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The effects of postnatal alcohol exposure and galantamine on the context pre-exposure facilitation effect and acetylcholine efflux using *in vivo* microdialysis

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Abstract

Fetal alcohol spectrum disorders (FASD) affect 2–5% of children. FASD have been shown to cause damage to multiple brain regions, but damage to the hippocampus specifically may explain deficits in learning and memory that are hallmark symptoms of FASD. The acetylcholine neurotransmitter system is a major input to the hippocampus and is a possible target of developmental alcohol exposure. Alcohol (3.0 g/kg/day) was administered via intragastric intubation to developing male rat pups (postnatal day [PD] 2–10; ethanol-treated [ET]), with controls receiving a sham intubation (IC) or no treatment (NC). In Experiment 1, *in vivo* microdialysis was used to measure acetylcholine efflux in adolescents (PD 32–35). During microdialysis, the effects of a high K^+/Ca^{2+} aCSF solution (PD 32–33) and an acute galantamine (acetylcholinesterase [AChE] inhibitor) injection (2.0 mg/kg; PD 34–35) on acetylcholine efflux were measured. Alcohol-exposed animals did not differ in acetylcholine efflux at baseline. However, alcohol-exposed animals had a decrease in K^+/Ca^{2+} -induced acetylcholine efflux compared to non-treated controls, and an enhanced acetylcholine response to galantamine compared to both control groups. Experiment 2 tested whether chronic administration of galantamine (2.0 mg/kg; PD 11–30) could attenuate alcohol-induced learning deficits in the context pre-exposure facilitation effect (CPFE; PD 30–32). Neither chronic galantamine nor postnatal alcohol exposure influenced performance in the CPFE task. Immunohistochemistry was used to measure expression of choline acetyltransferase (ChAT; medial septum), vesicular acetylcholine transporter (vAChT; ventral CA1), and the $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR; ventral CA1) following microdialysis (Exp. 1) or chronic galantamine and behavioral testing (Exp. 2). Neither alcohol exposure nor behavioral testing significantly altered the density of vAChT or $\alpha 7$ nAChRs in the ventral CA1 region of the hippocampus. The average number of

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ChAT⁺ cells was increased in the ET animals that displayed the context-shock association; there were no changes in the IC and NC animals that learned the context-shock association or in any of the animals that were in the control task that entailed no learning. 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.

Keywords

fetal alcohol spectrum disorder; context pre-exposure facilitation effect; galantamine; *in vivo* microdialysis

Introduction

Alcohol is a well-known teratogen, and the effects of developmental alcohol exposure are collectively referred to as fetal alcohol spectrum disorders (FASD) (Sokol, Delaney-Black, & Nordstrom, 2003). FASD include fetal alcohol syndrome (FAS) at the severe end and alcohol-related neuro-developmental disorders (ARNDs) at the less severe end of the spectrum (Astley, 2011; Sokol et al., 2003). FAS is a significant public health concern, representing an estimated annual cost of 3.6 billion dollars in the United States (Lupton, Burd, & Harwood, 2004). Even with the wealth of information available about the negative effects of developmental alcohol exposure, about 12.2% of pregnant women consume alcohol, with 1.9% engaging in binge drinking (CDC, 2009), indicating a significant need for therapeutic interventions that can be administered after alcohol exposure has occurred.

Rodent models have demonstrated that certain brain regions such as the hippocampus (reviewed in Berman & Hannigan, 2000) and cerebellum (see Norman, Crocker, Mattson, & Riley, 2009 for review) are especially sensitive to alcohol. This sensitivity remains even when alcohol exposure is limited to the period equivalent to the third trimester in humans (PD 1–10), referred to as the brain growth spurt (Bayer, Altman, Russo, & Zhang, 1993). Within the hippocampus, developmental alcohol exposure reduces the number of cells in area CA1 (Barnes & Walker, 1981; Bonthius & West, 1990, 1991; Greene, Diaz-Granados, & Amsel, 1992; Tran & Kelly, 2003), alters mossy fiber distribution (Fukui & Sakata-Haga, 2009; West & Hodges-Savola, 1983), alters dendritic spine density (Abel, Jacobson, & Sherwin, 1983; Tarelo-Acuña, Olvera-Cortés, & González-Burgos, 2000; West, 1990), and disrupts hippocampal function, as measured by electrophysiology (Hablitz, 1986; Sutherland, McDonald, & Savage, 1997; Swartzwelder, Farr, Wilson, & Savage, 1988; Tan, Berman, Abel, & Zajac, 1990; Varaschin, Akers, Rosenberg, Hamilton, & Savage, 2010). Furthermore, alcohol-induced behavioral deficits are often seen in tasks that rely on the hippocampus, such as spatial learning (Cronise, Marino, Tran, & Kelly, 2001; Goodlett & Johnson, 1997; Kelly, Goodlett, Hulsether, & West, 1988) and contextual fear conditioning (Allan, Chynoweth, Tyler, & Caldwell, 2003; Jablonski & Stanton, 2014; Murawski & Stanton, 2010, 2011). Elucidation of the neurochemical changes in this brain region

associated with alcohol exposure may help identify 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), and provides substantial input to the hippocampus via the septohippocampal pathway (Abreu-Villaça, Filgueiras, & Manhães, 2011; Drever, Riedel, & Platt, 2011). Acetylcholine is synthesized in the cytoplasm of cholinergic neurons via the action of ChAT and packaged into vesicles by vesicular acetylcholine transporter (vAChT) (Holler, Berse, Cermak, Diebler, & Blusztajn, 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 acetylcholine synthesis and neurotransmission. There are two types of ACh receptors: nicotinic (nAChR) and muscarinic (mAChR). Each of these receptors has multiple subtypes with differential distribution throughout the brain. For example, the $\alpha 7$ and $\alpha 4\beta 2$ nAChRs and the M1, M2, and M4 mAChRs are found at high levels in the hippocampus (Drever et al., 2011).

Few studies have examined the effects of developmental alcohol exposure on the cholinergic system, but those that have been conducted support the hypothesis that alcohol exposure disrupts the cholinergic system in the hippocampus. Prenatal alcohol exposure reduced acetylcholine content in fetal (GD 18 and 21) and neonatal whole brain (PD 5 and 10) (Rawat, 1977), and increased muscarinic receptor binding in CA3 (PD 4 and 30) and CA1 (PD 4) (Nio, Kogure, Yae, & Onodera, 1991). Alcohol administration (PD 4–10) increased the number of muscarinic receptors in the hippocampus in adulthood (Kelly, Black, & West, 1989). In a recent study, Monk, Leslie, & Thomas (2012) found that postnatal alcohol exposure (PD 4–9) significantly reduced the density of M₁ receptors and increased the density of M_{2/4} receptors in the hippocampus. Together, these results clearly demonstrate an alcohol-related increase in muscarinic receptor binding in the hippocampus, suggesting impairments in the cholinergic system, but few studies have examined alcohol-related changes in nicotinic acetylcholine receptors. In addition, choline administration has been repeatedly shown to attenuate learning deficits caused by postnatal alcohol exposure (Ryan, Williams, & Thomas, 2008; Thomas, Biane, O'Bryan, O'Neill, & Dominguez, 2007; Thomas & Tran, 2012), and part of the mechanism of choline could be via alterations of the cholinergic system. However, choline administration does not ameliorate motor coordination deficits caused by developmental exposure to alcohol (Thomas, O'Neill, & Dominguez, 2004), indicating that exploration of other therapeutic interventions is needed.

Galantamine is an acetylcholinesterase inhibitor that has been used to treat cognitive deficits associated with Alzheimer's disease (Ago, Koda, Takuma, & Matsuda, 2011). Galantamine is also an allosteric potentiating ligand at nicotinic acetylcholine receptors, where it is believed to bind to a specific site extracellularly on the N-terminal domain of nAChRs (Schrattenholz, Pereira, Roth, Weber, Albuquerque, et al., 1996), and potentiate the response of nAChRs to suboptimal levels of acetylcholine (Schrattenholz et al., 1996). Furthermore, galantamine protects against excitotoxic cell death (Kihara, Sawada, Nakamizo, Kanki, Yamashita, et al., 2004; Takada-Takatori, Kume, Sugimoto, Katsuki, Sugimoto, et al., 2006), is anti-apoptotic (Takada-Takatori et al., 2006), increases neurotrophic factor mRNA

(Kita, Ago, Takano, Fukada, Takuma, et al., 2013), and facilitates neurogenesis (Jin, Xie, Mao, & Greenberg, 2006). Importantly, many of these are also affected by developmental alcohol exposure, indicating that galantamine may be useful not only for the treatment of alcohol-induced learning deficits, but may help to counteract some of the negative effects of alcohol on the developing brain.

The context pre-exposure facilitation effect (CPFE) is a variation of contextual fear conditioning in which animals that are pre-exposed to the environment in which they will be shocked show enhanced freezing during testing (Rudy, Huff, & Matus-Amat, 2004). Performance in the CPFE paradigm is improved by acetylcholine agonists (e.g., nicotine; Kenney & Gould, 2008), suggesting a role for the acetylcholine neurotransmitter system in this form of learning. Furthermore, developmental alcohol exposure has been shown to impair performance on the CPFE task (Klintsova, Helfer, Calizo, Dong, Goodlett, et al., 2007; Murawski et al., 2010, 2011).

The current studies used an animal model of exposure to alcohol during the third trimester to test the overall hypothesis that developmental alcohol exposure causes a decrease in cholinergic function in the hippocampus. Alcohol was administered during early postnatal development, in a period equivalent to the third trimester in humans (PD 2–10). The purpose of this model is to mimic exposure to high levels of alcohol during the third trimester, a time when the hippocampus is undergoing rapid development (Bayer et al., 1993) and seems to be especially sensitive to alcohol. 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. These hypotheses were tested in three experiments. The first experiment measured acetylcholine efflux using *in vivo* microdialysis, with the hypothesis that animals exposed to alcohol during the hippocampal growth spurt (PD 2–10; Bayer et al., 1993) would have a significant reduction in hippocampal acetylcholine efflux. The second experiment tested the hypothesis that galantamine administration would attenuate alcohol-induced impairments in the context pre-exposure facilitation effect, a learned effect dependent in part upon the hippocampus. In the final experiment, immunohistochemistry was used to measure the expression of two cholinergic proteins in the hippocampus (vesicular acetylcholine transporter [vAChT] and the $\alpha 7$ nicotinic acetylcholine receptor [$\alpha 7$ nAChR]) and the expression of ChAT in the medial septum, with the hypothesis that developmental alcohol exposure would reduce ChAT in the medial septum and vAChT in the hippocampus, indicating impaired acetylcholine synthesis and release. Furthermore, we expected an increase in the expression of $\alpha 7$ nAChR to offset the reduction in acetylcholine synthesis and release.

Materials & methods

2.1 General methods

For these experiments, male 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 °C with a 12-h:12-h light:dark cycle (lights on at 7:00 AM). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina. Untimed (visibly) pregnant dams were purchased from Harlan

(Indianapolis, IN) and singly housed throughout pregnancy in polypropylene cages with bedding. Food and water were available *ad libitum*. All chemicals used in the experiments were from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

2.1b Pup Treatment—The day of birth (~GD 23) was designated as PD 1, and no treatments occurred on this day. Rat pups within a litter were assigned quasi-randomly to one of the three treatment groups, so that no more than one pup per litter was assigned to a particular group in a particular experiment. There were three treatment groups: ethanol-treated (ET), intubated control (IC), and non-treated control (NC). Pup treatment occurred during PD 2–10 at approximately 9:00 AM and 11:00 AM. Pups in the ET group were intubated daily with 3.0 g/kg of ethanol in 27.8 mL/kg of enriched milk (West, Hamre, & Pierce, 1984), using Intramedic™ PE 10 tubing dipped in corn oil. Two hours later (± 15 min), ET pups were given a second intubation of milk alone in order to account for lack of feeding during intoxication. IC pups were sham intubated daily using identical procedures, except that no solutions were administered. During PD 2–7, pups were identified using non-toxic permanent marker. On PD 7, all pups were tattooed for identification throughout the rest of the experiment (Animal Identification & Machine Systems, Inc.). Pups were housed with their dams until PD 21, at which time they were weaned and group-housed (2–3 animals per cage) 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- μ L blood samples were collected from ET and IC pups from a small nick in the tail 2 h after intubation in order to assess maximum BACs (Marino, Cronise, Lugo, & Kelly, 2002). Blood was taken from the intubated control animals to control for the stress of the blood sampling procedures. All blood samples were placed into 190 μ L of 0.53 N perchloric acid, neutralized with 200 μ L of 0.30 M potassium carbonate, vortexed, and centrifuged ($8700 \times g$). Supernatant was separated and frozen at -80°C until time of assay. At the time of sampling, standard ethanol samples with specific BACs (0–600 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).

2.2 Experiment 1: Microdialysis and characterization of hippocampal acetylcholine efflux

The purpose of Experiment 1 was to describe basal, potassium/calcium-stimulated, and galantamine-induced 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 would be no difference between groups in basal acetylcholine release, but when the acetylcholine system was stimulated, ET animals would show a decrease in acetylcholine release. To accomplish this goal, the following procedures were used:

2.2a Surgical procedures—Animals were handled daily 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 (BAS, West Lafayette, IN, USA) unilaterally in the ventral hippocampus, using the following coordinates relative to bregma: AP: -4.8 mm, 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 Experiment 2. Animals were allowed to recover for 2 days (~PD 28–29). During this time, animals were habituated to the microdialysis chambers (36 cm × 36 cm round bottom Plexiglas® cage; BAS, West Lafayette, IN) 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 chambers and placed back into the animal colony before the beginning of the dark cycle. A window of 2 days was given for each procedure (e.g., surgery, habituation) due to time constraints on 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), stylets were removed and microdialysis probes with a semipermeable membrane (BAS, West Lafayette, IN) were inserted into the guide cannulas. The microdialysis probes extended 2.0 mm beyond the tip of the guide cannula. Probes were continuously perfused with aCSF (2.0 µL/min) containing the following (mM): NaCl, 150.0; D-glucose, 5.0 (Fisher Scientific, Pittsburgh, PA); KCl, 3.0; CaCl₂, 1.7 (Fisher Scientific, Pittsburgh, PA); MgCl₂, 0.9; and an acetylcholinesterase inhibitor (neostigmine bromide, 50 nM; Moore, Stuckman, Sarter, & Bruno, 1996).

For the high potassium (K⁺)/high calcium (Ca²⁺) artificial cerebrospinal fluid solution, the concentrations were as follows (in mM): NaCl, 53.0; D-glucose, 5.0; KCl, 100.0; CaCl₂, 5.0; MgCl₂, 0.9 (containing neostigmine bromide, 50 nM). Neostigmine bromide was included in the aCSF for both microdialysis experiments, in order to allow reliable detection of baseline acetylcholine levels to be able to quantify the magnitude of the change in acetylcholine release following each manipulation. Three hours after the insertion of the probe, dialysate collection began, and occurred every 15 min. For the first microdialysis session (high K⁺/Ca²⁺), there were 10 dialysate collections: 4 baseline, 2 high K⁺/Ca²⁺, and 4 post-stimulation.

Two days following the first microdialysis session, the effects of an acute galantamine injection were measured. Briefly, 4 baseline samples were collected. Then, a subcutaneous injection of galantamine (2.0 mg/kg; Tocris Biosciences, Bristol, UK) 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 would increase acetylcholine release, and determine whether galantamine would differentially affect the alcohol-exposed animals. Dialysates were stored at –80 °C until analysis by high-performance liquid chromatography with electrochemical detection (HPLC-EC).

Within two days of microdialysis, animals were transcardially perfused with 4% paraformaldehyde. The brains were removed and post-fixed in 4% paraformaldehyde overnight, followed by 15% sucrose in 0.1 M tris-buffered saline overnight, and finally 30% sucrose in 0.1 M tris-buffered saline overnight. Brains were sectioned on a freezing microtome (Thermo Scientific; 40 µm) using serial sectioning procedures. Sections were

stored in anti-freezing solution (30% sucrose/30% glycerol in 0.1 M PB) at -20°C until time of staining (Experiment 3). Probe placement was verified using immunohistochemistry for ChAT (Experiment 3). Briefly, probes were classified as being correctly placed if they were located in the ventral CA1 region of the hippocampus (Paxinos & Watson, 1986; plates 38–42). Only animals whose probes were located in the correct brain region were used for high-performance liquid chromatography (HPLC) analyses.

2.2c High-performance liquid chromatography with electrochemical detection

—High-performance liquid chromatography with electrochemical detection (HPLC-EC) was used to quantify acetylcholine concentrations in dialysates. To do this, a dialysate sample (20 μL) was injected into the HPLC solvent delivery system (Bioanalytical Systems PM-92) that was coupled to a Bioanalytical Systems Epsilon electrochemical detector. Acetylcholine was separated from choline using an analytical column (Eicompak AC-GEL 2.0×150 mm; Eicom, San Diego, CA) with a mobile phase (pH 8.5). The mobile phase contained sodium 1-decanesulfonate (1.64 mM) and potassium bicarbonate (50 mM). After separation, the acetylcholine was broken down by acetylcholinesterase and choline oxidase using an acetylcholine enzyme reactor (Eicom, AC-Enzypak II) to generate stoichiometric quantities of hydrogen peroxide in a post-column derivatization step. Finally, current corresponding to acetylcholine and choline peaks was detected at a peroxidase-coated glassy carbon electrode. Acetylcholine concentrations were determined using chromatographic peaks compared with a standard curve (Stanley & Fadel, 2012).

2.3 Experiment 2: 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 was 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 $3 \times 2 \times 2$ (treatment $\times$ drug $\times$ testing condition) design, creating 12 experimental groups.

2.4a Drug administration—Beginning on PD 11, galantamine (2.0 mg/kg/day, subcutaneous [s.c.]) or isovolumetric vehicle (0.9% saline, s.c.) was administered daily, and this administration lasted until behavioral testing began on PD 30. Drug administration occurred at the same time every morning ($\sim 11:00$ AM $\pm$ 30 min).

2.4b Behavioral testing – apparatus—Behavioral testing was conducted in Plexiglas® boxes ($46 \times 24 \times 22$ 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 for ventilation. The stainless-steel rods were attached to a shock apparatus (Coulbourn Instruments; Allentown, PA) for delivering foot shocks (1.5 mA, 2 sec.). Behavioral testing was recorded by a video camera placed in the sound-attenuating chamber; videos were saved on a desktop computer. The computer was equipped with FreezeScan (CleverSys, Inc., Reston, VA), a program that can track and register freezing behavior in the testing apparatus. This behavioral testing paradigm required two distinct contexts. Context A consisted of the testing box described above, with no

modifications. Context B was a modification of the Plexiglas® box used for Context A. It contained a mesh floor to provide distinct somatosensory cues. The outside of the Plexiglas® box was covered with paper containing distinct patterns (black and white stripes, black and white checkerboard). In addition, between animals, context A was cleaned with a 5% ammonium hydroxide solution, while context B was cleaned with 70% ethanol to provide distinct olfactory cues.

2.4c Behavioral testing – protocol—Context pre-exposure facilitation effect testing occurred on PD 30–32, with pre-exposure on PD 30, training on PD 31, and testing on PD 32 (Murawski et al., 2010); see Fig. 4A for a schematic diagram of the testing paradigm. There were two conditions for behavioral testing: pre-exposure (PRE) and no pre-exposure (NO PRE). Animals in the PRE condition experienced pre-exposure in context A, while animals in the NO PRE condition experienced the pre-exposure phase in context B. The pre-exposure session was 5 min in duration. Twenty-four hours later (PD 31), all animals were trained in context A. During training, animals were given a brief (1.5 mA, 2 sec) foot shock, after which they were immediately removed from the context. Then, 24 h later (PD 32), animals were placed in context A and freezing was recorded for 5 min. This is the testing phase. In addition, freezing was measured during the pre-exposure phase to measure possible baseline differences in freezing that could explain differences during testing.

2.4 Experiment 3: Examination of hippocampal cholinergic immunoreactivity

Immunohistochemistry was used to identify cholinergic proteins and to examine group differences in hippocampal expression of these proteins (ChAT, vAChT, and $\alpha 7$ 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 and learning on these cholinergic proteins was examined using tissue from Experiment 2 (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 $\alpha 7$ nAChR. Within 2 days of the conclusion of experimental procedures, animals were perfused and brain tissue was sectioned and stored at -20°C until time of staining (see section 2.2b for details).

Sectioned tissue (40 μm) containing the ventral CA1 regions of the hippocampus (vAChT and $\alpha 7$ nAChR) or medial septum (ChAT) was processed through immunohistochemical procedures to assess expression of cholinergic proteins: ChAT, vAChT, and $\alpha 7$ nAChR. The antibodies that were used were: rabbit anti-ChAT (AB 143, Millipore Corp., Temecula, CA), goat anti-vAChT (AB 1588, Millipore Corp., Temecula, CA), rabbit anti-nicotinic acetylcholine receptor $\alpha 7$ (AB 23832, 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). Specificity of antibodies was verified from references provided on the manufacturer websites. Knockout mice for ChAT (Brandon, Lin, D'Amour, Pizzo, Dominguez, et al., 2003) and vAChT (de Castro, De Jaeger, Martins-Silva, Lima, Amaral, et al., 2009) are non-viable postnatally, but we observed the expected

pattern of staining. Mielke and Mealing (2009) verified the specificity of the alpha7 nAChR antibody using Western blotting and a blocking peptide.

Briefly, sections were washed in Tris-buffered saline (TBS), followed by rinsing in methanolic peroxide. Sections were then blocked and membranes were permeabilized by rinsing in TBS with Triton-X and horse serum (Fisher Scientific, Pittsburgh, PA). Tissue was incubated overnight with ChAT (1:1250), vAChT (1:5000), or nicotinic acetylcholine receptor alpha7 (1:1000) primary antibodies. Tissue was incubated for another day at 4 °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 α 7 nAChR) for 1.5 h. After rinsing in TBS, tissue was incubated with peroxidase-conjugated streptavidin for 1 h. Immunoreactivity was visualized using a nickel-cobalt enhanced diaminobenzidine tetrahydrochloride (DAB) reaction (Fisher Scientific).

Sections were mounted on gelatin-coated slides, and processed through a series of increasing concentrations of ethanol followed by HistoClear (Fisher Scientific) and xylene rinses before cover slipping with Cytoseal (Thermo Scientific). The ventral CA1 region of the hippocampus 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 animal 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 ChAT, immunoreactivity was measured at 20 $\times$ magnification using a Nikon E600 microscope with Neurolucida software (MBF Bioscience, Williston, VT). 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 20 $\times$ using a Nikon E600 microscope with IPLab software (Scanalytics, Inc., Fairfax, VA). Images were processed for densitometry analysis using ImageJ (Schneider, Rasband, & Eliceiri, 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 interpret and analyze, all values were expressed as a percent of total staining using a maximum density value of 5000, so that higher number indicate more dense patterns of labeling.

2.5 Statistical analyses

All statistical analyses were completed using 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, because there were some differences in freezing during pre-exposure.

To analyze group differences in freezing, a $3 \times 2 \times 2$ -way (treatment $\times$ drug $\times$ testing condition) ANOVA was used. Immunohistochemistry data were analyzed using a one-way ANOVA with treatment as the variable (Experiment 1) or a $3 \times 2 \times 2$ -way (treatment $\times$ drug $\times$ testing condition) ANOVA (Experiment 3). Tukey's HSD *post hoc* tests were used when necessary.

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 Fig. 1). 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). The assumption of sphericity was violated, 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 there was no main effect of treatment or interaction between age and treatment, indicating that all animals gained weight during the first 10 days, but that alcohol treatment did not significantly impact weight gain. All animals were weighed again before surgical procedures (PD 27–28) and before the second microdialysis session (PD 33–34). One-way ANOVAs with treatment as a factor were used to analyze body weights. There was no effect of treatment on weight on the day of surgery or the second microdialysis session. All physical parameters and BAC data for this experiment can be found in Table 1.

3.1b High-performance liquid chromatography: the effects of a high K^+/Ca^{2+} manipulation—Surgeries and microdialysis were conducted on 8 animals per group. Following HPLC and analysis of probe placement, the NC, IC, and ET groups contained 7, 6, and 3 animals, respectively (see Fig. 1). In order to ensure that the effects found were reliable in spite of the small sample sizes, effect sizes are reported for each test. To analyze the impact of a high K^+/Ca^{2+} administration, repeated-measures ANOVA was used with time point (4 baseline, 2 high K^+/Ca^{2+} , and 4 post-stimulation) 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 animal from each of the IC and NC groups was removed from analysis because of undetectable baseline acetylcholine values.

There were no group differences in baseline acetylcholine efflux as measured in pmol/20 μ L ($F[3,39] = 0.68, p = 0.57$; see Table 2). Analysis of the dialysis data as converted into percent of baseline indicated a significant effect of time point ($F[9,117] = 8.56, p < 0.001, \eta^2 = 0.41$). As expected, the administration of a high K^+/Ca^{2+} aCSF led to a significant increase in acetylcholine efflux in all animals. 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 two 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 ($F[1,8] = 7.62, p = 0.025, \eta^2 = 0.49$), as well as the first post-stimulation

collection ($F[1,7] = 8.24, p = 0.024, \eta^2 = 0.54$). By the second post-stimulation collection, the difference between the ET and NC groups had disappeared ($F[1,8] = 3.27, p = 0.11, \eta^2 = 0.29$). In both cases, the high K^+/Ca^{2+} -induced increase in acetylcholine efflux was smaller in the ethanol-exposed animals (see Fig. 2). Comparisons between the ET and IC groups at these three time points revealed no significant differences (p 's > 0.20).

3.1c High-performance liquid chromatography: the effects of acute galantamine

—For the second microdialysis session, there were 4 baseline collections, followed by an acute galantamine injection (2.0 mg/kg; s.c.) and 8 post-injection collections. Again, analysis of the acetylcholine efflux in pmol/20 μ L at baseline revealed no differences among groups ($F[3,33] = 0.22, p = 0.88$; see Table 2). 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. Data from one NC animal were removed from analysis because the acetylcholine levels were more than two standard deviations from the mean.

There was a significant main effect of time point ($F[11,99] = 14.24, p < 0.001, \eta^2 = 0.61$), as well as a significant interaction between time point and treatment ($F[22,99] = 3.06, p = 0.009, \eta^2 = 0.41$). To analyze the significant time point $\times$ 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. At the first time point after the galantamine injection, there was a significant effect of treatment ($F[2,12] = 9.29, p = 0.004, \eta^2 = 0.61$; see Fig. 3). 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); there was no significant difference between control groups. A similar pattern was observed for second and third time points after galantamine injection, so that there was a significant effect of treatment (p 's $< 0.05, \eta^2 > 0.55$), an effect that 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 ($p < 0.05, \eta^2 > 0.45$), 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: Galantamine and its effects on contextual fear conditioning

3.2a Body weights—A repeated-measures ANOVA with day as the repeated measure and treatment as the between-subjects measure was used to analyze all body weight data (Table 2). The assumption of sphericity was violated ($p < 0.05$), so Greenhouse-Geisser-adjusted degrees of freedom were used. For body weights during postnatal treatment (PD 2–10), 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 ($F[16,888] = 3.25, p = 0.02$), although *post hoc* analyses revealed no

differences between the treatment groups on any day, indicating that all animals gained weight throughout the postnatal period, regardless of treatment (see Table 3).

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 (PD 11-30). The assumption of sphericity was violated 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, indicating that neither postnatal alcohol nor galantamine treatment had long-lasting effects on body weights (see Table 3).

3.2b Blood alcohol concentrations—The average blood alcohol concentration ($\pm$ SEM) for alcohol-exposed animals in this study was 324.56 ± 14.11 (see Table 3). 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 2.

3.2c Context pre-exposure facilitation effect—Freezing was measured during all phases of testing and analyzed using a $3 \times 2 \times 2$ -way (treatment $\times$ drug $\times$ condition) ANOVA. During training, the animals were in the testing environment for less than 5 sec, and did not freeze when shocked. During the pre-exposure phase, 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 Fig. 4B). 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. For this reason, all testing values were expressed as a percent change from baseline.

A $3 \times 2 \times 2$ -way (treatment $\times$ drug $\times$ condition) 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 Fig. 4C). 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, indicative of the context pre-exposure effect.

3.3 Experiment 3: Examination of cholinergic immunoreactivity

To examine immunoreactivity of cholinergic proteins, animals from Experiments 1 and 2 were used. All animals from Experiment 1 were used for immunohistochemistry analysis, even if the microdialysis probe was off-target. Thus, physical parameters for these animals can be found in Table 4.

3.3a Choline acetyltransferase in the medial septum—For each animal, the number of ChAT+ 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; plates 16–18). 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. Fig. 5A–D shows representative photomicrographs for the animals that demonstrated the CPFE (Pre).

A $3 \times 2 \times 2$ -way (treatment $\times$ drug $\times$ condition) ANOVA was used to analyze ChAT data from animals that 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 toward a significant interaction between treatment, drug, and condition ($p = 0.053$). To analyze the two-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 ($\pm$ 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 (no learning) group, there were no significant main effects or interactions (Fig. 5A). In the pre-exposed group (learning), 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 ($p = 0.115$; Fig. 5B). 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 ($p = 0.055$). In addition, galantamine treatment led to a significant reduction in ChAT+ cells in the medial septum ($p < 0.05$).

A one-way ANOVA with treatment as the factor was used to analyze ChAT+ cells in the medial septum in the animals that were used for microdialysis. There was no significant effect of treatment (Table 5). 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 learning) or who were tested in microdialysis. Interestingly, among animals that demonstrate a CPFE, ethanol-exposed animals have an increased number of ChAT+ cells, while galantamine treatment decreased that number across all three treatment groups.

3.3b Alpha7 nicotinic acetylcholine receptor—For each animal, the density of staining was measured for two sections (if possible). Briefly, photomicrographs of each section were taken using a Nikon E600 microscope (20 $\times$) 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 animals used in Experiment 2 were analyzed using a $3 \times 2 \times 2$ -way (treatment $\times$ drug $\times$ condition) ANOVA. There was no main effect of treatment, drug, or condition, nor were there any interactions (see Table 6). Data from immunohistochemical analysis of the animals used in Experiment 1 were analyzed using a one-way ANOVA. Similarly, the data from animals used for microdialysis confirmed these findings, with no effect of treatment (see Table 5).

3.3c Vesicular acetylcholine transporter—To analyze vesicular acetylcholine transporter in the hippocampus, photomicrographs were obtained at 20 $\times$ 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. A $3 \times 2 \times 2$ -way (treatment $\times$ drug $\times$ condition) ANOVA was used to analyze the data from the animals tested for CPFE. There were no significant main effects, nor were there any interactions (see Table 6). A one-way ANOVA with treatment as the factor was used to analyze data from animals used for microdialysis. Confirming the findings from the animals tested behaviorally, there was no effect of treatment (see Table 5).

Discussion

The current experiments tested the hypothesis that developmental alcohol exposure significantly impairs the hippocampal acetylcholine system, and that this disruption may underlie learning deficits commonly seen in animal models of fetal alcohol syndrome (FAS). Although the animal model used in the current experiments does not recapitulate all aspects of FASD, alcohol exposure during the third-trimester equivalent did significantly affect acetylcholine efflux. We found that postnatal alcohol exposure significantly disrupted acetylcholine efflux following administration of high K^+/Ca^{2+} aCSF and significantly potentiated acetylcholine efflux following an acute galantamine administration. Furthermore, neither postnatal alcohol exposure nor galantamine affected performance in the context of pre-exposure facilitation effect. Finally, we observed an increase in ChAT in alcohol-exposed animals that learned to associate the shock with the context (Pre), but not in animals that did not learn this association (No Pre), nor in animals exposed to *in vivo* microdialysis only.

This is the first study to use *in vivo* microdialysis to measure acetylcholine release in the hippocampus following developmental alcohol exposure. We found that alcohol-exposed animals did not differ in baseline acetylcholine efflux, but differences appeared when the acetylcholine system was stimulated (e.g., galantamine). We used a combination of high K^+ and Ca^{2+} to produce sustained elevations in ACh release without extracellular Ca^{2+} availability being a limiting factor. While elevated Ca^{2+} may produce a ceiling effect on postsynaptic excitatory potentials, our primary purpose was to examine *presynaptic* capacity for ACh release via local depolarization. There is some evidence that these ions produce differing degrees of neurotransmitter release, perhaps by different mechanisms, but high concentrations of both K^+ and Ca^{2+} have independently been shown to increase release of ACh, DA, and 5-HT (Kawata, Okada, Murakami, Mizuno, Wada, et al., 1999; Zhu et al., 2002). We have previously used high K^+/Ca^{2+} to reveal age-related differences in

hippocampal glutamate and GABA release (Stanley, Fadel, & Mott, 2012). Finally, Moore et al. (1996) used a similar protocol to reveal age-related differences in prefrontal cortical ACh release. Importantly, in our work, these conditions did reveal differences in depolarization-induced hippocampal ACh release as a function of prenatal ethanol exposure, at least relative to non-treated control animals.

In animals that underwent microdialysis, there were no changes in ChAT in the medial septum or any changes in vAChT and $\alpha 7$ nAChR in the CA1 region of the hippocampus. It was surprising that the large differences in acetylcholine efflux were not accompanied by any changes in these markers of the acetylcholine system. However, changes in the expression or function of other cholinergic proteins that were not measured in this study, such as the high-affinity choline uptake transporter (CHT) or AChE, could account for the results. CHT is found presynaptically on cholinergic neurons and is responsible for transporting choline into the terminal for synthesis of acetylcholine. In fact, CHT function and choline uptake are rate-limiting steps for the synthesis of acetylcholine (Sarter et al., 2005). Furthermore, the function of CHT 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, Jansen, & Broekkamp, 1989). It is possible that alcohol exposure reduces the expression of CHT in the hippocampus, impairing acetylcholine synthesis, and thus contributing to the deficit in K^+/Ca^{2+} -induced acetylcholine efflux observed in the current study. Alcohol exposure during development may also affect the expression or function of AChE, because the enzyme serves as the primary mechanism for terminating the actions of ACh in the synapse. Specifically, there may be a down-regulation of acetylcholinesterase production in alcohol-exposed animals. In turn, galantamine is more effective as an AChE inhibitor in these animals, because there is less AChE to inhibit its effects. This may explain the alcohol-induced increase in extrasynaptic levels of acetylcholine observed following galantamine administration. In short, both capacity for ACh release (high K^+/Ca^{2+}) and response to AChE inhibition (galantamine) were impaired in ethanol-exposed rats, suggesting that behavioral or cognitive deficits in these animals may have a cholinergic basis, although full mechanisms of these impairments remain to be demonstrated.

Previous research has shown that alcohol-exposed animals are impaired in the context pre-exposure facilitation effect paradigm (Dokovna, Jablonski, & Stanton, 2013; Hamilton, Murawski, St Cyr, Jablonski, Schiffino, et al., 2011; Jablonski et al., 2014; Murawski et al., 2010, 2011). Since this paradigm is hippocampus-dependent (Rudy et al., 2004), and is influenced by the acetylcholine system (Kenney et al., 2008), these findings suggest that alcohol exposure disrupts the hippocampal acetylcholine system. The present studies found evidence of cholinergic dysfunction in the hippocampus, but alcohol-exposed animals were not impaired on the CPFE task. There are a number of possible explanations for this negative finding. First, 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) and prevent alcohol-induced hypothermia in males (Weinberg, Kim, & Yu, 1995). Although control animals were also handled daily for

injections, alcohol-exposed animals are differentially susceptible to stressors. Specifically, alcohol exposure causes dysregulation of the HPA axis and enhanced stress responsivity in animals prenatally exposed to alcohol (reviewed in Hellemans, Sliwowska, Verma, & Weinberg, 2010). Both injections and behavioral testing should elicit a stress response in all animals. However, in alcohol-exposed animals, daily handling for injections could habituate the animals to experimental procedures, such that they were less stressed when exposed to the behavioral testing and thus did not exhibit impaired performance. Future studies should use a non-injected control when testing treatments for alcohol-induced learning deficits in this task.

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. In a study by Murawski et al. (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 (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 et al. (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 mg/dL), administered from PD 4–9 impaired performance on the CPFE task in adolescents (PD 31–33). While the doses 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 BACs reached were comparable to those in the current experiments. This suggests that the difference in alcohol dose is not the cause for the negative findings. Previous studies have demonstrated the importance of peak BAC, and not total dose, in demonstrating alcohol-related changes in behavior (Goodlett & Johnson, 1997).

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, Zhu, & Ruan, 2011), MK-801 (Su, Huang, Wang, Li, & Si, 2014), maternal deprivation (Benetti, Mello, Bonini, Monteiro, Cammarota, et al., 2009), and L-kynurenine (Alexander, Pocivavsek, Wu, Pershing, Schwarcz, et al., 2013). However, the efficacy of galantamine as a treatment for alcohol-induced learning deficits was unknown. We found no evidence for a beneficial effect of chronic galantamine administration overall in the context pre-exposure facilitation paradigm; 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 et al., 2008). Physostigmine (0.01 mg/kg prior to all three phases), an AChE inhibitor, has even been shown to reverse the alcohol-induced deficit observed in this paradigm (Dokovna et al., 2013). Thus, 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, Schrattenholz, Samochocki, Radina, & Albuquerque, 2000), suggesting that the CPFE paradigm is more sensitive to higher levels of acetylcholine overall, rather than sensitive to potentiation of nicotinic receptors. We did

observe a larger increase in acetylcholine efflux in alcohol-exposed animals compared to control animals following an acute injection of galantamine, but it is unclear whether chronic galantamine administration would produce a similar result. It is possible that galantamine would enhance performance in the CPFE if given directly before testing, but it is important to keep in mind the relevance of this dosing regimen in a clinical population.

Finally, we found that postnatal alcohol exposure did not alter any of the cholinergic proteins measured in the animals that experienced microdialysis (Experiment 1). In addition, the expression of vAChT or $\alpha 7$ nAChR in the ventral CA1 was not affected by alcohol exposure, chronic galantamine, or CPFE testing. There were, however, 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. Furthermore, galantamine caused an overall reduction in ChAT, but only in the pre-exposure groups. 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-regulated 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. In addition, galantamine could interact with other neurochemical components of the hippocampus (not measured here) 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 (Drever et al., 2011) and glutamatergic neurons (Fabian-Fine, Skehel, Errington, Davies, Sher, et al., 2001; Gray, Rajan, Radcliffe, Yakehiro, & Dani, 1996) within the hippocampus. 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.

Although these data represent a novel contribution to the field of fetal alcohol syndrome, there were a few limitations to the current experiments. First, due to difficulties in cannula surgery and microdialysis procedures in the alcohol-exposed animals, we have a small sample size for these experiments ($n = 3$). It is important to note, however, that even with a small sample size we observed significant changes with large effect sizes in hippocampal acetylcholine content following postnatal alcohol exposure. Future studies should confirm and extend these results using larger sample sizes. Next, these experiments used only males, due to the complex experimental design in Experiment 2. Finally, immunohistochemistry was used to measure cholinergic proteins, but it is possible that this method was not sensitive enough to detect small changes in the expression of these proteins, and that more sensitive techniques would have yielded significant results.

In summary, this study describes significant alterations in hippocampal neurochemistry that may help to explain deficits in learning and memory commonly observed in animal models of fetal alcohol syndrome. The acetylcholine system in the hippocampus shows clear impairment under stimulated conditions, and in response to galantamine in alcohol-exposed animals compared to controls. While there were no specific effects of galantamine on

behavior or on any cholinergic markers, the results do suggest that investigation of other markers of the cholinergic system in the hippocampus and the impact of galantamine on other hippocampal-dependent behaviors may be fruitful avenues for future research. Pharmacological approaches to treatment of FASD are just beginning to be delineated and the current studies suggest that the cholinergic system in the hippocampus may be an important target.

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Abbreviations

CPFE	context pre-exposure facilitation effect
ChAT	choline acetyltransferase
ET	ethanol-treated
FASD	fetal alcohol spectrum disorder
IC	intubated control
nAChR	nicotinic acetylcholine receptor
NC	non-treated control
PD	postnatal day
vAChT	vesicular acetylcholine transporter

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Highlights

- Developmental alcohol exposure increased galantamine-induced acetylcholine efflux.
- Developmental alcohol exposure did not impair learning in CPFE.
- Rats exposed to alcohol during development and which acquired the CPFE showed increased ChAT in the hippocampus.
- Developmental alcohol exposure did not change vAChT or $\alpha 7$ nAChR in the hippocampus.

Figure 1.

Representative photomicrograph depicting probe placement in area CA1 of the hippocampus. Tissue was sectioned and stained using immunohistochemistry for ChAT. The arrow indicates the probe track. Probes were classified as being correctly placed if they were located in the ventral CA1 region of the hippocampus. CC: corpus callosum

Figure 2.

Average acetylcholine efflux ($\pm$ SEM) for all groups during the first session of *in vivo* microdialysis (expressed as a percent of baseline). There were four baseline collections, followed by two high K^+/Ca^{2+} , and four post-stimulation collections. There were no significant differences between groups during baseline collections. There was no significant interaction between time point and treatment group, but the ET and NC groups were compared directly due to the variability in the IC group. Following the administration of a high K^+/Ca^{2+} aCSF, ethanol-exposed animals had a smaller increase in acetylcholine, when compared to NC animals (a). Samples sizes for the NC, IC, and ET groups were 7, 6, and 3, respectively.

Figure 3.

Average acetylcholine efflux ($\pm$ SEM) for all groups during the second session of *in vivo* microdialysis (expressed as a percent of baseline). There were four baseline collections, followed by an acute galantamine injection (2.0 mg/kg; subcutaneous) and eight post-injection collections. There were no differences between groups during baseline. There were significant group differences following the galantamine injection. The ET group had enhanced extrasynaptic acetylcholine compared to the IC group in the first three post-injection samples (b) and compared to the NC group (a) at the first six post-injection samples. There were no differences between the groups at Post 7 or 8. Samples sizes for the NC, IC, and ET groups were 6, 7, and 3, respectively.

Figure 4.

A. Schematic diagram of CPFE testing. During the pre-exposure phase, animals are placed into context A (Pre) or context B (No Pre) for 5 min. Twenty-four hours later, all animals are placed into context A and given a brief footshock (1.5 mA, 2 sec). Testing consists of placing animals back into context A and recording freezing for 5 min. **B.** Percent freezing ($\pm$ SEM) for all groups during the pre-exposure phase. There was a significant main effect of condition (b), where animals in the No Pre group froze more than animals in the Pre group. There was also a main effect of treatment (a), where IC animals had significantly more freezing than the NC group; the ET group was not significantly different from either the ICs or NCs. **C.** Average freezing (expressed as a percent change from pre-training) during testing ($\pm$ SEM; 5 min). There was a significant main effect of condition (b), such that the Pre group froze more than the No Pre group, indicating the context pre-exposure facilitation effect. There were no differences due to treatment or drug administration.

Figure 5. Representative photomicrographs of ChAT immunoreactivity in the medial septum at 2 $\times$ (**A,C**) and 20 $\times$ (**B,D**) for the NC group (**A,B**) and the ET group (**C,D**). ET animals overall had significantly more ChAT+ cells than the control groups.

Figure 6.

Average number ($\pm$ SEM) of choline acetyltransferase-positive cells (ChAT+) in the medial septum. In the animals that did not learn (**A**), there were no effects of treatment or drug. However, in the animals that did learn (**B**), there were main effects of treatment and drug. Ethanol-exposed animals had significantly more ChAT+ cells than the NC group, but not the IC group (a). Galantamine treatment resulted in a reduction in ChAT+ cells, regardless of treatment (b).

Table 1

Physical parameters (mean $\pm$ SEM) for animals used for *in vivo* microdialysis experiments (Experiment 1). There were no significant differences among the treatment groups at any age.

Group	PD 2 (g)	PD 10 (g)	PD 27/28 (g)	PD 33/34 (g)	BAC (mg/dL)
NC (n)	6.67 $\pm$ 0.34 (7)	18.47 $\pm$ 0.75 (7)	73.70 $\pm$ 1.54 (7)	99.27 $\pm$ 2.49 (7)	n/a
IC (n)	6.59 $\pm$ 0.34 (7)	17.93 $\pm$ 0.68 (7)	74.10 $\pm$ 3.42 (7)	101.79 $\pm$ 5.59 (7)	n/a
ET (n)	6.43 $\pm$ 0.24 (3)	16.77 $\pm$ 1.27 (3)	67.13 $\pm$ 4.30 (3)	101.90 $\pm$ 11.64 (3)	318.09 $\pm$ 87.40 (4)**

** Note: BACs are included for all animals, regardless of probe placement.

Table 2

Average acetylcholine efflux ($\pm$ SEM; pmol/20 μ L) during baseline collections. There were no significant differences among the treatment groups.

Group	Session 1 (High K^+ /Ca ²⁺)				Session 2 (Galantamine)			
	Baseline 1	Baseline 2	Baseline 3	Baseline 4	Baseline 1	Baseline 2	Baseline 3	Baseline 4
NC (n)	0.016 $\pm$ 0.004 (7)	0.012 $\pm$ 0.002 (7)	0.011 $\pm$ 0.001 (7)	0.009 $\pm$ 0.002 (7)	0.008 $\pm$ 0.003 (5)	0.009 $\pm$ 0.003 (6)	0.009 $\pm$ 0.003 (6)	0.008 $\pm$ 0.002 (6)
IC (n)	0.015 $\pm$ 0.002 (6)	0.016 $\pm$ 0.003 (6)	0.014 $\pm$ 0.003 (6)	0.012 $\pm$ 0.002 (6)	0.006 $\pm$ 0.001 (7)	0.008 $\pm$ 0.003 (7)	0.010 $\pm$ 0.003 (6)	0.010 $\pm$ 0.002 (7)
ET (n)	0.008 $\pm$ 0.001 (3)	0.010 $\pm$ 0.002 (3)	0.010 $\pm$ 0.002 (3)	0.010 $\pm$ 0.001 (3)	0.004 $\pm$ 0.001 (3)	0.004 $\pm$ 0.001 (3)	0.005 $\pm$ 0.001 (3)	0.004 $\pm$ 0.001 (3)

Table 3

Physical parameters ($\pm$ SEM) for chronic galantamine and behavioral testing experiment. Neither alcohol exposure nor galantamine treatment significantly affected body weights.

Group	PD 2 (g)	PD 10 (g)	PD 21 (g)	PD 30 (g)	BAC (mg/dL)
NC-SAL (<i>n</i>)	7.4 $\pm$ 0.2 (20)	19.2 $\pm$ 0.5 (20)	44.5 $\pm$ 1.2 (20)	91.6 $\pm$ 1.8 (20)	n/a
NC-GAL (<i>n</i>)	7.1 $\pm$ 0.3 (20)	19.0 $\pm$ 0.6 (20)	44.5 $\pm$ 1.3 (20)	92.1 $\pm$ 2.2 (20)	n/a
IC-SAL (<i>n</i>)	7.1 $\pm$ 0.2 (20)	18.8 $\pm$ 0.4 (20)	44.3 $\pm$ 1.2 (20)	91.1 $\pm$ 1.7 (19)	n/a
IC-GAL (<i>n</i>)	7.1 $\pm$ 0.3 (20)	19.2 $\pm$ 0.6 (20)	43.5 $\pm$ 1.3 (20)	90.7 $\pm$ 1.9 (20)	n/a
ET-SAL (<i>n</i>)	7.2 $\pm$ 0.2 (21)	19.3 $\pm$ 0.6 (21)	45.5 $\pm$ 1.4 (21)	93.3 $\pm$ 2.1 (21)	356.65 $\pm$ 17.11 (21)
ET-GAL (<i>n</i>)	7.5 $\pm$ 0.2 (22)	20.8 $\pm$ 0.6 (22)	46.1 $\pm$ 1.5 (22)	95.5 $\pm$ 1.8 (22)	324.54 $\pm$ 14.57 (21)

Table 4

Physical parameters (mean $\pm$ SEM) for animals used in immunohistochemistry analysis from Experiment 1. There were no significant differences between treatment groups at any age.

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

Table 5

Data from immunohistochemistry (mean $\pm$ SEM) for choline acetyltransferase (number of cells), alpha7 nAChR (percent staining), and vAChT (percent staining) for animals used in microdialysis (Experiment 1). There were no significant differences between treatment groups in any measure of immunoreactivity.

Stain	Non-treated Control (n)	Intubated-Control (n)	Ethanol-Exposed (n)
Choline Acetyltransferase	56.24 $\pm$ 6.95 (8)	60.28 $\pm$ 13.46 (7)	64.01 $\pm$ 19.12 (7)
Alpha7 Nicotinic Acetylcholine Receptor	26.73 $\pm$ 3.03 (7)	24.53 $\pm$ 3.87 (7)	30.05 $\pm$ 2.05 (5)
Vesicular Acetylcholine Transporter	23.18 $\pm$ 2.76 (7)	24.71 $\pm$ 2.63 (7)	26.55 $\pm$ 2.66 (5)

Average density of staining (expressed as a percent) $\pm$ SEM from animals used in Experiment 2 (chronic galantamine and behavioral testing). There were no significant differences between treatment groups in alpha7 nAChR or vAChT immunoreactivity.

Table 6

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 $\pm$ 4.18 (9)	53.79 $\pm$ 4.62 (12)	56.60 $\pm$ 4.91 (12)	50.99 $\pm$ 4.48 (16)	55.83 $\pm$ 4.26 (13)	53.55 $\pm$ 4.01 (15)
Vesicular Acetylcholine Transporter	46.15 $\pm$ 4.37 (18)	47.17 $\pm$ 3.80 (19)	49.00 $\pm$ 4.15 (18)	47.71 $\pm$ 3.64 (17)	50.56 $\pm$ 2.37 (17)	52.50 $\pm$ 3.44 (19)