed to locate the platform during the trial period, the experimenter guided the animal to the platform, where the subject remained for 15 seconds. Each trial was again partitioned by a 30-second interval during which the animal was placed in a separate housing unit.

Spatial Working Memory Task

Spatial working memory was assessed by placing the submerged platform in a different quadrant on each day of a six-day testing period (See Table 4 for sequence of starting & platform positions; Morris, 1984). A matching to sample exercise was used wherein animals were administered two trials on each testing day, the first of which was designated as a sample trial and the second being the test trial in which the animal attempted to match behavior to the sample trial; latency/distance to target savings between Time 1 (Sample) and Time 2 (Test) was measured (Vorhees & Williams, 2010). Again, each trial allowed for a total of 90 seconds for the animal to reach the platform. Upon successful completion, the animal was allowed 15 seconds on the platform. Failure to reach the platform during the 90-second trial period again prompted the experimenter to guide the subject to the platform, where the animal remained for 15 seconds.

There was a 30-second inter-trial period during which the animal was housed in a separate unit to await the second trial (Morris, 1984).

Quantification: Morris Water Maze

As discussed previously, spatial reference and working memory was examined using the Morris water maze (MWM). Each examination requires four trials per day over the course of five days separately. Performance on the MWM (spatial reference & working memory) is assessed as average distance (meters) swam/time spent swimming (seconds) (± SEM) across time points (Obernier et al., 2002; Vorhees & Williams, 2000). Analyses of time spent/distance swam in the target quadrant, and in the target zone, of the maze are also provided as confirmatory evidence for either learning or lack thereof.

Reversal learning is assessed as the mean (± SEM) number of trials to reach criterion. As identified in Obernier et al. (2002), reversal learning criterion is expressed as the average distance/latency (across all animals) required to reach the platform on the last day of the spatial reference/acquisition task plus two standard deviations (Obernier et al., 2002). Additionally, time spent/distance swam in original and target quadrants, as well as the target zone, were measured for the reversal learning task (Obernier et al., 2002).

Planned Statistical Analysis

In the present study, data analysis was separated into a series of general linear model assessments: subject analysis, analysis of drug administration method, acquisition learning, probe trial, reversal learning, and working memory. The subject analyses compared the subject variables (weight, withdrawal behaviors, body weight loss) across the drug administration variable (ethanol & nicotine) in order to identify any potential confounding relationships. The subject analyses consisted of a series of either one-way ANOVAs or independent samples *t*-tests

with drug treatment as the categorical predictor and blood ethanol concentrations (BEC), behavioral intoxication score, drug dosage, and body weight (prior to and during drug exposure) as the dependent variables.

The analysis of drug administration method was conducted in order to assess the influence of intragastric intubation when evaluating cognitive performance. Independent samples *t*-tests were used in order to assess the potential difference between intubated and non-intubated control animals. Provided no significant differences were found, controls were collapsed into a single control group in order to maximize statistical power.

The main analyses were separated by learning period (acquisition, probe, reversal, working memory) and consisted of a series of 2 (diet) x 2 (injection) x 2 (cohort/abstinence period) repeated measures ANOVA to test interactions between drug exposure (ethanol & nicotine) and/or abstinence period with learning dependent variables (latency, path, zone, quadrant). If a significant interaction was found between the variables of interest, simple effects were conducted for each independent variable to identify the area of occurrence.

Chapter 4: Results

Subject Analysis

Adult male Sprague-Dawley rats (*N*=33, Mean age: 192.06 days, *SD* = 16.10) were administered a modified Majchrowicz (1975) model of binge ethanol with added chronic nicotine exposure consisting of ten (10) days of nicotine administration (0.3mg/kg) three times daily (7am, 3pm, 11pm) with the final four days of drug exposure combining binge modeled ethanol administration (25% in Vanilla Ensure Plus©). Treatment groups were separated into either independent drug exposure (ethanol only, nicotine only), dual drug exposure (ethanol and nicotine), or control (treatment control or stress control). Animal body weights were measured immediately prior to initial dosing (*M* = 696.83g, *SD* = 91.15). A one-way ANOVA, with treatment group as the between subjects factor, was used to assess potential differences in initial body weight. No differences in body weight between groups were observed prior to drug administration, $F(4, 40) = 0.47$, $p = .759$. As such, all experimental animals began drug administration with similar body weights.

During the binge ethanol administration a 26.6% mortality rate was observed, resulting in 33 animals surviving to complete MWM testing phases. The combined ethanol and nicotine exposure group had the largest rate of mortality (60%), indicating possibly either experimenter error or problems associated with the physiological interaction of nicotine and ethanol, such as the increased ethanol toxicity associated with combined exposure (See later details on BEC levels for complete description). As a result of the high mortality rate an analysis on the effect of treatment group on survival rate was conducted. A one-way ANOVA with treatment group as the categorical predictor revealed a significant effect of treatment on mortality, $F(4, 40) = 2.71$, $p = .044$. Fisher's LSD post-hoc analysis indicated that the animals that had received the dual ethanol

and nicotine exposure paradigm (M = 1.60, SE = .16) had a significantly greater rate of mortality than animals that had received nicotine only (M = 1.10, SE = .10), in addition to the non-intubated control group (M = 1.00, SE = .000). This effect may have been due to treatment order and/or practice effects as the ethanol and nicotine receiving animals received drug treatment at the beginning of each dosing session. Results, however, do suggest that the combined ethanol/nicotine administration paradigm is significantly more likely to induce mortality than is evident in a nicotine only exposed animal. Due to the loss of animal subjects prior to behavioral testing, all further analyses on spatial learning and memory were based on an N of 33.

An analysis of body weight during the binge ethanol administration was conducted in order to rule out the influence of a dietary deficiency resultant from the change of *ad libitum* access to regular rat chow to a restricted liquid diet of Vanilla Ensure Plus® on spatial learning and memory processes, as well as to monitor the general health of animals. Across the binge ethanol administration period, all animals experienced an expected proportion of body weight loss relative to pre-binge body weight (M = 0.07, SD = 0.04). No differences between treatment groups were observed in percentage of weight loss relative to pre-binge weight, $F(4, 29) = 0.91$, $p = .469$. Accordingly, all animals entered behavioral testing with a similar body weight, having experienced relatively comparable weight loss attributed to the change in diet.

In order to evaluate the drug intoxication level of each subject, independent samples t-tests or one-way ANOVAs were performed on drug dosage, ethanol intoxication behavior score, and blood ethanol concentration. A one-way ANOVA with treatment group as the categorical predictor was conducted to evaluate nicotine/saline dosage between injection receiving treatment groups. There were no significant differences between any treatment groups in nicotine/saline dosage received, $F(3, 23) = 0.54$, $p = 0.657$. It was identified that all injection-receiving animals

were administered equivalent nicotine or saline dosages. Refer to Figure 2 for a representation of mean injected volume.

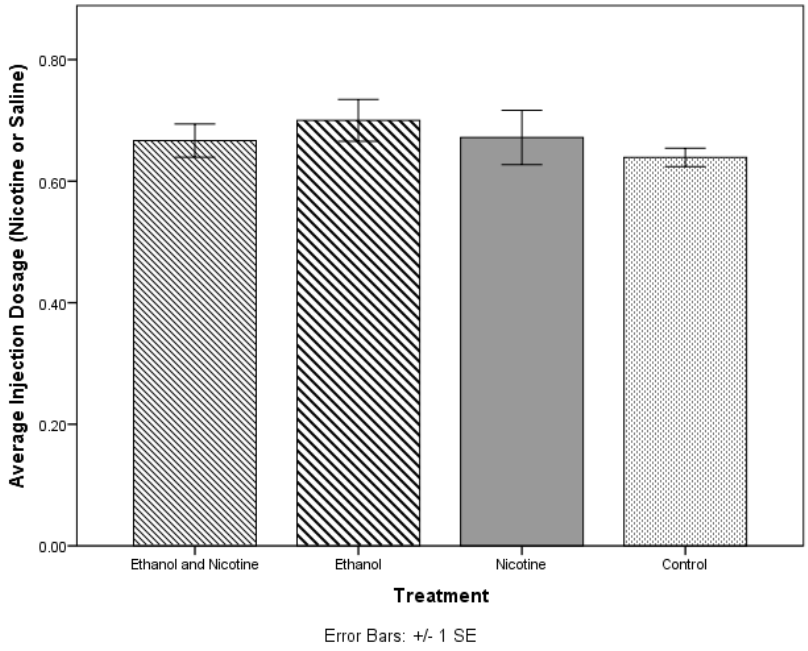

Figure 2. Representation of injected volumes between treatment groups.

To evaluate the ethanol intoxication levels between the ethanol only and the combined ethanol and nicotine exposure treatment groups, an independent samples *t*-test was used. There was no significant difference between the treatment groups on ethanol dosage, $t(10) = -0.67$, $p = 0.52$). Results indicated that the ethanol only treatment group ($M = 6.52$mL, $SD = 1.09$) was administered an equivalent dose of ethanol as the combined ethanol/nicotine treatment group ($M = 6.02$mL, $SD = 1.48$). An additional analysis of blood ethanol concentration was conducted in order to further evaluate ethanol toxicity between the ethanol receiving treatment groups. An independent samples *t*-test revealed no significant differences between the ethanol only treatment group ($M = 229.81$ mg/dl, $SD = 51.65$) and the ethanol/nicotine receiving treatment group ($M = 208.65$ mg/dl, $SD = 70.00$); $t(10) = 0.53$, $p = 0.607$. Results indicated that the ethanol-receiving treatment groups were similarly intoxicated during the drug administration period. This pattern of ethanol toxicity across ethanol receiving treatment groups was also evidenced behaviorally.

Another independent samples *t*-test was utilized to evaluate the behavioral intoxication ratings during the binge administration between ethanol receiving groups. There was no significant difference in behavioral intoxication ratings between the ethanol only treatment group ($M = 2.59$, $SD = 0.29$) and the ethanol/nicotine receiving group ($M = 2.65$, $SD = 0.64$); $t(10) = 0.20$, $p = 0.846$. As such, animals received similar volumes of ethanol during the binge administration period, as well as displayed relatively equivalent behavioral intoxication levels.

Following treatment cessation, ethanol withdrawal severity was monitored for 18hrs, at thirty-minute increments following the final ethanol dose (beginning 10 hours after final ethanol dose), in order to assess withdrawal severity. Withdrawal behaviors were scored on a scale of 0 (no WD behavior) to 4 (death), depending on the severity of the WD behavior, and were summed for each 30 min time point per animal. Averages of summed values for each time point across the 18hr period were then calculated and an independent samples *t*-test was used in order to assess potential differences between the withdrawing animals who received combined ethanol and nicotine, and the withdrawing animals who received only ethanol. There were no significant differences in withdrawal behaviors between the treatment groups, $t(10) = -1.44$, $p = .180$. Dual ethanol and nicotine receiving animals ($M = .32$, $SEM = .09$) experienced equivalent levels of withdrawal, as compared to ethanol-only receiving animals ($M = .70$, $SEM = .18$). Additional independent samples *t*-tests on each 30 minute observation time-point revealed no differences, as well ($p > .05$)

Analysis of Drug Administration Method

As previous work from our lab had suggested the potential influence of an intragastric gavage in deficits in hippocampal neurogenesis, an important determinant of spatial learning and

memory, an analysis of learning differences attributable to the drug administration method was necessary to rule out complications in the assessment of learning. As such, researchers developed two control groups; one (treatment Control) received control diet (Dextrose in Vanilla Ensure Plus®) and an isotonic saline (0.9% NaCl) solution via intragastric intubation and subcutaneous injection, respectively, the other (non-intubated control) received the control diet via a restricted self-administration avenue and was not administered the saline solution. An analysis of the method of administration (i.e. intragastric intubation and subcutaneous injection) toward spatial learning and memory was examined using independent samples *t*-tests, or repeated measures ANOVA (depending on variable) across all learning dependent variables. No differences between intubated and non-intubated controls were found on any learning dependent variables ($p > .05$; See Table 1). Results suggest that the method of drug administration did not influence learning in the present study. As such, the unhandled and experimental controls were collapsed into one overall control group; all subsequent analyses were conducted using the overall control group (hereby referred to as control).

Table 1. Pairwise Comparisons between Intubate/injected control and Unhandled control

Dependent Variable	*SS*	*df*	*MS*	*F*	*Sig.*
Acquisition Latency	310.947	1	310.947	1.69	.221
Acquisition Path	6.069	1	6.069	1.37	.266
Thigmotaxis Time (Days 1-12)	64.041	1	64.041	1.74	.229
Thigmotaxis Path (Days 1-12)	34.954	1	34.954	2.269	.176
Working Memory: LTS	.793	1	.793	.001	.974
Working Memory: DTS	1.724	1	1.724	.035	.856

Dependent Variable	*t*	*df*	*Sig.*	Mean Diff.	Std. Error
Acquisition: Zone A	.323	11	.753	.646	1.998
Acquisition: Zone B	-.408	11	.691	-1.515	3.715
Acquisition: Zone C	.404	11	.694	1.762	4.365
Reversal Learning: Trials to Criterion Latency	.610	11	.554	.262	.429
Reversal Learning: Trials to Criterion Path	.813	11	.433	.238	.293
Reversal Learning: Target Quad. Time	-.203	11	.843	-.815	4.021
Reversal Learning: Target Quad. Path	.069	11	.946	.222	3.211
Reversal Learning: Original Quad. Time	.371	11	.718	1.423	3.836
Reversal Learning: Original Quad. Path	.530	11	.607	1.992	3.761

Note. Top table analyses run as Repeated Measures ANOVA. Bottom table analyses run as independent samples *t*

Cohort/Abstinence Period

Abstinence period was assessed independently before the planned 2 x 2 x 2 experimental analyses in order to rule out potential complications that could not be attributed to drug induced withdrawal. The first cohort consisted of $N = 16$ and began spatial learning and memory assessment five days post cessation of drug administration and continued for the full 12 days of

MWM evaluation. The second cohort consisted of the remaining N =17, and began spatial learning and memory assessment on day 19 post drug cessation, and followed the same series of MWM evaluation as the first cohort. In order to determine whether the abstinence period impacted spatial abilities without regard to drug administration, a series of one-way ANOVAs or independent samples *t*-tests were conducted across all learning dependent variables with cohort as the categorical predictor variable. There were no significant differences between cohort distinction and the main learning dependent variables (i.e. latency, distance). See Table 2 for analyses. However, there was a singular difference in average time spent in the inner zone (zone A) of the water maze during the acquisition phase, $t(31) = 2.13$, $p = .041$. Animals in the shorter abstinence period (cohort 1) spent more time in the inner zone than those in the longer abstinence period (cohort 2). As time spent in inner areas of the water maze has been posited as indicative of reduced anxiety, this finding would suggest that animals in the shorter abstinence period had lower levels of anxiety related behaviors. This difference was, however, not apparent in any other learning dependent variables and did not appear to influence the rate of acquisition or the animal's time/distance spent searching for the target. Therefore, the abstinence period alone did not appear to influence any variables that may be indicative of an animal's overall learning pattern across any of the acquisition, reversal, or working memory phases of assessment.

Table 2. Pairwise Comparisons between abstinence periods (Cohort 1 & 2)

Dependent Variable	*SS*	*df*	*MS*	*F*	*Sig.*
Acquisition Latency	494.681	1	494.681	.944	.339
Acquisition Path	4.106	1	4.106	.202	.656
Working Memory: LTS	91.947	1	91.947	.125	.736
Working Memory: DTS	4.126	1	4.126	.098	.756

Dependent Variable	*t*	*df*	*Sig.*	Mean Diff.	Std. Error
Acquisition: Zone A	2.131	31	.041	2.352	1.104
Acquisition: Zone B	1.548	31	.132	4.269	2.757
Acquisition: Zone C	.404	11	.694	1.762	4.365
Reversal Learning: Trials to Criterion Latency	.397	31	.694	.1213	.3057
Reversal Learning: Trials to Criterion Path	-.024	31	.981	-.0074	.3034
Reversal Learning: Target Quad. Time	-.202	31	.841	-.5481	2.711
Reversal Learning: Target Quad. Path	-.232	31	.818	-.5440	2.347
Reversal Learning: Original Quad. Time	1.40	31	.171	3.845	2.746
Reversal Learning: Original Quad. Path	-.304	31	.763	-.0625	.2057

Note. Top table analyses run as repeated measures ANOVA. Bottom table analyses run as independent samples *t*-test

Acquisition Learning

Initial testing using the Morris Water Maze apparatus included a five-day acquisition period during which animals were assessed across four trials per day, with a different starting direction for each trial (See Table 3). A submerged platform remained in the SW corner through the acquisition period (days 1-5). In order to determine the effect of drug administration on

acquisition of spatial learning and memory, a series of 2 (ethanol or control) X 2 (nicotine or control) x 2 (cohort) repeated measures ANOVAs was conducted. As outlined by Vorhees & Williams (2000), analyses were conducted both across days using blocked averaged trials per days, as well as across trials for each individual day.

Table 3. Starting positions for MWM testing: Acquisition and reversal learning protocol

Day	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 4**
1	N	E	SE	NW
2	SE	N	NW	E
3	NW	SE	E	N
4	E	NW	N	SE
5	N	SE	E	NW
Day	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 4**
1	S	W	NW	SE

Note. Table based on Vorhees and Williams (2006)

To test the hypothesis that drug exposure would increase the time (i.e. latency) required to reach the submerged platform a 2 (diet) x 2 (injection) x 2 (cohort) repeated measures ANOVA was used. Contrary to hypothesized drug influences, there was no main effect of diet. Animals that had received ethanol performed consistently similarly to animals that had not received ethanol ($p > .05$). Thus, ethanol did not appear to impact learning during initial acquisition. However, a significant main effect of injection was identified across days on time (in seconds) spent swimming (i.e. latency), $F(1, 25) = 5.60$, $p = .026$, *partial* $\eta^2 = .183$. As hypothesized, animals that had received nicotine ($M = 35.89$, $SE = 3.03$) spent a significantly longer amount of time searching for the target platform than animals that had received saline and/or no injection ($M = 26.88$, $SE = 2.29$). See Figure 3 for a representation of means.

In order to identify whether certain days during the acquisition period were more impacted than others, individual 2 (diet) x 2 (injection) x 2 (cohort) repeated measures ANOVA analyses across trials on latency to target were conducted for days 1 – 5. Of particular import, the final day of acquisition learning was particularly problematic for animals that had received nicotine. A main effect of injection was observed, $F(1, 25) = 9.430$, $p < .001$, *partial* $\eta^2 = .274$. On the final day of acquisition learning, animals that received nicotine ($M = 18.34$, $SE = 1.94$) spent significantly more time swimming in order to find the platform than animals who had not received nicotine ($M = 10.87$, $SE = 1.94$). As such, despite having displayed evidence of learning across the acquisition period, albeit at a slower pace, nicotine-receiving animals were unable to maintain the same rate of learning displayed by non-nicotine receiving animals. Indeed, by the end of the acquisition learning phase, the difference between nicotine-receiving and non-nicotine receiving animals is greater than is observed at earlier periods of learning (see Figure 3). No effects, of either cohort or diet, were identified in the above analyses ($p > .05$). Results suggest that neither ethanol nor the abstinence period influenced escape latency on any individual day during the acquisition period.

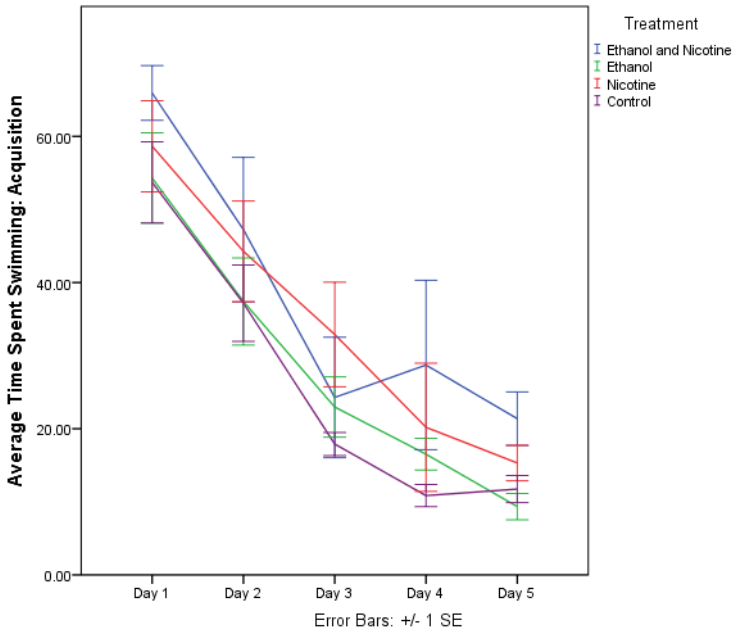

Figure 3. Average Time (s) Spent Swimming: Acquisition

In order to corroborate the finding that nicotine impaired spatial performance, analysis of path distance, an accepted method of identifying spatial learning (D'Hooge & De Deyn, 2001), was conducted. In keeping with the previous finding, a learning impairment due to previous nicotine administration was found in average distances swam (in meters) in order to reach the target platform. A 2 (diet) x 2 (injection) x 2 (cohort) across the acquisition period indicated a significant main effect of injection, $F(1, 25) = 6.34$, $p = .019$, *partial* $\eta^2 = .202$. Animals that had received nicotine ($M = 7.39$m, $SE = .447$) required a significantly longer path than animals that had been administered saline and/or no injection ($M = 5.57$m, $SE = .58$) to reach the submerged platform. See Figure 4 for a representation of means.

Further analysis of trial learning on individual days revealed substantive differences in distance swam on the final day of acquisition learning (Day 5), similar to the analysis on latency. A 2 (diet) x 2 (injection) x 2 (cohort) repeated measures ANOVA, across trials 1-4 on day 5 indicated a main effect of injection, $F(1, 29) = 8.79$, $p = .006$, *partial* $\eta^2 = .232$. On the final day of acquisition learning, animals that received nicotine ($M = 4.02$, $SE = .339$) required a

significantly longer swim path to find the platform than animals who had not received nicotine (M = 2.36, SE = .448). As such, nicotine administration resulted in a greater level of impairment on the final day of learning; despite learning the task, nicotine-receiving animals were unable to perform the task as efficiently as the other conditions. No effects, of either cohort or diet, were identified in any of the above analyses ($p > .05$). As such, results suggest that neither ethanol nor the abstinence period influenced the distance swam to reach the target platform.

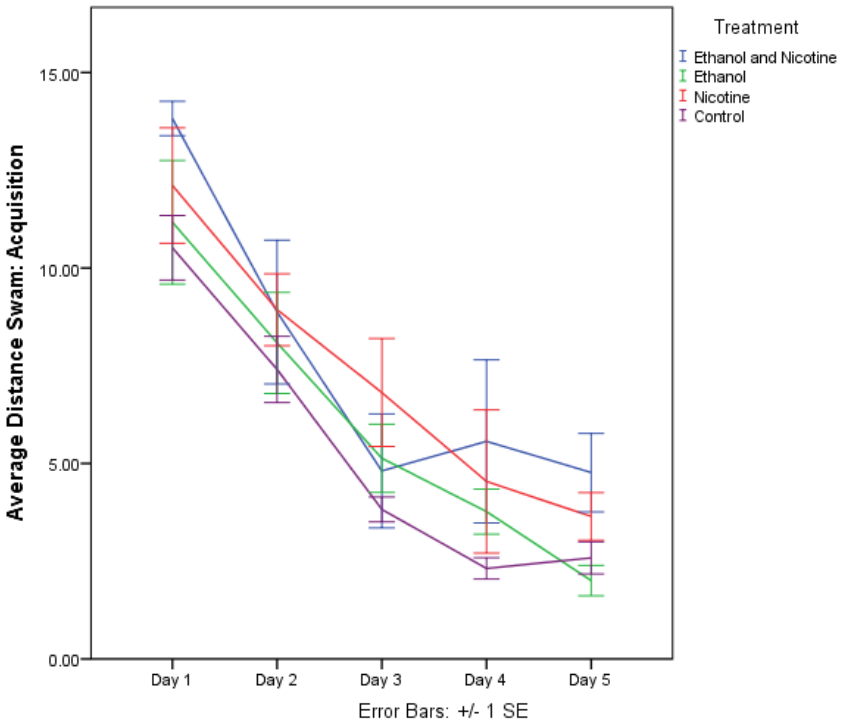

Figure 4. Average Distance Swam (m): Acquisition

As it was possible that the impairments in the distance and latency required to reach the submerged platform were due to drug-induced motor deficiencies, rather than any true deficit in learning, an analysis of swim speed was conducted. An increased swim speed may have accounted for the reduced escape latency and swim distance displayed by animals that had not experienced drug administration. Therefore, the effect of drug administration on spatial learning and memory might in fact be an effect of motor ability (Maei et al., 2009; Malleret, Hen, Guillou, Segu, & Behot, 1999). However, a 2 (Diet) X 2 (Injection) X 2 (Cohort) repeated

measures ANOVA revealed no effect of any condition on averaged swim speed, as calculated by average distance/average time per day ($p > .05$). See Figure 5 for a representation of means. Results suggest that the learning impairment attributed to nicotine administration was not a function of abnormal locomotor activity.

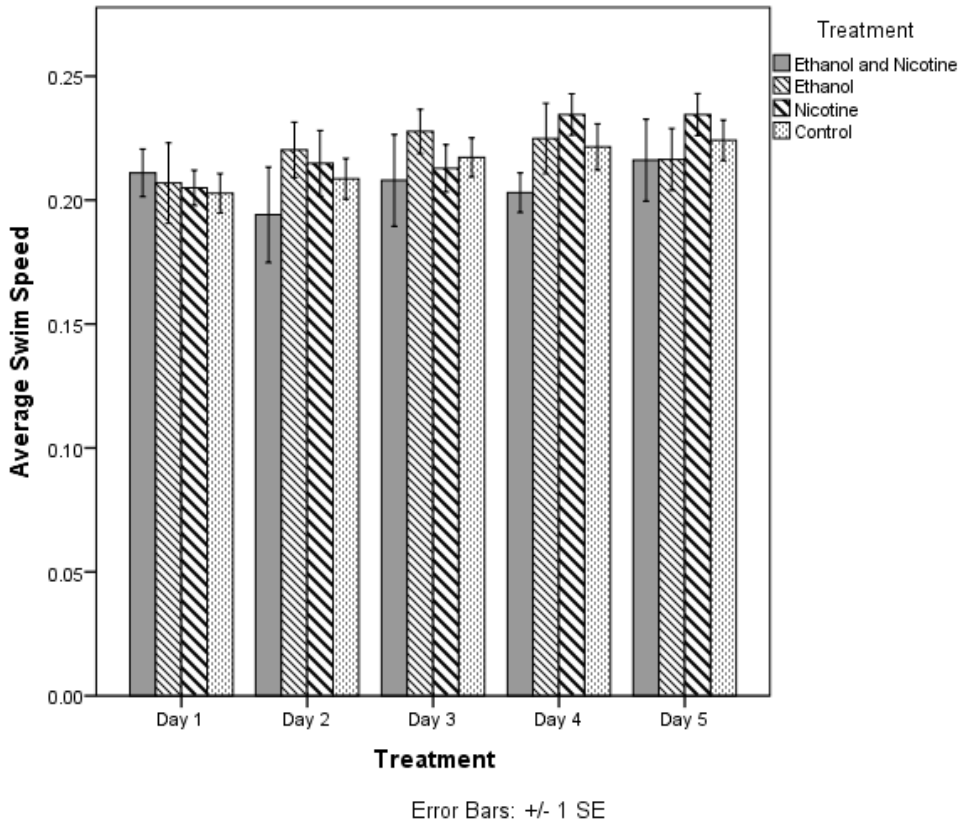

Figure 5. Average Speed: Acquisition

In order to further assess the effect of drug administration, analyses of percent time spent in each zone (A-C) were conducted (See Figure 6). Time spent in the target zone (B) has been identified as a sensitive measure of search accuracy, and so may be used to estimate the quality of learning across the assessment period (Maei, Zaslavsky, Teixeira, & Frankland, 2009). Conversely, time spent outside of the target zone (Zones A & C) is identified as an index of diminished efficacy in search accuracy (Maei et al., 2009). As such, a greater level of time spent in the target zone indicates a more effective learning strategy and suggests an increased level of

spatial performance. Further evidence toward a nicotine-induced learning deficit was observed in percent time spent in the target zone (B) and in the outer zone (C). See Figures 7 and 8. Following a 2 (diet) x 2 (injection) x 2 (cohort) repeated measures ANOVA, results indicated a significant main effect of injection {$F(1, 25) = 4.98$, $p = .035$, *partial* $\eta^2 = .172$}; animals that had been administered nicotine ($M = 37.91$, $SE = 2.18$) spent the least proportion of overall swim time, across days, in the target zone (B) as compared to animals not having received nicotine ($M = 43.97$, $SE = 1.62$). Additionally, a significant main effect of injection was found in percent time spent in the outer zone (C), $F(1, 25) = 5.37$, $p = .029$, *partial* $\eta^2 = .183$; animals that had received nicotine ($M = 54.17$, $SD = 2.83$) spent significantly more time, proportionally, in the outer zone (C) compared to animals that did not receive nicotine (M = 45.99, SE = 2.11). Results suggest that animals having been administered nicotine, while having learned the spatial task, had a less accurate search strategy and therefore a diminished quality of learning. No effects, of either cohort or diet, were identified in the above analyses ($p > .05$). As such, results suggest that neither ethanol nor the abstinence period influenced search accuracy.

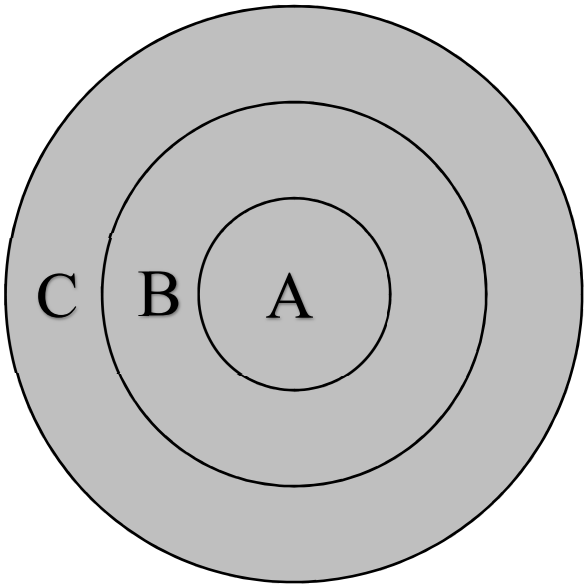

Figure 6. Depiction of Zone Areas for MWM task

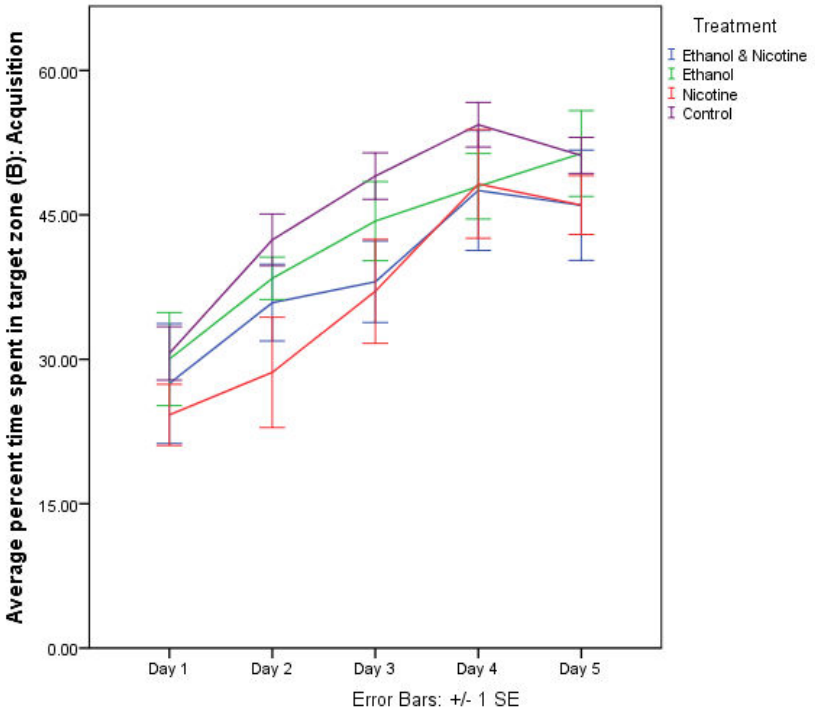

Figure 7. Average Proportion Time (s): Target Zone (B)

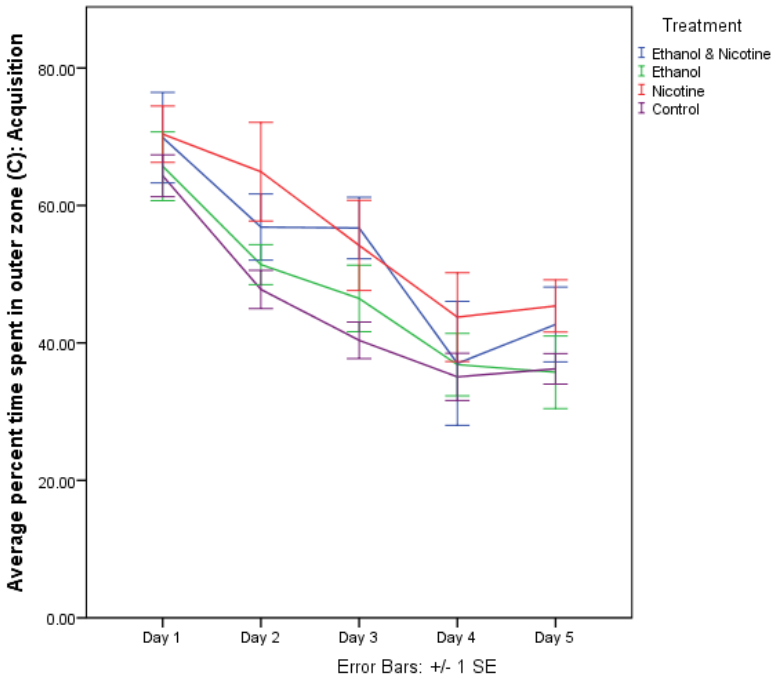

Figure 8. Average Proportion Time (s): Outer Zone (C)

Probe Trial

Following five days of acquisition learning, and immediately prior to reversal learning, animals underwent a single probe trial during which the submerged platform was removed from

the pool and the animal was given 90 seconds to explore the pool. The probe trial has generally been used as an assessment of whether an animal was able to acquire the spatial task during initial learning (Vorhees & Williams, 2004). Typically, researchers analyze time spent in the original target quadrant, in addition to passes through the area from which the platform was removed (Vorhees, & Williams, 2004). Animals who have successfully learned the spatial task are presumed to spend more time searching the target quadrant, as well as making more crosses over the platform area. In order to assess the acquisition of learning in the present study, a 2 (diet) x 2 (injection) x 2 (cohort) ANOVA was conducted on proportion of overall time spent in the target quadrant and number of crosses over original platform area. No main effects for any condition were found on either dependent variable ($p > .05$). However, a significant interaction (cohort*diet) was found in proportion of overall time spent in the target quadrant, $F(1, 25) = 8.29$, $p = .008$. Simple effects using Fisher's LSD post-hoc analysis was then used to determine the area of occurrence. For animals in the first cohort, ethanol administration diminished the proportion of time spent searching the target quadrant, $F(1, 25) = 5.87$, $p = .023$. For animals in the second cohort, ethanol administration had no effect, $F(1, 25) = 2.76$, $p = .109$. Accordingly, animals that had received ethanol spent significantly less time, proportionally, in the target quadrant during the shorter abstinence period; an effect that diminished as the withdrawal period lengthened (See Figures 9) for respective representation of means. Results suggest that a shorter ethanol abstinence period impacted spatial learning and memory when compared to a longer ethanol abstinence period. There was no effect of any condition on number of passes through the original target area ($p > .05$; See Figure 10).

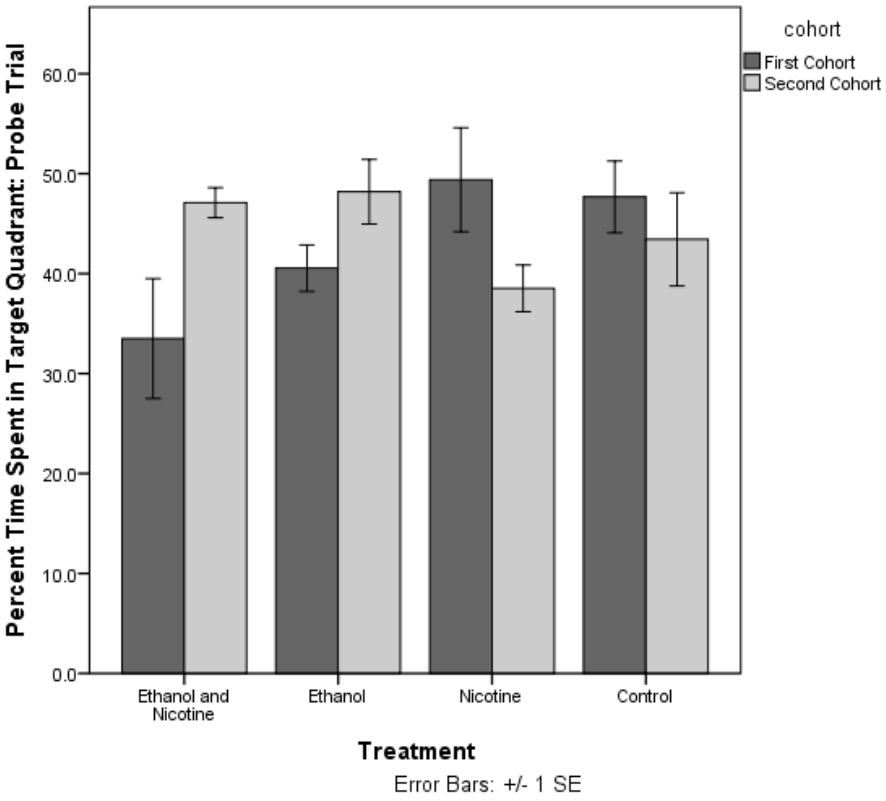

Figure 9. Average Proportion Time (s): Target Quadrant

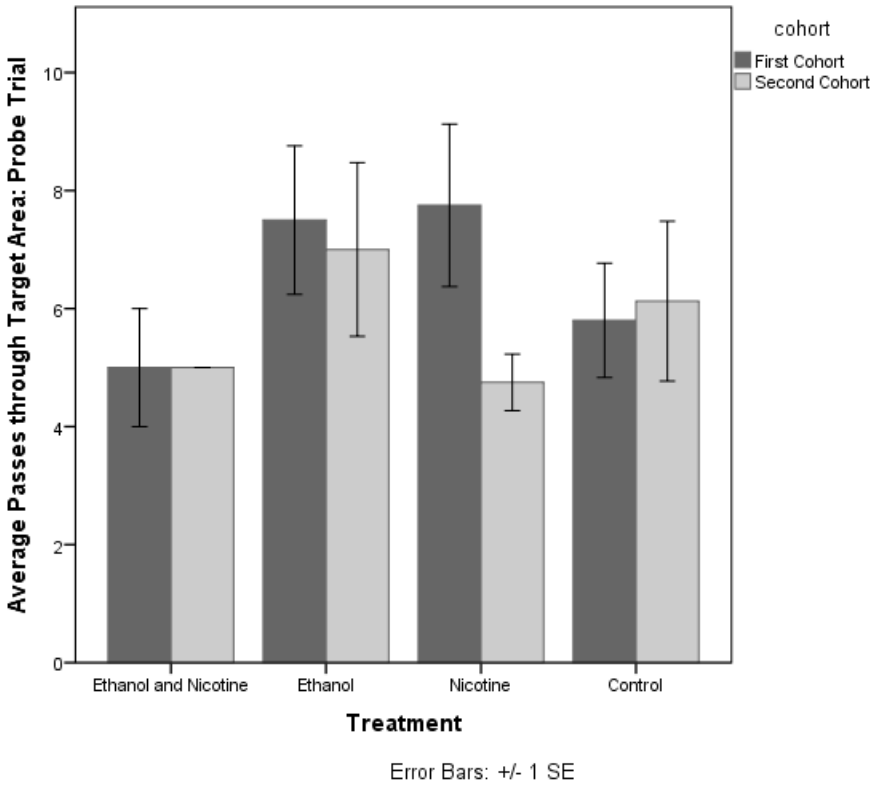

Figure 10. Passes through target platform area: Probe Trial

Reversal Learning

Following five days of acquisition learning, and immediately after the probe trial, animals underwent four trials of reversal learning wherein the submerged platform was moved to the opposite quadrant (NE) relative to initial learning platform placement (SW). Each animal was observed across four trials with varying start positions (See Table 3). In order to assess reversal learning, a criterion established according to Obernier et al. (2002) was set at two standard deviations above mean distance swam (M = 296.62 cm, SD = 170.74) and mean time spent swimming (M = 13.20s, SD = 7.21) on the final day of acquisition testing. A 2 (diet) x 2 (injection) x 2 (cohort) ANOVA was used to assess number of trials to reach criterion in both distance swam and time spent swimming. Results indicated a significant interaction (Diet*Injection) in trials to reach criterion (Distance), $F(1, 25) = 3.36$, $p = .029$, *partial* $\eta^2 = .177$. To determine where the area of occurrence was located, simple effects conducted using Fisher's LSD post hoc test were completed. Expected deficits were revealed in reversal learning by the nicotine-only group (M = 2.63, SD = 1.19). See Table 4. Animals that had received nicotine only required significantly more trials to reach criterion than controls ($p < .05$). Interestingly, contrary to hypothesized drug interactions, combined exposure of ethanol and nicotine required significantly fewer trials, on average, than exposure to nicotine alone ($p < .05$). Thus, results suggest that the combined exposure of ethanol and nicotine attenuated the learning deficit produced by nicotine administration (See Figure 11).

Table 4. Reversal Learning: Mean Trials to Reach Criterion Path

	Mean Difference			
	Ethanol & Nicotine	Ethanol	Nicotine	Control
Ethanol & Nicotine	***n* = 4** 1.50 (.577)	*-.375* .469 *p* = .430	*-1.13* .469 *p* = .023	*-.038* .438 *p* = .931
Ethanol	*.375* .469 *p* = .430	***n* = 8** 1.88 (.641)	*-.750* .383 *p* = .060	*.337* .344 *p* = .336
Nicotine	*1.13* .469 *p* = .023	*.750* .383 *p* = .060	***n* = 8** 2.63 (1.19)	*1.09* .344 *p* = .004
Control	*.038* .438 *p* = .931	*-.337* ..344 *p* = .336	*-1.09* .344 *p* = .004	***n* = 13** 1.54 (.519)

Note. Fisher's LSD Post hoc analyses. Standard deviations are included in parentheses below means. Mean Differences are provided between treatment groups.

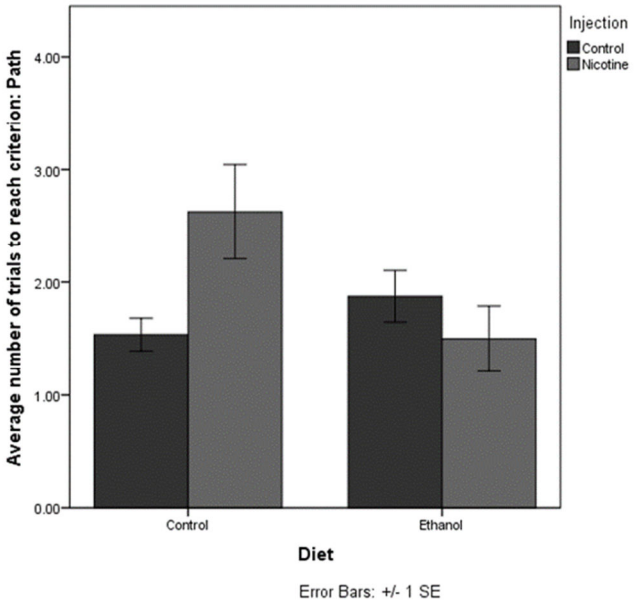

Figure 11. Number of trials to reach criterion: Path

There were no main effects of diet, $F(1, 25) = 0.10$, $p = .758$, or injection, $F(1, 25) = 2.17$, $p = .153$, on time spent swimming. Similarly, there were no main effects of diet, $F(1, 25) = .12$, $p = .734$, or injection, $F(1, 25) = 1.68$, $p = .206$, on distance swam.

In order to further assess cognitive flexibility during reversal learning, analyses on time and path spent searching the original quadrant (SW), in addition to the target quadrant (NE) were conducted. A 2 (diet) x 2 (injection) x 2 (cohort) ANOVA revealed an interaction effect (Diet*Injection) on proportion of overall distance spent searching the target quadrant (NE), $F(1, 25) = 4.76$, $p = .039$, partial $\eta^2 = .160$. Simple effects conducted by Fisher's LSD post hoc analysis supported the finding that dual ethanol/nicotine receiving animals spent a greater proportion of overall swim distance in the target quadrant ($M = 34.58$, $SD = 3.09$) compared to ethanol only ($p < .05$)($M = 28.94$, $SD = 2.26$), as well as nicotine only ($p < .05$) ($M = 29.83$, $SD = 2.19$) (See Figure 12). As such, results indicate that ethanol and nicotine receiving animals were more successful in reversing the task and therefore show a greater degree of cognitive flexibility than evidenced by nicotine and ethanol only receiving conditions.

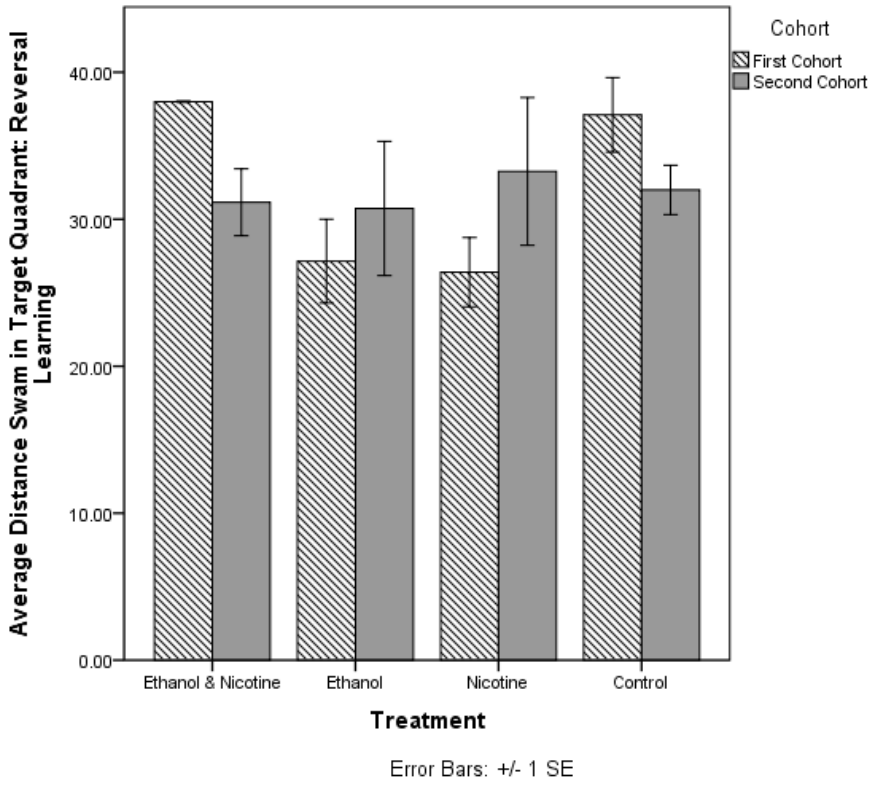

Figure 12. Average Proportion Distance: Target Quadrant

A 2 x 2 x 2 ANOVA conducted on proportion of overall time spent searching the target quadrant indicated a similar trend of interaction effect (Diet*Injection), $F(1, 25) = 3.50$, $p = .073$. Although not significant, this pattern does appear to support the finding discussed above with regard to distance swam.

Difficulty in reversing the task, attributable to nicotine administration, was also evidenced by a three way interaction effect (Diet*Injection*Cohort) identified by a 2 x 2 x 2 ANOVA conducted on proportion of overall swim time, $F(1, 25) = 5.84$, $p = .023$, and swim distance, $F(1, 25) = 6.65$, $p = .016$, in the original quadrant. See Figures 13 and 14 for a representation of means. Results indicated that animals that had received nicotine spent a greater proportion of overall swim time and distance searching the original quadrant area during acute withdrawal than controls, an effect that was diminished during protracted withdrawal.

Interestingly, Fisher's LSD post hoc analyses determined that the nicotine withdrawal period greatly impacted time and distance spent in the original quadrant, such that animals that experienced shorter term nicotine withdrawal spent significantly greater proportions of overall time and distance in the original quadrant than longer term nicotine withdrawal animals ($p < .05$). As such, a greater nicotine withdrawal period appeared to lessen the impact on cognitive flexibility attributed to prior nicotine administration.

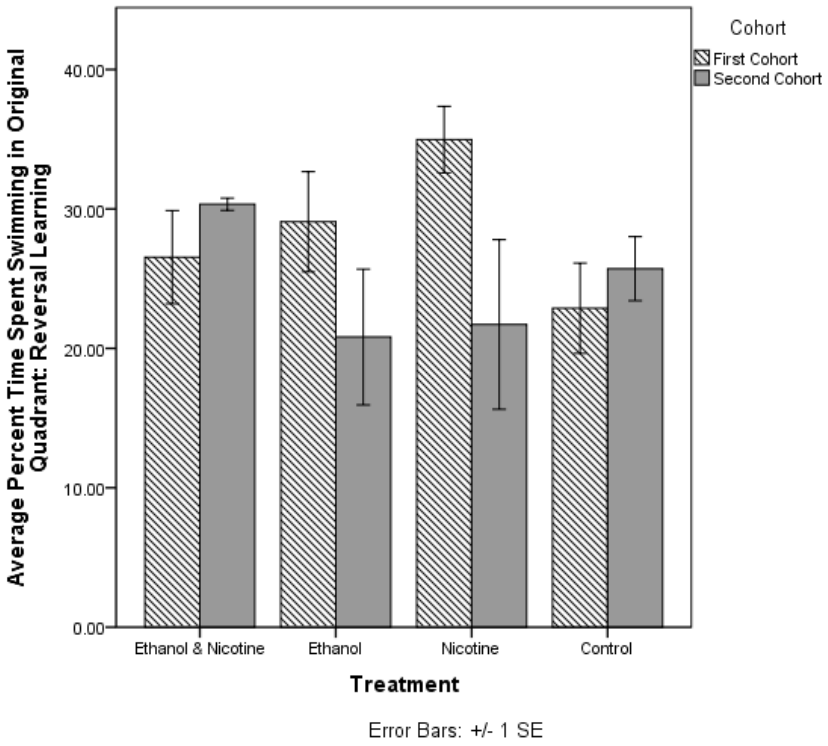

Figure 13. Average Proportion Time: Original Quadrant

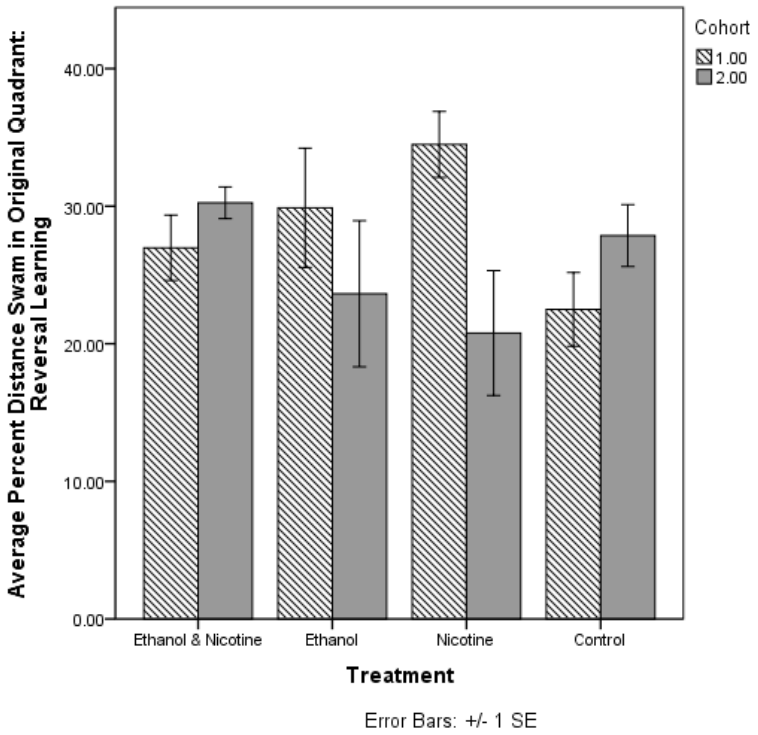

Figure 14. Average Proportion Distance: Original Quadrant

Interestingly, the differences between treatment groups on distance swam in the target quadrant appear to be more relevant to acute withdrawal, as evidenced by a 3-way interaction (cohort*diet*injection) across trials, $F(1, 25) = 5.57$, $p = .026$. Animals that had received dual ethanol/nicotine spent the largest proportion of distance swam in the target quadrant during acute withdrawal, exhibiting a greater degree of cognitive flexibility, an effect that was diminished in longer term (protracted) withdrawal. Reference Figure 15 for representation of means. No differences between protracted withdrawal groups were evidenced on proportion of swim path spent in target quadrant.

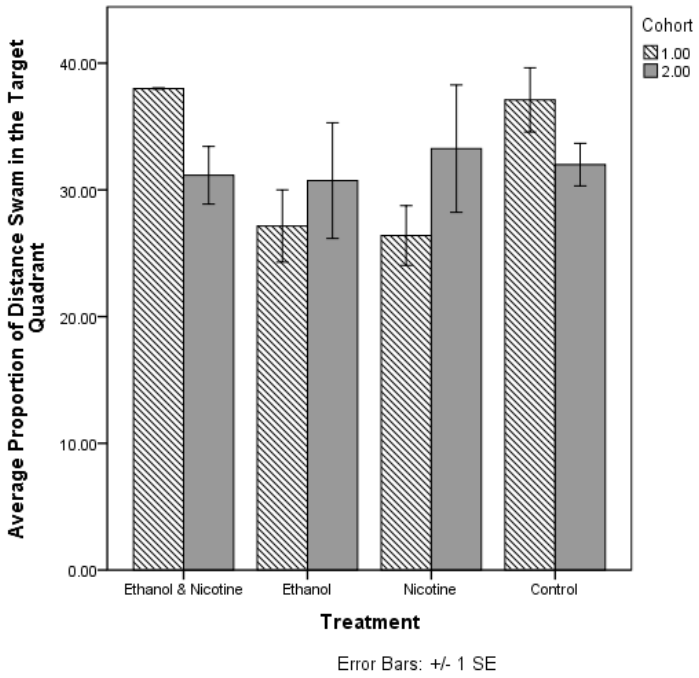

Figure 15. Average Proportion Distance: Target Quadrant Three-way

Surprisingly, despite animals receiving combined ethanol and nicotine exposure spending a greater proportion of overall swim distance searching the target quadrant, a perseverative behavior was evidenced during protracted withdrawal in original platform area crossing. A 3-way interaction, $F(1, 25) = 4.23$, $p = .05$, *partial* $\eta^2 = .145$, across trials revealed that nicotine and ethanol receiving animals ($M = 1.86$, $SD = .413$) had significantly more forays into the original quadrant target area. Thus, while the ethanol and nicotine animals, during protracted withdrawal, were successful in reversing the task, they also exhibited perseveration on the original requirements of the previous task (See Figure 16).

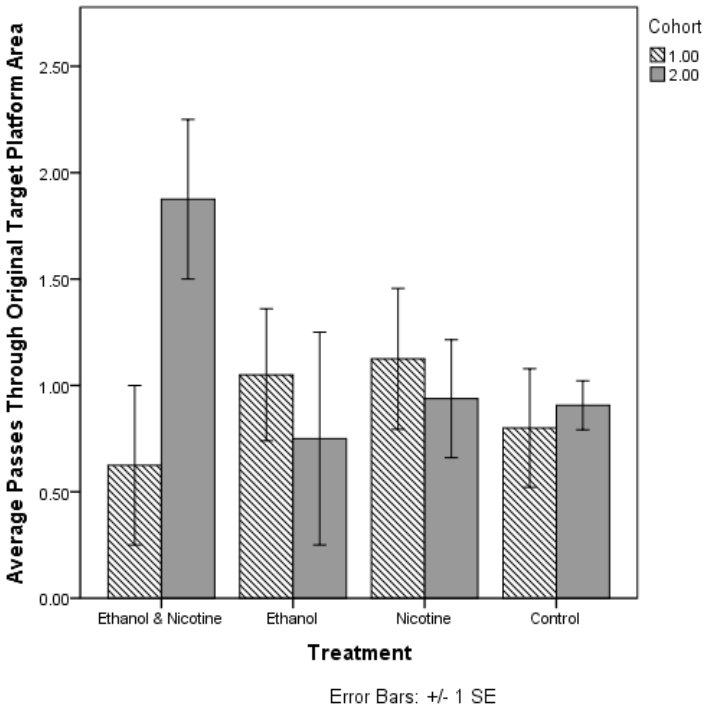

Figure 16. Average passes through original target area: Reversal Learning

Working Memory

Working memory was assessed across the final six days of MWM testing, during which animals were required to complete two trials (Sample/Test) per day with the same start and goal positions for each trial (Table 5). Savings (distance and time) from the initial sample trial observed during the test trial were analyzed using a 2 (diet) x 2 (injection) x 2 (cohort) repeated measures ANOVA, across days 1-6. Contrary to hypothesized results, there were no effects of Diet, Injection, or Cohort ($p > .05$) on either time or distance savings (See Figures 17 and 18). All subjects exhibited similar patterns of time/distance savings ($p > .05$), indicating there were no deficits in working memory attributable to drug administration.

Table 5. Directional placement of platform and animal for working memory assessment

Day of Acquisition	Start	Goal
7	N	SW
8	N	SE
9	E	NE
10	S	SW
11	W	SE
12	S	NE

Note. Two trials (Sample & Test) are given per day with 30s inter-trial period and will utilize the same start and goal point. Table based on Vorhees and Williams (2000).

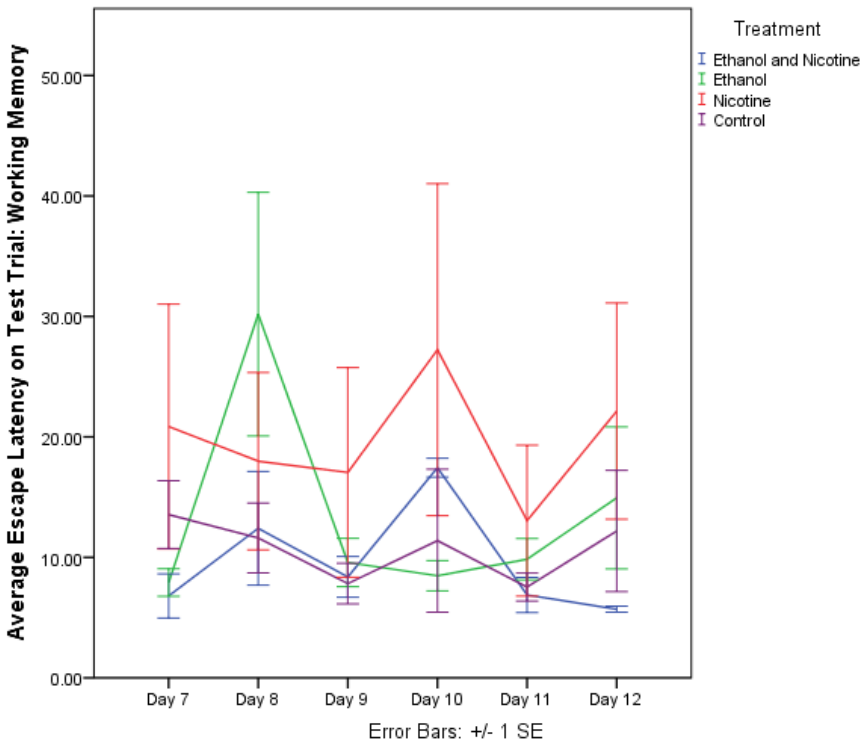

Figure 17. Escape Latency: Test Trial

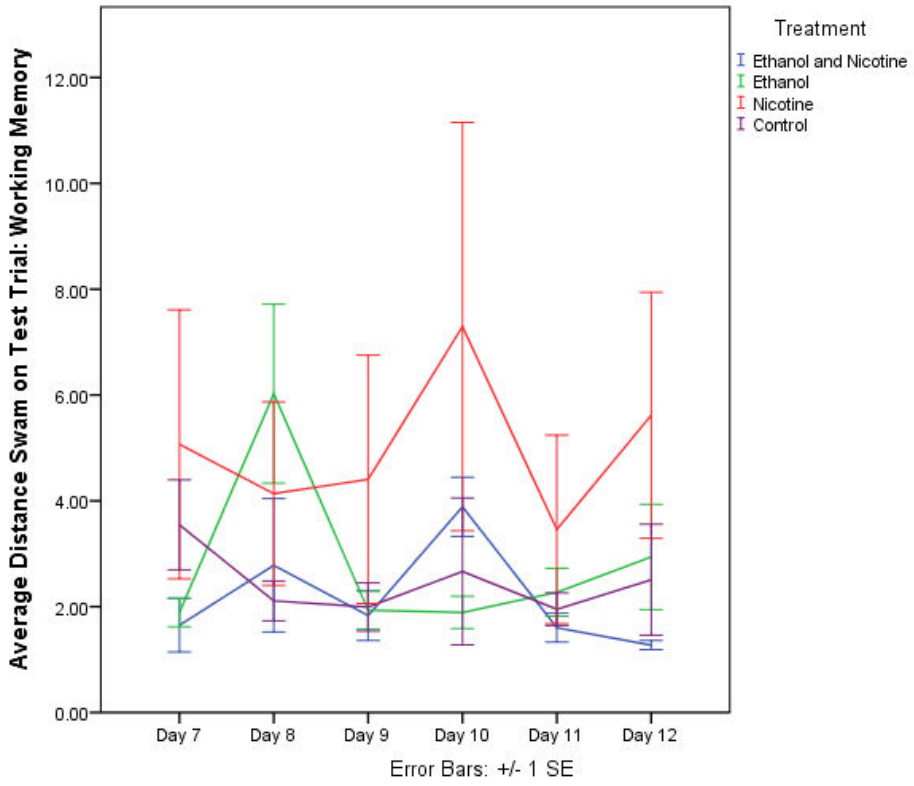

Figure 18. Average Distance Swam: Test Trial

Anxiety Related Behavior

Additional analyses on anxiety related behaviors were conducted in a post hoc fashion in order to determine potential confounding issues within the present study. Anxiety related behavior was measured during assessment in the Morris water maze across all learning phases, and was identified as the proportion of overall time spent and distance swam in thigmotaxis. Thigmotaxis is defined as movement during which the animal remains in contact with the outer wall of the water maze, and has been posited as an index of anxiety (Colwill, & Creton, 2011; Simon, Dupuis, & Costentin, 1994). Averages of percent time spent and distance swam in thigmotaxis across trials for each day during the acquisition period were calculated. A 2 (diet) x 2 (injection) x 2 (cohort) repeated measures ANOVA across days 1–5 was then utilized to analyze potential differences in thigmotaxis during initial acquisition. A significant main effect

of injection was identified in percent time spent in thigmotaxis, $F(1, 24) = 4.87$, $p = .037$, *partial* $\eta^2 = .145$. Animals that had received nicotine ($M = 20.75$, $SE = 2.58$) spent a significantly greater proportion of overall time in thigmotaxis than animals that had not received nicotine ($M = 13.66$, $SE = 1.92$). No main effects of diet or cohort on percent time spent in thigmotaxis were found ($p > .05$). A similar, a marginally significant main effect of nicotine was identified following a 2 x 2 x 2 repeated measures ANOVA in percent of distance swam in thigmotaxis, $F(1, 24) = 4.07$, $p = .055$, *partial* $\eta^2 = .129$. Animals that had received nicotine ($M = 15.51$, $SE = 2.41$) displayed an all but significantly greater proportion of overall swim distance in thigmotaxis than animals that had not received nicotine ($M = 9.45$, $SE = 1.80$). No main effects of diet or cohort on percent distance swam in thigmotaxis were found ($p > .05$).

Further, Pearson's *r* bivariate correlations were conducted in order to establish whether the above effect was associated with the assessment of spatial learning and memory. Averages of percent time spent and distance swam in thigmotaxis during the acquisition period were compared with averages across the acquisition period on escape latency and distance required to reach target platform. Significant positive correlations were found between average time spent in thigmotaxis and mean escape latency, $r(31) = .78$, $p < .001$, in addition to average percent of distance swam in thigmotaxis and average distance required to reach target, $r(31) = .81$, $p < .001$. Refer to Figures 19 and 20 for a visual representation of these relationships. Results indicate that as thigmotaxic behavior increased so did the time spent and distance swam required for an animal to escape the maze.

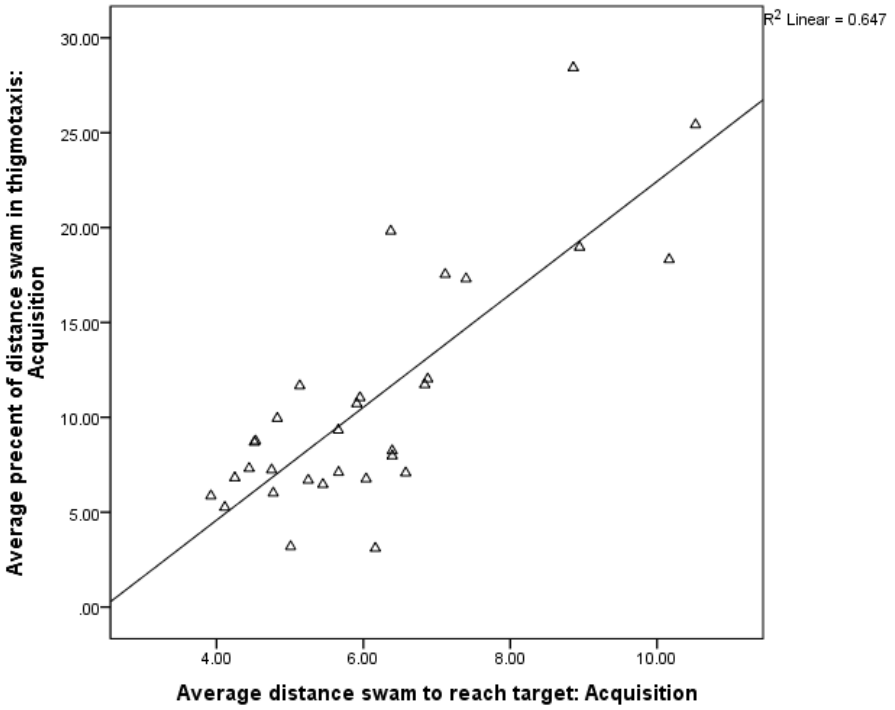

Figure 19. Thigmotaxis and Distance Required to Complete the MWM task

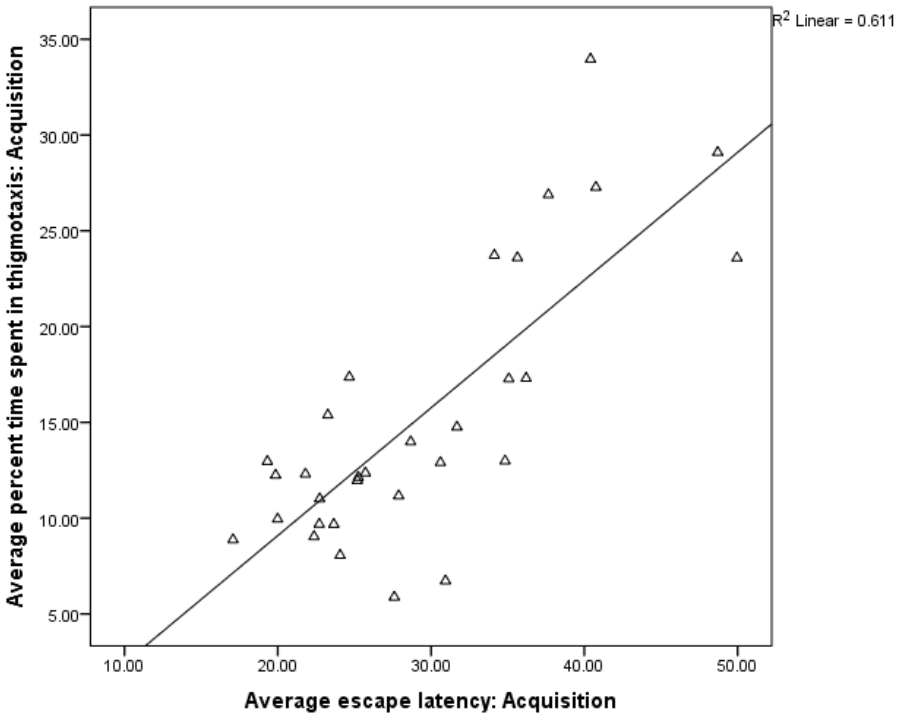

Figure 20. Thigmotaxis and Time Required to Complete the MWM task

Analyses of thigmotaxic behavior during the reversal and working memory phases were also conducted in order to assess the influence of anxiety. Accordingly, 2 x 2 x 2 repeated

measures ANOVAs across trials (reversal learning) and across days (working mem.) were utilized in order to evaluate potential differences in treatment groups. There were no main effects of diet, injection, or cohort for either learning period ($p > .05$). However, there was a singular interaction effect (injection*cohort) identified during reversal learning in percent time spent in thigmotaxis, $F(1, 25) = 5.30$, $p = .030$. Fisher's LSD post hoc analysis was then used to identify the area of occurrence. Accordingly, results indicated that animals that had received nicotine and experienced the longer term withdrawal phase spent a significantly greater proportion of overall time in thigmotaxis than all other treatment groups except for the animals that had received ethanol and nicotine and experienced the longer term withdrawal. Refer to Figure 21 for a representation of means. A similar effect was observed following the same analysis on percent of overall distance swam in thigmotaxis, $F(1, 25) = 4.54$, $p = .043$. Interestingly, Fisher's LSD post hoc analyses revealed that animals that had received nicotine and experienced the longer term withdrawal phase spent a significantly greater proportion of overall swim distance in thigmotaxis than all other treatment groups except for the animals that had received dual ethanol/nicotine and experienced the longer term withdrawal phase. See Figure 22 for a representation of means. No other effects were observed ($p > .05$). These results provide support for the prevalence of anxiety related behaviors during protracted withdrawal of nicotine.

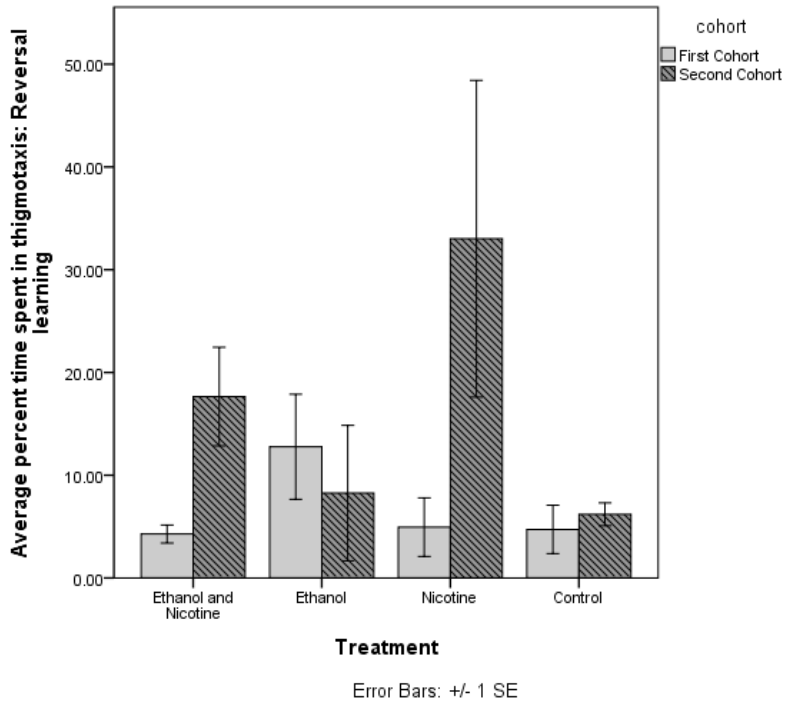

Figure 21. Average Percent Time Spent in Thigmotaxis: Reversal Learning

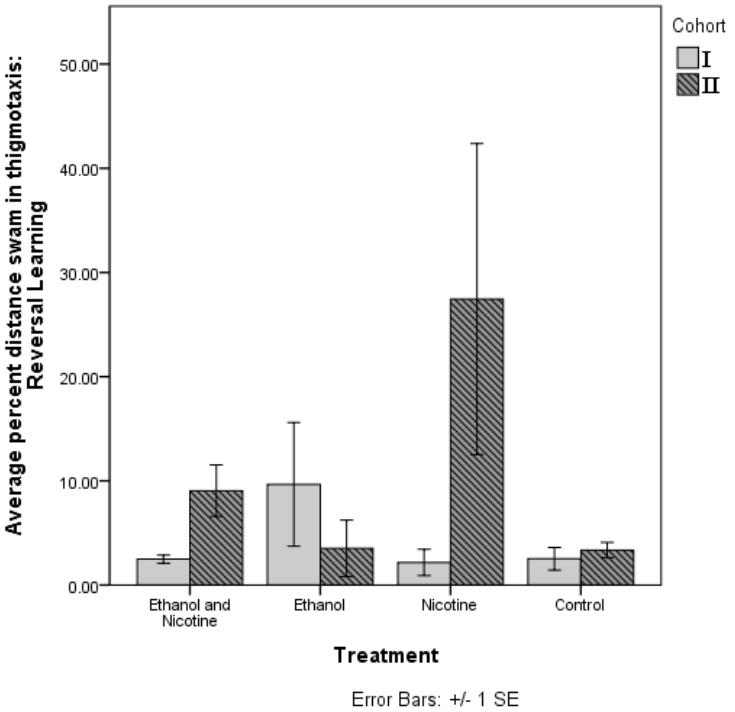

Figure 22. Average Percent Distance Swam in Thigmotaxis: Reversal Learning

Chapter 5: Discussion

The present study further examined the hypothesis that independent exposure to either ethanol or nicotine would impair spatial learning and memory, and that dual drug exposure (nicotine & ethanol) would do so more dramatically. It was hypothesized that across three aspects of learning (reference, cognitive flexibility, working memory) there would be significant main effects for injection (nicotine vs control) and diet condition (ethanol vs control) on learning dependent variables (latency/distance required to complete task, percent time spent in targeted quadrant/zone). Specifically, it was expected that animals administered nicotine or ethanol would experience a greater degree of cognitive difficulty, expressed by longer search times (i.e. latency) as well as longer paths required to find the submerged platform (i.e. distance). Additionally, it was hypothesized that there would be a significant interaction between diet and injection across learning dependent variables. The combination of ethanol and nicotine exposure was expected to potentiate the impairments attributed to either drug independently. Specifically, animals administered dual ethanol and nicotine were expected to require significantly longer periods of time, in addition to longer distances, in order to complete the spatial learning and memory task.

With regard to the hypothesis that independent exposure of nicotine would impair spatial learning and memory, results support the notion that prior nicotine administration inhibits performance during initial acquisition of a spatial learning task. Specifically, animals that received nicotine required a longer period of time, in addition to a longer swim path in order to reach the goal during initial acquisition. Thus, while animals did indeed exhibit learning during initial acquisition, the rate at which the task was learned was slower and continued to prove difficult throughout the acquisition learning phase. Consequently, our results suggest that nicotine use can have adverse consequences in cognitive functioning. It has been previously

shown that nicotine exposure results in reduced markers of cell development and migration (ex. PSA-NCAM expression), as well as inhibiting aspects of the neurogenic process, both of which have been posited to be influential determinants of synaptic functionality and the learning process (Abrous et al., 2002). As such, nicotine-induced neurological deficits appear to be outwardly observable in the behavioral inability to complete spatial learning and memory assessments at comparable levels to drug naïve subjects.

Interestingly, our results also suggest that the animals that had received nicotine developed a less effective search strategy based on swim patterns and therefore had a diminished quality of learning during the acquisition period. Indeed, animals that had received nicotine spent a greater proportion of their time in the maze searching a zone that would not allow for escape. Spatial navigation in the Morris water maze requires the identification and use of distal visual cues to approximate the location of the submerged platform; a process that requires an intact hippocampal formation (Morris, 1984; Brody, & Holtzman, 2006). It has been posited that numerous strategies are utilized in order to navigate toward such a goal; taxon and locale strategies are two examples of such strategies (Redhead & Hamilton, 2007). The taxon strategy refers to a direct heading toward extramaze cues, whereas the locale navigational strategy utilizes distal cues to inform relative distances and the spatial relationship between the approximate goal area and the location of the distal cue, the latter of which may be a more effective means of locating a hidden object (Kubik, Stucklik, & Fenton, 2005; Redhead & Hamilton, 2007). While rodents are quite capable of utilizing multiple search strategies, animals that have undergone hippocampal lesions display diminished capacity for consolidating numerous landmark cues into a coherent cognitive map of the maze environment, and thereby utilizing locale search strategies, which may be reflected in the present study by percent time spent in the targeted zone (Pearce,

Roberts, & Good, 1998). As such, the deficient spatial navigation displayed by animals in the present study that had received nicotine suggests the prevalence of hippocampal damage as a possible explanatory mechanism. Histological efforts on the present subject pool will be necessary to confirm this position.

However, an important consideration in the evaluation of the effect of nicotine is that it appears to be dependent on the dosage and treatment duration. Indeed, previous reports have outlined that a 0.07 mg/kg nicotine dose given once daily has been shown to be beneficial for learning acquisition among both young and older aged rats (Socci, Sandberg, & Arendash, 1995). Contrarily, a 0.2 mg/kg nicotine dose across two trials per day was shown to have no effect on cognitive performance in the Morris water maze (Attaway, Compton, & Turner, 1999). It should be noted that the present study utilized a 0.3 mg/kg dosage across three time points per day over a ten day period, a relatively moderate dose compared to typical nicotine administration researches. The differences in nicotine dosage will need to be further evaluated in order to determine the specific dose at which nicotine administration no longer provides cognitive benefits and in turn becomes a neurotoxic agent producing cognitive impairments.

Furthermore, as the present study utilized an abstinence period following drug administration and prior to behavioral testing, it is also possible that the learning impairment observed in the nicotine treatment group may in fact reflect some degree of a withdrawal effect. Such an estimation of the influence attributed to nicotine withdrawal in learning may be unwarranted, however. Previous efforts have shown that a 0.7 mg/kg nicotine dose administered two times a day for a single day and 10 days prior to training in the Morris water maze had a comparable effect to the same nicotine dose administered for 10 days, with MWM testing having begun 24 hours following the final dosage (Kenney & Gould, 2008). Our results appear to echo

this evaluation in that no effect of withdrawal period was identified during the acquisition phase. A short term nicotine withdrawal phase did not influence spatial learning any more or less than a longer term nicotine withdrawal phase. However, as both treatment periods in the present experienced a withdrawal phase it may be beneficial for future research to involve a continuing nicotine administration model, one that is maintained throughout behavioral testing.

Further analysis of the effects of nicotine on spatial learning and memory capacity was assessed during a spatial reversal task, which identifies whether an animal can extinguish initial learning requirements in order to perform a new task, indicative of cognitive flexibility (Garthe, Behr, & Kempermann, 2009). As hypothesized, nicotine exposure resulted in a greater degree of cognitive inflexibility, as evidenced by a significantly greater number of trials in order to establish the learning criterion, in addition to perseverative behavior on initial requirements of acquisition learning. Nicotine-only administration facilitated this impairment to a greater degree than all conditions except ethanol-only. Of particular import, the deficit to cognitive flexibility exhibited by nicotine administration was significantly greater than the dual nicotine and ethanol exposure treatment. Paradoxically, our results suggest that the nicotine-induced impairments on the reversal criterion and perseveration were attenuated by prior exposure to ethanol. Despite this seemingly contradictory report, previous research has outlined neurological correlates that may provide further explanation as to the interaction between nicotine and ethanol. It has been reported that concomitant exposure (nicotine and ethanol) will lessen the adverse effects of either drug independently (Oliveira-da-Silva et al., 2009). Specifically, cell degeneration, in addition to reductions of neuronal and glial cell densities attributed to either ethanol or nicotine exposure, may be ameliorated when compared to co-exposure models of administration (Oliveira-da-Silva et al., 2009). Our results appear to provide confirmatory evidence in that a diminished effect of

ethanol and nicotine was observed when combined, as compared to a singular nicotine exposure. Further, it is possible that when administered simultaneously the inhibitory action of ethanol may counteract the excitatory action produced by nicotine. Indeed, it has been demonstrated that ethanol exposure may have neuroprotective effects when administered prior to traumatic brain injury or an ischemic stroke, an effect that has been described as associated with glutamatergic inhibition by ethanol (Kelly, 1995). As nicotine administration facilitates the release of glutamate through indirect agonistic activation, glutamate excitotoxicity may be the mechanism by which nicotine induced damage is incurred. Therefore, it is possible that ethanol acts as a neuroprotective agent when administered with nicotine by inhibiting NMDA receptor mediated excitotoxicity, rather than potentiating the degenerative action of nicotine, as previously thought (Kelly, 1995).

Despite this possibility for neuroprotective effects of ethanol, the combination of ethanol and nicotine administration also induced an oddly unique perseverative behavior. Specifically, during extended withdrawal and despite spending significantly more time in the targeted northeast quadrant and requiring substantially less trials to establish the learning criterion than other treatment groups, dual ethanol and nicotine administered animals would have significantly more crosses over the original target area in the southwest quadrant. And so, despite being more successful than other treatment groups in reversing the task, the dual ethanol and nicotine treatment group exhibited what is presumed to be dysfunctional learning processes in an inability to completely extinguish a no longer relevant goal. This could indicate a deficit in response inhibition, or the suppression of formerly relevant behaviors when environmental demands require a modification (Obernier et al., 2002). Inhibited cognitive control, such as this, has been consistently associated with patients diagnosed with substance abuse or dependence issues and

may have indicated ongoing addictive pathologies within the present study sample (Gorman, James, Jentsch, 2009). This perseverative behavior, however, developed only during extended withdrawal and coincided with the development of a greater level of anxiety related behaviors, potentially as a product of nicotine withdrawal. Therefore, this development may reflect, to some degree, the influence of withdrawal from nicotine or ethanol and/or how anxiety may interact with learning processes. Further efforts aimed at elucidating the relationship between ethanol and nicotine on addictive pathology such as perseverative behavior would be necessary to clarify this effect.

Indeed, perhaps the nicotine-induced impairments in spatial learning and memory across both initial acquisition and reversal are reflective of anxiogenic developments associated with nicotine withdrawal. As ethanol-induced neurodegeneration has been extensively documented, it is unlikely that nicotine-induced impairments in spatial learning and memory are lessened by additional exposure to a neurotoxic agent (Crews et al., 2004; Nixon & Crews, 2002). Further, it has been demonstrated that withdrawal from nicotine abuse results in an increase in anxiety-related behaviors (Doremus et al., 2003). Similarly, the present researchers found a statistically significant pattern of anxiogenic behavior (i.e. thigmotaxis) resultant from nicotine withdrawal across the acquisition and reversal learning paradigms. Thigmotaxis is identified as an animal's tendency to remain near the periphery of the maze, which will typically lessen as the animal becomes more familiar with the maze environment (Simon, Dupuis, & Costentin, 1994). Thigmotaxic behavior will also increase, or decrease, depending on the administration of an anxiogenic or anxiolytic agent, respectively, and therefore has typically been considered an index of anxiety (Colwill, & Creton, 2011; Simon, Dupuis, & Costentin, 1994). Presently, animals exposed to a chronic model of nicotine exhibited a greater degree of thigmotaxic behavior during

initial acquisition of a spatial learning task, as well as during reversal of the task. Interestingly, ethanol exposure attenuated the nicotine induced anxiety response during initial acquisition when administered concomitantly. Ethanol exposure alone did not produce anxiety related behaviors during any learning phase. As such, the apparent deficit in spatial learning and memory, as assessed by latency and distance to target during Morris water maze testing, may indeed be a product of the greater degree of anxiety prevalent during nicotine withdrawal rather than any true deficit in learning.

Interestingly, as described above, during reversal learning there was evidence of interactive effects in that the ethanol and nicotine receiving animals that had experienced shorter term withdrawal demonstrated what appeared to be greater cognitive flexibility as compared to nicotine-only receiving animals having experienced longer term withdrawal. This effect was diminished when comparing long term nicotine withdrawal with that of long term dual ethanol and nicotine withdrawal; however, trending patterns would suggest that provided greater sample numbers, the effect may in turn be identified. As ethanol has demonstrated anxiolytic effects, attenuation of the learning impairment produced by nicotine withdrawal may be attributed to a reduction of anxiogenesis in co-exposure animals (Wilson et al., 2004). Indeed, our results support this hypothesis in that prior ethanol exposure appeared to ameliorate nicotine-induced anxiogenesis during a test of cognitive flexibility. Further exploration of this interaction using classical anxiety measures, such as the elevated plus maze and/or the open field would be useful in clarifying this relationship.

With respect to the researcher's other hypotheses, there was little support for the notion that ethanol contributed to learning impairments, except when evaluating learning on the probe trial and taking into account withdrawal periods. Specifically, for animals in the shorter ethanol

abstinence period, a diminished proportion of time spent searching the target quadrant was evident during the probe trial. As such, despite showing evidence of learning across the acquisition period, during short term withdrawal ethanol administration inhibited spatial performance; an effect that was not apparent at later periods of withdrawal. Interestingly, there was no evidence of a learning impairment across the acquisition period that could be attributed to ethanol administration, which may suggest differing search strategies for each assessment period. As previously discussed, rodents may use a variety of search strategies in order to escape the maze. The most effective search strategy, however, requires the use of distal cues to approximate the location of the platform and thereby spend the majority of time searching an area relatively close to where the platform had previously been; a task that requires an intact hippocampus and is generally described as a spatial strategy (Gallagher, Burwell, and Burchinal, 1993). This spatial navigation is contrasted with yet another search strategy wherein the animal swims in concentric circles around the water maze, generally at an approximate distance from the maze wall and which would allow for escape when the platform is present (Gallagher, Burwell, and Burchinal, 1993). Animals with hippocampal lesions will generally exhibit use of the concentric circle strategy. However, the concentric circle strategy is not observably less effective when analyzing escape latencies if the platform is present. Indeed, the escape latencies observed during initial acquisition across both search strategies often will be statistically equivalent and therefore may not necessarily indicate a learning impairment, when in fact there may be (Dalm, Grootendorst, Kloet, & Oitzl, 2000). Our results appear to support this, in that the ethanol administered animals had a diminished proportion of time spent in the target quadrant during the probe trial, which would indicate the use of a non-spatial strategy in locating the submerged

platform and may suggest hippocampal damage. Further histological efforts aimed at the neurogenic cycle would be necessary to validate this assumption.

Our efforts to examine the effects of ethanol, however, did not support previous findings of an ethanol induced impairment during the reversal learning phase of Morris water maze assessment. Specifically, ethanol administration independently did not affect the task of cognitive flexibility, and as previously discussed lessened the impact of nicotine when administered concomitantly. As such, prior ethanol exposure may be beneficial when considering the impact of prior nicotine use. It should be noted that our finding that three ethanol (25% EtoH in Vanilla Ensure Plus © w/v) doses per day did not significantly alter spatial reversal contrasts similar efforts by Obernier et al. (2002). Previously, Obernier et al. (2002) reported that comparable doses of ethanol disrupted spatial reversal, evidenced by a greater number of trials in order to successfully reach reversal criterion (mean distance and latency travelled on final day of acquisition + 2 *SD*). The disruption found in the previous study also evidenced a perseveration on the initial requirements of the task by ethanol treated animals (Obernier et al., 2002). These results are interesting in that they posit the potential deficit in executive functioning associated with modifying learning requirements during a changing task. Our results present contradictory findings in that ethanol treated animals did not exhibit either a decreased ability to reverse the requirements of the spatial learning task, or show evidence of perseveration, except when evaluated in conjunction with nicotine exposure and taking into account withdrawal periods. Specifically, researchers in the present study found an increased rate of time spent/distance swam in the original quadrant (SW) during reversal learning by dual ethanol and nicotine treatment, indicative of a perseverative behavior. However, this finding was only expressed following 2.5 – 3 weeks of drug abstinence, was dependent on additional nicotine

exposure, and was not observed along a comparable period of withdrawal as to that identified in Obernier et al. (2002).

There are several possibilities as to how the discrepancies between our studies may have developed. Firstly, Obernier et al. (2002) used surgical implantation of a polyethylene intragastric catheter in order to administer the ethanol dosages, whereas our study utilized an intragastric intubation method requiring an experimenter to physically administer a bulbous ended syringe into the abdominal cavity via the trachea and esophagus. As such, it is possible that the differing method of administration may have contributed to performance on the spatial reversal. Further, our study utilized a ~30-second intertrial period during learning assessments, whereas a 60-second intertrial period was utilized in the previous study. Importantly, an extended intertrial period has been demonstrated to be of a greater difficulty with regard to spatial performance than that of a shorter period (Vorhees & Williams, 2006). Therefore, it may be that the extended delay between trials contributes to the effect of ethanol on spatial learning.

Additionally, as previously mentioned, researchers in the present study utilized two differing periods of drug abstinence. As such, it is possible that the influence of ethanol was diminished because each cohort was necessarily composed of half the desired subjects ($n = 8$) and extended over a substantial period of ethanol abstinence (~30 days). While our acute abstinence period (5-17 days) was comparable to that of Obernier et al. (2002), researchers in the present study were restricted to an ethanol treatment group of $n = 4$ during this period, indicating deficient statistical power as a possible explanation for the lack of effect described by Obernier et al. (2002). Indeed, this cognitive recovery was demonstrated in the assessment of learning during the probe trial analysis of the present study. Specifically, short term ethanol abstinence reduced the proportion of time spent searching the target quadrant; an effect that is consistent

with the differing search strategy displayed by animals with ethanol induced hippocampal damage (Gallagher et al., 1993, Dalm et al., 2000). This effect was not present in the longer term ethanol abstinence group, which would suggest the possibility for a relatively complete recovery of cognitive processes, as compared to drug naïve subjects.

An important consideration here may be the neurogenic reversal accompanied by periods of abstinence (Nixon, 2004). Specifically, previous work has shown that abstinence from ethanol results in compensatory proliferative action along the subgranular zone of the dentate gyrus and therefore a possible recovery of cognitive functioning may be evident (Nixon, 2004). Thus, as the assessments of spatial learning and memory in the present study were conducted on days 5 – 17 and 19 – 31 post administration, it is likely that any observable behavioral abnormalities associated with ethanol induced neurological deficits, such as inhibited cell proliferation, have been diminished by the second week of abstinence. Further elaboration on the time-course associated with potential recovery of cognitive functioning would be useful in clarifying the effects of ethanol.

Analysis of working memory capacity in the present study presented no support for the notion of either an ethanol or nicotine induced impairment. Interestingly, the lack of drug effect was observed across both acute and protracted withdrawal and would suggest alternative neurological pathways associated with the maintenance of spatial working memory processing, when compared to reference memory correlates. Specifically, a nicotine-induced impairment was observed during acquisition and reversal across both periods of withdrawal. As such, it is unlikely that this pattern would be observed unless working memory processes were activating areas that were preserved from nicotine-induced damage. Indeed, it has been demonstrated that lesions to medial prefrontal cortical areas (PFC), specifically prelimbic areas, will disrupt

matching to sample exercises, potentially a unique function of the dorsolateral prefrontal cortex (DLPFC) as opposed to the ventromedial PFC (VmPFC; Nieuwenhuis & Takashima, 2011). Interestingly, activity within the ventromedial prefrontal cortex has demonstrated involvement in the consolidation of information, in addition to selectively attending to future consequences of behaviors, capacities that are disrupted when VmPFC damage is displayed and is implicated in nicotinic addictive pathology (Nieuwenhuis & Takashima, 2011). Further differences between VmPFC and DLPFC capacities are observed in emotional responses and higher order executive involvement as opposed to attentional biases and drug-related contextual cues, respectively (Goldstein & Volkow, 2011). As such, a differential activation of prefrontal regions may be implicated in the effect of nicotine on reference/acquisition capacities, as opposed to that of working memory processes. Examination of hippocampal connectivity to prefrontal cortical regions, and the associated influences of nicotine exposure would be necessary to elucidate any potential relationship, a task outside of the scope of the present study.

Additionally, as the present study utilized a matching to sample assessment of working memory, requiring only two trials per day, it is possible that this measure was not sensitive enough to identify behavioral differences between treatment groups. Specifically, it has been suggested that a comprehensive assessment of working memory capacity may include four trials per day, similar to that utilized during acquisition and reversal learning (Vorhees, & Williams, 2004). It may also be that working memory assessments require a longer overall period of testing. Indeed, it is possible that an animal requires numerous days of working memory testing in order to establish an understanding of the change in rules for the task. Specifically, water maze training up until the working memory assessment period involved four trials with a new starting direction for each trial, but the platform remained in the same area across testing days. This

process changed during working memory assessment in that animals were then required to use the same starting direction twice a day but a different goal area every day. Our working memory period of six days may not have been sufficient enough to establish the change in rule and then detect differences. Therefore, future endeavors may use an extended version of working memory assessment, one that may account for the establishment of the maze rule prior to assessment of differences. It may also be beneficial to consider a continuation of the four trial per day method rather than switching to the matching-to-sample exercise.

With regard to the hypothesis that intragastric intubation may contribute to cognitive difficulties, no support was found. Indeed, the present study provides clarification on the influence of the method of administration (i.e. intragastric gavage) when evaluating hippocampal dependent learning and/or processes. Prior research in our lab had suggested a potential role of intragastric gavage in the proliferative impairment observed following drug administration, and as such associated cognitive processes. Specifically, a previous study from our lab identified a reduction in the number of Ki67 + cells in the SGZ of the DG, a marker of cell proliferation, when comparing a non-injected/intubated control (unhandled) with an experimental control receiving intragastric intubation of a dextrose containing complete liquid diet (Vanilla Ensure Plus ©). Consequently, the method of administration was presumed to contribute to neurological deficits, and subsequently any cognitive impairments, observed following drug exposure. However, the previous study maintained an unhandled control on a regular rat chow diet throughout the drug administration period. As nutritional demands have been posited as likely determinants of proliferative action, the present study aimed to further evaluate this relationship. It has been demonstrated that being maintained on a liquid diet will be associated with reductions in hippocampal cell proliferation, as compared to a solid diet (Patten, Moller, Graham, Gil-

Mohapel, Christie, 2013). Accordingly, the present researchers introduced an unhandled control group, which did not receive an injection/intubation during the administration period but was rather given an equivalent volume of liquid diet (Dextrose in Vanilla Ensure Plus ©) as to the dosage administered to experimental controls (i.e. injection/intubation receiving control). Contrary to the researcher's hypothesis, the method of administration did not have an effect on any learning dependent variables. Specifically, spatial performance between unhandled and experimental controls was statistically equivalent. Thus, researchers posit that the prior finding of reduced proliferation between a liquid diet control and a regular solid food control was likely attributed to the differences in diet rather than an effect of drug administration method, as deficits to proliferation have been typically reflected in dysfunctional spatial learning and memory. It will be necessary to assess levels of neurogenic action among the animals used in the present study to clarify the validity of this presumption.

Future Directions

In conclusion, the present study provides evidence that exposure to a chronic model of nicotine will diminish cognitive performance across later periods of withdrawal (acute & protracted). However, whether this is a true deficit in spatial learning and memory, or rather a product of anxiogenic behavioral alterations remains to be identified. Future efforts should be aimed at clarifying the distinctions between anxiogenesis and memory performance in a co-administration model of drug abuse. As the Morris water maze is not a classic test of anxiety, it may be beneficial to investigate withdrawal behaviors during performance on the Open Field, and/or the Elevated Plus mazes.

On the topic of ethanol administration, the reduction of time spent searching the target quadrant during the probe trial indicated the possibility of differential search strategies that have

previously been associated with hippocampal damage. However, ethanol did not contribute to an impairment during any other phase of learning which may suggest that ethanol prompts the use of differing cognitive strategies no less effective in acquiring a goal, rather than specifically impairing learning processes.

It may be that multiple binges are necessary in order to identify a deficit in spatial learning. Indeed, a singular binge episode is not representative of the consumptive pattern engaged by many alcoholics (Courtney & Polich, 2009). And although a single binge ethanol episode has been shown to result in an increased neurodegenerative pattern, it is possible that cognitive recovery is quicker and less impacted during abstinence than would be evident across a more representative multiple binge pattern (Nixon et al., 2002; Nixon, 2004; Obernier et al., 2004). As such, it may be beneficial to implement a multiple binge paradigm during a replication study in order to assess differing levels of cognitive recovery following ethanol consumption.

Further, as the dual exposure (nicotine and ethanol) paradigm resulted in a substantially higher rate of mortality (60%) than singular exposure (20%), a further elaboration of the physiological interaction of nicotine and ethanol in this model would be necessary to future endeavors. Indeed, previous reports from our lab have identified that despite a similar ethanol dosage, animals receiving a dual ethanol/nicotine exposure may have a greater blood ethanol concentration than animals receiving only ethanol (Lingg, Hartless, & Hayes, 2014). Thus, the combined ethanol/nicotine exposure paradigm may potentiate ethanol toxicity to a greater degree than ethanol alone is capable of, via an as yet unidentified pharmacodynamic interaction. Furthermore, as a consequence of the high mortality rate among dual exposure animals, statistical power may have been adversely affected. It may be that the co-administration of ethanol and nicotine does in fact impact spatial learning and memory, a larger surviving sample

having been provided may reflect this hypothesis. At present, however, such a determination cannot be made and it must therefore be stated that the use of ethanol and nicotine simultaneously does not impair memory processes to any greater degree than independent drug use.

Final Message

Although the present researchers provide evidence for an impairment to spatial learning and memory following a chronic model of nicotine exposure, ambiguous determinations on the interaction between drug exposures (ethanol and nicotine), withdrawal, and the influence of anxiety on spatial learning and memory capacities highlight the importance for future efforts to continue investigation of these relationships. At present, our results show little support for an ethanol induced cognitive impairment but rather the induction of differential search strategies that appear to be equally effective in establishing the learning requirement. However, it is this differential search strategy (i.e., concentric circles) that has been previously associated with hippocampal damage and the inability to access spatial strategies and therefore may be indicative of ethanol induced damage. Further, the interaction between ethanol and nicotine continues to provide an interesting avenue of research, as it appears the combination of ethanol and nicotine will lessen the nicotine-induced cognitive impairment, despite a potentiation of neurodegenerative patterns.

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Appendix A: Behavioral Intoxication Scale (Majchrowicz, 1975)

Behavioral intoxication scale and concurrent ethanol dosages as determined by Majchrowicz (1975)

Behavioral State	Behavioral Indications	Ethanol Dose
0	Normal animal; neutrality	5 g/kg
1	Sedated; Hypoactive, Midly ataxic	4 g/kg
2	Ataxia 1; Elevated abdomen and pelvis	3 g/kg
3	Ataxia 2; Loss of abdominal and pelvis elevation, Delayed righting reflex	2 g/kg
4	Loss of righting reflex w/ a retained eye blink reflex	1 g/kg
5	Loss of righting reflex w/ a loss of eye blink reflex	0 g/kg

Appendix B: Observable Withdrawal Behaviors

Observable withdrawal behaviors following ethanol intoxication

General Hyperactivity	Frantic exploration of cage environment, excessive locomotor activity, enhanced startle reflex
Tail tremors and tail stiffness	Tail of the animals rigidly extended in a horizontal plane, usually the earliest signs of ETX. Maximum intensity occurs within six hours of zero level BEC
General tremors and spasticity	Tremors spread rostrally through the body and toward head, usually within 2 to 3 hrs. of ETX. Behavior is characterized by rigid posture throughout the body; associated with severe inflexibility and a marked resistance to handling. Head tremors will appear following ethanol elimination, however is only prevalent in animals with severe ETX syndrome.
Wet shakes and chattering teeth	Observed in animals experiencing severe ETX syndrome. Accompanied either by preceding or post clonic-tonic convulsions. Chattering of teeth may be observed by continual observation in a noise reduced environment.
Audiogenic seizures	Initially observed as wild running, which may be an indication of relatively low levels of seizure activity. If more severe, wild running behavior may be accompanied by a generalized clonus of limbs. Generalized tonic extension may then be observed if seizure activity is particularly severe.
Spontaneous convulsive seizures	Similar patterns of behavior as seen during AGS, in addition to facial and forelimb clonus that may precede rearing and falling behaviors. Spontaneous seizures may be observed following ethanol elimination. Following overt convulsive behavior the animals may display little or no signs of ethanol withdrawal; appearing relatively sedated. Occasional death may be observed, with multiple seizures normally fatal. Additionally, recurrent seizures may be observed up to and following 4 days of EtoH treatment.
Bizarre behavior	Atypical signs of increasingly dysfunctional neuropathophysiology. These behaviors are usually apparent in animals following at least 4 days of ethanol intox. Behaviors include retropulsion, apparently aimless locomotor activity, stereotyped body movement, head-search activity, aggressiveness, and uncontrolled running episodes.

Note. Table based on Majchrowicz (1975) as described in Faingold (2008).

Appendix C: Starting positions for MWM testing - Acquisition and reversal learning **protocol**

Day	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 4**
1	N	E	SE	NW
2	SE	N	NW	E
3	NW	SE	E	N
4	E	NW	N	SE
5	N	SE	E	NW
Day	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 4**
1	S	W	NW	SE

Note. Table based on Vorhees and Williams (2006)

Appendix D: Directional placement of platform and animal for working memory **assessment**

Day of Acquisition	Start	Goal
1	N	SE
2	E	NE
3	S	SW
4	W	SE
5	S	NE

Note. Two trials (Sample & Test) are given per day with 15s inter-trial period and will utilize the same start and goal point. Table based on Vorhees and Williams (2000).