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Cholinergic rescue of neurocognitive insult following third-trimester equivalent alcohol exposure in rats

Nicholas A. Heroux, **Colin J. Horgan**, **Jeffrey B. Rosen**, **Mark E. Stanton**

Department of Psychological and Brain Sciences, University of Delaware, Newark, DE 19716, United States

Abstract

Neonatal ethanol exposure during the third trimester equivalent of human pregnancy in the rat significantly impairs hippocampal and prefrontal neurobehavioral functioning. Postnatal day [PD] 4-9 ethanol exposure in rats disrupts long-term context memory formation, resulting in abolished post-shock and retention test freezing in a variant of contextual fear conditioning called the *Context Preexposure Facilitation Effect* (CPFE). This behavioral impairment is accompanied by disrupted medial prefrontal, but not dorsal hippocampal expression of the immediate early genes (IEGs) *c-Fos, Arc, Egr-1,* and *Npas4* [1]. The current experiment examined if systemic administration of the acetylcholinesterase inhibitor physostigmine (PHY) prior to context learning would rescue prefrontal IEG expression and freezing in the CPFE. From PD4-9, Long-Evans rats received oral intubation of ethanol (EtOH; 5.25g/kg/day) or sham-intubation (SI). Rats received a systemic injection of saline (SAL) or PHY (0.01mg/kg) prior to all three phases (Experiment 1) or just context exposure (Experiment 2) in the CPFE from PD31-33. A subset of rats were sacrificed 30min after context learning to assay changes in IEG expression in the medial prefrontal cortex (mPFC), dorsal hippocampus (dHPC), and ventral hippocampus (vHPC). Administration of PHY prior to all three phases or just context learning rescued both post-shock and retention test freezing in the CPFE in ETOH rats without altering performance in SI rats. ETOH-SAL rats had significantly reduced mPFC but not dHPC expression of *c-Fos, Arc, Egr-1,* and *Npas4.* ETOH-PHY treatment rescued mPFC expression of *c-Fos* in ethanol-exposed rats and increased *Arc* and *Npas4* regardless of dosing condition. While there was no effect of PHY on dHPC or vHPC expression of *Arc*, *Egr-1,* or *Npas4,* this treatment significantly boosted hippocampal expression of *c-Fos* regardless of ethanol treatment. These findings implicate impaired cholinergic and prefrontal function in cognitive deficits arising from 3rd-trimester equivalent alcohol exposure.

Keywords

cholinergic system; acetylcholine; prefrontal cortex; neonatal ethanol exposure; FASD; context fear; immediate early genes

Corresponding author: Nicholas A. Heroux, B.S., Department of Psychological and Brain Sciences, University of Delaware, Newark, DE 19716, 302-831-0687, nheroux@udel.edu.

1. Introduction

Fetal alcohol spectrum disorders (FASDs) represent a spectrum of physical and neurobehavioral impairments caused by gestational alcohol exposure in humans [2]. Alcohol acts as a teratogen in the developing nervous system, causing widespread decreases in brain volume, cortical thickness, neural activity, and altered functional connectivity [3-9]. Gestational exposure alters the normal development of structures such as the basal ganglia, cerebellum, hippocampus, and prefrontal cortex [3,6,7,9,10], resulting in disruptions in learning and memory engaging these structures in exposed children [8,11-20]. Animal models of FASD have proven instrumental in isolating the mechanisms underlying neurobehavioral impairments because of the ability to manipulate alcohol exposure window, pattern, and dosage across discrete phases of development [21]. Rats undergoing binge-like alcohol exposure during the third-trimester equivalent of human pregnancy (e.g., from postnatal day [PD] 4-9, 7-9, or 2-10) show similar neurobiological and behavioral impairments as in the human condition [2,21-24]. This exposure results in altered glutamatergic and cholinergic molecular signaling in the dorsal hippocampus (dHPC) that cannot fully be attributed to cell loss in rats [1,23,25-29]. In contrast, while there is no lasting prefrontal cell loss, neonatal ethanol exposure alters dendritic complexity, neurophysiological properties, and gene expression in the medial prefrontal cortex (mPFC) in rats [1,23,28,30-33].

Recent research in both animal models and humans has strongly suggested a link between acute and chronic alterations in cholinergic signaling in these structures across development as a mechanism underlying ethanol-induced insult [23,34-36]. During the brain growth spurt in rats (i.e., the first 1-2 weeks of life), alcohol interferes with bioavailability of choline and acetylcholine in the brain as well as glial and muscarinic-receptor cell signaling important for neuritogenesis in proliferating pyramidal neurons [37-40]. This exposure persistently alters acetylcholine efflux, muscarinic-receptor cell signaling, and DNA methylation in both the hippocampus and prefrontal cortex [36,41-43]. In animal models, developmental choline supplementation during alcohol exposure reduces ethanol-induced neuroanatomical and molecular insult and, in some cases, rescues behavioral performance in tasks such as the Morris water maze, trace fear conditioning, and eyeblink conditioning [23,44-50]. While more research is needed, these benefits of choline supplementation during gestation are efficacious in the human condition [51-53]. Despite this growing body of work, the effect of augmenting cholinergic system function on neural activity during behavior in ethanol-exposed rats is not well characterized.

Our lab has shown that a variant of Pavlovian contextual fear conditioning, called the *Context Preexposure Facilitation Effect* (CPFE), is particularly sensitive to the effects of developmental alcohol exposure in rats [1,27,28,54-57]. In the CPFE, learning about the context, acquiring a context-shock association, and retrieval of contextual fear is temporally dissociated across three phases (context preexposure, immediate-shock training, and retention). The CPFE depends on activity and cholinergic muscarinic-receptor cell signaling in the dHPC and mPFC [58-60]. Neonatal ethanol exposure from either PD4-9 or PD7-9 abolishes retention test freezing in the CPFE [28,55-57,61]. We've recently shown that PD4-9 ethanol exposure disrupts the consolidation of the context representation during

context preexposure, subsequently resulting in abolished post-shock and retention test freezing in the CPFE [1]. This behavioral impairment is accompanied by disrupted medial prefrontal, but not dorsal hippocampal expression of the immediate early genes (IEGs) *c-Fos, Arc, Egr-1,* and *Npas4* during context preexposure. Interestingly, administration of the acetylcholinesterase inhibitor physostigmine (PHY) prior to all three phases rescues retention test freezing in PD7-9 ethanol-exposed rats [62]. The specific phase(s) of the CPFE in which PHY rescues performance and how PHY alters regional IEG expression during the CPFE in ethanol-exposed animals is not known.

The purpose of the current study was to examine the effects of augmenting cholinergic signaling on behavioral performance and medial prefrontal, dorsal hippocampal, and ventral hippocampal IEG expression in ethanol-exposed rats. In Experiment 1, we extended our earlier findings [62] by examining whether or not systemic administration of the acetylcholinesterase inhibitor physostigmine prior to all three phases would rescue freezing in rats receiving PD4-9 ethanol exposure. In Experiment 2, we sought to determine if this same injection prior to just context learning would rescue disrupted prefrontal immediate early gene expression and freezing in the CPFE that we have previously observed [1,28]. To our knowledge, this is the first study to examine the effects of boosting cholinergic function (via PHY) on neural activity during behavior in rats receiving neonatal ethanol exposure during the brain growth spurt.

2. Materials and Methods

2.1. Subjects

Animal husbandry was as described in our previous reports [60,63]. In Experiment 1, there was 43 adolescent (PD31) Long Evans rats (22 females and 21 males), derived from 8 separate litters bred in-house. In Experiment 2, there was 154 PD31 rats (75 females and 79 males), derived from 22 separate litters. The animal housing facility was maintained on a 12:12 h light/dark cycle with lights on at 7:00 am. Litters were culled on PD3 to eight pups (4 males and 4 females when possible), alcohol or sham dosing occurred through PD4-9, and pups were weaned from their mother on PD21 and housed with same-sex littermates. On PD29, rats were individually housed in small clear cages for the remainder of the experiment (until PD33-34). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Delaware following guidelines established by the National Institute of Health.

2.2. Neonatal alcohol dosing (PD4-9, 5.25g/kg/d split into two daily doses)

Neonatal ethanol dosing via intragastric intubation occurred over PD4-9 with methods that have been described previously [1]. Littermates were randomly assigned to receive either ethanol (EtOH group) or sham intubations (SI group), with an equal number of males and females in each litter whenever possible. Same-sex littermates assigned to the same dosing condition (EtOH or SI) were assigned to different experimental groups so that no more than one same-sex littermate was assigned to any particular condition. Briefly, on PD4, pups were separated from their mothers and placed into weigh boats set over a heating pad that provided warmth during the separation. Pups were weighed prior to the first intubation

session (occurring daily at 9am ± 1hr). The intubation process involved passing PE10 tubing lubricated with corn oil down the esophagus and into the stomach of the rat pup. Rats in the SI group received intragastric intubations on the same schedule as the EtOH group, and the tube was removed after approximately 6-8 seconds during each scheduled intubation without the infusion of any solution. Rats in the EtOH group were intubated and given a daily dose of 5.25 g/kg of alcohol, [11.9% v/v ethanol (made from 95% ethanol)] in a custom milk formula previously described (Kelly & Lawrence, 2008). This dose was divided into two feedings each day, separated by 2hr. The formula was delivered in a volume of 0.02778 ml/g body weight. A third intubation of the milk formula (containing no ethanol) was administered two hours after the second daily alcohol dosing. After each intubation was completed (<20 minutes per litter), pups were returned as a litter to their mothers. Importantly, although intragastric intubation using these parameters results in a transient reduction in body weight and growth [56,57,64], this reduction is absent by adolescence and thus likely does not contribute to behavioral deficits resulting from exposure [see 64].

2.3. Blood alcohol concentrations (BACs)

On PD4, 90 min following the second alcohol intubation, pups received a small tail-clip and a 20µ1 blood sample was collected using a capillary tube. Blood samples from Group SI were discarded and those from alcohol-exposed pups were saved for further blood alcohol analysis. Blood samples from alcohol-exposed pups were centrifuged, and the plasma was collected and stored at −20°C. Blood alcohol concentrations were determined using an Analox GL5 Analyzer (Analox Instruments, Luneburg, MA) as previously described [55]. Briefly, the rate of oxidation of alcohol in each plasma sample was measured. BACs (expressed in mg/dl) were calculated based on comparisons to known values of an alcohol standard solution.

2.4. Apparatus and stimuli

The apparatus and stimuli used have been described previously [55,60,63]. Briefly, fear conditioning occurred in four clear Plexiglas chambers within a fume hood (Context A – Pre group), with grid floors connected to shock scramblers (Med Associates, Georgia, VT-ENV-414S). The alternate context (Context B – Alt-Pre group) consisted of the same Plexiglas chambers with a convex wire mesh insert that covered the back wall and floor of the chamber and a white paper sleeve that covered the outside walls of the chamber. The unconditioned stimulus (US) was two, 1.5 mA foot-shocks, each 2s in duration, and presented 1s apart immediately upon chamber entry. Videos of each session (preexposure, training, testing) were recorded using Freeze-Frame 3.0 software (Actimetrics, Wilmette IL) with freezing defined as a bout of 0.75 s or longer without a change in video pixilation.

2.5. Context Preexposure Facilitation Effect (CPFE)

The CPFE procedure consisted of three phases (context preexposure, immediate-shock training, retention testing) and took place over the course of three days from PD31 to PD33 as described previously [58-60,63]. In Experiment 1, all rats were preexposed to the same context they were later trained in (Pre group, Context A). In Experiment 2, rats were assigned to this Pre group or an alternate preexposure condition (Alt-Pre group, Context B). Rats in the Pre group received exposure to Context A, the training context, while rats in the

Alt-Pre group received exposure to Context B (see section 2.4). Alt-Pre rats serve as non-associative behavioral controls as they demonstrate the immediate-shock deficit (ISD), which reflects an inability to form a context-shock association without prior exposure to Context A [65].

Rats received an intraperitoneal (i.p.) injection of 1ml/kg sterile saline (SAL group) or 0.01mg/ml/kg physostigmine (PHY group) dissolved in saline. This injection occurred 30min prior to all three phases in Experiment 1, and 30min prior to only context preexposure in Experiment 2. During context preexposure on PD31, rats were placed in Context A or B and underwent multiple context preexposure, consisting of one initial 5 min exposure to the chamber, followed by five 1 min exposures, with a 1 min interval between exposures. In Experiment 2, a subset of rats in the Pre group (across both drug conditions) were sacrificed via live decapitation and tissue was collected for RNA extraction and qPCR 30min after context preexposure (see sections 2.6 and 2.7). Alt-Pre rats were not sacrificed as there is no difference between Pre and Alt-Pre gene expression on the preexposure day of the CPFE [63]. In addition, a behaviorally naïve home-cage condition (HC group) was also sacrificed to establish baseline gene expression after drug injection. During immediate-shock training on PD32, rats were carried into the testing room, placed in their respective Context A training chamber, and within 3s, were given two 1.5mA foot-shocks separated by 1s. Rats remained in the training chamber for a 3min post-shock freezing test after foot-shock presentation to measure the immediate acquisition of the context-shock association. During testing on PD33, rats were returned to the same Context A chamber in which they were trained for a 5min retention freezing test. Rats were returned to their home-cages within 1min of the termination of any session (preexposure, training, and testing).

2.6. Brain removal and tissue dissection

In Experiment 2, rats were taken from their home cages 30 min after context preexposure and rapidly decapitated without anesthesia. Brains were removed and bathed in ice-cold saline for about 5-8 sec to increase tissue firmness. Coronal brain slabs (1-1.5 mm) were cut out using a .5mm coronal rat brain matrix. The medial prefrontal cortex (mPFC), dorsal hippocampus (dHPC), and ventral hippocampus (vHPC) were dissected out of the coronal slabs, checking both sides for anterior-posterior boundaries. Consistent with and extending a previous study [63], dissection boundaries were approximately as follows: mPFC, +4.20mm to +2.52mm from bregma; dHPC, 2.16mm to −3.84mm from bregma, vHPC, −4.56 to −6.12 from bregma (using the Paxinos & Watson [2017] rat brain atlas as a guide). Dissected tissue was immediately flash frozen on dry ice and subsequently stored at −80 °C until the time of analysis.

2.7. Quantitative Real-time PCR

The quantitative real-time PCR procedure used has been described previously [63,66]. RNA was extracted from frozen tissue samples using TRIzol Reagent (Cat. No. 15596018, Invitrogen). Genomic DNA was eliminated and cDNA was synthesized from extracted RNA (1000ng/μL) using the QuantiTect® Reverse Transcription Kit (Cat. No. 205314, Qiagen). Relative gene expression was quantified by real-time PCR using the GREEN FASTMIX PERFECTA-SYBR Kit (Cat. No. 101414-270, Quantabio) in 10μL reactions on a

CFX96Touch real time PCR machine. Expression of *Egr-1* was analyzed using a QuantiTect® Primer Assay (Cat. No. QT00182896, Qiagen) and diluted according to protocol. All other primers were ordered through Integrated DNA Technologies and diluted to a final concentration of 0.13 μM (*18s, Arc, c-Fos,* and *Npas-4*). The gene *18s* is a ribosomal housekeeping gene and was used as a control for all experimental groups as it did not differ significantly across any groups or manipulations. Samples were numbered, blinded to treatment group and run in duplicate on real-time PCR plates. For each reaction, the average quantitative threshold amplification cycle number (C_q) value was determined from each duplicate, and the $2^{-\Delta\Delta Cq}$ method was used to calculate the relative gene expression for each gene relative to *18s.*

2.8. Data analysis and statistics

2.8.1. Analysis of body weight—Neonatal body weight was analyzed with repeated measures ANOVA with a between-subjects factor of dosing condition (SI vs. EtOH) and the within-subjects factor of age (PD4 vs. PD9). There were no main effects or interactions involving sex in the PD4 and PD9 weights (*p*s > .05) so the data were collapsed across this variable at these ages. Body weight at PD31 was analyzed with a 2 (Sex; male vs. female) × 2 (Dosing condition; SI vs. EtOH) factorial ANOVA. Post-hoc contrasts were performed with Newman–Keuls tests.

2.8.2. Analysis of behavioral data—Behavioral data processing procedures have been described previously [58,63]. A human observer blind to the experimental groups verified the freezing threshold setting with Freeze View 3.0 (Actimetrics, Wilmette IL). The software program computes a “motion index” that was adjusted to set a freezing threshold separately for each animal (per software instructions) by a blind observer who verified from the video record whether or not small movements were scored as freezing. Once set, the threshold did not change during a session. We have validated this procedure against other scoring methods (e.g., hand scoring of video records by two blind observers). Freezing behavior was scored as the total percent time spent freezing longer than .75s bins (defined as the cessation of all movement except breathing) in each respective session bin (context exposure, post-shock freezing, and a 24 h retention test).

The data were imported into STATISTICA 64 data analysis software and freezing behavior was analyzed by ANOVA. There were no main effects or interactions involving sex on freezing behavior (*p*s > .05), so the data were collapsed across this variable. In Experiment 1, freezing data were analyzed using 2 (Dosing condition; SI vs. EtOH) × 2 (Drug condition; SAL vs. PHY) × 2 (within subjects; phase of testing; Post-shock vs. Retention) repeated measures ANOVA. In Experiment 2, consistent with previous reports [59,60], to reduce animal usage, the Alt-Pre group was pooled across drug but not dosing condition (Pooled-Alt-Pre group) as there was no significant difference between the two drug groups and they froze at uniformly low levels ($p > .34$). Therefore, freezing data were analyzed using 2 (Dosing; SI vs. EtOH) × 3 (Condition; SAL vs. PHY vs. Pooled-Alt-Pre) × 2 (within subjects; phase of testing; Post-shock vs. Retention) repeated measures ANOVA. Post-hoc contrasts were performed with Newman–Keuls tests. Rats were excluded from analysis as an outlier if they had a score of ± 1.96 standard deviations from the group mean, however, the

Z-score of removed outliers averaged ± 3.91 (± 0.97 SEM). We remove outliers in this manner in all of our studies without regard to how it affects the experimental outcome. Outliers removed were as follows: Experiment 1 [(Post-shock: SI-PHY=1, SI-SAL=1)] and Experiment 2 [(Post-shock: EtOH-Pooled-Alt-Pre=1, EtOH-PHY=1, EtOH-SAL=1, SI-PHY=1, SI-SAL=1; Retention: EtOH-Pooled-Alt-Pre=1, SI-Pooled-Alt-Pre=1, EtOH-PHY=2, EtOH-SAL=2)].

2.8.3. Analysis of qPCR data—Relative gene expression for the IEGs *c-Fos, Arc, Egr-1,* and *Npas4* in the mPFC, dHPC, and vHPC was determined (see section 2.7). The relative gene expression value was obtained by normalizing the data to the reference gene (*18s*) and to the average delta C_T of the home-cage control group for each gene. Consistent with previous findings (Heroux et al., 2018; 2019), there were no interactions involving sex (ps > .30), so the data were collapsed across this variable. There was also no difference between the raw data in HC group injected with SAL or PHY, so the HC group was collapsed across drug condition (*p*s > .35). Gene expression was analyzed using a 2 (Dosing condition; SI vs. EtOH) × 2 (Drug condition; SAL vs. PHY) factorial ANOVA for each gene (*c-Fos, Arc, Egr-1,* and *Npas4*) in the mPFC, dHPC, and vHPC. Post-hoc contrasts were performed with Newman–Keuls tests and a Dunnett's test that contrasted the four experimental groups with the HC control group. The average Z-score of removed outliers was ± 3.14 (± 0.12 SEM; see Table 2).

3. Results

3.1. Body Weights and BACs (both experiments)

Body weight averages for sham-intubated and alcohol-exposed rats at PD4 and PD9 appear in Table 1. Both the SI and EtOH groups gained substantial weight during the dosing period (PD4-PD9) up until the age of testing (PD31). A 2 (Dosing; SI vs. EtOH) × 2 (Age; PD4 vs. PD9) repeated measures ANOVA revealed significant main effects of Dosing condition [$F(1, 195) = 67.59$, $p < .001$], Age [$F(1, 195) = 7213.30$, $p < .001$], as well as a Dosing × Age interaction [$F(1, 195) = 316.35$, $p < .001$]. Newman-Keuls tests revealed no difference between group weights on PD4 ($p > .70$), but on PD9, EtOH rats weighed about 15% less than SI rats (*p*s < .001). Transient growth retardation in ethanol treated rats over this dosing period has been reported previously (Murawski et al., 2012; Hamilton et al., 2011; Heroux et al., 2019). Ethanol did not alter body weight at the time of behavioral testing (Table 1). A 2 (Dosing; SI vs. EtOH) × 2 (Sex; male vs. female) factorial ANOVA performed on PD31 body weights revealed a significant main effect of Sex [$F(1, 193) = 56.33$, $p < .001$] but not Dosing condition [$F(1, 193) = 2.31$, $p > .13$], with no interaction between these two variables [$F(1, 193) = 0.00$, $p > .96$]. Females had lower body weights compared to males at PD31 regardless of dosing condition (see Table 1). Finally, the average BAC value taken from the blood samples of the EtOH group in each experiment can be seen in Table 1.

3.2. Experiment 1: Systemic administration of physostigmine prior to every phase rescues the CPFE in EtOH-exposed rats

The purpose of Experiment 1 was to determine whether impaired post-shock and retention test freezing in ethanol-exposed rats could be rescued by systemic administration of the

acetylcholinesterase inhibitor physostigmine prior to each phase of the CPFE. The behavioral procedure and results for Experiment 1 can be seen in Figure 1A-C. A 2 (Dosing: EtOH vs. SI) × 2 (Drug: SAL vs. PHY) × Phase (Post-shock vs. Retention) between-within factorial design assessed rats exposed only to Context A (Pre, see Section 2.5). We predicted that alcohol would impair freezing in SAL- but not PHY-treated rats.

Analyses for Experiment 1 were run on 41 rats distributed across the following groups: SI-SAL-Pre (n=11), SI-PHY-Pre (n=11), EtOH-SAL-Pre (n=9), and EtOH-PHY-PRE (n=10). ANOVA revealed a significant main effect of Dosing [$F(1, 35) = 23.91$, $p < .001$], Drug [$F(1, 35) = 8.40$, $p < .01$], and a significant Dosing × Drug interaction [$F(1, 35) = 18.23$, $p < .001$]. There was no main effect or any interactions involving the repeated measure of Phase ($ps > .45$). EtOH-SAL rats showed abolished post-shock and retention test freezing relative to SI-SAL rats ($p < .01$). PHY treatment eliminated this effect by restoring freezing in the EtOH-PHY group to control levels. Importantly, freezing levels during the preexposure session were uniformly low across experimental conditions; SI and EtOH animals freezing levels were 2.53 ± 0.54 SEM and 1.41 ± 0.21 SEM, respectively. These results demonstrate that systemic PHY administration prior to all three phases of the CPFE rescues impaired context conditioning in ethanol-exposed rats.

3.3. Experiment 2: Systemic administration of physostigmine prior to context preexposure rescues the CPFE and boosts IEG expression in EtOH-exposed rats

3.3.1. Behavioral results—The purpose of Experiment 2 was to examine the effects of physostigmine given only prior to context preexposure on IEG expression and impaired freezing in ethanol-exposed rats. The behavioral procedure and results for Experiment 2 can be seen in Figure 2A-C. Freezing behavior was assessed with a 2 (Dosing: EtOH vs. SI) × 3 (Condition: SAL vs. PHY vs. Pooled-Alt-Pre) × 2 (Phase of testing: Post-shock vs. Retention) between-within factorial design. We predicted that alcohol would impair freezing in SAL- but not PHY-treated rats, with no difference in freezing between EtOH-SAL rats and the non-associative Alt-Pre control group pooled across drug (Pooled-Alt-Pre; see section 2.5).

Analyses for Experiment 2 were run on 76 rats distributed across the following groups: SI-Pooled-Alt-Pre (n=14), SI-SAL-Pre (n=13), SI-PHY-Pre (n=12), EtOH-Pooled-Alt-Pre (n=12), EtOH-SAL-Pre (n=12), and EtOH-PHY-PRE (n=13). ANOVA revealed a significant main effect of Dosing [$F(1, 63) = 16.58$, $p < .001$], Condition [$F(2, 63) = 64.56$, $p < .001$], and a significant Dosing × Condition interaction [$F(2, 63) = 21.82$, $p < .001$]. There was no main effect or any interactions involving the repeated measure of Phase ($ps > .05$). Freezing above the non-associative Pooled-Alt-Pre control group was present in SI rats regardless of drug, but was only present in EtOH rats given PHY ($ps < .01$). Consistent with Experiment 1, SI and EtOH freezing levels during the preexposure were 2.91 ± 0.75 SEM and 3.23 ± 0.51 SEM, respectively. These results demonstrate that PHY treatment prior to context learning rescues abolished freezing in the CPFE.

3.3.1. IEG results—Littermates of the behavior group were sacrificed 30 min after context exposure on the preexposure day of the CPFE (see sections 2.6 and 2.7). The IEG

results can be seen in Figure 3A-C. Gene expression was analyzed using a 2 (Dosing condition; SI vs. EtOH) × 2 (Drug condition; SAL vs. PHY) factorial ANOVA for each gene (*c-Fos, Arc, Egr-1,* and *Npas4*) in the mPFC, dHPC, and vHPC. Post-hoc contrasts were performed with Newman–Keuls tests and a Dunnett's test that contrasted the four experimental groups with the HC control group. The number of outliers removed in each sampling condition can be found in Table 2.

In the mPFC (Fig. 3A), the EtOH-SAL group showed significantly reduced mRNA expression of the IEGs *c-Fos, Arc,* and *Npas4* compared to Group SI-SAL ($p < .001$). Interestingly, PHY administration in EtOH rats specifically rescued prefrontal expression of *c-Fos* (i.e., the increase occurred in EtOH-PHY but not SI-PHY rats; ps < .01). Both SI and EtOH rats given PHY showed significantly elevated expression of the IEGs *Arc* and *Npas4* above their respective saline-treated groups (ps < .01), indicating a failure of PHY to reverse ethanol effects on these IEGs. While PHY treatment did raise prefrontal *egr-1* expression in SI rats ($p < .05$), there was no difference in expression between the EtOH groups (ps > .20). Dunnett's tests revealed that every group was significantly elevated above HC levels (ps < .01) with the exception of EtOH-SAL for *Arc*; and EtOH-SAL and EtOH-PHY for *egr-1* expression ($p > .05$).

In the dHPC (Fig. 3B), no main of interaction effects involving alcohol (dosing) were found for any IEG and a main effect of drug was found only for *c-Fos* (Table 2). The EtOH-PHY and SI-PHY groups showed significantly higher *c-Fos* expression than their saline-treated counterparts ($p < .01$), with no difference between the two groups ($p > .40$). Dunnett's tests revealed that SI and EtOH rats given either drug had significantly elevated expression of all IEGs above HC levels. Finally, in the vHPC (Fig. 3C), there were no main or interaction effects of dosing or drug (Table 2). While Dunnett's tests revealed that all four treated groups had significantly higher expression of *c-Fos* and *Arc* than HC levels (ps < .05), there was no elevation above HC in vHPC *egr-1* and *Npas4* (ps > .10).

Taken together, the results of Experiment 2 demonstrate that systemic PHY administration prior to context preexposure in the CPFE rescues context freezing in ethanol-exposed rats. A similar effect on IEG expression was found only for *c-Fos* in mPFC. PHY administration increased prefrontal *Arc* and *Npas4* expression, and dHPC *c-Fos* expression regardless of alcohol treatment. Consistent with previous findings (Heroux et al., 2018) alcohol impaired IEG expression only in mPFC.

4. Discussion

The current set of experiments examined the effects of systemic administration of the acetylcholinesterase inhibitor physostigmine on disruptions in contextual fear conditioning and regional immediate-early gene expression in adolescent rats receiving neonatal ethanol exposure during the brain growth spurt. Consistent with our prior reports [1,27,55,56], high binge-like doses of ethanol given over PD4-9 abolished both post-shock and retention test freezing in the CPFE. This behavioral disruption in ethanol-exposed rats was rescued by PHY administration prior to all three phases (Experiment 1) or just prior to context preexposure (Experiment 2), with no effects of PHY in sham-intubated rats. Furthermore,

PHY treatment prior to context learning selectively rescued ethanol-induced disruptions in prefrontal expression of *c-Fos* but not *Npas4* or *Arc,* or *Egr-1*. Prefrontal expression of *Npas4* and *Arc* was non-specifically boosted by PHY in both ethanol-exposed and sham-intubated rats, whereas PHY increased *Egr-1* in sham but not ethanol-exposed rats. Hippocampal IEG expression was not impaired by alcohol or altered by PHY, except for increased *c-Fos* expression in dHPC regardless of alcohol exposure. Taken together, augmenting cholinergic signaling rescues neonatal-alcohol induced impairment of configural learning, memory, and prefrontal gene expression.

The present study informs the psychological and neural mechanisms through which neonatal alcohol impairs cognition. The CPFE is a variant of contextual fear conditioning in which learning about the context, acquiring a context-shock association, and retention of contextual fear is separated across three days. The CPFE depends on the encoding of contextual cues on the preexposure day that are subsequently consolidated into a conjunctive context representation [67,68]. During training, pattern completion allows this retrieved conjunctive representation to be associated with immediate foot-shock (i.e. occurring less than 3s upon chamber entry) to yield context fear learning [67]. Rats preexposed to an alternate context are unable to form a context-shock association because of insufficient exposure to the training context [69]. The CPFE develops between PD17 and PD24 in the rat, after which it depends on activity and cholinergic muscarinic-receptor cell signaling in the dHPC and mPFC during all three phases [58-60,68,70]. While impairment of the CPFE is robust across dosing scenarios, neonatal ethanol exposure has no effect on hippocampal-dependent single-trial standard contextual fear conditioning, in which learning about the context and acquiring a context-shock association occur within the same session [1,71,72]. Neonatal ethanol exposure impairs the consolidation of the conjunctive context representation on the preexposure day of the CPFE [1,61], resulting in abolished post-shock and retention test freezing [see Experiment 1 and 2]. Moreover, ethanol-exposed rats have significantly reduced prefrontal but not hippocampal immediate early gene expression during context learning in the CPFE [1,13; see Experiment 2]. In summary, neonatal alcohol impairs incidental context learning or consolidation through a mechanism that may involve reduced prefrontal activity or plasticity.

In the current study, systemic administration of physostigmine prior to all three phases or just prior to context preexposure rescued post-shock and retention test freezing deficits in ethanol-exposed rats. This extends our prior finding that PHY treatment prior to all three phases rescues retention freezing in PD7-9 ethanol-exposed rats [62]. In both cases, the behavioral rescue was specific to ethanol-exposed rats, without any non-specific boost in performance in sham-intubated rats. In addition to rescuing behavioral performance, PHY treatment rescued prefrontal *c-Fos* expression in ethanol-exposed rats while boosting prefrontal *Arc* and *Npas4* and hippocampal *c-Fos* expression in both dosing groups. Taken together, these results further support our conclusion that impaired context learning or consolidation by PD4-9 ethanol exposure reflects impaired prefrontal activity or plasticity involving cholinergic signaling.

Developmental alcohol exposure during the brain growth spurt significantly impairs neurobehavioral maturation of the hippocampus and prefrontal cortex in rats (see [1] for

extended discussion). Ethanol exposure during the third trimester equivalent of human pregnancy (i.e., PD4-6, PD7-9, PD4-9, or PD2-10) decreases hippocampal CA1 pyramidal cell counts but has no lasting effect on prefrontal cell number in rats [25-27,29,30,54,73]. In addition to cell loss, ethanol exposure disrupts cholinergic and glutamatergic cell signaling, gene expression, and neuroplasticity in the hippocampus [23,27,29,36,74-76]. Although the current study failed to find any alcohol-induced disruption in hippocampal IEG expression during context learning, this does not rule out the possibility that hippocampal dysfunction could contribute to the current findings (see [1] for extended discussion). It's possible that the current study did not observe disrupted hippocampal IEG expression due to sampling the entire dHPC (with PCR) vs. distinct sub-regions (with *in situ* hybridization or stereology), examining one time-point, and differences in dosing window or cell-type specificity. In contrast to the hippocampus, neonatal ethanol exposure results in altered gene expression, dendritic complexity, voltage-gated Ca^{2+} channel activity, and increased DNA methylation in the prefrontal cortex [1,23,28,30-32,77]. Importantly, ethanol-induced disruptions in behavioral performance cannot be fully accounted for by hippocampal cell loss [26,27]. For example, rescuing hippocampal CA1 cell loss via Vitamin E supplementation after PD7-9 ethanol exposure does not normalize performance in the Morris Water Maze (MWM) in rats [26]. Indeed, that PHY treatment prior to only context preexposure rescues the CPFE suggests that the lasting effects of ethanol on behavior are likely due to altered connectivity or molecular signaling of neurons rather than being solely due to developmental injury or loss of neurons themselves. Moreover, our data suggest that reduced activity or plasticity in the prefrontal cortex significantly contributes to ethanol-induced disruptions in behavior that have traditionally been solely attributed to the hippocampus.

Emerging evidence in both human and animal models has demonstrated a link between disrupted cholinergic function across development as a mechanism and potential therapeutic target underlying ethanol-induced insult [see 34 for discussion]. In rats, neonatal ethanol exposure reduces acetylcholine efflux and bioavailability of choline [39,40,43]. Ethanol-induced disruption of muscarinic-receptor cell signaling during development interferes with neuritogenesis and alters muscarinic-receptor cell composition in the hippocampus [35,36,39,41]. Developmental choline supplementation during and after alcohol exposure mitigates many of the neurobiological and behavioral disruptions caused by developmental alcohol exposure. For example, choline reverses increased global DNA methylation in the hippocampus and prefrontal cortex caused by PD2-10 ethanol exposure in rats [23]. Ethanol-induced disruptions in the MWM, fear conditioning, spatial discrimination, working memory, and motor behavior are attenuated by developmental choline supplementation in rats [44-46,48-50,78,79]. Thus far, research has focused on examining the effects of prolonged choline supplementation on ethanol-induced disruptions in behavior later in life. Here, we show that boosting cholinergic signaling via acute treatment with an acetylcholinesterase inhibitor prior to learning attenuates ethanol-induced behavioral disruptions in contextual fear conditioning in adolescent rats [62]. Interestingly, deficits in trace fear conditioning in rats receiving PD4-9 ethanol exposure are also dose-dependently rescued by systemic PHY treatment prior to training [44]. While more research is needed, some mechanisms by which PHY treatment may rescue neurobehavioral deficits by

decreasing neuroinflammation, lowering the threshold for LTP induction, and increasing bioavailability of acetylcholine [36,80-82].

The current study supports and extends previous literature examining the neurobiology of contextual fear conditioning and the role of IEGs in learning and memory. We chose to examine the IEGs *c-Fos, Arc, Egr-1,* and *Npas4* because we have recently reported that neonatal ethanol exposure impairs expression of these IEGs during context learning [1,28]. Moreover, context exposure and fear training induces expression of these IEGs in the prefrontal cortex, hippocampus, and amygdala in rats [1,27,83]. These IEGs are also expressed during spatial learning, as well as cued, trace, and contextual fear conditioning across various stages of development [63,83-98]. The IEGs *c-Fos, Egr-1,* and *Npas4* are transcription factors that regulate the expression of other plasticity-associated late-response genes that support long-term memory, whereas *Arc* encodes a protein that directly regulates dendritic synaptic plasticity [99-101]. While their role is likely universal in supporting memory, the IEGs *Arc* and *Npas4* have been suggested to be important for consolidation of contextual and spatial memories [84,89,91,98,102], and *c-Fos* and *Egr-1* have been studied in relation to the consolidation of fear memories [94,103-108]. Despite emerging evidence supporting a role of the prefrontal circuitry across learning paradigms, especially those relevant to alcohol-induced insult [1,60,88,109-111], examination of the role of prefrontal IEG-expressing neurons in memory has been largely ignored in favor of the hippocampus. Although the mechanism is not known, we show that PHY treatment in ethanol-exposed rats differentially elevates expression of *c-Fos, Arc,* and *Npas4* in the prefrontal cortex and hippocampus. PHY treatment eliminated the alcohol-induced deficit in context fear and in prefrontal *c-Fos* expression. For other prefrontal IEG expression as well as for dHPC *c-Fos* expression, PHY treatment elevated IEG expression but did not rescue the alcohol-induced deficit. While the current study cannot fully establish causality between gene expression and behavior, the data suggest a mechanistic role for prefrontal *c-Fos* expression. Increased acetylcholine bioavailability via systemic PHY treatment elevates *c-Fos* expression by activating neuronal nicotinic and muscarinic receptors across receptor-populated brain regions, including the prefrontal cortex [112-114]. While there is a clear disruption in dHPC cholinergic receptor function after neonatal alcohol exposure in rats, this disruption likely extends beyond the dHPC and might inform our findings in the mPFC [36]. Given that *c-Fos* expression is linked to neuronal activity and plasticity necessary for cellular consolidation of long-term memory [99,100], PHY treatment likely recues alcohol-induced deficits by augmenting plasticity-related protein expression. It is also possible that elevated prefrontal *Arc* and hippocampal *c-Fos* expression in ETOH-PHY rats contributed to the rescue by exceeding a threshold of expression that was not met in EtOH-SAL rats. Another contributing factor might be IEG expression differences between neuronal sub-regions (e.g., IL vs. PL) and sub-types between dosing conditions, which may contribute to the cholinergic rescue of neurobehavioral effects of alcohol. More research is needed to establish mechanistic roles of cholinergic signaling, regional expression of specific IEGs, and behavioral performance in normally developing and ethanol-exposed rats.

In summary, our findings demonstrate that the acetylcholinesterase inhibitor physostigmine rescues ethanol-induced disruptions in context memory and prefrontal expression of some immediate early genes in adolescent rats. While the current study cannot establish a causal

link between IEG expression and behavior, these results suggest that ethanol disrupts behavioral performance by altering activity and/or plasticity induced by cholinergic signaling in the prefrontal cortex during configural learning and memory. Taken together with our recent reports [1,28,62], these findings are important because prefrontal dysfunction is an integral hallmark of FASD in humans, but animal models have thus far largely failed to capture prefrontal dysfunction after third-trimester equivalent exposure. Building upon an emerging body of animal and human research linking alcohol and cholinergic dysfunction, future experiments should characterize the effects of intra-cranial infusions of drugs that augment cholinergic signaling into mPFC or other discrete brain regions on behavioral performance of alcohol-exposed rats across the lifespan.

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Highlights

- Neonatal alcohol exposure disrupts context and contextual fear memory in rats
- Alcohol exposure disrupts prefrontal but not hippocampal IEG expression
- Systemic acetylcholinesterase inhibition rescues behavioral deficits in exposed rats
- This treatment specifically reverses deficits in prefrontal *c-Fos* in alcohol-exposed rats
- This treatment nonspecifically elevates prefrontal *Arc, Npas4,* and hippocampal *c-Fos*

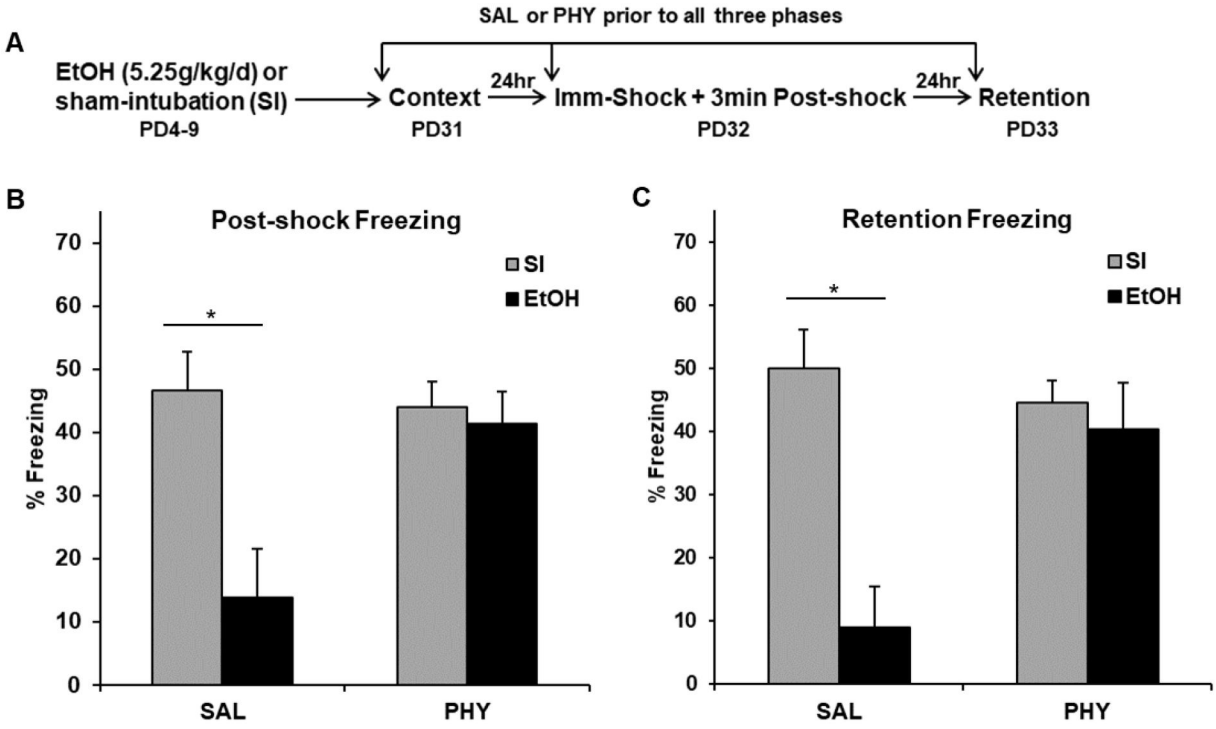

Figure 1.

Behavioral design **(A)** and mean percent freezing (± SEM) for the 3min post-shock **(B)** or 5min retention **(C)** freezing tests in Experiment 1. **(A)** Rats were given alcohol (EtOH) or sham-intubation (SI) from PD4-9, and given physostigmine (PHY) or saline (SAL) prior to each phase of the CPFE procedure occurring from PD31-33. **(B-C)** SAL-treated EtOH group rats showed abolished post-shock and retention test freezing compared to SI rats regardless of drug treatment. PHY treatment prior to each phase of the CPFE restored freezing in EtOH rats compared to their SAL-treated counterparts. * indicates $p < .05$

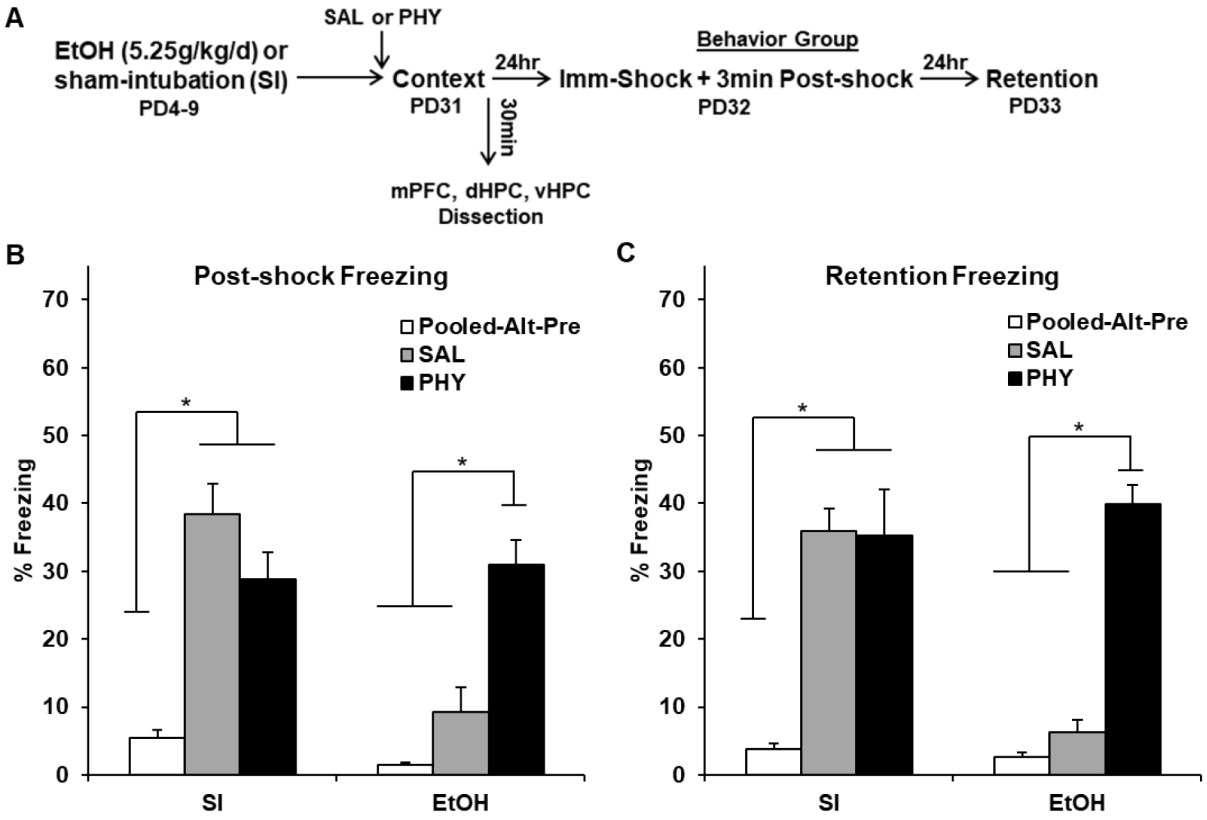

Figure 2.

Behavioral design **(A)** and mean percent freezing (± SEM) for the 3min post-shock **(B)** or 5min retention **(C)** freezing tests in Experiment 2. **(A)** Rats were given alcohol (EtOH) or sham-intubation (SI) from PD4-9, and given physostigmine (PHY) only prior to context preexposure in the CPFE. **(B-C)** Freezing above the non-associative Pooled-Alt-Pre control group was present in SI rats regardless of drug, but was only present in EtOH rats given PHY but not SAL. * indicates $p < .05$

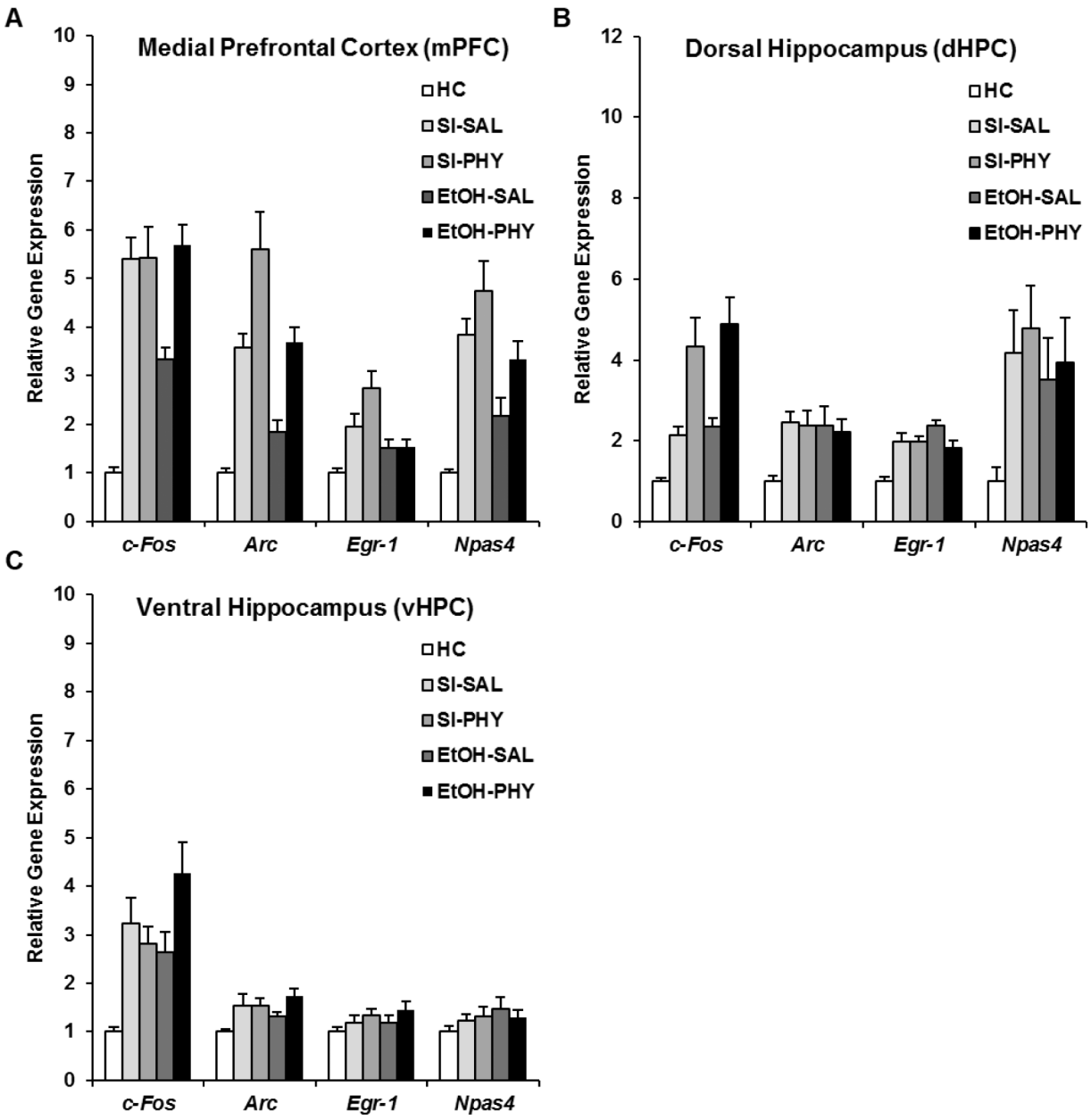

Figure 3.

mRNA expression of *c-Fos, Arc, Egr-1,* and *Npas4* in the mPFC **(A)**, dHPC **(B)**, or vHPC **(C)** in SI and EtOH rats treated with SAL or PHY sacrificed 30min after context exposure in the CPFE. **(A)** The EtOH-SAL group showed significantly reduced mRNA expression of every IEG compared to Group SI-SAL. PHY treatment rescued *c-Fos* expression in the mPFC of EtOH rats, but elevated Arc and npas4 regardless of ethanol treatment. **(B)** PHY treatment elevated *c-Fos* expression in the dHPC in both SI and EtOH rats. **(C)** PHY treatment elevated *c-Fos* expression in the vHPC in EtOH rats.

Table 1

Body weights and BACs for Experiment 1 and 2. Average body weights (in grams ± SE) are given from the SI and EtOH groups at the first and last day of the dosing period (PD4 and PD9, respectively) and the first day of behavioral training (PD31). BACs (in mg/dl ± SE) were taken from blood samples collected on PD4 from the EtOH group. * indicates a significant difference between the SI and EtOH groups.

Experiment	Dose	n =	Body weight (Grams ± SEM)				PD4 BACs (mg/dl)
			PD4	PD9	PD31 (males)	PD31 (females)	
Experiment 1	**SI**	23	12.1 ± 0.27	20.8 ± 0.36	115.42 ± 2.57	108.55 ± 2.87	N/A
	EtOH	20	12.06 ± 0.26	17.68 ± 0.46*	110.55 ± 3.24	101.27 ± 1.69	389.33 ± 4.78
Experiment 2	**SI**	77	11.49 ± 0.14	21.23 ± 0.21	116.17 ± 1.46	106.16 ± 1.57	N/A
	EtOH	77	11.42 ± 0.15	17.79 ± 0.20*	115 ± 1.21	105.76 ± 1.39	407.80 ± 2.86
Experiment 1 and 2 Collapsed	**SI**	100	11.63 ± 0.16	21.13 ± 0.18	116 ± 1.26	106.70 ± 1.37	N/A
	EtOH	97	11.56 ± 0.14	17.77 ± 0.19*	114.17 ± 1.16	104.75 ± 1.16	403.99 ± 2.58

Table 2

Final group numbers (*n*), number of outliers removed (*HC, SI-SAL, SI-PHY, EtOH-SAL, EtOH-PHY*), and statistical results for all factorial ANOVAs (see *F* and *p* values) for each gene (*c-Fos, Arc, Egr-1,* and *Npas4*) in each region (mPFC, dHPC, and vHPC) for Experiment 2.

	Medial Prefrontal Cortex (mPFC)				Dorsal Hippocampus (dHPC)				Ventral Hippocampus (vHPC)			
	F	***p***	***Final n***	***Outliers***	***F***	***p***	***Final n***	***Outliers***	***F***	***p***	***Final n***	***Outliers***
Genes			(*HC, SI-SAL, SI-PHY, EtOH-SAL, EtOH-PHY*)				(*HC, SI-SAL, SI-PHY, EtOH-SAL, EtOH-PHY*)				(*HC, SI-SAL, SI-PHY, EtOH-SAL, EtOH-PHY*)	
c-Fos												
Dosing	4.53	< .05			0.58	> .45			0.73	> .39		
Drug	7.63	< .01	23, 11, 8, 12, 12	1, 1, 1, 0, 0	21.59	< .01	21, 10, 10, 11, 11	2, 1, 1, 0, 0	1.53	> .23	22, 10, 10, 11, 9	2, 1, 1, 1, 0
Dosing × Drug	7.56	< .01			0.12	> .70			4.37	< .05		
Arc												
Dosing	20.26	< .01			0.11	> .74			0.01	> .90		
Drug	18.13	< .01	22, 11, 9, 10, 11	2, 1, 0, 2, 1	0.1	> .74	21, 10, 10, 10, 10	2, 1, 1, 1, 1	1.55	>.22	22, 10, 10, 11, 10	2, 1, 1, 1, 1
Dosing × Drug	0.04	> .80			0.03	> .87			1.71	> .19		
Egr-1												
Dosing	12.03	< .01			0.54	> .45			0.14	> .70		
Drug	2.94	> .10	22, 11, 9, 11, 10	2, 1, 0, 1, 2	2.83	> .10	20, 11, 10, 10, 10	1, 1, 1, 1, 1	1.85	> .18	22, 10, 10, 11, 10	2, 1, 1, 1, 1
Dosing × Drug	2.51	> .12			2.75	> .10			0.08	> .77		
Npas4												
Dosing	19.2	< .01			0.22	> .64			0.35	> .56		
Drug	9.55	< .01	23, 12, 9, 11, 11	1, 0, 0, 1, 1	0.49	> .48	21, 11, 11, 11, 11	2, 0, 0, 1, 0	0.07	> .79	22, 10, 10, 12, 10	2, 1, 1, 0, 1
Dosing × Drug	0.49	> .48			0.01	> .90			0.43	> .50		