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Impaired Trace Fear Conditioning and Diminished ERK1/2 Phosphorylation in the Dorsal Hippocampus of Adult Rats Administered Alcohol as Neonates

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Utilizing a rat model of fetal alcohol spectrum disorder (FASD), ethanol was administered over postnatal days (PD) 4 to 9. As adults, control and ethanol rats underwent trace fear conditioning (TFC), in which a tone conditioned stimulus (CS) and footshock unconditioned stimulus (US) were repeatedly paired, though the two stimuli never overlapped in time. Following training in Experiment 1, conditioned fear (freezing) to the tone CS was dose-dependently reduced in ethanol rats relative to controls. Experiment 2 was designed to test whether the TFC deficit varied based on the duration of the trace interval (TI; time from CS offset to US onset). Holding the time separating CS onset from US onset constant at 20 sec, control and ethanol rats were trained with a 5 or 15 sec tone CS, followed 15 or 5 sec later, respectively, by the US. Conditioned fear to the tone CS was significantly reduced in high dose ethanol rats trained with the 15 sec TI only. Acquisition and consolidation of trace fear memories relies on forebrain *N*-methyl-D-aspartate receptor (NMDAR) signaling, including the downstream phosphorylation of extracellular signal-regulated kinase1/2 (pERK1/2). Separate rats were trained with the 5 or 15 sec TI and then sacrificed 1 hr later. Significant reductions in pERK1/2-positive neurons were seen in areas CA1 and CA3 of the dorsal hippocampus (DH) following training at both TIs in ethanol rats. The disruption of DH learning-dependent plasticity appears tied to freezing behavior in ethanol rats, but only when the training stimuli are separated by more than 5 sec.

Keywords: fetal alcohol spectrum disorder, trace fear conditioning, hippocampus, NMDA receptor, ERK1/2

Exposure to alcohol during central nervous system (CNS) development can induce a variety of neuroanatomical, behavioral, and cognitive deficits (Jones, 2011; Mattson et al., 2011), collectively known as Fetal Alcohol Spectrum Disorder (FASD). The disorder's severity depends on the timing of maternal alcohol consumption and the resulting peak blood alcohol concentration (BAC) (Hannigan, 1996). Early postnatal life in rodents is a period of high-alcohol vulnerability, approximating the third-trimester "brain growth spurt" in human development (Bayer et al., 1993; Gil-Mohapel et al., 2010). Alcohol administration produces many of the same neurodevelopmental deficits seen in individuals with FASD (Driscoll et al., 1990), including impaired learning and memory. In the two experiments below, ethanol was administered over postnatal days (PD) 4 to 9 in a binge-like manner, which is known to produce higher peak BACs than chronically consumed alcohol (Bonthius et al., 1988) and better mimics the consumption patterns of alcoholic pregnant women (Stephens, 1985). Alcohol's teratogenic effects on forebrain development, the medial prefrontal cortex and hippocampus in particular, can be seen via reduced cell numbers (Livy et al., 2003; Mihalick et al., 2001) and constrained or altered spine density and dendritic complexity (Hamilton et al., 2010; Tanaka et al., 1991). Postnatal exposure is reported to diminish or alter hippocampal neurogenesis, structural plasticity, *N*-methyl-D-aspartate receptor (NMDAR)-dependent long-term potentiation (LTP), and CA1 learning-dependent excitatory unit activity (Bonthius & West, 1991; Klintsova et al., 2007; Lindquist et al., 2013; Livy et al., 2003; Puglia & Valenzuela, 2010).

Pavlovian fear conditioning entails the presentation of a neutral conditioned stimulus (CS), usually a light or tone, followed by an aversive unconditioned stimulus (US), a footshock. Over training, the CS and US become associated such that the CS, alone, can elicit an emotional (fear) conditioned response (CR). In the amygdala, NMDAR-dependent LTP provides the physiological mechanism by which a wide array of fear CRs are acquired and expressed (Sigurdsson et al., 2007). Delay fear conditioning is relatively simple to learn, with the CS and US typically overlapping in time. Trace fear conditioning (TFC) demands more attention and cognitive processing (Beylin et al., 2001; Han et al., 2003), with a stimulus-free interval of time separating CS offset from US onset—necessitating, Pavlov (1927) proposed, a CS sensory trace be maintained across the trace interval (TI) to suc-

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cessfully associate the two stimuli. TFC is considered an analogue of declarative memory (Clark & Squire, 1998) and, like working memory, requires stimulus information be actively sustained in memory over variable intervals of time (Goldman-Rakic, 1995; Woodruff-Pak and Disterhoft, 2008). TFC relies on a spatially distributed memory system that includes, among other structures, the prelimbic (PL) region of the medial prefrontal cortex, and the dorsal and ventral hippocampus (DH and VH) (Czerniawski et al., 2012; Runyan et al., 2004).

Forebrain NMDARs are necessary for both the development and retrieval of trace fear memories, as evidenced by diminished cue-dependent freezing after pretraining and/or pretesting infusions of (2R)-amino-5-phosphonovaleric acid (APV; NMDAR antagonist) in the PL, DH, or VH (Czerniawski et al., 2012; Gilmartin & Helmstetter, 2010; Quinn et al., 2005; Wanisch et al., 2005). NMDAR-gated calcium influx initiates a variety of downstream molecular cascades (Hunt & Castillo, 2012), including the activation and phosphorylation of extracellular signal-related kinase1/2 (pERK1/2). ERK1/2 is a member of the mitogen activated protein kinase (MAPK) superfamily and is prominently found in the amygdala, cerebellum, hippocampus, neocortex, and striatum—all areas implicated in the learning and memory process (Flood et al., 1998). In the hippocampus, ERK1/2 is localized to the soma and dendrites of CA1, CA3, and dentate gyrus neurons (Fiore et al., 1993) and its phosphorylation plays a well-documented role in the consolidation of new learning (Peng et al., 2010).

Several studies have demonstrated the disruptive effects of postnatal ethanol exposure on visual TFC in both adolescent (Hunt et al., 2009; Wagner & Hunt, 2006) and young adult (Schreiber & Hunt, 2013; Schreiber et al., 2013) rats, with poorest performance observed in rats exposed to the highest ethanol dose (5.0 or 5.25 g/kg/day). Experiment 1 sought to extend these findings to auditory TFC and investigate the long-term neurotoxic effects of low, moderate, or high doses of ethanol. Young adult (~PD70) rats were trained with a 15 sec tone CS followed 30 sec later by the footshock US (Figure 1A). We were particularly interested in determining whether the auditory TFC paradigm is sufficiently sensitive to detect deficits following administration of the low dose of alcohol.

In fear conditioned ethanol-exposed adolescent rats, Hunt et al. (2009) reported significantly less CS freezing in rats trained with a 10 sec versus 0 sec TI, with intermediate decreases in freezing seen with 1 and 3 sec. The same 10 sec light CS was used for all subjects, however, resulting in a longer interstimulus interval (ISI; time from CS onset to US onset) as the TI was lengthened. It remains to be verified that the ethanol-induced disruption in TFC is due, specifically, to the insertion and duration of the TI. Maintaining a constant 20 sec ISI in Experiment 2, control and high dose ethanol rats were trained as adults in auditory TFC with a short (5 sec) or long (15 sec) TI—that is, a 15 sec tone CS and 5 sec TI or a 5 sec tone CS and 15 sec TI (Figure 1B). Holding the training ISI constant, we propose, allows for a more direct assessment of postnatal ethanol and TFC, based on the amount of time separating the two training stimuli.

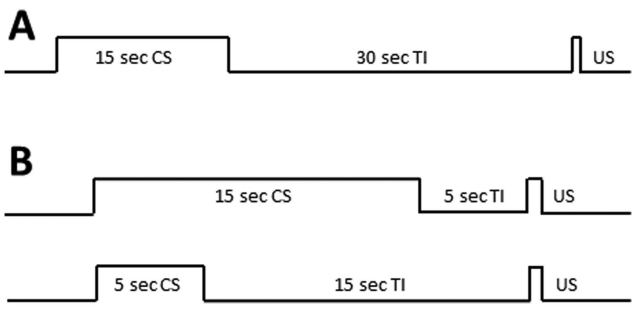

Figure 1. Schematics illustrating the CS-US temporal relationship during auditory TFC. (A) In Experiment 1, rats were trained with a 15 sec tone CS followed 30 sec later by a 1 sec footshock US. (B) Experiment 2 utilized a 15 sec tone CS and 5 sec TI (top) or a 5 sec tone CS and 15 sec TI (bottom). CS: conditioned stimulus; TI: trace interval; US: unconditioned stimulus.

Indeed, the time between CS offset and US onset is proposed to recruit different brain structures, with the DH preferentially engaged by long TIs (Chowdhury et al., 2005; Misane et al., 2005). It was recently reported, for example, that the medial prefrontal cortex was required for TFC with both short (5 sec) and long (40 sec) TIs, whereas the DH was required with the long interval only (Guimarãis et al., 2011). In Experiment 2, ERK1/2 phosphorylation within the DH was used as a molecular correlate of long-term memory storage, enabling a between-subjects comparison of the trace fear memory's strength (based on freezing behavior at test) and a critical step in the NMDAR-dependent downstream signaling cascade that underlies memory consolidation. DH tissue sections, from adult rats sacrificed 1 hr after TFC, were submitted to immunohistochemistry (IHC) for the pERK1/2 antibody in CA1 and CA3 neurons. If DH involvement in TFC is contingent on TI duration, we reasoned, the expression of conditioned auditory fear would be selectively reduced in ethanol rats trained with the 15 sec interval, accompanied (in separate rats) by fewer pERK1/2-positive neurons.

Materials and Methods

Experiment 1

Subjects. Long-Evans male and female breeder rats were purchased from Harlan Laboratories (Indianapolis, IN) and housed in the vivarium at the University of Kansas. The animals were maintained on a 12 hr light/dark cycle (lights on at 0700 hr) with ad libitum access to food and water. One male and one female breeder were pair-housed for 1 week. Beginning 3 weeks after initial placement, female rats were checked morning and evening for parturition. After birth, litters were culled to 10–12 pups, retaining equal numbers of males and females when possible. Rats were weaned on PD21 and same sex housed through PD45, then singly housed throughout training and testing. All behavioral procedures were conducted during the light phase and were in strict compliance with the University of Kansas animal care guidelines. All necessary measures were taken to minimize pain and discomfort. In total, 45 experimentally naïve Long-Evans rats from 16 litters were used in Experiment 1.

Neonatal treatment. For all procedures below, rat pups were removed from the dam and huddled together in a small plastic container atop a heating pad. On PD3, pups were injected in one or more paws with nontoxic black ink for identification purposes and assigned to one of five treatment groups: unintubated control (UC), sham-intubated (SI), or three ethanol groups, 3 g/kg/day (3E), 4

g/kg/day (4E), and 5 g/kg/day (5E). From PD4–9 all pups were weighed daily. The ethanol pups also experienced three intragastric intubations per day—twice with an ethanol/milk solution and a third, milk-alone solution. The first two intubations consisted of 6.80% (3E), 9.06% (4E), or 11.33% (5E) ethanol in a nutritive milk formula (vol/vol), based on the recipe of West et al. (1986). PE10 tubing was lubricated with corn oil and lowered down the esophagus into the stomach. The other end of the tube was connected to a 1 mL syringe containing the correct volume (0.02778 ml/g of body weight) of the ethanol/milk or milk-alone solution. The SI pups were intubated three times daily but never received ethanol or milk. The UC pups were removed from the dam three times daily but never intubated.

Blood alcohol concentration. Immediately before the final intubation on PD 4, tail clips were done on SI, 3E, 4E, and 5E pups and 20 μl of blood collected using a heparinized capillary tube. Samples from the SI rats were discarded while samples from ethanol rats were dispensed into microcentrifuge tubes, centrifuged, and plasma separated. BACs were determined using an oximetric assay procedure. An Analox GL5 Analyzer (Analox Instruments, Lunenberg, MA) was used to measure the rate of oxygen consumption resulting from oxidation of ethanol in the sample. Peak BAC was calculated by comparing an experimental sample to a known alcohol standard (100 or 300 mg/kg) used in the calibration procedure.

Apparatus. All training and testing occurred in standard operant boxes (Coulbourn Instruments, Allentown, PA), enclosed in sound-attenuating chambers. Each operant box had two stainless steel walls, two Plexiglas walls, and a grid floor composed of 0.5 cm stainless steel bars placed approximately 1.5 cm apart. A small animal shock generator (model H13–15; Coulbourn Instruments) and neon grid scrambler connected to the grid floor provided the footshock US.

Trace fear conditioning. All rats were handled a minimum of two times in the 5 days preceding the start of TFC. Two rats were transported in their individual home cage, carried one on top of the other, and brought into a well-lit room. Each chamber was wiped with a vinegar-water (1:5) solution and the rat placed inside. TFC consisted of 10 paired CS-US presentations. The CS was a 15 sec, 2.8 kHz, 85 dB tone presented through a speaker located at the top of each chamber and the US was a 1 sec, 1.0 mA scrambled footshock. The US followed CS offset by 30 sec, resulting in a 45 sec ISI. The intertrial interval (intertrial interval [ITI]) was 240 ± 30 sec.

Over the next 2 days all rats experienced a context and tone retention test in counterbalanced order. For the context test, two rats were brought into the running room and individually placed in their respective conditioning chamber for 14 min. The purpose of the context test was to measure contextual fear in the absence of the tone CS. After a 2 min baseline, freezing was analyzed for 10 min and rats removed 2 min later. Conversely, the tone test was designed to measure conditioned auditory fear independent of contextual cues. Rats were presented with an 8 min tone CS in a novel context, including new transport cues. Specifically, rats were transported in their home cage atop a metal wheeled cart covered with a towel. The testing room was dark except for a single red light bulb. The testing chamber was illuminated by a hanging 12 W light, scented with Windex® (S. C. Johnson & Son, Inc., Racine, WI), and the grid bars were covered by a sheet of Plexiglas. The 8 min tone CS began 3 min after placement of each rat and they were removed 3 min after CS termination. Freezing during the initial 3 min interval was examined to verify no differences in baseline freezing to the novel context between treatment groups.

Freezing analysis. Freezing was defined as cessation of all movement except that required for respiration (Blanchard & Blanchard, 1969). Freezing behavior was recorded with a black-and-white video camera (Model WDSR-2005SC; Circuit Specialists, Inc., Mesa, AZ) mounted at the top of the conditioning chamber. The interior was illuminated by an infrared light source to observe freezing behavior. The video signal was inputted to FreezeScan (CleverSys, Inc., Reston, VA), a video-based tool that can detect and quantify when subjects are motionless. Freezing was measured throughout the context and tone tests.

Experiment 2

Subjects. Long-Evans male and female breeder rats were purchased from Harlan Laboratories and housed in The Ohio State University vivarium. Subjects were maintained on a 12 hr light/dark cycle (lights on at 0600 hours) with ad libitum access to food and water. Breeding was done in the manner described above. All pups were weaned on PD21, same-sex housed through PD60, then individually housed through the completion of the study. All procedures were done in accordance with The Ohio State University Institutional Animal Care and Use Committee (IACUC), and all necessary measures were taken to minimize pain and discomfort. In total, 77 rats from 28 litters were used in Experiment 2.

Neonatal treatment. Rat pups were paw-marked on PD3 and assigned to one of three treatment groups: UC, SI, or 5E. The weighing, intubation, tail clip, and BAC procedures across PD4–9 were identical to Experiment 1. Treatment group differences in adult body weight could have affected shock saliency or learning in Experiment 1, thus body weights were further recorded on PD10, 15, 21, 30, 45, and 60.

Trace fear conditioning. All rats were handled a minimum of two times in the 5 days preceding the start of TFC. Subjects were transported and run two at a time in separate operant chambers, as described above. All rats were presented with 10 CS-US pairings. The CS was a 2.8 kHz, 80 dB tone followed by a 1 sec, 0.8 mA footshock US. The shock intensity was lowered from Experiment 1 because of the shorter TIs (5 or 15 sec). The ITI was 240 ± 30 sec.

All rats underwent context and tone testing in counterbalanced order. Freezing was measured across the 14 min context test. The tone CS was initiated 3 min following placement as described above, with minor changes. Two rats in their home cages were placed on a metal wheeled cart, covered with a towel, and transported to the darkened running room. The chamber was scented with Windex® and illuminated by a hanging 15 W light, the grid floor was covered by Plexiglas, and a laminated pink figure was attached to the front door. The tone CS in Experiment 1 was 8 min in duration, which could, over time, lead to its incorporation into the background context. Consequently, the tone CS was broken into four 2 min blocks, each separated by 1 min, to better "discretize" the auditory stimulus and distinguish it from other permanent features of the novel context. Rats were removed ~2 min after termination of the last tone. Pretone freezing was analyzed to verify no treatment group differences in baseline freezing.

Freezing analysis. Freezing was measured using the FreezeScan system, as described above. For training, freezing was measured across each of the 10 trials during the tone CS and the TI before US onset (i.e., the ISI). Over the next 2 days freezing was analyzed across min 2–12 of the context test and, for the CS-alone test, before and during each of the four 2 min tone presentations.

Immunohistochemistry. Separate groups of SI and 5E rats underwent TFC (5 or 15 sec TI) and were then sacrificed 1 hr later. Rats were euthanized with 200 mg/kg of Euthasol (Virbac, Fort Worth, TX) and immediately perfused with 0.9% saline followed by 4% paraformaldehyde. The brain was extracted and fixed in 4% paraformaldehyde for 48 h. A vibratome (VT1000S, Lecia Microsystems, Buffalo Gove, IL) was used to section (50 μm) brains in the coronal plane; tissue collection began just before the dorsal tip of the DH, when the corpus callosum begins to form, and ended when the DH and VH merge.

For each subject, a pseudo-random tissue section (~2.30 mm posterior to bregma) was selected. Moving caudally, every sixth section thereafter was used for IHC (~6 sections per animal). All reactions took place on an agitator at room temperature and all rinses (3 × 10 min) were done in TBST, except where noted. Initially, sections were rinsed and then submerged in a sodium citrate buffer and heated in a steam bath for 20 min at 37°C. Sections were rinsed and quenched for 1 h with 0.3% H_2O_2 in methanol. The tissue was rinsed, blocked in 5% normal goat serum for 1 h, then submerged in a 1:100 dilution of pERK1/2 (p44/42 MAPK antibody; 9102L, Cell Signaling, Danvers, MA) and left to incubate overnight at 4°C. The next day sections were rinsed and incubated in a 1:200 dilution of anti-rabbit secondary (BA-1000; Vector Laboratory, Burlingame, CA) for 90 min. The tissue was next incubated for 1 h with avidin-biotinylated horseradish peroxidase (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA), rinsed, incubated with 0.05% 3,3,-diaminobenzidine tetrahydrochloride (DAB kit, Vector Laboratories), then rinsed in 1X PBS to stop the reaction. Sections were mounted on subbed slides and left to dry overnight. Sections were then dehydrated in 70%, 95% and 100% ethanol for 2 min each, followed by 5 min in xylene and cover-slipped using Permount® (Fisher Scientific, Waltham, MA). Two tissue sections from each treatment group were briefly incubated in a neuronal nuclear counterstain, hemotoxylin-eosin (sc-24973; Santa Cruz Biotechnology, Inc., Dallas, TX), prior to cover-slipping.

Stain quantification. The hippocampus in each hemisphere was multiply imaged at 10× via the motorized stage of a high-resolution Nikon 90i microscope (Nikon Instruments, Melville, NY), with automatic stitching of the final image using NIS Elements software (Nikon Instruments). Each image was saved with a new identifier, ensuring all cell counts were done by raters blind to each subject's ID, treatment group, and TI. Blind raters selected the four best tissue sections from each subject. For each DH image, cells were quantified within defined regions of interest (ROI), areas CA1 and CA3, using NIS Elements software. The transition zone between CA1 and CA3 was located at the point where the smaller cell bodies of CA1 pyramidal cells, layered four to five deep, merge with the larger and more loosely packed CA3 neurons. Area CA3 was further broken down into different subregions, CA3ab and CA3c, based on the anatomical drawings in Lorente de Nó (1934), as illustrated and discussed in Kesner (2007). The two CA3 subregions are proposed to differ both anatomically (Scharfman, 2007) and functionally (Lee & Kesner, 2004). Each DH image was magnified 75% and pERK1/2 labeled cells counted within each ROI by one author and a research assistant. The interrater coefficient of determination, based on a subset of ROIs counted by both, was $r^2 = 0.96$. Cell counts are expressed per unit area (square millimeters), as a ratio of total pERK1/2 cells divided by the ROI area.

Data analysis. BAC, body weight, and freezing data from both experiments were analyzed using single-factor, multifactorial, or repeated measures ANOVAs. IHC results were analyzed with multifactorial ANOVAs. Significant main effects or interactions were followed by Tukey's post hoc tests and a significant effect implies $p < .05$.

Results

Experiment 1

Blood alcohol concentration. The mean (±*SE*) BAC for each treatment group was 245.5 ± 6.3 mg/dl (3E rats), 309.3 ± 7.4 mg/dl (4E rats), and 356.8 ± 11.1 mg/dl (5E rats). The single-factor (Treatment) ANOVA resulted in a significant main effect, $F(2, 24) = 43.12$, $p < .0001$. Tukey's post hoc group comparisons indicate the mean BAC for each treatment group was significantly different from the remaining two groups.

Trace fear conditioning: Retention testing. Freezing behavior (mean ± *SE*) during the context and tone tests was analyzed in UC ($n = 10$), SI ($n = 8$), 3E ($n = 9$), 4E ($n = 9$), and 5E ($n = 9$) rats. The two tests were presented in counterbalanced order. Collapsing across treatment groups, single-factor ANOVAs revealed no significant effect of test order on context test freezing ($p = .48$) or tone test freezing ($p = .29$). Mean freezing during the context test, as shown in Figure 2A, did not significantly differ between treatment groups ($p = .49$), whereas mean freezing during the 8 min tone test did, $F(4, 40) = 5.63$, $p < .001$. Post hoc analyses indicate the 5E rats froze significantly less than the UC, SI, and 3E rats. Figure 2B illustrates freezing percentages for the 3 min pretone interval and the 8 min tone, broken into 1 min bins. Freezing behavior did not differ between treatment group during the pretone period ($p = .23$). For the tone CS, a 5 (Treatment) × 8 (Time) repeated measures ANOVA revealed significant main effects for treatment, $F(4, 280) = 5.67$, $p < .001$ and time, $F(7, 280) = 13.50$, $p < .0001$, but not their interaction. Neonatal treatment significantly influenced freezing behavior to the tone CS, with the 5E rats again impaired relative to UC, SI, and 3E rats. The significant effect for time indicates reduced freezing across the 8 min tone. Though sex effects have been seen in ethanol-exposed rats trained in visual TFC (Schreiber & Hunt, 2013; Schreiber et al., 2013), sex was not examined in this or the next experiment as a fuller analysis of postnatal ethanol, sex, and auditory TFC is in progress.

Experiment 2

BAC and body weights. The mean BAC in 5E rats was 347.6 ± 8.8 mg/dl, comparable with that seen in 5E rats in Experiment 1. Table 1 lists body weights for all rats (behavior only) across PD4 to PD60. Treatment group effects were examined across the intubation period (PD4–9) via a 3 (Treatment) × 6

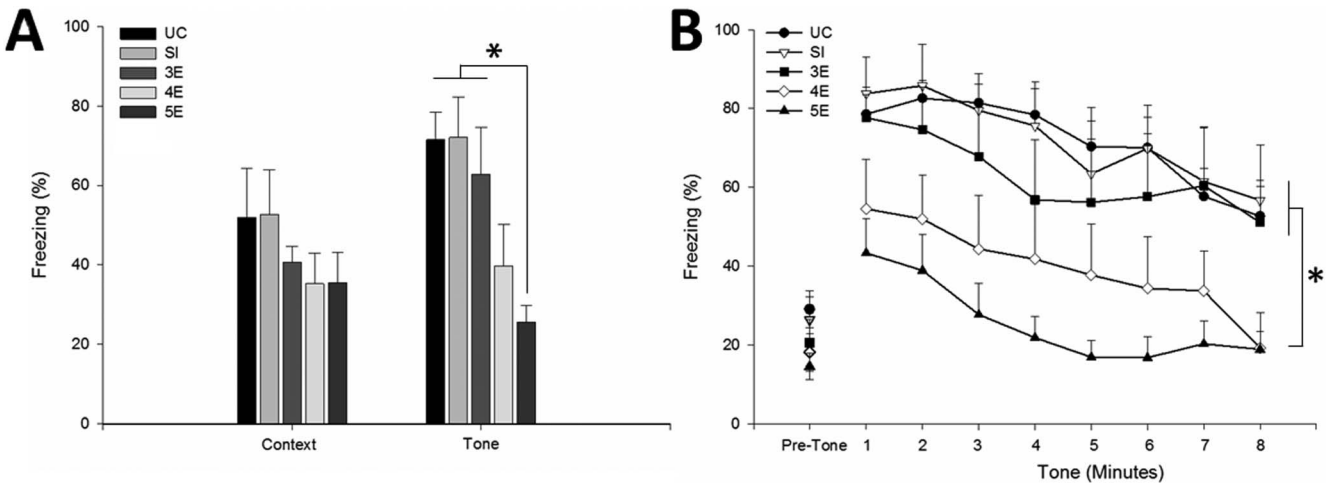

Figure 2. Percent freezing (mean ± *SE*) during the context and tone tests in Experiment 1. (A) Mean freezing during the context test did not significantly differ between treatment groups. Mean freezing during the tone test did differ between groups, with significantly less freezing in 5E than UC, SI, or 3E rats (asterisk). (B) Mean freezing during the 3 min pretone period and over the 8 min tone test. No significant differences were noted in baseline freezing between treatment groups. Freezing across the 8 min tone was significantly reduced in the 5E rats relative to the UC, SI, and 3E rats (asterisk).

(Day) repeated measures ANOVA, resulting in a significant treatment × day interaction, $F(10, 270) = 15.37$, $p < .0001$. Contrasts were performed on each day via Bonferroni-corrected one-way ANOVAs, requiring (at 6 contrasts) $p < .0083$ for significance (maintaining a family wise $\alpha = .05$). The 5E rats weighed significantly less than UC rats on PD8 and PD9. Treatment group effects were also analyzed across development (PD10, 15, 21, 30, 45, and 60) though, because of substantial heterogeneity of variance, each day was analyzed separately via one-way (Treatment) ANOVAs and, as required, follow-up contrasts. The 5E rats again weighed significantly less than UC rats on PD10 and, along with SI rats, on PD21. Results indicate 5E (and to a lesser degree, SI) rats lagged behind UC rats through weaning, though the differences in weight were mitigated as rats grew toward adulthood.

Trace fear conditioning and retention testing. Freezing behavior (mean ± *SE*) was analyzed as a function of Treatment (UC, SI, or 5E) and TI (5 or 15 sec), resulting in six groups of rats: UC/5s ($n = 8$), UC/15s ($n = 9$), SI/5s ($n = 10$), SI/15s ($n = 10$), 5E/5s ($n = 10$), and 5E/15s ($n = 10$). Freezing percentages during TFC and testing were submitted to two-factor ANOVAs (Treatment × TI). For training, neither factor significantly influenced freezing during the tone CS (Figure 3A) or TI (Figure 3B), indicating no treatment group differences in TFC acquisition. Over the next 2 days, no significant differences in freezing were found based on test order for the context ($p = .47$) or tone ($p = .82$) tests. As seen in Figure 3C, mean freezing during the context test was unaffected by neonatal treatment ($p = .61$) or TI ($p = .20$), whereas mean freezing during the tone test was significantly affected by both treatment ($F(2, 51)$ 3.76, $p < .05$) and TI, $F(1, 51) = 4.69$, $p < .05$, though the interaction was not significant (Figure 3D). The freezing data indicate the 5E rats froze significantly less than SI rats and, across all rats, those trained with the 15 sec TI froze significantly less than those trained with the 5 sec TI.

Figure 3E and F illustrate mean (±*SE*) freezing during the pretone period and across each of the four tone presentations in UC, SI, and 5E rats trained with the 5 or 15 sec TI, respectively. Neonatal treatment did not significantly influence baseline freezing in rats trained with the 5 sec TI ($p = .69$) or 15 sec TI ($p = .49$). A 3 (Treatment) × 4 (Tone) repeated measures ANOVA applied to CS freezing percentages in the 5 sec TI rats resulted in a main effect for tone only, $F(3, 75) = 5.25$, $p < .001$, with reduced freezing across tone presentations (Figure 3E). Results establish that adult 5E rats can process and associate the CS and US and, at test, freeze at levels similar to controls. The repeated measures ANOVA for the 15 sec TI rats revealed significant main effects for both treatment, $F(2, 78) = 5.20$, $p < .05$ and tone, $F(3, 78) = 5.06$, $p < .01$, but not their interaction. CS-mediated freezing was selectively disrupted in 5E rats, relative to SI rats (Figure 3F). The change in test tone parameters (8 min vs. 4 × 2 min) seemed to have little effect on the pattern of freezing, with similar extinction curves seen in control rats trained with the 5, 15, or 30 sec TI (Figures 2B and 3E and 3F).

Immunohistochemistry. No differences in freezing behavior were observed during training or testing between control (UC and SI) rats (Figure 3A–D). To minimize animal numbers, therefore, IHC was performed on DH tissue extracted from SI and 5E rats trained with the 5 or 15 sec TI only, resulting in four groups of rats: SI/5s ($n = 5$), SI/15s ($n = 5$), 5E/5s ($n = 5$), and 5E/15s ($n = 5$). ROI areas in each DH image ($n = 40$ per treatment/TI group) were initially analyzed via single-factor (Treatment) ANOVAs. No significant differences were seen in area CA1 ($p = .99$) or CA3 ($p = .50$), indicating postnatal ethanol did not induce significant hippocampal atrophy relative to controls. We cannot, however, rule out ethanol-induced apoptosis, which is more severe in area CA1 than CA3 of adult rats (Livy et al., 2003; Tran & Kelly, 2003), or changes in total ERK1/2—either one of which could have influenced the current results.

Table 1
Experiment 2: Mean Body Weights (Grams ± SE) Over Development for Each Treatment Group, Collapsed Across Trace Interval

	PD4	PD5	PD6	PD7	PD8*	PD9*	PD10*	PD15	PD21*†	PD30	PD45	PD60
UC (n = 17)	10.5 ± 0.3	12.4 ± 0.4	14.2 ± 0.5	16.2 ± 0.5	18.2 ± 0.6	20.4 ± 0.6	22.9 ± 0.7	36.6 ± 2.4	57.7 ± 2.6	109.8 ± 5.0	211.3 ± 8.2	295.0 ± 14.6
SI (n = 20)	10.4 ± 0.3	12.5 ± 0.4	14.1 ± 0.4	15.8 ± 0.5	17.6 ± 0.5	19.4 ± 0.6	21.4 ± 0.6	30.5 ± 2.2	49.0 ± 2.4	94.7 ± 4.6	192.6 ± 7.5	269.9 ± 13.4
5E (n = 20)	10.9 ± 0.3	11.5 ± 0.4	12.7 ± 0.4	14.2 ± 0.5	15.8 ± 0.5	17.6 ± 0.6	19.6 ± 0.6	30.0 ± 2.2	49.4 ± 2.4	97.1 ± 4.6	190.9 ± 7.5	265.1 ± 13.4

Significant differences in body weights were noted between UC and 5E rats on PD8, PD9, PD10, and PD21, as signified by the asterisk. The exclamation mark on PD21 indicates a significant difference in body weight between UC and SI rats.

For each DH region, pERK1/2 mean (±*SE*) cell counts were submitted to two-factor (Treatment × TI) ANOVAs (Figure 4A–D). For area CA1, cells were not counted in three sections from Group 5E/5s because of tissue damage. As illustrated in Figure 4A–C, significant neonatal treatment effects were seen in areas CA1, $F(1, 153) = 11.14$, $p < .001$, CA3, $F(1, 156) = 19.72$, $p < .001$, and CA3ab, $F(1, 156) = 20.26$, $p < .001$, with significantly fewer pERK1/2 cells in 5E than SI rats in each region. The TI significantly affected pERK1/2 labeling in area CA3, $F(1, 156) = 6.35$, $p < .05$, with fewer stained cells in 15 sec than 5 sec TI rats (Figure 4B). A significant treatment × TI interaction was seen in one region only, area CA3c, $F(1, 156) = 3.99$, $p < .05$. Post hoc contrasts revealed significant reductions in pERK1/2 labeled cells in SI/15s, 5E/5s, and 5E/15s rats compared with SI/5s rats (Figure 4D).

Discussion

Early developmental ethanol exposure can induce a multitude of CNS abnormalities, including long-lasting deficits in TFC, a cognitively challenging form of Pavlovian conditioning (Weike et al. 2007). Relative to control subjects, the behavioral results from both experiments show significant reductions in CS-dependent freezing in 5E rats trained with a long (15 or 30 sec) but not short (5 sec) trace interval (Figures 2 and 3), in general agreement with previous findings based on visual TFC in adolescent and adult rats (Hunt et al., 2009; Schreiber & Hunt, 2013; Schreiber et al., 2013; Wagner & Hunt, 2006). Taken together, the results suggest little recovery of function from adolescence to adulthood in rats administered alcohol as neonates. The pattern of results is also similar to that seen in rats exposed to a high dose of postnatal ethanol (5.0 or 5.25 g/kg/day) and trained in trace eyeblink conditioning, a simple form of cerebellar-dependent motor learning with much shorter CS and US durations. Acquisition of the trace eyeblink CR, which depends on the DH (Moyer et al., 1990), was significantly impaired in ethanol rats trained with a 500 ms TI (Murawski et al., 2013; Thomas & Tran, 2012) but not a 300 ms TI (Lindquist, 2013).

In Experiment 1, conditioned fear tied to the tone CS, but not training context, was diminished in a dose-dependent manner in adult ethanol rats (Figure 2A and B). The 5E rats were significantly impaired relative to UC, SI, and 3E rats, whereas the reduction in 4E rats was more modest and nonsignificant. Alcohol administration had no discernible effect on TFC acquisition or expression in 3E rats. Importantly, during TFC in Experiment 2 the control and 5E rats froze at similar rates throughout the CS-US ISI (Figure 3A and B), suggesting normal acquisition of the trace fear memory. The 5E rats were specifically impaired when trained with the 15 sec TI; treatment group differences were not seen with the 5 sec TI (Figure 3E and F). The ISI, which was held constant, can therefore be ruled out as the primary instigator of the current TFC impairment in 5E rats. Rather, in accord with previous results (Hunt et al., 2009), the freezing data indicate TI duration is the feature most responsible for the 5E rats impaired performance.

The 5E rats were selectively impaired after training with the 15 sec TI, whereas the IHC results revealed significant reductions in CA1 and CA3 pERK1/2 immunoreactivity in 5E rats trained at both TIs (Figure 4A and B). Consistent with other reports using pretraining inactivation (muscimol) or NMDAR blockade

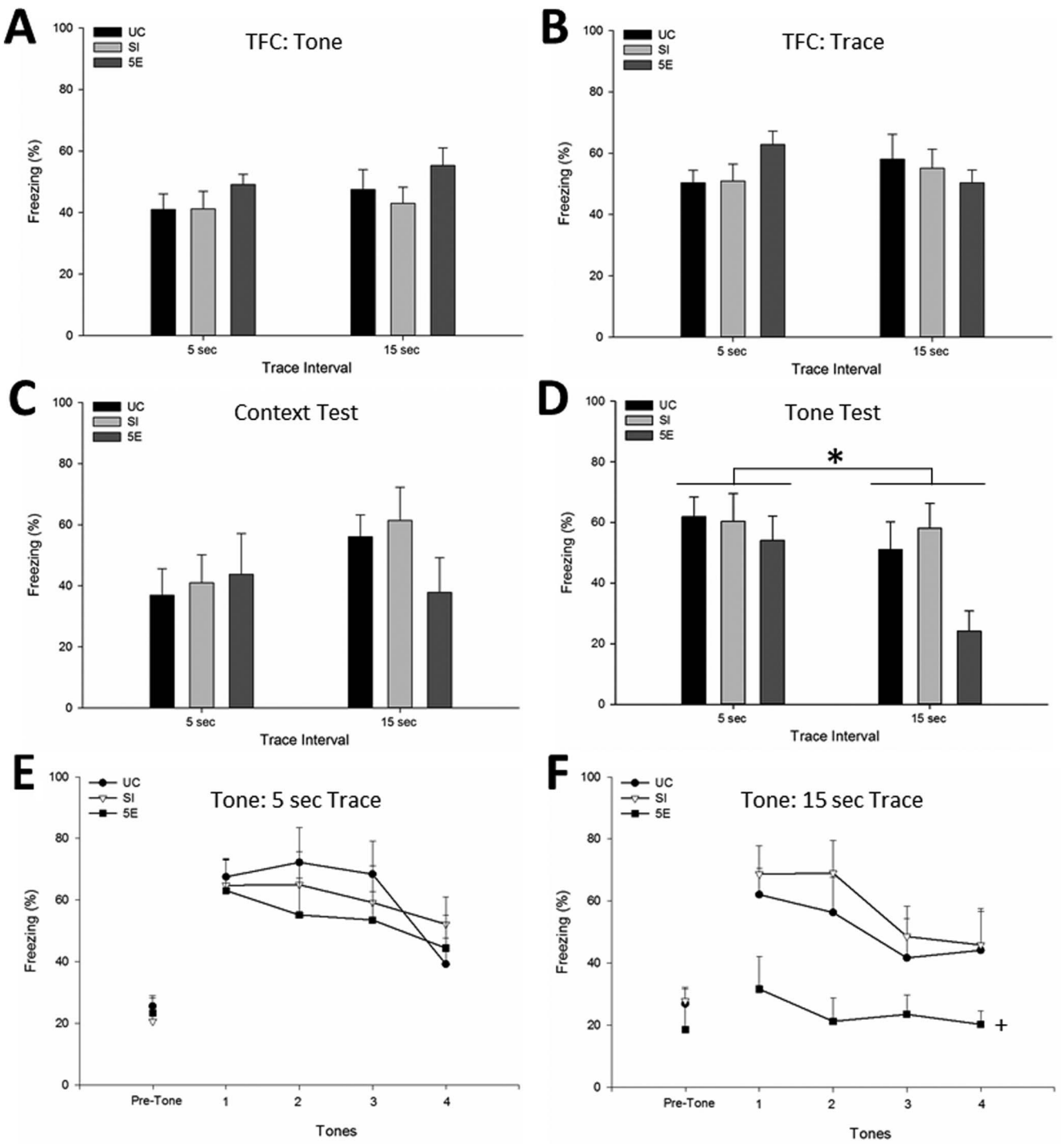

Figure 3. Percent freezing (mean ± *SE*) during acquisition and expression of TFC in Experiment 2. During training, neonatal treatment had no significant effect on freezing during the tone CS (A) or trace interval (B). For testing, neonatal treatment had no significant effect on context freezing (C). In (D) mean freezing during the tone was significantly influenced by neonatal treatment and the trace interval. Significantly less freezing was seen across all groups of rats trained with the 15 sec versus 5 sec trace interval (asterisk), and 5E rats froze significantly less than SI rats (not shown). Freezing during the pretone period and across each of the four 2 min tones is illustrated below. In (E) neonatal treatment had no significant effect on CS-dependent freezing in rats trained with the 5 sec trace interval. In (F) CS-dependent freezing was significantly reduced in 5E rats, compared with SI rats, when trained with the 15 sec trace interval (plus sign).

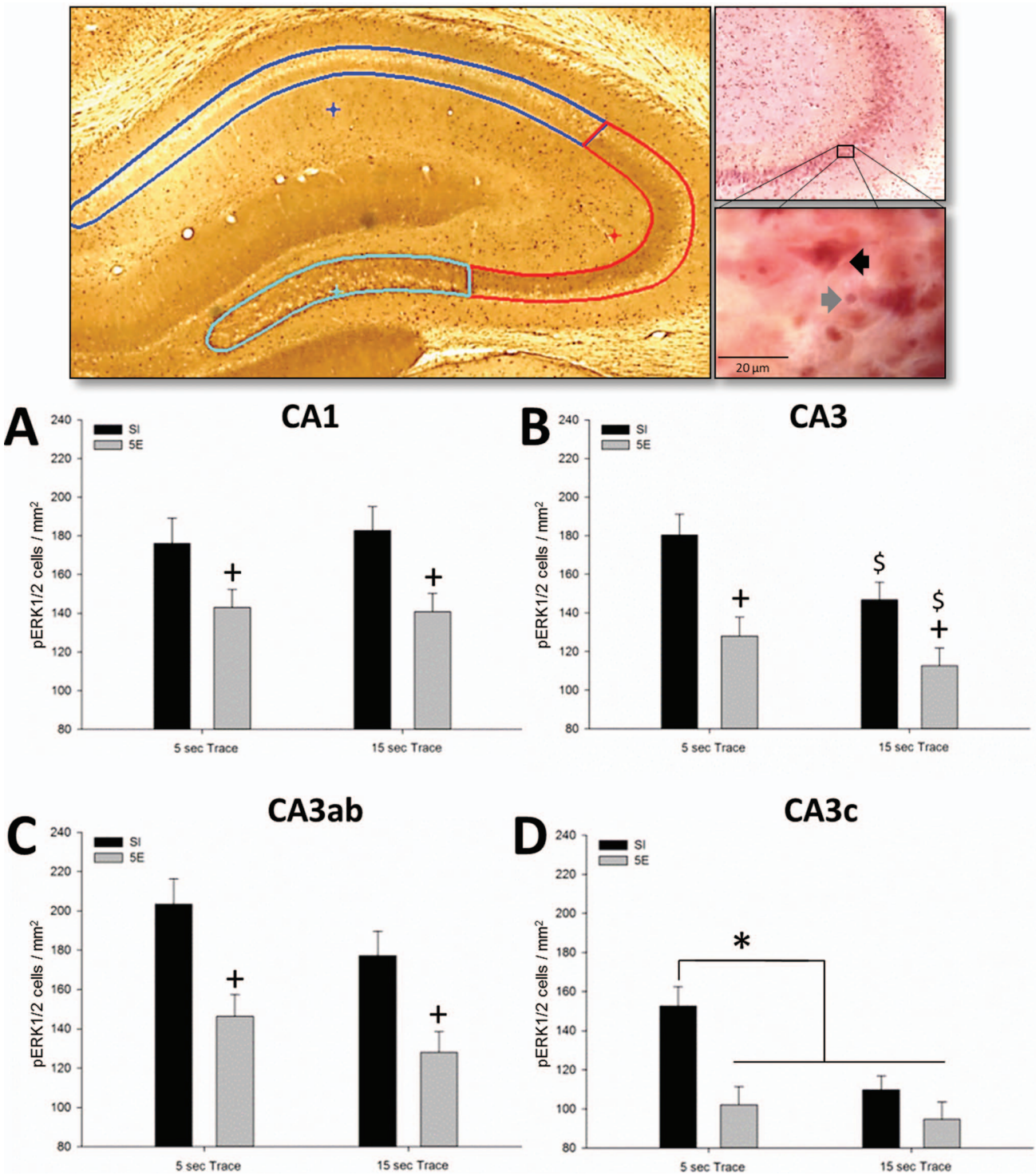

Figure 4. At top are representative photomicrographs of pERK1/2 and hemotoxylin-eosin stained DH tissue sections. At left, pERK1/2 labeled cells (from a SI rat) can be seen within the ROIs for CA1 (dark blue), CA3ab (red), and CA3c (light blue). The image at right on top shows a portion of CA3ab, at 10× magnification, in dual-labeled pERK1/2 and hemotoxylin cells. At right on the bottom is a 100× magnified image of the box above showing a hemotoxylin-stained neuronal nucleus (gray arrow) and a dual-labeled cell (black arrow). Scale bar = 20 µm. The number of pERK1/2 labeled cells is presented per unit area for CA1 (A), CA3 (B), CA3ab (C), and CA3c (D). Plus signs indicate significant treatment group effects; dollar signs indicate significant trace interval effects. The asterisk indicates significant contrasts. See text for details on statistical analyses.

(Guimarãis et al., 2011; Misane et al., 2005), the long-term trace fear memory in 5E rats appears intact with the short (5 sec) TI despite DH dysfunction. Only in 5E rats trained with the 15 sec TI do the decreases in pERK1/2-positive neurons correspond to diminished CS freezing, suggesting the DH is not required or other regions can compensate when the CS and US are separated by 5 sec or less.

Among the structures engaged by TFC, PL neurons have reciprocal connections with both the amygdala and VH (Hoover & Vertes, 2007), and display learning-related increases in CS-evoked spiking that can be actively sustained through presentation of the aversive US (Gilmartin & Helmstetter, 2010; Gilmartin & McEchron, 2005b). The VH is also necessary for TFC (Cox et al., 2013; Gilmartin et al., 2012), providing a relay between the DH and amygdala (Chiba, 2000). As for the DH, Gilmartin and McEchron (2005a) reported an early increase and later decrease in CS-evoked spiking in CA1 neurons during training with a 20 sec TI, whereas dentate gyrus neurons showed a progressive increase in spiking activity across training. Neither region fired reliably across the TI, however (discussed in Gilmartin et al., 2013).

As shown in Figure 4A–D, pERK1/2 immunoreactivity in trace fear conditioned subjects was generally consistent between CA1 and CA3, with significantly lower levels in 5E rats. Cell counts were also significantly reduced in area CA3 of the 15 sec TI rats compared with the 5 sec TI rats, suggesting less plasticity as the TI was lengthened. Area CA3 is a heterogeneous structure and the pERK1/2 expression profile differed somewhat between subregions. The number of pERK1/2-positive cells in CA3ab was significantly reduced in 5E compared with SI rats, whereas significant reductions in CA3c were seen in SI/15s, 5E/15s, and 5E/5 sec rats relative to the SI/5s rats. The differences could have functional relevance. Area CA3ab is thought to be more closely related to area CA1 (Lee & Kesner, 2004), with both regions involved in processing new or novel information (perhaps early in TFC, see above). Area CA3c, on the other hand, has inhibitory backprojections to the dentate gyrus (Scharfman, 2007), and plays a greater role in processing spatial information (Hunsaker et al., 2008). Potential differences in function aside, diminished pERK1/2 immunoreactivity in CA1 and CA3 neurons is proposed to weaken the trace fear memory's durability. In fact, ERK1/2 activation and phosphorylation is regulated by NMDAR-dependent activity (Carasatorre & Ramirez-Amaya, 2013) and, in 5E rats, upstream deficits in receptor function could have contributed to observed decreases in DH learning-dependent plasticity.

NMDARs, ERK1/2, and Ethanol

In a series of studies, Otto and colleagues have demonstrated successful TFC acquisition with long (28 or 30 sec) TIs following DH lesions or inactivation but not NMDAR antagonism (Czerniawski et al., 2012; Czerniawski et al., 2009; Yoon & Otto, 2007), indicating that if intact at the time of training then NMDAR signaling is required to properly encode and/or consolidate the trace fear memory. Forebrain NMDARs are expressed at the neuronal surface as tetramers, comprised of two NR1 subunits and two NR2 subunits. In adult animals, NR1/2B diheteromeric receptors are enriched in extrasynaptic sites, whereas NR1/2A and triheteromeric NR1/2A/2B receptors are principally embedded within the postsynaptic density (Foster et al., 2010; Paoletti, 2011). TFC is impaired following infusion of a NR2B antagonist (Ro25–6981) in either the PL (Gilmartin et al., 2013) or DH (Gao et al., 2010), with observed reductions in tone test freezing. The results suggest forebrain NR2B-containing NMDARs are required to associate the temporally discontinuous CS and US signals.

The NR2B subunit confers distinct gating and pharmacological properties to the NMDAR (Traynelis et al., 2010), which in turn regulates the recruitment of downstream plasticity-related signaling molecules, including Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Barria & Malinow, 2005; Delaney et al., 2013; Paoletti, 2011). The interaction between NR2B and CaMKII directly activates ERK1/2 and leads to its prolonged phosphorylation, promoting structural increases in spine volume and increased α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking (El Gaamouch et al., 2012). Accordingly, ERK1/2 plays a well-recognized role in the maintenance of synaptic plasticity and long-term memory consolidation (Giese & Mizuno, 2013; Peng et al., 2010). Villarreal and Barea-Rodriguez (2006), for instance, reported significant increases in hippocampal ERK1/2 phosphorylation immediately after TFC with a 30 sec TI, while pretraining inhibition (via SL327) impaired CS-dependent fear when tested 24 hr later. Combined with the effects of NR2B antagonism on TFC discussed above, NR2B-containing NMDARs appear to regulate the formation and consolidation of trace fear memories via the activation of CaMKII and the ERK/MAPK pathway.

Perinatal ethanol exposure alters the expression of NR2A and NR2B subunits in the forebrain of preweanling rats (Hughes et al., 1998; Nixon et al., 2002, 2004), possibly as a protective mechanism against alcohol's neurotoxic effects (Lovinger, 1995). In adult mice exposed to prenatal alcohol, PSD-95-associated NR2B subunit levels are downregulated (Samudio-Ruiz et al., 2010), likely reflecting fewer synaptic NR1/2A/2B-NMDARs. Prenatal exposure is further reported to reduce NMDAR-dependent LTP in the dentate gyrus of adult rats, with males more impaired than females (Sickmann et al., 2014), as well as decrease ERK1/2 activation in the dentate gyrus of adult mice (Samudio-Ruiz et al., 2009). Only a single study, to our knowledge, has examined alcohol's effects on ERK1/2 after postnatal exposure. Alcohol administration on PD7, via subcutaneous injection, inhibited pERK1/2 in the cortex and hippocampus when measured 4 to 24 hr later (Subbanna et al., 2013). The IHC results from Experiment 2 extend these findings to PD4–9 ethanol-exposed adult rats, with diminished ERK1/2 phosphorylation in CA1 and CA3 hippocampal neurons 1 hr after TFC.

Taken together, we hypothesize that early developmental ethanol exposure induces long-lasting alterations in NMDAR subunit composition and function, which, in turn, limits the downstream activation and phosphorylation of ERK1/2 and other molecules involved in long-term memory consolidation—albeit the aberrant DH plasticity mirrors observed deficits in freezing only when the TI exceeds 5 sec. A better understanding of the deleterious consequences of perinatal ethanol exposure, as relates to forebrain NMDAR subunit-selective function and putative disruptions in memory encoding and consolidation, is expected to inform future research into a number of higher-order cognitive impairments seen in children and adults with FASD (e.g., Pei et al., 2008), as well as aid in the search for novel treatments.

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