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Characterization of the Hippocampal Acetylcholine System in a Rodent Model of Fetal Alcohol Syndrome

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ABSTRACT

Fetal alcohol spectrum disorders (FASD) are a major public health concern, as it is estimated that 2-5% of children are exposed to alcohol at some point during prenatal development. FASD have been shown to cause damage to multiple brain regions, but research shows that the hippocampus is especially sensitive to alcohol exposure. This damage to the hippocampus explains, in part, deficits in learning and memory that are hallmark symptoms of FASD. The acetylcholine neurotransmitter system plays a major role in learning and memory, and the hippocampus is one of its main targets. This experiment used a rodent model of Fetal Alcohol Syndrome to examine neurochemical and behavioral changes as a result of developmental alcohol exposure, with a focus on the hippocampal acetylcholine system. Alcohol (3.0 g/kg) was administered via intragastric intubation to developing rat pups (PD 2-10). There were three treatment groups: ethanol-exposed, intubated control, and non-treated control. In Experiment 1, *in vivo* microdialysis was used to measure acetylcholine release in adolescents (PD 32 and 34). During microdialysis, the effects of a high K^+/Ca^{2+} aCSF solution (PD 32) and the effects of an acute galantamine (2.0 mg/kg; PD 34) injection on acetylcholine release were measured. Experiment 3 tested whether chronic administration of galantamine (2.0 mg/kg; PD 11-30), an acetylcholinesterase inhibitor, could attenuate alcohol-induced learning deficits in the context pre-exposure facilitation effect (CPFE; PD 30-32). Experiment 2 utilized brain tissue from Experiments 1 and 3 to measure the impact of developmental alcohol exposure and galantamine treatment on expression of choline

acetyltransferase (ChAT; medial septum), vesicular acetylcholine transporter (vAChT; ventral CA1) and the α7 nicotinic acetylcholine receptor (α7 nAChR; ventral CA1). We found that alcohol-exposed animals did not differ in acetylcholine release at baseline or following administration of a high K^+/Ca^{2+} aCSF solution. However, alcohol exposure during development significantly enhanced acetylcholine content following an acute injection of galantamine. Neither chronic galantamine nor alcohol exposure influenced performance in the CPFE task. Finally, the average number of ChAT+ cells was increased in alcohol-exposed animals that displayed the context-shock association (Pre), but not in any of the animals that were in the control task which entailed no learning. Neither alcohol exposure, nor learning, significantly altered the density of vAChT or α7 nAChRs in the ventral CA1 region of the hippocampus. Taken together, these results indicate that the hippocampal acetylcholine system is significantly disrupted under conditions of pharmacological manipulations (e.g. galantamine) in alcohol exposed animals. Furthermore, ChAT was up-regulated in alcohol-exposed animals that learned to associate the context and shock, which may account for their ability to perform this task. Developmental alcohol exposure may disrupt learning and memory in adolescence via a cholinergic mechanism.

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LIST OF ABBREVIATIONS

FAS ..... Fetal Alcohol Syndrome

FASD ..... Fetal Alcohol Spectrum Disorders

ARND ..... Alcohol-Related Neurodevelopmental Disorders

ND/AE ..... Neurobehavioral Disorder/Alcohol Exposed

ACh ..... Acetylcholine

vAChT ..... Vesicular Acetylcholine Transporter

nAChR ..... Nicotinic Acetylcholine Receptor

ChAT ..... Choline Acetyltransferase

ADHD ..... Attention Deficit/Hyperactivity Disorder

GD ..... Gestational Day

PD ..... Postnatal Day

BAC ..... Blood Alcohol Concentration

LTP ..... Long-Term Potentiation

CS ..... Conditioned Stimulus

CPFE ..... Context Pre-Exposure Facilitation Effect

PRE ..... Pre-exposure

NO PRE ..... No Pre-exposure

MS ..... Medial Septum

mAChR ..... Muscarinic Acetylcholine Receptor

AChE ..... Acetylcholinesterase

APL ..... Allosteric Potentiating Ligand

aCSF..... Artificial Cerebrospinal Fluid

ET..... Ethanol Treatment Group

IC..... Intubated Control Group

NC ..... Non-treated Control Group

HPLC ..... High Performance Liquid Chromatography

TBS ..... Tris-Buffered Saline

CHT..... High-Affinity Choline Transporter

CHAPTER 1

INTRODUCTION

The first clinical diagnosis of Fetal Alcohol Syndrome (FAS) was in 1973 by Jones and Smith after they reviewed case histories of newborn babies exposed to alcohol during gestation (Jones & Smith, 1973a; Jones, Smith, Ulleland, & Streissguth, 1973b). They observed that patients exposed to alcohol experienced growth retardation and craniofacial malformations. FAS is defined specifically by the presence of three characteristics: facial dysmorphology, central nervous system dysfunction, and growth retardation (Sokol et al., 2003). Researchers have also noted that prenatal alcohol exposure results in a variety of cognitive and behavioral symptoms, including intellectual disability and impaired attention and social skills (Mattson & Riley, 1998; Streissguth, Landesman-Dwyer, Martin, & Smith, 1980; Streissguth & O'Malley, 2000). This constellation of physical and cognitive symptoms has come to be known as Fetal Alcohol Spectrum Disorders (FASD), an umbrella term which includes FAS at the more severe end of the spectrum, and alcohol-related neurodevelopmental disorders (ARNDs) or neurobehavioral disorder/alcohol exposed (ND/AE) at the less severe end of the spectrum (Sokol et al., 2003; Astley, 2011).

FASD are a major health concern, affecting about 1 in every 100 live births (Sampson et al., 1997). In addition, it is estimated that the cost to treat individuals with Fetal Alcohol Syndrome (FAS) is 3.6 billion dollars annually in the U.S., with the cost to treat FASD much higher (Lupton et al., 2004). Even with the wealth of information

available about the negative effects of prenatal alcohol exposure, an estimated 12.2 % of pregnant women consume alcohol, with 1.9% engaging in binge drinking (CDC, 2009). Given these findings, it is crucial to develop effective treatments for alcohol-induced brain damage and behavioral dysfunction.

Structural abnormalities as a result of *in utero* alcohol exposure have been studied extensively in humans (see Norman et al., 2011, for review) and by using animal models (Berman & Hannigan, 2000). For example, microcephaly (Coulter et al., 1993) and dysmorphology of specific brain regions, such as the corpus callosum, have repeatedly been observed in autopsy studies of children prenatally exposed to alcohol (Clarren et al., 1978; Coulter et al., 1993), and more recently by using neuroimaging (Normal et al., 2011).

In rodent models, the hippocampus has been shown to be especially sensitive to alcohol-induced damage including decreased cell number (Barnes & Walker, 1981; Bonthius & West, 1990; Bonthius & West, 1991; Miki et al., 2000; Tran & Kelly, 2003), decreased dendritic spine density (Berman et al., 1996; Tarelo-Acuna et al., 2000; West, 1990), impaired neurogenesis (Hamilton et al., 2011; Klintsova et al., 2007) and changes in the electrophysiological properties of hippocampal neurons (Hablitz, 1986; Swartzwelder et al., 1988; Tan et al., 1990). Notably, behavioral deficits are often seen in tasks that depend heavily on the hippocampus, indicating the structural abnormalities noted above likely have a functional impact. Because of the consistent finding of damage by alcohol exposure during development, the hippocampus may be very useful as a target brain region to understand the mechanism of alcohol-induced deficits. In addition, elucidation of the neurochemical changes in this brain region associated with alcohol

exposure may help indicate potential pharmacological therapies for these behavioral deficits.

The acetylcholine (ACh) neurotransmitter system plays a role in many important cognitive functions including learning, memory, and attention (Micheau & Marighetto, 2011; Sarter & Parikh, 2005). Previous research has shown that developmental alcohol exposure impacts some aspects of the acetylcholine system, such as the muscarinic receptor subtype (Kelly et al., 1989; Monk et al., 2012; Nio et al., 1991), but no studies have examined acetylcholine release or described alcohol-induced changes to the nicotinic receptor subtype.

The current studies tested the hypothesis that alcohol exposure during development causes a decrease in cholinergic function in the hippocampus. Furthermore, it is hypothesized that increasing the functioning of the cholinergic system can mitigate deficits in behaviors dependent upon the hippocampus seen in animals exposed to alcohol during development. The first two experiments characterized the cholinergic system in the hippocampus in animals exposed to alcohol during development and the third experiment tested the effectiveness of a drug that potentiates the cholinergic synapse for treating behavioral deficits in animals exposed to alcohol during the third trimester. Specifically, in the first experiment, acetylcholine efflux was measured in order to characterize acetylcholine release in an awake, behaving animal exposed to alcohol during the third trimester of development. The second experiment assessed hippocampal expression of two cholinergic proteins, namely vesicular acetylcholine transporter (vAChT) and the nicotinic acetylcholine receptor alpha 7 (α7 nAChR) and expression of choline acetyltransferase (ChAT) in the medial septum. The final experiment tested

whether galantamine can attenuate alcohol-induced impairments in the context pre-exposure facilitation effect, a learned effect dependent in part upon the hippocampus. Galantamine is a drug which acts on the acetylcholine system to enhance cholinergic signaling, both by preventing the breakdown of acetylcholine and by making cholinergic receptors more responsive to the presence of acetylcholine.

This introduction will provide an overview of FASD, followed by an overview of animal models of FASD. Then, a summary of neurological and behavioral changes associated with *in utero* alcohol exposure is provided, with a focus on the hippocampus and cholinergic system. Finally, there will be an overview of the research on pharmacological interventions in animal model of FASD, followed by a description of the drug galantamine and the rationale suggesting that it might be a good treatment for alcohol-related learning deficits.

1.1 Overview of FASD

Fetal Alcohol Spectrum Disorders (FASD) refers to a range of impairments observed in patients whose mothers consumed alcohol during pregnancy. FAS is the most severe of the FASD, and requires diagnostically that a patient present with facial abnormalities, growth retardation, and central nervous system abnormalities (Sokol et al., 2003; Astley, 2011). Symptoms of FASD vary widely, and central nervous system dysfunction can be observed in individuals who fail to present with outward physical abnormalities, making diagnosis of this disorder very difficult (Astley, 2011). Central nervous system dysfunction can include a number of impairments in domains such as language, learning, attention, and social functioning (Astley, 2011). This section will provide an overview of neuroanatomical changes observed in patients with FAS,

followed by a discussion of the neurobehavioral impairments that are consistently observed in this population.

1.1a Neuroanatomical Changes in Individuals with FASD

With the advent of neuroimaging technology, considerable advancements have been made in characterizing brain abnormalities in individuals with FASD. Overall reductions in the volume of the brain have been consistently observed (Mattson et al., 1994; Archibald et al., 2001), along with abnormalities in the size of specific brain regions, such as the cerebral cortex, corpus callosum, basal ganglia, and cerebellum (reviewed in Normal et al., 2009).

Using structural magnetic resonance imaging, Archibald and colleagues (2001) described a study in which individuals with Fetal Alcohol Syndrome, prenatal exposure to alcohol (non-dysmorphic), and controls were compared. The results showed that individuals with FAS had significant microcephaly and a reduction in the volume of the caudate nucleus and parietal lobe, even after controlling for reductions in overall brain volume in FAS patients. However, there was no significant reduction in the volume of the hippocampus, controlling for reductions in the volume of subcortical structures. In addition, alcohol exposed individuals had significant reductions in gray and white matter volumes in the cerebrum and cerebellum, with larger decreases in cerebral white matter volume. A recent study found that while the overall volumes of the caudate nucleus and hippocampus were unchanged by alcohol exposure, there were specific differences in the shapes of these structures, and these differences correlated to the amount of alcohol to which patients were exposed (Joseph et al., 2012), suggesting that these regions are extremely sensitive to alcohol.

Using voxel-based morphometric analyses on children and adolescents prenatally exposed to alcohol, Sowell et al. (2001) demonstrated structural abnormalities in the left hemisphere, specifically in the temporal and parietal lobes, as evidenced by an increase in gray matter and a reduction in white matter. In a recent study, Yang et al. (2012) used structural magnetic resonance imaging to examine the brains of a large number of individuals with FASD (n=82) and controls (n=71). Consistent with previous studies (Riley et al., 1995), the authors found a reduction in collosal thickness, which was localized to the genu and splenium in FASD patients. Interestingly, these reductions were correlated with reduced palpebral fissure length, one of the hallmark facial features of FAS.

These studies suggest that certain brain regions, namely the parietal lobe, corpus callosum, hippocampus, and basal ganglia, are especially impacted by prenatal alcohol exposure, as measured by structural magnetic resonance imaging. Importantly, some of these changes are correlated to amount of alcohol consumed by the mother (Joseph et al., 2012) and facial dysmorphology (Yang et al., 2012), indicating that these structural changes may represent a specific effect of prenatal exposure to alcohol and are not due to the many other differences between alcoholic women and non-alcoholic women who are pregnant.

1.1b Behavioral Changes Observed in Individuals with FASD

FASD are characterized behaviorally by intellectual disability, as well as specific impairments in memory, language, attention, executive functioning, and social skills (reviewed in Kodituwakku, 2009). Importantly, the magnitude of some of these deficits is not different between individuals diagnosed with FAS and individuals who do not meet

the full criteria for FAS (Kodituwakku et al., 2001), indicating that individuals with moderate exposure to alcohol may be impaired on some aspects of cognitive functioning.

One of the goals of clinical research is to develop a neurocognitive profile of these individuals, in order to be able to accurately diagnose individuals who do not meet the full criteria for FAS, but who are otherwise impaired. Intellectual disability as measured by IQ tests is a common observation in patients with FASD, and about 24% of individuals with FAS have IQ scores below 70 (Streissguth et al., 2004), and may be related to the amount of alcohol exposure (Streissguth et al., 1989).

Attention is an aspect of cognitive functioning that is commonly impaired in patients with FASD. In fact, up to 95% of individuals with FAS also qualify for a diagnosis of attention deficit/hyperactivity disorder (ADHD) (Streissguth et al., 1996; Fryer et al., 2007). Coles et al. (2002) utilized a Continuous Performance Task program to explore alcohol-related deficits in sustained attention, in which participants must identify a target letter among non-target letters. In this study, there were four experimental groups: control, dysmorphic, alcohol exposed (but non-dysmorphic), and special education. The results showed that the special education group was impaired across all domains of sustained attention, but the alcohol-exposed group was specifically impaired in the visual domain, suggesting that visual attention may be uniquely affected by alcohol.

In another study designed to explore FASD-related attention impairments, Mattson et al. (2006) used a paradigm with three conditions: visual focus, auditory focus, and auditory-visual shift. The focus conditions required that the participants respond to a target stimulus (visual or auditory), while the shift conditions required that the

participants respond only when the target stimulus shifted modality, making this task much more difficult. Children prenatally exposed to alcohol were impaired in the visual focus condition to a greater degree than the auditory focus condition. In contrast, in the shift condition, alcohol exposed children were not different from controls on accuracy, but did have slower reaction times. Taken together, these results suggest that children prenatally exposed to alcohol may have specific impairments in visual attention and this finding may be useful in a diagnostic and treatment setting.

Another aspect of cognitive functioning in which alcohol-exposed individuals are impaired is executive functioning. Executive functioning is a term that refers generally to the ability to use cognitive resources effectively to obtain a specific goal (Riley et al., 2005). Importantly, children and adolescents prenatally exposed to alcohol are impaired across multiple domains of executive functioning: cognitive flexibility (Mattson et al., 1999), response inhibition (Mattson et al., 1999), set shifting (Mattson et al., 1999; Schonfeld et al., 2001), nonverbal fluency (Schonfeld et al., 2001) and concept formation (Mattson et al., 1999; Schonfeld et al., 2001). Ware et al. (2012) compared three groups of children: those with a history of heavy prenatal alcohol exposure, children without a history of alcohol exposure, but diagnosed with ADHD, and typically developing controls on the Delis-Kaplan Executive Function System. Executive function was a significant predictor of adaptive abilities, defined as the ability to respond and adjust to the environment, in the ADHD and FAS group. The effect was general in children with ADHD, with 3 out of the 4 measures of executive functioning predicting adaptive abilities. In contrast, only nonverbal executive functioning significantly predicted

adaptive functioning in children with FAS, indicating that these executive functioning impairments significantly affect functioning in social settings, such as school.

Impairments in learning and memory are another common observation in children with prenatal exposure to alcohol. Mattson et al. (1996) demonstrated alcohol-related impairments in verbal learning and memory, using the California Verbal Learning Test-Children's Version. This task utilizes a word list to assess recall and recognition memory. Children prenatally exposed to alcohol had impairments in recall and demonstrated increased perseveration errors. Importantly, these impairments persisted even after accounting for mental age. In another study, Green and colleagues (2009) used the Cambridge Neuropsychological Tests Automated Battery to characterize functioning in children with Fetal Alcohol Syndrome (FAS), partial FAS, or alcohol-related neurodevelopmental disorder (ARND). Alcohol exposed children had longer reaction times and impairments in planning and spatial working memory. Importantly, these deficits became increasingly larger as the task load increased, indicating that alcohol-exposed individuals are only impaired on difficult tasks. This is consistent with the animal literature, where alcohol-exposed rodents are especially impaired on difficult behavioral tasks.

Additionally, in a study designed to assess spatial learning in alcohol exposed children, Uecker & Nadal (1996) asked children to sit in front of a testing board with objects placed onto 16 dots. After the children had observed the objects, the objects were removed and the children were asked to recall the items (immediate object recall). Then, the children were asked to recall where the items were located (immediate spatial recall) on the testing board. To assess delayed memory, twenty four hours later, children were

placed in front of the testing board, and asked to recall the names (delayed object recall) and locations (delayed spatial recall) of items that were on the board the previous day. Children prenatally exposed to alcohol were not impaired on the immediate object recall task, but had difficulty with the delayed object recall. However, prenatal exposure to alcohol impaired performance on both the immediate and delayed spatial recall task.

More recently, Hamilton et al. (2003) used a virtual water maze task to assess spatial navigation impairments in eight adolescent males with FAS. The non-virtual task is used frequently in animal studies to assessed hippocampal function (Morris, Garrud, Rawlins, & O'Keefe, 1982). In this task, there are two conditions: place learning and cued-navigation. In the place learning condition, a virtual platform is located in a fixed position, and the participants must use extra-maze cues to find the platform. In the cued-navigation trials, the platform is located above the water, and the participants must navigate to the platform. During the probe trial, the virtual platform is removed from the maze, and the navigation through the maze is recorded. Individuals with FAS were impaired on the place learning trials, as indicated by an increased distance traveled to reach the platform, but were no different from controls on the cued navigation trials. During the probe trial, children with FAS spent less time in the target quadrant of the maze, and indication of impaired spatial navigation. These studies demonstrate that patients with FAS have spatial navigation deficits, and indicate impaired hippocampal functioning (Morris et al., 1982; Logue, Paylor, & Wehner, 1997).

Behavioral dysfunction is common in individuals diagnosed with FAS, and is even apparent in individuals who do not meet the full criteria for a diagnosis of FAS (Astley, 2011). Alcohol exposed children and adolescents show impairments on an

assortment of cognitive tasks assessing attention, executive function, verbal learning, and spatial navigation. Researchers have been trying to define a specific neurobehavioral profile in individuals with FAS, in order to distinguish these individuals from individuals with other developmental disabilities and neurodevelopmental disorders, such as attention-deficit/hyperactivity disorder (reviewed in Kodituwakku, 2009). However, this has been a slow process which may be due to the multiple factors that can affect behavior, such as prenatal exposure to drugs other than alcohol. For this reason, animal models can be useful to isolate the neurobehavioral deficits produced by alcohol exposure alone, and to begin to delineate the changes in brain regions that play a role in these deficits.

1.2 Animal Models of FASD

Fetal alcohol spectrum disorders (FASD) is a term that refers to the symptoms that arise following exposure to alcohol *in utero* (Sokol et al., 2003). Alcohol exposure can produce a variety of symptoms that vary greatly in their severity. In humans, there are a number of factors that can interact with alcohol to determine clinical outcomes, such as nutritional effects, genetic factors, and the use of other drugs besides alcohol (Abel & Hannigan, 1995). By using animal models, researchers have tight control over the timing and dose of alcohol administered to the developing organism. Animal models were developed early in the field of FASD research, but the specific method of alcohol administration has evolved substantially. Before discussing these models of FASD, it is important to note that there are three major periods of rodent development: gestational days (GD) 1-10, GD 11-22, and postnatal days (PD) 1-10. These periods of development correspond closely with the three trimesters of development in humans (Bayer et al.,

1993; Dobbing & Sands, 1979). This section will discuss the various animal models of FASD, while providing justification for the choice of animal model in the proposed experiments.

Animal models of FASD can vary according to the timing of alcohol administration: prenatal, postnatal, or both. Some studies expose animals to a dose of alcohol on a specific gestational day (e.g. GD 7-8), which results in a precise pattern of craniofacial abnormalities (Lipinski et al., 2012; Parnell et al., 2009). A majority of studies employ a more chronic alcohol administration procedure, in which alcohol is administered over a specific period of time during prenatal development, postnatal development, or over the combination of prenatal and postnatal periods. Alcohol-induced morphological and behavioral alterations have been shown to vary substantially based on the timing of alcohol administration, and these findings are reviewed below.

Importantly, the timing of alcohol administration determines the methods that can be used to administer alcohol to the developing organism. For example, to administer alcohol to rodents during the first and second-trimester equivalent periods of development, alcohol must be given directly to the dam. To do so, a variety of methods can be used: liquid diet, intubation, injection, and inhalation. In the liquid diet paradigm (Savage et al., 1991; Varaschin et al., 2010), the dam is given alcohol through the diet, which consists of a liquid solution containing alcohol. The dam drinks the solution over time, which prevents the researcher from having control over the precise dose of alcohol administered. This procedure produces relatively low blood alcohol concentrations (BACs; ~80 mg/dl). Another way in which alcohol can be administered is through intraperitoneal injection (Lipinski et al., 2012). This procedure is ideal for researchers

wishing to explore the effects of alcohol exposure over a short period of time (e.g. a single gestational day), but are not practical for repeated exposures, due to the stress of the daily injections and damage to tissues outside of the digestive system. Inhalation of alcohol is also another common administration procedure, and involves the placement of the dam into a chamber filled with ethanol vapors (Granato et al., 2012; Moore et al., 2004). Finally, intragastric intubation involves insertion of a tube into the esophagus of the dam, and administering a solution containing ethanol directly into the stomach. This procedure must be done daily at a consistent time, but is relatively quick and allows the researchers to control the dose of alcohol that is given.

The period of rodent development equivalent to the third trimester in humans occurs postnatally outside the maternal environment (Bayer et al., 1993). Multiple brain regions develop substantially during this time period, such as the hippocampus and cerebellum (Bayer et al., 1993), and behaviors that rely on these regions are impacted by alcohol (see Berman & Hannigan, 2000, for review). This makes it important to have methods, such as artificial rearing and intubation, to administer alcohol to the developing animal during this developmental period. Artificial rearing refers to a procedure in which an animal is surgically implanted with a cannula directed into the stomach (Samson & Diaz, 1981). A milk solution which contains a specific amount of alcohol can then be administered through this cannula. This procedure is quite stressful, and can result in decreased body weights in alcohol-exposed offspring (Thomas et al., 2004b). In addition, the procedures are time consuming and very labor intensive, making many researchers choose to use other administration procedures. Inhalation is another method that can be used to administer alcohol, but the pups must be placed into the inhalation chamber with

the dam, making it hard to control the precise dose of alcohol that the animals receive, since they receive it directly, through the ethanol vapors, and indirectly through milk. Furthermore, the dam is also exposed to the vapors and her maternal behavior is likely altered by the procedure. Finally, intragastric intubation involves insertion of a tube into the esophagus of the pup, and administering a milk solution containing ethanol directly into the stomach. This procedure allows precise control over the timing and dose of alcohol which the pups receive and requires minimal separation from the dam (Kelly & Lawrence, 2008). This procedure is stressful to the pup and so a control condition for the intubation must be included.

The goal of the present studies is to assess ethanol-induced changes in the hippocampal acetylcholine system. The hippocampus undergoes rapid growth during the third trimester equivalent period (Bayer et al., 1993) and significant levels of choline acetyltransferase (ChAT), the enzyme which catalyzes the synthesis of acetylcholine, do not appear in the hippocampus at until GD 18 (Schambra et al., 1989). This suggests that hippocampal cholinergic function would not be impacted by alcohol exposure during the first- and second-trimester equivalent periods of development, which occur from GD 1-10 and GD 11-22, respectively. However, alcohol exposure during the third-trimester equivalent, which occurs postnatally in the rat, may impair the function of this neurotransmitter system. For this reason, alcohol was administered postnatally, to capture this potential window of vulnerability. Moreover, intragastric intubation has been used repeatedly in this laboratory (e.g. Cronise et al., 2001; Marino et al., 2004; Tran & Kelly, 2003; Otero et al., 2012), and has been chosen as the administration method for these studies for the following reasons: 1) it allows precise control over the timing and dose of

alcohol administered, 2) it produces relatively high BACs, and 3) it is quick, which reduces the stress to the developing organism.

1.3 Alcohol-Induced Morphological and Functional Changes in Animal Models of FAS

Considerable advancements have been made in describing brain damage following *in utero* alcohol exposure, both in humans and by using animal models. Much of the research with animal models has focused on two brain regions, the cerebellum (Idrus & Napper, 2012) and hippocampus (Tran & Kelly, 2003). This is because these brain regions are undergoing rapid development during the third trimester equivalent period of development, a time at which organisms are quite sensitive to alcohol exposure. As mentioned above, it is crucial to develop effective treatments for alcohol-induced brain damage, and behavioral studies suggest that the hippocampus may be the best target for these interventions. This section will provide an overview of the morphological and functional changes observed in the hippocampus of animals exposed to alcohol during development, and these research findings are divided into two categories: ultrastructural and electrophysiological.

1.3a Alcohol-Induced Ultrastructural Changes in Animal Models of FASD

Developmental ethanol exposure produces altered hippocampal morphology, as evidenced by reductions in cell number and altered connectivity. Specifically, hippocampal neurons are lost as a consequence of prenatal ethanol exposure, particularly in area CA1 of the hippocampus in rats (Barnes et al., 1981; Bonthius et al., 1990; Bonthius et al., 1991). Early postnatal exposure to alcohol produced a significant reduction in pyramidal cell number in CA1, but only when alcohol administration (10.2%, artificial rearing) occurred in a “binge”-like manner (Greene et al., 1992). Using

unbiased stereological procedures, Tran et al. (2003) examined critical periods for ethanol-induced cell loss in the hippocampus using intragastric intubation of the dam (4.5 g/kg/day) and pup (3.0 g/kg/day). A 17% reduction in the number of pyramidal and granule cells was observed in the hippocampal CA1 region, but not CA3 or DG, in animals exposed to alcohol during the third trimester alone and during all three trimesters equivalent. What can be taken away from these studies is that the hippocampus and area CA1 specifically, is sensitive to alcohol exposure, especially when it is administered in a condensed, binge-like fashion.

Furthermore, there are a number of findings suggesting altered synaptic connectivity in the hippocampus. In the hippocampus, mossy fibers are responsible for the transmission of information from the dentate gyrus to area CA3 (Gould & Leach, 2014). Alterations in hippocampal mossy fiber distribution have been shown following prenatal ethanol exposure using liquid diet (West & Hodges-Savola, 1983; Fukui & Sakata-Haga, 2009), specifically in the dorsal region (Sakata-Haga, Sawada, Ohta, Sui, Hisano,& Fukui, 2003), suggesting that ethanol disrupts the transfer of information from the dentate gyrus to area CA3. In addition, hippocampal spine density has been shown to be reduced following exposure to ethanol during development (Abel, Jacobson, & Sherwin, 1983; West, 1990). Tarelo-Acuna et al. (2000) exposed animals to 20% ethanol (v/v in water) throughout gestation and until weaning and examined the hippocampus at 15, 21, 40, and 90 days of age. Ethanol exposure during development led to a reduction in the number of thin dendritic spines (PD 15 and PD 40), accompanied by an increase in the proportion of stubby (PD 15 and PD 40) and wide (PD 15) dendritic spines in the hippocampus (Tarelo-Acuna et al., 2000). Collectively, these findings indicate that

alcohol exposure results in altered synaptic structure, and it is likely that these structural abnormalities result in impaired synaptic plasticity.

1.3b Alcohol-Induced Changes to Hippocampal Electrophysiology in Animal Models of FASD

Administration of alcohol to developing organisms also leads to changes in hippocampal function, as measured by electrophysiology. Long-term potentiation (LTP), which is thought to be a model of synaptic plasticity, has consistently been shown to be disrupted in animal models of FASD. Using *in vivo* electrophysiology techniques, Varaschin et al. (2010) demonstrated no effect of alcohol using a 10-tetanus stimulus train, but significant impairments using a 3-tetanus stimulus train in the dentate gyrus of the hippocampus of animals prenatally exposed to ethanol (peak BAC 84 mg/dl). Specifically, animals exposed to ethanol show an increase in field EPSPs following a 10-tetatnus stimulus train, but fail to maintain the excitatory state following 3 stimulus trains. In another study, Sutherland et al. (1997) used a liquid diet procedure (5% ethanol; 83 mg/dl) to examine the impact of moderate alcohol consumption during pregnancy on synaptic plasticity in adult offspring. The results demonstrated that there was a reduction in field EPSPs and population spikes in the dentate gyrus in response to high-frequency stimulation of the perforant path of the hippocampus. Research has also demonstrated an alcohol-induced decrease in the magnitude of long-term potentiation observed in CA1, along with a decrease in the frequency of spontaneous bursts from CA3 when cells were tested in a high-potassium medium (Swartzwelder et al., 1988). Furthermore, animals treated with ethanol prenatally (35% ethanol-derived calories) have increased paired-pulse potentiation as juveniles (Hablitz, 1986) and adults (Tan et al., 1990) compared to

controls. These results consistently demonstrate impaired hippocampal synaptic plasticity following exposure to alcohol during development, even when the exposure is relatively low (Sutherland et al., 1997; Varaschin et al., 2010). These impairments are likely involved in learning deficits associated with developmental alcohol exposure.

1.3c Summary of Alcohol-Induced Morphological Changes in Animal Models of FASD

Overall, it has been consistently demonstrated that exposure to alcohol during development results in damage to the hippocampus, a brain structure implicated in learning and memory deficits that are common to animal models of FASD. Specifically, both cell number and dendritic spines are reduced, suggesting a decrease in synaptic connectivity in the hippocampus. Moreover, long-term potentiation has been shown to be disrupted following exposure to alcohol during development. These changes are extensive and suggest that there is a disruption in hippocampal function that may underlie behavioral deficits associated with FASD.

1.4 Alcohol-Induced Behavioral Changes in Animal Models of FASD

Cognitive impairments are a hallmark feature of Fetal Alcohol Spectrum Disorders (FASD), and deficits are seen in a range of domains, including executive function, learning and memory, social behavior, language, visual-spatial ability, attention, and motor function (Mattson, Crocker, & Nguyen, 2011). Animal models have allowed researchers to reliably reproduce deficits in some behaviors (e.g. learning and memory), and begin to reveal the mechanisms responsible for those deficits. Clinical data indicate impairment in behaviors relying on the frontal lobes, but examining frontal lobe function using animal models is difficult, due to the time required and the complexity of the behavioral paradigms. For the most part, research using animal models has focused on

learning and memory deficits, in part due to the ability to reliably test learning and memory. Notably, learning impairments are often observed on tasks which depend in part upon the hippocampus, indicating that this brain region is a good target for understanding the mechanisms of alcohol-induced behavioral impairments. This section will provide a review of the most consistent behavioral findings using animal models of FASD.

Spatial learning is an important aspect of every day functioning, and allows for the creation of mental representations of the environment. Importantly, spatial learning seems to be particularly affected in animal models of FASD (Kelly, Goodlett, Hulsether, & West, 1988; Cronise et al., 2001; Murawski & Stanton, 2010). It is important to note that lesions of the hippocampus impair performance on the Morris water maze (Morris, Garrud, Rawlins, & O'Keefe, 1982; Logue, Paylor, & Wehner, 1997), a task commonly used to assess spatial learning in animal models, indicating that the hippocampus is essential for spatial learning. Rodent models of FASD also consistently demonstrate impairments in other cognitive tests (reviewed in Berman & Hannigan, 2000), with specific deficits in water maze acquisition (Goodlett & Johnson, 1997), contextual fear conditioning (Allan et al., 2003; Murawski et al., 2010), spontaneous alternation (Thomas et al., 2010), and serial spatial discrimination reversal learning (Thomas et al., 2004b). Importantly, these tasks all depend on hippocampal activity, and damage to the hippocampus is suggested to be involved in alcohol-induced deficits on these tasks. This supports the idea that alcohol impacts this brain structure, and highlights the need for focusing research on the hippocampus with respect to the specific damage by alcohol and for potential treatment. It is important to use behavioral measures that are sensitive to

hippocampal damage, in order to test potential therapeutic interventions to treat alcohol-induced learning deficits.

A variety of factors influence the observation of spatial learning deficits in animal models of FASD, including age of testing, dose, and timing of exposure. For example, exposure to ethanol during the prenatal period leads to impaired water maze performance in 40 day old rats, but these effects were not apparent in 60 and 90 day old rats (Gianoulakis, 1990). In addition, Cronise et al. (2001) observed deficits in water maze performance in juveniles, but not adults, developmentally exposed to alcohol. Goodlett et al. (1987) examined water maze performance in juvenile rats following postnatal ethanol treatment (6.6 g/kg; PD 4-10) and found that a condensed dose of alcohol (4 feedings; "binge" exposure) led to an increase in escape latency and path length, both indicative of impaired performance. Interestingly, the same dose (6.6 g/kg) given over time (12 feedings; "uniform" exposure) failed to produce these deficits, indicating that spatial learning is sensitive to high blood alcohol concentrations rather than simply dose of alcohol.

In another study, Goodlett & Johnson (1997) conducted two experiments to test the effects of timing and dose on spatial navigation in juvenile rats (PD 26-31). Alcohol was given during three developmental time periods (PD 4-6, PD 7-9, or PD 4-9). Significant impairments in water maze acquisition were seen for the groups treated with alcohol during PD 7-9 and PD 4-9, but not PD 4-6. In the second experiment, two doses of alcohol were administered (4.5 g/kg/day and 5.25 g/kg/day) on PD 7-9. Both doses led to significant reductions in the amount of time spent in the correct quadrant on the probe trial, indicating impaired memory for the escape platform. Cronise et al. (2001) examined

the critical periods for alcohol-induced spatial learning deficits in juveniles and found that both pre-and postnatal exposure were required. From these studies, three major conclusions can be drawn: 1) "Binge" exposure is needed to reliably produce deficits in the water maze (Goodlett et al., 1987), 2) Spatial learning impairments are not consistently observed in adult animals exposed to alcohol during development (Cronise et al., 2001), and 3) Postnatal treatment alone (Goodlett & Peterson, 1995; Goodlett et al., 1997; Marino, Aksenov, & Kelly, 2004) or exposure during all three trimesters equivalent (Cronise et al., 2001) impair water maze performance in juvenile rats, suggesting that third trimester equivalent exposure is required to impair spatial navigation. This is consistent with the observation that the hippocampus is undergoing rapid growth during the third trimester equivalent, and is required for spatial navigation.

Another task that is sensitive to developmental alcohol exposure is contextual fear conditioning. Contextual fear conditioning is a behavioral test that consists of two phases: training and testing. During training, an animal is placed into a testing environment, or context, and receives a series of brief foot shocks. The animal associates the context with the foot shock, and will show increased behavioral signs of fear (e.g. freezing) during the testing phase. Rodents developmentally exposed to alcohol are impaired on this task, demonstrating a reduction in freezing to the context (Allan et al., 2003; Murawski & Stanton, 2010). Importantly, alcohol exposed animals fail to show impairments on a similar task involving the association of a tone (auditory CS) with a foot shock (Murawski & Stanton, 2010). This suggests that alcohol exposed animals have difficulty with contextual fear conditioning specifically, and that these differences are not due to other factors, such as differential sensitivity to pain.

Developmental alcohol exposure has also been shown to impair performance on the context pre-exposure facilitation effect (CPFE), a variation of contextual fear conditioning (Murawski & Stanton, 2010; Murawski & Stanton, 2011; Klintsova et al., 2007). In the CPFE paradigm, there are two conditions: pre-exposure (PRE) and no pre-exposure (NO PRE) and three stages of testing: pre-exposure, training, and testing. Animals are pre-exposed to a context, either the one in which they will undergo training (PRE), or a different one (NO PRE). Training occurs on the following day, and consists of placement into a context, followed by an immediate shock and removal from the context. Testing occurs the next day, and studies have demonstrated that animals pre-exposed to the context in which they were shocked have an increase in freezing during testing. This is called the context pre-exposure facilitation effect (CPFE) and occurs because the animals pre-exposed to the context have a mental representation of the testing environment, and are able to recall that representation during training. However, animals that were not pre-exposed to the context have no prior mental representation with which to associate the shock. It is important to note that during training, the animal experiences a foot shock, and is immediately removed from the context, before they have time to create a new mental representation of the context. Thus, the animals must recall previous experiences, making the task quite difficult.

In a study designed to examine conditioned fear deficits in alcohol exposed animals, Murawski & Stanton (2010) compared three behavioral testing paradigms: CPFE, standard contextual fear conditioning, and fear conditioning to a tone. In all three experiments, animals were exposed to high doses of ethanol, administered as a single daily "binge" dose (5.25 g/kg/day) from postnatal days 4-9. The results demonstrated that

alcohol-exposed animals did not show impaired conditioning to a discrete auditory CS, a task which relies on activation of the amygdala, and not the hippocampus (LeDoux, 2000). Ethanol exposure did cause impaired freezing in standard contextual fear conditioning. Finally, ethanol-exposed animals were found to be impaired on the CPFE task, and these impairments were not sex-specific. However, the magnitude of the alcohol effect was much larger in the CPFE task than in the standard contextual fear conditioning task, an observation that is likely due to the increased difficulty of the CPFE. Importantly, lesions of the hippocampus impair performance on both standard contextual fear conditioning and CPFE (LeDoux, 2000) suggesting this behavioral paradigm is sensitive to hippocampal damage.

In another study by the same group, the dose and timing of ethanol administration were varied, and animals were tested using the CPFE paradigm (Murawski & Stanton, 2011). The authors determined that high (4.0 and 5.25 g/kg), but not low (2.75 g/kg) doses of alcohol administered as a single daily "binge" exposure on PD 4-9 impaired learning when animals were tested from PD 31-33. Moreover, exposure to ethanol from postnatal days 7-9, but not PD 4-6, produced impaired CPFE performance. This study demonstrates that the task is sensitive to ethanol exposure during a period in which the hippocampus is undergoing rapid growth (Bayer et al., 1993).

In summary, learning deficits are dependent upon the timing of ethanol exposure, age of testing, and dose. A few consistent patterns can be observed: postnatal exposure (Cronise et al., 2001; Goodlett et al., 1987; Goodlett & Johnson, 1997), as well as combined pre and postnatal treatment (Cronise et al., 2001), impair water maze performance in juveniles. The effects of alcohol exposure on adult spatial learning are not

as consistent as the effects on learning by juvenile animals. Moreover, alcohol exposure during the postnatal period also disrupts the context pre-exposure facilitation effect (Murawki & Stanton, 2010; Murawski & Stanton, 2011), and this effect is dependent on dose and timing of ethanol administration (Murawski & Stanton, 2011). The findings on the behavioral deficits caused by alcohol exposure during development clearly implicate the hippocampus as a site of damage, particularly in juveniles. This proposal will utilize the novel and sensitive testing paradigm, CPFE, and examine group differences in performance in juvenile rats (PD 30-32). Furthermore, this study will test the ability of a novel drug, galantamine, to ameliorate alcohol-induced impairments.

1.5 Alcohol-Induced Neurochemical Changes in Animal Models of FASD

Research regarding the impact of alcohol exposure on the developing brain, particularly the hippocampus, has mainly focused on structural changes, as described above. While overt structural changes are useful as markers of alcohol-induced damage, it is quite difficult to manipulate brain structure. However, it is possible manipulate neurochemistry, making it important to understand neurochemical changes that occur as a result of *in utero* alcohol exposure, in order to effectively treat learning deficits caused by alcohol exposure.

Much of the research on neurochemical changes in animal models of FASD has focused on the glutamatergic and GABAergic systems, as these systems are most directly impacted by ethanol (reviewed in Olney et al., 2002). However, some recent data suggests that other systems, namely the cholinergic system, may warrant more detailed investigation. It has been demonstrated that acetylcholine receptors are located presynaptically on both glutamatergic (Gray et al., 1996; Fabian-Fine et al., 2001) and

GABAergic neurons in the hippocampus (Drever et al., 2011), suggesting they may modulate glutamatergic and GABAergic neurotransmission, and in turn, synaptic plasticity. Importantly, in developing pharmacological therapies, it is difficult to target the glutamate and GABA systems, due to the diffuse nature of these neurotransmitter systems. Although the acetylcholine system has widespread projections, the side effects from cholinergic drugs are significantly reduced, when compared to glutamatergic and GABAergic drugs. This section will provide an overview of research focusing on alcohol-induced neurochemical changes, with an emphasis on the glutamate, GABA, and acetylcholine neurotransmitter systems. These systems play a major role in synaptic plasticity, and disruptions in the functioning of these systems explains, in part, impaired performance on many learning tasks.

1.5a Alcohol-Induced Changes to the Glutamate and GABA Neurotransmitter Systems in the Hippocampus

In regards to alcohol's impact on glutamate receptors, the research is fairly consistent in showing that alcohol exposure decreases expression of NMDA receptors and glutamate binding in the hippocampus (reviewed in Costa et al., 1999). For example, Farr et al. (1988) examined hippocampal tissue from 45-day old rats exposed to alcohol during prenatal development (3% or 6%) and found a reduction in ^{3}H-glutamate binding in the dorsal hippocampus. In another study, Savage et al. (1991) used a moderate exposure paradigm to subject animals to alcohol prenatally (3%, peak BAC 39 mg/dl), and measured ^{3}H glutamate binding in the hippocampus of 45 day old rats, and determined that alcohol reduced binding density in the dentate gyrus, CA1, and subiculum of the dorsal hippocampus. In a study designed to examine critical periods for

alcohol-induced changes to the glutamate system, Diaz-Granados et al. (1997) exposed rats to alcohol during the prenatal period (35%, intubation), the postnatal period (10.2%, artificial rearing), or both the prenatal and postnatal periods of development and examined ^{3}H-MK-801 binding in the hippocampus. The results showed a decrease in NMDA receptors density in the hippocampus following both prenatal and postnatal exposure to alcohol.

In a more recent study, Samudio-Ruiz et al. (2010) used a two bottle choice paradigm (0.066% saccharin-sweetened water or 0.066% saccharin-sweetened water with 5% ethanol) to expose mice to moderate amount of ethanol (5%, two bottle choice) during prenatal development, and examined hippocampal NMDA receptor subunit expression. They compared different membrane preparations to characterize membrane localization of these receptor subunits, and found a reduction of synaptosome-associated NR2B levels, indicating a reduction in the number of NMDA receptors containing these subunits in the hippocampus. Taken together, these findings clearly demonstrate that alcohol exposure during either prenatal or postnatal development leads to a reduction in glutamate receptor binding, and suggests that these reductions may contribute to alcohol-related impairments in synaptic plasticity.

Alcohol exposure during development has also been shown to alter GABAergic neurotransmission (Sari et al., 2010; Everett et al., 2012), and these changes may be involved in alcohol-induced changes in plasticity, by disrupting the normal excitatory/inhibitory balance in the alcohol-exposed brain. A study by Ikonomidou et al (2000) demonstrated that alcohol exposure produced a pattern of apoptosis that was similar to that caused by NMDA antagonists and GABA agonists, suggesting that alcohol

is mediating cell death through these mechanisms. Alcohol (2.5 g/kg, s.c.) was administered during synaptogenesis (PD 7) and caused physiological cell death in multiple brain regions, including the hippocampus, as evidenced by TUNEL staining. The authors examined the impact of various doses (2.5 g/kg, 3 g/kg, or 5 g/kg) and dosing regimens (1, 2, or 5 injections) of alcohol on apoptotic neurodegeneration and found that a pattern of alcohol exposure that produced high BACs also produced apoptosis. In addition, NMDA antagonists caused apoptosis, but the pattern of apoptosis caused by ethanol exposure was more extensive, suggesting an additional mechanism.

In an attempt to determine the mechanism of alcohol-induced apoptosis, the authors administered other drugs to animals and characterized the pattern of neurodegeneration. They found that both NMDA antagonists and GABA agonists produced neurodegeneration, and that together the pattern was similar to that of ethanol, suggesting that alcohol-induced neurodegeneration is the result of antagonism of NMDA receptors and agonist of GABA receptors. Although NMDA antagonists and GABA agonists are not typically thought of as being able to cause apoptosis, it is important to remember that this is occurring during development. GABA acts as an excitatory factor in immature neurons, undergoing a switch to inhibitory function once neurons become mature (Dieni et al., 2012)

Developmental alcohol exposure also results in impaired GABAergic neurotransmission. Specifically, Sari et al. (2010) utilized a mouse model of prenatal alcohol exposure (25%, GD 7-13) and examined fetal brains for neurotransmitter content and found a reduction in GABA in fetal brains of alcohol-exposed animals. In a recent study by Everett et al. (2012), GABA-mediated spontaneous network activity in the

hippocampus was examined following postnatal exposure to ethanol, and the results showed an alcohol-induced decrease in the frequency and an increase in the amplitude of GABA-mediated postsynaptic currents. These alterations in GABAergic neurotransmission, along with altered glutamatergic neurotransmission, may contribute to FASD-related impairments in synaptic plasticity.

1.5b Alcohol-Induced Changes to the Acetylcholine Neurotransmitter System in the Hippocampus

As mentioned above, alcohol causes structural and functional changes to the hippocampus. These functional changes may be due, in part, to the glutamatergic and GABAergic neurotransmitter systems. However, it is very difficult to modulate these systems without serious side effect because of their role in a variety of other processes. Recent research suggests that the cholinergic system, specifically the α7 nicotinic receptor subtype, may modulate glutamatergic and GABAergic neurotransmission and play an important role in synaptic plasticity (Fabian-Fine et al., 2001). It is critical to characterize the effects of developmental alcohol exposure on the cholinergic system, in order to more clearly understand the mechanisms of impaired synaptic plasticity in animal models of FASD. A better characterization of this system may lead to more effective treatments for alcohol-induced learning deficits. This section will provide a brief overview of the acetylcholine system, followed by a review of the research on alcohol's impact on the acetylcholine system in the hippocampus.

The acetylcholine (ACh) neurotransmitter system plays a role in many important functions, including learning, memory, and attention (Micheau & Marighetto, 2011; Sarter & Parikh, 2005). There are four main areas of cholinergic cell bodies, numbered

Ch1-4 (Mesulam et al., 1983). Of interest here are neurons located in the medial septal nucleus (MS) and vertical limb nucleus of the diagonal band of Broca (Abreu-Villaca et al., 2011), which provide cholinergic innervation to the hippocampus (Drever et al., 2011). Cholinergic input to the DG and CA3 regions of the hippocampus comes from the medial septum and diagonal band, with the dorsal hippocampus receiving a large input from the medial septum and the ventral hippocampus receiving most of its input from the diagonal band, with some input from the septum (Gould & Leach, 2014). Acetylcholine is synthesized in the cytoplasm of cholinergic neurons via the action of the enzyme choline acetyltransferase (ChAT) and packaged into vesicles by vesicular acetylcholine transporter (vAChT) (Holler et al., 1996; Van der Zee & Keijser, 2011). ChAT and vAChT are both found in ACh producing neurons, and can provide an indication of the capacity for cholinergic synthesis and neurotransmission. There are two types of ACh receptors: nicotinic (nAChR) and muscarinic (mAChR), both of which are abundant in the hippocampus. Each of these receptors has multiple subtypes, which have differential distribution throughout the brain. For example, the α7 and α4β2 nAChRs and the M1, M2, and M4 mAChRs are found in high levels in the hippocampus (Drever et al., 2011).

The development of the acetylcholine system has been described in a recent review (Abreu-Villaca et al., 2011) and it has been suggested that acetylcholine plays a direct role in development of the nervous system (Abreu-Villaca et al., 2011). Specifically, acetylcholine is involved in neurite outgrowth, synaptogenesis, neurogenesis, and cell survival (Abreu-Villaca et al., 2011; Drever et al., 2011), all of which are affected by fetal alcohol exposure (Riley et al., 2011). Choline acetyltransferase (ChAT) expression is often used as a marker of choline producing

neurons. In a study of the regional expression of ChAT in the mouse, Schambra et al. (1989) found that the vertical limb nucleus of the diagonal band of Broca and the medial septum, both of which project cholinergic fibers into the hippocampus, did not display significant levels of ChAT immunoreactivity until GD 17-18. In addition, a study by Thal et al. (1992) examined regional and development-specific activity of ChAT and found that ChAT activity is not detected in the hippocampus until GD 18. Since acetylcholine cannot be synthesized and released into the hippocampus until late during the second trimester equivalent, alcohol exposure during the third-trimester equivalent could impact acetylcholine synthesis and release.

Vesicular acetylcholine transporter (vAChT) mRNA expression shows a developmental pattern that is slightly different from ChAT, with levels reaching about 60% of maximum around birth, and levels maxing out around postnatal day 24 (Holler et al., 1996). Expression of vAChT protein in brain is slightly delayed, compared to mRNA expression, with steady increases from about PD 8, reaching maximum around PD 50 (Holler et al., 1996). One of the ways that developmental alcohol exposure could impact the acetylcholine system is through interference with the normal expression of vAChT mRNA, which would be reflected in alcohol-induced reductions in vAChT protein expression, and impaired acetylcholine release.

Some nAChR subtypes (e.g. α7) appear as early as GD 12 and peak on PD 7 in the hippocampus (Abreu-Villaca et al., 2011), indicating that these receptors may be involved in early developmental processes such as hippocampal pyramidal cell proliferation, migration, and synaptogenesis. Muscarinic acetylcholine receptors (mAChRs) appear as early as GD 13 in the rat, but do not reach adult levels until after PD

14 (M1 and M3). The high-affinity choline transporter, which functions to retrieve choline from the synaptic cleft (Sarter & Parikh, 2005), does not appear in significant levels until PD 15 (Abreu-Villaca et al., 2011). To summarize, many aspects of the cholinergic system (e.g. nicotinic receptor subtypes, vesicular transporters) appear early in neural development (GD 12), but other aspects (e.g. muscarinic receptors and choline transporters) appear later in development. This suggests that while acetylcholine itself may be involved in early developmental processes, the acetylcholine neurotransmitter system is not fully developed until later. Since we are interested in alcohol-induced changes in the acetylcholine system, we examined the impact of third-trimester equivalent alcohol exposure on the development of the hippocampus. In addition, changes to the cholinergic system would likely cause functional deficits on learning and memory processes which rely on cholinergic neurotransmission.

The hippocampus receives its major cholinergic input via the septo-hippocampal pathway (Drever et al., 2011), and there is evidence that cholinergic input influences learning and memory. Drugs that antagonize acetylcholine receptors (e.g. scopolamine) impair performance on spatial learning tasks in and rodents (reviewed in Deiana et al., 2011). In the hippocampus (area CA1), Fabian-Fine et al. (2001) demonstrated that alpha7 nAChRs are located both pre-synaptically and post-synaptically at a majority of glutamatergic and GABAergic synapses, suggesting that they may be important in modulating glutamatergic and GABAergic neurotransmission (Drever et al., 2011).

In a study designed to examine the impact of nicotine on GABA and glutamate release, Radcliffe et al. (1999) found that presynaptic nicotinic acetylcholine receptors facilitate the release of GABA and glutamate through the enhancement of calcium influx.

There is evidence the cholinergic input to the hippocampus can modulate hippocampal theta activity, thereby regulating the rhythmic firing in this brain region (Stewart & Fox, 1990). In addition, drugs that facilitate cholinergic neurotransmission (e.g. nicotine, choline) enhance learning (Loy et al., 1991; Wong-Goodrich et al., 2008) while drugs that reduce cholinergic neurotransmission (e.g. scopolamine, methyllycaconitine) impair learning (Pocivavsek et al., 2006). Few studies have examined the effects of developmental alcohol exposure on the cholinergic system, but those that have support the proposed hypothesis that alcohol exposure impairs cholinergic neurotransmission in the hippocampus. Rawat (1977) examined fetal (GD 18 and 21) and neonatal brains (PD 5 and PD 10) for ACh content following prenatal alcohol exposure (6% w/v in liquid diet) and found decreased levels of ACh at both ages. In another study, Nio et al. (1991) exposed pups to 30% ethanol from GD 7-22, and measured hippocampal muscarinic binding in neonatal (PD 4) and juvenile (PD 30) rats. The authors found an increase in muscarinic receptor binding in CA3 (PD 4 and PD 30) and CA1 (PD 4).

In another study, Kelly et al. (1989) exposed pups to alcohol (6.6 g/kg/day, artificial rearing) from postnatal days (PD) 4-10 (equivalent to the third trimester) and found that alcohol exposure resulted in an increased cyclic GMP response to high concentrations of a muscarinic agonist (bethanechol) in adults, as measured by radioimmunoassay. Moreover, the impact of alcohol exposure on hippocampal muscarinic receptors was analyzed by radioimmunoassay using QNB as the muscarinic ligand, which does not distinguish between muscarinic subtypes. Alcohol led to a significant increase in the dissociation constant and the number of muscarinic cholinergic

receptors in the hippocampus, which is indicative of impaired cholinergic neurotransmission.

Monk et al. (2012) utilized radioligands that are specific to receptor subtypes to characterize hippocampal M_1 (^{3}H-pirenzepine) and $M_{2/4}$ (^{3}H-AF-DX 384) muscarinic receptors in a rodent model of fetal alcohol syndrome. Using intragastric intubations, male pups were exposed to 5.25 g/kg of ethanol a day from PD 4-9. The results showed that alcohol exposure reduced the density of muscarinic M_1 receptors, but increased $M_{2/4}$ receptors in the hippocampus following alcohol exposure during the early postnatal period. The ratio of M_1:$M_{2/4}$ receptors was also reduced by postnatal alcohol exposure. M_1 receptors are primarily excitatory, while $M_{2/4}$ receptors are primarily inhibitory (Drever et al., 2011), indicating an overall reduction in activity of muscarinic receptors in the hippocampus following postnatal alcohol exposure.

Together, these results clearly demonstrate an alcohol-related increase in muscarinic receptor binding in the hippocampus, suggesting impaired cholinergic neurotransmission. However, no studies to date have examined the nicotinic subtype of acetylcholine receptors in an animal model of FASD, and the α7 nAChR subtype has been implicated in synaptic plasticity in the hippocampus (Fabian-Fine et al., 2001; Drever et al., 2011). Importantly, the acetylcholine system modulates performance on the CPFE, a task which shows sensitivity to alcohol exposure (Kenney & Gould, 2008).

1.5c Summary of Alcohol-Induced Neurochemical Changes in Animal Models of FASD

A few major conclusions can be made from the research on the neurochemical impact of alcohol exposure during development: 1) Alcohol exposure causes excitotoxic cell death, likely through a glutamate- and GABA-mediated mechanism (Ikonimodou et

al., 2000), 2) Developmental alcohol exposure leads to a reduction in glutamate receptor binding in the hippocampus (Costa et al., 1999) 3) Alcohol exposure reduces GABA-mediated neurotransmission in the hippocampus (Sari et al., 2010), and 4) Alcohol exposure produces an increase in muscarinic receptor binding in the hippocampus (Kelly et al., 1989), which is indicative of impaired cholinergic neurotransmission. These changes in neurotransmission are likely involved in alcohol-mediated disruptions in synaptic plasticity, which might help to explain alcohol-related impairments in learning paradigms that rely on the hippocampus. Critically, the acetylcholine neurotransmitter system may modulate glutamatergic and GABAergic neurotransmission in the hippocampus (Drever et al., 2011), making it a possible target for pharmacological intervention in individuals with FASD. However, a more detailed characterization of alcohol-induced changes to the hippocampal cholinergic system is needed.

1.6 Pharmacological Interventions in Animal Models of FASD

As mentioned above, a small percentage of women (~2%, CDC, 2009) fail to stop drinking heavily during pregnancy, making it important to develop effective interventions to treat alcohol-induced brain damage and learning deficits. There is a wide range of literature attempting to fill this gap in the research. A number of studies using animal models have utilized novel pharmacological interventions in an effort to ameliorate alcohol-induced brain damage and behavioral deficits. For the most part, studies have utilized behavioral paradigms which require an intact hippocampus for performance, due to the observations that this brain region is a critical target of developmental alcohol exposure.

Alcohol-induced reductions in cell number have been hypothesized to result from increased production of free radicals and oxidative stress (Abel & Hannigan, 1995). The hippocampus is especially sensitive to oxidative stress because levels of endogenous antioxidants, such as Vitamin E, are very low (Abel & Hannigan, 1995). This suggests that pharmacological agents which reduce oxidative stress (e.g. antioxidants) can attenuate alcohol-induced cell loss in the hippocampus. In a study by Marino et al. (2004), rats were exposed to alcohol (5.25 g/kg/day) on PD 7-9, and some were also given an antioxidant, vitamin E (2 g/kg). Rats were tested on a spatial navigation task as juveniles (PD 22-29), and their brains were examined for hippocampal cell number and protein carbonyl formation. Alcohol-exposed juveniles were impaired on a spatial navigation task, showing an increase in the latency to find the hidden platform. Moreover, alcohol exposure led to a reduction in CA1 cell number and an increase in protein carbonyl formation. While the impact of alcohol on cell number and oxidative stress was rescued by vitamin E, spatial navigation deficits were not.

Another antioxidant, resveratrol, has been shown to alleviate behavioral impairments following postnatal alcohol exposure (5 g/kg; PD 7-9) (Tiwari & Chopra, 2011). A study by Tiwari & Chopra (2011) examined the impact of resveratrol treatment on alcohol-induced hippocampal damage and behavioral deficits. Resveratrol is a natural antioxidant found in grapes, nuts, and berries. Due to its antioxidant properties, the authors were interested in whether resveratrol would reverse alcohol-induced impairments in spatial navigation and the elevated plus maze. Indeed, resveratrol (10 or 20 mg/kg/day) rescued behavioral impairments caused by postnatal exposure to alcohol (5 g/kg, PD 7-9). However, antioxidant administration must occur concurrently with

alcohol exposure (Marino et al., 2004; Tiwari & Chopra, 2011), making this treatment approach impractical in a clinical setting.

There have been a few other studies utilizing novel treatment paradigms in an attempt to attenuate FASD-related learning deficits. For example, Idrus, Happer, & Thomas (2012) examined the ability of cholecalciferol (PD 2-30), a vitamin D3 supplement, to ameliorate perseverative behavior in rats exposed to alcohol (5.25 g/kg/day) during the third trimester equivalent period of development (PD 4-9). Rats were tested on a serial spatial discrimination reversal learning test, and alcohol exposed animals had an increase in the number of perseverative errors, an effect which was ameliorated with cholecalciferol treatment. In another study, Savage et al. (2010) administered a histamine (H_3) receptor antagonist (ABT-239) to animals prenatally exposed to moderate levels of alcohol (mean BAC: 84 mg/dl) and examined performance on two hippocampus dependent tasks: contextual fear conditioning and spatial navigation of a water maze. ABT-239 was administered 30 minutes prior to behavioral testing. Alcohol exposed animals were impaired on both tasks, and ABT-239 treatment ameliorated those deficits.

At this time, the most effective and most widely researched treatment for alcohol-induced behavioral deficits in animal models is choline. Choline has been shown to facilitate memory performance (Williams et al., 1998), alter hippocampal cellular structure (Li et al., 2004), increases DNA methylation (Meck & Williams, 2003), and enhance hippocampal long term-potentiation (Pyapali et al., 1998) in untreated animals. More importantly, it has been shown to reduce the severity of alcohol-induced working memory deficits in the Morris water maze (Ryan et al.,2008; Thomas et al., 2007) and

trace eye blink conditioning (Thomas & Tran, 2011), when given *after* alcohol exposure. However, choline does not ameliorate alcohol-related impairments in motor coordination on a parallel bar motor task (Thomas et al., 2004a) indicating that exploration of other therapeutic interventions is needed. Importantly for this proposal, choline supplementation facilitates (Koppen et al., 1997), while choline restriction reduces (Nakamura et al., 2001), acetylcholine release in the hippocampus.

Importantly, all of the tasks that are impacted by choline treatment of alcohol-induced deficits mentioned above rely on the functioning of the hippocampus. Choline administration ameliorates alcohol related learning deficits when administered along with alcohol (Thomas et al., 2000; Thomas et al., 2004b) and when administered after alcohol exposure (Thomas et al., 2007; Thomas & Tran, 2012). It is unclear how choline is impacting the developing brain, but a recent study by Monk et al. (2012) found that choline administration ameliorated alcohol-induced increases in M2/4 receptor density in the hippocampus, suggesting it impacts cholinergic neurotransmission. Furthermore, alcohol exposure increases DNA methylation in the prefrontal cortex and hippocampus, and this effect is reduced following choline supplementation (Otero et al., 2012). In addition, choline has been shown to be a selective agonist of α7 nicotinic acetylcholine receptors, which play an important role in synaptic plasticity (Alkondon et al., 1997). Although choline may be an effective treatment, the mechanisms of its impact on the developing brain are unclear, and more research is needed to determine *how* choline facilitates learning. Due to choline's impact on cholinergic neurotransmission, and acetylcholine's role in synaptic plasticity, it may be that the acetylcholine system is an especially good target for FASD-related learning deficits. This highlights the need to look

at other cholinergic drugs, such as galantamine, in an effort to isolate the key components of for treatment of effects caused alcohol exposure during development.

1.7 Galantamine as a Potential Therapy for FASD-Related Learning Deficits

Disruptions in cholinergic neurotransmission are associated with cognitive dysfunction, which is a hallmark symptom of many neurological disorders, including Alzheimer's disease, schizophrenia, and alcohol dependence (Ago et al., 2011). Galantamine is a drug that has been used to attenuate cognitive deficits associated with Alzheimer's disease (Ago et al., 2011) and chronic alcohol consumption (Iliev et al., 1999), and acts by facilitating cholinergic transmission (Ago et al., 2011). This section will review the pharmacological mechanisms of galantamine, and propose galantamine as a potential therapy for alcohol-induced learning deficits.

Galantamine is a weak inhibitor of acetylcholinesterase (AChE), the enzyme responsible for breaking down acetylcholine (ACh) in the synaptic cleft (Ago et al., 2011). It is an allosteric potentiating ligand (APL) at nicotinic acetylcholine receptors (nAChRs), where it is believed to bind to a specific site extracellularly on the N-terminal domain of nAChRs (Schrattenholz et al., 1996), and potentiate the response of nAChRs to suboptimal levels of acetylcholine (Schrattenholz et al., 1996).

Using cell lines expressing subtypes of human mAChR (M1-M5), Samochocki et al. (2003) demonstrated that galantamine does not act on muscarinic receptors, and is instead, a selective potentiating ligand at all nAChR subtypes tested (α4β2, α3β4, and α7/5-HT_3 chimeric). The allosteric potentiating ligand action at human α7 nAChRs could not be isolated in this study because α7 receptors could not be stably expressed in the cell line. However, cells expressing a chimeric α7/5-HT_3 receptor did show a galantamine-

induced potentiation of the response to nicotinic agonists, suggesting that galantamine does indeed act at these receptors. In addition, a study by Dajas-Bailador et al. (2003) determined that galantamine does not compete for ligand binding sites at the α4β2, α3, or α7 nAChRs, as indicated by a lack of displacement of radiolabeled ^{3}H-nicotine, ^{3}H-epibatidine, or ^{3}H-methyllycaconitine (MLA), respectively. It was demonstrated that administration of galantamine (0.5, 1, and 3 µM, *in vitro*) increases the intracellular release of Ca^{2+} in response to nAChR activation, and this effect was blocked by mecamylamine (nonspecific nAChR antagonist).

Galantamine has many other unique properties which make it an interesting candidate for treatment of alcohol-induced learning impairments, such as its neuroprotective effects (Kihara et al., 2004; Takada-Takatori et al., 2006), its impact on anti-apoptotic signaling cascades (Takada-Takatori et al., 2006), its effects on neurotrophic factors (Kita et al., 2013), its ability to increase neurogenesis (Jin et al., 2006), and its impact on neurotransmitter release (Santos et al., 2002). These properties of galantamine are discussed in detail below.

Galantamine is able to protect neurons against glutamate-related neurotoxicity (Akaike et al., 2010; Kihara et al., 2004; Takada-Takatori et al., 2006; Takada-Takatori et al., 2009).Using primary cultures of cortical neurons, Takada-Takatori et al. (2006) demonstrated that galantamine (2 µM) prevented glutamate neurotoxicity, as evidenced by a reduction in nuclear fragmentation, indicating a reduction in apoptosis. Specifically, glutamate (1µM) reduced cell viability from ~80% to ~50%, and treatment with galantamine caused a recovery of cell viability. The neuroprotective effect of galantamine was blocked by mecamylamine (10 µM), a non-specific nAChR antagonist, DhβE (10

nM), a specific α4 nAChR antagonist, and by methyllycaconitine, a selective α7 nAChR antagonist.

Activation of α7 nAChRs activates the phosphatidylinositol 3-kinase (PI3K)-Akt system, which is neuroprotective, and might help to explain galantamine's role in neuroprotection (Kihara et al., 2001). Activation of the Bcl-2 family of anti-apoptotic proteins by PI3K-Akt system protects against apoptosis. Indeed, in the study by Takada-Takatori et al. (2006), galantamine's neuroprotective effect was blocked by PP2, an inhibitor for the Src family tyrosine kinase Fyn, AG490, an inhibitor for tyrosine kinase JAK2, and by LY294002, a PI3K inhibitor. These findings indicate that galantamine may be acting downstream to inhibit apoptosis pathways and prevent neurotoxicity.

Kita et al. (2013) explored the effects of galantamine on cortical and hippocampal neurotrophic factor levels. Galantamine impacted neurotrophic levels in the hippocampus, and did so in a dose- and time-dependent manner. Specifically, galantamine (0.3, 1, and 3 mg/kg) was administered acutely and tissue was examined 0, 3, 6, and 12 hours after injection. Galantamine (0.3, 1, and 3 mg/kg) increased mRNA levels of insulin-like growth factor 2 (IGF2) at 3, 6, and 12 hours, and increased fibroblast growth factor 2 (FGF; 3 mg/kg only) at 3 hours in the hippocampus. Galantamine (3 mg/kg) also decreased BDNF mRNA at 3 hours after injection in the hippocampus. There were no effects of galantamine on neurotrophic factors in the prefrontal cortex. The effect of galantamine on IGF2 mRNA levels was blocked by the administration of methyllycaconitine, a selective α7 nAChR antagonist, but not by telenzepine, a M1 mAChR antagonist, suggesting that this effect of galantamine was due to its impact on α7 nAChRs (Kita et al., 2013).

An important study by Jin et al. (2006) examined the ability of galantamine to facilitate neurogenesis in the subgranular zone of the dentate gyrus. Rats were administered galantamine (5 mg/kg, i.p.) for 14 days and also received BrdU (50 mg/kg, i.p.) for the last 3 days. Brains were examined for BrdU, βIII tubulin (immature cells of neuronal lineage), and doublecortin (DCX; immature neurons) using immunohistochemistry. Galantamine administration increased BrdU immunoreactivity in the subgranular zone by ~36%, and some of these cells also expressed βIII-tubulin. This suggests that some of the newly produced cells became neurons, and were incorporated into the existing neuronal networks.

Using patch clamping, Santos et al. (2002) demonstrated that galantamine impacts neurotransmitter release. Specifically, administration of galantamine (1μM) to rat hippocampal slices increased the amplitude of evoked EPSCs, and these currents were driven by AMPA/kainate receptors, since they could be blocked by APV and CNQX, antagonists of the AMPA and kainate receptors, respectively. Galantamine was also shown to increase the frequency of ACh-induced spontaneous IPSCs in interneurons. Taken together, these findings suggest that galantamine modulates acetylcholine-induced neurotransmitter release, measured by the characteristics of EPSCs and IPSCs in cultured hippocampal neurons.

Not only does galantamine impact neurotransmission (Santos et al., 2002), protect against excitotoxicity caused by glutamate (Kihara et al., 2004; Takada-Takatori et al., 2006), and increase neurogenesis (Jin et al., 2006), but it has been shown to ameliorate learning impairments. For example, Luo et al. (2011) exposed rat pups to lead (0.2%) through the dam's milk, and evaluated the effect of galantamine on lead-induced

impairments in synaptic plasticity. Galantamine was administered via intraperitoneal injection 0.1 mg/kg/day for two weeks, and then electrophysiological recordings were taken in the dentate gyrus of anesthetized animals at 60-90 days of age. Galantamine treatment ameliorated lead-induced impairments in EPSP slope and population spike amplitude. Furthermore, lead exposure reduces the amplitude of long-term potentiation and depotentiation in the dentate gyrus, and this was ameliorated with galantamine. In another study, Woodruff-Pak et al. (2003) exposed rats to mecamylamine, a nonspecific nicotinic receptor antagonist, and observed mecamylamine-induced impairments on a delay eye blink conditioning paradigm (decreased percentage of conditioned responses). This impairment was reversed when the animals were administered galantamine (3.0 mg/kg).

In summary, galantamine is a unique drug which has been approved for treatment of Alzheimer's disease (reviewed in Ago et al., 2011). However, it has other important properties which suggest it may be helpful to treat cognitive impairments following prenatal exposure to alcohol in humans. To test this hypothesis, an animal model of FASD was used in which alcohol is administered to the developing animal during the third-trimester equivalent, and performance on a hippocampal learning paradigm, the CPFE, was examined.

1.8 Conclusions and Rationale

It has been shown that a small percentage of women continue to consume high levels of alcohol while pregnant (~2%, CDC, 2009), making it crucial to develop effective interventions for children prenatally exposed to alcohol. Developmental exposure to alcohol results in learning impairments, especially in behavioral paradigms

that rely heavily on the hippocampus. For example, rodents exposed to high doses of alcohol during the third trimester equivalent are impaired on contextual fear conditioning (CPFE), but not fear conditioning to an auditory stimulus (Murawski & Stanton, 2010). Importantly, the CPFE requires the hippocampus, while auditory fear conditioning requires the amygdala (LeDoux, 2000). This suggests that the hippocampus is especially impacted by developmental alcohol exposure, and that this brain region is a good target to assess therapeutic intervention. What is more, developmental alcohol exposure produces significant impairments in neurotransmitter systems, specifically glutamate (Diaz-Granados et al., 1997; Farr et al., 1988; Samudio-Ruiz et al., 2010; Savage et al., 1991), GABA (Everett et al., 2012; Sari et al., 2010), and acetylcholine (Kelly et al., 1989; Monk et al., 2012; Nio et al., 1991; Rawat, 1977).

The hippocampus receives major acetylcholine input from the septum and acetylcholine modulates synaptic transmission at glutamate and GABA synapses through muscarinic and nicotinic acetylcholine receptors (reviewed in Cobb & Davies, 2005). For example, presynaptic nAChRs enhance GABA and glutamate release by increasing calcium influx (Radcliffe et al., 1999). Drugs that modulate cholinergic neurotransmission, such as galantamine, can impact synaptic plasticity, and in turn impact behavioral performance on tasks which recruit the hippocampus.

One of the considerations for this study was whether to target the dorsal or ventral hippocampus, each proposed to have different functions. The dorsal hippocampus is associated with many spatial and cognitive tasks, whereas the ventral hippocampus is thought to play a role in cognitive tasks with a strong emotional component (Fanselow & Dong, 2010). Fear conditioning, and the context pre-exposure facilitation effect paradigm

specifically, recruit the ventral hippocampus, due to the large emotional component (Fanselow & Dong, 2010). Evidence for this comes from studies in which the activity of the ventral hippocampus is blocked via NMDA antagonists. Deficits are seen in acquisition of contextual, but not cued, fear conditioning (Zhang et al., 2001). Due to these findings, neuroanatomical and *in vivo* microdialysis studies were targeted to the ventral hippocampus, specifically in area CA1, which has been shown previously to be heavily impacted by developmental alcohol exposure (Greene et al., 1992; Tran & Kelly, 2003).

1.9 Overview of Proposed Experiments and Hypotheses

In order to test the overall hypothesis that cholinergic dysfunction is a feature of FASD and that cholinergic dysfunction underlies learning impairments that are a hallmark feature of FASD, there were three experiments.

Experiment 1 tested the hypothesis that postnatal alcohol exposure causes impaired cholinergic neurotransmission. This study utilized an animal model in which alcohol was administered during the postnatal period, or third-trimester equivalent, and a technique (microdialysis) was used to measure acetylcholine efflux, or release, in an awake, behaving animal. There were two parts to this experiment: High K^+/Ca^{2+} and Galantamine. In the first part, a high K^+/Ca^{2+} artificial cerebrospinal fluid (aCSF) was administered during microdialysis to measure the capacity for acetylcholine efflux. It was hypothesized that there would be no difference between groups in basal acetylcholine release, but when the acetylcholine system is stimulated using a high potassium/high calcium solution, ethanol exposed animals would show a decrease in acetylcholine release. This hypothesis arises from the observation that alcohol exposed individuals, and

animals, perform well on simple cognitive tasks, but are impaired when task difficulty is increased. In the second part, galantamine injections occurred during microdialysis to look at the acute impact of this drug on acetylcholine efflux. It was hypothesized that there would be no baseline differences in acetylcholine efflux, but when stimulated with galantamine, alcohol-exposed animals would have a smaller increase in acetylcholine efflux, compared to controls.

Experiment 2 utilized immunohistochemical techniques to measure expression of three cholinergic proteins: ChAT, vAChT, and α7 nAChR and examine the impact of galantamine on these measures. ChAT is a marker of acetylcholine synthesis, and served as a control to determine whether alcohol exposure causes an overall reduction of cholinergic neurons. It was hypothesized that alcohol exposure would reduce vAChT immunoreactivity, and increase α7 immunoreactivity, in the hippocampus, which would indicate an impairment in acetylcholine release. Importantly, an increase in α7 nAChR expression is associated with receptor desensitization, a process that is related to a reduction in the response to acetylcholine (Williams et al., 2011). Galantamine treatment was expected to increase the presence of acetylcholine in the synapse, which would not impact vAChT (a presynaptic marker), but would decrease α7 nAChR immunoreactivity.

Experiment 3 tested the hypothesis that galantamine would ameliorate FASD-induced deficits in the context pre-exposure facilitation effect (CPFE) paradigm. This study utilized the same alcohol exposure paradigm as Experiment 1 and galantamine was administered chronically *after* alcohol exposure. Following drug administration, animals were tested in a learning paradigm that shows the context pre-exposure facilitation effect

(CPFE). It was hypothesized that alcohol exposed animals would be impaired in the CPFE task, and that galantamine would ameliorate these deficits.

CHAPTER 2

METHODS

2.1 General Methods

2.1a Subjects

For these experiments, Long-Evans rats were used. All subjects were housed in the animal colony of the University of South Carolina School of Medicine. Temperature was maintained at 22^{o} C with a 12h:12hlight:dark cycle (lights on at 0700). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina. Untimed (visibly) pregnant dams were ordered and singly housed throughout pregnancy in polypropylene cages with bedding.

2.1b Pup Treatment

The day of birth (~GD 23) was designated as postnatal day (PD) 1 and no treatments were given on this day. Rat pups within a litter were assigned quasi-randomly to one of the three treatment groups such that no more than one pup per litter was assigned to a particular group in a particular experiment. Beginning on PD 2, ethanol treated (ET) pups were intubated using Intramedic PE 10 tubing dipped in corn oil with 3.0 g/kg of ethanol in 27.8 ml/kg of enriched milk (West, Hamre, & Pierce, 1984) between 9:00 am and 11:00 am. Two hours later, a second intubation of milk alone was given to control for differences in feeding behavior due to ethanol intoxication. IC pups were intubated twice daily with Intramedic PE 10 tubing, but no solutions were administered. Intubations continued through PD 10. From PD 2-7, pups were identified

using non-toxic permanent marker. On PD 7, all pups were tattooed for identification purposes (Animal Identification & Machine Systems, Inc.). Pups were housed with their dams until PD 21, at which time they were weaned and housed in same-sex groups (2 or more animals) until commencement of the experimental procedures. This study used only males, and the females from each litter were assigned to other experiments.

2.1c Blood Alcohol Concentrations (BACs)

On PD 10, 10-µl blood samples were collected from all pups (except the NC group) from a small nick in the tail 2 hours after intubation. This time has been shown to be optimal for assessing maximum BACs (Marino et al., 2002). All blood samples were placed into 190 µl of 0.53N perchloric acid, neutralized with 200 µl 0.30 M potassium carbonate, vortexed, and centrifuged (8700 g). Supernatant was separated and frozen at -80^{o} C until time of assay. At the time of sampling, standard ethanol samples with specific BACs (0, 50, 100, 200, 300, 400, 500, and 600 ng/ml) were made, in order to have samples with which to compare experimental animals. BACs were analyzed using an enzymatic procedure with a 96-well plate (Dudek & Abbott, 1984). Briefly, 400 µl of 1.87 mM Tris-NAD stock and 50 µl of alcohol dehydrogenase were added to each well, including standards. Samples were thawed and 50 µl of supernatant was added into each sample's respective well. The plate was then briefly placed onto an orbital shaker to make sure the samples were mixed then it was incubated for one hour before being read on a plate reader at 320 nm, which provided absorbance measurements for each well. Using the absorbance values from the standards, a standard curve was made. Blood alcohol concentrations for experimental samples were calculated using the standard curve obtained from the standard samples.

2.2 Experiment 1: Microdialysis and characterization hippocampal acetylcholine efflux

The purpose of Experiment 1 was to describe basal and potassium-stimulated acetylcholine release in an animal model of FASD. There were three treatment groups (ET, IC, and NC), with 3-8 animals per group. It was hypothesized that there were no difference between groups in basal acetylcholine release, but when the acetylcholine system is stimulated, ET animals will show a decrease in acetylcholine release. To accomplish this goal, the following procedures were used:

2.2a Surgical Procedures

Animals were handled prior to surgery (PD 21-27). On the day of surgery (~PD 27), all rats were anesthetized with isoflurane. All animals underwent surgical procedures to implant a guide cannula unilaterally in the ventral hippocampus, using the following coordinates relative to Bregma: AP: -4.8mm, ML: +5.0 mm, DV: -4.0 mm. The ventral hippocampus has been shown to play a role in contextual fear conditioning (Fanselow & Dong, 2010), so it was chosen to coincide with Experiments 2 and 3. Animals were allowed to recover for 2 days (~PD 28-29). During this time, animals were habituated to the microdialysis bowls for a total of 9 hours (~PD 28-30). When possible, habituation was evenly divided between days. However, in some cases, habituation was conducted for a set of animals after another set had completed microdialysis. In this case, animals were removed from microdialysis bowls and placed back into the animal colony before the lights went out. A window of 2 days was given for each time point (e.g. surgery, habituation) due to the number of animals being processed through the experimental procedures.

2.2b Microdialysis

There were two microdialysis sessions, with a day in between for recovery. The ages for microdialysis were chosen to coincide with the age of the animals used for behavioral testing (Experiment 3). On the day of microdialysis (~PD 30-31 or PD 32-33), microdialysis probes with a semipermeable membrane were inserted into the guide cannulae. The microdialysis probes extend 2.0 mm beyond the tip of the guide cannulae. Probes were continuously perfused with artificial cerebrospinal fluid containing an acetylcholinesterase inhibitor (neostigmine bromide; 0.5 nm; Moore et al., 1996) or a high potassium (K^+)/high calcium (Ca^{2+}) artificial cerebrospinal fluid solution (containing neostigmine bromide, 50 nM) at a rate of 2.0 µl/min. Three hours after the insertion of the probe, dialysate collection began, and occurred every 15 min. On the first day of microdialysis, 4 baseline samples were collected, followed by two high K^+/Ca^{2+} samples, and 4 post-stimulation samples, for a total of 10 dialysate collections. On the second day of microdialysis (2 days after session 1), 4 baseline samples were collected. Then, a subcutaneous injection of galantamine (2.0 mg/kg) was given, followed by 8 post-injection dialysate collections, for a total of 12 collections. The purpose of the injection was to confirm that galantamine will increase acetylcholine release, and determine whether galantamine will differentially impact the alcohol exposed animals. Microdialysis samples were stored at -80^o C until analysis by liquid chromatography with electrochemical detection (HPLC).

Within two days of microdialysis, animals were transcardially perfused with 4% paraformaldehyde. The brains were removed and postfixed 4% paraformaldehyde overnight, followed by 15% sucrose overnight, and finally 30% sucrose overnight. Brains

were sectioned a freezing microtome (Thermo Scientific; 40 µm) using serial sectioning procedures. Sections were stored in cryoprotecting solution at -20^{o}C until time of staining (Experiment 2). Probe placement was verified using immunohistochemistry for ChAT (Experiment 2). Briefly, probes were classified as being correctly placed if they were located in the ventral CA1 region of the hippocampus (Plates 38-42; Paxinos & Watson, 1986). Only animals whose probes were located in the correct brain region were used for analyses.

2.2c High Performance Liquid Chromatography with Electrochemical Detection

High performance liquid chromatography with electrochemical detection (HPLC-EC) is a technique that can be used to quantify neurotransmitter concentrations in dialysate collections. HPLC was used to separate the dialysate sample in order to identify the substituent chemicals in the biological fluid. Then, electrochemical detection was used to measure acetylcholine. To do this, a dialysate sample (20 µl) was injected into the HPLC solvent delivery system (Bioanalytical Systems PM-92) which was coupled to a Bioanalytical Systems Epsilon electrochemical detector. Acetylcholine was separated from choline using an analytical column (Eicompak AC-GEL 2.0 x 150 mm; Eicom, USA) with a mobile phase (pH 8.5). The mobile phase contains sodium 1-decanesulfonate (1.64 mM) and potassium bicarbonate (50 mM). After separation, the acetylcholine was reacted with choline oxidase using an acetylcholine enzyme reactor (Eicom, AC-Enzympak II) to produce hydrogen peroxide in a post-column derivatization step. The production of hydrogen peroxide was completed using a peroxidase coated glassy carbon electrode. Acetylcholine concentrations were determined using chromatographic peaks compared with a standard curve (Stanley & Fadel, 2012a).

2.3 Experiment 2: Examination of hippocampal cholinergic immunoreactivity

Immunohistochemistry was used to identify cholinergic proteins, and examine group differences in hippocampal expression of these proteins (ChAT, vAChT and α7 nAChR). Alcohol-induced differences in immunoreactivity were examined using tissue from Experiment 1 (microdialysis) and there were three treatment groups (ET, IC, and NC). Then, the impact of galantamine on these cholinergic proteins was examined using tissue from Experiment 3 (galantamine and CPFE testing). The reason that both sets of animals were used was to control for the possible impact of behavioral testing on ChAT, vAChT and α7 nAChR. This study used 3 (treatment) x 2 (drug) x 2 (testing condition) design, creating 12 experimental groups. Within two days of the conclusion of experimental procedures, animals were perfused and brain tissue was sectioned and stored at -20^{o}C until time of staining (see section *2.2b* for details)

Sectioned tissue (40 μm) containing the hippocampus (vAChT and α7 nAChR) or medial septum (ChAT) was processed through immunohistochemical procedures to assess expression of cholinergic proteins: ChAT, vAChT, and α7 nAChR. The antibodies that were used are: rabbit anti-ChAT (AB143, Millipore Corp., Temecula, CA), goat anti-vAChT (AB 1588, Millipore Corp., Temecula, CA), rabbit anti-nicotinic acetylcholine receptor alpha7 (ab23832, Abcam), biotinylated horse anti-goat IgG (Vector Laboratories, Inc., Burlingame, CA), biotinylated horse anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA), and peroxidase conjugated streptavidin (Jackson Immunoresearch Laboratories, Inc., West Grove, PA).

Briefly, sections were washed in Tris-buffered saline (TBS; Sigma), followed by rinsing in methanolic peroxide. Sections were then blocked and membranes were

permeabilized by rinsing in TBS with Triton-X (Sigma) and horse serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Tissue was incubated overnight with ChAT (1:1250), vAChT (1:5000), or nicotinic acetylcholine receptor alpha 7 (1:1000) primary antibodies. Tissue was incubated for another day at 4^{o}C. Then, sections were rinsed in TBS, followed by incubation with biotinylated horse anti-goat IgG (vAChT) or biotinylated horse anti-rabbit IgG (ChAT and α7 nAChR) for 1.5h. After rinsing in TBS, tissue was incubated with peroxidase-conjugated streptavidin for 1h. Immunoreactivity was visualized using a nickel-cobalt enhanced diamino-benzidinetetrahydrochloride (DAB) reaction (Sigma).

Sections were mounted on gelatin coated slides, and processed through a series of ethanol washes followed by Histoclear (Fisher Scientific) and xylene rinses before coverslipping with Cytoseal (Thermo Scientific). The hippocampus (area CA1) was defined anatomically by plates 38-42 and the medial septum was defined by plates 16-18 of the atlas of Paxinos and Watson (1986). Two sections per animals were measured and averaged for each brain region. Slides were labeled with numbers, and the experimenter was blind to treatment groups during quantification of immunohistochemistry.

2.3a Microscopy Analysis

For choline acetyltransferase (ChAT), immunoreactivity was measured at 20x magnification using a Nikon E600 microscope with Neurolucida software (MBF Bioscience). Vesicular acetylcholine transporter is found at terminals, and produces a dense staining of fibers, making it difficult to quantify by counting. Similarly, alpha7 nAChR produces terminal labeling in the hippocampus. For this reason, for the vAChT and alpha7 immunohistochemistry, pictures were taken at 20x using a Nikon E600

microscope with IPLab software (Scanalytics, Inc., Fairfax, VA). Images were processed for densitometry analysis using ImageJ (Schneider et al., 2012). ImageJ produces density measurements by counting the number of pixels that encompass a range of values from white (highest) to black (lowest), thus producing a density measurement in which high numbers indicate less dense staining and low numbers indicate more dense staining. To make this easier to analyze, all values were expressed as a percent of total staining using a maximum density value of 5000. Percentage values were calculated using the following formula:

$$\text{percent of staining: } \frac{5000\text{-density}}{5000} \text{ x } 100$$

2.4 Experiment 3: Galantamine and its effects on contextual fear conditioning

Subjects were exposed to alcohol during the third trimester, according to the methods described above (in section *2.2 Pup Treatment*). Then, galantamine was administered after alcohol exposure ceased and until behavioral testing began. The hypothesis as that animals exposed to ethanol during development would be impaired on the CPFE task, and galantamine treatment would restore performance to control levels. This experiment utilized a 3 (treatment) x 2 (drug) x 2 (testing condition) design, creating 12 experimental groups.

2.4a Drug Administration

Beginning on PD 11, galantamine (2.0 mg/kg/day, s.c.) or vehicle (0.9% saline, s.c.) was administered daily, and this administration will last until behavioral testing began on PD 30. Drug administration occurred at the same time every morning (~11 a.m. ±30m).

2.4b Behavioral Testing—Apparatus

Behavioral testing was conducted in Plexiglas boxes (46 x 24 x 22 cm), with stainless steel rods (1.9 cm apart) on the floor (Burghardt, Pasumarthi, Wilson, & Fadel, 2006). The testing boxes were located inside sound attenuating chambers equipped with fans and cameras to record behavior. The stainless steel rods were attached to a shock apparatus (Coulborn Instruments; Allentown, PA), in order to deliver a foot shock (1.5 mA, 2 sec.). Behavioral testing was recorded by the camera, and saved on a desktop computer. The computer was equipped with FreezeScan (CleverSys, Inc., Reston, VA), a program which can track and register freezing behavior in the apparatus.

This behavioral testing paradigm required two distinct contexts. Context A consists of the testing box described above, with no modifications. However, Context B was a modification of the Plexiglas box used for Context A. It contained a mesh floor to provide distinct somatosensory cues. Also, the outside of the Plexiglas box was covered with paper containing distinct patterns (black and white stripes, black and white checkerboard), see *Figure 2.1*.

2.4c Behavioral Testing—Protocol

Context pre-exposure facilitation effect (CPFE) testing occurred on PD 30-32, with pre-exposure on PD 30, training on PD 31, and testing on PD 32 (Murawski & Stanton, 2010). There were two conditions for behavioral testing: PRE and NO PRE. Animals in the PRE condition will undergo pre-exposure in context A, while animals in the NO PRE condition will experience the pre-exposure phase in context B. All animals were trained in context A. During training, all animals were given a brief (1.5mA, 2 sec) foot shock, after which they were immediately removed from the context. During testing,

animals were placed in context A and freezing was recorded for 5 minutes. In addition, freezing were measured during the pre-exposure phase to make sure that there were no baseline differences in freezing that could explain differences during testing.

Figure 2.1 Picture of Context B that was used in context pre-exposure facilitation effect testing.

2.5 Statistical Analyses

All statistical analyses were completed using the SPSS Statistical Package (Version 22; International Business Machines Corp., Armonk, NY). For microdialysis data, acetylcholine content of the baseline samples was averaged, and this value served as the baseline value for each subject. ACh efflux values were expressed as a percent change from baseline. A repeated-measures ANOVA was used, with sample as the repeated measure, and treatment as the between-subjects measure. For CPFE data, freezing values were expressed as a percent change from baseline, since pilot studies indicated baseline differences in freezing during pretraining. To analyze group differences in freezing, a 3 (treatment) x 2 (drug) x 2 (testing condition) ANOVA was used. Immunohistochemistry data was analyzed using a one-way ANOVA with treatment

as the variable (Experiment 1) or a 3 (treatment) x 2 (drug) x 2 (testing condition) ANOVA (Experiment 3). Tukey's HSD post hoc tests were used when necessary.

CHAPTER 3

RESULTS

3.1 Experiment 1: Microdialysis and Hippocampal Acetylcholine Efflux

3.1a Body Weights and Blood Alcohol Concentrations

For all microdialysis data, only the animals with correct probe placement were included, see *Figure 3.1*. In addition, body weight data was missing for one litter on PD 7 and one litter on PD 10 because of inclement weather. A mixed-design ANOVA with day as the repeated measure and treatment as the between-subjects measure was used to analyze body weight data during pup treatment (PD 2-10). When conducting repeated-measures analyses, Mauchly's Test of Sphericity is used to determine whether the data meet the assumption of sphericity, meaning that the all groups have equal variances. This assumption was violated ($p < 0.05$), so Greenhouse-Geisser adjusted degrees of freedom were used for determining significance. There was a significant main effect of age, F (8, 112) = 76.55, $p < 0.001$, but no main effect of treatment or interaction between age and treatment (p's = 0.52 and 0.87), indicating that all animals gained weight during the first 10 days, but that alcohol treatment did not significantly impact weight gain, see *Figure 3.2.*

All animals were weighed before surgical procedures (PD 27-28) and again before the second session of microdialysis (PD 33-34). One-way ANOVAs with treatment as a factor were used to analyze body weights. There was no effect of treatment on the day of surgery or the second microdialysis session, see *Figure 3.3*. The average

blood alcohol concentration (±SEM) for ethanol-exposed animals was 304.90 ± 132.14 (n=2), see *Table 3.1*.

3.1b High-Performance Liquid Chromatography: The Effects of a High K^+/Ca^{2+} Manipulation

To analyze the impact of a high K^+/Ca^{2+} administration (4 Baseline, 2 High, and 4 Post-Stimulation), a repeated-measures ANOVA was used with time point as the within-subjects variable and treatment as the between-subjects variable. Sphericity was violated, so Greenhouse-Geisser adjusted degrees of freedom were used for determining significance. One IC animal was removed from analysis because the acetylcholine was undetectable in the samples. One NC animal was removed from analysis because the pump was set at 5 µl/min for all baseline collections and was changed to 2 µl/min for all other collections, making it difficult to interpret the data.

There was a significant main effect of time point, F (9, 117) = 8.56, $p < 0.001$. Pairwise comparisons (LSD) showed that the four baseline collections were not significantly different from one another, but that the first high K^+/Ca^{2+} collection (H1) had significantly more acetylcholine compared to baselines 2 and 4, but not baselines 1 and 3. However, the second time point with the high K^+/Ca^{2+} manipulation had significantly more acetylcholine compared to all baseline samples. The first high K^+/Ca^{2+} collection was significantly higher P1 and P2. The second high K^+/Ca^{2+} collection had significantly more acetylcholine than the first K^+/Ca^{2+} collection. The second high K^+/Ca^{2+} collection had significantly more acetylcholine than all post-stimulation time points. The post-stimulation time points were not significantly different from one another, see *Figure 3.4*. The interaction between time point and treatment was not

significant, but examination of the data led us to perform post-hoc tests on the second High K^+/Ca^{2+} collection, as well as the 2 post-stimulation collections. Since the variability in the IC group was high, the ET group was compared to the NC group only. There was a significant difference between the ET and NC groups at the first High K^+/Ca^{2+} collection (F (1, 8) = 7.62, p = 0.025) as well as the first post-stimulation collection (F (1, 7) = 8.24, p = 0.024). In both cases, the High K^+/Ca^{2+}-induced increase in acetylcholine efflux was smaller in the ethanol-exposed animals, see *Figure 3.5*.

3.1c High Performance Liquid Chromatography: The Effects of Acute Galantamine

For the second microdialysis session, there were four baseline collections, followed by an acute galantamine injection (2.0 mg/kg; s.c.) and eight post-injection collections (labeled B1-4 and P1-8). A repeated measures ANOVA with treatment as a between-subjects variable and time point as the within-subjects variable was conducted. Since the data violated the assumption of sphericity ($p < 0.05$), Greenhouse-Geisser adjusted degrees of freedom were used to determine significance. One NC animal was removed from analysis because of issues with the HPLC analysis.

There was a significant main effect of time point, F (11, 99) = 14.24, $p < 0.001$ (*Figure 3.6*), as well as a significant interaction between time point and treatment, F (22, 99) = 3.06, p = 0.009. To analyze the significant time point x treatment interaction, a one-way ANOVA with treatment as the between-subjects variable was used for each time point separately with Tukey post-hoc analyses as needed. For time points 1-4 (baseline), there were no significant effects of treatment. At the first time point after the galantamine injection, there was a significant effect of treatment (F (2, 12) = 9.29, p = 0.004), see *Figure 3.7*. Tukey post-hoc analysis revealed that the ET group had a significantly larger

galantamine-induced increase in acetylcholine, compared to both the IC and NC groups (*p's* = 0.004 and 0.006, respectively), but there was no significant difference between control groups. A similar pattern was observed for second and third time points after galantamine injection, such that there was a significant effect of treatment (*p's* < 0.05), an effect which was driven by a larger increase in extracellular concentrations of acetylcholine in the ET group, compared to both control groups (*p's* < 0.05), but controls were not different from each other. For the fourth, fifth, and sixth time points after galantamine injection, there was a main effect of treatment ($p < 0.05$), but Tukey post-hoc tests revealed that the ET group had a significantly larger increase in acetylcholine when compared to the NC group only. Again, the control groups were not significantly different. There were no significant differences between the treatment groups at the remaining time points.

3.2 Experiment 2: Examination of hippocampal cholinergic immunoreactivity

3.2a Choline Acetyltransferase (ChAT) in the Medial Septum

For each animal, the number of choline acetyltransferase (ChAT) positive cells was counted using Neurolucida for two sections (if possible), and the average number of cells was used for analysis. Briefly, for each section, a trace of the medial septum was made using the atlas of Paxinos and Watson (1986). Then, the number of cells was counted within the trace, and all data normalized to the size of that trace. The data from two sections was averaged to create a mean value for each animal. *Figure 3.8* shows representative photomicrographs for the animals that demonstrated the CPFE (Pre).

A 3 x 2 x 2 (treatment x drug x condition) way ANOVA was used to analyze ChAT data from animals which were tested for CPFE in Experiment 3. There was a

significant interaction of drug and condition (F (1, 87) = 5.23, p = 0.025), and a trend towards a significant interaction between treatment, drug, and condition (p = 0.053). To analyze the 2-way interaction between drug and condition, a one-way ANOVA was conducted with drug as the variable in each testing condition. In the no pre-exposure group, there was no difference between the saline-exposed (43.27 ± 3.89) and galantamine-exposed (45.28 ± 3.82) groups. In the pre-exposed group, saline treated animals had significantly more ChAT+ cells than the galantamine treated group, F (1, 44) = 5.96, p = 0.02. The average number (±SEM) of ChAT+ cells for the saline and galantamine treated groups was 51.29 ± 4.08 and 36.89 ± 4.26, respectively.

To analyze the trending 3-way interaction between treatment, drug, and condition, separate two-way ANOVAs were conducted with drug and treatment as variables in each testing condition. In the no pre-exposure group, there were no significant main effects or interactions, *Figure 3.9.* In the pre-exposed group, there was a significant main effect of drug (F (1, 40) = 7.01, p = 0.012) and treatment (F (2, 40) = 7.14, p = 0.002), but no interaction between drug and treatment (p = 0.115), *Figure 3.10*. Tukey post-hoc tests revealed that alcohol exposed animals had significantly more ChAT+ cells than the NC group (p = 0.001), but not the IC group (p = 0.055).

A one-way ANOVA with treatment as the factor was used to analyze ChAT positive cells in the medial septum in the animals that were used for microdialysis. There was no significant effect of treatment, see *Figure 3.11*. These data confirm the findings in the animals in the no pre-exposure group.

In summary, there is no effect of ethanol exposure or galantamine on the number of ChAT cells in animals that do not have the CPFE (no pre-exposure) or who were

tested in microdialysis. Interestingly, among animals that demonstrate a CPFE (see Experiment 3), ethanol exposed animals have an increased number of ChAT positive cells and galantamine decreases that number across all three treatment groups.

3.2b Alpha7 Nicotinic Acetylcholine Receptor

For each animal, the density of staining was measured for two sections (if possible) (*see Figure 3.12*). Briefly, photomicrographs of each section were taken using a Nikon E600 microscope (20x) within area CA1 of the hippocampus. Photos were then imported into ImageJ, where images were used to analyze density of staining. The two sections were averaged to create a mean staining density value for each animal. Data from immunohistochemical analysis of the animals used in Experiment 3 was analyzed using a 3 (treatment) x 2 (drug) x 2 (condition) ANOVA. There was no main effect of treatment, drug or condition nor were there any interactions, see *Table 3.5*. Similarly, the data from animals used for microdialysis confirmed these finding with no effect of treatment, see *Table 3.4.*

3.2c Vesicular Acetylcholine Transporter

To analyze vesicular acetylcholine transporter in the hippocampus, photomicrographs were obtained at 20x using a Nikon E600 camera. Images were imported into ImageJ for densitometry measurement. For each animal, density for two sections (where possible) was averaged to create a mean staining density, see *Figure 3.13*). A 3 x 2 x 2 (treatment x drug x condition) way ANOVA was used on the data from the animals tested for CPFE. There were no significant main effects, nor were there any interactions, see *Table 3.5*. A one way ANOVA with treatment as the factor was used to

analyze data from animal used for microdialysis. Confirming the findings from the animals tested behaviorally, there was no effect of treatment, see *Table 3.4*.

3.3 Experiment 3: Galantamine and its effects on contextual fear conditioning

3.3a Body weights

A repeated-measures ANOVA with day as the repeated measure and treatment as the between-subjects measure was used to analyze body weight data from PD 2-10. The assumption of sphericity was violated ($p < 0.05$), so Greenhouse-Geisser adjusted degrees of freedom were used. There was a significant effect of day (F (8, 960) = 4239.39, $p <$ 0.001), indicating that all animals gained weight over the intubation period. There was a significant interaction between day and treatment (F (16, 888) = 3.25, p = 0.02). To analyze the interaction, one-way ANOVAs were conducted for each day separately, with treatment as a factor. There were no differences between treatment groups on any day, suggesting that all animals gained weight throughout the postnatal period, regardless of treatment, see *Figure 3.14.*

A repeated-measures ANOVA with day as the repeated measure and treatment and drug as the between subjects measures was conducted to determine if galantamine injections or postnatal treatment affected body weights. The assumption of sphericity was violated according to Mauchly's test of sphericity ($p < 0.05$) so Greenhouse-Geisser adjusted degrees of freedom were used. There was a significant effect of day on body weight (F (19, 2090) = 3106.8, $p < 0.001$, but no interaction between day and any other variables, see *Figure 3.15*. This indicates that postnatal treatment did not have long-lasting effects on body weight, nor did galantamine injections.

3.3b Blood Alcohol Concentrations

The average blood alcohol concentration (±SEM) for alcohol-exposed animals in this study was 324.56 ± 14.11, see *Table 3.2*. Blood samples were obtained at PD 10 before treatment started, but it is possible that even though animals were randomly assigned to experimental groups, there was a difference in BACs between groups. A two-way ANOVA with drug and condition as the variables was used to test this. There was no main effect of drug or condition, nor was there an interaction between the two variables. Using a one-way ANOVA with experiment as the independent variable, there was no significant difference in blood alcohol concentrations between Experiments 1 and 3.

3.3c Context Pre-exposure Facilitation Effect

Freezing was measured during all phases of testing and analyzed using a 3(treatment) x 2 (drug) x 2 (condition) way ANOVA. During training, the animals were in the testing environment for less than 5 seconds, and did not freeze when shocked. During pretraining, there was a significant main effect of treatment (F (2, 109) = 3.88, p = 0.024) and condition (F (1, 109) = 12.67, p = 0.001), but no main effect of drug, or interactions between any of the variables see *Figure 3.16*. Tukey post-hoc tests were used to describe the main effect of treatment. Intubated controls froze significantly more than non-treated controls during pretraining (p = 0.017), but there were no differences between the ethanol-exposed animals and controls. The average freezing (percent) for the Pre and No-Pre groups was 7.67 ± 0.77 and 11.58 ± 0.78, respectively, indicating that Context B elicited more freezing than Context A. For this reason, all testing values were expressed as a percent change from baseline. To calculate this, the following equation was used:

$$\left[\frac{\text{(Percent freezing during testing)- (Percent freezing during pretraining)}}{\text{(Percent freezing during pretraining)}}\right] \text{ x } 100$$

A 3 (treatment) x 2 (drug) x 2 (condition) way ANOVA was used to analyze behavioral data. There was a significant main effect of condition, F (1, 102) = 18.59, $p <$ 0.001, but no main effect of treatment or drug or interaction between any conditions, see *Figure 3.17*. In the no pre-exposure group, there was an average 218.84% (± 33.94) increase in freezing over baseline, while in the pre-exposure condition, there was an average 594.61% (±80.63) increase in freezing over baseline.

Table 3.1

Physical Parameters (mean ±SEM) for animals used for in vivo microdialysis experiments (Exp. 1).

Group	PD 2 (g)	PD 10 (g)	PD 27/28 (g)	PD 33/34 (g)	BAC (mg/dl)
NC (n)	6.67 ± 0.34 (7)	18.47 ± 0.75 (7)	73.70 ± 1.54 (7)	99.27 ± 2.49 (7)	n/a
IC (n)	6.59 ± 0.34 (7)	17.93 ± 0.68 (7)	74.10 ± 3.42 (7)	101.79 ± 5.59 (7)	n/a
ET (n)	6.43 ± 0.24 (3)	16.77 ± 1.27 (3)	67.13 ± 4.30 (3)	101.90 ± 11.64 (3)	304.90 ± 132.14 (2)

Table 3.2

Physical parameters (mean ±SEM) for animals used in immunohistochemistry analysis (Exp. 1).

Group	PD 2 (g)	PD 10 (g)	PD 27/28 (g)	PD 33/34 (g)	BAC (mg/dl)
NC (n)	6.69 ± 0.30 (8)	18.47 ± 0.75 (7)	72.71 ± 1.66 (8)	99.01 ± 2.17 (8)	n/a
IC (n)	6.70 ± 0.32 (8)	17.93 ± 0.68 (7)	73.63 ± 3.00 (8)	101.24 ± 4.87 (8)	n/a
ET (n)	6.64 ± 0.36 (7)	17.85 ± 0.92 (6)	70.98 ± 3.20 (6)	103.60 ± 5.79 (6)	318.09 ± 87.41 (4)

Table 3.3

Physical Parameters (±SEM) for Experiment 3

Group	PD 2 (g)	PD 10 (g)	PD 21 (g)	PD 30 (g)	BAC (mg/dl)
NC-SAL (n)	7.4 ± 0.2 (20)	19.2 ±0.5 (20)	44.5 ± 1.2 (20)	91.6 ± 1.8 (20)	n/a
NC-GAL (n)	7.1 ± 0.3 (20)	19.0 ± 0.6 (20)	44.5 ± 1.3 (20)	92. 1 ± 2.2 (20)	n/a
IC-SAL (n)	7.1 ± 0.2 (20)	18.8 ± 0.4 (20)	44.3 ± 1.2 (20)	91.1 ± 1.7 (19)	n/a
IC-GAL (n)	7.1 ± 0.3 (20)	19.2 ± 0.6 (20)	43.5 ± 1.3 (20)	90.7 ± 1.9 (20)	n/a
ET-SAL (n)	7.2 ± 0.2 (21)	19.3 ± 0.6 (21)	45.5 ± 1.4 (21)	93.3 ± 2.1 (21)	356.65 ± 17.11
ET-GAL (n)	7.5 ± 0.2 (22)	20.8 ± 0.6 (22)	46.1 ± 1.5 (22)	95.5 ± 1.8 (22)	324.54 ± 14.57

Table 3.4

Average density of staining (expressed as a percent) ± SEM for Experiment 1

Stain	Non-treated Control (n)	Intubated-Control (n)	Ethanol-Exposed (n)
Alpha7 Nicotinic Acetylcholine Receptor	25.11 ± 3.02 (6)	25.41 ± 4.46 (6)	30.05 ± 2.05 (5)
Vesicular Acetylcholine Transporter	20.52 ± 0.86 (6)	24.71 ± 2.63 (7)	26.55 ± 2.66 (5)

Table 3.5

Average density of staining (expressed as a percent) ± SEM for Experiment 3

Stain	NC-SAL (n)	NC-GAL (n)	IC-SAL (n)	IC-GAL (n)	ET-SAL (n)	ET-GAL (n)
Alpha7 Nicotinic Acetylcholine Receptor	49.91 ± 4.18 (9)	53.79 ± 4.62 (12)	56.60 ± 4.91 (12)	50.99 ± 4.48 (16)	55.83 ± 4.26 (13)	53.55 ± 4.01 (15)
Vesicular Acetylcholine Transporter	46.15 ± 4.37 (18)	47.17 ± 3.80 (19)	49.00 ± 4.15 (18)	47.71 ± 3.64 (17)	50.56 ± 2.37 (17)	52.50 ± 3.44 (19)

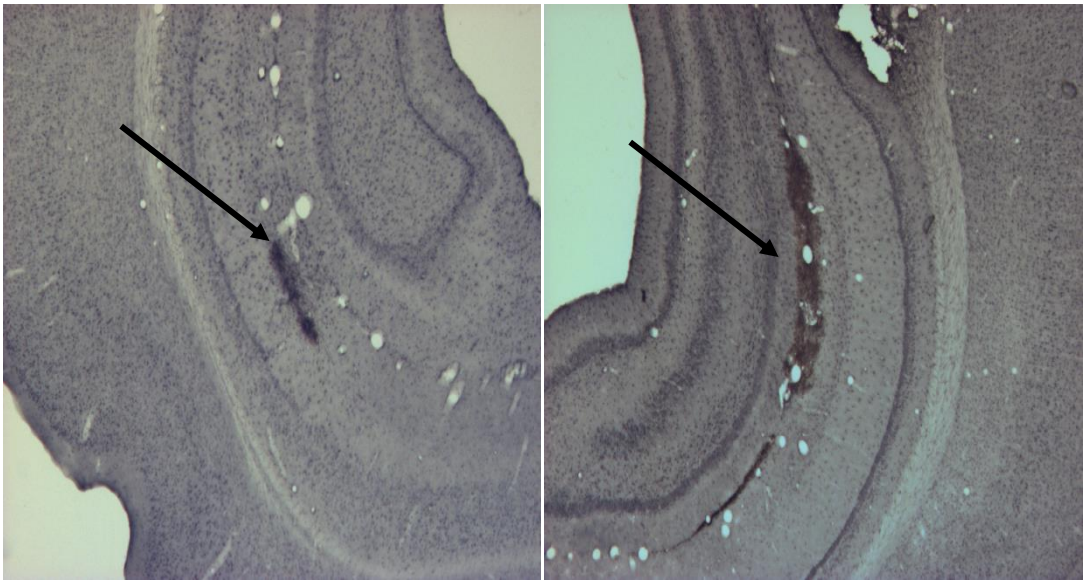

Figure 3.1 Representative photomicrographs (2x magnification) depicting probe placement in area CA1 of the hippocampus. Tissue was sectioned and stained using immunohistochemistry for ChAT. Probes were classified as being correctly placed if they were located in the ventral CA1 region of the hippocampus (Plates 38-42; Paxinos & Watson, 1986).

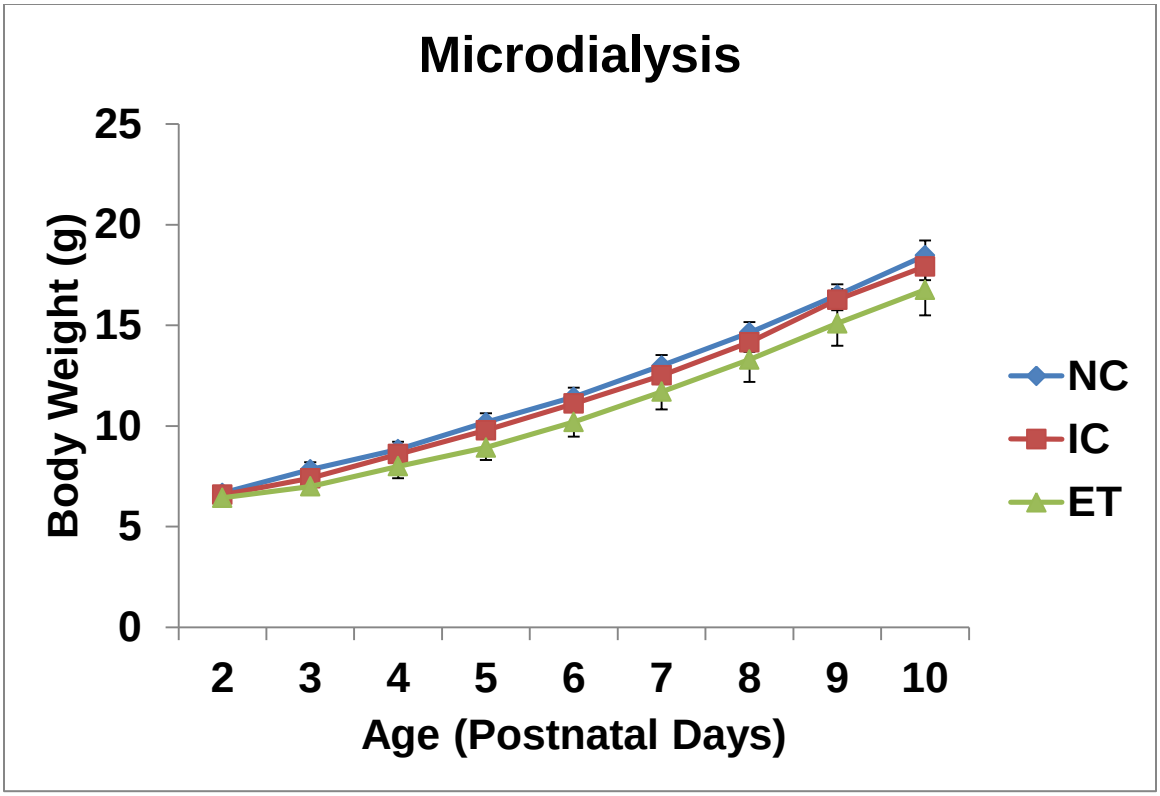

Figure 3.2 Average (±SEM) body weights (g) throughout pup treatment. All animals gained weight over the treatment period ($p < 0.001$), but there was no effect of treatment, nor was there an interaction between age and treatment. Only the animals that were included in the final HPLC data are included in the graph.

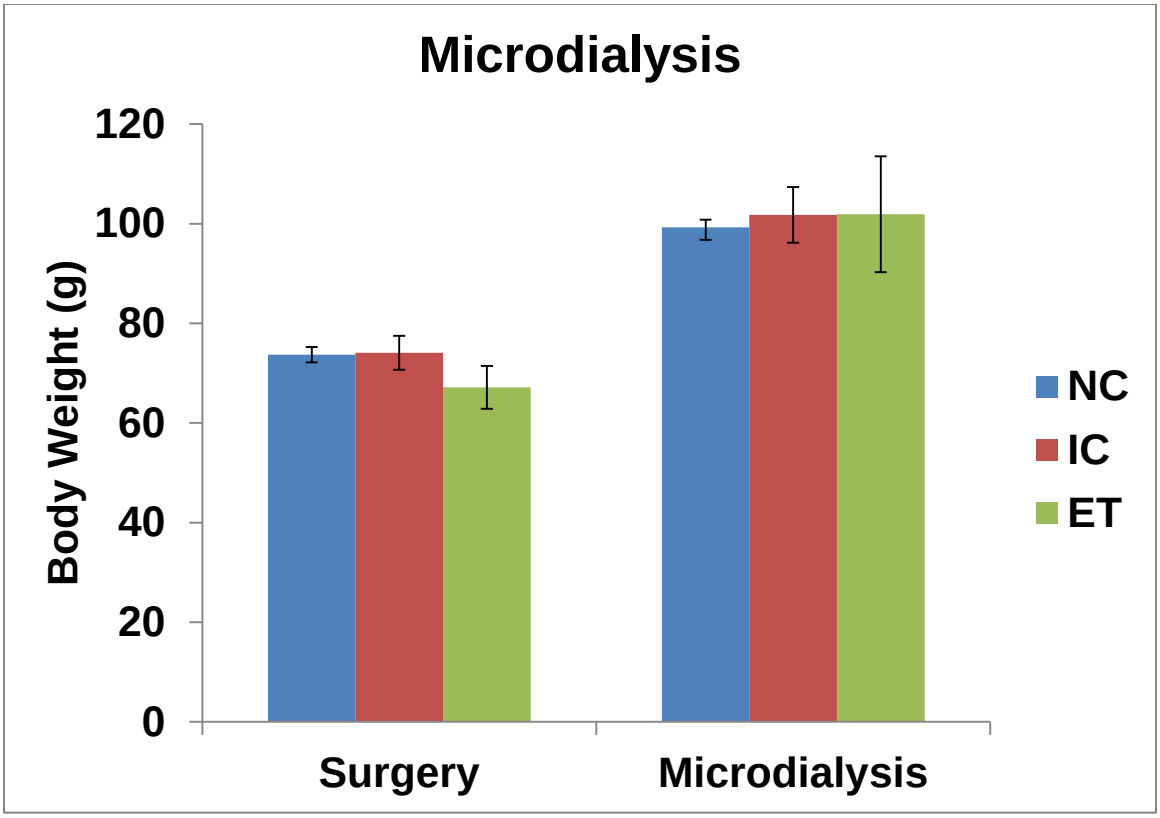

Figure 3.3 Average (±SEM) body weight (g) on the day of cannula surgery (PD 27 or 28) and microdialysis (PD 33 or 34). There were no effects of treatment on either day, indicating that postnatal alcohol exposure did not significantly impact growth. Only the animals that were included in the final HPLC data are included in the graph.

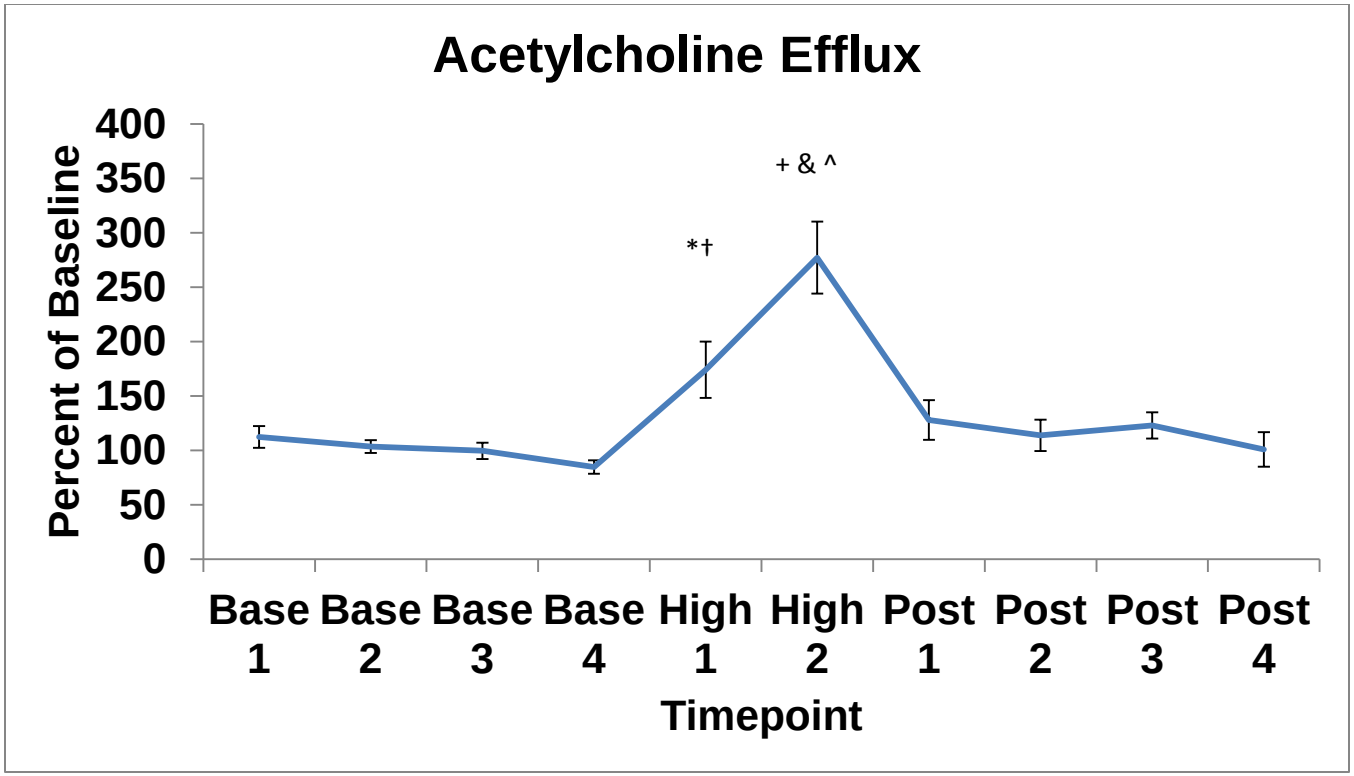

Figure 3.4 Average acetylcholine efflux (±SEM) in area CA1 of the hippocampus for all treatment groups. * indicates a significant increase over baselines 2 and 4 ($p < 0.05$). $^{+}$ indicates a significant increase over all baseline samples. $^{\&}$ indicates a significant increase over the first high K^{+}/Ca^{2+} collection. † indicates a significant increase compared to P1 and P2. $^{\wedge}$ indicates a significant increase over all post-stimulation time points.

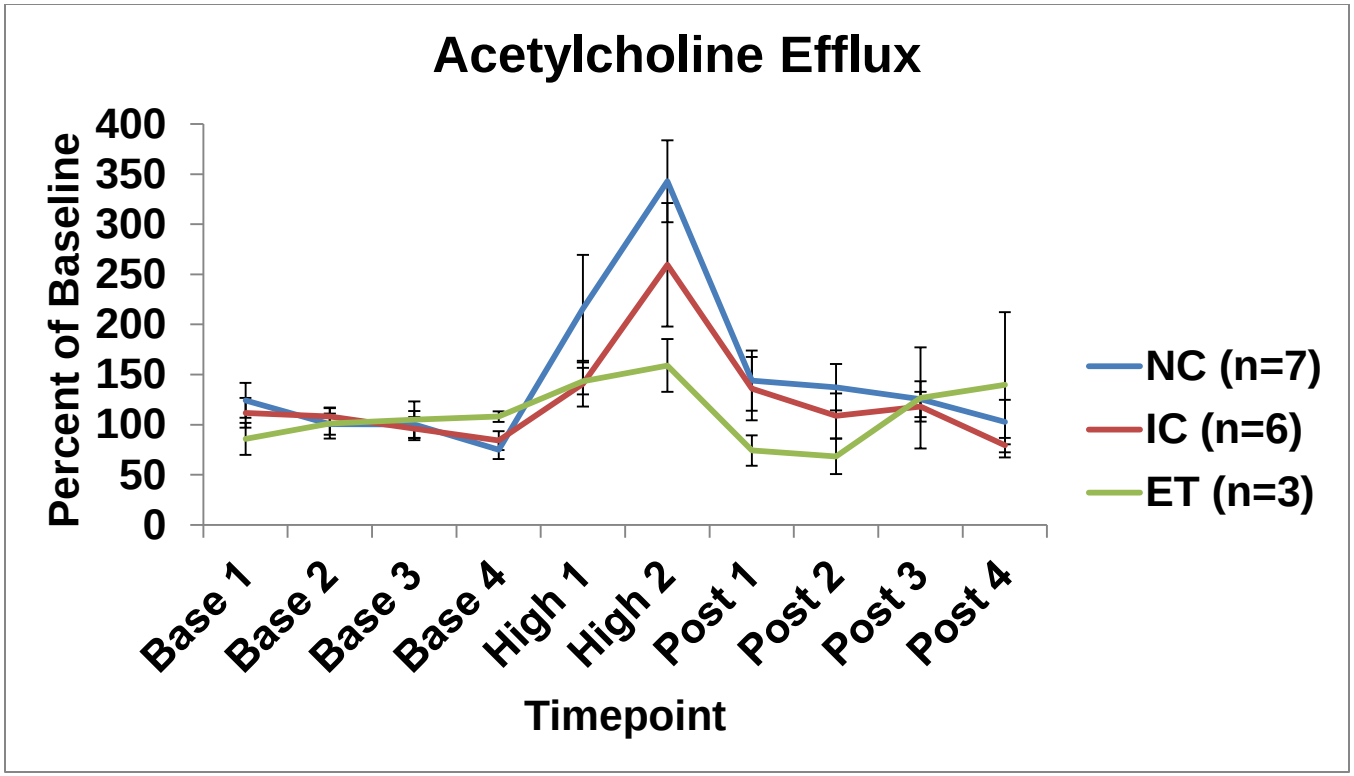

Figure 3.5 Average acetylcholine efflux (±SEM) for all treatment groups for the first microdialysis session. Infusion of a high K^+/Ca^{2+} aCSF solution through the microdialysis probe significantly increased acetylcholine efflux, but the interaction with treatment group was not significant.

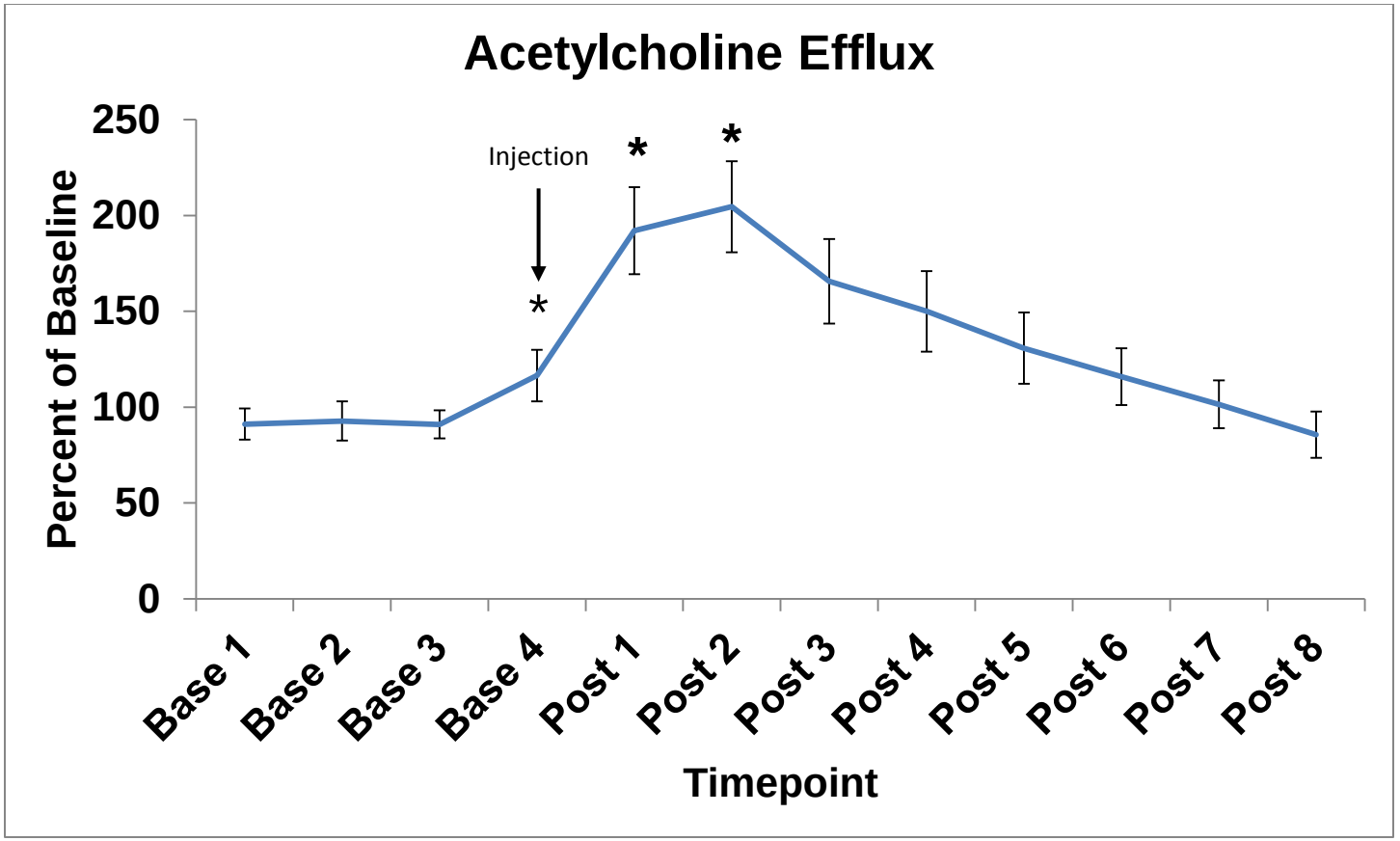

Figure 3.6 Average acetylcholine efflux for the second microdialysis session (percent of baseline; ±SEM), collapsed across treatment groups. Galantamine administration led to a significant increase in extrasynaptic levels of acetylcholine, compared to baseline (*).

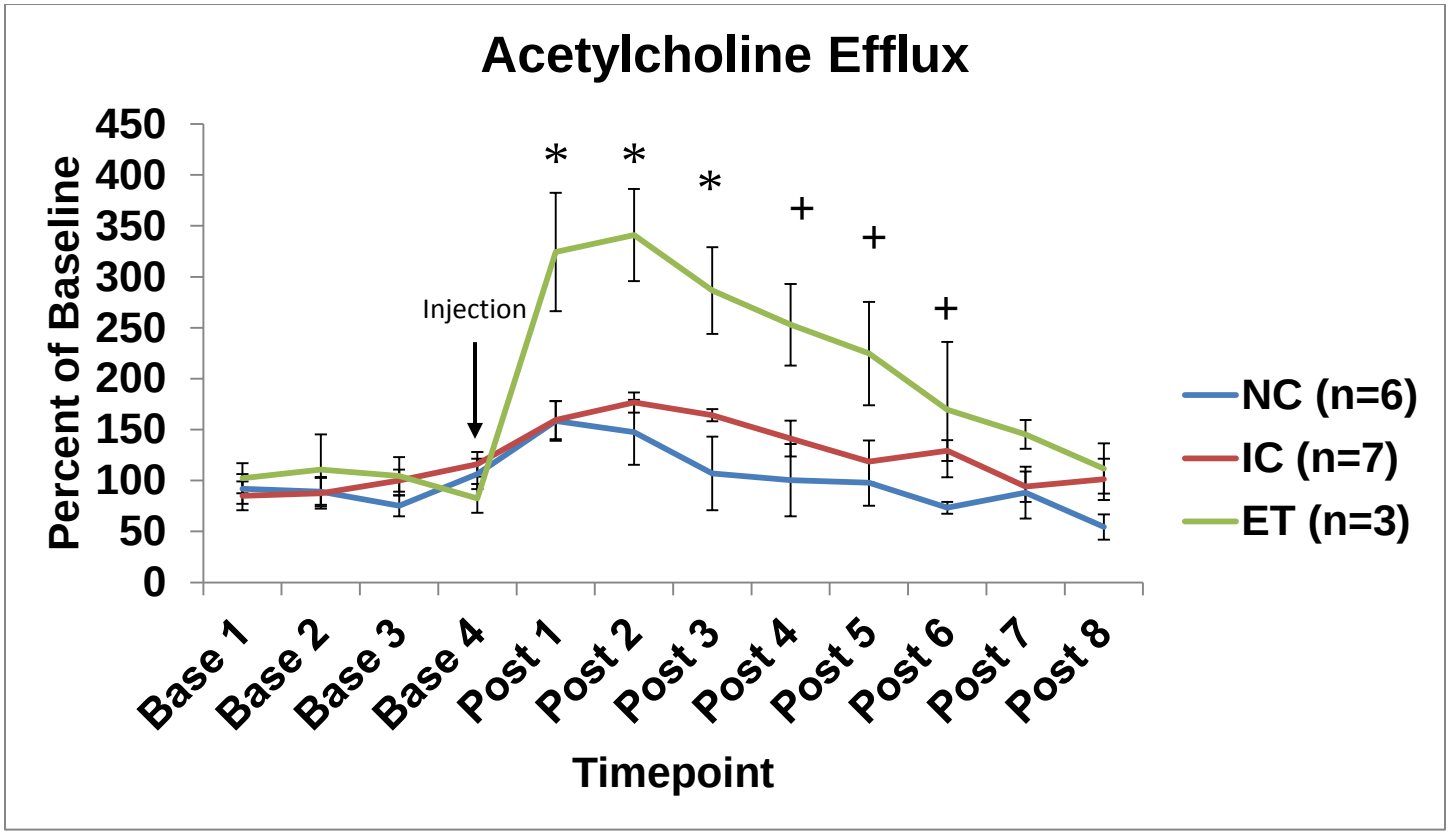

Figure 3.7 Average extracellular acetylcholine in CA1 (percent of baseline; ±SEM) for all treatment groups. There was a significant time point x treatment interaction, such that alcohol-exposed animals had a significantly larger galantamine-induced increase in acetylcholine. * indicates a significant difference between ET and controls (IC and NCs) at $p < 0.05$. $^{+}$ indicates a significant difference between ET and NC groups at $p < 0.05$.

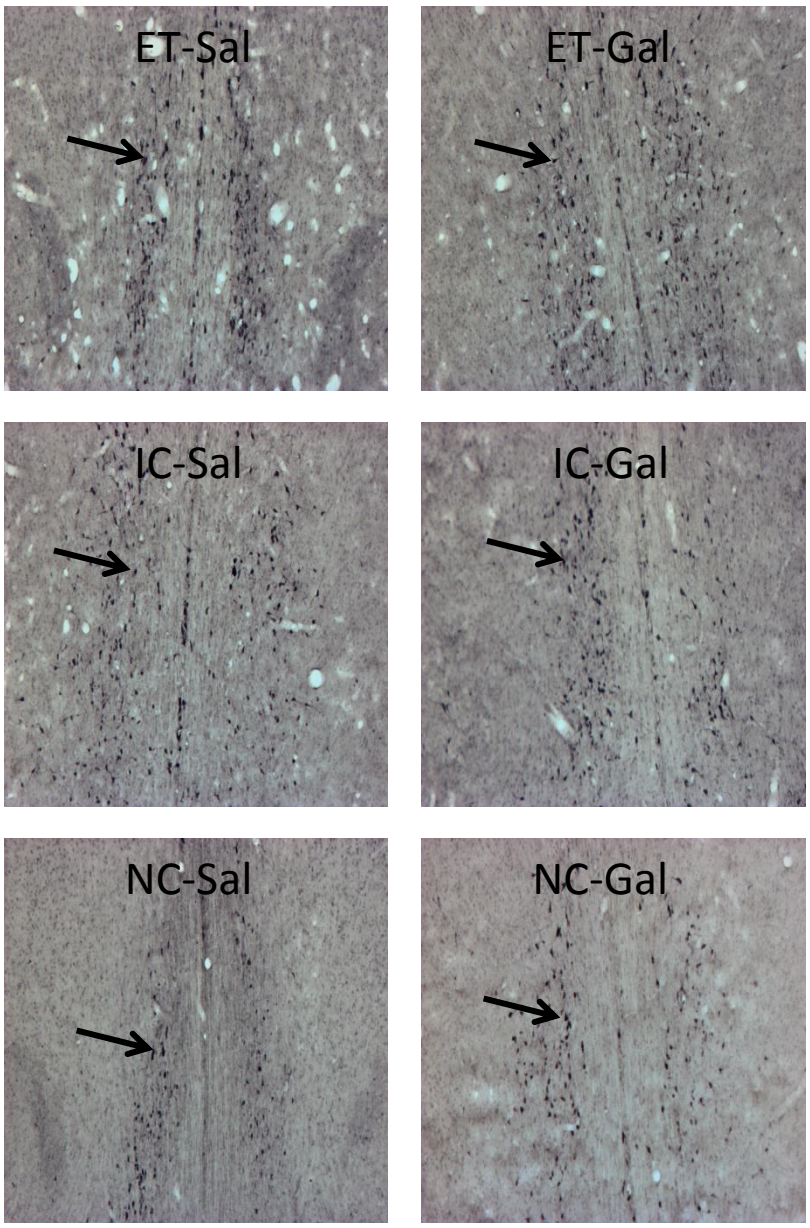

Figure 3.8 Representative photomicrographs of choline acetyltransferase (ChAT) in the medial septum (4x magnification) from animals in the pre-exposure group (Experiment 3) showing an up-regulation of ChAT in alcohol-exposed animals and a down-regulation of ChAT in animals treated chronically with galantamine.

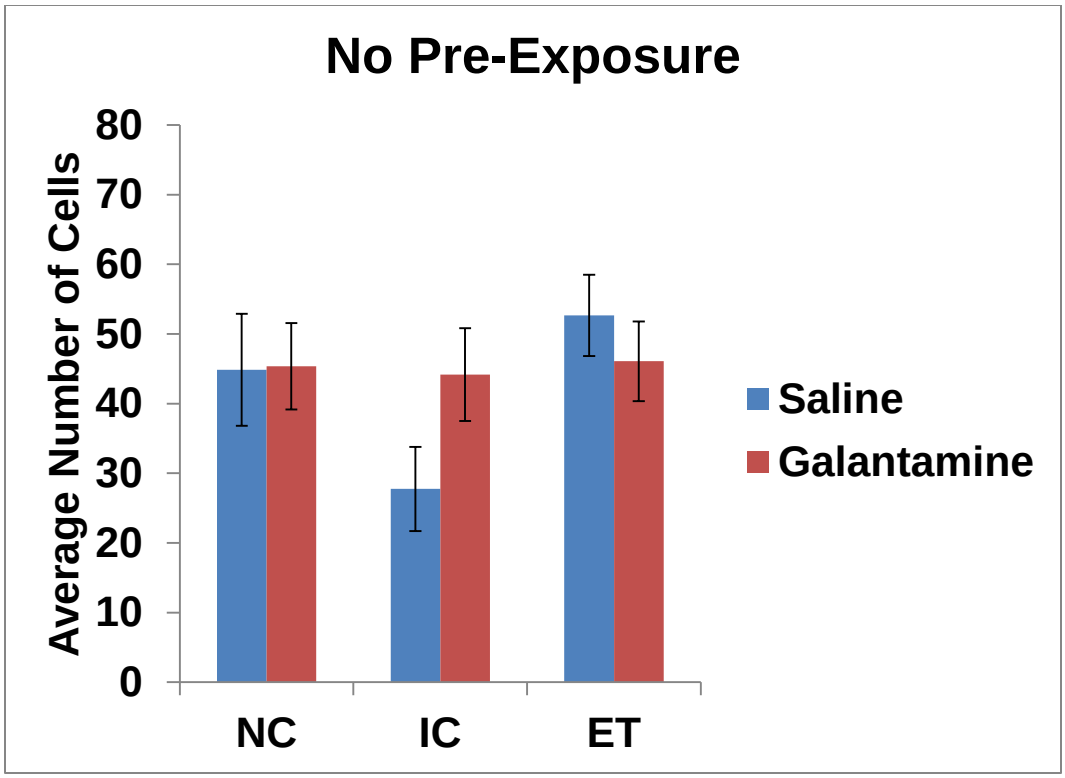

Figure 3.9 Average number of ChAT+ cells in the medial septum (± SEM) in the no pre-exposure testing condition. There were no main effects, nor was there an interaction between treatment and drug. Data represent the average of two sections per animal, when possible.

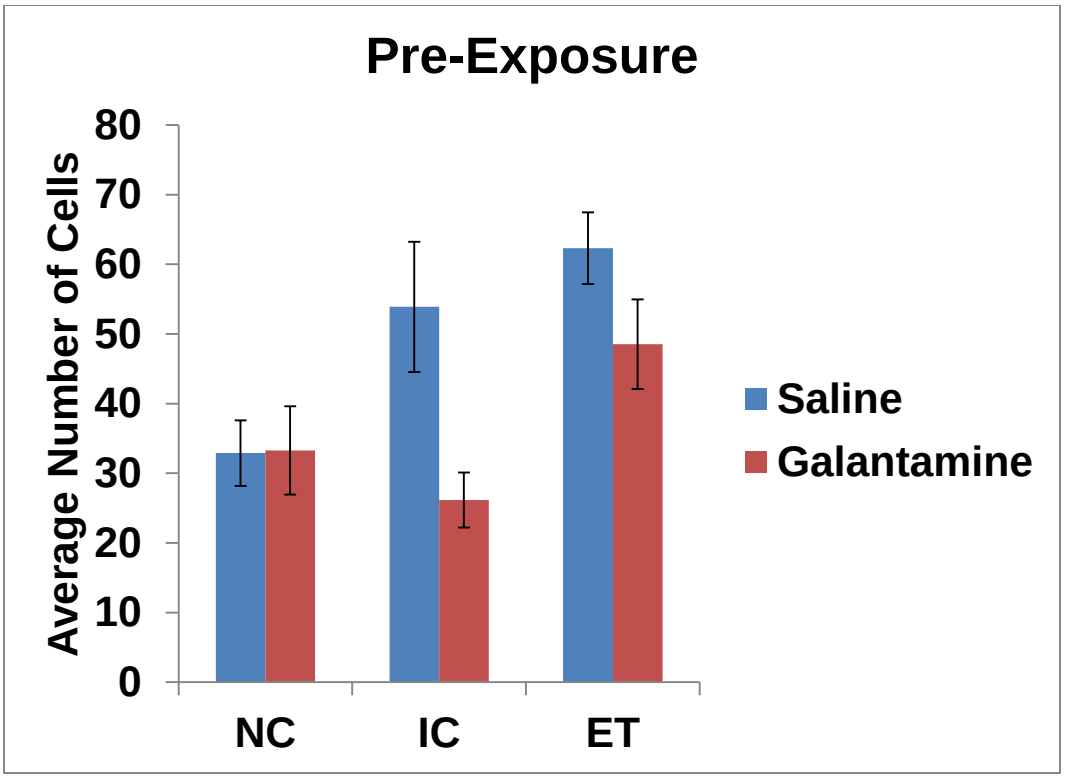

Figure 3.10 Average number of ChAT+ cells (± SEM) in the medial septum in the pre-exposure group testing condition. There was a significant main effect of both treatment and drug, where alcohol-exposed animals had significantly more ChAT+ cells compared to the NC, but not the IC group. Galantamine treatment reduced the number of ChAT+ cells, regardless of treatment. Data represent the average of two sections per animal, when possible.

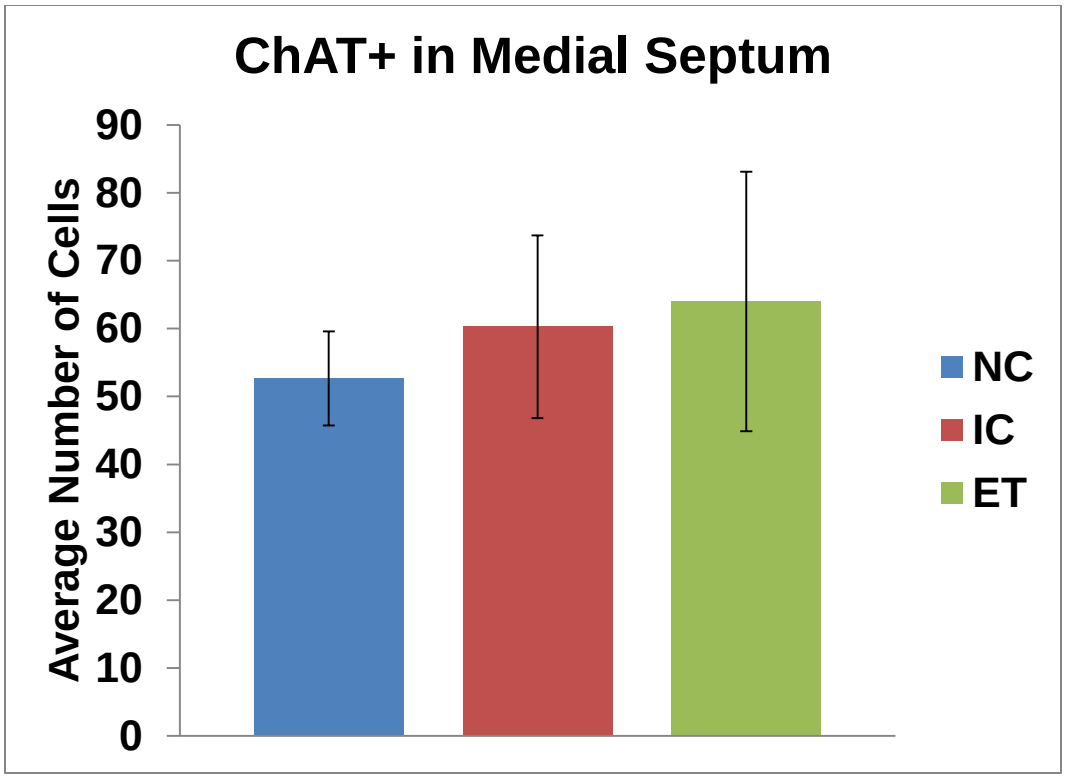

Figure 3.11 Average number (±SEM) of ChAT+ cells in the medial septum from Experiment 1 (Microdialysis). There was no effect of treatment. N's for NC, IC, and ET groups were 9, 7, and 6, respectively. Data represent the average of two sections per animal, when possible.

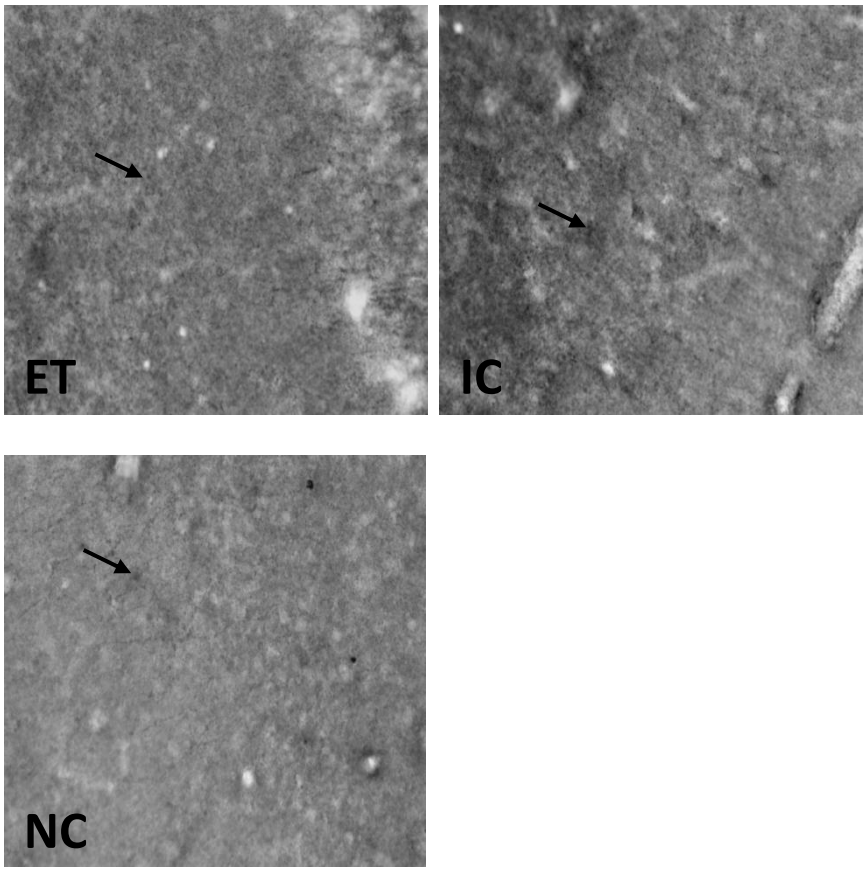

Figure 3.12 Representative photomicrographs of the Alpha7 nicotinic acetylcholine receptor in area CA1 of the ventral hippocampus (Plates 38-42; Paxinos & Watson, 1986) from Experiment 1 (Magnification = 20x).

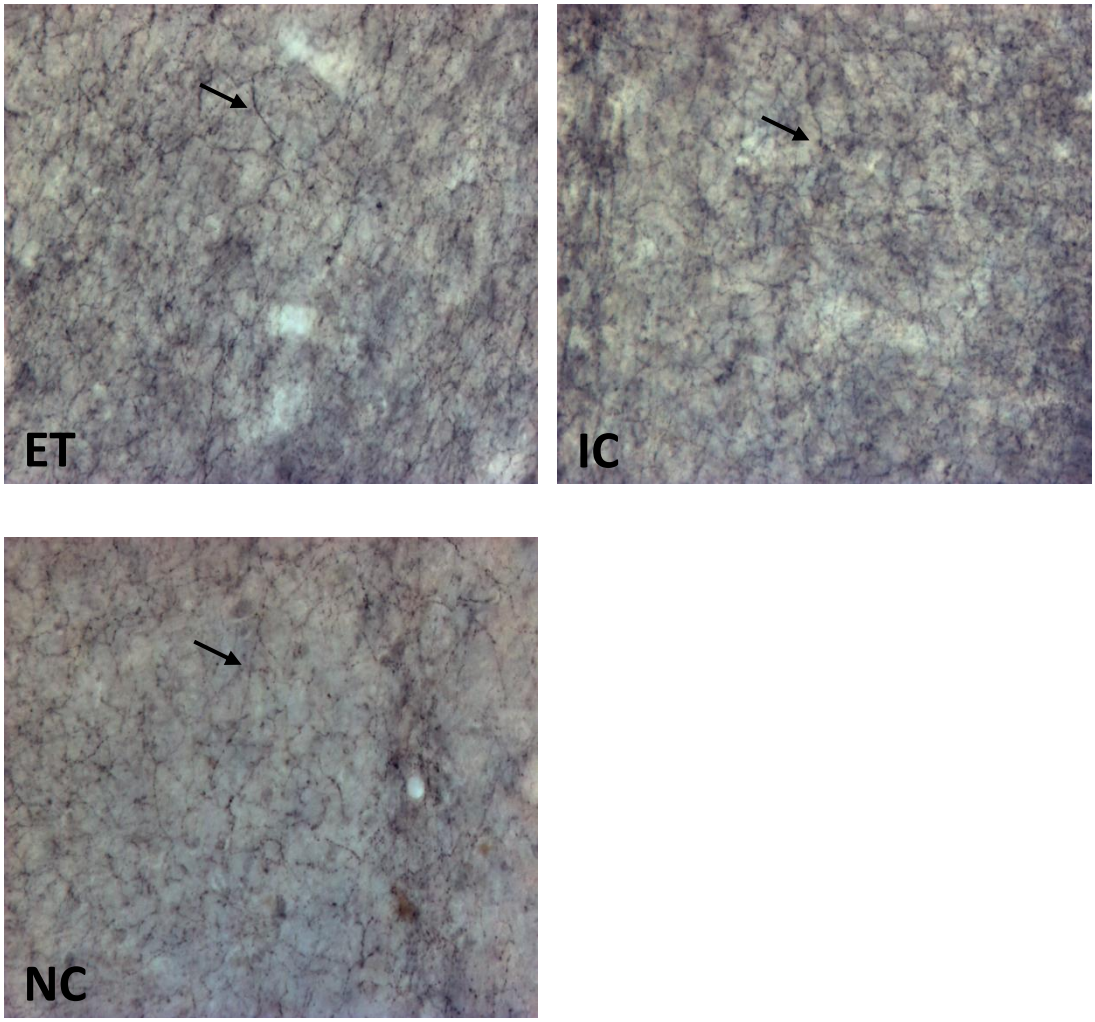

Figure 3.13 Representative photomicrographs (20x magnification) of the vesicular acetylcholine transporter in area CA1 of the ventral hippocampus (Plates 38-42; Paxinos & Watson, 1986) for each treatment group. There was no significant effect of treatment, drug, or condition.

Figure 3.14 Average (±SEM) body weight (g) during pup treatment for Experiment 3. There was no significant effect of treatment, indicating that all animals gained weight throughout the early postnatal period. N's for the NC, IC, and ET groups were 40, 40, and 43, respectively)

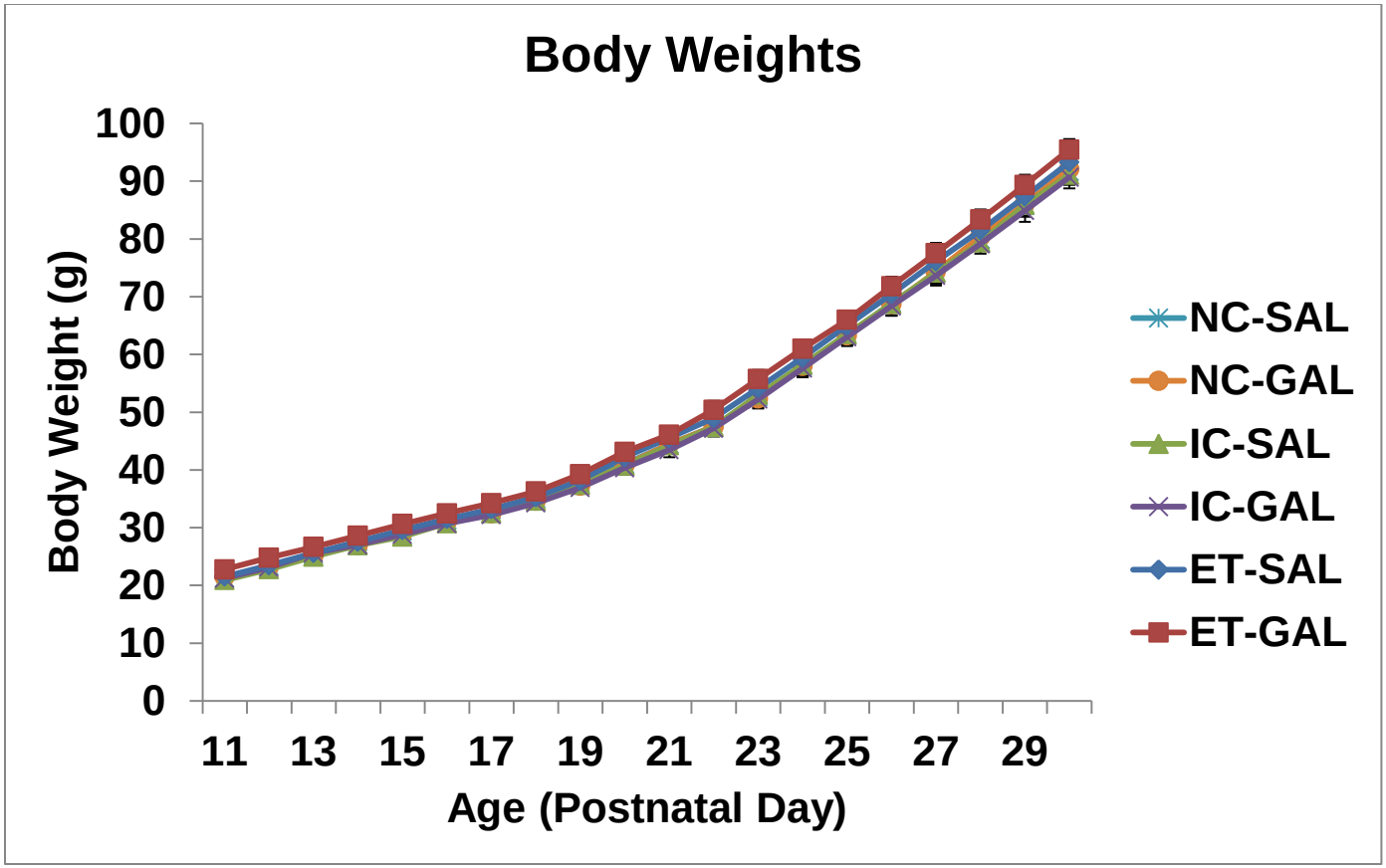

Figure 3.15 Average body weight (g; ±SEM) for all treatment groups. There were no significant effects of alcohol exposure or drug treatment on body weight gain throughout adolescence. Sample sizes for each group can be found in *Table 3.3*.

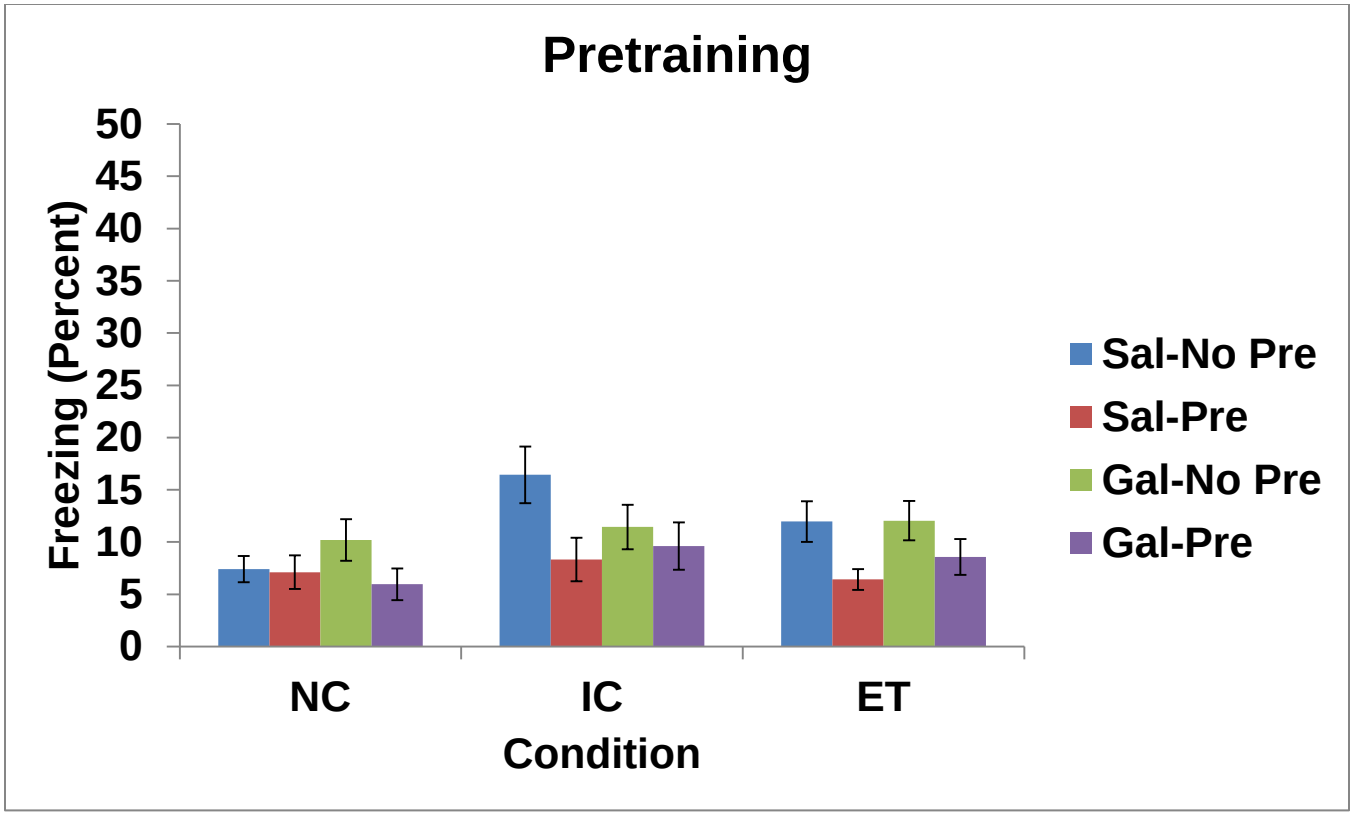

Figure 3.16 Average freezing (percent; ±SEM) for the pretraining session. There was a main effect of treatment, where IC animals froze significantly more during pretraining than NC animals. There was also a main effect of testing condition, where animals in the No-Pre group froze significantly more than animals in the Pre group.

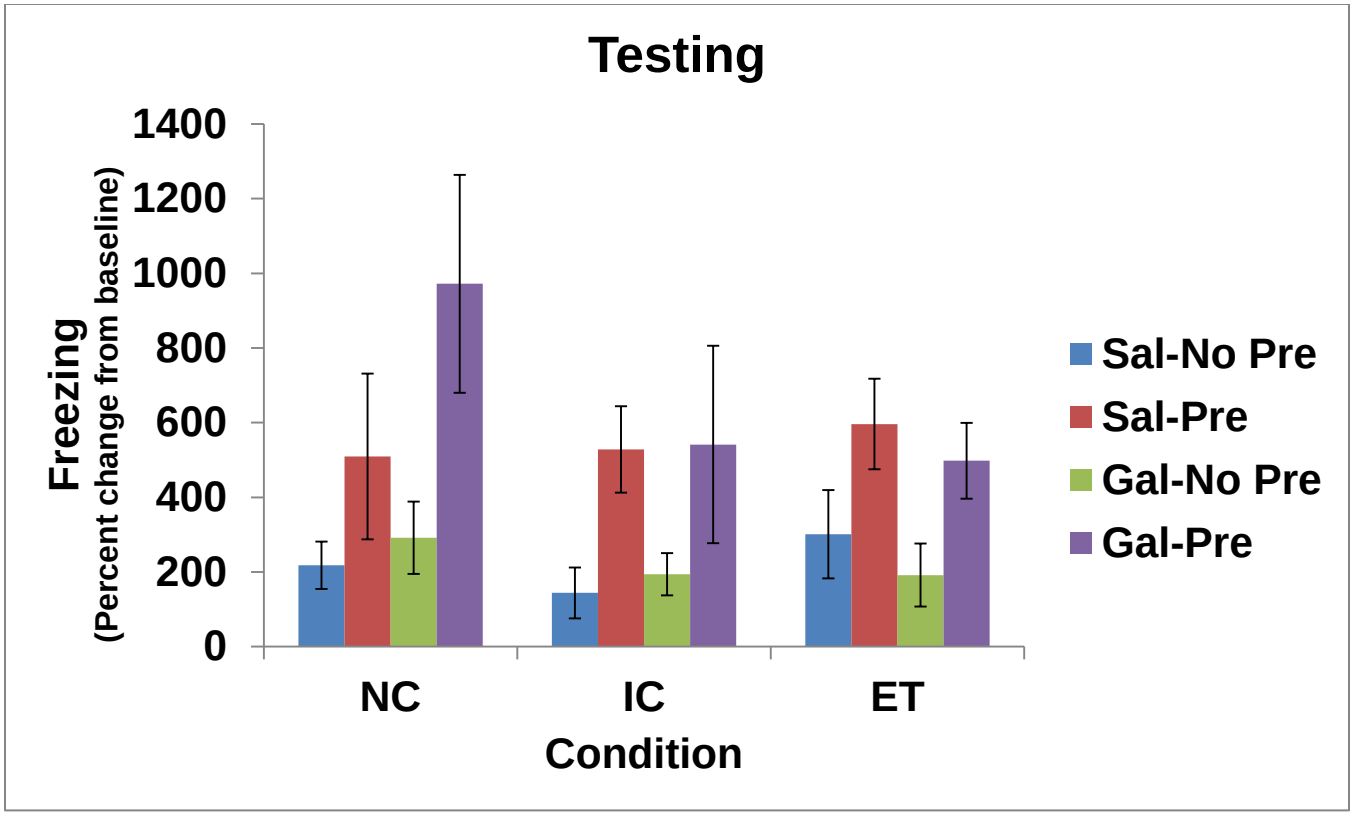

Figure 3.17 Average freezing (percent of baseline; ±SEM) for all groups. There was a main effect of condition, where the animals that were pre-exposed (Pre) to the context froze significantly more than those that were not (No Pre), indicating the context pre-exposure facilitation effect. There were no effects of treatment or drug.

CHAPTER 4

DISCUSSION

4.1 Summary of Findings

The goal of the present set of experiments was to test the hypothesis that developmental alcohol exposure significantly impairs the hippocampal acetylcholine system and that this disruption may influence learning. The current results provide an important piece to the puzzle of how alcohol exposure impacts the developing hippocampus. There were five main findings from these experiments: 1) Developmental alcohol exposure did not influence baseline acetylcholine efflux, 2) Developmental alcohol exposure led to a smaller increase in acetylcholine efflux during a high K^+/Ca^{2+} administration, 3) Developmental alcohol exposure significantly increased extrasynaptic acetylcholine levels following an acute injection of galantamine, 4) Developmental alcohol exposure significantly increased the number of ChAT+ cells in the medial septum in animals that demonstrate the CPFE, without affecting vAChT or α7 nAChRs in the hippocampus, and 5) Neither galantamine nor developmental alcohol exposure impacted performance on the CPFE task. Taken together, these results indicate that developmental exposure to alcohol causes a significant disruption in cholinergic signaling within the hippocampus.

The present findings represent a novel contribution to research on Fetal Alcohol Syndrome in multiple respects. To begin with, this is the first study to use *in vivo*

microdialysis to measure acetylcholine release in the hippocampus following developmental alcohol exposure. Second, this is the first study to examine multiple cholinergic proteins within the same animal, in an effort to describe how alcohol might impact the cholinergic system as a whole. Third, these data show a *lack* of impairment on the context pre-exposure facilitation effect following postnatal alcohol exposure which contradicts previous findings (Dokovna et al., 2013; Hamilton et al., 2011; Jablonski & Stanton, 2014; Murawski & Stanton, 2010; Murawski & Stanton, 2011). Finally, the data suggest that chronic galantamine administration during adolescence does not influence freezing during contextual conditioning.

4.2 Developmental Alcohol Exposure Impairs Hippocampus-Dependent Learning

Alcohol-induced learning impairments have been well characterized using animal models (see Berman & Hannigan, 2000, for review). The impact of developmental alcohol exposure varies based on experimental variables such as timing of alcohol exposure (e.g. prenatal vs. postnatal), age of testing, and sex. For the most part, impairments are seen in learning paradigms that require, or rely on, the hippocampus (e.g. Morris Water Maze, contextual fear conditioning, etc.). Some common findings from the literature and reviewed in the introduction are: 1) Alcohol exposure must occur in a "binge-like" fashion to impair water maze performance, 2) Spatial learning impairments are often seen in juveniles, but are not consistently observed into adulthood, and 3) Exposure to alcohol during the period equivalent to the third trimester may be required to impair spatial navigation. These findings indicate that exposure to alcohol when the hippocampus is undergoing rapid growth (e.g. third trimester) significantly impairs hippocampal function later in life. This section will summarize the current

findings using the context pre-exposure facilitation effect paradigm, and incorporate those findings into the literature.

4.2a Context Pre-Exposure Facilitation Effect

Previous research has shown that alcohol-exposed animals are impaired on the context pre-exposure facilitation effect paradigm (Dokovna et al., 2013; Hamilton et al., 2011; Jablonski & Stanton, 2014; Murawski & Stanton, 2010; Murawski & Stanton, 2011). Since this paradigm is hippocampus-dependent (LeDoux, 2000), and is influenced by the acetylcholine system (Kenney & Gould, 2008), these findings suggested that alcohol exposure disrupts the hippocampal acetylcholine system. The present studies found evidence of cholinergic dysfunction in the hippocampus, but did not find that alcohol-exposed animals were impaired on the CPFE task. There are a number of possible explanations for this negative finding and this discussion will focus on three very likely possibilities: 1) The CPFE task is sensitive to handling during development, 2) The dose of alcohol (3 g/kg) used in the current study was significantly lower than the previous studies, and 3) Alcohol was administered from PD 2-10 in the current study, whereas the previous studies administer alcohol from PD 4-9.

Alcohol-exposed animals are sensitive to postnatal handling, and it is possible that the daily injections for the administration of galantamine or saline (on PD 11-30) were sufficient to ameliorate an alcohol-induced impairment in CPFE performance. In fact, neonatal handling has been shown to reduce alcohol-induced deficits in passive avoidance learning (Gallo & Weinberg, 1982). In addition, Weinberg et al. (1995) demonstrated a sex-specific effect of handling; males and females given prenatal alcohol exposure exhibited increased hypothermia following an acute injection of alcohol and

this effect was ameliorated by neonatal handling in males, but not females. The present study used only males and it is possible that they are especially susceptible to the effects of handling that result from daily injections.

Although control animals were also handled daily for injections, alcohol-exposed animals are differentially susceptible to stressors. Research by Weinberg and colleagues (reviewed in Hellemans et al., 2010) has repeatedly demonstrated dysregulation of the HPA axis in animals prenatally exposed to alcohol. This dysregulation of the HPA axis results in an enhanced response to stressors. Both injections and behavioral testing should elicit a stress response in all animals. However, in alcohol-exposed animals, daily handling for injections can habituate the animals to experimental procedures, such that they are less stressed when exposed to the behavioral paradigm used in this study. Future studies should use a non-injected control when testing treatments for alcohol-induced learning deficits in this task. On the other hand, daily choline, but not vehicle, administration has been shown to ameliorate learning deficits caused by developmental alcohol exposure using another hippocampus-dependent task-the Morris water Maze (Ryan et al., 2008; Thomas et al., 2007). Perhaps the Morris water maze would be a better behavioral task to test the impact of chronic galantamine administration on learning in alcohol-exposed animals.

Another possible explanation for the current findings is that the dose of alcohol used in the present study (3.0 g/kg) was not sufficient to produce contextual fear conditioning deficits. A study by Allan et al. (2003) utilized a voluntary drinking paradigm (blood alcohol concentrations (BACs) ~120 mg/dl) and found that prenatal alcohol exposure disrupted freezing to the context in adult mice. However, the contextual

fear conditioning paradigm used in the study was a standard paradigm in which there were multiple tone-shock pairings within a context. In a study by Murawski & Stanton (2010), alcohol exposure (5.25 g/kg; BACs ~410 mg/dl) impaired performance on the CPFE paradigm, but did not disrupt post-shock freezing or freezing to a tone (CS). Goodfellow & Lindquist (2014) utilized a postnatal alcohol exposure model (PD 4-9) to test the impact of 3 g/kg, 4 g/kg, and 5 g/kg doses (BACs of ~200, ~300, and ~350 mg/dl, respectively) on contextual fear conditioning in adult rats. There was a reduction in freezing during testing in alcohol exposed adults, but only in the 4 g/kg and 5 g/kg conditions (Goodfellow et al., 2014). Similarly, Murawski & Stanton (2011) determined that high doses of alcohol (4.0 and 5.25 g/kg; BACs ~370 and 450 mg/dl, respectively), but not low doses (2.75 g/kg; BACs ~ 230), administered from PD 4-9 impaired performance on the CPFE task. While the dose of alcohol that produced conditioning deficits in the studies mentioned above were higher (4.0-5.25 g/kg; BACs 300-410 mg/dl) than the dose used in the current studies (3.0 g/kg; ~320 mg/dl), the average BACs were comparable, suggesting that the difference in alcohol dose is not the cause for the current negative findings. Previous studies have demonstrated the importance of BAC, and not dose, in demonstrating alcohol-related changes in behavior (Goodlett et al., 1987).

Finally, the previous studies (Murawski & Stanton 2010; Murawski & Stanton, 2011; Goodfellow et al., 2014) utilized an alcohol exposure paradigm in which alcohol was administered to the developing pup from PD 4-9. In the current experiments, alcohol exposure occurred from PD 2-10, encompassing a wider range of development. Importantly, both of these exposure periods cover the time of maximal hippocampal growth, likely producing significant alterations in hippocampal development.

Furthermore, the current exposure paradigm has been used repeatedly to demonstrate spatial navigation impairments in juveniles (Cronise et al., 2001; Marino et al., 2004) and reduction in CA1 cell number (Marino et al., 2004; Tran et al., 2003), indicating that exposure to alcohol from PD 2-10 is sufficient to produce long-lasting alterations in the hippocampus. It is possible that alcohol administration from PD 2-10 induced some compensatory changes in the hippocampus that prevent alcohol-induced deficits on the CPFE task and shorter administration periods (e.g. PD 4-9 or PD 7-9) does not allow these compensatory changes to occur. However, we did observe altered hippocampal neurotransmitter release in alcohol-exposed animals, indicating that our exposure paradigm did cause significant changes in the function of the hippocampus. Future studies could compare these different exposure paradigms (PD 2-10, PD 4-9) on the CPFE task to confirm and extend the findings from these experiments.

In summary, the present research suggests that alcohol-exposed animals are not impaired on the CPFE task. These findings are contrary to previous research showing an alcohol-induced deficit in freezing to the context (Dokovna et al., 2013; Hamilton et al., 2011; Jablonski & Stanton, 2014; Murawski & Stanton, 2010; Murawski & Stanton, 2011). However, it is unlikely that differences in dose and exposure paradigm can account for the differences between studies. It is most likely that the handling that results from daily injections was able to facilitate performance in alcohol-exposed animals and result in no observable deficits.

4.2b Galantamine Administration and Context Pre-exposure Facilitation

Galantamine, an acetylcholinesterase inhibitor and allosteric potentiating ligand at nicotinic acetylcholine receptors, has been shown to improve learning and memory, in

addition to being a treatment for learning impairments caused by developmental lead exposure (Luo et al., 2011), MK-801 (Su et al., 2014), maternal deprivation (Benetti et al., 2009), and L-kynurenine (Alexander et al., 2013). However, the efficacy of galantamine as a treatment for alcohol-induced learning deficits was unknown. The present studies tested whether galantamine could be administered chronically *after* alcohol exposure had occurred. We found no evidence for a beneficial effect of chronic galantamine administration overall in the context pre-exposure facilitation paradigm as it did not improve contextual freezing in control or alcohol-exposed animals.

Performance in the CPFE paradigm is enhanced by acetylcholine agonists (e.g. nicotine; Kenney & Gould, 2008), and physostigmine (0.01 mg/kg prior to all three phases) has even been shown to reverse the alcohol-induced deficit observed in this paradigm (Dokovna et al., 2013), indicating that this task is sensitive to cholinergic manipulation. Physostigmine is a stronger AChE inhibitor than galantamine, but is a weaker allosteric potentiating ligand at nicotinic receptors (Maelicke et al., 2000), suggesting that the CPFE paradigm is more sensitive to higher levels of acetylcholine overall, rather than binding and potentiation of nicotinic receptors.

It is possible that acute galantamine administration, rather than chronic administration, would have enhanced freezing to the conditioned context. In fact, administration of MK-801, an NMDA receptor antagonist, impaired spatial navigation in adult rats, an effect that was reversed with administration of galantamine (1 mg/kg) 30 minutes before each testing session (Su et al., 2014). In addition, Benetti et al. (2009) showed that maternal deprivation caused impairments in novel object recognition and social recognition. These impairments were blocked by treatment with galantamine (1

mg/kg) 30 minutes prior to training. Galantamine (3.0 mg/kg) can also ameliorate attentional set-shifting impairments caused by perinatal treatment with $_{L}$-kynurenine (precursor for kynurenic acid, an α7 nAChR antagonist) when administered acutely before testing (Alexander et al., 2013). Finally, galantamine has also been shown to reduce the impairments in learning caused by nicotine withdrawal (Wilkinson et al., 2011) and mecamylamine treatment (Woodruff-Pak et al., 2003) when given prior to testing.

However, there is some evidence that suggests chronic galantamine is effective in improving learning and memory processes. A study by Luo et al. (2011) exposed pups to lead (0.2%) and administered galantamine (0.1 mg/kg) two weeks prior to measurement of synaptic plasticity in adulthood. Galantamine was able to rescue deficits in synaptic plasticity caused by lead, but it should be noted that this study did not measure behavior, so it is unknown whether these changes in synaptic plasticity would be reflected in better learning. In a study by Barnes et al. (2000), osmotic minipumps containing galantamine (0.277 mg/day) were implanted subcutaneously in aged rats. Galantamine significantly increased the density of nicotinic receptors in hippocampus and frontal cortex (measured by [^{3}H-nicotine] binding) while also increasing the maintenance of LTP. In addition, galantamine treatment (3 mg/kg, 10 days) significantly improved performance on a trace eye blink conditioning task, as measured by increased acquisition of the CS-US association in aged rabbits (Weible et al., 2004). These studies using animal models indicate that chronic galantamine is effective in changing synaptic plasticity (Luo et al., 2011), nicotinic receptors (Barnes et al., 2000), and trace eye blink conditioning (Weible et al., 2004). However, the change in learning was only observed in adults and it is

possible that chronic galantamine may only be effective in changing behavior in adulthood, although chronic galantamine administration has not been tested in adolescents.

In a double-blind placebo controlled study, galantamine was shown to improve global functioning and working memory in humans who exhibited mild cognitive impairment (Koontz et al., 2005). In another study, Gron et al. (2006) demonstrated a significant improvement in multiple measures of cognitive function (episodic learning, delayed recall, and recruitment of the hippocampus for spatial navigation) following galantamine treatment for 7 days in patients with mild cognitive impairment. Goekoop et al. (2004) conducted a study in which patients with mild cognitive impairment were tested for cognitive function as well as brain activation using fMRI. Patients were tested at baseline, following a single dose of galantamine, and following 5 days of galantamine treatment. There was a significant increase in brain activation (left prefrontal cortex, left hippocampus, and left medial occipital gyrus) while performing an episodic memory task with multiple doses, but not a single dose, of galantamine. All in all, these findings indicate that chronic treatment with galantamine can improve learning and memory, but it may be only effective in adulthood, when there is an aging-related decline in cholinergic tone and cognitive function.

Although the purpose of the experimental design was to test chronic galantamine treatment as a potential pharmacological therapy for alcohol-induced deficits, acute galantamine could have produced positive results. In fact, acute galantamine administration during *in vivo* microdialysis significantly increased extracellular levels of acetylcholine, an effect that was enhanced in alcohol-exposed animals. This suggests that

alcohol exposure alters the hippocampal acetylcholine system in such a way that acute galantamine could possibly enhance performance in the CPFE task.

Galantamine administration from PD 11-30 did not significantly alter vesicular acetylcholine transporter (vAChT) or α7 nAChR density in CA1. There was a significant effect of galantamine on choline acetyltransferase (ChAT), where galantamine treatment significantly decreased ChAT+ immunoreactivity in the medial septum. However, this effect was only seen in animals that demonstrated the CPFE (PRE), suggesting an impact of behavioral testing on this measure. This down-regulation of ChAT suggests a reduction in acetylcholine synthesis. Galantamine administration did not differentially affect performance, or ChAT immunoreactivity, in alcohol-exposed animals; the alcohol-exposed animals also showed the galantamine-induced reduction in ChAT+ immunoreactivity.

In summary, previous research shows that chronic galantamine is effective in treating cognitive deficits due to mild cognitive impairment in humans (Goekoop et al., 2004; Gron et al., 2006; Koontz et al., 2005). However, one study that compared young and aged rabbits found no enhancement of learning in young rabbits following galantamine treatment (Weible et al., 2004), suggesting that the cholinergic system must be impaired in some way for galantamine to be effective. On the other hand, acute galantamine (e.g. 30 min before testing) has been shown to effectively improve performance following a number of manipulations that result in cognitive impairment (e.g. MK-801, maternal deprivation) (Alexander et al., 2013; Benetti et al., 2009; Su et al., 2014; Woodruff-Pak et al., 2003). No study has examined chronic galantamine administration in these paradigms. Thus, it may be that chronic galantamine is only

effective when there is an impairment in the cholinergic system (e.g. aging), such that galantamine does not facilitate performance in controls. In alcohol-exposed animals, we did not observe a deficit in learning, making it difficult to determine whether galantamine was effective.

There are a number of experiments that can be done to determine if galantamine is an effective treatment for alcohol-induced learning deficits. First, it is important to establish a model in which alcohol-exposed animals are impaired either by reducing the handling of animals and testing for impairment in CPFE or by examining performance in a different behavioral task, such as spatial navigation. Chronic galantamine could be administered via a mini-pump or with injections in a task not affected by handling. Previous research indicates that acute galantamine administration can significantly improve learning in a number of behavioral paradigms (Alexander et al., 2013; Benetti et al., 2009; Su et al., 2014; Woodruff-Pak et al., 2003) and it is likely that we would have seen a similar pattern in the CPFE. However, the clinical relevance of these experimental designs should be taken into consideration. It does not make much sense to test the effectiveness of acute galantamine administration, since that would require individuals with Fetal Alcohol Spectrum Disorders to take galantamine before any cognitive test. So, the next logical step is to test galantamine in other learning paradigms, such as spatial navigation, a task which in which alcohol-exposed animals are impaired, and can benefit from chronic treatment (e.g. choline; Ryan et al., 2008; Thomas et al., 2007).

4.2c Stages of Conditioning and the Potential Impact of Developmental Alcohol Exposure

The context pre-exposure facilitation paradigm allows researchers to separate which aspects of learning are impacted by developmental exposure to alcohol. Since

context learning (pretraining) occurs separately from the context-shock association (training), performance on each day of testing can inform which aspects of learning are influenced by these stimuli. A study by Jablonski & Stanton (2014) sought to determine what aspects of conditioning (encoding of context, encoding of context-shock association, retrieval of context-shock association) are impacted by alcohol exposure. To do this, sham-intubated and alcohol-exposed pups (5.25 g/kg) were tested for freezing immediately following training (immediate shock followed by 5 min test) and then 24h later by re-exposing the animals to the context (5 min test). If alcohol-exposed animals show a deficit in freezing following training, it suggests impaired consolidation of the original pre-exposure phase or impaired encoding of the context-shock association. If alcohol-exposed animals show a deficit in freezing during testing only, it suggests a failure to consolidate or retrieve the context-shock association. Alcohol-exposed animals showed no deficit in freezing immediately following the shock (training phase), suggesting that alcohol exposure does not influence consolidation of the mental representation of the context or encoding of the context-shock association. However, alcohol-exposed females (but not males) did show a deficit in freezing when re-exposed to the context during the testing phase (24h following shock), suggesting an impairment in consolidation or retrieval of the context-shock association. This is the first study by this group to show a sex-specific effect, suggesting it might be a spurious finding. In the current study, only males were used so it is possible that we may have seen an ethanol-induced conditioning deficit in females.

Although there is only one study examining the impact of developmental alcohol exposure on the phases of conditioning using the CPFE paradigm, there are a number of

studies that test how nicotine influences these phases. Since nicotine is an agonist for nicotinic acetylcholine receptors, administration of nicotine prior to specific phases of testing can speak to the effect of the cholinergic system on these phases. For example, nicotine administration prior to pretraining and testing, but not prior to training and testing, enhances freezing during testing, indicating that nicotine influences consolidation and retrieval of the context memory, but not the association of the context with the shock (Kenney & Gould, 2008). Furthermore, context learning is thought to rely on hippocampus-cortex circuits, whereas the context-shock association is thought to rely on hippocampus-amygdala circuits (Rudy et al., 2004). The data from Kenney & Gould (2008) suggest that nicotine administration impacts the hippocampus-cortex circuits, or even intra-hippocampal circuitry, instead of the hippocampus-amygdala circuits. Interestingly, Jablonski & Stanton (2014) show that alcohol exposure impairs consolidation or retrieval of the context-shock association, suggesting that there may be alcohol-induced dysfunction in hippocampus-amygdala circuits instead of hippocampus-cortex circuits (Rudy et al., 2004).

Indeed, there is research indicating that developmental alcohol exposure influences the amygdala. It is hypothesized that developmental alterations in amygdalar function may explain deficits in social behavior observed in individuals with FASD. Developmental alcohol exposure during all three trimester equivalent periods of development disrupts social recognition memory in both male and female rats and reduces oxytocin receptor binding in the amygdala in female rats (Kelly et al., 2009). Using the same alcohol exposure paradigm, Lugo et al. (2006) found increased met-enkephalin levels in the hypothalamus and decreased levels in the central nucleus of the

amygdala. Alcohol exposure during the third-trimester equivalent significantly alters social interactions in both males (5g/kg) and females (3 g/kg & 5 g/kg), while significantly reducing DNA concentrations in the amygdala of males only (Kelly & Dillingham, 1994). Exposure to alcohol during the third-trimester equivalent (PD 2-12) significantly altered the dopaminergic influence of inhibitory GABA-mediated transmission in the basolateral amygdala (Diaz et al., 2014). Using an intermittent alcohol exposure paradigm (5.0 g/kg/day on PD 7, 15, and 20), Balaszczuk et al. (2011) found that alcohol exposure induced significant apoptotic cell death in the central amygdala at PD 7 and 20. Altogether, these findings indicate that developmental exposure to alcohol significantly disrupts amygdalar function, likely through multiple systems, including oxytocin, opioids, and dopamine. It is possible that developmental alcohol exposure disrupts hippocampal-amygdalar connections, which in turn explains impairments in contextual fear conditioning as well as social recognition memory.

In summary, although there are a number of studies showing a deficit on the CPFE task in alcohol-exposed animals (Dokovna et al., 2013; Hamilton et al., 2011; Jablonski & Stanton, 2014; Murawski & Stanton, 2010; Murawski & Stanton, 2011), we did not find evidence for a deficit in this study. One likely explanation is the alcohol-exposed animals are especially sensitive to handling, and that daily injections (saline or galantamine) were able to attenuate alcohol-induced deficits in this experiment. From the literature, it is clear that alcohol exposure disrupts performance on this task, and recent data suggest that this is due to a failure in consolidation or retrieval of the context-shock association (Jablonski et al., 2014). This suggests that developmental alcohol exposure disrupts hippocampal-amygdala circuits (Rudy et al., 2004). This is consistent with

previous research indicating abnormal function of the amygdala (Diaz et al., 2014) and increased cell death in the central amygdala (Balaszczuk et al., 2011) in animals exposed to alcohol during the early postnatal period. Future studies could examine whether developmental alcohol exposure disrupts hippocampal-amygdala circuits, perhaps by measuring neurotransmitter release in both regions.

4.3 Immunohistochemical Findings

The hypothesis for the current studies was that developmental alteration of the acetylcholine system might account for changes in hippocampus-dependent learning, and these changes in the acetylcholine system were measured using immunohistochemistry. Since it was possible that behavioral testing itself could alter the cholinergic system, animals that underwent *in vivo* microdialysis served as controls in the current experiments. Postnatal alcohol exposure did not alter any of the cholinergic proteins measured in the animals that experienced microdialysis (Experiment 1). In addition, neither alcohol exposure, chronic galantamine, nor CPFE testing impacted expression of vAChT or α7 nAChR in the CA1 region of the ventral hippocampus.

There were changes in the expression of ChAT in the medial septum. Alcohol exposure significantly increased the average number of ChAT+ cells in the medial septum, but only in animals that displayed the context pre-exposure facilitation effect (CPFE). Furthermore, galantamine caused an overall reduction in ChAT+, but only in the pre-exposure group. In the no pre-exposure group, there were no changes in ChAT as a result of alcohol exposure or galantamine treatment. It is possible that alcohol-exposed animals up-regulate the production of ChAT in order to perform the task, and this may help to explain the lack of ethanol impairment on this specific behavioral paradigm.

Surprisingly, there was a significant reduction in ChAT+ in all groups as a result of chronic galantamine administration, but only in the animals that learned the CPFE. Moreover, this change in ChAT was not reflected in CPFE performance; a decrease in ChAT with other neurochemical components stable would be expected to impair performance. So, all animals learned to perform the CPFE, but galantamine did not significantly enhance or impair learning in any group. Thus, there is a disconnect between a neurochemical change and behavior. There are a few possibilities for why this might occur: 1) The CPFE task was not demanding enough to show an effect of galantamine, and 2) Galantamine administration interacts with another neurochemical component to negate the effects of decreased ChAT, and 3) The dose of galantamine was sufficient to induce a change in brain, but not behavior.

As to the first possibility, that the CPFE paradigm is not difficult enough to show an effect of galantamine, previous research has suggested that the CPFE paradigm is quite difficult, especially when compared to standard contextual fear conditioning. (Murawski & Stanton, 2010). In a study by Murawski & Stanton (2010),within the pre-exposure group, alcohol exposed animals displayed an average of about 5% freezing during testing, while controls displayed about 20% freezing. On average, the animals in this study froze about 20-50% of the time during the five minute testing period, which is significantly more than in previous studies (Jablonski et al., 2014; Murawski & Stanton, 2010; Murawski & Stanton, 2010). It is possible that something about the testing procedure elicited more freezing from all animals, covering up any impairment in alcohol-exposed animals. For example, the animals were transported into the testing

room by riding in an elevator, and that may have influenced behavior in some way, although they were given an hour to recover from transportation before testing.

Secondly, galantamine could interact with other neurochemical components of the hippocampus in a way that negated the effect of decreased ChAT. For example, in rats that learned to perform the CPFE task, galantamine could result in an increase in the efficiency of vesicular acetylcholine transporters, leading to an increase in the packaging of acetylcholine for release. This increase in acetylcholine release could result in an increase in GABAergic and glutamatergic signaling, due to the location of nAChRs on GABAergic and glutamatergic neurons. As a result, synaptic plasticity would be enhanced, leading to better consolidation and retrieval of the context-shock association and this could negate any impairment in performance due to the decrease in ChAT.

Finally, it is possible that the dose of galantamine used in the current studies was sufficient to cause a change in brain, but not behavior. The dose was chosen based on the literature that demonstrated galantamine's ability to rescue learning and memory deficits (0.1-5 mg/kg; Benetti et al., 2009; Jin et al., 2006; Luo et al., 2011; Woodruff-Pak et al., 2003). However, since there are a wide range of doses used in the literature, a dose of 2.0 mg/kg was chosen; it is within the range that was effective in the previous studies. Also, this dose was successful in increasing extracellular concentrations of acetylcholine (Exp. 1) and decreasing ChAT in animals that learned the CPFE (Exp. 2). Nevertheless, a different dose may have produced changes in behavior.

It is surprising that there was not an increase in vAChT in the PRE group, as previous data has shown significant correlation between ChAT and vAChT (van der Zee et al., 2011). However, ChAT was measured in the medial septum and vAChT was

measured in the hippocampus. Perhaps measuring ChAT in the hippocampus, at terminals, would have produced similar results as vAChT. While there were few changes in the *number* or *density* of cholinergic proteins as a result of developmental alcohol exposure, we cannot rule out the possibility that alcohol exposure disrupts the *function* of these proteins. In fact, developmental alcohol exposure alters the epigenome both globally and in the hippocampus (Otero et al., 2012; Perkins et al., 2013). Thus, alcohol exposure may influence the expression, through an epigenetic mechanism, of proteins involved in the synthesis, packaging, release, or detection of acetylcholine that were not measured here.

In addition, it was surprising that chronic administration of galantamine, an allosteric potentiating ligand at nicotinic receptors, did not alter the expression of α7 nAChRs in CA1. This is consistent with findings by Svedberg et al. (2004) who demonstrated up-regulation of CA1 and CA3 α4 receptor binding, but not α7 receptor binding (in either region) following 10 days of galantamine administration, indicating that there may not be an effect of galantamine on α7 nAChR expression, regardless of which region of the hippocampus is examined. There are also a few alternative possible explanations for this finding: 1) Galantamine induces altered α7 nAChR expression in other hippocampal sub regions and 2) α7 nAChRs are expressed on many neuronal subtypes and alcohol exposure causes differential changes in expression on these neuronal populations. In regards to the first possibility, there is little research on the effects of galantamine exposure on the expression of α7 nAChRs within the hippocampal sub regions except for the paper by Svedberg et al., 2004, so it is difficult to speculate on this possibility.

The second alternative explanation is more likely. The α7 nAChR is highly expressed throughout the hippocampus (Drever et al., 2011), not only on neurons, but also glial cells. In fact, the α7 nAChR can be found on microglia (Shytle et al., 2004) and hippocampal astrocytes (Sharma & Vijayaraghavan, 2001). Nicotinic receptors can also be found presynaptically on GABAergic (Drever et al., 2011) and glutamatergic (Fabian-Fine et al., 2001; Gray et al., 1996) neurons within the hippocampus. Since α7 nAChRs are expressed on so many different cell types within the hippocampus, it is possible that postnatal alcohol exposure did influence expression of α7 nAChRs, but only did so in some cell types. Thus, any effect of alcohol exposure was masked by a lack of change (or an opposite change) on other cell types. Future studies can examine the impact of alcohol exposure of α7 nAChRs by phenotyping the cells using double-labeled immunohistochemistry in order to address this possibility.

It is also possible that alcohol does indeed impact vAChT, α7 nAChR and ChAT but that the effects have normalized by adolescence. Since the acetylcholine system is known to regulate many aspects of brain development, such as neurite outgrowth, synaptogenesis, neurogenesis, and cell survival (Abreu-Villaca et al., 2011; Drever et al., 2011), any dysfunction during development could have significant consequences for neuronal function later on. In fact, a study by Nio et al. (1991) demonstrated an increase in muscarinic receptor binding in CA1 at PD 4, but not at PD 30. Perhaps there is a similar pattern for nicotinic receptors in CA1, where alcohol would cause significant changes in expression early in development, but these changes are not long lasting, thus explaining why we did not see any alcohol-induced changes in α7 nAChR expression in CA1.

The current experiments measured multiple proteins associated with the cholinergic system, but there are others that could be impacted by developmental exposure to alcohol. For example, the high-affinity choline uptake transporter which recycles choline from the extrasynaptic space could be measured (Sarter & Parikh, 2005). Alcohol exposure could cause a down-regulate of the expression of this transporter, leading to a deficit in choline uptake from the synaptic cleft.

On the other hand, alcohol exposure during development could impact the expression of other nicotinic receptor subtypes, such as $\alpha_4\beta_2$. This receptor is densely expressed throughout the hippocampus. Within area CA1 of the hippocampus, $\alpha_4\beta_2$ nAChRs make up 99% of the nAChRs (Perry et al., 2002), although this number varies widely by mouse strain (Gahring & Rogers, 2008). Galantamine administration for 10 days significantly up-regulates α/β-subunit (heteromeric), but not α7 homomeric, receptor binding in whole rabbit hippocampus (Woodruff-Pak et al., 2010). Similarly, Svedberg et al., (2004) found that galantamine administration (4 mg/kg/day for 10 days) significantly increased α4 receptor binding in CA1 and CA3 in mice. However, galantamine administration did not significantly alter α7 receptor binding in the hippocampus. Reid & Sabbagh (2008) compared α4β2 and α7 nAChR receptor binding in cortex, hippocampus, and striatum following 14 days of treatment with galantamine and found that galantamine (0.3 mg/kg and 0.9 mg/kg) induced significant up-regulation of α4β2 receptor binding in hippocampus and cortex, while only significantly up-regulating α7 receptor binding in the cortex. Taken together, these studies indicate that galantamine treatment affects expression of α4β2, but not α7, nAChRs in the hippocampus. Future studies should examine whether galantamine administration (PD 11-30) would significantly impact

α4β2 nAChRs, and whether this effect would be differentially affected by postnatal alcohol exposure.

In summary, we did not observe alcohol-induced changes in hippocampal vAChT or α7 nAChR density following microdialysis (Exp. 1) or behavioral testing (Exp. 3). There was also no effect of alcohol exposure on the average number of ChAT+ cells in animals who were tested in microdialysis or in animals that do not display the CPFE (No Pre group). Interestingly, among animals that do show the CPFE (Pre group), alcohol exposure significantly increased, while galantamine significantly decreased, the number of ChAT positive cells in the medial septum. These results suggest a specific effect of pre-exposure to the context, rather than a general impact of alcohol exposure or galantamine. Since alcohol exposed animals were able to perform the CPFE task (increased freezing in the Pre group) as well as controls, it is possible that there was an up-regulation in ChAT that enabled them to better associate the context and shock.

4.4 Postnatal Alcohol Exposure Significantly Alters Acetylcholine Efflux

The current experiments utilized a dynamic method of measuring neurotransmitter release (*in vivo* microdialysis) in an animal model of FAS. These are the first experiments describing acetylcholine release in the hippocampus following developmental alcohol exposure. In fact, very little research has been done using *in vivo* microdialysis, or other dynamic neurochemical techniques, in the FASD field. The results of these experiments provided three important pieces of information: 1) Alcohol exposure does not influence acetylcholine content at baseline, 2) Alcohol exposure significantly reduced the capacity for acetylcholine release, as measured by high K^+/Ca^{2+} administration, and 3) Alcohol exposure significantly increased extrasynaptic levels of

acetylcholine in response to an acute injection of galantamine. Taken together, these findings indicate significant disruption in hippocampal cholinergic signaling that may underlie alcohol-induced learning deficits. Since there were no baseline differences, it appears that alcohol-induced disruptions are only apparent after manipulation of the neurochemical environment (e.g. high K^+/Ca^{2+}, galantamine).

Administration of a high K^+/Ca^{2+} aCSF solution during microdialysis allowed us to examine the maximum *capacity* for acetylcholine release, as K^+ and Ca^{2+} should cause vesicles to dock and release their contents into the extrasynaptic space. Consistent with our hypotheses, K^+/Ca^{2+} significantly increased acetylcholine efflux in all animals (see *Figure 3.4*). Furthermore, animals exposed to alcohol during the early postnatal period had a reduction in K^+/Ca^{2+}-induced acetylcholine efflux (see *Figure 3.5*). Alcohol exposure also significantly impacted the cholinergic response to an acute injection of galantamine. Specifically, galantamine administration significantly increased extrasynaptic concentrations of acetylcholine, an effect that was greatly exaggerated in alcohol-exposed animals (see *Figure 3.7*).

Following microdialysis, alcohol-exposed animals did not exhibit any changes in ChAT in the medial septum nor did we see any changes in vAChT or α7 nAChR in the CA1 region of the hippocampus (see section *4.3*). It is surprising that we observed such large changes in acetylcholine content following postnatal alcohol exposure, but we did not see any changes in markers of the acetylcholine system. However, there are other cholinergic proteins that were not measured in this study that could account for the results, specifically the high-affinity choline uptake transporter (CHT). CHT is found presynaptically on cholinergic neurons, and is responsible for transporting choline into

the terminal for synthesis of acetylcholine. Expression of CHT in the prefrontal cortex is related to attentional processes (Sarter & Parikh, 2005). Furthermore, its expression has been shown to be related to spatial learning, as the administration of hemicholinium-3 (HC-3), which blocks CHT, disrupts spatial discrimination learning (Hagan et al., 1989). It is possible that alcohol exposure significantly reduces the expression of CHT in the hippocampus, impairing acetylcholine synthesis, and thus contributing to the observed deficit in K^+/Ca^{2+}-induced acetylcholine efflux observed in the current study. Furthermore, developmental alcohol exposure may disrupt the function, but not the number or density of the cholinergic proteins measured.

Alcohol exposure during development may also impact the production of acetylcholinesterase (AChE), an enzyme present in cholinergic synapses that functions to break down acetylcholine. Alcohol exposure may lead to a significant down-regulation of acetylcholinesterase production. In turn, galantamine is more effective as an AChE inhibitor in these animals, because there is less AChE to inhibit. This may serve to explain the alcohol-induced increase in extrasynaptic levels of acetylcholine observed following galantamine administration.

In summary, these data indicate a significant impact of developmental alcohol exposure on the hippocampal cholinergic system. Alcohol exposure decreases the *capacity* for acetylcholine release, while also enhancing acetylcholine in the presence of galantamine. We did not observe changes in cholinergic proteins that can explain the current results, although there are other cholinergic proteins likely involved (e.g. CHT, AChE). Perhaps alcohol exposure significantly reduces the production of CHT, leading to

decreased acetylcholine synthesis. In turn, there may be a down-regulation in the production of AChE to counteract the reduction in acetylcholine synthesis and release.

4.5 Working Model of Hippocampal Neurochemistry in Alcohol-Exposed Animals

To summarize, the current results indicate that developmental alcohol exposure significantly disrupts the development of the cholinergic system in the hippocampus, disturbing acetylcholine efflux in adolescence (PD 32-34). Furthermore, alcohol exposure did not impair performance on the CPFE, a hippocampus-dependent behavior. While the present studies do not describe alcohol-induced cholinergic dysfunction entirely, they provide an important piece of the puzzle. This section will review the research examining cholinergic dysfunction following developmental alcohol exposure, while incorporating the current results, as well as describe a proposed model of hippocampal neurochemistry in alcohol-exposed animals.

Alcohol-induced changes in neurochemistry within the hippocampus have been well documented using animal models (see *Section 1.6* for a summary), although there is still much work to be done. Understanding more about the neurochemical changes induced by developmental alcohol exposure can lead to targeted pharmacological treatments. Generally, research has focused on the GABA and glutamate systems within the hippocampus, due to their role in synaptic plasticity. However, developing pharmacological interventions targeting the GABA and glutamate systems is difficult, because of their location throughout the brain. Thus, targeting these systems would influence brain function in many brain regions, likely producing negative side effects. Drugs targeting the acetylcholine system, such as acetylcholinesterase inhibitors, have fewer negative side effects and are more promising as pharmacological treatments.

Presynaptically, there are a number of proteins involved in acetylcholine release. For example, ChAT is the enzyme that catalyzes the synthesis of acetylcholine, while vAChT packages acetylcholine into vesicles for release. The high affinity choline uptake transporter (CHT) is also situated on the presynaptic terminal and plays a vital function in that it retrieves choline from the synaptic cleft, from which acetylcholine is synthesized. In the synaptic cleft, acetylcholinesterase (AChE) is responsible for breaking down acetylcholine into its substituent parts: choline and acetyl-CoA. At the post synaptic site, there are many acetylcholine receptors, divided primarily into nicotinic and muscarinic. Both the nicotinic and muscarinic receptors have a variety of receptor subtypes that contribute to the variety of cholinergic signaling.

Alcohol exposure during development has been previously shown to impact some of these cholinergic markers, and data from the current experiments serves to help fill in the gaps. At the presynaptic level, alcohol exposure was shown to increase the expression of ChAT in the medial septum (the primary cholinergic input to the hippocampus) in the CPFE and galantamine experiment, but not the microdialysis experiment. Furthermore, this effect was only observed in animals that learned the context-shock association (PRE), suggesting that it may be due to the learning paradigm, and not a general effect of developmental alcohol exposure. The alcohol-exposed group did not have any changes in the expression of vAChT in the hippocampus.

At the postsynaptic level, previous experiments have shown alcohol-induced changes in the expression and binding of muscarinic acetylcholine receptors, but no study has examined nicotinic acetylcholine receptors. Specifically, Kelly et al. (1989) found that alcohol exposure during the third trimester equivalent (PD 4-10) induced a

significant increase in muscarinic receptor binding within the whole hippocampus in adult animals. Nio et al. (1991) demonstrated increased muscarinic receptor binding in CA3 at PD 4 and PD 30 and in CA1 at PD 4. Both of these studies used radioligands that did not distinguish between muscarinic receptor subtypes. In a more recent study, Monk et al. (2012) showed that alcohol exposure (PD 4-9) resulted in a significant reduction in M_1 and a significant increase in $M_{2/4}$ receptors in the dorsal hippocampus. However, no study has examined muscarinic receptor expression within the ventral hippocampus. The current studies indicate that alcohol exposure does not affect the expression of the α7 nAChR within area CA1 of the ventral hippocampus, although altered expression within the dorsal hippocampus or other hippocampal sub regions (e.g. dentate gyrus) cannot be ruled out. Furthermore, alcohol exposure may impact the expression of other nicotinic receptor subtypes.

In summary, the current studies, incorporated with previous findings, indicate that the development of the hippocampus is severely impacted by alcohol exposure. The hippocampal acetylcholine system, in particular, appears to be sensitive to alcohol. A proposed model for alcohol-induced cholinergic dysfunction within the hippocampus can be found in *Figure 4.1*. Briefly, developmental exposure to alcohol has been shown previously to cause a reduction in overall acetylcholine content (whole brain; Rawat, 1977) as well as leading to an increase in muscarinic receptor binding. Some of these studies indicate that the effects of alcohol exposure on the muscarinic system in the hippocampus are region specific (e.g. CA1 vs. CA3; Nio et al., 1991), as well as being sensitive to the age at which the tissue was taken (early postnatal period: Nio et al., 1991; adulthood: Kelly et al., 1989). The current experiments add to the story by measuring α7

nicotinic acetylcholine receptors, as well as focusing on the ventral hippocampus. In conclusion, the results show that developmental alcohol exposure does not alter the expression of ChAT (see section *4.3* for full description), vAChT, or α7 nAChR. However, developmental alcohol exposure significantly disrupts hippocampal acetylcholine efflux and extrasynaptic concentrations of acetylcholine (Exp. 1). These results represent a novel contribution to the research on the neurochemical impact of developmental alcohol exposure.

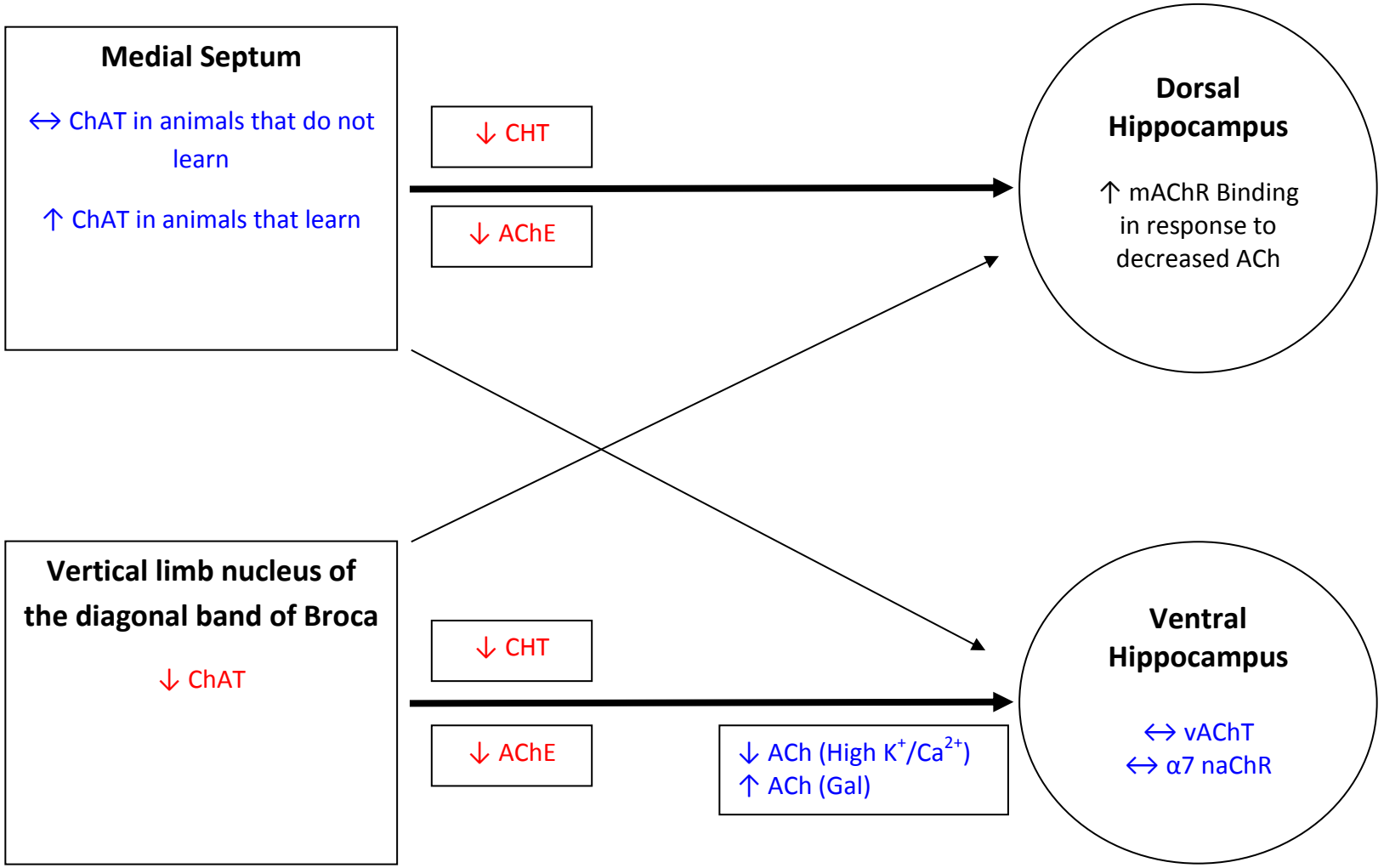

Figure 4.1 Proposed Model of Alcohol-Induced Changes to the Septohippocampal Acetylcholine System. Previous research has shown that the medial septum provides the main cholinergic input to the dorsal hippocampus, while the vertical limb nucleus of the diagonal band of Broca provides the main cholinergic input to the ventral hippocampus (represented by the size of the arrows). Black text indicates findings from published research studies. Blue text indicates results from the current experiments (↔ indicates no change). Red text indicates hypothetical changes to the cholinergic system as a result of developmental alcohol exposure. The current experiments indicate that in alcohol-exposed animals that do not show learning (No Pre), there are no changes in ChAT, α7 nAChR, or vAChT. However, alcohol-exposed animals that learn the CPFE (Pre) show an up-regulation in ChAT, therefore increasing the amount of acetylcholine available. A down-regulation in acetylcholine synthesis via a decrease in the expression CHT would lead to a decrease in acetylcholine release (Exp 1). A related down-regulation in the production of AChE would help to offset decreased ACh release. **Abbreviations:** acetylcholine (ACh), acetylcholinesterase (AChE), choline acetyltransferase (ChAT), context pre-exposure facilitation effect (CPFE), galantamine (Gal), high affinity choline transporter (CHT), muscarinic acetylcholine receptor (mAChR), Pre-exposure (Pre), nicotinic acetylcholine receptor (nAChR), No Pre-exposure (No Pre), vesicular acetylcholine transporter (vAChT)

4.6 Limitations of Experimental Design

While every effort was made to have a balanced experimental design, there were some limitations to the current experiments. First, sex differences were not examined. Murawski & Stanton (2010) did not find sex differences in CPFE testing, but pilot studies from our laboratory suggested there may be sex differences in this behavior. However, examination of sex differences was just not feasible in this experiment, due to the cost and number of animals required to complete such experiments. The females from each litter were assigned to other experiments.

In addition, it would have ideal to have a group of animals not exposed to microdialysis (Experiment 1) or the behavioral testing procedures in Experiment 3 in order to fully characterize the impact of galantamine and alcohol on the developing cholinergic system. We observed an up-regulation of ChAT in alcohol exposed animals, but only in those that learned the CPFE task (Pre) indicating that learning itself changed the expression of ChAT. However, we did not observe any changes in cholinergic proteins in the no pre-exposure group or in the animals exposed to microdialysis.

There are also limitations with the neuroanatomical measures. Immunohistochemistry was chosen as the technique to measure cholinergic proteins. Other techniques such as western blotting and in situ hybridization could have been used, and future studies could confirm and extend the current results by exploring other methods for the measurement of these proteins. Using immunohistochemistry has many advantages, such as the ability to localize proteins. However, immunohistochemical staining can be hard to quantify, especially if the protein of interest is located at the synapse (vAChT and α7 nAChR). For this reason, we had to measure immunoreactivity

for vAChT and α7 nAChR using densitometry, making it difficult to detect subtle changes in expression.

Finally, for the microdialysis study, probe placement was off-target in a majority of the alcohol-exposed animals. Surgery was completed for eight animals, but only three animals were used in the final data. One animal was removed as an outlier because of abnormal behavior (e.g. vocalizing and excessive activity) in the home cage. The other animals were removed because the probe was located on the border between the hippocampus and cortex. HPLC was conducted for these samples, but the acetylcholine content was significantly different from the three animals whose probes were in the correct location, so they were removed from analysis. The poor probe placement is likely due to differences in the size of the brain following developmental alcohol exposure, and future studies should use different coordinates for alcohol-exposed animals in order to accurately sample from the region of interest.

4.7 Future Directions

While the current studies focused on learning and memory impairments in animal models of Fetal Alcohol Syndrome, a variety of behavioral processes are negatively affected by developmental alcohol exposure, such as attention and executive control. The prefrontal cortex is involved in attention processes, as well as being important for executive control (Sarter & Parikh, 2005). Furthermore, the prefrontal cortex receives dense cholinergic input that is involved in attention (reviewed in Sarter & Parikh, 2005). Future studies should measure acetylcholine efflux in the prefrontal cortex in alcohol-exposed animals in an effort to understand more about alcohol-related attention deficits.

Within the hippocampus, there is still much to do to understand how developmental alcohol exposure disrupts cholinergic neurotransmission. First of all, future research should measure the expression of cholinergic proteins not covered by these experiments (e.g. CHT, AChE, and other nicotinic receptor subtypes). Second, acetylcholine efflux should be measured in other hippocampal sub regions that are known to be impacted by developmental alcohol exposure (e.g. CA3). Third, *in vivo* microdialysis techniques should be paired with behavioral testing to begin to understand how these changes in neurotransmitter release influence learning and memory processes. Finally, electrophysiological techniques could be paired with pharmacological manipulations of the acetylcholine system (e.g. MLA administration to block α7 nAChRs, muscarinic antagonists) to determine how alcohol exposure and the cholinergic system interact to influence synaptic plasticity.

These experiments would provide important information on the neurochemical impact of developmental alcohol exposure. Knowing more about the effect of alcohol exposure on the neurochemical environment within the hippocampus will illuminate possible pharmacological interventions for individuals affected by Fetal Alcohol Spectrum Disorders. There are a number of cholinergic drugs available that act presynaptically to affect the synthesis and packaging of acetylcholine (e.g. AChE inhibitors, choline) and postsynaptically on the nicotinic (e.g. choline, lobeline, varenicline) and muscarinic acetylcholine receptors (e.g. choline, pilocarpine). By identifying which part(s) of the cholinergic system are impacted by developmental alcohol exposure, more targeted preclinical experiments can be conducted to determine if

any of these cholinergic drugs can be effective treatments for learning and memory deficits.

4.8 Summary

In summary, the results presented here represent an important contribution to the field of Fetal Alcohol Syndrome research. The neuroanatomical experiments did not yield very many positive results, but it is still important to know what cholinergic proteins *are not* impacted by developmental alcohol exposure, in order to fully describe the acetylcholine system in this animal model. We also determined that chronic galantamine treatment did not enhance learning and memory on the CPFE task, although the effects of acute galantamine treatment are unknown. These are the first experiments to use *in vivo* microdialysis to begin to understand the neurochemical impact of developmental alcohol exposure within the hippocampus. Future studies are needed to fully describe the hippocampal acetylcholine system and begin to understand how these changes can influence learning and memory. We found that alcohol exposure significantly decreased the capacity for acetylcholine release, while also significantly increasing acetylcholine content following an acute galantamine injection. These data indicate that while alcohol-exposed animals do not exhibit reduced acetylcholine efflux at baseline, when the acetylcholine system is manipulated in some way, we begin to see significant disruption in cholinergic neurotransmission. Taken together, these data begin to explain the learning and memory deficits that are commonly observed in animals exposed to alcohol during development and indicate that the cholinergic system is indeed a good target for the development of effective pharmacological intervention for these deficits.

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