Published in final edited form as:

Behav Neurosci. 2018 December ; 132(6): 497–511. doi:10.1037/bne0000272.

Impairment of the Context Pre-exposure Facilitation Effect in Juvenile Rats by Neonatal Alcohol Exposure is Associated with Decreased Egr-1 mRNA Expression in the Prefrontal Cortex

Sarah A. Jablonski, Patrese A. Robinson-Drummer, William B. Schreiber, Arun Asok, Jeffrey B. Rosen, and Mark E. Stanton

University of Delaware, Department of Psychology, Newark, DE, 19716. USA

Abstract

The context preexposure facilitation effect (CPFE) is a variant of contextual fear conditioning in which learning about the context (preexposure) and associating the context with a shock (training) occur on separate occasions. The CPFE is sensitive to a range of neonatal alcohol doses (Murawski & Stanton, 2011). The current study examined the impact of neonatal alcohol on Egr-1 mRNA expression in the infralimbic (IL) and prelimbic (PL) subregions of the mPFC, the CA1 of dorsal hippocampus (dHPC), and the lateral nucleus of the amygdala (LA), following the preexposure and training phases of the CPFE. Rat pups were exposed to a 5.25 g/kg/day single binge-like dose of alcohol (Group EtOH) or were sham intubated (SI; Group SI) over postnatal days (PD) 7–9. In behaviorally tested rats, alcohol administration disrupted freezing. Following context preexposure, Egr-1 mRNA was elevated in both EtOH and SI groups compared to baseline control animals in all regions analyzed. Following both preexposure and training, Group EtOH displayed a significant decrease in mPFC Egr-1 mRNA expression compared to Group SI. However, this decrease was greatest after training. Training day decreases in Egr-1 expression were not found in LA or CA1 in Group EtOH compared to Group SI. A second experiment confirmed that the EtOH-induced training-day deficits in mPFC Egr-1 mRNA expression were specific to groups which learned contextual fear (versus non-associative controls). Thus, memory processes that engage the mPFC during the context-shock association may be most susceptible to the teratogenic effects of neonatal alcohol.

Keywords

Neonatal alcohol exposure; Contextual fear conditioning; Prefrontal cortex; Zif268; Immediate early genes

Introduction

Fetal alcohol spectrum disorders (FASDs) describe a continuum of neurobehavioral impairments resulting from prenatal exposure to alcohol. In children, classical behavioral phenotypes of FASDs involve an array of higher-order cognitive deficits, such as impaired

spatial processing (Coles et al., 1991; Hamilton, Kodituwakku, Sutherland, & Savage, 2003; Uecker & Nadel, 1996), impaired attention (Mattson, Crocker, & Nguyen, 2011), and impaired executive functioning, including poor response inhibition (Fryer et al., 2007; O'Brien et al., 2013), adaptive behavior (Ware et al., 2012), concept formation and set-shifting (Mattson et al., 2011; Schonfeld, Mattson, Lang, Delis, & Riley, 2001; Vaurio, Riley, & Mattson, 2008). Human imaging studies have shown that the effects of prenatal alcohol on the developing nervous system are not uniform since some brain regions or cell populations are more vulnerable than others (Alfonso-Loeches & Guerri, 2011). For example, the hippocampus, cerebellum and frontal cortices are especially sensitive to early alcohol insult (Fryer et al., 2007; Norman, Crocker, Mattson, & Riley, 2009; Norman et al., 2013; Spadoni et al., 2009).

In rat models of FASDs, alcohol exposure during the perinatal period, a stage of brain maturation that corresponds to the third trimester of human development (Dobbing & Sands, 1979) leads to a wide-variety of morphological and physiological consequences to the developing hippocampus (e.g., Livy et al., 2003; Marino, Aksenov, & Kelly, 2004; Puglia & Valenzuela, 2010a;b; Savage et al., 1992; Tran & Kelly, 2003). In addition, neonatally-exposed subjects are impaired in various hippocampal-dependent memory tasks which require spatial learning and/or trace memory retention (Goodlett & Johnson, 1997; Jablonski & Stanton, 2014; Murawski et al., 2012; Murawski & Stanton, 2010; Schreiber, St Cyr, Jablonski, Hunt, Klintsova & Stanton, 2013; Thomas & Tran, 2012; Tomlinson, Wilce, & Bedi, 1998). Our lab has consistently demonstrated significant impairments in rats exposed to alcohol on both PD4–9 and PD7–9 on the hippocampal-dependent variant of contextual fear conditioning called the context pre-exposure facilitation effect (CPFE; Dokovna, Jablonski, & Stanton, 2013; Jablonski & Stanton, 2014; Murawski & Stanton, 2011; Murawski et al., 2012; Murawski & Stanton, 2010). The CPFE is a 3-day procedure. First, animals explore the conditioning context during pre-exposure. A single unified representation of that context is then stored and consolidated (Jablonski & Stanton, 2014; Jablonski, Schiffino, & Stanton, 2012; Rudy, 2009; Rudy & O'Reilly, 1999). On the second day during training, animals use “pattern completion” during the brief (3–7 s) training episode to recall the entire contextual representation from the pre-exposure day. This retrieved representation of the context is subsequently associated with immediate foot shock (Rudy, 2009). During a test of contextual freezing which occurs on the final day, rats pre-exposed to the conditioning context retrieve the context-shock association and freeze more than control rats that were initially pre-exposed to an alternate context (Fanselow, 1990). Animals exposed to an alternate context on the pre-exposure day serve as non-associative controls. They exhibit the “immediate shock deficit,” a failure to associate the training context with the shock because of insufficient time to encode the context on the training day (Fanselow, 1990). Importantly, by separating context learning from context-shock associative learning, the CPFE paradigm can be used to further elucidate which memory processes are impaired by neonatal alcohol (Jablonski & Stanton, 2014). Recently, Murawski et al. (2012) demonstrated significant reductions in the number of CA1 pyramidal cells in rats exposed from PD4–9 to only the high dose (5.25 g/kg/day), but not a lower dose (4.00 g/kg/day) of alcohol compared to controls. The CPFE is sensitive to a range of neonatal alcohol doses (Murawski & Stanton, 2011) and the overall loss of CA1 pyramidal

cells counts do not fully explain the dose-dependent alcohol-induced behavioral deficits observed in the CPFE (Murawski et al., 2012). Similarly, alcohol co-administered with vitamin E reverses the reduction in CA1 pyramidal cell number following alcohol exposure, without affecting the alcohol-induced deficit in spatial learning (Marino et al., 2004). It may be, rather than cell loss per se, altered neuronal signaling mechanisms following alcohol exposure in the hippocampus, or more importantly in other structures, is what accounts for the behavioral deficits seen in the CPFE.

We have begun to investigate the activation patterns of the immediate-early gene (IEG) early-growth response gene 1 (Egr-1, Krox 24, NGFI-A, Zif268, TIS8) following the pre-exposure and training phases of the CPFE in developing rats (Asok et al., 2013; Robinson-Drummer, Chakarabarty, Heroux, Rosen & Stanton, 2018; Schreiber, Asok, Jablonski, Rosen, & Stanton, 2014). Egr-1 is an inducible regulatory transcription factor linked to neural plasticity and is broadly associated with learning and memory (for reviews, see Alberini, 2009; Bozon, Davis, & Laroche, 2002; Bozon et al., 2003; Davis, Bozon, & Laroche, 2003; Rosen & Donley, 2006; Valenzuela, Morton, Diaz, & Topper, 2012). In the rat, Egr-1 mRNA is evident by the 2nd and 3rd postnatal weeks (Herms, Zurmöhle, Schlingensiepen, Brysch, & Schlingensiepen, 1994; Sanders, Happe, Bylund, & Murrin, 2008). Egr-1 expression (both mRNA and protein) is increased in the hippocampus and amygdala following acquisition and/or retention of contextual fear conditioning (Lee, 2010; Malkani & Rosen, 2000; Malkani, Wallace, Donley, & Rosen, 2004; Rosen, Fanselow, Young, Sitcoske, & Maren, 1998). Interestingly, following context-shock training in the CPFE, animals exposed to the conditioning context (Group Pre) show an increase in Egr-1 mRNA expression over control animals pre-exposed to an alternate context (Group Alt-Pre) in the prelimbic (PL) and infralimbic (IL) subregions of the mPFC (Asok et al., 2013). Elevated Egr-1 expression in the PL for Group Pre extends to a multiple pre-exposure paradigm which provides an increased amount of context exploration on the pre-exposure day, suggesting that this training day increase persists even when context novelty is reduced (Schreiber et al., 2014). These findings, then, are consistent with other studies identifying a role of Egr-1 in context-shock and auditory cue-shock associations in the lateral amygdala (Malkani et al., 2004; Maddox, Monsey, & Schafe, 2011), however, in the most recent studies the mPFC has emerged as an important area for evaluating plasticity-related alterations in contextual fear conditioning (Asok et al., 2013; Robinson-Drummer et al., 2018; Schreiber et al., 2014). Our recent studies involving localized drug infusions confirm that the CPFE depends on neural activity and cholinergic signaling in the mPFC (Heroux, Robinson-Drummer, Sanders, Rosen & Stanton, 2017; Robinson-Drummer, Heroux & Stanton, 2017).

The current study examined how neonatal alcohol exposure influences Egr-1 mRNA expression during the CPFE in adolescent rats. We hypothesized that neonatally-exposed animals would show less Egr-1 mRNA expression, relative to sham-intubated controls, following the context acquisition and/or context-shock association phases of the CPFE. Particularly, this decrease would be evident in the PL and IL subregions. Because the CPFE correlates with differential activation of mPFC Egr-1 mRNA in the Pre- vs. Alt-Pre groups on the training day, it may be that behavioral deficits in alcohol-exposed animals would correspond to decreases in training day Egr-1 in the mPFC. Neonatal alcohol exposure in

rats has no effect on cell number but reduces mPFC dendritic complexity (Granato, Di Rocco, Zumbo, Toesca, & Giannetti, 2003; G. F. Hamilton, Whitcher, & Klintsova, 2010), reduces mPFC spine density and distribution (Lawrence, Otero, & Kelly, 2012; Whitcher & Klintsova, 2008), suppresses synaptic release mechanisms (Barr, Hofmann, Phillips, Weinberg, & Honer, 2005; Zink et al., 2009) and leads to a lower number and shorter duration of dendritic spikes in mPFC pyramidal neurons (Granato, Palmer, De Giorgio, Tavian, & Larkum, 2012). Others have demonstrated a correlation between gestational alcohol exposure, prefrontal Egr-1 expression and impaired cognitive function on a spatial T-maze alternation task (Nagahara & Handa, 1995). Together, these findings suggest reduced synaptic targets and impaired plasticity-associated functions in the mPFC are a result of developmental alcohol exposure. Experiment 1 examined regional gene expression on the preexposure and training days of the CPFE protocol and behavioral performance on the testing day. Experiment 2 replicated and extended the training-day and behavioral effects reported in Experiment 1 by adding a non-associative (Alt-Pre) control group to the design. The current report is the first to examine whether neonatal alcohol disrupts Egr-1 mRNA expression in the hippocampus, amygdala and/or prefrontal cortex of adolescent rats following context exposure and/or context-shock training in a manner that could underlie the alcohol-induced behavioral deficits in the CPFE.

Experiment 1

Methods

Subjects—Subjects were 110 (60 males and 50 females) Long-Evans rats derived from 17 time-bred dams in the University of Delaware breeding colony. Of these, 32 (20 males; 12 females) were assigned to the behavioral assay, 39 (19 males; 20 females) were assigned to the pre-exposure day Egr-1 mRNA assay, and 39 (21 males 18 females) were assigned to the training day Egr-1 mRNA assay (see Table 1).

As previously described (Jablonski & Stanton, 2014; Murawski & Stanton, 2011), time-mated female rats were housed with breeder males in the animal housing facility of the Office of Laboratory Animal Medicine (OLAM) at the University of Delaware. The following day, rats were examined for the presence of an ejaculatory plug and if found, that day was designated as gestational day (GD) 0. Pregnant females were housed in polypropylene cages (45 × 42 × 21 cm) with standard bedding and *ad libitum* access to food and water. The animal housing facility was maintained on a 12:12h light/dark cycle. The age of the litters was determined by checking for births during the illuminated period. The date of birth was designated as PD0 (all births occurred on GD22). On PD2, litters were brought to the lab's local colony room. On PD3 litters were culled to 8 pups (usually 4 males and 4 females) and were given subcutaneous injections of non-toxic black ink into one or more paws for identification. On PD21 pups were weaned and housed in 45 × 24 × 17 cm cages with same-sex littermates and had *ad libitum* access to water and food. On PD28–29, (2 days prior to experimentation) rats were individually housed in smaller opaque cages (24 × 18 × 13 cm) throughout the duration of the experiment. All subjects were treated in accordance with guidelines of the Institutional Animal Care and Use Committee at the University of Delaware.

Alcohol Dosing—As described previously (e.g., Murawski & Stanton, 2011), pups were administered a single binge-like dose of alcohol from PD7 through PD9 (5.25g/kg/day). Within each litter, pups were assigned to receive either ethanol (Group EtOH) or sham intubations (Group SI), with an equal number of males and females in each group, whenever possible. Same-sex littermates assigned to the same Treatment (SI or EtOH) were placed in distinct Sampling Conditions (Pre-exposure, Training, Behavior) and/or assigned as a home-caged control (HC) during either pre-exposure or training (see Table 1), so that no more than one same-sex littermate was assigned to a particular experimental condition. We did not include a “normally reared” control group in this study because this group does not differ from the SI group behaviorally (Murawski & Stanton, 2011) and we have already reported on behavior and *Egr-1* expression in normal rats tested under identical conditions (Asok et al., 2013), which the SI controls in this study replicate (see *Results*, below).

Beginning on PD7, pups (typically 8/litter) were separated from their dams and placed together on an anti-static weigh boat situated above a heating pad, which was set to a low setting in order to provide warmth in absence of the dam. Each pup was weighed prior to intubation (around 9:00 am). While each pup was gently restrained, intragastric intubations consisted of a thin (PE10) tube lubricated with corn oil, which was passed over the tongue down the esophagus and into the stomach of each pup. For Group EtOH, pups received a single binge-like dose of alcohol (5.25g/kg/day; 23.94% v/v) in a custom milk formula (Kelly & Lawrence, 2008). The formula was delivered in a volume of 0.02778 ml/g body weight for about 10–15 seconds. For Group SI, pups were intubated the same way, however no milk formula was infused (to prevent abnormally high weight gain, Goodlett & Johnson, 1997). After the dosing/intubation was completed (typically 20 min/litter), pups were returned back as a litter to the dam. Two hours (± 10 min) after the first intubation, pups were taken from the dam in an identical manner for the second intubation. This time, each pup received a small tail clip in which blood samples were collected into a heparinized capillary tube. Samples from Group SI were discarded while those from Group EtOH were frozen and stored for analysis (See “Blood Alcohol Concentration”). The second intubation procedure was identical to the first, however Group EtOH received an infusion of milk alone (without alcohol) and Group SI received another sham intubation. A third milk-only or sham intubation occurred after the second dosing session. The additional calories of the supplementary milk help to maintain normal body weight throughout the dosing period (Marino et al., 2004). The identical intubation procedure occurred on PD8 and PD9 except no blood samples were collected and only 1 milk-only or sham intubation occurred after the first administration.

Blood Alcohol Concentration—Blood samples taken from Group EtOH on PD7 were centrifuged and plasma was stored at -20°C . Blood alcohol concentration (BAC) was determined using an Analox GL5 Analyzer (Analox Instruments, Luneberg, MA) as previously described (Brown, Calizo, Goodlett, & Stanton, 2007; Green, Johnson, Goodlett, & Steinmetz, 2002). Briefly, the rate of oxidation of alcohol was measured from each plasma sample. BAC was determined by comparing the plasma alcohol concentration (mg/dl) to the known values of an alcohol standard.

Apparatus and Stimuli—The apparatus and stimuli were as described in previous reports from our laboratory (e.g., (Asok et al., 2013; Murawski & Stanton, 2010; Schiffino, Murawski, Rosen, & Stanton, 2011). Context conditioning (i.e. preexposure, training and testing) occurred in one of four clear Plexiglas chambers ($16.5 \times 21.1 \times 21.6$) situated under a fume hood, which provided minimal background noise and overhead lighting. The sides and ceilings of each chamber were transparent except for one opaque wall, which prevented viewing of the adjacent rat. Stainless steel bars (0.5-cm diameter, 1.25-cm apart) constituted the floor of each chamber and were connected to a shock generator. The unconditioned stimulus (US) was a single 2s 1.5 mA footshock. Testing day freezing was recorded with a video camera connected to a Dell computer running FreezeFrame software (Coulbourn Instruments, Whitehall, PA). Offline analysis of animal movement was determined by measuring changes in pixel luminance.

Design and Procedures—The general training procedure has been described previously (Asok et al., 2013; Murawski et al., 2012). There were eight experimental conditions with one littermate assigned to each condition (Table 1). Typically, three littermates (from both Group SI and Group EtOH) were sacrificed on the Pre-exposure day, three on the Training Day, and 2 littermates were retained for behavioral testing on the final day, to confirm the alcohol-induced deficit in test-day freezing seen in our previous studies (e.g., Dokovna et al., 2013; Jablonski & Stanton, 2014; Murawski & Stanton, 2010). In order to streamline the design, the No-Pre/Alternate-Pre group from our typical CPFE studies was not excluded in Experiment 1. We've consistently shown low levels of context fear in rats exposed to an alternate context, at various developmental ages (Asok et al., 2013; Burman, Murawski, Schiffino, Rosen, & Stanton, 2009; Jablonski et al., 2012; Schiffino et al., 2011; Schreiber et al., under review). Additionally, freezing in this group is not influenced by neonatal alcohol exposure (Murawski & Stanton, 2011; Murawski et al., 2012; Murawski & Stanton, 2010). The No-Pre/Alt-Pre group was included in Experiment 2, which replicated and extended the training-day phase and behavioral data from Experiment 1.

Behavior

Pre-exposure: During pre-exposure on PD30–31 (between 2–6pm), rats were weighed and placed individually in Plexiglas cages ($11 \times 11 \times 18$ cm) covered with opaque paper on each side. For each “load” of animals, rats were transported (typically 4 at a time) to the conditioning context. Once animals arrived to the behavioral testing area, the rats waited while the experimenter cleaned each chamber with 5% ammonium hydroxide (approximately 2-min). Rats were then brought into the testing room and placed in the chamber where exploration occurred for 5-min. Rats were then removed from the chamber, placed in the transport container and immediately returned to the colony room. Pre-exposure chamber location was counterbalanced across each experimental condition (see Table 1). For the pre-exposure sampling condition, rats were sacrificed 30 min (± 3) after removal from the chamber. HC rats were sacrificed immediately after removal from their home cage (they were not previously weighed), while littermates underwent pre-exposure.

Training: Twenty-four (± 1) hours after pre-exposure, rats were again weighed and transported to the behavioral testing area in a manner identical to the previous day.

Following chamber cleaning (approximately two minutes), rats were brought into the testing room one at a time and were placed in their respective training chamber (the identical chamber as pre-exposure). Upon placement, rats received an immediate (<5s) 2s, 1.5 mA footshock. Rats were immediately removed following unconditioned stimulus (US) offset and were returned to the colony room. Again, for the training sampling condition, rats were sacrificed 30 ($\pm$ 3) min after removal from the chamber and HC rats were sacrificed immediately after removal from their home cage while littermates underwent training.

Testing: Twenty-four ($\pm$ 1) hour after training, rats in the behavioral sampling condition were returned to their respective chamber where freezing to the context was measured for 5-min. All other procedures were identical to the pre-exposure and training phases.

As described previously (Schiffino et al., 2011), behavioral freezing was measured with FreezeFrame software. It was defined as the cessation of all movement, lasting 0.75 s or longer, except for respiration. The software program computes a “motion index” separately for each animal (per software instructions) and a freezing threshold for each subject based on changes in pixel luminance. A human observer blind to the conditions of the subjects inspected the threshold and, if necessary, made offline adjustments by slightly increasing or decreasing the threshold in order to ensure that small motor movements were not recorded as freezing. Adjustments occurred only once during analysis of a given animal. We have validated this procedure against other scoring methods (e.g., hand scoring of video records by two blind observers) and found that it is very reliable ($r = 0.99$; $p < .001$; unpublished observations). When freezing scores fell ± 1.96 standard deviations from the group mean, that score was defined as an outlier and was excluded from further analysis. Freezing behavior is reported as a percent (%) of the total time spent freezing over the 5-min testing phase.

Brain Collection—As previously described (Asok et al., 2013), animals were sacrificed 30 $\pm$ 3 min after removal from the chamber and as mentioned above, HC animals were sacrificed directly from their home cages while their littermates were undergoing pre-exposure or training. Care was taken so that HC rats had the same experimental history as their experimental group counterparts, up to the time of sacrifice, and were comprised about equally of EtOH and SI rats. Rats were sacrificed by rapid decapitation, and brains were immediately removed and frozen in isopentane at -45°C . Brains were stored at -80°C until sectioned. Using the Paxinos and Watson stereotaxic brain atlas as a guide (Paxinos & Watson, 2007) sixteen micrometer coronal brain sections corresponding to the dorsal hippocampus, lateral nucleus of the amygdala, and medial prefrontal cortex were sectioned on a cryostat (Leica Inc., Deerfield, IL). Two brain sections were placed on each slide and were stored at -80°C until processed for in situ hybridization.

In situ Hybridization—All in situ hybridization and image analyses were conducted identically to that described in (Asok et al., 2013). An antisense RNA probe (riboprobe) was transcribed from a plasmid containing a sense cDNA coding for a 230 bp sequence of Egr-1 (gift from J. Milbrandt, Washington University, St. Louis, MO). The transcribed riboprobe incorporated a radioactively labeled ^{35}S UTP (approximately 1×10^6 dpm) using a T7 RNA polymerase Maxiscript kit, according to the manufacturer’s instructions (Life

Technologies, Grand Island, NY). Following hybridization and washing, dry slides were exposed to Kodak Biomax MR film for 2 days.

In situ Hybridization Image Analysis—As described previously (Asok et al., 2013), autoradiograms of brain sections were captured and digitized to 8-bit gray values via a Dage CCD video camera with ImageJ 1.48 program (Wayne Rasband, NIMH) on an Apple computer. ImageJ was used to subtract the background (2D-rolling ball radius of 50.0 pixels) and measure the mean density (mean gray value) within the regions of interest. The mean density of all mRNA labeling was analyzed for the LA and CA1 (using Plate 57 of Paxinos and Watson as a guide) and the PL and IL (using Plate 11). Two brain slices per animal were hybridized and the mean gray value of the left and right side of the brain was averaged within slices and then within slides. A ^{14}C standard with known amounts of radioactivity was exposed to the film and captured along with the slides. The standard was used to generate a 3rd degree polynomial equation and convert the unknown mean gray values obtained from the slides to a known amount of radioactivity (nCi/g). The nCi/g value was then normalized against the average nCi/g of all HC animals in that region in order to obtain a proportionate score (for each of the pre-exposure and training phases). When nCi/g scores fell ± 1.96 standard deviations from the nCi/g group mean for a particular region, that score was defined as an outlier and was excluded from the calculation of proportionate scores and further analysis. To collapse across films, the proportionate scores were averaged together and multiplied by 100 so that the average of all HC animals would equal 100%.

Statistical Analysis—Total freezing during the 5-min testing session was analyzed with a sex x treatment ANOVA.

Consistent with our past reports (Asok et al., 2013; Schreiber et al., 2014; Robinson-Drummer et al., 2018) in situ hybridization data (proportionate scores) were analyzed by one-way ANOVA performed separately on each brain region with Condition (HC, SI, EtOH) as a factor. Post-hoc analyses included Dunnett's tests to contrast each Treatment (SI, EtOH) with the HC baseline and Newman-Keuls tests to contrast Group SI and Group EtOH (Schreiber et al., 2014) with statistically significant differences, set at $p < 0.05$.

Results

Body Weights and BACs, Table 2—Body weight averages from each of the 3 assays (Pre-exposure, Training and CPFE Behavior) appear in Table 2. All groups gained a significant amount of weight over the dosing period (PD7–9; p 's $< .001$) and up to the age of testing (PD31). A 2 (Treatment) x 2 (Days) repeated measures ANOVA on PD7 and PD9 body weights revealed a main effect of Days ($F_{1,102}=1525.25$, $p<.001$), a main effect of Treatment ($F_{1,102}=33.08$, $p<0.001$), and a Treatment x Days interaction ($F_{1,102}=655.75$, $p<0.001$). Newman-Keuls post hoc test showed that although body weights did not differ between Treatment at PD7 (p 's > 0.60), at PD9, Group EtOH body weights were significantly lower than Group SI ($p<0.001$).

A 2 (Treatment) x 2 (Sex) factorial ANOVA on PD31 body weights (combined across all sampling and behavioral conditions) revealed a significant main effect of Sex $F_{1,86}=34.57$, $p<.001$), with males weighing more than females. No main effect of Treatment or Sex x

Treatment interaction was found ($F_{s>.81}$), indicating that, at the age of testing, growth was not altered by alcohol exposure. BACs were obtained from blood samples taken on PD7 from alcohol-exposed rats (Table 1). Group BACs are similar to those we and others have previously reported at this alcohol dose (e.g., Johnson and Goodlett, 2002; Jablonski & Stanton, 2014; Murawski & Stanton, 2011).

CPFE Behavior – Retention Testing

Subjects: After outlier exclusion (Table 1), behavioral analyses were completed on the remaining 29 rats [$n_{SI} = 16$ ($n_{male} = 11$, $n_{female} = 5$), $n_{EtOH} = 13$ ($n_{male} = 7$, $n_{female} = 6$)].

Contextual Fear Conditioning: There was no main effect [$F_{(1, 25)} = .63$, $p = .43$] or interaction [$F_{(1, 25)} = .04$, $p = .84$] involving sex. Alcohol impaired contextual freezing ($F_{(1,25)} = 4.39$, $p < .05$; Figure 1); percent freezing scores showed that Group EtOH froze significantly less to the conditioning context compared to Group SI.

CPFE Post-Pre-exposure Egr-1 mRNA expression

Subjects: Sixteen animals in the CA1 and LA HC ($n_{SI} = 10$, $n_{EtOH} = 6$), 14 animals in the PL and IL ($n_{SI} = 9$, $n_{EtOH} = 5$) and 23 animals in the pre-exposure sampling condition ($n_{SI} = 12$, $n_{EtOH} = 11$ for all regions) were assayed. HC controls consisted of both SI and EtOH treatment groups, for each region. Two subjects were excluded from PL and IL analyses because of tissue damage and labelling issues. After outlier exclusion, the final group sizes for each region and condition appear in Table 1.

Egr-1 mRNA expression: For each region, 2 (Sex: Male, Female) x 3 (Condition: HC, EtOH, SI) factorial ANOVA revealed no main or interaction effects of sex in the PL, IL or LA (all p 's $> .23$) except in the CA1 (see below). Following pre-exposure, Group SI and Group EtOH displayed significantly elevated Egr-1 in CA1, LA, PL, and IL, compared to HC controls. Group SI displayed significantly greater Egr-1 expression compared to Group EtOH in IL and less so in PL (Figure 2). These results were confirmed statistically in separate ANOVAs performed on each region.

For CA1, there was no interaction ($F_{2,30} = 0.83$, $p > .44$) but a main effect of sex ($F_{1,30} = 5.03$, $p < .04$) due to increased ($p < .01$) expression in males (148.85 ± 12.22) relative to females (111.86 ± 8.51) overall, although both sexes showed similar gene expression patterns (i.e. increased SI and EtOH expression over HC). A one-way ANOVA of the proportionate scores from HC revealed a main effect of Condition (HC, SI, EtOH; $F_{2,33} = 7.04$, $p < .003$). Dunnett's test showed that both Group SI and Group EtOH differed significantly from HC ($p < .04$). Newman-Keuls indicated no difference between Group SI and Group EtOH. Thus, differential Egr-1 activation in CA1 on the preexposure day cannot account for greater fear conditioning in Group SI relative to Group EtOH (Figure 1).

In the LA, a one-way ANOVA revealed a main effect of Condition ($F_{2,33} = 14.17$, $p < .001$). Dunnett's test revealed that both Group SI and Group EtOH differed significantly from HC (p 's $< .001$). Newman-Keuls analysis showed that gene expression between Group SI and

Group EtOH did not differ from one another ($p>0.25$). Again, alcohol did not alter activation of Egr-1 in LA during context pre-exposure.

In the PL, there was a significant main effect of Condition ($F_{2,30}=25.73$, $p<.001$). Dunnett's test showed that both Group SI and Group EtOH differed significantly from HC (p 's < 0.001). Newman-Keuls analysis showed that Egr-1 expression in Group SI and Group EtOH failed to differ from one another, but by a narrow margin statistically ($p>0.054$).

Similarly, in the IL, there was a significant main effect of Condition ($F_{2,29}=37.98$, $p<.001$). Dunnett's test showed that both Group SI and Group EtOH differed significantly from HC (p 's < 0.001) and Newman-Keuls indicated that gene expression in Group SI was significantly higher than in Group EtOH ($p< 0.02$).

In summary, context pre-exposure increased Egr-1 mRNA expression in all regions for both treatment conditions relative to HC, however increases in Group SI compared to Group EtOH was only observed in IL.

CPFE Post-Training Egr-1 mRNA expression

Subjects: Fifteen animals in the HC ($n_{SI} = 9$, $n_{EtOH} = 6$) and 24 animals from the training sampling condition ($n_{SI} = 14$, $n_{EtOH} = 10$) were assayed. One subject was excluded from all analyses because of experimental error (HC, Group SI) and 2 subjects were excluded from PL and IL analyses due to tissue damage and labelling issues (Pre, Group SI; Pre, Group EtOH). After outlier exclusion, the final number of subjects in each group appear in Table 1.

Egr-1 mRNA expression: As on the preexposure day, significant sex differences were generally absent (p 's $> .05$) except where noted. Group SI and Group EtOH showed training day elevations of Egr-1 expression in the LA, PL and IL, compared to HC controls. In CA1, Group EtOH did not differ from Group SI, although there was a trending increase relative to HC. Egr-1 in Group SI was higher than Group EtOH in the PL and IL and Group EtOH showed higher Egr-1 compared to Group SI in LA (Figure 3).

In CA1, 2 (Sex: Male, Female) x 3 (Condition: HC, EtOH, SI) factorial ANOVA revealed no interaction ($F_{2,28} = 1.56$, $p> .23$) but a main effect of sex ($F_{1,28} = 4.61$, $p< .02$) however post-hoc Newman-Keuls found no difference ($p> .08$) between males (103.12 ± 5.59) and females (118.00 ± 7.77). One-way ANOVA (collapsed across sex) on proportionate scores showed a nonsignificant trend toward a main effect of Condition ($F_{2,31}=2.8$, $p>.076$) in CA1.

Similar to CA1, a main effect of sex ($F_{1,30} = 4.65$, $p< .04$) but no sex x condition interaction ($F_{2,30} = 0.57$, $p> .57$) and no post-hoc difference ($p> .44$) between males (122.40 ± 7.05) and females (128.54 ± 8.28) was observed in the LA. Collapsed across sex, there was a significant main effect of Condition ($F_{2,33}=13.25$, $p<.001$). Dunnett's test showed that both Group EtOH and Group SI significantly differed from HC (p 's $<.009$). Newman-Keuls analysis showed that gene expression in Group EtOH was higher than Group SI ($p< 0.03$).

In the PL, there was a significant main effect of Condition ($F_{2,30}=28.03$, $p<.001$). Dunnett's test showed that both SI and EtOH groups were higher than HC (p 's < 0.002) and Newman-Keuls showed that gene expression in Group SI was higher ($p< 0.006$) than Group EtOH.

Similarly, in the IL, there was a main effect of Condition ($F_{2,29}=32.52, p<.001$). Dunnett's test showed elevated Egr-1 in both Group SI and Group EtOH over HC (p 's < 0.002). Newman-Keuls analysis showed again, that gene expression in Group SI was higher ($p< 0.002$) than Group EtOH.

In summary, these results indicate greater Egr-1 expression in Group SI relative to Group EtOH in the IL and PL subdivisions of the mPFC, following the context-shock association phase of the CPFE. However, context-shock training resulted in an opposite treatment effect in LA with Group EtOH displaying greater Egr-1 compared to Group SI.

Experiment 2:

Experiment 1 indicated that training-day Egr-1 expression in mPFC was reduced by neonatal alcohol exposure. However, a non-associative (Alt-Pre) control condition was not included in the design, precluding a strong conclusion that this reduction was related to contextual fear conditioning as opposed to other aspects of the training experience. This limitation was addressed in Experiment 2, which replicated the training-day and behavioral testing phases of Experiment 1, but with the addition of the Alt-Pre control condition.

Methods

Subjects—Subjects were 97 (50 males and 47 females) Long-Evans rats derived from 12 time-bred dams in the University of Delaware breeding colony. Of these, 37 (20 males; 17 females) were assigned to the behavioral assay and 60 (30 males; 30 females) were assigned to the training day Egr-1 mRNA assay (see Table 3).

Design and Procedures—The general training design was the same as that described in Experiment 1. There were eight experimental conditions with one littermate assigned to each condition (Table 3). Typically, five littermates (from both Group SI and Group EtOH) were sacrificed on the Training Day, and three littermates were retained for behavioral testing on the final day. Both SI and EtOH animals sacrificed following training contained home cage controls (HC), animals preexposed to the training context (Group Pre) or preexposed to an alternate context (Group Alt-Pre).

Apparatus, Alcohol Dosing, Behavioral Procedures, Egr-1 Assays, Statistics.

—These were the same as described for Experiment 1 with the exception of the Alt-Pre condition (see previous paragraph). Group Alt-Pre rats were preexposed to an alternate context (Murawski & Stanton, 2011) consisting of the same Plexiglas chambers used for Group Pre with modifications. Wire mesh inserts, which protruded into the chambers, changed both the texture of the floor and the dimensions of chamber. In addition, white opaque coverings were added such that only the wall facing the camera remained unobscured. Training and testing occurred in the same conditioning chambers as Group Pre.

Results

Body Weights and BACs, Table 2—Body weight averages for Experiment 2 appear in Table 2. All groups gained a significant amount of weight over the dosing period (PD7–9; p 's $< .001$) and up to the age of testing (PD31). A 2 (Treatment) x 2 (Days) repeated

measures ANOVA on PD7 and PD9 body weights revealed no a main effect of Treatment ($F_{1,95}=2.56$, $p=.11$), however there was a main effect of Days ($F_{1,95}=458.40$, $p<.001$), and a Treatment x Days interaction ($F_{1,95}=44.97$, $p<0.001$). Newman-Keuls post hoc test showed that although body weights did not differ between Treatment at PD7 ($p's>0.36$) whereas, at PD9, Group EtOH body weights were significantly lower than Group SI ($p<0.001$).

A 2 (Treatment) x 2 (Sex) factorial ANOVA on PD31 body weights (combined across all sampling and behavioral conditions) revealed a significant main effect of Sex $F_{1,93}=41.32$, $p<.001$, with males weighing more than females. No main effect of Treatment or Sex x Treatment interaction was found ($F's<.51$), indicating lack of growth effects of alcohol at the time of testing (as in Experiment 1). BACs were obtained from blood samples taken on PD7 from alcohol-exposed rats (Table 2). Group BACs were similar to Experiment 1 and other studies in the literature.

CPFE Behavior – Retention Testing

Subjects: After outlier exclusion (see Table 3), behavioral analyses were completed on the remaining 34 rats.

Contextual Fear Conditioning: There was no difference in freezing scores between SI and EtOH Alt-Pre animals ($t(11)=-0.81$, $p<.44$) so this group was collapsed into a Pooled Alt-Pre group. A 2 (Sex) x 3 (Condition) ANOVA revealed no main [$(F_{1,28}) = 0.65$, $p>.42$] or interaction ($F_{2,28}=0.12$, $p>.88$) effects of sex so all groups are collapsed across this variable. One-way ANOVA revealed a significant main effect of Condition [$(F_{2,28}) = 10.64$, $p<.001$]. SI-Pre freezing was significantly higher than both EtOH Pre ($p<.001$) and Pooled Alt-Pre ($p<.001$) however freezing did not differ between EtOH Pre and Pooled Alt-Pre ($p>.62$). Neonatal alcohol exposure abolished the CPFE in adolescent rats.

CPFE Post-Training Egr-1 mRNA expression

Subjects: Twelve animals in the HC ($n_{SI}=6$, $n_{EtOH}=6$) and 48 animals from the training sampling condition ($n_{SI\ Pre}=12$, $n_{SI\ Alt-Pre}=12$, $n_{EtOH}=12$, $n_{EtOH\ Alt-Pre}=12$) were assayed. One subject from the PL and 6 subjects from the CA1/LA were removed from analyses due to tissue damage or labeling issues. Group sizes after outlier exclusion are shown in Table 3.

Egr-1 mRNA expression: Similar to Experiment 1, significant sex differences were generally absent ($p's>.38$) except where noted. Training day *Egr-1* expression was elevated in the PL, IL and CA1 of the experimental groups compared to HC controls but not in the LA. (Figure 5).

For CA1, 2 (Sex: Male, Female) x 5 (Condition: HC, EtOH_{Pre}, EtOH_{Alt-Pre}, SI_{Pre}, SI_{Alt-Pre}) factorial ANOVA revealed no main effect of sex ($F_{1,39}=3.84$, $p>.05$) but there was a significant interaction ($F_{4,39}=3.40$, $p>.02$) driven by a group difference ($p<.01$) between SI_{Pre} males (96.34 ± 8.12) and SI_{Pre} females (204.66 ± 34.21) although no other groups differed across sex ($p's>.70$).

There was a main effect of Condition ($F_{4,44}=3.66$, $p<.02$) in CA1 however, Dunnett's test showed that only Group EtOH Alt-Pre showed a significant increase in expression relative to HC ($p<.01$) in CA1. In the LA, ANOVA revealed no main effect of Condition ($F_{4,44}=2.10$, $p<.098$).

There was a main effect of Condition in both the PL ($F_{4,48}=13.16$, $p<.001$) and IL ($F_{4,47}=18.06$, $p<.001$). Dunnett's test showed that all experimental groups in the PL ($p's < 0.05$) and IL ($p's < 0.02$) were higher than HC. For both regions, Newman-Keuls showed that gene expression in Group SI Pre was higher than all other experimental groups ($p< 0.02$) however no difference in expression ($p's > .05$) was observed between SI Alt-Pre, EtOH Pre and EtOH Alt-Pre groups in either region.

In summary, the inclusion of Alt-Pre non-associative controls in Experiment 2 indicate that the alcohol effect on prefrontal *Egr-1* expression occurred in the Pre-but not the Alt-Pre groups. This suggests that the learning failure observed in the EtOH-treated animals during memory retention testing may be the result of disrupted, learning-related, regional activation in the mPFC during the training phase of the CPFE.

Discussion

The current experiment examined the impact of neonatal alcohol on 1) contextual fear learning in the CPFE and 2) experience-induced *Egr-1* mRNA expression following the context exposure and context-shock association phases of the CPFE, in juvenile rats. In agreement with our earlier findings (Jablonski & Stanton, 2014; Murawski & Stanton, 2011), a high dose of alcohol (5.25g/kg/d) administered from PD7–9 disrupted conditioned freezing in the CPFE on PD33. *Egr-1* mRNA expression in mPFC also varied as a result of developmental treatment and phase of the CPFE protocol. Following training, and to a lesser extent following pre-exposure, alcohol-exposed rats (Group EtOH) displayed a significant decrease in mPFC *Egr-1* mRNA expression compared to controls (Group SI). Thus, the alcohol-induced modification of mPFC *Egr-1* mRNA expression, may contribute to the observed contextual fear deficits seen in alcohol-exposed animals in the CPFE.

The CPFE paradigm occurs over 3 days, providing an approach for temporally dissociating learning about the context (forming a contextual representation; day 1) from associating the contextual memory representation with shock (day 2; Fanselow, 2000; Jablonski & Stanton, 2014; Rudy, 2009). The current findings suggest that the mPFC is active following exposure to a novel context and shows learning-related activation following immediate-shock training. Thus mPFC seems to play a role in forming a contextual representation on the pre-exposure day and in context-shock learning or consolidation on the training day. Indeed, mPFC infusions of muscimol (Heroux et al., 2017) or scopolamine (Robinson-Drummer et al., 2017) on either day disrupts the CPFE (retention-test freezing) so both processes could be impaired by neonatal alcohol. However, previous behavioral studies from our lab suggest that training-day processes are more likely impaired. PD7–9 alcohol exposure impairs 24hr retention of context-shock association however immediate measures of learning (i.e. post-shock freezing test) reveal that EtOH animals can momentarily retrieve and associate a previously acquired context memory with foot-shock (Jablonski & Stanton, 2014). These results demonstrate a susceptibility of training day memory processes (e.g., consolidation of

the context-shock association), rather than pre-exposure day processes (e.g., forming and consolidating a contextual representation) to alcohol-induced deficits in contextual fear conditioning in the CPFE. Taken together, preserved post-shock freezing and differential Egr-1 expression in the mPFC suggests an inability of alcohol-exposed animals to maintain for retrieval the updated contextual memory following the context-shock association, likely as a consequence of impaired long-term plasticity mechanisms.

There is some evidence that neuronal plasticity within the mPFC is persistently impaired in animal models of FASDs. For example, Nagahara and Handa (1995) showed that following testing in a T-maze spatial alternation task in adulthood, rats exposed to alcohol during the last week of gestation displayed a significantly lower elevation of c-fos and jun B mRNA expression in the PL compared with controls. Hamilton, et al. (2010a, b) found that although the expression of c-fos and Arc was increased in the mPFC of normally-developing rats after social experience, IEG expression remained unaltered in animals prenatally exposed to alcohol. Finally, prenatal alcohol exposure reduces the number of c-fos⁺ cells in the IL of juvenile rats relative to non-exposed rats following exposure to a novel environment (Fabio et al., 2013). Collectively, these findings demonstrate a link between prenatal alcohol exposure and experience-induced mPFC IEG expression. To our knowledge, though, this is the first examination of the effect pre- or postnatal alcohol exposure on mPFC experience-induced gene expression in fear conditioning.

Alcohol exposure triggers widespread apoptotic neurodegeneration in the developing cortex, in part, by functional impairment of ionotropic N-methyl-D-aspartate glutamate receptors (NMDARs), which are critically involved in brain development and synaptic plasticity. Importantly, this effect is restricted to the neonatal period of synaptogenesis (Ikonomidou et al., 2000). Early ethanol exposure alters prefrontal cortical NMDAR subunit composition, inhibits ion flux through the receptor, and affects cell surface expression (Hughes, Kim, Randall, & Leslie, 1998; Incerti, Vink, Roberson, Wood, Abebe, & Spong, 2010; Y. H. Lee, Spuhler-Phillips, Randall, & Leslie, 1994; Nixon, Hughes, Amsel, & Leslie, 2002; Olney, Wozniak, Jevtovic-Todorovic, & Ikonomidou, 2001; Pian, Criado, Milner, & Ehlers, 2010). Following NMDA activation in normal-developing animals, Ca²⁺ influx initiates a series of events leading to activation of transcription factors, such as CREB, within the nucleus. CREB binds to the cAMP response element (CRE), triggering the expression of plasticity-related genes (Medina, 2011; Orsini & Maren, 2012; Veyrac, Besnard, Caboche, Davis, & Laroche, 2014). Third trimester alcohol exposure disrupts CREB phosphorylation (Krahe, Wang, & Medina, 2009), which may then disrupt the expression of immediate early genes that are important for neuronal plasticity, such as Egr-1 (Medina, 2011; Soares-Simi et al., 2013). Indeed, intracerebroventricular (i.c.v.) antagonism of NMDARs abolishes retention of contextual fear conditioning and the concomitant increase in Egr-1 mRNA expression in the LA (Malkani & Rosen, 2001). Thus, alcohol-related disturbances in neural circuit function and later cognitive processing may be partially caused by ethanol interference with NMDA-dependent synaptic development (den Hartog et al., 2013; Sadrian, Wilson, & Saito, 2013).

In addition to its effects on NMDARs, it is well known that early alcohol exposure leads to long-lasting alterations in neocortical GABAergic transmission by altering the number, distribution, development and molecular properties of GABAergic neurons (Cuzon, Yeh,

Yanagawa, Obata, & Yeh, 2008; Davidson, Matsumoto, Shanley, & Wilce, 1996; Ikonomidou et al., 2000; Sanderson, Donald Partridge, & Fernando Valenzuela, 2009). Developmental alcohol also disrupts the balance of excitatory/inhibitory circuitry (Bavelier, Levi, Li, Dan, & Hensch, 2010; Sadrian et al., 2013), interferes with voltage-gated Ca^{2+} channels (Granato et al., 2012), alters AMPA receptor functioning (Bellinger, Davidson, Bedi, & Wilce, 2002; Medina, 2011; Sadrian et al., 2013; Sanderson et al., 2009; Valenzuela, et al., 2012), interferes with vascular development (Jégou et al., 2012) and induces modification of epigenetic regulators (Otero, Thomas, Saski, Xia, & Kelly, 2012; Perkins, Lehmann, Lawrence, & Kelly, 2013). All of these factors may contribute to the plasticity deficits seen in animal models of FASDs.

The current findings expand upon a large body of work implicating the mPFC in acquiring context-specific extinction memories, fear storage, and fear memory retrieval (e.g., Frankland & Bontempi, 2005; Frankland & Bontempi, 2006; Knapska & Maren, 2009; Maren, Phan, & Liberzon, 2013; Morrow, Elsworth, Inglis, & Roth, 1999; Sotres-Bayon & Quirk, 2010). Recently, Zelikowsky et al. (2013) showed that both the IL and PL are critically involved in contextual fear and fear renewal when dorsal hippocampal function was compromised. This compensatory context fear correlated with a rearrangement in the balance of activity between PL and IL in amygdala-projecting cells. Additionally, IL lesions alone caused an increase in context generalization. These findings, combined with studies showing alterations in mPFC neuronal spiking activity following re-exposure to an already acquired fear-evoking context (Baeg et al., 2001) or re-exposure to an already experienced context (Hyman, Ma, Balaguer-Ballester, Durstewitz, & Seamans, 2012) suggest that the mPFC disturbances caused by early ethanol exposure are sufficient to disrupt the neural circuitry necessary for retrieving context-specific memories on the training day. Indeed, neonatal alcohol exposure causes alterations in mPFC spine density, distribution and morphology (Hamilton et al., 2010; Lawrence et al., 2012; Whitcher & Klintsova, 2008), as well as reductions in basilar dendritic length and branching without altering cell number (Granato et al., 2003; Hamilton et al., 2010). Thus, these alcohol-induced disturbances in the mPFC, but not cell loss *per se*, could reduce the level of Egr-1 expression in alcohol-exposed versus control animals during the training phase of the CPFE. Whether this reflects impaired retrieval of the context representation or impaired learning of the context-shock association remains to be determined.

A recent study from our lab examined the number of CA1 c-Fos⁺ cells following the pre-exposure phase of the CPFE in rats exposed from PD4–9 to different levels of alcohol (4.00 and 5.25 g/kg/d). Compared to SI controls, experience-dependent increases in the number of CA1 c-Fos⁺ cells were lower in alcohol-exposed rats given the highest alcohol dose indicating a decrease in IEG expression in the hippocampus following exposure to a novel environment (Murawski et al., 2012). However, we demonstrate no treatment effect (SI vs. EtOH) in CA1 following the pre-exposure phase. This discrepancy may be due to the narrower developmental window of alcohol exposure (PD7–9 vs. PD4–9), to the particular IEG examined, differences in the time-course of IEG expression, or because we analyzed mRNA, and not protein, expression in the present study. Similarly, in the LA, both treatment groups showed elevated Egr-1 after pre-exposure, suggesting that the LA may be activated by novelty or by forming a context representation (Asok et al., 2013), which remains

unaltered by developmental alcohol. Indeed, intact standard contextual fear conditioning (sCFC) in alcohol exposed animals, which does not include a separate day of pre-exposure, suggests that the amygdala may not be targeted by our exposure protocol (Murawski & Stanton, 2010). After the context-shock association phase, however, Group EtOH showed elevated expression compared to both baseline and Group SI in LA and marginally differed from baseline in CA1 ($p < .052$). This unexpected increase could occur because alcohol-exposed animals continue to process the context as similar to that of a novel context on the second day or because the LA and CA1 are working to compensate for alcohol-induced mPFC damage. Future research addressing these possibilities may help to clarify the effects of neonatal alcohol on cell signaling in these regions.

We report an association between contextual fear conditioning deficits in alcohol-exposed animals with a decrease in Egr-1 mRNA expression in the infralimbic and prelimbic subregions of the mPFC. By using the CPFE paradigm, which allows for separating learning about the context from its association with shock, we conclude that memory processes in mPFC associated with the context-shock association phase are the most susceptible to the teratogenic effects of neonatal alcohol. These findings indicate that ethanol exposure during development may lead to persistent deficits in mPFC neuronal plasticity which could be partly responsible for the long-lasting dysregulation in neuronal circuits and behavioral function associated with human FASDs (El Shawa, Abbott, & Huffman, 2013). Egr-1 is regulated by the MAPK/ERK signaling pathway, specifically by the SRE and/or the CRE binding sites, via the transcription factors Elk-1 and CREB, respectively (Bozon et al., 2002; Davis et al., 2003). Targeting components of these pathways in the mPFC may be beneficial for reversing deficits in synaptic plasticity and improving behavioral outcomes, providing a potential mechanism of intervention for FASDs.

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Figure 1.

Mean percent freezing during the 5-min testing phase of the CPFE paradigm. Data are from animals that completed the full behavioral paradigm (groups 7 and 8 from Table 1). Group SI froze significantly more than Group EtOH. Error bars represent $\pm$ SEM, $*p < .05$.

Figure 2.

Mean percent expression of Egr-1 mRNA compared to HC following context pre-exposure in the CPFE paradigm (groups 1, 2 and 3 from Table 1). (Upper Panel) Group SI and Group EtOH displayed significantly elevated Egr-1 mRNA expression in all areas, compared to HC. Compared to Group EtOH, Group SI showed increased Egr-1 only in IL. This effect was only marginally significant in PL. (Lower Panel) Digitized enhanced contrast images of animals in the HC, EtOH and SI conditions containing all brain regions analyzed. Error bars are $\pm$ SEM. * $p < .05$, ** $p < .01$, *** $p < .001$

Figure 3.

Mean percent expression of Egr-1 mRNA compared to HC following immediate shock training in the CPFPE paradigm (groups 4, 5 and 6 from Table 1). (Upper Panel) Group SI displayed significantly higher Egr-1 mRNA than Group EtOH in the PL and IL. (Lower Panel) Digitized enhanced contrast images of animals in HC, EtOH and SI conditions containing all brain regions analyzed. Error bars are $\pm$ SEM. * $p < .05$, ** $p < .01$, *** $p < .001$

Figure 4.

Mean percent freezing during the 5-min testing phase of Experiment 2 (groups 6, 7 and 8 Table 2). Group SI froze significantly more than Group EtOH and Group Pooled Alt-Pre, which did not differ, replicating the results of Experiment 1 and extending them to include a non-associative control group. Error bars represent $\pm$ SEM, *** $p < .001$.

Figure 5.

Mean percent expression of Egr-1 mRNA compared to HC following immediate shock training in experiment 2 (groups 1–5 from Table 2). Group SI Pre displayed significantly higher Egr-1 mRNA than all other groups in the PL and IL. In the PL and IL, groups SI Alt-Pre, EtOH Pre and EtOH Alt-Pre did not differ significantly in their relative expression although all groups were significantly elevated above HC. There were no group differences in the LA or CA1 except that the group EtOH Alt-Pre was significantly elevated above HC. Error bars are are was $p < .05$, ** $p < .01$

Table 1.

Subject assignment and experimental design for Experiment 1. M/F, male/female (counterbalanced in each condition); SI, sham-intubated; TRN CXT, training context; ‘x’, denotes time of sacrifice (sac), *Baseline animals include those from both treatment conditions (SI & EtOH). Group sizes are indicated after outliers were removed. The number of outliers appear in parentheses.

M/F	Condition		Phase		Testing	CAI n (outliers)	LA n (outliers)	PL n (outliers)	IL n (outliers)	Behavior n (outliers)
Littermate	Sampling	Treatment	Preexposure	Sac						
1	Preexposure	5.25g	TRN CXT	X		12(0)	12(0)	11(1)	11(1)	
2		SI	TRN CXT	X		10(1)	11(0)	10(1)	10(1)	
3		Baseline*	Home cage	X		14(3)	12(3)	12(2)	11(3)	
4	Training	5.25g	TRN CXT		TRN CXT	9(1)	10(1)	8(1)	8(1)	
5		SI	TRN CXT		TRN CXT	13(1)	13(1)	12(1)	12(1)	
6		Baseline*	Home cage		Home cage	12(2)	13(2)	13(1)	12(2)	
7	Behavior	5.25g	TRN CXT		TRN CXT					13(2)
8		SI	TRN CXT		TRN CXT					16(1)

Table 2.

Average body weights (in grams, $\pm$ SE) for animals in each sampling condition (pre-exposure, training, CPFE behavior) are given from the two treatment groups (SI, sham intubated; EtOH, 5.25 g/kg/d over PD7–9) during the first (PD7) and last (PD9) day of dosing for Experiments 1 and 2. Weights were taken on the first day of behavioral training (PD30–31, pre-exposure). Average blood alcohol concentration (BAC) obtained from blood samples collected on PD7 from alcohol-exposed rats are given in mg/dL. Group EtOH body weights were significantly less than Group SI only at PD9.

Exp.	Treatment	Body Weight (g)				BAC (mg/dL)
		PD7	PD9	PD31–32 (males)	PD31–32 (females)	PD7
1	SI	16.05 $\pm$ 0.20	21.06 $\pm$ 0.27	111.64 $\pm$ 2.06	98.86 $\pm$ 2.82	n/a
	EtOH	16.22 $\pm$ 0.21	17.27 $\pm$ 0.25	110.45 $\pm$ 1.66	95.84 $\pm$ 2.70	465.8 $\pm$ 7.87
2	SI	16.52 $\pm$ 0.24	20.78 $\pm$ 0.34	106.27 $\pm$ 1.99	94.67 $\pm$ 1.24	n/a
	EtOH	16.90 $\pm$ 0.26	17.27 $\pm$ 0.25	104.50 $\pm$ 2.05	94.17 $\pm$ 1.27	417.5 $\pm$ 14.54

Table 3.

Subject assignment and design for Experiment 2. M/F, male/female (counterbalanced in each condition); SI, sham-intubated; TRN CXT, training context; ALT CXT, Alternate preexposure context ‘x’, denotes time of sacrifice (sac), *Groups include subjects from both treatment conditions (SI & EtOH). Group sizes are indicated after outliers were removed. The number of outliers appear in parentheses.

M/F	Condition		Phase		Testing	CA1 n (outliers)	LA n (outliers)	PL n (outliers)	IL n (outliers)	Behavior n (outliers)
	Littermate	Sampling Treatment	Preexposure	Sac						
1	Training		5.25g	TRN CXT	X	10(1)	9(1)	11(1)	10(2)	
2			5.25g	ALT CXT	X	10(0)	9(1)	11(1)	11(1)	
3			SI	TRN CXT	X	10(1)	10(1)	9(1)	10(2)	
4			SI	ALT CXT	X	10(2)	11(1)	11(1)	11(1)	
5	Behavior		Baseline*	TRN/ALT CXT	X	9(1)	10(0)	11(1)	10(2)	
6			5.25g	TRN CXT						10(1)
7			SI	TRN CXT						11(1)
8			5.25g/SI*	ALT CXT						13(1)