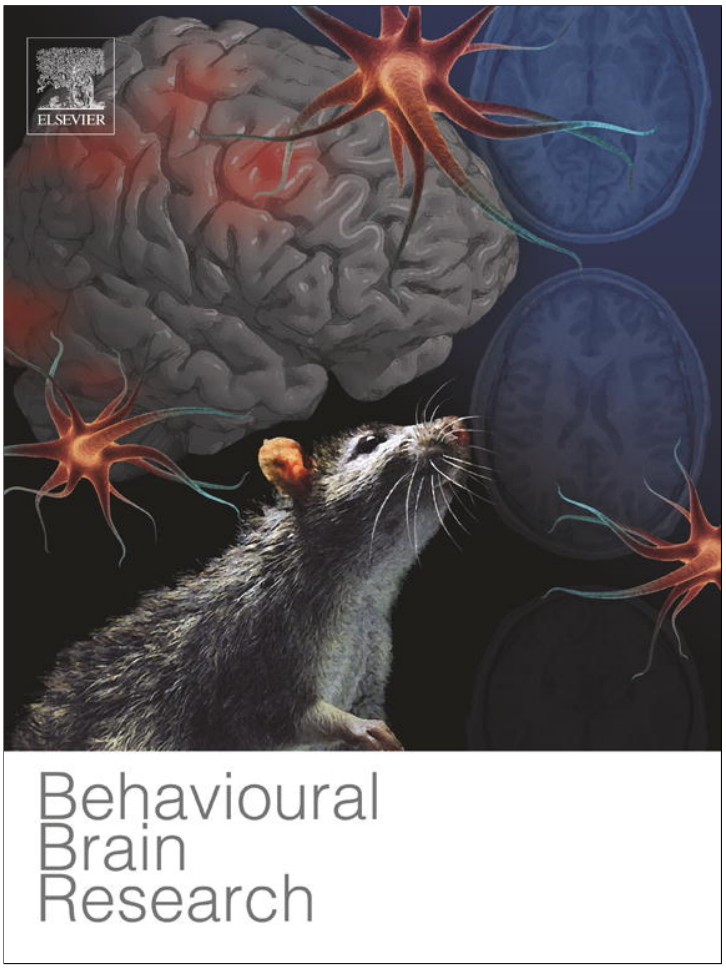

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Research report

Hippocampal-dependent Pavlovian conditioning in adult rats exposed to binge-like doses of ethanol as neonates

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HIGHLIGHTS

- Early ethanol exposure damages the brain, including the cerebellum and hippocampus.
- Hippocampal function assessed via trace eyeblink conditioning.
- Trace eyeblink conditioning was unimpaired by early ethanol exposure.
- Hippocampal function assessed via contextual gating of eyeblink conditioned response.
- Contextual control of conditioned response was unimpaired by early ethanol exposure.
- Results best explained by ethanol-induced cerebellar damage.

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ABSTRACT

Binge-like postnatal ethanol exposure produces significant damage throughout the brain in rats, including the cerebellum and hippocampus. In the current study, cue- and context-mediated Pavlovian conditioning were assessed in adult rats exposed to moderately low (3E; 3 g/kg/day) or high (5E; 5 g/kg/day) doses of ethanol across postnatal days 4–9. Ethanol-exposed and control groups were presented with 8 sessions of trace eyeblink conditioning followed by another 8 sessions of delay eyeblink conditioning, with an altered context presented over the last two sessions. Both forms of conditioning rely on the brainstem and cerebellum, while the more difficult trace conditioning also requires the hippocampus. The hippocampus is also needed to gate or modulate expression of the eyeblink conditioned response (CR) based on contextual cues. Results indicate that the ethanol-exposed rats were not significantly impaired in trace EBC relative to control subjects. In terms of CR topography, peak amplitude was significantly reduced by both doses of alcohol, whereas onset latency but not peak latency was significantly lengthened in the 5E rats across the latter half of delay EBC in the original training context. Neither dosage resulted in significant impairment in the contextual gating of the behavioral response, as revealed by similar decreases in CR production across all four treatment groups following introduction of the novel context. Results suggest ethanol-induced brainstem-cerebellar damage can account for the present results, independent of the putative disruption in hippocampal development and function proposed to occur following postnatal ethanol exposure.

1. Introduction

First defined four decades ago, fetal alcohol syndrome (FAS) represents a pattern of growth retardation, facial dysmorphology, and cognitive impairment in infants born to women who drink while pregnant [1]. The teratogenic effects of alcohol are now recognized to result in a spectrum of physical and mental dysfunction, collectively termed fetal alcohol spectrum disorders (FASD) [2]. FASD is a recognized major public health problem [3], and one of the leading preventable causes of mental retardation [4]. The most severely afflicted children along the FASD continuum are diagnosed with FAS, which has a birth prevalence of 2–7 per 1000 births in the United States [4]. At the opposite end of the spectrum, alcohol-related neurodevelopmental disorder (ARND) refers to children that express the mental but not physical abnormalities associated with early alcohol exposure [5]. In fact, the estimated number of FASD children may be up to 10 times higher than reported due to difficulties in diagnosing ARND children in the absence of physical dysmorphology [6,7]. Across the five established FASD categories, afflicted children may exhibit pervasive deficits in learning, memory, attention, and executive function [8–10].

A FASD animal model, in which rodents are treated with pre- and/or postnatal alcohol, has been successfully used over the last several decades to investigate the neurotoxic effects of alcohol

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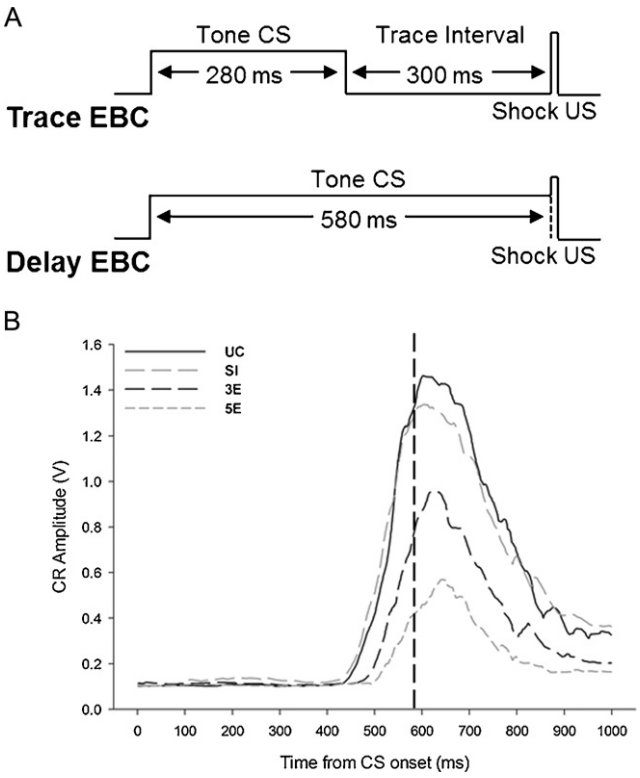

Fig. 1. Schematic illustrating the CS and US temporal parameters for trace and delay EBC (A). Trace EBC consisted of a 280 ms tone CS followed 300 ms later by a 10 ms periorbital shock US. Delay EBC consisted of a 590 ms tone CS and a co-terminating 10 ms shock US. The training ISI for both paradigms was 580 ms. (B) Integrated and averaged electromyogram (EMG) eyeblink traces for one subject in each of the four neonatal treatment groups during CS-alone probe trials on D05 and D06, the fifth and sixth sessions of delay EBC in the original context. The dashed line indicates when the US would have occurred on paired trials.

on the developing brain [11–13]. The extent of ethanol-mediated dysfunction depends on the interaction of two factors: the developmental timing of ethanol exposure and the resulting blood alcohol concentration (BAC). Importantly, the rat brain across the first 10 days of life—during the so-called "brain growth spurt" [14]—is roughly equivalent to the human brain during the third trimester [15,16]. Binge-like ethanol exposure across postnatal days (PD) 4–9 produces structural and functional abnormalities throughout the brain, including the cerebellum and hippocampus [17–20], with the most significant impairment seen with peak BACs above 300 mg/dl. Determining which behavioral paradigms are sufficiently sensitive to detect ethanol-induced dysfunction remains an important goal as researchers seek to uncover and understand the cellular and molecular anomalies associated with perinatal alcohol exposure.

Ethanol-exposed rats have been tested in a variety of spatial and configural learning tasks in order to study hippocampal-dependent learning. Postnatal ethanol exposure results in deficient place learning, for example, with males significantly impaired relative to females [21,22], indicating the neurodevelopmental consequences of ethanol may be sex dependent. The current study assessed the impact of postnatal (PD4-9) ethanol exposure in male and female rats on two forms of Pavlovian conditioning, delay and trace eyeblink conditioning (EBC; see Fig. 1A). Delay EBC, the simpler of the two, entails the repeated presentation of a neutral light or tone conditioned stimulus (CS) paired with a mildly aversive corneal air-puff or periorbital shock unconditioned stimulus (US). The CS precedes presentation of the US, though both stimuli typically overlap and co-terminate. After repeated CS-US pairings, subjects learn to produce an eyeblink conditioned response (CR) to the CS alone. In well-trained subjects, the eyeblink CR is generated such that the eyelid (or nictitating membrane) is fully extended at the time of US onset. Acquisition of delay EBC is critically reliant on the brainstem and cerebellum [reviewed in [23]]. Interestingly, while the hippocampus is not necessary for the acquisition of delay EBC [24,25], the dorsal hippocampus is rapidly engaged during training, with an almost immediate increase in pyramidal cell activity in response to the US and learning-related activity tied to the CS that models the emergence, amplitude, and time course of the conditioned blink [26,27].

In trace EBC the CS is turned on and off prior to presentation of the US, forming a stimulus-free gap of time between the two stimuli. In addition to the brainstem-cerebellar circuit, if the trace interval separating CS offset from US onset is long enough—at least 250 ms for rodents [28,29] or 500 ms for rabbits [30]—the hippocampus is also required for successful learning [31]. Lesions of the hippocampus impair acquisition of the eyeblink CR and disrupt the organism's ability to properly time the response in relation to the US [30,32]. The hippocampus is critical for successful trace but not delay EBC, even when the CS-US interstimulus interval (ISI; time between CS onset and US onset) is held constant across both learning paradigms [33,34]. Thus, the lack of temporal contiguity between the CS and US and not the CS-US interval is thought to be the critical factor that necessitates hippocampal involvement [35].

While many functions have been ascribed to the hippocampus and its role in trace EBC, one possibility is that the hippocampus is specifically needed when the CS and US are temporally separated in time. Early in conditioning, as the rodent explores its environment, spatial and sensory cues are thought to be combined within the hippocampus into a unitary contextual representation [36,37], which in turn forms an association with the aversive US [38,39]. As conditioning continues, the hippocampus is proposed to discriminate the "background" contextual cues from the "foreground" sensory cue (i.e., tone CS) that is predictive of the US, facilitating their association [40]. The dissociation between context-US and CS-US associative learning was observed in rabbits trained to criterion in delay EBC, which showed reduced levels of conditioned responding following the introduction of a novel context [41]. Specifically, unoperated control rabbits and those with pre-training lesions of the neocortex showed a decrease in CR frequency in the switched context, which differed along olfactory, visual, and tactile dimensions, whereas rabbits with pre-training lesions of the hippocampus continued to show robust responding. The results support the idea that the hippocampus provides spatial-contextual information that, once associated with the emotional attributes of the US, can modulate behavioral responding to the CS [42,43]. This information might not be necessary for delay conditioning but it might be essential in trace conditioning.

The goal of the current study was to ascertain whether early developmental ethanol exposure impairs hippocampal function in adult rats as assessed by eyeblink CR production—during acquisition of trace EBC and later, in well-trained subjects, in the presence of a novel context. Rats were exposed to moderately low (3 g/kg/day) or high (5 g/kg/day) doses of ethanol across PD4 to PD9. Beginning on about PD70, the two ethanol groups and two control groups were presented with 8 sessions of trace EBC (T01-T08) followed by another 6 sessions of delay EBC (D01-D06) in the same context. Contextual modulation of the behavioral response was examined across a further two delay EBC sessions (C01–C02) by altering the context, including transport cues and sensory cues within the conditioning chamber. The training ISI was held constant at 580 ms for both trace and delay EBC. The subject's sex was examined as a function of CR production and topography. We hypothesized that postnatal ethanol exposure would induce a dose-dependent disruption in hippocampal function, relative to control subjects, such that the ethanol-exposed rats would exhibit retarded

trace EBC acquisition and show less consequence of the context shift—i.e., they would exhibit less drop in conditioned responding following introduction of the novel context.

2. Materials and methods

2.1. Subjects

Long-Evans male and female breeders were housed in the vivarium at the University of Kansas. The animal colony was maintained on a 12 h light/dark cycle (lights on at 0700 hrs) with *ad-lib* access to food and water. One male and one female were housed together for one week. Beginning three weeks following their initial placement, female rats were checked morning and evening for parturition. Following birth, litters were culled to 10 pups, retaining equal numbers of males and females when possible. On PD3 a small amount of nontoxic black ink was subcutaneously injected into one or more paws for identification purposes. Rats were weaned on PD21 and same sex housed through PD45, then individually housed. Forty-one experimentally naïve Long-Evans rats (female = 19; male = 22) were used in the present experiment. Surgical and behavioral procedures were conducted during the light phase. All procedures, including surgery and postoperative care, were in strict compliance with the University of Kansas animal care guidelines, and all necessary measures were taken to minimize pain and discomfort.

2.2. Neonatal treatment

Beginning on PD4, rat pups were removed from the dam, weighed, and placed in a small plastic container atop a heating pad to minimize heat loss. Pups were pseudo-randomly assigned to one of four treatment groups: unintubated control (UC), sham-intubated (SI), or two ethanol groups: 3 g/kg/day (3E) and 5 g/kg/day (5E). Pups in the ethanol-exposed treatment groups underwent intragastric intubations three times daily beginning at 0900 h from PD4 to PD9, each intubation separated by 2 h. The rat pups were intubated two times with an ethanol/milk solution and a third, milk alone, intubation. The first two intubations consisted of 6.80% (3E) or 11.33% (5E) ethanol in a nutritive milk formula (vol/vol), based on the recipe of West et al. [44]. PE10 tubing, lubricated with corn oil, was 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, though no formula was ever given. UC pups were removed from the dam three times daily but never intubated. Once grown, the SI rats were the critical control group for assessing ethanol-induced dysfunction.

2.3. Blood alcohol concentration (BAC)

For SI, 3E, and 5E rat pups, 20 μl of blood was collected in heparinized capillary tubes from a tail clip immediately before the final intubation on PD 4. Blood samples from the SI rats were discarded. Blood samples for the 3E and 5E 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. BAC was calculated by comparing an experimental sample to a known alcohol standard used in the calibration procedure.

2.4. Surgery

All surgical procedures were performed under aseptic conditions. Adult rats, approximately 65 days of age, were anesthetized using intraperitoneal (I.P.) injections of an anesthetic cocktail (2.2 ml/kg), consisting of physiological saline (9.0 mg/kg), ketamine (74.0 mg/kg), xylazine (3.7 mg/kg), and acepromazine (0.74 mg/kg). Ketamine boosters were administered as required to maintain anesthesia. Each subject was surgically prepared with a pair of differential electromyographic (EMG) wires and a bipolar periocular stimulator (Plastics One, Roanoke, VA). EMG activity was recorded in the orbicularis oculi (OO) muscle surrounding the eye by passing two ultrathin (0.003 in.) Teflon-coated stainless steel wires subdermally beneath the anterior portion of the upper eyelid. Gold-coated stainless steel wires were implanted in the dorso-caudal portion of the OO muscle for delivery of the periorbital electrical shock US. A ground wire was connected to one of three stainless steel skull screws. The two EMG wires and ground wire all terminated in gold pins inside a 6-pin plastic connector (M363; Plastics One Inc. Roanoke, Va). The headstage and bipolar stimulating electrodes were fixed in dental cement. The wound was salved with antibiotic ointment (Povidone), and the animals were given at least 7 days to recover. All rats were handled for two days immediately prior to the start of training.

2.5. Behavioral training and testing

Rats were placed in standard operant boxes (Coulburn Instruments, Allentown, PA), contained within 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. Electrode leads attached to each subject's head swiveled freely on a 10-channel commutator connected to a counter-balanced pivoting arm, allowing subjects to move freely about in the conditioning chamber.

All subjects received one session of adaptation (45 min), with the commutator attached but no stimuli presented. The first of 16 conditioning sessions began the next day. Trace EBC was presented across the first eight training sessions (T01–T08), followed by another six sessions of delay EBC (D01–D06) in the same context, and, finally, two sessions of delay EBC in an altered context (C01–C02). For trace EBC, the CS was a 280 ms, 2.8 kHz, 85 dB SPL tone delivered from an overhead speaker. The trace interval was 300 ms followed by a 10 ms, 4.0 mA, 60 Hz, constant-current square wave, periocular stimulation US. For delay EBC, the same CS, now 590 ms in duration, co-terminated with the 10 ms periocular shock US (Fig. 1A). Both trace and delay EBC consisted of 10 blocks of 10 trials: 9 CS-US paired trials and 1 CS-alone trial. The CS-US interstimulus interval (ISI) was 580 ms for all conditioning sessions and the intertrial interval (ITI) was 25 ± 5 s. Data from one SI, two 3E, and one 5E subject were excluded due to poor EMG quality.

Two distinct training contexts were used. Across the initial 15 sessions (adaptation and 14 training sessions) two animals were removed from their home cage and individually carried in a small plastic cage ($11\ 3/4 \times 8 \times 5$ in.) covered with a metal grate, one on top of the other, to a room adjacent to the running room. Inside the running room, an overhead fluorescent light was turned on. A fan inside each conditioning chamber (providing 65 dB background masking noise) was turned on and the inside was wiped down with a vinegar/water (1:5) solution. Each rat was removed from its cage and attached to the commutator tether while standing adjacent to the appropriate conditioning chamber. The conditioning session commenced approximately 2 min following placement of the second rat.

For the final two delay EBC sessions (C01–C02), two rats were transported in their individual home cage placed atop a metal cart covered with a towel. The animals were again placed in the room adjacent to the running room, but in a different location. Inside the running room, the overhead fluorescent light was turned off and a single red bulb was turned on. The fan inside each chamber was turned off and a 15 W fluorescent bulb, located at the top of the sound attenuating chamber, was turned on. A Plexiglas sheet was placed over the grid bars of each chamber and the inside was wiped down with Windex®. The commutator tether was removed from the conditioning chamber and attached to the subject's headstage in the adjacent room, after which the animal was brought into the running room and the tether reattached. As before, conditioning commenced approximately 2 min following placement of the second rat.

2.6. EMG data analysis

Throughout each EBC session, eyelid EMG activity was amplified (10000X), band-pass filtered (300–1000 Hz), digitized (500 Hz), rectified, smoothed (10 ms time constant), time-shifted (10 ms, to compensate for smoothing), and stored for offline analysis using the Spike 2 waveform analysis system (CED Limited, Cambridge, England). EMG data from CS-US paired trials were used to compute the percentage of CRs produced during each session and CS-alone trials were used to compute the amplitude and timing of the averaged CR [Datamunch program; [45]]. Amplitude of the reflexive unconditioned eyeblink response (UR) was calculated based on CS-US paired trials. On each trial, EMG activity from the OO muscle was sampled for 1500 ms, divided into three periods: (i) a 350 ms pre-CS period, prior to CS onset; (ii) a 580 ms CS-US period, between CS onset and US onset; and (iii) a 570 ms post-US period, following US onset.

The averaged EMG activity during the pre-CS period was used as a baseline for classifying behavior and scoring trials. Trials in which CS-elicited EMG activity exceeded pre-CS mean EMG activity by 8 standard deviations (SDs) or more, beginning 100 ms after CS onset, were counted as CRs. Elevated EMG activity (>8 SDs) during the first 100 ms of the CS was considered an alpha response and not counted as a CR. Trials with elevated EMG activity (> 8 SDs) during the 100 ms period immediately preceding CS onset were deemed bad trials and excluded from analysis. Data were analyzed using single-factor, mixed design, and repeated measures ANOVAs, and, when appropriate, Tukey-Kramer post hoc tests. A significant post hoc effect implies $p < 0.05$.

3. Results

The mean (±SE) blood alcohol concentration was 247.1 ± 16.2 mg/dl for the 3E rats and 343.7 ± 11.0 mg/dl for the 5E rats. A two factor (Group × Sex) ANOVA yielded a significant main effect for treatment Group, $F(1, 14) = 24.35$, $p < 0.0001$, but not Sex ($p = 0.16$), indicating the averaged peak BAC was significantly higher in group 5E than group 3E.

Fig. 2 depicts the percentage of eyeblink CRs generated across 16 sessions of sequential trace-delay EBC. A mixed-design $4 \times 2 \times 16$ repeated measures ANOVA, with two between-subjects factors

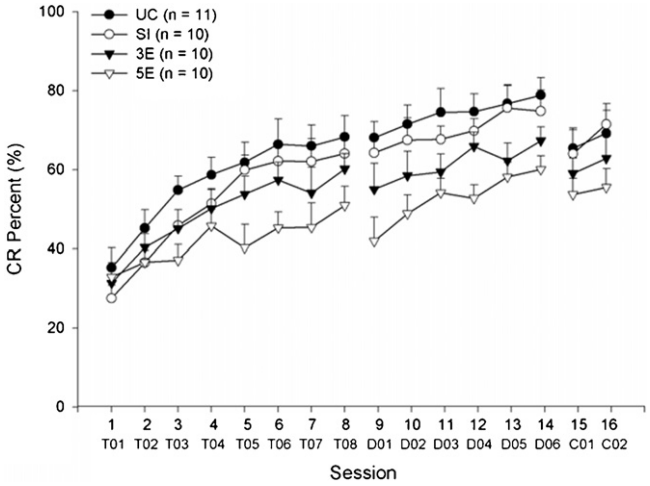

Fig. 2. Percentage conditioned responding (mean ± SE) across 16 training sessions as a function of neonatal treatment. Trace EBC was presented across the initial 8 training sessions (T01–T08) followed by another six sessions of delay EBC (D01–D06) in the same context. The final two delay EBC sessions (C01–C02) were conducted in a novel context. CR production was significantly impaired in the 5E rats compared to the UC and SI rats across all 16 training sessions.

(Group and Sex), and one within-subjects factor (Session), was applied to the entire data set. No interaction reached significance. There was a statistically significant main effect for treatment Group, $F(3, 495) = 4.75$, $p < 0.01$, but not Sex ($p = 0.42$). The main effect for Session was also significant, $F(15, 495) = 33.30$, $p < 0.001$, indicating increased responding across training. Tukey-Kramer post hoc analyses found that the UC and SI rats produced significantly more eyeblink CRs across all 16 sessions than the 5E, but not 3E, rats. One aim of the current experiment was to uncover potential group or sex differences across trace EBC specifically. Limiting the analysis to the eight trace EBC sessions (T01-08), a 4 (Group) × 2 (Sex) × 8 (Session) repeated measures ANOVA revealed a significant main effect for Session only, $F(7, 231) = 37.82$, $p < 0.001$. No other main effect or interaction reached significance, indicating the ethanol-exposed rats were not significantly impaired in trace EBC relative to control subjects. Representative electromyographic eyeblink traces are illustrated in Fig. 1B.

In order to directly compare the two tasks (trace vs. delay EBC), further analyses on CR frequency were limited to the last six trace EBC sessions (T03–T08) and the first six delay EBC sessions (D01–D06). This was done in order to minimize initial trace EBC learning, which obscures treatment group effects, and the effect of the context shift, which affects delay more than trace EBC. Independent Bonferroni-corrected ($p < 0.025$) single factor ANOVAs over the initial two trace EBC sessions confirmed that no treatment group differences exist, T01 ($p = 0.53$) and T02 ($p = 0.35$). Consequently, CR percentage was analyzed using a mixed-design 2 (Task) × 4 (Group) × 2 (Sex) × 6 (Session) repeated measures ANOVA. All interactions were non-significant. Main effects were statistically significant for Task, $F(1, 330) = 10.49$, $p < 0.01$, treatment Group, $F(3, 330) = 9.08$, $p < 0.001$, and Session, $F(5, 330) = 25.74$, $p < 0.001$, but not Sex ($p = 0.36$). Post hoc analyses indicated significantly more eyeblink CRs were generated during delay than trace EBC. Significantly greater responding in the UC rats was also seen relative to both 3E and 5E rats, whereas the SI rats produced more CRs than the 5E rats only. The subject's sex did not affect the frequency of emitted CRs. The data suggests that all treatment groups improved their performance across the multiple training sessions in both tasks, with greater levels of responding during delay EBC than trace EBC. Only group 5E was significantly impaired relative to group SI, however, indicating impaired learning beyond that induced by the intubation procedure.

Topography of the eyeblink CR was examined next across the same 12 sessions described above (trace EBC: T03–T08; delay EBC: D01–D06) on each of the ten CS-alone trials per session (Fig. 3). CRs were examined from CS onset through the end of the trial (1150 ms). CR onset latencies, peak latencies, and peak amplitudes were grouped into 3 session bins (T03–T05, T06–T08, D01–D03, and D04–D06) in order to increase power and the detection of potential treatment group or sex differences (via two-factor ANOVAs). On some CS-alone trials (less than 5% of the total) no CR was recorded, in which case the cell was left blank and not used in the statistical analyses.

There were no main or interactive effects of sex on any outcome measure. Consequently, Fig. 3 illustrates CR topography as a function of treatment group only. For CR onset latency (Fig. 3A), a significant treatment group main effect was seen for the last three delay and trace EBC sessions only—T06–T08, $F(3, 114) = 3.46$, $p < 0.05$, and D04–D06, $F(3, 115) = 6.52$, $p < 0.001$. The averaged onset latencies were significantly longer in the 5E rats compared to the UC rats for T06–T08 and significantly longer in the 3E and 5E rats compared to the UC rats for D04–D06. For the latter sessions, the onset latencies in the 5E rats were also significantly longer than the latencies in SI rats. There were no statistically significant between-group differences in CR peak latency across any binned sessions (Fig. 3B), though latencies in all four groups generally increased following the switch from trace to delay EBC. CR peak amplitude varied significantly across treatment groups for T03–T05, $F(3, 111) = 42.46$, $p < 0.0001$, T06–T08, $F(3, 114) = 37.37$, $p < 0.0001$, D01–D03, $F(3, 113) = 25.88$, $p < 0.0001$, and D04–D06, $F(3, 115) = 17.51$, $p < 0.0001$. Post hoc pair-wise comparisons indicated that the 5E rats produced blinks with lower peak amplitudes than the UC, SI, and 3E rats across all binned sessions, similar to other reports [46–50]. The 3E rats also produced smaller amplitude blinks relative to the UC rats across all four binned sessions and relative to the SI rats across all binned sessions except D01–D03 (Fig. 3C).

UR peak amplitude in response to the periorbital shock was examined next to determine whether reflexive responding to the US differed among the four neonatal treatment groups (Fig. 3D). The UR data, based on the 90 CS-US paired trials per session, was analyzed in the same manner described above for CR topography. Neither sex nor neonatal treatment significantly affected UR magnitude across any of the binned training sessions. Reflexive responding to the periorbital shock must be interpreted cautiously, however, when preceded by the tone CS, as the UR is known to be influenced by CS-dependent CRs and to be modified as a function of learning, increasing in amplitude across training [51]. Nonetheless, the results provisionally indicate, in line with previous research, that the ethanol-exposed rats were not deficient in processing or responding to the US during classical eyeblink conditioning [13,49,50,52].

Finally, the percentage of eyeblink CRs was analyzed following presentation of novel transport and contextual cues. Sex was not considered in this analysis or those below, based on the null results above. Bonferroni-corrected single factor (Group) ANOVAs were applied to the final two delay EBC sessions. Neonatal treatment did not significantly affect CR production following the context switch on sessions C01 ($p = 0.33$) or C02 ($p = 0.17$). Thus, while treatment group differences were not observed following the context shift, the critical analysis involves changes in CR frequency across D06 to C01. A single factor ANOVA applied to the CR difference scores, in which the averaged percent CR for each subject on session D06 (last session of delay EBC in original context) was subtracted from the averaged percent CR on session C01 (first session of delay EBC

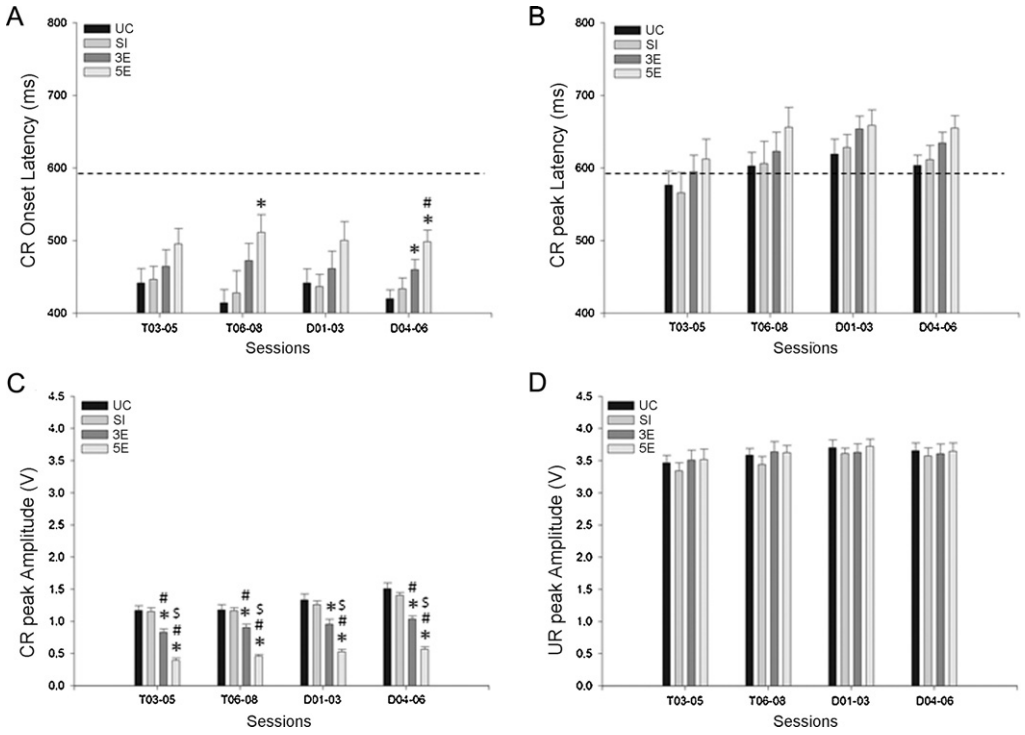

Fig. 3. Conditioned and unconditioned response topography (mean ± SE) for each of the four treatment groups across four blocks of binned training sessions: T03–T05, T06–T08, D01–D03, and D04–D06. (A) CR onset latencies. The 5E rats produced conditioned blinks with significantly longer onset latencies relative to the UC rats for T06–T08 and significantly longer onsets relative to UC and SI rats for D04–D06. For the latter sessions, onset latencies in the 3E rats were also significantly lengthened relative to UC rats. (B) CR peak latencies. No treatment group differences were observed. The dashed line indicates the time of US onset. (C) CR peak amplitudes. The 5E rats produced conditioned blinks with significantly smaller amplitudes than the UC, SI, and 3E rats across all four binned sessions. The blinks produced by the 3E rats were also significantly smaller than those produced by SI rats across all binned sessions, except D01–D03. (D) UR peak amplitudes. No treatment group differences were observed. For all figures, an asterisk indicates a significant difference relative to the UC rats, the pound sign indicates a significant difference relative to the SI rats, and the dollar sign indicates a significant difference relative to the 3E rats.

in new context), did not reveal any significant effect of neonatal treatment ($p = 0.76$).

To enhance potential context-mediated differences in CR production, analyses were restricted to the final two and initial two blocks of 10 trials (18 CS-US paired trials) on D06 and C01, respectively, similar to the procedure of Penick and Solomon [41]. Fig. 4A shows the averaged percentage of emitted CRs across the last 18 paired trials on session 14 and the first 18 paired trials on session 15. The resulting difference scores are illustrated in Fig. 4B (left side), with CR frequency on D06 subtracted from the CR frequency on C01 for each treatment group subject. A single factor ANOVA revealed no significant between-group differences in the reduction in CR frequency that followed introduction of the novel context ($p = 0.57$). The control rats clearly learned faster and generated more CRs at the conclusion of D06 (Figs. 2 and 4A), however, accounting perhaps for the larger drop in CR frequency relative to the 3E and 5E rats. In Fig. 4B (right side), the C01 – D06 difference scores for each treatment group were normalized to the percentage of CRs emitted across the last 18 paired trials on D06: UC (16.4/74.1), SI (16.0/75.0), 3E (11.0/65.5), and 5E (10.5/60.5). Again, neonatal treatment did

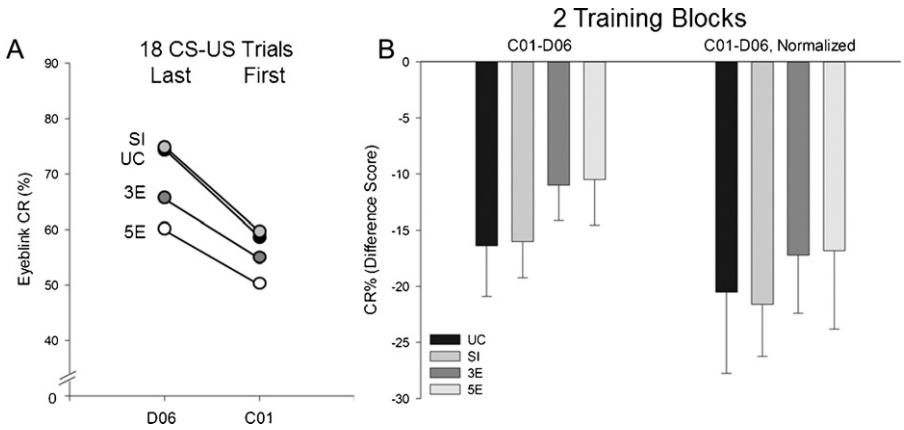

Fig. 4. Changes in conditioned responding (mean ± SE) were examined across D06 (last session of delay EBC in original context) and C01 (first session of delay EBC in novel context). (A) CR frequencies were computed based on the final 18 and initial 18 CS-US training trials on D06 and C01, respectively. All groups of rats produced fewer CRs following introduction of the novel context. (B) On the left, CR difference scores for the last 18 and first 18 CS-US trials on D06 and C01, respectively. On the right, CR difference scores were normalized to the percentage of emitted blinks on the last 18 trials on D06. See text for details.

not significantly affect the context-dependent drop in conditioned responding ($p = 0.93$)—and, in fact, the normalized decrease in CR frequency was quite comparable across the four treatment groups, provided the subjects' prior learning rate was taken into account.

4. Discussion

The current study investigated the effects of postnatal binge-like ethanol exposure, at two different dosages, on hippocampal function in young adult rats. All subjects were submitted to 8 sessions of trace EBC followed by another 8 sessions of delay EBC, with an altered context presented on the final two delay EBC sessions (Fig. 2). Across all 16 training sessions the 5E rats produced significantly fewer CRs than both the UC and, critically, the SI rats. The effect of neonatal treatment was non-significant, however, when analyses were limited to the eight trace EBC sessions. The CRs produced by the 5E rats had significantly longer onset latencies than those observed in the SI rats across the last three delay EBC sessions (D04–06) in the original conditioning context (Fig. 3A). By far, however, the biggest impairment related to neonatal ethanol exposure was on CR peak amplitude. The 5E rats produced conditioned blinks across both trace and delay EBC that were significantly smaller in amplitude than those generated by the SI rats (Fig. 3C). As did the 3E rats, except for sessions D01–D03. Early ethanol exposure did not significantly affect CR production following introduction of a novel context, with all four treatment groups exhibiting similar drops in conditioned responding (Fig. 4). In no case did the sex of the subject significantly influence CR production or topography. Contrary to our hypothesis, therefore, neither alcohol dosage significantly affected trace EBC or the contextual modulation of the eyeblink CR, suggesting ethanol-induced damage to the brainstem-cerebellar circuit can account for the present results, independent of hippocampal dysfunction.

Classical eyeblink conditioning is arguably one of the best understood forms of learning in terms of the behavioral response [53] and the underlying neurocircuitry [23]. The critical brain regions lie within the brainstem and cerebellum, areas that are highly susceptible to the neurotoxic effects of postnatal alcohol. Granule cells, which relay CS information, are reduced from approximately 20–50% [54,55], whereas inferior olive cells, which relay US information, are reduced by up to 25% [56,57]. Two regions of the cerebellum where CS-US associative plasticity is known to occur, Purkinje cells in the cortex and deep cerebellar neurons in the interpositus (IP) nucleus, are also reduced by up to 50% [11,46,58–60]. Multiple- and single-unit recordings in the dorsal lateral region of the anterior IP have revealed populations of cells that discharge just prior to CR onset, preceding and predicting the temporal form of the behavioral response in both the rabbit [61–63] and rat [64,65]. The increase in spiking actually precedes onset of the eyeblink CR by 30–60 ms, suggesting IP action potentials are responsible for "driving" the behavioral response [66]. It has been proposed that fewer plastic cells are available in the IP of ethanol-exposed rats that are capable of encoding the CS-US association, which consequently results in less drive on downstream motor nuclei responsible for CR production during delay EBC [67]. Ethanol-induced Purkinje cell loss may also contribute to the observed reductions in CR amplitude and, though less pronounced, changes in timing. Fewer plastic cells may diminish the ability of Purkinje cells to properly sculpt the behavioral response, which is proposed to occur via precisely timed inhibition and disinhibiton of excitatory IP unit activity across the CS-US interval [23].

The hippocampus also clearly interacts with the cerebellum across both delay and trace EBC. Hippocampal pyramidal cell activity models the amplitude and time course of the behavioral CR, an effect blocked by lesions of the cerebellar nuclei [68,69], whereas hippocampal lesions prevent or abolish trace eyeblink CRs [30,70]. The hippocampus is also sensitive to ethanol exposure during the third-trimester equivalent period in rats, with significant region-specific neuronal depletion [reviewed in [71]]. Binge-like ethanol exposure (peak BACs > 350 mg/dl), significantly reduced CA1 cell numbers immediately following the intubation procedure [18] and later on, once the rats reached adulthood [72]. In contrast, cell numbers in hippocampal regions CA3 and the dentate gyrus were reduced when counted on PD10 but not in adult rats [18,72], suggesting compensatory mechanisms may promote recovery in these two brain regions. Postnatal ethanol exposure also decreases morphological plasticity, alters synaptic plasticity, and reduces neurogenesis in the hippocampus [18,72–74].

Trace EBC is proposed to require structures beyond the basic brainstem-cerebellar circuit in order to bridge the temporal gap separating the CS and US and associate the discontiguous stimuli. Neuronal recordings have revealed populations of cells that continue to fire following CS offset in both the hippocampus [75–77] and the medial prefrontal cortex (mPFC) [78]. Reverberating N-methyl-D-aspartate receptor (NMDAR) synaptic excitation in the recurrent collateral fibers of the principal cells of area CA3 and/or the mPFC is proposed to provide the participating microcircuit with the necessary positive feedback to continue firing following termination of the transient stimulus [79,80]. NMDAR-dependent synaptic plasticity is also thought necessary for successful association of the CS and US, as indicated by impaired acquisition of trace EBC following infusion of NMDAR antagonists into the hippocampus [81,82]. Pre- and/or postnatal ethanol exposure alters the subunit composition and function of forebrain NMDARs across development [83–85], limiting perhaps the ability of forebrain neurons to sustain activation following termination of the transient CS and/or the induction of synaptic plasticity and learning. Structurally, perinatal alcohol exposure also disrupts mPFC neurodevelopment, with prenatal exposure reducing cell numbers in layers II and V in the mPFC of adult rats [86], and postnatal exposure decreasing dendritic complexity and spine density in layers II/III and layer III, respectively, of adolescent (PD26-30) rats [20,87].

Two recent studies have examined trace EBC in juvenile rats (∼PD30–34) exposed to binge-like ethanol across PD4-9 [88,89]. Both reported a significant impairment in trace EBC in rats dosed with a high concentration of alcohol (5.25 g/kg/day). In contrast, the present experiment did not reveal significant deficits in trace EBC in adult (∼PD70) rats exposed to 5.0 g/kg/day of ethanol across PD4-9. Several reasons might account for the disparate results. First, a higher alcohol concentration was used in the two previous studies, although peak BACs (346 ± 22 mg/dl) in [88] were comparable to those reported herein. Second, the former studies used a longer trace interval (500 ms) than the 300 ms interval used in the current experiment, which may amplify ethanol-induced hippocampal learning deficits. Finally, previous research has found that alcohol's adverse effects may be differentially expressed based on the age of the subject—e.g., PD4-9 ethanol-exposed male rats were significantly impaired relative to females in T-maze reversal learning when tested on PD28, whereas the females were more impaired when tested on PD63 [90].

All groups of subjects in the current study do appear capable of encoding the new context and discriminating it from the original context, as revealed by the resulting drop in eyeblink CR production on C01. Penick and Solomon [41] reported a decrease in behavioral responding of approximately 35–40% in sham and neocortical lesion rabbits, but not hippocampal lesion rabbits, following the context shift (relative to the response rate just prior to the context shift; Fig. 2 in [41]). The current results replicate and extend these findings to adult rats. As illustrated in Fig. 2, all rats produced fewer CRs when switched to a novel context on C01. To emphasize exploratory context-dependent changes in CR frequency, the

analysis was restricted to the final 2 blocks of trials on D06 and the initial two blocks of trials on C01 (Fig. 4A). The normalized data shown in Fig. 4B indicates the UC rats generated 20.5 ± 7.3% (mean ± SE) fewer CRs on C01, comparable to the decrease observed in the SI (21.6 ± 4.6%), 3E (17.2 ± 5.2%), and 5E (16.8 ± 7.0%) rats. Unlike hippocampal lesions, therefore, the putative hippocampal damage that followed early ethanol exposure in the present study was not sufficient to prevent the context-dependent decrease in eyeblink CR production.

While the data seems to suggest that the ethanol-exposed rats could discriminate between the two conditioning contexts, it is also possible that the result is due to other factors, possibly unrelated to hippocampal function. For example, the novelty of the context shift across C01 and C02 may have increased distraction and/or exploration on the part of the animal, contributing to the drop in conditioned responding. The transport cues were also altered which may have contributed to the CR drop, independent of the conditioning context. That said, research into a variant of one trial context fear conditioning (the context pre-exposure facilitation effect) indicates that retrieval of context memories can be achieved by a subset of contextual cues [91] or by the transport cues used to bring the rat to the context [92,93], suggesting the transport and contextual cues may be bound up in a single conjunctive representation.

The current results add to the existing literature detailing the adverse effects of early developmental ethanol exposure, particularly at high doses. Findings indicate that trace-delay EBC, across all 16 sessions, was significantly impaired by 5 g/kg/day of ethanol across PD4-9 when measured in adult rats compared to the SI controls. CR peak amplitude was also consistently reduced by both doses of alcohol across trace and delay EBC. Neonatal treatment did not affect CR production, however, when analyses were limited to the eight trace EBC sessions. Contextual modulation of the eyeblink CR was also unaffected by early ethanol exposure, which is thought to be specifically dependent on the hippocampus' role in forming contextual memories. Inasmuch, the current results are perhaps best explained by the well-documented damage that follows postnatal ethanol exposure in the developing brainstem and cerebellum, apart from ethanol-induced hippocampal dysfunction.

Trace EBC remains a valuable paradigm for assessing the teratogenic effects of alcohol on the developing hippocampus, however—e.g., a recent report found impaired trace and delay EBC in FAS children [94]. One crucial advantage of classical eyeblink conditioning, in fact, is that the results can be directly related to the underlying neural circuitry, which is conserved across multiple mammalian species, allowing direct comparisons between the FASD rodent model and FASD individuals [e.g., 94, 95]. For future studies, the trace interval may need to be lengthened in order to detect impaired learning in ethanol-exposed rats, similar to other recent reports that did observe deficient trace EBC [88,89]. More research is also required to determine precisely how contextual encoding might influence or gate the conditioned eyeblink response in ethanol-exposed subjects.

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