

# Neonatal Ethanol Exposure Impairs Trace Fear Conditioning and Alters NMDA Receptor Subunit Expression in Adult Male and Female Rats

Molly J. Goodfellow, Khalid A. Abdulla, and Derick H. Lindquist

**Background:** Ethanol (EtOH) exposure in neonate rats during a period comparable to the human third trimester, postnatal days (PD) 4 to 9, leads to persistent deficits in forebrain-dependent cognitive function—modeling the dysfunction seen in individuals diagnosed with fetal alcohol spectrum disorders. EtOH-exposed adult rats are impaired in auditory trace fear conditioning (TFC), a form of Pavlovian conditioning in which a neutral conditioned stimulus (CS; tone) is followed by an aversive unconditioned stimulus (US; footshock), with both stimuli separated in time by a stimulus-free “trace” interval (TI). TFC acquisition depends on *N*-methyl-*D*-aspartate NMDA receptor (NMDAR) activation in the dorsal hippocampus (DH), ventral hippocampus (VH), and medial prefrontal cortex (mPFC).

**Methods:** Male and female rat pups were sham-intubated (SI) or intragastrically intubated with EtOH (5E; 5 g/kg/d) over PD 4 to 9 and, as adults, submitted to TFC with a 15-second tone CS and 30-second TI. Whole-cell tissue lysates from the DH, VH, and mPFC of TFC rats and DH synaptic/extrasynaptic membrane fractions from experimentally naïve animals were analyzed via Western blot for NMDAR subunit (GluN1, GluN2A, GluN2B) expression.

**Results:** Freezing behavior during CS-alone test trials was significantly reduced in both male and female 5E rats, relative to same-sex controls. Western blot results based on DH tissue samples revealed a greater proportion of GluN2A to GluN2B subunits in 5E rats, relative to SI rats, and significantly reduced synaptic GluN2B and PSD-95 expression.

**Conclusions:** EtOH-induced changes in DH NMDAR subunit expression—particularly synaptic GluN2B, which is critical for TFC—are proposed to weaken long-term memory consolidation and, during behavioral testing, diminish CS-evoked freezing behavior.

**Key Words:** Fetal Alcohol Spectrum Disorders, Trace Fear Conditioning, NMDA Receptors, Hippocampus, Memory Consolidation.

FETAL ALCOHOL SPECTRUM disorders (FASD) encompass a range of persistent physical and cognitive abnormalities that can occur following gestational alcohol exposure (Streissguth, 2007). Neuroimaging studies have revealed structural and functional abnormalities in the hippocampus and prefrontal cortex of children with FASD (Davis et al., 2011), contributing to the impaired performance seen in both children and adults with FASD when tested in a variety of forebrain-dependent cognitive (e.g., learning and memory) tasks (Mattson et al., 2011). Ethanol (EtOH) exposure over postnatal days (PD) 4 to 9 in rodents,

a period comparable to the human third trimester (Bayer et al., 1993), also impairs forebrain neurodevelopment, producing significant reductions in dendritic spine density, neurogenesis, and long-term potentiation (LTP) (Hamilton et al., 2010; Klintsova et al., 2007; Puglia and Valenzuela, 2010).

We recently reported significant learning and memory deficits in PD 4 to 9 EtOH-exposed (5E) rats, when tested as adults (DuPont et al., 2014). Relative to controls, adult 5E rats were impaired in auditory trace fear conditioning (TFC), in which a 15-second tone conditioned stimulus (CS) was followed, 30 seconds later, by a footshock unconditioned stimulus (US). TFC depends on a spatially distributed neural circuit that includes the medial prefrontal cortex (mPFC) and ventral hippocampus (VH) (Czerniawski et al., 2012; Gilmartin and Helmstetter, 2010). When the “trace” interval (TI; time separating CS offset from US onset) is longer than ~10-seconds, the dorsal hippocampus (DH) is required as well (Chowdhury et al., 2005).

The toxic effects of ETOH on the developing hippocampus rely, at least in part, on subject sex. Following PD 4 to 9 exposure, for example, Schreiber and Hunt (2013)

From the Department of Psychology (MJG, KAA, DHL), The Ohio State University, Columbus, Ohio; and Department of Neuroscience (KAA, DHL), The Ohio State University, Columbus, Ohio.

Received for publication March 14, 2015; accepted November 4, 2015.

Reprint requests: Derick H. Lindquist, PhD, Departments of Psychology & Neuroscience, The Ohio State University, Psychology Building, Room 45, 1835 Neil Avenue, Columbus, OH 43210; Tel.: 614-292-2236; Fax: 614-688-4733; E-mail: lindquist.40@osu.edu  
Copyright © 2016 by the Research Society on Alcoholism.

DOI: 10.1111/acer.12958



found that adolescents of both sexes and adult females, but not adult males, were impaired in visual TFC with a 10-second TI. Conversely, Johnson and Goodlett (2002) reported deficits in the Morris water maze, another DH-dependent task, in adult males (but not females) exposed to EtOH across PD 7 to 9. Such variability following postnatal EtOH exposure suggests the underlying mechanisms likely vary by age of testing and the behavioral task employed. In the current study, adult (~PD 70) control and 5E rats were submitted to DH-dependent TFC (30-second TI)—training in female rats was initiated during proestrus, when estradiol and progesterone are at their highest levels (Woolley and Schwartzkroin, 1998).

Forebrain *N*-methyl-D-aspartate receptors (NMDARs) are heterodimers composed of 2 GluN1 and 2 GluN2 subunits, GluN2A or GluN2B. The GluN2 subunits primarily determine the voltage dependence of the  $Mg^{2+}$  channel block, receptor kinetics, and downstream signaling (Erreger et al., 2005). Early in life, GluN2B-containing NMDARs, which gate more calcium but open less often than GluN2A-NMDARs (Erreger et al., 2005; Monyer et al., 1994), are ubiquitously expressed. Across postnatal development, GluN2A expression is up-regulated and GluN2B expression is slightly down-regulated (Monyer et al., 1994), producing a more “stable” phenotype that requires greater excitation to induce synaptic plasticity (Yashiro and Philpot, 2008). Aberrant NMDAR subunit expression has been documented in preweanling rats exposed to EtOH during the pre- and/or postnatal period (Nixon et al., 2002, 2004), as well as in adult mice following prenatal exposure (Samudio-Ruiz et al., 2010).

In the DH and mPFC, NMDARs play an important role in the encoding and consolidation of TFC. For instance, pre-training infusions of an NMDAR antagonist into either region impairs TFC, as evidenced by reduced freezing behavior during context and CS retention testing (Gilmartin and Helmstetter, 2010; Quinn et al., 2005). DuPont and colleagues (2014) also found significant reductions in phosphorylated extracellular signal-related kinase (ERK)1/2 expression in CA1 and CA3 neurons from 5E rats sacrificed 1 hour after TFC, which was interpreted to reflect abnormal NMDAR-dependent plasticity. It has not been previously established, however, whether postnatal EtOH exposure induces long-lasting changes in forebrain NMDAR subunit expression when measured in adult rats. In addition to NMDAR subunit composition, the location of membrane-bound GluN2A- and GluN2B-containing NMDARs (i.e., synaptic vs. extrasynaptic compartments) can also influence learning-dependent synaptic plasticity (Hardingham and Bading, 2010). Altogether, this study was designed to evaluate DH-dependent TFC in adult control and 5E rats (of both sexes) and quantify the expression of GluN1, GluN2A, and GluN2B subunits in whole-cell lysates (WCLs) from the DH, VH, and mPFC and, separately, in DH synaptic membrane fractions (SMFs) and extrasynaptic membrane fractions (EMFs).

## MATERIALS AND METHODS

### *Subjects and Neonatal Treatment*

Long-Evans rats were bred and housed in The Ohio State University vivarium and submitted to EtOH treatment as previously described (Goodfellow and Lindquist, 2014). On PD 3, litters were culled to 10 to 12 pups and paw-marked for identification. Across PD 4 to 9, pups were administered EtOH (11.33%) in milk solution via intragastric intubation twice daily, 2 hours apart (5 g/kg/d; 5E), followed 2 hours later by milk alone. Sham-intubated (SI) control rats received identical treatment except no milk or EtOH was given. On PD 4, blood samples for determining blood alcohol concentration (BAC) were taken via tail clip just prior to the milk-alone intubation. Samples from the SI rats were discarded; the 5E samples were centrifuged and plasma separated. Peak BACs were determined using an Analox GL5 Analyzer (Analox Instruments, Lunenburg, MA), which measures the rate of oxygen consumption resulting from oxidation of EtOH in the sample.

Rats were weaned on PD 21, same-sex group housed until PD 60 and, consistent with our previous studies, individually housed until sacrificed (DuPont et al., 2014; Goodfellow and Lindquist, 2014). Testing occurred between PD 65 to 75 around midday (~1100 to 1500) during the light phase (12-hour light/dark cycle—lights on at 0600). Prior work has demonstrated that SI and unhandled/unintubated control rats exhibit similar patterns of freezing behavior during TFC and retention testing with TIs ranging from 5 to 30-seconds (DuPont et al., 2014; Hunt et al., 2009). Considering SI rats are the critical comparison group for 5E rats (Schreiber and Hunt, 2013), and to conserve animals, only SI and 5E rats were used in the current study. A total of 89 rats were used; in cases where more than 1 male or female per treatment group per litter were used (SI  $n = 8$  males, 6 females; 5E  $n = 4$  males, 8 females), data were averaged (i.e., 26 rats yielded 13 data points), resulting in  $N = 76$ .

### *Estrous Cycle Tracking*

From PD 61 to 65, at ~0930 females were vaginally swabbed with a sterile cotton swab soaked in 0.9% saline. Samples were applied to a subbed slide and stained with toluidine blue to determine stage of estrous based on cell morphology, as described in Shors (1998). Those in proestrus underwent TFC 2 to 4 hours later or, separately, were sacrificed to quantify membrane-bound NMDAR protein expression. Females not in proestrus continued to be swabbed daily until they entered proestrus. As a control, male genitals were also swabbed until 1 male could be conditioned (or sacrificed) at the same time as 1 female littermate. For all females, stage of estrous was numerically coded (1 = estrus, 2 = diestrus 1, 3 = diestrus 2, and 4 = proestrus) and averaged from PD 61 to 64. A normal estrous cycle would result in a mean of 2.5.

### *Apparatus*

Standard operant boxes (Coulbourn Instruments, Allentown, PA) in sound-attenuating chambers were composed of 2 stainless steel and 2 plexiglass walls, and a floor with 0.5-cm stainless steel bars spaced 1.5 cm apart. A shock generator (model 82400; Lafayette Instruments, Lafayette, IN) and neon grid scrambler (model 58020; Lafayette Instruments) delivered the footshock US. The interior was illuminated with infrared lights, and freezing behavior was recorded with a video camera (Model WDSR-2005SC; Circuit Specialists, Inc., Mesa, AZ) mounted to the top of the conditioning chamber. The video signal was inputted to FreezeScan (CleverSys, Inc., Reston, VA), which quantified freezing behavior within experimenter-defined time bins.

### Trace Fear Conditioning

Rats were carried in their home cages, 2 at a time, to a well-lit testing room and placed in an operant box illuminated by a 15-W bulb and scented with a vinegar/water (1:5) solution. After a 240 ± 30-second baseline, rats were presented with a 15-second, 2.8 kHz, 80 dB tone CS followed 30 seconds later by a 1-second, 0.8 mA footshock US, resulting in a 45-second interstimulus interval (ISI; time from CS onset to US onset). In all, rats received 10 CS-US presentations, separated by an intertrial interval (ITI) of 240 ± 30-seconds. Approximately 24 and 48 hours later, rats underwent context and CS-alone testing in counterbalanced order.

Context fear was assessed in the conditioning chamber. After a 120-second baseline, freezing was measured for 600-seconds and the rat removed 120-seconds later. CS-evoked freezing was assessed in a novel context. Rats were wheeled into a dark testing room (lit only with a red overhead light) on a metal cart, with each cage covered by a towel. The chamber was scented with Windex® (S.C. Johnson & Son, Inc., Racine, WI), a gray opaque plexiglass sheet covered the grid bars, an interior fan provided 60 dB white noise, a small magnet was placed on 1 metal wall, and a pink geometric figure was attached to the door. Freezing was measured for 1 minute prior to the first tone CS to verify the context switch was successful. The 15-second tone CS was presented only 5 times (90 ± 10-second ITI) to minimize extinction learning. Freezing was recorded during each CS-alone trial and the 30-second period following CS offset (i.e., the TI). Rats were returned to the vivarium 120-seconds after the final trial.

### Whole-Cell Lysate Tissue Preparation

At least 3 days after behavioral testing, rats were deeply anesthetized with isoflurane and decapitated. Brains were removed and the DH, VH, and mPFC rapidly dissected and stored at -80°C. While TFC requires the prelimbic, but not infralimbic, region of the mPFC (Gilmartin and Helmstetter, 2010), we were unable to confidently separate them—thus, prelimbic and infralimbic cortices were analyzed together. Tissue was homogenized in 95°C buffer (1% sodium dodecyl sulfate [SDS], 10 mM Tris [pH 7.4], 1 mM Na<sub>3</sub>VO<sub>4</sub>) and centrifuged at 29,000×g at 15°C for 10 minutes. The supernatant (WCL) was stored at -80°C.

### Membrane Fraction Tissue Preparation

Potential sex differences in NMDAR subunit expression were examined in experimentally naïve rats under the same hormonal conditions (i.e., proestrus for female rats) as at the start of TFC. SMFs and EMFs were isolated via a protocol adapted from Samudio-Ruiz and colleagues (2010) and Goebel-Goody and colleagues (2009). Tissue was lysed in homogenization buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 320 mM sucrose, 20 mM

Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 10 mM NaF, 20 mM β-glycerophosphate, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>) and centrifuged (1,000×g, 10 minutes). The supernatant was centrifuged again (15,000×g, 30 minutes), and the resulting pellet was homogenized with distilled/deionized cold water containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). HEPES-NaOH buffer (pH 7.4, 7.5 mM) was added and kept on ice 30 minutes before centrifugation (22,000×g, 20 minutes). Pellets were resuspended in Triton X-100 buffer (1% Triton, 10 mM Tris, pH 7.4, 5 mM NaF, 1 mM EDTA, 0.5 mM EGTA, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>), kept on ice for 30 minutes and centrifuged (100,000×g, 60 minutes) to yield the triton-insoluble pellet (SMF). Acetone was added to the supernatant, incubated 4 hours (-20°C), and centrifuged (15,000×g, 10 minutes) to obtain the Triton-soluble (EMF) pellet. The EMF and SMF were each combined with homogenization buffer containing 1% SDS, heated (95°C, 5 minutes), and stored at -80°C. Bradford assays determined protein concentrations (ThermoScientific, Rockford, IL); due to low yields, total protein loaded and antibody concentrations were titrated prior to data collection (see Table 1).

### Western Blotting

Samples containing 20 μg (WCL), 7 μg (SMF), or 5 μg (EMF) protein were combined 1:1 with loading buffer and heated (95°C, 5 minutes). Samples were loaded into a 7.5% TGX gel (Bio-Rad, Hercules, CA), electrophoresed (150V, ~70 minutes), and transferred to a nitrocellulose membrane (Bio-Rad) at 100V for 60 minutes. Membranes were cut to separate GluN2A or GluN2B from GluN1, PSD-95, and the loading control (GAPDH or actin) and equilibrated in 5% nonfat dry milk (NFDM; Bio-Rad) for 60 minutes at room temperature. Membranes incubated overnight at 4°C in primary antibody in NFDM. The next day, membranes were rinsed in TBST 4 × 7 minutes and equilibrated in secondary antibodies in NFDM for 60 minutes. Membranes were rinsed in TBST 4 × 7 minutes and covered in Clarity Western Blot Substrate (Bio-Rad) for 5 minutes. Blots were imaged with a FluorChem M system and analyzed with AlphaView software (ProteinSimple, Santa Clara, CA).

### Data Analysis

Data were analyzed via 1-way, multifactorial, and repeated-measures analysis of variance (ANOVA), and (when required) Bonferroni-corrected 1-way ANOVAs. Tukey's post hoc tests were used to study significant main effects and interactions. A significant post hoc test implies  $p < 0.05$ . As determined prior to the start of the experiment, behavioral data were excluded if it was >2 standard deviations from the group mean (see Broadwater and Spear, 2013; Dokovna et al., 2013)—2 5E females, 2 5E males, and 1 SI female were dropped.

**Table 1.** Antibodies Used for Western Blotting Including Their Host Animal, Molecular Weight, Manufacturer, and the Concentrations Used for Whole-Cell Lysate (WCL), Synaptic Membrane Fraction (SMF), and Extrasynaptic Membrane Fraction (EMF)

Target	Host	Molecular weight	Manufacturer	WCL concentration	SMF concentration	EMF concentration
GluN2A	Rabbit	180 kD	PhosphoSolutions, Aurora, CO	1:3,000	1:1,000	1:200
GluN2B	Rabbit	180 kD	Millipore, Billerica, MA	1:1,500	1:2,000	1:400
GluN1	Mouse	120 kD	BD Biosciences, San Jose, CA	1:3,000	1:1,250	1:250
PSD-95	Rabbit	95 kD	Cell Signaling, Danvers, MA	—	1:1,000	1:1,000
Actin	Mouse	42 kD	PhosphoSolutions, Aurora, CO	—	1:1,500	1:1,500
GAPDH	Mouse	38 kD	Novus, Littleton, CO	1:5,000	—	—
Rabbit	Goat	—	Pierce, Rockford, IL	1:5,000	1:5,000	1:5,000
Mouse	Goat	—	BD Biosciences, San Jose, CA	1:2,000	1:2,000	1:2,000

## RESULTS

### Blood Alcohol Concentration

Peak BACs (mean  $\pm$  SE) in 5E rats were  $351.7 \pm 6.2$  mg/dl in females and  $344.5 \pm 7.2$  mg/dl in males—no significant sex differences were found ( $p = 0.45$ ).

### Estrous Cycle

Based on our numerical coding, normal cycling would yield a value of 2.5. Treatment group means ( $\pm$ SE) were  $2.36 \pm 0.09$  in SI rats and  $2.24 \pm 0.07$  in 5E rats. A 1-way (Treatment) ANOVA failed to find significant treatment group differences ( $p = 0.31$ ; data not shown), suggesting the SI and 5E female rats cycled similarly prior to TFC. While the estrous cycle was not tracked following TFC, the normal cycling seen between treatment groups suggests all females were likely in estrus or diestrus 1 during the 2 test sessions.

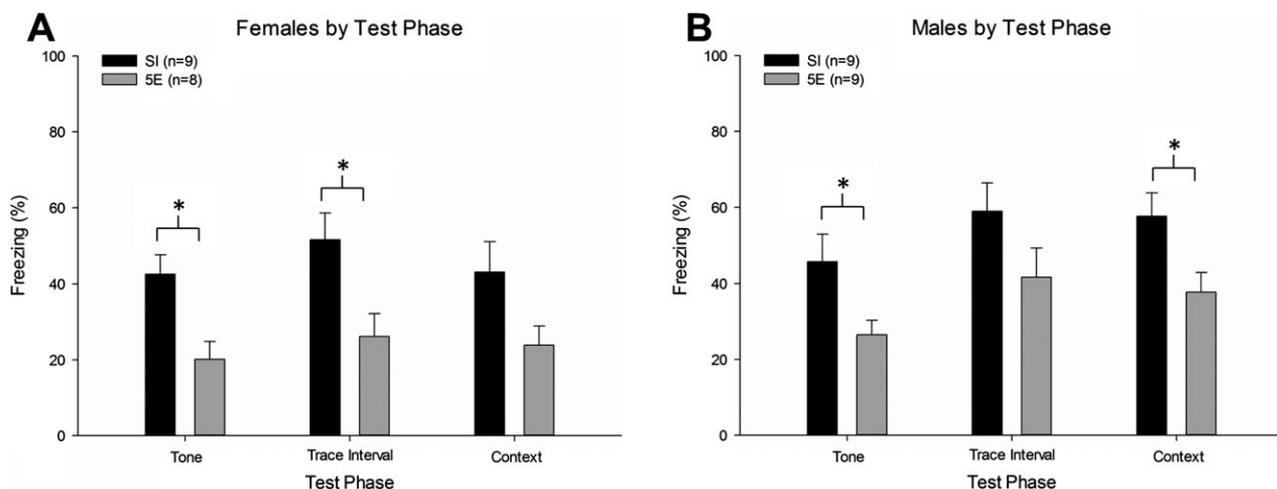
### Trace Fear Conditioning

Previous research from our laboratory demonstrated no differences in CS-evoked freezing behavior between control and 5E treatment groups during the TFC session (DuPont et al., 2014), indicating intact sensory/motor responding and associative learning for both groups. The current study expands on our previous results by comparing SI and 5E freezing percentages during the 15-second tone CS and, following its offset, the 30-second TI. Context-dependent freezing was measured during a separate test. The 2 (Treatment)  $\times$  2 (Sex) ANOVAs revealed significant Treatment group main effects for the CS,  $F(1, 31) = 15.19$ ,  $p < 0.001$ , TI,  $F(1, 31) = 8.90$ ,  $p < 0.01$ , and context,  $F(1, 31) = 9.75$ ,  $p < 0.01$ . Post hoc analyses indicate the 5E rats froze less than SI rats during all 3 test phases. A significant Sex effect was found for context only,  $F(1, 31) = 5.13$ ,

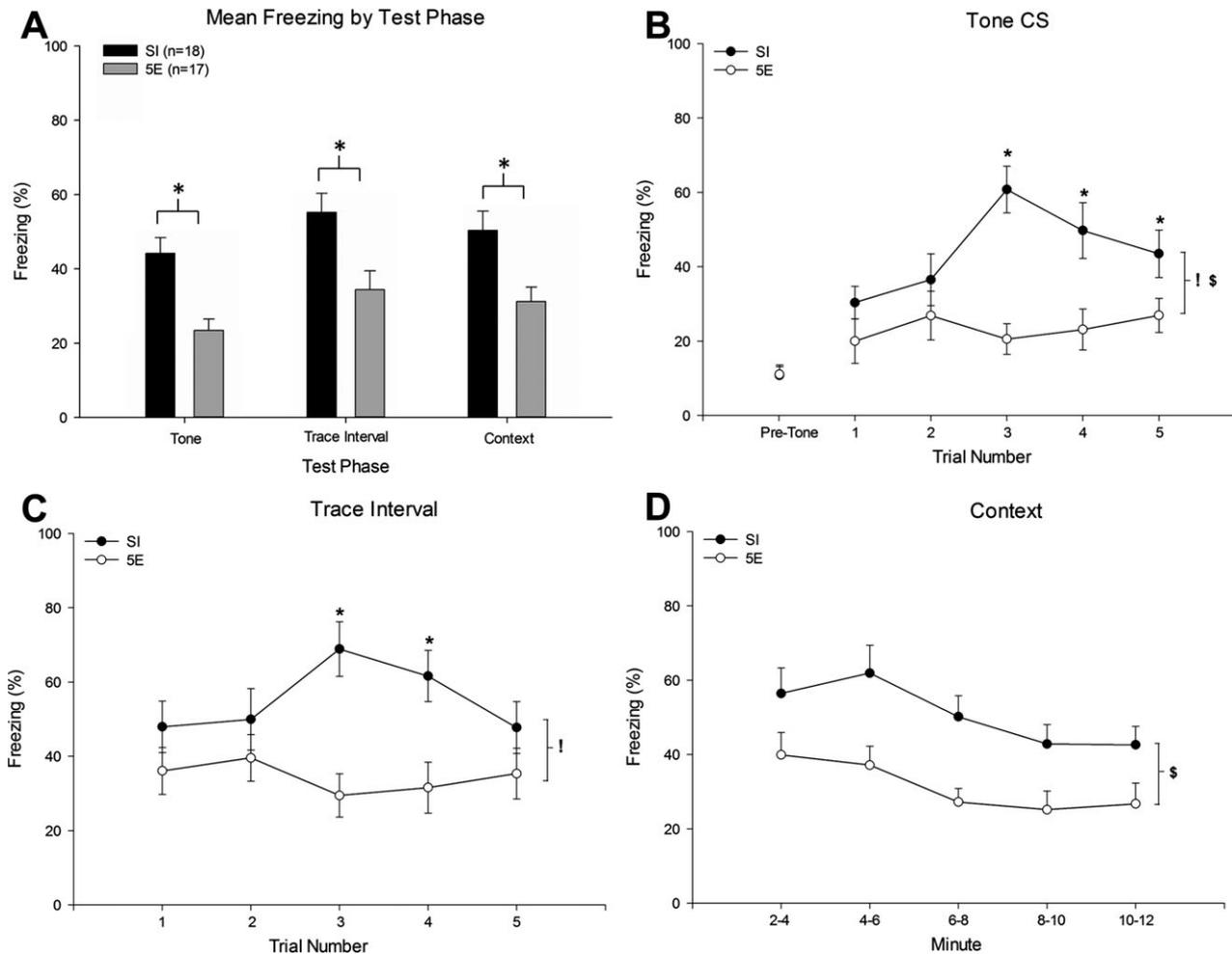
$p < 0.05$ , with females freezing less than males, regardless of neonatal treatment. The Treatment  $\times$  Sex interaction was not significant for any test phase.

Mean freezing rates in males and females were separately analyzed across all 3 test phases with 1-way (Treatment) ANOVAs. In females (Fig. 1A), there was a significant main effect for the tone CS,  $F(1, 15) = 10.47$ ,  $p < 0.01$ , and TI,  $F(1, 15) = 7.24$ ,  $p < 0.05$ , with 5E females impaired relative to SI females. Significant treatment group differences for context test freezing just missed significance ( $p = 0.06$ ). In males (Fig. 1B), there were significant main effects for the tone CS,  $F(1, 16) = 5.71$ ,  $p < 0.05$ , and context,  $F(1, 16) = 6.07$ ,  $p < 0.05$ , but not to the TI ( $p = 0.12$ ). The 5E rats were again impaired relative to SI rats.

Due to the relatively modest sex differences, behavioral data were subsequently collapsed across sex and analyzed via 1-way (Treatment) ANOVAs. Results revealed significant main effects for the tone CS,  $F(1, 33) = 15.48$ ,  $p < 0.001$ , TI,  $F(1, 33) = 8.32$ ,  $p < 0.01$ , and context,  $F(1, 33) = 8.55$ ,  $p < 0.01$ , with 5E rats freezing less than SI rats (Fig. 2A). The significant context freezing impairment differs from our previous study (DuPont et al., 2014) and is likely attributable to the larger group sizes and higher power in the current study. Next, 2 (Treatment)  $\times$  5 (Trial) repeated-measures ANOVAs were used to examine freezing during and following each CS-alone trial or, for the context test, across five 2-minute bins. As seen in Fig. 2B, the low level of freezing in both treatment groups during the pre-tone period in the novel context indicates a successful context switch (i.e., little context fear generalization). Across the 5 CS presentations, results revealed significant effects for Trial,  $F(4, 132) = 2.49$ ,  $p < 0.05$ , and the Treatment  $\times$  Trial interaction,  $F(4, 132) = 3.06$ ,  $p < 0.05$ . Separate 1-way ANOVAs on each trial found that 5E rats froze significantly less during trials 3 to 5. During the TI (Fig. 2C), a significant Treatment  $\times$  Trial interaction was also found,  $F(4, 132) = 3.19$ ,



**Fig. 1.** Percent freezing at test (mean  $\pm$  SE), by treatment group and sex. **(A)** Female ethanol-exposed (5E) rats were impaired relative to female sham-intubated (SI) rats in freezing to the tone conditioned stimulus (CS) and trace interval (TI). **(B)** The 5E male rats were also impaired relative to SI male rats in freezing to the tone CS and training context. An asterisk (\*) denotes a significant treatment group effect ( $p < 0.05$ ).



**Fig. 2.** Percent freezing at test (mean  $\pm$  SE), collapsed across sex. **(A)** Freezing was significantly reduced in ethanol-exposed (5E) rats, relative to sham-intubated (SI) rats, to the tone conditioned stimulus (CS), trace interval (TI), and training context. **(B)** No treatment group differences were seen in freezing prior to the first CS-alone trial (pre-tone period), indicating a successful context switch. Across the 5 CS-alone trials, the 5E rats froze significantly less over trials 3 to 5. **(C)** Following CS offset, freezing was measured during the 30-second TI. The 5E rats froze significantly less on trials 3 and 4. **(D)** Across the 10-minute context test (shown in 2-minute bins), the 5E rats were significantly impaired relative to SI rats. An asterisk (\*) denotes a significant treatment group effect; a dollar sign (\$) represents a significant trial effect; an exclamation point (!) indicates a significant Treatment  $\times$  Trial interaction ( $p < 0.05$ ).

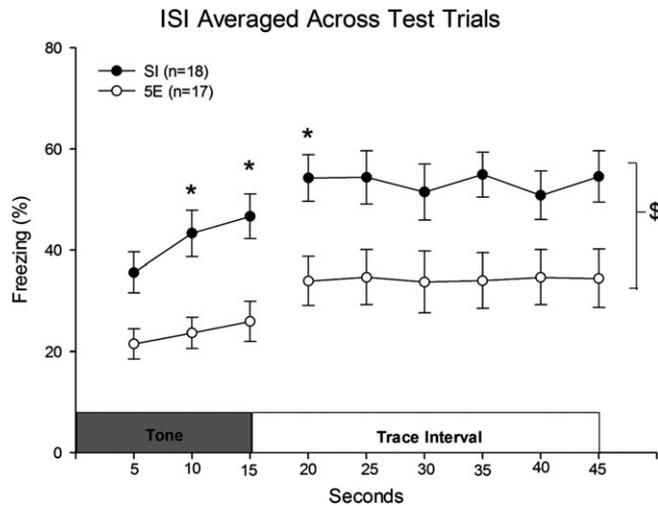
$p < 0.05$ , with 5E rats significantly impaired during trials 3 and 4. For the context test (Fig. 2D), only the Trial main effect was significant,  $F(4, 132) = 7.48$ ,  $p < 0.001$ , with both groups freezing less over time.

Finally, we sought to characterize freezing behavior across the entire 45-second ISI (15-second CS + 30-second TI; Fig. 3). Freezing was contrasted between treatment groups during each of nine 5-second bins (CS: bins 1 to 3, TI: bins 4 to 9). A repeated-measures ANOVA revealed significant main effects for Treatment,  $F(1, 33) = 10.83$ ,  $p < 0.01$ , and Time,  $F(8, 264) = 9.31$ ,  $p < 0.001$ , but not their interaction ( $p = 0.77$ ). Post hoc testing verified the 5E rats were impaired relative to SI rats. Bonferroni-corrected 1-way ANOVAs were applied to each of the nine 5-second bins—requiring (at 9 contrasts)  $p < 0.0056$  for statistical significance, maintaining a family-wise  $\alpha = 0.05$ . Results indicate that 5E rats froze significantly less than SI rats across bins 2 and 3 (the final 10-seconds of the tone CS) and bin 4 (first 5-seconds of the TI).

As seen in Fig. 3, the 5E rats were clearly impaired relative to SI rats but the pattern of freezing was similar for both groups across the averaged 45-second trial.

#### Whole-Cell Lysate Immunoblots

Following the completion of testing, brain tissue was analyzed for NMDAR subunit expression via Western blotting. Note, tissue was lost due to a  $-80^{\circ}\text{C}$  freezer malfunction, reducing the sample size of both treatment groups. The optical density (OD) values of individual subunits were first normalized to OD values of loading control expression ([target OD/loading control OD]  $\times$  loading control mean). As we did not control for estrous cycle on the day of sacrifice, data were collapsed across sex and analyzed via 1-way (Treatment) ANOVAs in the DH, VH, and mPFC (SI  $n = 3$  males, 4 females; 5E  $n = 3$  males, 3 females). Importantly, immunoreactivity for the GAPDH loading control did not



**Fig. 3.** Averaged percent freezing (mean  $\pm$  SE) over the 45-second interstimulus interval (ISI; 15-second conditioned stimulus + 30-second trace interval). Each data point (5-second bin) is the average of 5 test trials. Ethanol-exposed (5E) rats froze significantly less than sham-intubated (SI) rats across the whole ISI. Post hoc testing indicates the 5E rats froze significantly less than SI rats across bins 2 to 4 (seconds 6 to 20). An asterisk (\*) denotes a significant treatment group effect and a dollar sign (\$) indicates a significant effect of time ( $p < 0.05$ ).

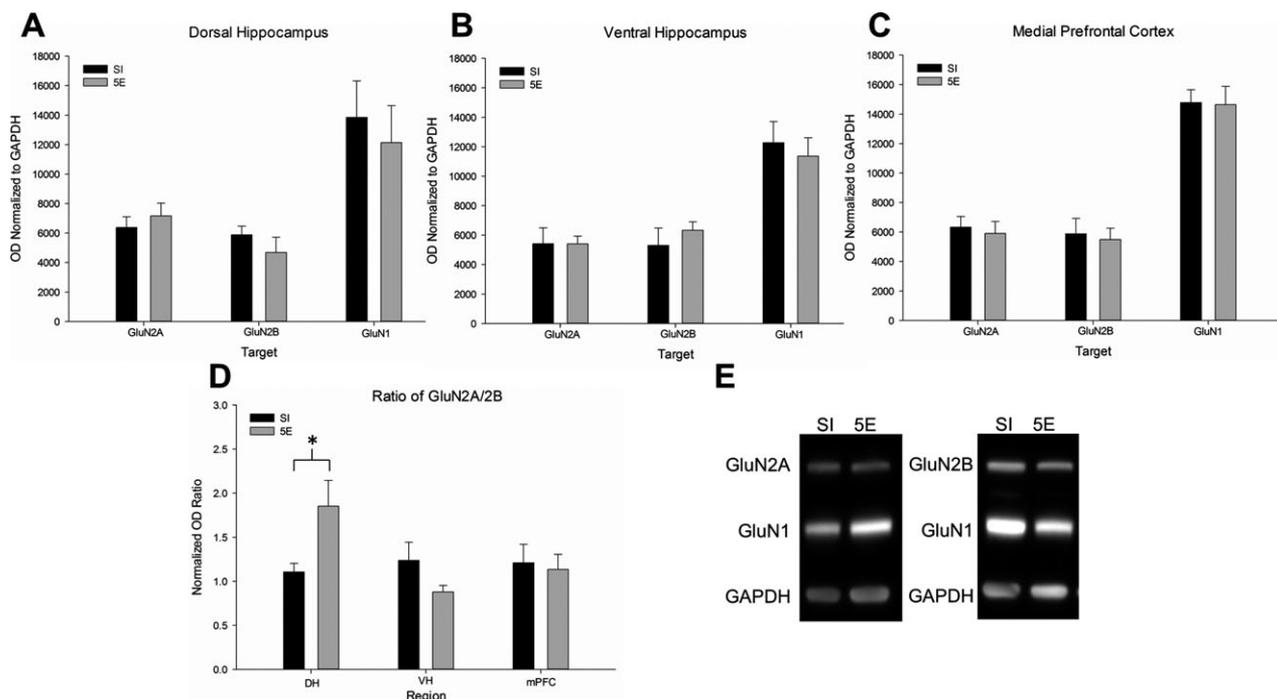
differ by treatment group. No significant treatment group differences were found in GluN2A, GluN2B, or GluN1 expression in any brain region (Fig. 4A–C). Closer examination of the DH results, however, revealed a significant

increase in the proportion of GluN2A expression relative to GluN2B in 5E rats,  $F(1, 11) = 4.86$ ,  $p = 0.05$  (Fig. 4D). The difference cannot be specifically attributed to TFC, however. All animals were sacrificed at least 3 days following the completion of testing.

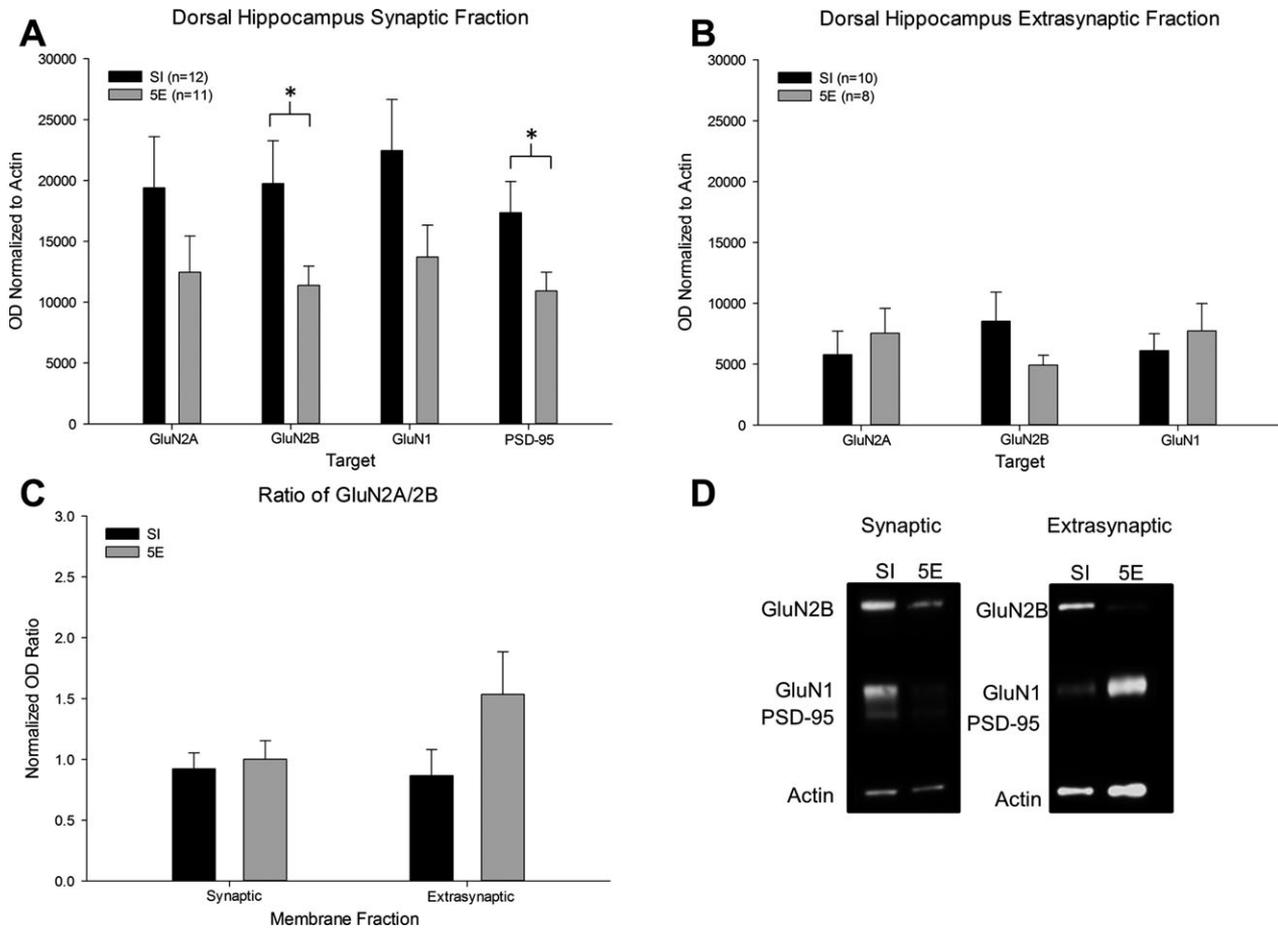
#### Subcellular Fraction Immunoblots

For subcellular fractions, experimentally naive females were sacrificed during proestrus along with a male littermate. Expression of the loading control, actin, did not significantly differ between treatment groups or sex in any region or fraction. Treatment  $\times$  Sex ANOVAs applied to DH synaptic fractions (SI  $n = 6$  males, 6 females; 5E  $n = 5$  males, 6 females) and DH extrasynaptic fractions (SI  $n = 5$  male, 5 females; 5E  $n = 4$  males, 4 females) failed to uncover significant effects for any protein. For the main effect of Sex, all  $p$ -values were  $>0.40$ . The data suggest NMDAR subunits are expressed at comparable levels in male and female adult rats.

Immunoblot data were thus collapsed across sex and analyzed via 1-way (Treatment) ANOVAs. For DH synaptic fractions, significant treatment group differences were seen for GluN2B,  $F(1, 21) = 4.42$ ,  $p < 0.05$ , and PSD-95,  $F(1, 21) = 4.46$ ,  $p < 0.05$ , with expression decreased in 5E rats relative to SI rats (Fig. 5A). The Treatment main effect failed to reach statistical significance for GluN1 ( $p = 0.10$ ) or GluN2A ( $p = 0.20$ ). VH synaptic fractions were also analyzed, with no significant Treatment group differences seen



**Fig. 4.** Western blot optical density (OD; mean  $\pm$  SE), normalized to GAPDH OD, for *N*-methyl-D-aspartate receptor (NMDAR) subunits. Results are based on whole-cell lysates from ethanol-exposed (5E) and sham-intubated (SI) rats taken from the (A) dorsal hippocampus (DH), (B) ventral hippocampus (VH), and (C) medial prefrontal cortex (mPFC). No treatment group differences were noted for NMDAR subunit expression in any brain region. (D) Ratio of GluN2A relative to GluN2B by region—a significant increase in the GluN2A/GluN2B ratio was found in DH only. (E) Representative DH immunoblot images. An asterisk (\*) denotes a significant treatment group effect ( $p < 0.05$ ).



**Fig. 5.** Western blot optical density (OD; mean  $\pm$  SE), normalized to actin OD, for *N*-methyl-D-aspartate receptor (NMDAR) subunits. Results are based on (A) dorsal hippocampus (DH) synaptic membrane fractions and (B) DH extrasynaptic membrane fractions, taken from ethanol-exposed (5E) and sham-intubated (SI) rats. In 5E rats, GluN2B and PSD-95 were significantly reduced in the synaptic membrane fraction only. (C) The proportion of GluN2A relative to GluN2B did not significantly differ between treatment groups in either membrane fraction. (D) Representative synaptic and extrasynaptic GluN2B immunoblots—note the absence of PSD-95 expression in the extrasynaptic fractions. An asterisk (\*) denotes a significant treatment group effect ( $p < 0.05$ ).

for any protein (data not shown). Figure 5B shows results from DH extrasynaptic fractions, which are characterized by a lack of PSD-95 expression (Fig. 5D). One-way (Treatment) ANOVAs did not uncover significant effects for any protein of interest. Interestingly, analysis of the GluN2A/GluN2B ratio found no treatment group differences for DH synaptic fractions ( $p = 0.69$ ), while a modest trend toward statistical significance ( $p = 0.11$ ) was seen with the extrasynaptic fractions (Fig. 5C). Protein levels from extrasynaptic VH and both synaptic and extrasynaptic mPFC were insufficient for analysis.

## DISCUSSION

In the current study, TFC performance and Western blot analyses of NMDAR subunit expression in the DH, VH, and mPFC were used to study the deleterious effects of postnatal binge-like EtOH exposure in adult subjects. Following TFC, the 5E rats demonstrated significant freezing deficits to the CS, TI, and context (Figs 2 and 3). Only modest sex differences were seen in behavior (Fig. 1), and none were

detected biochemically. A significant increase in the GluN2A/GluN2B ratio (based on WCL immunoblots) was seen in the DH, but not the VH or mPFC, of 5E rats (Fig. 4). Treatment group differences were also seen in DH synaptic but not extrasynaptic fractions, with synaptic GluN2B and PSD-95 significantly reduced in 5E rats (Fig. 5). Changes in NMDAR subunit composition and receptor kinetics are proposed to impede the maintenance of TFC-dependent synaptic plasticity, restricting the consolidation of long-term trace fear memories.

### Trace Fear Conditioning

Forebrain cognitive function was assessed in adult rats via TFC, a Pavlovian paradigm that depends on higher order cognition, including attention-dependent associative learning and working memory (Raybuck and Lattal, 2014). During retention testing, the 5E rats froze significantly less than SI rats during the tone CS and ensuing TI in a novel context, and when re-exposed to the conditioning context (Fig. 2). The results are consistent with prior research documenting

impaired forebrain-dependent associative learning following PD 4 to 9 EtOH administration (DuPont et al., 2014; Schreiber and Hunt, 2013) and support the proposition that early-life EtOH exposure can induce persistent deficits in cognitive function. The effect is likely not due to treatment group differences in somatic development—adult control and 5E rats do not differ in terms of body weight (DuPont et al., 2014; Goodfellow and Lindquist, 2014).

Results indicate that, following TFC, 5E rats of both sexes froze significantly less during CS-alone test trials (Figs 1 and 2). In comparison, Schreiber and Hunt (2013) reported that adult females, but not males, exposed to EtOH over PD 4 to 9 were significantly impaired in CS-evoked freezing following visual TFC. There are a number of methodological differences between the 2 studies—for example, Schreiber and Hunt (2013) used freely cycling females, a light CS, a 10-second TI, and group-housed animals throughout testing—that likely contribute to the disparate results. In the current study, 5E female rats also froze less during the TI (but not in the training context) relative to SI females, whereas 5E male rats froze less in the training context (but not during the TI) relative to SI males (Fig. 1). That said, the sex effects were still modest—both male and female 5E rats demonstrated a clear trend toward reduced freezing during the TI and in the training context relative to same-sex controls.

DuPont and colleagues (2014) found significant reductions in 5E rats' freezing behavior to the tone CS following TFC with a 15- and 30-second TI, but not a 5-second TI. As previously discussed, the mPFC seems to be required for TFC across all TIs, whereas the DH is only engaged when the TI exceeds ~10-seconds (Chowdhury et al., 2005). Such results suggest the current TFC deficit seen in 5E rats depends, principally, on DH dysfunction. To better relate putative deficits in DH function to freezing behavior, we broke down the 45-second ISI (15-second CS + 30-second TI) into nine 5-second bins. When freezing was averaged across all 5 CS-alone test trials, both SI and 5E rats showed increased freezing as the time of the expected US approached (Fig. 3). Despite the 5E rats freezing less overall, both treatment groups demonstrated a similar pattern of freezing behavior across the 45-second ISI. Results support our hypothesis that the memory retrieved by 5E rats at the time of testing is weaker (i.e., less consolidated) than the memory retrieved by SI rats (Goodfellow and Lindquist, 2014).

TFC acquisition relies on GluN2B-containing NMDARs in both the mPFC (prelimbic cortex) and DH. Pretraining administration of a GluN2B-specific antagonist (Ro25-6981) into either region selectively blocks TFC acquisition, while delay fear conditioning (in which the CS and US overlap and coterminate) and context fear conditioning are unaffected (Gao et al., 2010; Gilmartin et al., 2013). DuPont and colleagues (2014) also reported significant reductions in DH phosphorylated ERK1/2 in 5E rats, which depends on the activation of GluN2B-containing NMDARs (Krapivinsky

et al., 2003). NMDAR-gated calcium flux stimulates the translocation and binding of  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II to the synaptic GluN2B c-terminus domain, which leads to the activation of plasticity-related signaling molecules essential to LTP maintenance (Lisman and Raghavachari, 2015). Lower synaptic GluN2B expression in 5E rats is proposed to hinder LTP maintenance following TFC, disrupting long-term memory consolidation of the newly learned information.

### *NMDA Receptors*

Western blot results based on WCLs revealed a significant elevation in GluN2A subunits relative to GluN2B in the DH, but not VH or mPFC, of adult 5E rats (Fig. 4D), which has the potential to limit or perturb NMDAR-gated calcium flux and synaptic plasticity in 5E rats (Shouval et al., 2002). NMDAR subunit expression is known to be altered as a function of development (Monyer et al., 1994), which may explain prior studies that failed to find lasting differences in NMDAR subunit expression following perinatal EtOH exposure. In Nixon and colleagues (2002), for example, the effects of PD 4 to 9 EtOH exposure on cortical and hippocampal NMDAR subunits were assessed on PD 10, 14, and 21. Results from EtOH-exposed rats revealed a selective increase in cortical GluN2A expression on PD 21 only. Zink and colleagues (2011) argue that early-life EtOH exposure may induce greater (perhaps cumulative) changes in GluN2A and GluN2B gene expression when measured in adults versus neonates. Current results suggest a similar developmental trajectory for membrane-bound GluN2A and GluN2B protein expression.

EtOH-induced changes in the estrous cycle could also contribute to observed changes in NMDAR subunit expression and TFC performance. Proestrus is correlated with increases in dendritic spine density and synapse number (Gould et al., 1990; Woolley and McEwen, 1992), as well as a decrease in the LTP induction threshold (Cordoba Montoya and Carrer, 1997). No differences were seen between SI and 5E female rats in day-to-day cycling—however, we cannot rule out potential treatment group differences in ovarian hormonal levels. Estradiol has been previously shown, for instance, to increase GluN1 translation in CA1 neurons of ovariectomized rats (Gazzaley et al., 1996) and to increase both spine numbers and PSD-95 expression in the DH of ovariectomized mice (Li et al., 2004). Given that male and female 5E rats were both impaired in TFC and showed no differences in NMDAR subunit expression, we suggest the estrous cycle and ovarian secretions are only minimally affected, if at all, by postnatal EtOH exposure.

Results based on DH membrane fractions found significant treatment group differences in synaptic GluN2B and PSD-95 expression only (Fig. 5A). Adult mice exposed to EtOH before birth also had significant reductions in GluN2B (though not PSD-95) in SMFs (Samudio-Ruiz et al., 2010). In contrast, Kervern and colleagues (2015) reported an up-

regulation in synaptic GluN2B protein expression following combined pre- and postnatal EtOH exposure, when measured in young adult rats. The reason for the discrepant results is unclear—the extended period of EtOH exposure in Kervern and colleagues (2015), and their focus on area CA1, likely contribute to the different levels of protein expression seen between studies. While only GluN2B and PSD-95 were significantly reduced in 5E rats, all synaptic NMDAR subunits showed a general trend toward reduced expression, relative to SI rats. Potentially contributing to these effects, postnatal EtOH exposure is known to induce apoptosis, albeit cell loss in adult animals is typically restricted to area CA1, while areas CA3 and the dentate gyrus seem more resistant to EtOH's toxic effects (Bonthius and West, 1991; Tran and Kelly, 2003). Moreover, perinatal EtOH exposure has also been reported, in PD 70 rats, to significantly reduce the density of synapses within the hippocampus (Kuge et al., 1993). Notwithstanding the widespread consequences of early-life EtOH exposure on hippocampal neuronal and synaptic development, the significant (and selective) reduction in synaptic GluN2B is proposed to underlie, at least in part, the TFC deficits observed in 5E rats.

NMDARs are localized within synaptic and extrasynaptic compartments, which governs the receptors coupling to specific intracellular signaling pathways (Hardingham, 2006). WCL immunoblot results revealed no significant treatment group differences for any NMDAR subunit suggesting intracellular stores are not significantly altered by postnatal EtOH administration, although NMDAR trafficking to and from the synapse could be disrupted (Samudio-Ruiz et al., 2010). Extrasynaptic NMDARs are principally activated under conditions of high glutamate release, which induces opposing effects to synaptic NMDAR activation (Ivanov et al., 2006). As discussed above, synaptic NMDARs play a well-documented role in LTP induction and maintenance (Lisman and Raghavachari, 2015; Lu et al., 2001), and postnatal EtOH is known to inhibit LTP (Puglia and Valenzuela, 2010). Extrasynaptic NMDARs, on the other hand, are thought to dephosphorylate ERK1/2 and promote long-term depression (Ivanov et al., 2006; Liu et al., 2013). Thus, the TFC impairments seen in 5E rats may be attributable to impaired LTP and/or an increase in LTD mediated through extrasynaptic NMDARs, whose expression was less affected by postnatal EtOH than synaptic NMDARs. It is not clear, however, to what degree extrasynaptic NMDARs are activated in adult 5E rats—to our knowledge, hippocampal glutamate release has not been measured in older animals following perinatal EtOH exposure. Acutely, early-life EtOH exposure is reported to rapidly suppress glutamatergic transmission (e.g., Mameli et al., 2005). Additional research is required to better characterize how learning-dependent synaptic plasticity is altered in EtOH-exposed rodents, based on NMDAR compartmental localization and the post translational modifications (e.g., phosphorylation) that occur downstream of NMDAR activation.

## CONCLUSIONS AND FUTURE DIRECTIONS

Current results extend our prior research and confirm the disruptive effects of postnatal EtOH exposure on forebrain-dependent cognitive function in male and female adult subjects. Following TFC, the 5E rats froze significantly less during the tone CS and following its offset, due, we suggest, to EtOH-induced alterations in hippocampal NMDAR subunit composition and function. The finding that synaptic GluN2B expression, in particular, is significantly reduced in 5E rats is proposed to induce persistent deficits in LTP maintenance and long-term memory consolidation. Results are anticipated to further research aimed at developing novel pharmacological treatments targeted at glutamatergic signaling and plasticity, with the goal of improving cognitive function in FASD rodent models and, ultimately, children and adults exposed to gestational alcohol.

## ACKNOWLEDGMENTS

The authors thank Jennifer Coppola, Shelby Parsons, and Gabriel Boyle for their assistance with running and analyzing the behavioral experiments. We also thank Dr. Kevin Caldwell for his advice on subcellular fractionation and Dr. Kevin Foust for use of his ultracentrifuge. Funding support generously provided by ABMRF, The Foundation for Alcohol Research, grant 60034780. The authors declare no conflicts of interest.

## REFERENCES

- Bayer SA, Altman J, Russo RJ, Zhang X (1993) Timetables of neurogenesis in the human brain based on experimentally determined patterns in the rat. *Neurotoxicology* 14:83–144.
- Bonthius DJ, West JR (1991) Permanent neuronal deficits in rats exposed to alcohol during the brain growth spurt. *Teratology* 44:147–163.
- Broadwater M, Spear LP (2013) Age differences in fear retention and extinction in male Sprague-Dawley rats: effects of ethanol challenge during conditioning. *Behav Brain Res* 252:377–387.
- Chowdhury N, Quinn JJ, Fanselow MS (2005) Dorsal hippocampus involvement in trace fear conditioning with long, but not short, trace intervals in mice. *Behav Neurosci* 119:1396–1402.
- Cordoba Montoya DA, Carrer HF (1997) Estrogen facilitates induction of long term potentiation in the hippocampus of awake rats. *Brain Res* 778:430–438.
- Czerniawski J, Ree F, Chia C, Otto T (2012) Dorsal versus ventral hippocampal contributions to trace and contextual conditioning: differential effects of regionally selective NMDA receptor antagonism on acquisition and expression. *Hippocampus* 22:1528–1539.
- Davis K, Desrocher M, Moore T (2011) Fetal alcohol spectrum disorder: a review of neurodevelopmental findings and interventions. *J Dev Phys Disabil* 23:143–167.
- Dokovna LB, Jablonski SA, Stanton ME (2013) Neonatal alcohol exposure impairs contextual fear conditioning in juvenile rats by disrupting cholinergic function. *Behav Brain Res* 248:114–120.
- DuPont CM, Coppola JJ, Kaercher RM, Lindquist DH (2014) Impaired trace fear conditioning and diminished ERK1/2 phosphorylation in the dorsal hippocampus of adult rats administered alcohol as neonates. *Behav Neurosci* 128:187–198.

- Erreger K, Dravid SM, Banke TG, Wyllie DJ, Traynelis SF (2005) Subunit-specific gating controls rat NR1/NR2A and NR1/NR2B NMDA channel kinetics and synaptic signalling profiles. *J Physiol* 563:345–358.
- Gao C, Gill MB, Tronson NC, Guedea AL, Guzman YF, Huh KH, Corcoran KA, Swanson GT, Radulovic J (2010) Hippocampal NMDA receptor subunits differentially regulate fear memory formation and neuronal signal propagation. *Hippocampus* 20:1072–1082.
- Gazzaley AH, Weiland NG, McEwen BS, Morrison JH (1996) Differential regulation of NMDAR1 mRNA and protein by estradiol in the rat hippocampus. *J Neurosci* 16:6830–6838.
- Gilmartin MR, Helmstetter FJ (2010) Trace and contextual fear conditioning require neural activity and NMDA receptor-dependent transmission in the medial prefrontal cortex. *Learn Mem* 17:289–296.
- Gilmartin MR, Kwapis JL, Helmstetter FJ (2013) NR2A- and NR2B-containing NMDA receptors in the prelimbic medial prefrontal cortex differentially mediate trace, delay, and contextual fear conditioning. *Learn Mem* 20:290–294.
- Goebel-Goody SM, Davies KD, Alvestad Linger RM, Freund RK, Browning MD (2009) Phospho-regulation of synaptic and extrasynaptic N-methyl-D-aspartate receptors in adult hippocampal slices. *Neuroscience* 158:1446–1459.
- Goodfellow MJ, Lindquist DH (2014) Significant long-term, but not short-term, hippocampal-dependent memory impairment in adult rats exposed to alcohol in early postnatal life. *Dev Psychobiol* 56:1316–1326.
- Gould E, Woolley CS, Frankfurt M, McEwen BS (1990) Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood. *J Neurosci* 10:1286–1291.
- Hamilton GF, Whitcher LT, Klintsova AY (2010) Postnatal binge-like alcohol exposure decreases dendritic complexity while increasing the density of mature spines in mPFC layer II/III pyramidal neurons. *Synapse* 64:127–135.
- Hardingham GE (2006) 2B synaptic or extrasynaptic determines signalling from the NMDA receptor. *J Physiol* 572:614–615.
- Hardingham GE, Bading H (2010) Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders. *Nat Rev Neurosci* 11:682–696.
- Hunt PS, Jacobson SE, Torok EJ (2009) Deficits in trace fear conditioning in a rat model of fetal alcohol exposure: dose-response and timing effects. *Alcohol* 43:465–474.
- Ivanov A, Pellegrino C, Rama S, Dumalska I, Salyha Y, Ben-Ari Y, Medina I (2006) Opposing role of synaptic and extrasynaptic NMDA receptors in regulation of the extracellular signal-regulated kinases (ERK) activity in cultured rat hippocampal neurons. *J Physiol* 572:789–798.
- Johnson TB, Goodlett CR (2002) Selective and enduring deficits in spatial learning after limited neonatal binge alcohol exposure in male rats. *Alcohol Clin Exp Res* 26:83–93.
- Kervern M, Silvestre de Ferron B, Alaux-Cantin S, Fedorenko O, Antol J, Naassila M, Pierrefiche O (2015) Aberrant NMDA-dependent LTD after perinatal ethanol exposure in young adult rat hippocampus. *Hippocampus* 25:912–923.
- Klintsova AY, Helfer JL, Calizo LH, Dong WK, Goodlett CR, Greenough WT (2007) Persistent impairment of hippocampal neurogenesis in young adult rats following early postnatal alcohol exposure. *Alcohol Clin Exp Res* 31:2073–2082.
- Krapivinsky G, Krapivinsky L, Manasian Y, Ivanov A, Tyzio R, Pellegrino C, Ben-Ari Y, Clapham DE, Medina I (2003) The NMDA receptor is coupled to the ERK pathway by a direct interaction between NR2B and RasGRF1. *Neuron* 40:775–784.
- Kuge T, Asayama T, Kakuta S, Murakami K, Ishikawa Y, Kuroda M, Imai T, Seki K, Omoto M, Kishi K (1993) Effect of ethanol on the development and maturation of synapses in the rat hippocampus: a quantitative electron-microscopic study. *Environ Res* 62:99–105.
- Li C, Brake WG, Romeo RD, Dunlop JC, Gordon M, Buzescu R, Magarinos AM, Allen PB, Greengard P, Luine V, McEwen BS. (2004) Estrogen alters hippocampal dendritic spine shape and enhances synaptic protein immunoreactivity and spatial memory in female mice. *Proc Natl Acad Sci USA* 101:2185–2190.
- Lisman J, Raghavachari S (2015) Biochemical principles underlying the stable maintenance of LTP by the CaMKII/NMDAR complex. *Brain Res* 1621:51–61.
- Liu DD, Yang Q, Li ST (2013) Activation of extrasynaptic NMDA receptors induces LTD in rat hippocampal CA1 neurons. *Brain Res Bull* 93:10–16.
- Lu W, Man H, Ju W, Trimble WS, MacDonald JF, Wang YT (2001) Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. *Neuron* 29:243–254.
- Mameli M, Zamudio PA, Carta M, Valenzuela CF (2005) Developmentally regulated actions of alcohol on hippocampal glutamatergic transmission. *J Neurosci* 25:8027–8036.
- Mattson SN, Crocker N, Nguyen TT (2011) Fetal alcohol spectrum disorders: neuropsychological and behavioral features. *Neuropsychol Rev* 21:81–101.
- Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12:529–540.
- Nixon K, Hughes PD, Amsel A, Leslie SW (2002) NMDA receptor subunit expression following early postnatal exposure to ethanol. *Dev Brain Res* 139:295–299.
- Nixon K, Hughes PD, Amsel A, Leslie SW (2004) NMDA receptor subunit expression after combined prenatal and postnatal exposure to ethanol. *Alcohol Clin Exp Res* 28:105–112.
- Puglia MP, Valenzuela CF (2010) Repeated third trimester-equivalent ethanol exposure inhibits long-term potentiation in the hippocampal CA1 region of neonatal rats. *Alcohol* 44:283–290.
- Quinn JJ, Loya F, Ma QD, Fanselow MS (2005) Dorsal hippocampus NMDA receptors differentially mediate trace and contextual fear conditioning. *Hippocampus* 15:665–674.
- Raybuck JD, Lattal KM (2014) Bridging the interval: theory and neurobiology of trace conditioning. *Behav Processes* 101:103–111.
- Samudio-Ruiz SL, Allan AM, Sheema S, Caldwell KK (2010) Hippocampal N-methyl-D-aspartate receptor subunit expression profiles in a mouse model of prenatal alcohol exposure. *Alcohol Clin Exp Res* 34:342–353.
- Schreiber WB, Hunt PS (2013) Deficits in trace fear conditioning induced by neonatal alcohol persist into adulthood in female rats. *Dev Psychobiol* 55:352–361.
- Shors TJ (1998) Stress and sex effects on associative learning: for better or for worse. *Neuroscientist* 4:353–365.
- Shouval HZ, Bear MF, Cooper LN (2002) A unified model of NMDA receptor-dependent bidirectional synaptic plasticity. *Proc Natl Acad Sci USA* 99:10831–10836.
- Streissguth AP (2007) Offspring effects of prenatal alcohol exposure from birth to 25 years: the Seattle Prospective Longitudinal Study. *J Clin Psychol Med Settings* 14:81–101.
- Tran TD, Kelly SJ (2003) Critical periods for ethanol-induced cell loss in the hippocampal formation. *Neurotoxicol Teratol* 25:519–528.
- Woolley CS, McEwen BS (1992) Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. *J Neurosci* 12:2549–2554.
- Woolley CS, Schwartzkroin PA (1998) Hormonal effects on the brain. *Epilepsia* 39:2–8.
- Yashiro K, Philpot B (2008) Regulation of NMDA receptor subunit expression and its implications for LTD, LTP, and metaplasticity. *Neuropharmacology* 55:1081–1094.
- Zink M, Ferbert T, Frank ST, Seufert P, Gebicke-Haerter PJ, Spanagel R (2011) Perinatal exposure to alcohol disturbs spatial learning and glutamate transmission-related gene expression in the adult hippocampus. *Eur J Neurosci* 34:457–468.