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Effects of Exercise and Environmental Complexity on Deficits in Trace and Contextual Fear Conditioning Produced by Neonatal Alcohol Exposure in Rats

ABSTRACT: *In rodents, voluntary exercise and environmental complexity increases hippocampal neurogenesis and reverses spatial learning and long-term potentiation deficits in animals prenatally exposed to alcohol. The present experiment extended these findings to neonatal alcohol exposure and to delay, trace, and contextual fear conditioning. Rats were administered either 5.25 g/kg/day alcohol via gastric intubation or received sham-intubations (SI) between Postnatal Day (PD) 4 and 9 followed by either free access to a running wheel on PD 30–41 and housing in a complex environment on PD 42–72 (wheel-running plus environmental complexity; WREC) or conventional social housing (SHSH) from PD 30 to 72. Adult rats (PD 80 ± 5) received 5 trials/day of a 10-s flashing-light conditioned stimulus (CS) paired with .8 mA footshock either immediately (delay conditioning) or after a 10-s trace interval (trace conditioning) for 2 days. Neonatal alcohol exposure impaired context and trace conditioning, but not short-delay conditioning. The WREC intervention did not reverse these deficits, despite increasing context-related freezing in ethanol-exposed and SI animals.* © *2012 Wiley Periodicals, Inc.* Dev Psychobiol 55: 483–495, 2013.

Keywords: *fetal alcohol spectrum disorder; neonatal alcohol exposure; trace fear conditioning; hippocampus; environmental complexity; voluntary exercise*

INTRODUCTION

Fetal alcohol spectrum disorder (FASD) describes a wide array of cognitive, behavioral, and neuroanatomical deficits that result from prenatal (or in utero) exposure to ethanol (EtOH; for recent review, see Riley, Infante, & Warren, 2011). Among the many symptoms evident in human FASD are impairments in working memory, executive function, and emotional regulation, which share a qualitative correspondence with deficits in learning and attention measured in animal models of FASD (Driscoll, Streissguth, & Riley, 1990; Rasmussen, 2005). One of the primary goals of FASD research is to develop treatments and interventions for FASD, particularly those effective during late adolescence and early adulthood that are applicable after diagnosis of FASD (Kully-Martens, Denys, Treit, Tamana, & Rasmussen, 2012). Behavioral interventions, such as rehearsal training and child friendship training, attenuate some of the working memory and social skills deficits in children diagnosed with FASD (Keil, Paley, Frankel, & O'Conner, 2010; Loomes, Rasmussen, Pei, Manji, & Andrew, 2008; O'Connor

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et al., 2006). Neonatal choline treatment overlapping and following alcohol exposure has also reversed some of the behavioral deficits observed in rodent models of FASD (Thomas, Abou, & Dominguez, 2009; Thomas, Idrus, Monk, & Dominguez, 2010; Thomas & Tran, 2012; Wagner & Hunt, 2006). In addition to offering treatment, identifying postnatal interventions for FASD may also provide a better understanding of the functional significance of neural plasticity and its impact on the development of brain and behavior.

One prominent animal model of FASD involves gastric intubation of alcohol in rodent neonates from postnatal day (PD) 4 to 9, a model of binge-like alcohol exposure during late pregnancy in humans. Treatment resulting in high blood alcohol concentrations (BAC) reduces hippocampal volume and pyramidal cell density in areas CA1 and CA3 as well as granule cell density and number (Bonthius & West, 1990; Greene, Diaz-Granados, & Amsel, 1992; Livy, Miller, Maier, & West, 2003; Marino, Aksenov, & Kelly, 2004; Murawski, Klintsova, & Stanton, 2012; Tran & Kelly, 2003). Additionally, binge-like neonatal alcohol exposure produces long-term effects on the hippocampus, including a reduction in the number of new neurons generated in adulthood and the survival of newly generated cells (Hamilton et al., 2011; Helfer, Goodlett, Greenough, & Klintsova, 2009; Klintsova et al., 2007). Complementing these neuroanatomical findings, neonatal alcohol intubation from PD 4 to 9 produces learning deficits across several hippocampus-dependent tasks, such as the Morris water maze, the context preexposure facilitation effect (CPFE), and trace fear conditioning (Goodlett & Johnson, 1997; Murawski & Stanton, 2011; Wagner & Hunt, 2006). Higher doses of alcohol cause more dramatic disruptions of behavior in trace fear conditioning than lower doses (Hunt, Jacobson, & Torok, 2009), and Murawski and Stanton (2011) reported a significant negative correlation between BAC level and context freezing, indicating that the disruption of behavior on hippocampus-dependent tasks is related to the potency of the alcohol exposure itself.

Trace fear conditioning is a form of Pavlovian conditioning in which a conditioned stimulus (CS) and unconditioned stimulus (US) are separated in time (trace interval) and contrasts with short-delay conditioning, in which CS and US occur successively in time. The hippocampus has been implicated as being crucial for learning temporally discontiguous associations, confirming the importance of its participation in trace but not short-delay conditioning (Wallenstein, Eichenbaum, & Hasselmo, 1998). Post-training lesions or pharmacological manipulations of the dorsal hippocampus disrupts trace fear conditioning, especially at longer trace intervals (Chowdhury, Quinn, & Fanselow, 2005; Quinn, Loya, Ma, & Fanselow, 2005; Quinn, Oommen, Morrison, & Fanselow, 2002). These findings were extended by Hunt et al. (2009) who showed that animals exposed neonatally to EtOH suffer more pronounced learning deficits in trace fear conditioning when trained with relatively long, but not short, trace intervals. Other cortical areas, such as the anterior cingulate cortex, are also implicated in trace but not short-delay fear conditioning (Han et al., 2003). Complementing the different neural substrates of these tasks, short-delay conditioning has an earlier developmental onset than trace fear conditioning, reflecting the delayed maturation of brain regions necessary for trace fear conditioning (Barnet & Hunt, 2005; Moye & Rudy, 1987).

Similarities between the behavioral dysfunctions associated with hippocampal lesions and those evident following neonatal alcohol exposure combined with the known neurotoxic effects of alcohol exposure on the developing hippocampus have led to the hypothesis that many of the behavioral deficits associated with FASD in both humans and animals may be caused by disruption of hippocampal development (e.g., Hamilton et al., 2011; Livy et al., 2003; Miki, Harris, Wilce, Takeuchi, & Bedi, 2003; Miki, Harris, Wilce, Takeuchi, & Bedi, 2004; Murawski & Stanton, 2010, 2011; Wagner & Hunt, 2006). Thus, manipulations that result in an enhancement of physiology, plasticity, and/or functional capacity of the hippocampus have been explored as interventions to attenuate or reverse the learning and anatomical deficits apparent in FASD (Hamilton, Whitcher, & Klintsova, 2010; Hamilton et al., 2011; Marino et al., 2004; Thomas et al., 2009, 2010; Thomas & Tran, 2012; Wagner & Hunt, 2006).

One of these manipulations is voluntary exercise, which reverses impairment of cell proliferation, neurogenesis, and spatial memory following prenatal alcohol exposure. Exercise also reduces LTP induction threshold, increases dendritic length, and increases new cell numbers in the dentate gyrus (Christie et al., 2005; Eadie, Redila, & Christie, 2005; Farmer et al., 2004; Redila et al., 2006). Additionally, voluntary exercise causes an up-regulation in brain-derived neurotrophic factor (BDNF) and enhances performance in the Morris water maze following traumatic brain injury (Griesbach, Hovda, Molteni, Wu, & Gomez-Pinilla, 2004). In neonatal animal models of FASD, 24-hr voluntary access to a running wheel (WR) transiently increases neurogenesis by PD 42, although the long-term survival of newly generated cells was impaired relative to controls at PD 72. This finding indicates that the benefits of voluntary exercise on brain development may also be impaired by early-life alcohol exposure (Helfer et al., 2009).

Environmental enrichment has also provided a means to increase neurogenesis in the dentate gyrus of the hippocampus, improve survival of newly generated neurons, enhance LTP in area CA1, and enhance contextual fear conditioning (Duffy, Craddock, Abel, & Nguyen, 2001; Kempermann, Kuhn, & Gage, 1997; Olson, Eadie, Ernst, & Christie, 2006). Environmental enrichment can help alleviate some of the detrimental effects of early-life alcohol exposure (for reviews, see Hannigan & Berman, 2000; Hannigan, O'Leary-Moore, & Berman, 2007) and enhances performance on hippocampus-dependent tasks following prenatal alcohol exposure, such as spatial learning in a Morris water maze (Hannigan, Berman, & Zajac, 1993). Although voluntary exercise and environmental enrichment are each independent methods of increasing hippocampal neurogenesis, combinations of environmental enrichment with voluntary exercise may lead to more dramatic enhancements than either presented individually (Fabel et al., 2009; Olson et al., 2006; Van Praag, Kempermann, & Gage, 2000).

The long-term enhancement of hippocampal function and anatomy by environmental enrichment and voluntary exercise provides a promising manipulation for reversing deficits in hippocampus-dependent tasks resulting from developmental alcohol exposure, such as trace and context fear conditioning. It was recently demonstrated that neonatal alcohol exposure in rodent models of FASD results in trace fear conditioning deficits that persist beyond adolescence and into adulthood (Schreiber & Hunt, 2012). Utilizing trace fear conditioning as an experimental paradigm presents the opportunity to measure the potentially beneficial effects of exercise and enrichment procedures encompassing adolescence and early-adulthood on the performance of hippocampus-dependent tasks as young adults, as well as the ability to contrast these findings against nonhippocampal performance control tasks utilizing similar training parameters only without the trace interval.

The present study examined the effects of WR followed by environmental complexity (EC) on trace fear conditioning and contextual fear conditioning in a neonatal alcohol rodent model of FASD. Rats were exposed to 5.25 g/kg/day of alcohol (EtOH) or sham-intubated (SI) over the PD 4–9 period. From PD 30 to 41, rats were either allowed access to a running wheel or remained in social housing (SH). WR rats were then exposed to a complex environment (EC) from PD 42 to 71, while SH rats remained in social housing. Behavioral training began on PD 80 ($\pm$5) and lasted 5 days. We predicted that rats in the neonatal alcohol group would show deficits in trace and context fear conditioning relative to SI rats. Additionally, we predicted that this deficit would not be present in wheel-running plus environmental complexity (WREC) rats (i.e., there would be no difference between EtOH and SI), nor would there be any effect of treatment or intervention on short-delay fear conditioning.

METHODS

Subjects

The subjects were 159 offspring (82 male and 77 female) of 29 time-pregnant Long-Evans dams bred in the University of Delaware Animal Care Facility. Dams were individually housed on a 12-hr light/dark cycle (8:00 am/8:00 pm) and given free access to food and water. On PD 3 each litter was culled to eight pups (4 male and 4 female) and paw marked with nontoxic black ink. The University of Delaware Institutional Animal Care and Use Committee (IACUC) reviewed and approved all animal procedures following guidelines established by the National Institutes of Health.

Alcohol Dosing

The dosing procedure has been described previously (Hamilton et al., 2010, 2011). On PD 4, pups were randomly assigned to receive either EtOH or SI, with equal numbers of males and females in each group in most cases. Intubations were performed from PD 4 to 9. SI pups received a 10-s intubation without gastric infusion of solution. EtOH pups received three doses daily, 2 hr apart. A milk–ethanol solution was administered on the first two occasions and the last was a milk-only dose. On PD 4 only, an additional milk only dose was given 2 hr after the first milk only dose. The milk–ethanol solution, containing 11.9% (v/v) ethanol, yielded 5.25 g/kg delivered to each EtOH pup daily. Weights for all animals, including SI pups, were measured each day before the first dose. Same-sex littermates in the same dosing condition (EtOH vs. SI) were assigned to different behavioral groups (Trace or Short-Delay, WREC or SHSH) so that no more than one same-sex littermate was assigned to a particular experimental condition (dosing condition $\times$ intervention $\times$ task). In two instances in which same-sex littermates were inadvertently assigned to the same experimental condition, their behavioral and blood alcohol data were averaged together.

Blood Alcohol Concentration

To assess peak BAC, blood was taken from all intubated rats (both ethanol-exposed and SI). On PD 4, approximately 90 min after the last milk–ethanol dose, blood samples were collected into heparinized tubes via tail clip and blood samples from the EtOH animals were centrifuged. Plasma was extracted and stored at -20°C until assay. BACs from EtOH rats were measured using an Analox GL-5 Alcohol Analyzer (Analox Instruments, Boston, MA), which measures oxygen consumption during the oxidation of ethanol.

Intervention Conditions

After weaning on PD 23, all rats were group housed with same-sex littermates 2–3 per cage, dispersing postnatal

condition (treatment × behavioral task) as evenly as possible. On PD 30, rats were randomly assigned to one of two intervention conditions, WREC or socially housed control (SHSH). Animals began the intervention 1 week after they were weaned (PD 23) to allow them to grow and acclimate to their new cages and cage-mates. Those assigned to the SHSH group remained in their current cages until PD 72. Those assigned to the WREC group were group housed and given 24-hr access to a stainless steel running wheel (Wahmann Manufacturing Co., Baltimore, MD) for voluntary exercise on PD 30–42 and then housed in complex environments on PD 42–72. Each running wheel was equipped with a counter measuring the number of revolutions, which was recorded every 24 hr at the onset of light. All animals were also weighed on PD 30. On PD 42, WREC animals were moved from WR cages, and, maintaining separation by sex, combined into larger groups ($n = 9$–12) for housing in EC cages. These cages consisted of a 30″ × 18″ × 36″ galvanized three-story steel cage (model: R-695; cagestore.com) with three ramps, two balconies, a full middle floor, and a drop-in 3½″ plastic pan filled with bedding. Cages were equipped with a variety of objects, such as buckets, blocks, PVC piping, plastic toys, etc. All objects were completely removed and replaced with new items every 2 days at the onset of the light cycle. WREC animals were housed in these cages for a period of 30 days (until PD 72). All animals were weighed on PD 30, 41, 50, 58, 66, and 80 (±5).

BrdU Administration

On PD 41, all animals were weighed and received a single intraperitoneal (ip) injection of synthetic thymidine analog 5-bromo-2-deoxyuridine (BrdU; 200 mg/kg in .9% sterile saline). The purpose of this injection was to make the developmental manipulations of rats in the present (behavioral) study comparable to rats in other studies of dentate granule cell neurogenesis (Hamilton et al., 2011).

Apparatus and Stimuli

The fear conditioning paradigm employed in this study occurred in five successive phases: 2 days of handling/pre-exposure, 2 days of training, and 1 day of testing. The training context was a Med Associates (Med Associates Inc., Georgia, VT) ENV-008 Standard Modular Chamber with interior dimensions of 30.5 cm × 24.1 cm × 21.0 cm, outfitted with a modified ENV-227 house light enclosed within a red cap which provided background illumination. The modular chamber was housed within an ENV-022MD medium-density fiberboard cubicle (22″ × 15″ × 16″) with a 25-W A19 Frost light bulb mounted parallel to the modular chamber, which served as the CS (see below). Chambers used during training had fans and house lights unplugged to avoid interference with testing. The testing context was distinctly different from the training context and consisted of a Plexiglas and wire chamber (23.5 cm × 23.2 cm × 29 cm) housed within a foam-lined open animal chamber (BRS/LVE, Laurel, MD). Background illumination was provided by two red 5 W light bulbs and the CS was produced by a white 25-W light bulb, all mounted outside the wire chamber on a wood plank. All chambers utilized on a particular day were cleaned with 5% ammonium hydroxide solution prior to the first load of animals. Between sessions, chambers were cleaned with water and the paper towels in the collection pans beneath the chambers were replaced. At the end of the day, chambers were again cleaned with 5% ammonium hydroxide solution.

Four video cameras (Panasonic SDR-H80 and SDR-H85) were used to record activity during training and testing. Behavioral data were scored on personal computers using the open-source VLC media player (VideoLAN organization; www.videolan.org/vlc/).

Handling and Pre-Exposure

Animals were handled for 3 min in the colony room approximately 15 min before pre-exposure began and then returned to their home cages. Rats were pre-exposed to the training or testing chambers, counterbalanced such that pre-exposure to the training chamber always occurred at the same time as the actual training session. Starting on PD 80 (±5), rats were weighed and transported to the training/testing room in their home cages. Rats were placed in and allowed to explore the chamber for a 5-min period, after which they were returned to their home cages and transported back to the colony room.

The second handling and pre-exposure session occurred 24 hr after the first session. This procedure was identical to those of the first handling and pre-exposure day.

Training

Twenty-four hours after the second pre-exposure day, rats were again weighed and transported in their home cages to the training/testing room. Training sessions began with a 5-min "baseline" period in the chamber without any stimuli. For trace training, rats received five trials consisting of a 10-s CS followed by a 10-s trace interval that terminated with a 1-s US (.8 mA footshock). Inter-trial intervals were 200 s. For short-delay training, rats received five trials consisting of the 10-s CS followed by the 1-s US (CS offset coincided with US onset). Inter-trial intervals were increased by 10 s in the Short-Delay group to equate CS-to-CS intervals with trace groups (Schreiber & Hunt, 2012). Sessions lasted a total of 20 min. Rats were left in the chambers for approximately 1 min following the last US, returned to their home cages, and transported back to the colony room.

The second training session occurred 24 hr after the first training session. The procedures were identical to those of the first training day and included the same number of training trials. There were a total of 10 trials (5 per day) that occurred across the two training sessions.

Testing

Twenty-four hours after the second day of training, rats were tested in an alternate context (see Apparatus and Stimuli, above). Testing sessions included a 1-min baseline period in the chamber without any stimuli. Test trials consisted of five presentations of the 10-s nonreinforced CS, separated by 90 s. Sessions lasted a total of 8 min.

Behavioral Analysis

Video recordings made on the second training day and during the test sessions were scored for freezing behavior. Freezing was defined as the absence of all visible movements except those associated with breathing (Fanselow, 1980). Videos were scored by two independent observers blind to the experimental condition of the animals and inter-rater agreement on instances of freezing was 91.5%.

Context conditioning was assessed on Training Day 2. Animals were observed in the training chambers every 6 s during the 300-s adaptation period prior to the first CS onset. During CS presentations on the testing day freezing behavior was scored once every 2 s during three trial epochs relative to the first CS onset: 10 s prior to CS onset (''Pre''), 10 s during the CS (''CS''), and 10 s after CS offset (''Post''). Percent freezing during each epoch (Pre, CS, and Post) was then calculated.

Data Analysis

Body weights on PD 4 and 9 were analyzed using a 2 (Treatment; EtOH vs. SI) × 2 (Intervention; WREC vs. SHSH) × 2 2 (Sex; Male vs. Female) × 2 (Day; PD 4 and 9) mixed factorial ANOVA. To avoid violating the homogeneity of variance assumptions of ANOVA, body weights on PD 30, 41, 50, 58, 66, and 80 (±5) were analyzed separately using a 2 (Treatment) × 2 (Intervention) × 2 (Sex) factorial ANOVAs. Freezing behavior was analyzed separately by Task (Trace or Short-Delay) because the ''Post'' epoch was only a relevant conditioning measure for animals that had been trained in the trace fear conditioning group. Data from short-delay conditioning animals were analyzed with a 2 (Treatment) × 2 (Intervention) × 2 (Sex) × 2 (Epoch: Pre, CS) mixed factor ANOVA. Data from the Trace conditioning animals were analyzed with a 2 (Treatment) × 2 (Intervention) × 2 (Sex) × 3 (Epoch: Pre, CS, Post) mixed factorial ANOVA. Context freezing was analyzed with a 2 (Treatment) × 2 (Intervention) × 2 (Sex) × 2 (Task; Trace, Short-Delay) factorial ANOVA. Post hoc analyses (Newman–Keuls) were used to characterize interaction effects. Statistical significance was set to $p < .05$.

Rats were excluded from analysis if they showed 100% baseline freezing or if their conditioned freezing scores were statistical outliers. Outliers were defined as an individual rat's score that differed by at least ±1.96 *SD* from the means for the other subjects in a given group. For the Short-Delay group, these scores were the difference between CS freezing minus baseline. For the Trace group, these scores were the difference between CS freezing minus baseline and post-CS freezing minus baseline. Rats in the Trace group were removed only if their scores were outliers on both measures. The number of rats so excluded is reported below.

RESULTS

After excluding animals with 100% ''Pre'' freezing ($N = 8$ males: 4 EtOH, 4 SI; 2 females: 1 EtOH, 1 SI) and statistical outliers ($N = 4$ males: 3 EtOH, 1 SI; 5 females: 4 EtOH, 1 SI), behavioral analyses were conducted on the remaining 140 subjects. The number of rats remaining in each group is indicated in Table 1 (right column).

Body Weights and BACs

Body weights at PD 4, 9, 30, 41, 50, 58, 66, and 80 (±5) are summarized in Table 1 (with group sizes).

From PD 4 to 9, ethanol-treated subjects gained less weight than SI subjects, and females weighed less than males (Combined Groups). These effects were analyzed statistically with a mixed factorial ANOVA which revealed main effects for treatment [$F(1, 132) = 44.14$, $p < .01$], sex [$F(1, 132) = 7.44$, $p < .01$], and day [$F(1, 132) = 2170$, $p < .01$] as well as a significant Day × Treatment interaction [$F(1, 132) = 119.43$, $p < .01$]. Newman–Keuls tests showed that EtOH and SI groups did not differ on PD 4, that both groups gained weight from PD 4 to 9, and that EtOH rats weighed less than shams on PD 9 (all p's $< .0001$).

On PD 30 and 41, the onset and conclusion of WR, EtOH animals weighed less than SI animals [$F(1, 132) = 17.171$, PD 30; 19.13, PD 41, p's $< .001$], and females weighed less than males [$F(1, 132) = 20.204$, PD 30; 115.68, PD 41, p's $< .0001$]. There was a tendency for the treatment effect to attenuate between PD 30 and 41 in SHSH but not WREC rats, although this was only marginally significant ($F = 3.32$, $p < .08$). Otherwise, WR did not affect body weight during this period. During the EC phase (PD 50, 58, and 66) there remained effects of treatment [$F(1, 132) = 11.79$, PD 50; 9.39, PD 58; 7.77, PD 66, all p's $< .01$] and sex [$F(1, 132) = 332.55$, PD 50; 498.43, PD 58; 656.52, PD 66, all p's $< .00001$]. Additionally, during this period WREC animals weighed less than SHSH controls [$F(1, 132) = 5.72$, PD 50, $p < .02$; $F(1,132) = 7.19$, PD 58; 9.92, PD 66, $p < .01$]. Finally, on the first day of training [PD 80 (±5)], male subjects weighed more than females ($F = 912.44$, $p < .00001$) and there was a significant Intervention × Sex interaction [$F(1, 132) = 4.27$, $p < .05$]. Newman–Keuls test showed that males in SHSH weighed more than males in WREC (SHSH = 401.46 ± 5.51, WREC = 383.83 ± 5.19) although in females there were no weight differences between intervention groups (SHSH = 249.88 ± 3.84, WREC = 252.64 ± 3.74). Importantly, there were no main effects or interactions involving treatment when training began (F's < 1.32).

The results in Table 1 show that during the neonatal dosing procedure, EtOH treatment caused a significant reduction in weight relative to SI controls, an effect which persisted until PD 80 (±5). After WR, animals

Table 1. Mean (±SEM) Body Weights (g) for Animals by Postnatal Day (PD)

	PD 4	PD 9	PD 30	PD 41	PD 50	PD 58	PD 66	PD 80 ± 5	N
All groups									
Females									
WREC									
ETOH (BAC = 433.85 ± 19.13)	9.31 (.30)	14.16 (.74)	80.14 (3.64)	135.14 (4.82)	168.14 (4.82)	196.00 (5.08)	218.21 (4.99)	244.93 (6.55)	14
SI	9.50 (.21)	17.83 (.42)	88.64 (1.65)	150.73 (2.20)	182.86 (2.42)	211.27 (2.67)	230.95 (3.27)	257.55 (4.27)	22
SHSH									
ETOH (BAC = 387.45 ± 17.42)	9.32 (.26)	14.20 (.57)	80.56 (2.61)	140.56 (3.41)	178.06 (3.77)	207.72 (3.93)	229.22 (4.81)	248.94 (5.66)	18
SI	9.23 (.21)	16.73 (.49)	86.69 (2.36)	148.94 (3.87)	186.13 (5.87)	215.38 (8.11)	237.56 (8.59)	250.94 (5.27)	16
Males									
WREC									
ETOH (BAC = 385.97 ± 13.04)	9.44 (.24)	14.29 (.56)	86.44 (3.57)	161.50 (5.02)	226.06 (6.78)	280.69 (8.49)	319.19 (9.39)	380.13 (8.12)	16
SI	9.94 (.22)	18.44 (.45)	96.00 (2.22)	178.80 (3.53)	244.00 (4.32)	300.85 (4.97)	341.15 (4.62)	386.80 (6.83)	20
SHSH									
ETOH (BAC = 381.31 ± 14.83)	10.02 (.21)	15.63 (.39)	90.16 (2.16)	172.03 (3.77)	241.84 (4.72)	301.31 (5.57)	346.00 (5.51)	403.69 (7.37)	16
SI	9.88 (.23)	18.10 (.54)	95.94 (2.35)	177.19 (3.67)	247.00 (4.99)	307.67 (6.05)	350.97 (6.59)	399.47 (8.25)	18
Combined groups									
Females	9.35 (.12)	15.91 (.33)	84.41 (1.29)	144.59 (1.84)	179.43 (2.15)	208.24 (2.56)	229.47 (2.77)	251.30 (2.66)	70
Males	9.83 (.11)	16.76 (.32)	92.46 (1.36)	172.89 (2.11)	240.18 (2.71)	298.10 (3.29)	339.76 (3.51)	392.39 (3.90)	70
WREC	9.57 (.12)	16.50 (.34)	88.54 (1.45)	157.89 (2.60)	206.58 (4.25)	248.61 (5.85)	278.69 (6.91)	318.24 (8.41)	72
SHSH	9.61 (.12)	16.16 (.31)	88.33 (1.36)	159.63 (2.61)	213.21 (4.52)	258.00 (6.43)	290.89 (7.74)	325.67 (9.84)	68
EtOH (BAC = 396.23 ± 8.38)	9.52 (.13)	14.57 (.29)	84.34 (1.56)	152.48 (2.79)	203.84 (4.61)	246.80 (6.37)	278.50 (7.62)	319.55 (9.79)	64
SI	9.65 (.11)	17.82 (.24)	91.89 (1.14)	164.01 (2.28)	214.83 (4.12)	258.54 (5.89)	289.77 (7.06)	323.78 (8.56)	76

Neonatal treatment occurred from PD 4 to 9; wheel-running/social-housing occurred from PD 30 to 42; environmental complexity/social housing occurred from PD 42 to 72. Animals were trained on PD 80 (±5).

in the intervention procedure weighed less than their socially housed counterparts, but this effect was only apparent in males by the training day. When training began, neither the treatment nor the intervention had an effect on body weight of either sex, suggesting that changes in freezing as a result of treatment and/or intervention effects were not related to body weight.

BACs obtained from 61 of 64 EtOH-exposed rats (three samples were lost due to technical difficulties) are also summarized in Table 1. There was no significant effect of sex on BAC level ($p = .14$).

Cued Fear

Cued fear conditioning data were taken 24 hr following the second training session. A 2 (Treatment) × 2 (Intervention) × 2 (Sex) × 2 (Epoch) mixed factorial ANOVA revealed that animals in the short-delay conditioning task (Fig. 1) froze more during the CS than before the CS ("Pre"), (Pre = 10.46 ± 2.52%, CS = 64.62 ± 3.50%), indicating that conditioning to the CS occurred [$F(1, 57) = 241.89$, $p < .00001$]. Also, EtOH animals froze more overall than SI animals [$F(1, 57) = 5.71$, $p < .05$; EtOH = 44.29 ± 4.99%, SI = 32.43 ± 4.12%], however this reflected a difference in baseline freezing rather than conditioning (Epoch × Treatment interaction, $F < .01$). Newman–Keuls tests of the Epoch × Intervention interaction [$F(1, 57) = 7.57$, $p < .01$] showed that SHSH animals exhibited a larger increase in freezing from Pre to CS (Pre = 6.45 ± 3.13%, CS = 71.61 ± 4.02%) than animals in the WREC intervention (Pre = 14.12 ± 3.83%, CS = 58.24 ± 5.43%). There was also an Epoch × Treatment × Sex interaction [$F(1, 57) = 4.43$, $p < .05$] for which Newman–Keuls revealed a greater increase in freezing from Pre to CS epochs in males (Pre = 5 ± 2.86%, CS = 69 ± 5.52%) than in females (Pre = 7.06 ± 2.94%, CS = 47.06 ± 8.21%) among SI animals, although there were no sex differences between Pre to CS changes in EtOH animals.

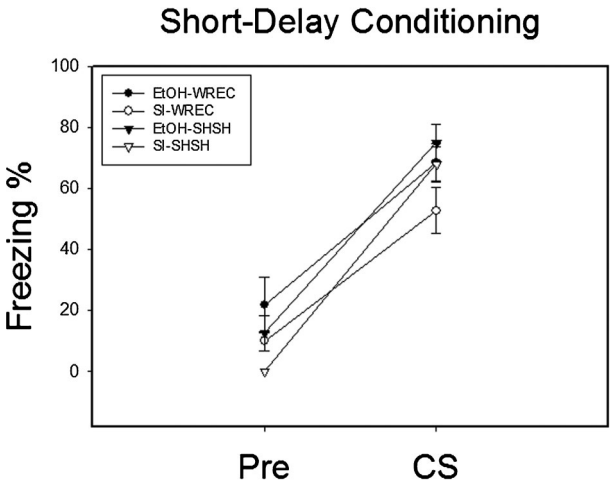

FIGURE 1 Freezing scores during "Pre" (10 s prior to CS onset) and "CS" (10 s during CS onset) epochs as a function of neonatal alcohol exposure and juvenile intervention condition in adult rats trained on short delay fear conditioning.

In summary, EtOH treatment had no effect on short delay conditioning. Short-delay conditioning in SHSH animals was more robust than in WREC animals, and among the SI controls males exhibited more robust conditioning than females. Owing to the lack of an Epoch × Treatment interaction, our results indicate that neonatal EtOH treatment did not disrupt short-delay conditioning.

A 2 (Treatment) × 2 (Intervention) × 2 (Sex) × 3 (Epoch; Pre, CS, Post) mixed factorial ANOVA conducted on the data from animals in the Trace conditioning task (Fig. 2a) indicated that animals froze more during the CS and Post epochs than during the Pre epoch (Pre = 9.47 ± 2.14%, CS = 27.47 ± 3.17%, Post = 33.47 ± 4.07%), indicating that conditioning to the CS occurred [$F(2, 134) = 27.01$, $p < .01$]. EtOH animals froze less overall than SI animals [$F(1, 67) = 12.17$, $p < .01$; EtOH = 14.72 ± 2.16% SI = 31.54 ± 3.06%]. A significant Epoch × Treatment interaction confirmed the trace fear conditioning deficit in EtOH animals relative to controls, as evidenced by smaller changes from baseline freezing to the CS and Post epochs compared to the SI controls [$F(2, 134) = 3.72$, $p < .05$] (EtOH treatment did not change baseline freezing significantly, $p > .16$). An Epoch × Treat- Treatment × Sex interaction was also significant [$F(2, 134) = 3.73$, $p < .05$]. Newman–Keuls analysis showed that in females, EtOH-induced conditioning deficits were only significant during the Post epoch (SI = 47.62 ± 8.56%, EtOH = 11.58 ± 5.99%), while in males the EtOH-induced conditioning deficits were only significant during the CS Epoch (SI = 41.67 ± 7.33%, EtOH = 17.06 ± 5.93%).

In summary, trace fear conditioning was significantly disrupted in EtOH animals relative to SI controls (Fig. 2b); these differences were most apparent during the CS epoch in males, and the Post epoch in females. The WREC intervention procedure did not reverse deficits in trace fear conditioning caused by alcohol exposure, as indicated by the lack of a Treatment × Intervention × Epoch interaction.

Context Fear

Context fear conditioning data were taken 24 hr following the first training session during the 5-min pre-exposure of the second training session. A 2 (Treatment) × 2 (Intervention) × 2 (Sex) × 2 (Task; Trace vs. Short-Delay) factorial ANOVA revealed that

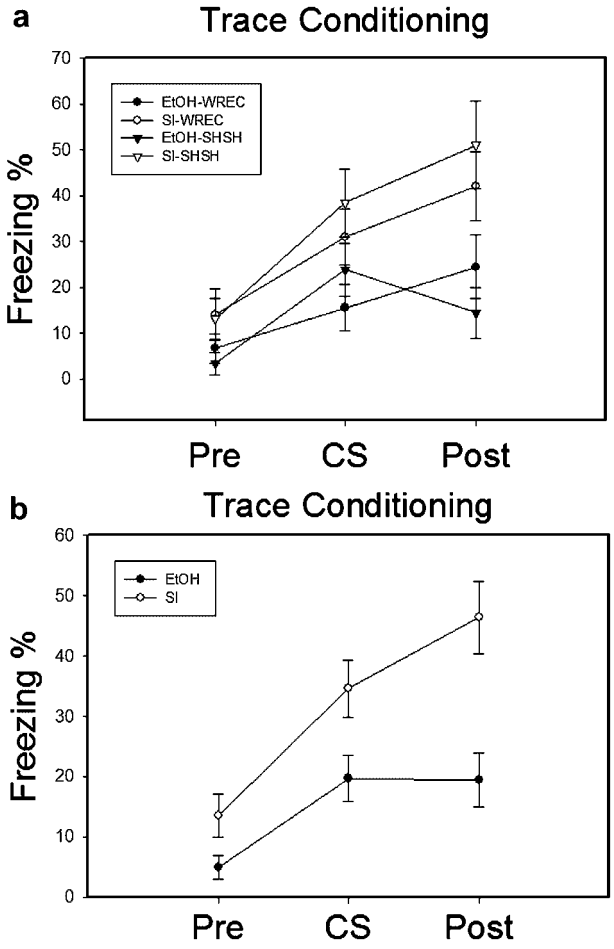

FIGURE 2 (a) Freezing scores during "Pre" (10 s prior to CS onset), "CS" (10 s during CS onset), and "Post" (10 s following CS offset) epochs, as a function of neonatal alcohol exposure and juvenile intervention condition in adult rats trained on short delay or trace fear conditioning. (b) Freezing scores during "Pre" (10 s prior to CS onset), "CS" (10 s during CS onset), and "Post" (10 s following CS offset) epochs, as a function of neonatal alcohol exposure in adult rats trained on trace fear conditioning [data from (a) are shown collapsed across intervention condition].

EtOH animals exhibited less context-related freezing on training day 2 than SI animals [Fig. 3a and b; $F(1, 124) = 18.33$, $p < .0001$]. Additionally, animals subject to the WREC intervention exhibited higher levels of freezing than those in the SHSH intervention [Fig. 3c; $F(1, 124) = 22.29$, $p < .00001$]. There was a

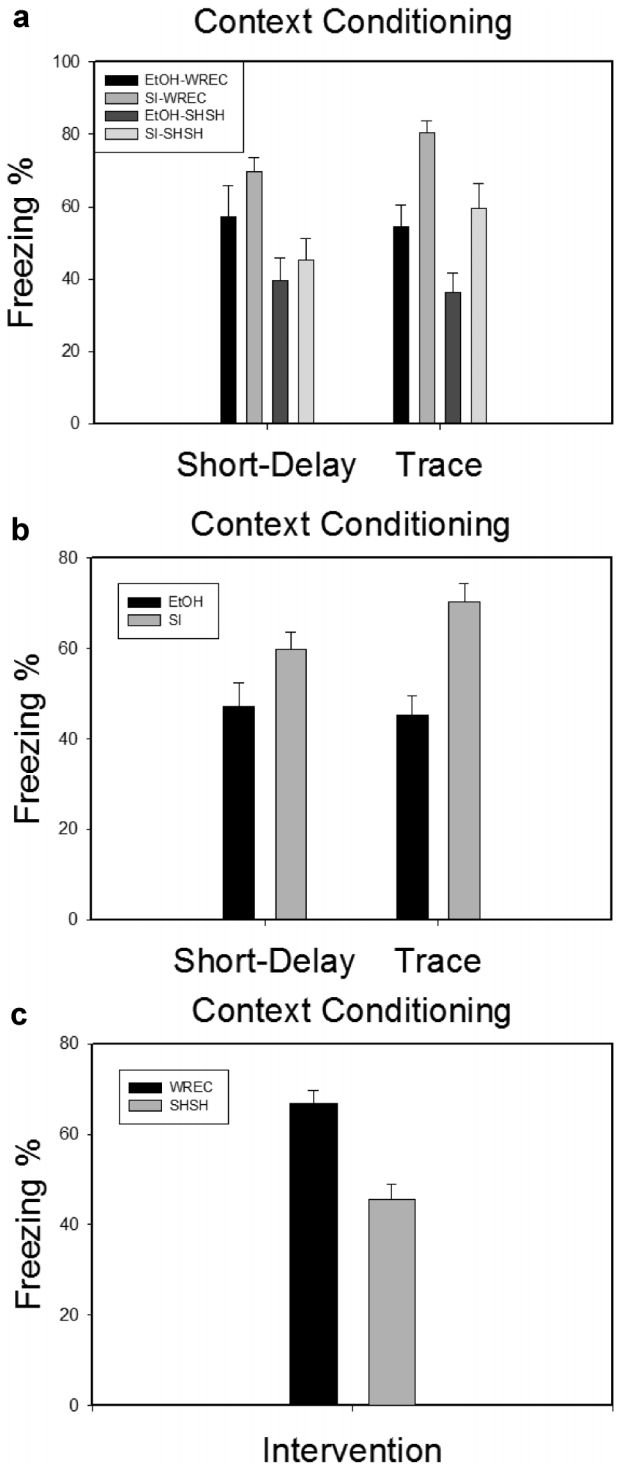

FIGURE 3 (a) Context-related freezing scores recorded during the 5-min adaptation period on training day 2, as a function of neonatal alcohol exposure and juvenile intervention condition in adult rats trained on delay or trace fear conditioning. (b) Context-related freezing scores recorded during the 5-min adaptation period on training day 2 [data from (b) are shown collapsed across intervention groups], collapsed across intervention groups. (c) Context-related freezing scores recorded during the 5-min adaptation period on training day 2 [data from (c) are shown collapsed across treatment and task].

tendency for differences in context freezing between EtOH and SI animals trained in the trace conditioning task to be larger than those trained in the short-delay task (Fig. 3b) although the Treatment × Task interaction was only marginally significant [$F(1, 124) = 2.79$, $p < .10$].

EtOH rats showed less context-related freezing than SI controls (Fig. 3b), and WREC animals showed more context-related freezing than SHSH controls (Fig. 3c). These treatment-based deficits and intervention-based enhancements on context learning do not reflect a reversal of alcohol-induced context learning deficits, but rather an overall increase in context conditioning across all experimental groups following WREC intervention.

Summary of Findings

In Trace and Context conditioning, animals exposed to EtOH from PD 4 to 9 exhibited less fear conditioning than SI controls. There were no significant conditioning differences between treatment groups in short-delay conditioning. WREC intervention did not reverse trace or context fear deficits in EtOH animals relative to SHSH controls. This negative finding cannot be attributed to ineffectiveness of the WREC manipulation because the intervention significantly increased context freezing.

DISCUSSION

The present study sought to examine whether a combination of exercise and environmental complexity during adolescence and early adulthood would reverse deficits in hippocampus-dependent tasks that result from neonatal alcohol exposure. Consistent with previous reports (Hunt et al., 2009; Schreiber & Hunt, 2012; Wagner & Hunt, 2006), neonatal alcohol exposure from PD 4 to 9 disrupted trace but not short-delay conditioning. The selective impairment of hippocampus-dependent trace conditioning confirms that the functional capacity of the rat to acquire and express aversive learning to fearful stimuli is not disrupted by neonatal alcohol administration. Thus, changes in sensory, motor, or motivational processes (e.g., shock sensitivity, hyperactivity) cannot account for the alcohol-induced deficits. Rather, alcohol disrupts only those neurobehavioral systems that are selectively involved in trace but not short-delay conditioning. Context conditioning was also impaired following neonatal alcohol exposure, consistent with previous findings (Murawski & Stanton, 2010). The WREC intervention caused an increase in context conditioning in both EtOH and SI groups, although contrary to our hypothesis, it had no effect on trace fear conditioning, and it did not reverse the effects of alcohol on trace or context conditioning.

Previous reports on adolescent visual-CS cued learning indicate that neonatal exposure to alcohol significantly disrupts trace fear conditioning relative to SI controls (Hunt et al., 2009; Wagner & Hunt, 2006), although CS-elicited freezing among SI animals is higher than was measured in the present study. Our findings replicate and extend those of Schreiber and Hunt (2012), which indicate that deficits in trace fear conditioning resulting from neonatal alcohol exposure are measurable both during adolescence and adulthood. Schreiber and Hunt (2012) also reported a sex effect in which adult trace fear deficits induced by neonatal ethanol were specific to females, with males showing some recovery of function between the juvenile period and adulthood. The present study indicates the opposite effect: males exposed to alcohol exhibit impaired CS-elicited freezing, and deficits in females are more prominent during the 10-s following the CS (trace interval). Our results suggest that even though ethanol-exposed rats may show sex differences in responding to the CS, the permanency of the neonatal alcohol deficits themselves may not be sex-specific. Conflicting reports of sex-specific deficits following early-life alcohol exposure on other hippocampus-dependent tasks further complicates the conclusion that sex-specific ethanol effects on learning are robust or consistent (Johnson & Goodlett, 2002; Kelly, Goodlett, Hulsether, & West, 1988). Failure to control for estrous cycle during testing could contribute to these conflicting findings. When we have controlled for stage of estrous in female rats, we have not found sex differences in the effect of neonatal alcohol on hippocampus-dependent context conditioning during adulthood when pre-exposure began during proestrus (Hamilton et al., 2011).

The results of the present study also replicate and extend the findings of Kohman et al. (2012), who tested the effects of voluntary exercise on trace and contextual fear conditioning. Consistent with our findings, Kohman et al. report that mice given access to WR show enhanced contextual fear conditioning relative to sedentary controls when testing occurred 1 day following training. For the mice in the context experiment, WR also increased the number of new granule cells in the dentate gyrus. Additionally, WR did not enhance trace fear conditioning, although when animals trained in trace fear conditioning were returned to their training chambers, WR mice exhibited higher context conditioning than sedentary mice. The results of the present study taken with those of Kohman et al. suggest that adult neurogenesis is enhanced by WR or a combination of WREC but is not sufficient to cause an increase in trace fear conditioning, confirming that the cognitive

benefits of exercise are selective to only certain hippocampus-dependent tasks. Furthermore, the present research also eliminates a caveat issued by Kohman et al. by demonstrating that this lack of an effect persists across large numbers of training trials.

Pavlovian trace fear conditioning is a hippocampus-dependent task (McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998; Quinn et al., 2002). The hippocampus is damaged by developmental alcohol exposure (Greene et al., 1992; Hamilton et al., 2011; Helfer et al., 2009; Miller, 1995), which in turn disrupts performance on hippocampus-dependent tasks (Greene et al., 1992; Hunt et al., 2009; Murawski & Stanton, 2011). Dorsal hippocampus NMDA receptor antagonists disrupt both acquisition and expression of trace fear conditioning (Quinn et al., 2005), indicating that the dorsal hippocampus in particular is critical for trace fear conditioning, especially CA1 pyramidal cells (Huerta, Sun, Wilson, & Tonegawa, 2000). Cell recordings in the medial prefrontal cortex (mPFC), which receives input via glutamatergic projections from the hippocampal formation (Gigg, Tan, & Finch, 1994), respond to a CS by showing transient increases in the firing rate in fast spiking cells (inhibitory interneurons) and decreases in firing rate in regular spiking cells (pyramidal cells) in the prelimbic and infralimbic cortex, suggesting that mPFC neuronal activity changes in anticipation of a US (Baeg et al., 2001). Quinn, Ma, Tinsely, Koch, and Fanselow (2008) demonstrate that lesions to the mPFC disrupt remotely acquired trace and context memories but have no effect on recently acquired trace fear conditioning, suggesting that the mPFC also participates in the long-term storage of fear memories, both cued and contextual. Early-life alcohol exposure has measurable effects on the mPFC as well, such as reduced cell numbers in layers II and V of the mPFC following prenatal alcohol treatment (Mihalick, Crandall, Langlois, Krienke, & Dube, 2001) and behavioral deficits on reversal learning (Mihalick et al., 2001; Thomas, Fleming, & Riley, 2002). Additionally, Nagahara and Handa (1995) found decreased IEG mRNA expression in prelimbic cortex and behavioral deficits on delayed-alternation in a T-maze following prenatal alcohol exposure. It is possible that the combined effect of alcohol on the hippocampus and the mPFC, both of which hold importance in the acquisition and retrieval of trace fear conditioning and context conditioning, accounts for some of the behavioral deficits measured in the present study.

Voluntary exercise has also been reported to enhance adult hippocampal neurogenesis (Brown et al., 2003; Helfer et al., 2009; Kempermann, Kuhn, & Gage, 1998; Olson et al., 2006; Rizzi, Bianchi, Guidi, Ciani, & Bartesaghi, 2011) as well as performance on hippocampus-dependent tasks, such as spatial memory (Rosenzweig & Bennet, 1996), the Morris water maze (Paylor, Morrison, Rudy, Waltrip, & Wehner, 1992), object recognition (Bruel-Jungerman, Laroche, & Lampon, 2005), and context learning (Barbelivien et al., 2006). Techniques for achieving adult neurogenesis similar to those employed in the present study report measurable increases in the granule cell layer of the dentate gyrus (Hamilton et al., 2011). On spatial learning variants of the Morris water maze, voluntary exercise can be beneficial to the extent that animals exposed prenatally to alcohol perform at the same level as controls (Christie et al., 2005). These findings suggest that exercise cannot only be used as a means of enhancing cognitive function in normal animals, but also as a means of reversing deficits in animal models of developmental disorders. The present study does not extend the generality of these findings. The present study differs from that of Christie et al. (2005) in many ways, including period of alcohol exposure, nature of the intervention, and the behavioral task. More research is needed to determine how these variables influence the ability of exercise to reverse memory deficits produced by developmental alcohol.

Our data indicate an overall main effect of intervention in which there is an increase in context conditioning in WREC animals relative to SHSHs. The nature of this effect is not a reversal of context conditioning deficits in ethanol-exposed animals to the levels of SI controls, but rather an overall increase in context conditioning across all groups regardless of treatment or task. The effects measured in the present study are similar to those demonstrated by Thomas, Sather, and Whinery (2008) who examined the effects of WR as a post-treatment intervention for neonatal alcohol-induced deficits in the Morris water maze. Thomas et al. found that voluntary exercise from PD 21 to 51 not only improved performance among ethanol-exposed animals, but sham and nonintubated controls as well, decreasing path lengths to find a hidden platform. Although the mechanisms through which these cognitive benefits occur has not been definitively examined, these results, taken with those of the present study, suggest that despite the various neuroanatomical insults known to be associated with early-life alcohol exposure, ethanol-exposed animals maintain neuronal plasticity and can thus benefit from postnatal interventions similar to controls. Additionally, our combined research suggests that context conditioning and spatial navigation share similar neurological underpinnings (e.g., the hippocampal formation) such that they are both similarly impaired by alcohol exposure and enhanced by voluntary exercise (and the nature of the enhancement is the same). Further research is warranted in describing the

function of the hippocampus in both of these tasks, as well as characterizing the neurobiological dissociations between commonly employed hippocampus-dependent forms of learning that benefit from voluntary exercise (e.g., spatial learning, context fear conditioning) and those that do not (e.g., trace fear conditioning).

In conclusion, a combination of voluntary exercise and EC did not reverse deficits produced by neonatal alcohol-exposure and measured in trace fear conditioning but did increase context conditioning across all treatment groups, consistent with previous reports (Kohman et al., 2012). Although these findings do not support the conclusions of previous research showing that voluntary exercise attenuates behavioral deficits in animals exposed to alcohol prenatally, they do provide evidence for dissociation between context and cued learning such that the beneficial effects of adult neurogenesis only selectively enhance one of these two similar forms of hippocampus-dependent learning. Further research is warranted to understand the complex neural mechanisms of alcohol's effects on learning and memory, as well as how these effects can be attenuated by various interventions occurring during adolescence and early adulthood.

NOTE

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