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Exercise and Environment as an Intervention for Neonatal Alcohol Effects on Hippocampal Adult Neurogenesis and Learning

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Abstract

Neonatal alcohol exposure impairs cognition and learning in adulthood and permanently damages the hippocampus. Wheel running (WR) improves hippocampus-associated learning and memory and increases the genesis and survival of newly generated neurons in the hippocampal dentate gyrus. WR significantly increases proliferation of newly generated dentate granule cells in alcohol-exposed (AE) and control rats on Postnatal Day (PD) 42 but only control rats show an increased number of surviving cells thirty days after WR (Helfer et al., 2009b). The present studies examined whether proliferation-promoting WR followed by survival-enhancing environmental complexity (EC) during adolescence could increase survival of new neurons in AE rats. On PD4–9, pups were intubated with alcohol in a binge-like manner (5.25g/kg/day, AE), were sham-intubated (SI), or were reared normally (suckle control, SC). On PD30 animals were assigned to WR (PD30–42) followed by EC (PD42–72; WR/EC) or were socially housed (SH/SH) for the duration of the experiment. All animals were injected with 200 mg/kg BrdU on PD41. In Experiment 1, survival of newly generated cells was significantly enhanced in the AE-WR/EC group in comparison with AE-SH/SH group. Experiment 2A examined trace eyeblink conditioning. In the SH/SH condition, AE impaired trace eyeblink conditioning relative to SI and SC controls. In the WR/EC condition, AE rats performed as well as controls. In Experiment 2B, the same intervention was examined using the context preexposure facilitation effect (CPFE); a hippocampus-dependent variant of contextual fear conditioning. Again, the WR/EC intervention reversed the deficit in conditioned fear to the context that was evident in the SH/SH condition. Post-weaning environmental manipulations promote cell survival and reverse learning deficits in rats that were exposed to alcohol during development. These manipulations may provide a basis for developing interventions that ameliorate learning impairments associated with human fetal alcohol spectrum disorders.

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Keywords

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1. INTRODUCTION

In humans, *in utero* alcohol exposure results in a multitude of long-term detrimental effects to the unborn fetus. In particular, this exposure damages select regions of the central nervous system including, but not limited to, the cerebellum, corpus callosum and dentate gyrus of the hippocampus (Riley et al., 1995, Mattson et al., 1996, Archibald et al., 2001, Autti-Ramo et al., 2002). Adverse outcomes of *in utero* alcohol exposure are commonly classified under the umbrella term: Fetal Alcohol Spectrum Disorders (FASD). FASD occurs in about 1–2% of live births each year in the United States (Abel, 1998), and with the large number of false negative diagnoses of FASD, this number of FASD positive patients may be at least 8% higher in specific populations (Denny et al., 2009, Ethen et al., 2009). FASD is defined, in part, by the presence of a wide range of neurobehavioral deficits, which include physical, cognitive, learning and behavioral disabilities. In humans, prenatal alcohol exposure alters hippocampal anatomy (for review see (Berman and Hannigan, 2000) and is associated with deficits in hippocampus-associated learning and memory (Mattson et al., 2001, Kooistra et al., 2010, Crocker et al., 2011, Mattson et al., 2011). Rodent work demonstrates that third trimester alcohol exposure leads to loss of hippocampal CA1 pyramidal cells, along with several pathological and plastic events in the dendritic arborization of these neurons (González-Burgos et al., 2006) as well as decreased cell numbers in the hippocampal CA1, CA3, and dentate gyrus regions (Livy et al., 2003). Further, third-trimester alcohol exposure leads to decreased neurogenesis in the adult hippocampal dentate gyrus (Hamilton et al., 2011, Hamilton et al., 2012) as well as impaired hippocampal functioning (Murawski and Stanton, 2010, Hamilton et al., 2011, Murawski et al., 2012).

The dentate gyrus is unique as it is one of two known regions in the entire brain in which neurons are continuously generated throughout life and are known to be functionally significant (van Praag et al., 1999, Deisseroth et al., 2004, Emsley et al., 2005, Dupret et al., 2007, Snyder et al., 2009, Marrone et al., 2012). In rodents, alcohol exposure during a period (gestational days 6–21) equivalent to the first- or second-trimester in humans has no effect on total number of granule cells in the dentate gyrus on postnatal day (PD) 30–35 (Christie et al., 2005, Redila et al., 2006a). In contrast, high, binge-like alcohol exposure during the third trimester equivalent (PD 4–12) reduces the number of dentate granule cells on PD 30–35 (Miller, 1995). Further, binge-like alcohol exposure on PD 4–9 decreases survival of newly generated dentate neurons in adolescent and adult rats (Klintsova et al., 2007, Hamilton et al., 2011, Hamilton et al., 2012). Together, the data indicate that third trimester equivalent alcohol exposure produces significant and long-lasting damage to neurons in the dentate gyrus.

Behavioral interventions may provide a novel treatment to simultaneously combat the neuroanatomical damage and behavioral impairment associated with FASD. Voluntary

exercise enhances adult neurogenesis (van Praag et al., 1999, Holmes et al., 2004, Clark et al., 2011). Previous work by our lab and others indicates that voluntary exercise alone is not sufficient to overcome the long-term decreases in adult neurogenesis evident in rats exposed to neonatal alcohol (Helfer et al., 2009b, Boehme et al., 2011). However, wheel running improves spatial memory and long-term potentiation in rats exposed to alcohol during gestation (Christie et al., 2005). In addition, rearing rats in an enriched environment (environmental complexity, EC) enhances adult neurogenesis (Beauquis et al., 2010, Rizzi et al., 2011, Mustroph et al., 2012). Further, EC rescues prenatal alcohol deficits in motor training and hippocampus-dependent tasks such as the Morris Water Maze (Hannigan et al., 1993). We recently demonstrated that voluntary exercise (wheel running, WR) from PD 30–42 followed by exposure to a complex environment from PD 42–72 (WR/EC) was sufficient to rescue decreases in adult neurogenesis produced by PD 4–9 alcohol exposure, while WR alone did not promote the survival of adult-born neurons in alcohol-exposed (AE) rats (Hamilton et al., 2012). The current study expands on this previous work by adding a standard social housing control group and by examining the effect of the WR/EC intervention on alcohol-induced learning deficits.

Specifically, this study determines the impact of a behavioral intervention consisting of twelve days of voluntary access to a running wheel followed by a thirty-day exposure to a complex environment (WR/EC). Comparisons are made relative to socially housed controls (SH/SH) on alcohol-induced deficits at both the anatomical and behavioral level. In Experiment 1, the impact of PD 4–9 binge-like alcohol exposure on adult hippocampal neurogenesis was examined. We hypothesized that neonatal alcohol exposure would decrease cell survival and that WR/EC would mitigate this deficit. In Experiment 2, we explored the effect of PD 4–9 binge-like alcohol exposure on hippocampus-dependent behavior of the littermates of animals from Experiment 1. We used two tasks from our lab that are impaired by PD 4–9 alcohol exposure: trace eyeblink conditioning (Murawski et al., 2013) and a variant of contextual fear conditioning termed the context preexposure facilitation effect (CPFE; (Murawski and Stanton, 2010, Hamilton et al., 2011). We predicted that neonatal alcohol exposure would impair both tasks and that WR/EC would rescue these alcohol-induced deficits. Taken together, these studies show that a behavioral therapeutic treatment ameliorates both neural and behavioral effects of neonatal alcohol exposure.

2. EXPERIMENTAL PROCEDURES

Experiment 1 explored the long-term effects of neonatal alcohol exposure on adult neurogenesis and determined whether the behavioral intervention of WR/EC (see below) could alleviate alcohol-induced deficits in adult neurogenesis. Experiments 2A and 2B assessed trace eyeblink conditioning and the CPFE, respectively, in littermates of the rats that were used to measure neuroanatomical outcomes in Experiment 1. In trace eyeblink conditioning, the conditioned stimulus (CS) precedes the unconditioned stimulus (US) and there is a stimulus free period (trace interval) between CS offset and US onset. In the CPFE, immediate-shock training increases freezing behavior of animals given prior exposure to the conditioning context (Group Pre) relative to control animals given prior exposure to an alternate context (Group No-Pre). Figure 1 illustrates the study design and timeline for the

procedures that are described more fully below. Briefly, pups received postnatal treatments on PD4–9, were weaned on PD23, and on PD30 animals were pseudorandomly assigned to one of two housing conditions, WR/EC and SH/SH. In the WR/EC condition, half the animals from each Postnatal Treatment group were housed from PD30–42 in cages (45 × 24 × 21cm) with voluntary access to a running wheel. Then, from PD42–72, these same animals were moved to EC cages for 30 days. In the SH/SH condition, animals from each Postnatal Treatment group were housed together in cages (45 × 24 × 21cm) for the duration of the experiment. Thus, the design of this study was a 3 (Postnatal Treatment) × 2 (Intervention) factorial that yielded 6 experimental groups: AE-WR/EC, sham intubated (SI)-WR/EC, suckle control (SC)-WR/EC, AE-SH/SH, SI-SH/SH and SC-SH/SH.

2.1 Subjects

All procedures were done in accordance with the protocol approved by the Institutional Animal Care and Use Committee at the University of Delaware based on NIH standards. For all experiments, timed-pregnant litters from the animal housing colony at the University of Delaware's Office of Laboratory Animal Medicine facility were used. Female Long-Evans rats were housed overnight with breeder males and were checked for an ejaculatory plug the following morning. If a plug was found, that day was designated as gestational day 0. Pregnant females were housed in clear polypropylene cages (45 × 24 × 21cm) with standard bedding and ad lib access to water and rat chow and maintained on a 12:12h light:dark cycle. Gestational age was used as a reference for the developmental timing of all treatments. Gestational day 22 was considered to be the day of birth, PD 0. On PD 3, litters were culled to eight pups (4 female, 4 males whenever possible) and pups received subcutaneous injections of a non-toxic black ink into one or more paws to aid in pup identification. On PD 4 pups were assigned to one of three Treatment conditions: AE, SI or SC; see below for full description). A total of 58 litters (both male and female rats) were used in this study. Brain tissue was collected from both male and female rats, however only findings from male rats are reported here. Twenty-four litters were SC and 34 were mixed SI/AE litters (resulting in a total of 68 SI males and 69 AE males). Twelve male AE pups died due to improper intubations, five pups (1 SC, 4 AE) died due to other factors and 14 SC males were sacrificed randomly to maintain even distribution of pups between conditions. Of the 24 SC litters, 23 males were used for neuroanatomical studies, 42 for behavioral studies, and the rest were used for other experiments or sacrificed. All pups were weaned on PD 23 and subsequently housed in social conditions of 2–3 same-sex rats per cage (45×24×17 cm) with ad lib access to water and rat chow.

In Experiment 1, a total of 70 male Long-Evans rat pups were used: AE-WR/EC n=10; SI-WR/EC, n=8; SC-WR/EC, n=9; AE-SH/SH, n=11; SI-SH/SH n=14; and SC-SH/SH n=18. Several brains were lost due to poor perfusion and sectioning. This resulted in a total of 56 animals used for Ki67 analysis: AE-WR/EC, n=7; SI-WR/EC, n=7; SC-WR/EC, n=8; AE-SH/SH, n=8; SI-SH/SH, n=11; SC-SH/SH, n=15. In addition, several animals had to be excluded from the BrdU cell counting due to apparent bad injection and absence of BrdU+ cells in the tissue, bringing the animal number to a total of 47 animals (AE-WR/EC n=7; SI-WR/EC, n=6; SC-WR/EC, n=7; AE-SH/SH, n=6; SI-SH/SH n=8; and SC-SH/SH n=13).

In Experiment 2A and 2B, no more than one same-sex littermate was assigned to a particular experimental condition (Postnatal Treatment × Intervention Condition × Behavioral Group) except in a few inadvertent case were data from littermates were averaged together. In the eyeblink conditioning study, data from 10–15% of the rats were excluded because of poor EMG recording quality or when reflex response to the US were difficult to measure, as is conventional in our lab (cf. (Murawski et al., 2013), for details). After these exclusions, a total of 74 male Long-Evans rats were used in Experiment 2A. Of these, data from 12 rats were excluded from analyses for meeting the criteria as a statistical outlier. Outliers were defined as subjects with mean scores on measures of adaptive CR% and CMA that both exceeded ± 2 standard deviations from the group mean. These exclusions included one rat from Group AE-SH/SH, 2 rats from AE-WR/EC, 2 rats from SI-SH/SH, 2 rats from SI-WR/EC, 3 rats from group SC-SH/SH, and 2 rats from group SC-WR/EC. The analyses were conducted on the remaining 62 subjects (AE-SH/SH, n= 8; SI-SH/SH, n=15; SC-SH/SH, n=13; AE-WR/EC, n=7; SI-WR/EC, n=9; SC-WR/EC, n=10). For Experiment 2B, the subjects included those from Experiment 2A (85 males; as well as 87 additional females which also previously underwent eyeblink conditioning). Females were used because sex does not alter the alcohol effect on this task (Hamilton et al., 2011) or the effect of the WR/EC intervention (see Results, below) and extra subjects were needed to provide sufficient group sizes in the large, 12-group design. Data from 12 rats were removed as a statistical outliers (SC-SH/SH-NoPre-M, n=1; SC-SH/SH-Pre-F, n=1; SC-WR/ EC-NoPre-M, n=1; SI-SH/SH-NoPre-F, n=2; SI-SH/SH-Pre-M, n=1; SI-WR/EC-NoPre-M, n=1; AE-SH/SH-NoPre-M, n=1; AE-SH/SH-Pre-F-, n=1; AE-WR/EC-NoPre-M, n=2; AE-WR/EC-Pre-M, n=1). The analyses were conducted on the remaining 160 subjects (SC-SH/SH-NoPre, n= 7M, 8F; SC-SH/SH-Pre, n= 8M, 8F; SC-WR/EC-NoPre, n= 6M, 6F; SC-WR/EC-Pre, n= 7M, 7F; SI-SH/SH-NoPre, n= 7M, 6F; SI-SH/SH-Pre, n= 8M, 7F; SI-WR/EC-NoPre, n= 6M, 7F; SI-WR/EC-Pre, n= 8M, 8F; AE-SH/SH-NoPre, n= 5M, 6F,; AE-SH/SH-Pre, n= 5M, 8F; AE-WR/EC-NoPre, n= 2M, 6F; AE-WR/EC-Pre, n= 8M, 6F).

2.2 Neonatal Alcohol Exposure

On PD 4, animals were assigned to one of three Treatment groups: SC, SI or AE as previously described (e.g., (Hamilton et al., 2011). Litters were randomly assigned to either provide solely SC rats or to provide both SI and AE rats, wherein half the littermates of each sex were randomly assigned to either the SI or AE treatment group. SC animals were removed from the mother briefly each day in order to be weighed, but otherwise were left undisturbed. Both SI and AE animals received intragastric intubations from PD 4–9. SI animals were intubated, on the same schedule as the AE animals, and the tube was removed after approximately ten seconds without the infusion of any solution. AE animals were intubated and given a daily dose of 5.25 g/kg of alcohol, [11.9% v/v ethanol (made from 95% ethanol, Fisher Scientific) in milk formula] which was divided into two feedings each day, two hours apart. Pups were returned to their mothers between the intubations to reduce the time without maternal interaction. Preparation of milk formula followed the protocol described previously (Kelly and Lawrence, 2008). A third intubation of milk (no ethanol) was administered two hours after the second alcohol dose. Solely on PD 4, a fourth intubation of milk (no ethanol) was given four hours after the second alcohol dose in order to compensate for reduced milk intake.

2.3 Blood Alcohol Concentrations (BAC)

On PD 4, 90 minutes following the second alcohol intubation, blood samples were collected from a tail clip of each AE and SI pup. SI samples were discarded immediately, while samples collected from AE animals were utilized to determine BAC levels. BACs were assayed from the plasma of each blood sample using an Analox GL5 Alcohol Analyzer (Analox Instruments, Boston, MA) as described previously (Green et al., 2002).

2.4 Behavioral Intervention

Beginning on PD30, half the animals from each Treatment group were housed, 3 per cage, one of each postnatal treatment group, but no littermates, in the same cage, for 12 days in cages (45 × 24 × 21 cm) with free 24-hour voluntary access to stainless steel running wheels (Helfer et al., 2009a, Hamilton et al., 2012). Each morning at the onset of the light cycle (9:00am), the number of wheel rotations was recorded from counters installed on the running wheels in order to determine running distance (pooled for the 3 rats in a given cage) across days, as well as across the entire WR experience. On PD42, WR animals were removed from cages containing wheels and subsequently housed in EC for 30 days. Each EC cage housed 9–12 animals (Hamilton et al., 2012). The EC 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 3 ½ inch plastic pan filled with bedding. Cages were equipped with a variety of objects, such as hammocks, buckets, blocks, etc. that were changed every other day. Every four days, rats were removed from the EC cage and placed in regular 45 × 24 × 21 cm cages for 15 minutes while toys, bedding, food, and water were replaced in the EC cage. Animals not exposed to the behavioral intervention remained in standard group housing (n = 2–3 same-sex rats, usually one from each treatment group) throughout the duration of the experiment.

2.5 Bromodeoxyuridine (BrdU) Injections

At the onset of the light cycle (9:00hrs) on PD 41, the last full day of WR, all rats were weighed and received a single injection of BrdU [200 mg/kg in 0.9% sterile saline solution (20 mg/ml), i.p.; Sigma]. Perfusions for animals from Experiment 1 occurred 31 days after injection on PD 72 in order to obtain cell survival measures (Figure 1).

2.6 Tissue Preparation

All animals assigned to Experiment 1 were anesthetized with a dose ranging between 0.4ml–0.7ml of a ketamine/xylazine mixture [1.5 ml of xylazine (100mg/ml, VEDCO) was added to 8.5ml of ketamine (100mg/ml, VEDCO)] on PD 72. Animals were transcardially perfused with heparinized 0.1M phosphate buffer followed by 4% paraformaldehyde (Fisher Scientific) in 0.1M phosphate buffer. Brains were stored at 4°C in 30% sucrose in 4% paraformaldehyde solution. Serial horizontal sections (40μm) were cut on the cryostat, collected maintaining order throughout the entire dorso-ventral extent of hippocampus (starting at Bregma ~−3.0mm and ending at Bregma ~−9.0mm) and placed in wells containing a cryoprotectant solution (30% sucrose and 30% ethylene glycol in 0.1M phosphate buffer solution, all chemicals supplied by Fisher Scientific). Brain tissue was stored at −20°C for approximately one month.

2.7 Immunohistochemistry

Similar to previous work in our lab (Hamilton et al., 2011; 2012), every 16th section (640 μm apart) of the entire dentate gyrus was chosen in a systematic random manner (the first section was chosen randomly from the first sixteen consecutive sections that contained the dentate gyrus) and processed for immunocytochemistry.

Briefly, sections were washed in Tris Buffer Solution (TBS, Sigma-Aldrich) followed by incubation in 0.6% hydrogen peroxide and then washed in TBS. For the BrdU staining only, sections were then incubated in 50% Formamide (Fisher Scientific) in 10 mM saline-sodium citrate buffer (SSC, Sigma-Aldrich) at 65°C for 2hrs followed by a 10mM SSC wash after which sections were quickly transferred into 2N hydrochloric acid (Fisher Scientific) for 30 min at 37°C. This was followed by a 10 min wash in 0.1M Boric acid (Fisher Scientific) in TBS wash and then a TBS wash. Next, for both stainings, sections were placed in blocking solution (3% normal goat serum-0.1% Triton X100 in 50mM TBS; all components supplied by Sigma-Aldrich) for 1 hour. Sections were transferred into primary antibody [1:100 dilution, rabbit anti-Ki67 (NCL-L-Ki67-MM1) Novacastra, Norwell, MA; or 1:500 dilution, rat anti-BrdU (OBT0030) Accurate, Westbury, NY] in washing solution (3% normal goat serum in TBS) and left for 48hrs (Ki67) or for 72hrs (BrdU) at 4°C. Next, sections were washed in TBS and then washing solution after which sections were incubated in secondary antibody [1:1000, biotinylated goat anti-rabbit (BA-1000) Vector, Burlingame, CA] for 1hr or [1:250, biotinylated goat anti-rat (BA-9400) Vector, Burlingame, CA] for 2hrs at RT. This was followed by two TBS washes and one wash in washing solution (for Ki67 staining protocol) or blocking solution (for BrdU). Next, sections were incubated in ABC solution (PK-6200, Vector Laboratories, Burlingame, CA) for 1hr. Sections were then rinsed in TBS and then incubated in 0.05% DAB (Sigma-Aldrich) – 0.015% H_2O_2 (Fisher Scientific) in TBS for at least five minutes (until reaction was visible under microscope). Sections were then rinsed quickly (1–2 sec) in TBS followed by two five min TBS washes. Sections were then mounted onto slides and left to dry. The following day, slides were counterstained with 0.1% Pyronin Y and coverslipped using DPX mountant (Sigma-Aldrich).

To assess the double labeling of BrdU+ cells with DCX, NeuN or GFAP, additional sections from animals from each treatment group were immunostained with relevant primary antibodies (DCX: 1:200, anti-DCX made in goat, sc-8066; Santa Cruz; NeuN: 1:500, monoclonal anti-NeuN antibody made in mouse, MAB377, Chemicon, Temecula, CA; or GFAP: 1:200, anti-GFAP antibody made in rabbit, G4546; Sigma) followed by the incubation in the corresponding fluorescent-tagged secondary antibodies as described previously (Helfer et al., 2009b). 4–7 animals per experimental group were used for phenotyping [as in (Steiner et al., 2004, Snyder et al., 2009)]. Tissue was incubated in a cocktail of two primary antibodies, one of them being one of the antibodies above and the other – anti-BrdU antibody. Anti-BrdU primary antibody [1:500 dilution, anti-BrdU made in rat (OBT0030) Accurate, Westbury, NY] was visualized by incubation in the biotinylated donkey anti-rat secondary antibody (Biotin SP-conjugated anti-rat IgG, 1:1000, Jackson ImmunoResearch Laboratories, Bar Harbor, ME) followed by Cy2-conjugated streptavidin (Jackson ImmunoResearch Laboratories) and DCX, NeuN or GFAP were visualized by incubation with Cy3-labeled secondary antibody (Jackson ImmunoResearch Laboratories)

(previously described in (Helfer et al., 2009b). Sections were mounted on slides and coverslipped using antifade media (Prolong Gold; Molecular Probes, Grand Island, NY). To assess staining specificity, negative controls were performed by omitting primary antibodies.

2.8 Unbiased Stereology and Cell Phenotyping

Quantification of the total number of BrdU+ and Ki67+ cells was performed in accordance with an unbiased stereology approach. A series of sections (every 16th section, 8–9 sections per animal) throughout the entire dorso-ventral extent of the dentate gyrus was used for each staining batch. All cell counts were made on coded slides by an investigator blind to the treatment conditions. Counts were made in an unbiased manner within a known volume of the dentate using the optical fractionator workflow (Stereo Investigator, Micro Bright Field Inc., Williston, VT). The grid frame was set to 200 × 200 μm and the counting frame set to 200 × 200 μm. A guard zone of 2 μm and a dissector height of 18 μm were used. The frozen sections were originally cut at a nominal thickness of 40 μm. Immunostaining and mounting result in altered section thickness, which was measured at each counting site. An average section thickness was computed by the software and used to estimate the total volume of the DG sample region and total number of BrdU+ and Ki67+cells. At least 100 cells per animal were counted, the actual number of counted cells was significantly higher: e.g., for BrdU these counts were 187+/−15 (SC), 213+/−16 (SI), 168+/−19(AE).

Analysis of BrdU+ cells phenotype was performed by using a high speed, line scanning confocal microscope (LSM 5 DUO confocal microscope, Zeiss, Thornwood, NY) equipped with a LD C-Apochromat 40× (1.1 NA) water objective and the AIM software. Laser excitation wavelengths of 488 nm and 561 nm and filters BP495–555 and BP575–615 were used to localize Cy2 and Cy3-conjugated secondary antibodies, respectively. Phenotyping of BrdU+ cells was performed on the 3D digital reconstructions and orthogonal representations from a series of confocal images taken at 0.5-μm intervals, 0.6 zoom and no averaging. Cells were identified as co-labeled if an overlap of the Cy2 and Cy3 labels was observed within a given cell in each of the xy-, xz-, and yz-planes in the orthogonal view. At least twenty-five (and as many as fifty) BrdU+ cells per animal were analyzed in NeuN and DCX stained tissue to further assess the possibility of a mature or immature neuronal phenotype and at least 50 BrdU cells per animal were examined in glial stained tissue.

2.9 Eyeblink Surgery

Subjects allocated for behavioral testing underwent eyeblink surgery on PD 73–79. This procedure has been extensively described in previous reports from this lab (e.g., (Murawski et al., 2009). Following administration (i.p. injection) of a ketamine/xylazine cocktail (.85–1.0 ml/kg) and buprenorphine (0.5 ml/kg), anesthetized rats were prepared for head stage implantation. The scalp was cleaned with betadine and 70% isopropyl alcohol prior to incision. The head stage consisted of two electromyography (EMG) electrodes. Differential electrodes were implanted in the upper eyelid of the left eye with a ground wire placed subdermally to the back of the neck. A bipolar stimulating electrode was placed caudal to the left eye for US delivery. The head stage was secured to the skull with dental acrylic via three skull screws situated around the base. Following surgery, an antiseptic erythromycin ointment was placed onto the eyes. Subjects were returned to individual cages which rested

on a heating pad set to the lowest setting where they were monitored for ~45 min during recovery from anesthesia. Rats were housed in individual cages with ad libitum food and water for the remainder of the experiment. During the post-surgery recovery period, animals were handled for a minimum of three days (3 min each) until the day before the conditioning sessions began.

2.10 Eyeblink Apparatus, Design and Procedures

Trace eyeblink conditioning occurred in one of sixteen individual sound-attenuating conditioning chambers (BRS/LVE, Laurel, MD), as previously described (Ivkovich et al., 2000, Murawski et al., 2013). Rats were placed in a stainless steel wire mesh cage within the conditioning chamber. Each chamber was equipped with a fan, which provided 70 db of background noise during each conditioning session. A cable connected to peripheral equipment passed through the opening of the conditioning chamber and was secured to rat's head stage. The cable was connected to a commutator, which enabled non-restricted movement throughout the chamber during the conditioning sessions. A customized Eyeblink Conditioning System (JSA Designs, Raleigh, NC) controlled CS and US presentations and recorded electromyogram (EMG) eyeblink activity from individual subjects. Each chamber included a speaker producing the auditory CS (an 80 dB, 2.8 kHz tone presented for 380 ms.). The US was produced by a constant-current, 60 Hz square wave stimulator (World Precision Instruments, Sarasota, FL) set to deliver a 2.0 mA, 100 ms periocular shock.

On the day prior to behavioral conditioning (PD 78–83), subjects were weighed and connected to the conditioning equipment for a "puff test" in which the experimenter gently blew three quick puffs of air directed at the left eye and visually inspected the quality of the EMG recording signal on an oscilloscope independently of US presentation.

Beginning on PD 79–84, subjects received six trace eyeblink conditioning sessions as described previously (Murawski et al., 2013). Each session consisted of 100 trials with a variable inter-trial interval averaging 30 sec (ranging from 18–42 sec, in pseudorandom order). Each session consisted of ten blocks of 100 trials. Each block consisted of nine paired trials and one CS-alone (non-reinforced) trial. In a given conditioning trial, the tone CS (380 ms) was presented and followed by a 500 ms trace period terminating with onset of the perioccular shock US, for an 880 ms inter-stimulus interval (the time between CS and US onset). Subjects received two conditioning sessions per day separated by 5 hours ± 30 minutes, typically between 9–11 am and 2–4 pm daily. Prior to the first conditioning session, subjects were weighed and placed into the conditioning chambers for a 5 min acclimation period.

For Experiment 2A, data collected during each conditioning sessions included a 1400 ms trial epic acquired from rectified EMG signals, sampled in 3.5 ms bins (Ivkovich et al., 2000, Ivkovich and Stanton, 2001). On paired CS-US trials, each trial epoch was further divided into five components: 1) a 280 ms pre-CS baseline period; 2) an 80 ms startle response period following CS onset; 3) an 800 ms conditioned response (CR) period; 4) a 200 ms "adaptive CR" period (prior to US onset); and 5) a 140 ms unconditioned response (UR) period following US offset. Data were not collected during the 100 ms US period in order to avoid stimulus artifacts. The CS-alone trials were as described above except that the

CR period extended to the end of the trial epoch. The dependent measures include startle responses (SRs) and SR maximum amplitudes (SMA), URs and UR maximum amplitudes (UMA), percentage of trials with conditioned responses (CR%), adaptive conditioned responses (aCR%), and conditioned response maximum amplitudes for both CR% and aCR % (CMA & adaptive CMA, respectively). Since the adaptive CR% measure has been found to be especially sensitive to hippocampal insult (e.g., (Ivkovich and Stanton, 2001), all reported data are taken from this measure.

The baseline amplitude for individual trials was determined by the average EMG activity recorded during the pre-CS period. An EMG response was recorded if the maximum amplitude exceeded the baseline amplitude by forty arbitrary units (Skelton, 1988). Additionally, in order to eliminate "noisy" trials from statistical analyses, trials with high baselines or those with EMG activity that crossed this baseline prior to CS onset were excluded. If greater than 30% of trials for any given animal were omitted in this manner, all data from that animal were excluded (see Subjects above). All CR percentage and amplitude measures are reported from paired CS-US trials.

2.11 Contextual Fear Conditioning, Design and Procedures

The apparatus, stimuli, and procedure were as described in our previous study reporting that neonatal alcohol impairs neurogenesis and the CPFE in adult rats (Hamilton et al., 2011). Context A consisted of four clear Plexiglas chambers (16.5 × 21.1 × 21.6 cm). Context B consisted of separate wire mesh cages (22 × 22 × 26 cm) enclosed in larger sound-attenuated chambers (BRS/LVE, Laurel, MD; (Murawski et al., 2012). Context B was located in a separate room from those of Context A. All training and testing occurred in Context A. The CPFE paradigm occurred over three consecutive days. First, rats were given a 5 min preexposure session to either Context A (the conditioning context; Group Pre) or Context B (an alternate context; Group No-Pre). Twenty-four hours later, all rats were given an immediate 2 sec 1.5 mA shock upon placement in Context A. Finally, all rats were tested for conditioned fear to Context A for 5 min on the following day. Freezing behavior on the test day was recorded with a video camera connected to a Dell computer, which ran the FreezeFrame software (Wilmette, IL). This program provided offline analysis for determining the percentage of time the rats spent freezing.

Subjects from each of the dosing (SC, SI, AE) and treatment condition (SH/SH, WR/EC) were assigned to each behavioral group (Pre, No-Pre), yielding 12 experimental groups. No more than one same-sex littermate per dosing and treatment condition was assigned to any behavioral group.

For Experiment 2A and 2B, vaginal swabs were collected from female rats ranging from PD 73–112 (for a range of 9–18 consecutive days), as previously described (Hamilton et al., 2011). For the males, the genital area was wiped with a saline-moistened cotton-tipped applicator for ~5 sec. Vaginal smears were analyzed after collection in order to monitor estrous cycle. Stages were determined based on the predominance of given cell types. Swabs were collected to ensure that rats were cycling regularly and also to determine if the estrous cycle phase influenced the amount of conditioned fear displayed during the CPFE.

Following trace eyeblink conditioning, the CPFE procedure began on PD 82–112. Swabbing continued each day until the preexposure day and for a subset of rats, on the day in which female rats were found to be in proestrus, they were preexposed to either Context A or B. In these cases, male rats were yoked to females so that both sexes were preexposed on the same days (Hamilton et al., 2011).

During preexposure, rats were transported in yellow ice buckets to either Context A or Context B, in which they were allowed to freely explore for 5 min before being returned to their home cage. During the training phase rats received a 2 sec, 1.5 mA footshock in Context A (<5 sec following chamber placement), were immediately removed, placed back in the transport bucket and returned to their home cage. During testing, rats were returned to Context A for 5 min. Rats were run one or two animals per load in any given context. For each load, Context A was cleaned with 5% ammonium hydroxide prior to chamber placement.

For Experiment 2B, testing session data were analyzed with a “hand scoring” method. This involved a rater, blind to the experimental condition of the animals, inspecting video records of the test session for freezing behavior. Freezing was defined as the cessation of all movement, except for respiration. If freezing was observed, the rater advanced the video for 0.80 sec and documented the time spent freezing. Each freezing bout was totaled and scored as a percentage across the 5 min testing session. Any individual freezing bout lasting less than 0.75 sec was excluded from the total.

2.12 Statistical Analysis

Body weight was analyzed with Treatment × Day ANOVA followed by posthoc tests (Tukey’s). Analysis of other dependent measures from each experiment consisted of 3 (Postnatal Treatment) × 2 (intervention) analysis of variance (ANOVA) followed by planned comparisons of the intervention effect at each level of postnatal treatment and postnatal treatment effects at each level of intervention. These comparisons were based on the *a priori* hypotheses that alcohol treatment effects would occur in the SH/SH condition (replicating previous work) but not in the WR/EC condition and that intervention effects might occur in the different prenatal treatment conditions. In addition, step-down ANOVAs examining treatment effects separately for each Intervention group were also performed to further describe/confirm “simple effects” that were predicted by our hypothesis. The behavioral analyses were run with Statistica 12 and neuroanatomical data were analyzed with SPSS Statistics (Version 21) software.

3. RESULTS

3.1 Body Weights, BACs and Exercise

The mean peak BAC (±SEM) measured on PD 4 for all AE animals in Experiment 1 was 409.06 ± 13.96 mg/dl and in Experiment 2 it was 406.30 ± 14.69 mg/dl. These BACs are similar to previously published studies using the same alcohol dose and delivery approach (Hamilton et al., 2011). For both experiments, all animals continued to gain weight throughout treatments as summarized in Table 1. The influence of Treatment on body

weights was determined using a repeated measures ANOVA with Treatment (SC, SI, AE) as the between subjects factor and Day (4, 9, 31, 42, 68–72) as a within subjects factor, followed by post hoc analysis (Tukey's test). Note, a main effect of sex on neonatal body weights [$F_{1,155} = 5.705$, $p < 0.05$] and adolescent and adulthood body weights [$F_{1,155} =$, $p < 0.0$] was evident in Experiment 2B, therefore data are presented separately for both sexes. To ensure that the assumption of ANOVA concerning homogeneity of variance was not violated, body weights from the neonatal period (PD 4 and 9) versus adolescence and adulthood (PD 31, 42, 68–72) were analyzed separately.

In all experiments, examination of neonatal body weights revealed a Day × Treatment interaction [Exp. 1, $F_{2,67} = 33.181$, $p < 0.01$; Exp. 2a, $F_{2,65} = 44.329$, $p < 0.01$; Exp. 2b, females: $F_{2,82} = 50.910$, $p < 0.01$, males: $F_{2,73} = 49.648$, $p < 0.01$] as well as main effects of Day [Exp. 1, $F_{1,67} = 1711.43$, $p < 0.01$; Exp. 2a, $F_{1,65} = 1688.48$, $p < 0.01$; Exp. 2b, females: $F_{1,82} = 1933.665$, $p < 0.01$, males: $F_{1,73} = 1886.548$, $p < 0.01$] and of Treatment [Exp. 1, $F_{2,67} = 47.697$, $p < 0.01$; Exp. 2a, $F_{2,65} = 7.687$, $p < 0.01$; Exp. 2b, females: $F_{2,73} = 6.890$, $p < 0.05$, males: $F_{2,73} = 10.888$, $p < 0.01$]. In all experiments, post hoc tests of the treatment main effect revealed that AE animals weighed significantly less than both SI ($p < .01$) and SC ($p < .01$), which did not differ between themselves. A univariate ANOVA on PD4 weights revealed no main effect of Treatment in any experiment ($Fs < 1$), while the same type of analysis on PD9 weights revealed a significant main effect of Treatment [Exp. 1, $F_{2,67} = 88.389$, $p < 0.01$; Exp. 2a, $F_{2,65} = 17.815$, $p < 0.01$; Exp. 2b, females: $F_{2,82} = 17.098$, $p < 0.01$, males: $F_{2,73} = 22.253$, $p < 0.01$,]. Again, in all experiments, post hoc tests demonstrated that AE animals weighed significantly less than both SI ($p < .01$) and SC ($p < .01$), which did not differ between themselves.

Body weights from experiment 2A and the females of Experiment 2B, ANOVA of adolescent and adulthood showed a main effect of day [Exp. 1, $F_{1, 67} = 5623.69$, $p < 0.01$; Exp. 2a, $F_{1, 62} = 6844.18$, $p < 0.01$; Exp. 2b, females: $F_{1,82} = 4111.318$, $p < 0.01$] but neither the main effect of Treatment nor the Treatment × Day interaction were significant (ps > .05). In contrast, the body weights of males from Experiment 2B show a main effect of Day [$F_{1, 73} = 9187.546$, $p < 0.01$] as well as a main effect of Treatment [$F_{2, 73} = 6.419$, $p < 0.01$]. Univariate ANOVAs revealed a main effect of Treatment on PD30 [$F_{2, 73} = 4.930$, $p < 0.01$], wherein AE animals weighed significantly less than SC ($p<.01$) but not SI animals, a main effect of Treatment on PD 42[$F_{2, 73} = 6.172$, $p < 0.01$] wherein AE animals weighed significantly less than SC ($p<.05$) and SI ($p<.05$) animals and a main effect of Treatment on PD 72[$F_{2, 73} = 4.951$, $p < 0.01$], wherein AE animals weighed significantly less than SC ($p<.01$) but not SI animals.

Taken together, the body weight data show that in most cases alcohol transiently reduced body weight during dosing but this effect recovered by the time the WR/EC intervention began and continued throughout adult assessment of neuroanatomy and behavior.

On average, the numbers of wheel revolutions (±SEM) per 24-hour period was 2520.77±248 (approximately 3.1km) per cage. Effects of postnatal treatment on the running activity could not be analyzed because it (activity) was pooled across all rats in a given cage, and rats from three postnatal treatment groups were housed in a given cage.

3.2 BrdU Cell Counts

Neonatal alcohol exposure decreased the number of cells born on PD 41–42 that survived in PD 72 adult rats, an effect that was mitigated by WR/EC (Figure 2 and 3). A two-way ANOVA was performed to examine the role of both Treatment (SC, SI, AE) and Intervention (SH/SH, WR/EC) on BrdU+ cell survival in adult male rats. ANOVA revealed a main effect of Treatment ($F_{2,51} = 9.687$, $p < .01$) and Intervention ($F_{1, 51} = 6.882$, $p < .05$), wherein WR/EC animals had significantly greater levels of surviving BrdU+ cells than SH/SH animals (6757 ± 463 vs.5751 ± 415). Although the interaction of Treatment × Intervention was not statistically significant, planned pairwise comparisons showed that the WR/EC intervention significantly increased BrdU+ cells only in the AE condition ($p < .01$). In addition, step-down ANOVAs of the treatment effect performed separately on each intervention condition revealed a main effect of Treatment ($F_{2, 26} = 8.714$, $p < .01$) in the SH/SH condition but not the WR/EC condition ($p = .14$). Taken together, these findings suggest that the WR/EC ameliorates the adverse effect of alcohol primarily by improving survival of BrdU+ cells in AE animals.

3.3 Ki67 Cell Counts

Assessment of the long-term influence of postnatal alcohol exposure and housing condition on cell proliferation levels using Ki67 (Figure 2, C and D) revealed no difference across Treatment group or Intervention in the number of proliferating (Ki67+) cells at the time of perfusion (PD72, Figure 4). A two-way ANOVA showed no main or interactive effects of Treatment or Intervention ($Fs < 1$). Further, no difference between groups was revealed through planned pairwise comparisons. These results suggest that PD4–9 alcohol exposure does not have a long lasting effect on the proliferation of newly generated neurons in male rats, nor does exposure to WR/EC.

3.4 BrdU+ Cells Phenotypes

To determine the phenotype of the BrdU+ labeled cells located in the dentate gyrus, the percentage of co-labeling with NeuN (mature neuronal marker), DCX (immature neuronal marker), and GFAP (astrocyte marker) was estimated in at least 25 BrdU+ cells in each animal (Mirescu et al., 2004). A “Calculated neurogenesis” approach (e.g., (Kempermann and Gage, 2002, McClain et al., 2011) was used to estimate the total number of double labeled mature neurons by multiplying the number of BrdU+ cells by the neuronal phenotype percentage for each animal.

The number of double-labeled mature neurons (BrdU+/NeuN+) was significantly influenced by both Treatment and Intervention (Figure 5A). A two-way ANOVA on the number of BrdU+/NeuN+ cells (Figure 5A) revealed main effects of Treatment ($F_{2, 31} = 6.907$, $p < .05$) and Intervention ($F_{1, 31} = 10.528$, $p < .05$); however, no interaction was found. Planned comparisons revealed AE-SH/SH animals had fewer co-labeled cells than their intervention counterpart (AE-WR/EC) or their treatment counterparts (SC-SH/SH and SI-SH/SH, $p < .05$). Intervention did not significantly increase double-labeled neurons in the SC and SI conditions ($ps \geq .06$). Step-down ANOVAs demonstrated a main effect of Treatment ($F_{2, 16} = 5.463$, $p < .05$) in the SH/SH condition but not the WR/EC condition ($p > .1$).

The number of double-labeled immature neurons (DCX+/BrdU+) cells was not influenced by either Treatment or Intervention (Figure 5B). A two-way ANOVA revealed a marginal main effect of Intervention ($F_{1, 23} = 3.092$, $p < .092$), but no main effect or interaction involving Treatment. Planned pairwise comparisons indicated no differences between the AE-SH/SH group and the SC-SH/SH ($p = 0.61$) and SI-SH/SH ($p = .056$) groups. Step-down ANOVAs found no significant main effect of Treatment in either the SH/SH or WR/EC conditions.

Postnatal treatment and Intervention influenced the number of astrocytes (GFAP+/BrdU+; Figure 5C). A two-way ANOVA showed significant main effects of Treatment ($F_{2, 25} = 7.698$, $p < .01$) and Intervention ($F_{1, 25} = 8.730$, $p < .01$) but no interaction ($F < 1$). Planned comparisons showed AE-SH/SH differed from SI-SH/SH ($p < .01$) but not SC-SH/SH ($p = .076$). Additionally, the SC-WR/EC group had more astrocytes than their SC-SH/SH counterparts ($p < .05$). Step-down ANOVAs revealed a significant main effect of Treatment ($F_{2, 12} = 4.254$, $p < .05$) in both the SH/SH animals, and the WR/EC animals ($F_{2, 13} = 4.554$, $p < .05$).

3.5 Eyeblink Conditioning

3.5.1 Performance Measures – SR and UR Amplitudes—A Treatment × Intervention factorial ANOVA was performed on SMA across Session 1 and UMA across the first 10 trials of Session 1 in order to examine the contribution of CS-processing, US-processing and motor competence to learned performance (UMA Means ± SEs) for the SH/SH intervention condition: AE, 889.32 ± 29.83; SI; 716.41 ± 37.23; SC, 801.17 ± 42.11 and WR/EC intervention condition: AE, 781.16 ± 76.95; SI, 736.46 ± 55.68; SC, 758.82 ± 66.76. SMA Means ± SEs for the SH/SH intervention condition: AE, 31.85 ± 21.8; SI, 235.53 ± 70.15; SC, 168.69 ± 53.19 and the WR/EC intervention condition: AE, 125.58 ± 64.94; SI, 242.41 ± 96.02; SC, 128.63 ± 63.85.) No effects of any variables were found on either measure (all ps >.09). Thus, the pattern of performance effects suggests that treatment or intervention effects on CR measures reflect effects on associative learning processes.

3.5.2 CR Percentage—Alcohol impaired trace conditioning in the SH/SH condition (Figure 6A) but not in the WR/EC condition (Figure 6B). A 3 (Treatment) × 2 (Intervention) × 6 (Sessions) mixed ANOVA revealed main effects of Treatment ($F_{2, 280} = 5.87$, $p< 0.005$) and Sessions ($F_{5, 280} = 21.32$, $p < 0.001$) as well as a Treatment × Intervention interaction ($F_{2, 280} = 4.18$, $p < 0.021$). Planned comparisons revealed a significant increase in CR% responding by AE-WR/EC relative to AE-SH/SH animals ($p < .05$). Additionally, there was a significant difference between AE-SH/SH and SI-SH/SH groups ($p < .001$) but not between AE-WR/EC and SI-WR/EC ($p > .68$), indicating intact performance in all groups except AE-SH/SH. Step-down Treatment × Sessions ANOVA on the SH/SH condition revealed a significant main effect of Treatment ($F_{2, 165} = 8.72$, $p < 0.001$) and Sessions ($F_{5, 165} = 11.03$, $p < 0.001$), but not a significant interaction ($p = 0.097$), indicating that AE rats showed significant reductions in CR% compared to controls (SC and SI; Figure 6A). Step-down ANOVA on the WR/EC condition (Figure 6B) revealed only a main effect of Sessions ($F_{5, 115} = 10.98$, $p < 0.001$). No other statistical effects were significant (all p-

values >.67). Taken together, these findings indicate that the WR/EC intervention reversed the alcohol-induced deficit in trace conditioning that is evident in AE-SH/SH control rats.

3.5.3 CR Peak Amplitude—The CR amplitude measure also revealed a trace conditioning deficit in the SH/SH condition but not the WR/EC condition (Figure 7). Three-way ANOVA revealed only main effects of Sessions ($F_{5, 280} = 34.84$, $p < 0.001$), and an interaction of Sessions × Treatment ($F_{10, 280} = 2.25$, $p < 0.02$). In contrast to the CR% measure, the Treatment × Intervention interaction was not significant ($p > .36$). Further, planned comparisons showed no significant difference in aCMA responding between AE-SH/SH and AE-WR/EC animals was evident ($p > .63$) nor was any difference between SI-SH/SH and SI-WR/EC animals ($p > .10$; Figure 7). Additionally, there was a significant difference between AE-SH/SH and SI-SH/SH ($p < .008$) without a significant difference between AE-WR/EC and SI-WR/EC ($p > .59$). Step-down ANOVAs on the SH/SH condition revealed only a main effect of Sessions ($F_{5, 165} = 18.06$, $p < .001$) and a Treatment by Session interaction ($F_{10, 165} = 2.84$, $p < .003$; Figure 7A). In contrast, step-down ANOVA on the WR/EC condition revealed only a main effect of Sessions, ($F_{5, 115} = 18.68$, $p < .001$) (Figure 7B).

In summary, neonatal alcohol exposure impaired trace conditioning in SH/SH control rats and this effect was reversed by voluntary exercise followed by environmental complexity. This effect was not secondary to effects of WR/EC on CS- or US-processing or motor performance but rather reflected an action of this intervention on associative or cognitive factors necessary for trace conditioning performance.

3.6 Contextual Fear Conditioning

Neonatal alcohol impaired the CPFE in SH/SH control rats (Figure 8A) but not in WR/EC rats (Figure 8B), as reflected by higher levels of freezing in preexposed groups (Pre) relative to their non-preexposed (No-Pre) controls in every condition, except the AE-SH/ SH condition (Figure 8A). As in our previous report, (Hamilton et al., 2011) there were no main or interaction effects involving sex (all *Fs* < 2.02) and so subsequent ANOVAs pooled data across this factor. A 3 (Treatment) × 2 (Intervention) × 2 (Preexposure) factorial ANOVA revealed a main effect of treatment condition ($F_{2, 148} = 3.06$, $p < .05$) and preexposure group ($F_{1, 148} = 56.87$, $p < .001$) but no main or interaction effects involving Intervention (ps > .082). Planned comparisons indicated no preexposure group difference among group AE-SH/SH ($p > .5$), however group Pre froze more than group No-Pre in the AE-WR/EC intervention condition ($p < .003$), indicating that impairment of the CPFE in AE-SH/ SH animals was reversed by exposure to the WR/EC intervention. In contrast, Pre animals froze more than No-Pre controls in both the SI and SC conditions, regardless of Intervention condition (all ps < .04).

Step-down 3 (Treatment) × 2 (Preexposure) ANOVA for the SH/SH intervention condition revealed a main effect of preexposure group ($F_{1, 77} = 22.65$, $p < .0001$) and a Preexposure × Treatment interaction ($F_{2,77} = 4.68$, $p < .02$), whereas, for the WR/EC intervention condition, ANOVA revealed a main effect of preexposure group ($F_{1, 71} = 33.14$, $p < .0001$), but no main or interactive effects of treatment ($p > .91$).

4. DISCUSSION

This study demonstrates that a combination of voluntary exercise followed by living in a complex environment (WR/EC) is a behavioral intervention that not only partially restores adult neurogenesis in the dentate gyrus (Experiment 1) but also improves hippocampus-dependent behavior (Experiments 2a,b) in animals neonatally exposed to alcohol. Previously, we demonstrated that voluntary exercise limited to twelve-days is not sufficient to increase adult neurogenesis in AE rats (Hamilton et al., 2012). Here we expand on our previous neuroanatomical work by showing the beneficial influence of a "super-intervention", or WR/EC, in comparison to a standard housing (SH/SH) control condition. It also extends our previous report that neonatal alcohol impairs neurogenesis and behavior (Hamilton et al., 2011) by showing that the WR/EC intervention can alleviate both outcomes in littermates.

Third trimester binge-like alcohol exposure on PD4–9 does not affect cell proliferation in adolescent rats (PD 42) (as shown in (Hamilton et al., 2012) but significantly decreases survival of those cells when analyzed thirty days post mitosis (PD 72). These results replicate previous work in our lab and add to the growing literature that demonstrates a negative impact of neonatal alcohol exposure on cell survival (Redila et al., 2006b, Ieraci and Herrera, 2007, Klintsova et al., 2007, Hamilton et al., 2011, Hamilton et al., 2012). Still, there are also conflicting reports that neonatal alcohol exposure does not impair cell survival (Wozniak et al., 2004, Helfer et al., 2009b, Gil-Mohapel et al., 2011). Methodological differences across these studies - using mice as subjects (Wozniak et al., 2004), multiple BrdU injections (Helfer et al., 2009b) or using both male and female rats (Gil-Mohapel et al., 2011) - may explain discrepancies in the data relative to the current results. For example, sex hormones influence cell proliferation and it is possible that females are injected on the day of proestrus (when estrogen levels are high), which would result in a significantly higher number of proliferating cells being labeled (Tanapat et al., 1999). Although more work is needed to elucidate these issues, the present findings add to our knowledge about the long-lasting negative effect of alcohol exposure during development on hippocampal adult neurogenesis.

Interestingly, in the rat model neonatal alcohol exposure caused decreases in distinct cell populations. In particular, neonatal alcohol exposure decreased the number of mature neurons (BrdU+/NeuN+; Figure 5A); while the number of immature neurons expressing DCX and labeled with BrdU remained constant thirty days post mitosis (Experiment 1). Previously, we have shown that neonatal alcohol exposure does not have a long lasting effect on DCX+/BrdU+ labeled immature neurons in the adolescent animal (Helfer et al., 2009b). The current data indicate that a long-lasting (up to PD72) effect of neonatal alcohol is specific to the process of neuronal survival but does not seem to affect proliferation per se and differentiation.

As noted above, this study extended a previous one that examined the impact of a WR alone (WR/SH) intervention compared to a WR/EC intervention (Hamilton et al., 2012). In particular, we conclude that exposure to WR/EC enhanced survival of BrdU+ cells and rescued the alcohol-induced deficits, relative to both normal control (SH/SH) animals

(Experiment 1) as well as to animals exposed to a WR alone intervention (Hamilton et al., 2012). There is an ongoing discussion in the field of adult neurogenesis about the specific impact of exercise vs exposure to components of environmental complexity (i.e., social interaction, sensory stimulation and physical activity) on new neurons generation in the adult brain. Two recent studies (Kobilo et al., 2011, Mustroph et al., 2012) that addressed this question have shown that housing mice in an enriched environment that contains toys and running wheels produced the highest level of hippocampal neurogenesis, followed by the group exposed to an environment with running wheels only. It is possible that if additional group of rats with continuous access to running wheels (during all 42 days of the intervention) were added to the experimental design we would also see the benefit of exercise on adult neurogenesis in AE animals.

The ability of WR/EC to rescue alcohol-induced deficits may occur through an increase in neurotrophic factors, such as vascular endothelial growth factor (VEGF) and brain derived neurotrophic factor (BDNF), which alcohol has been previously shown to decrease (Crews and Nixon, 2003, Olson et al., 2006). Exercise upregulates β-endorphin expression, as well as the expression of VEGF and BDNF (Fabel et al., 2003, Adlard et al., 2004, Olson et al., 2006). Increases in these factors may play a role in the higher number of proliferating cells. In parallel, EC also increases neurotrophic factors (Brown et al., 2003, During and Cao, 2006, Olson et al., 2006). Along with increased levels of neurotrophic factors (Olson et al., 2006, Angelucci et al., 2009), EC has been associated with increased levels of adult neurogenesis. Specifically, EC appears to enhance cell survival (Brown et al., 2003, Olson et al., 2006). The combination of WR followed by EC provides the potential for an optimal intervention that would lead to the highest levels of adult neurogenesis. The current data supports this as WR/EC provided a more substantial rescue of alcohol-induced decreases in levels of cell survival than wheel running alone (Hamilton et al., 2012). Previous work indicated WR alone was not sufficient to enhance the survival of newly generated neurons in rats exposed to alcohol postnatally (Helfer et al., 2009b). Further, exposure to EC is not sufficient to rescue alcohol-induced deficits in adult neurogenesis in mice (Choi et al., 2005). This suggests a possible synergistic effect of WR/EC that is necessary as an intervention for adverse effects of neonatal alcohol exposure.

Binge-like alcohol exposure from PD 4–9 results in significant cell loss in the hippocampus (Livy et al., 2003) and leads to various behavioral deficits in tasks associated with hippocampal function (e.g., (Goodlett and Johnson, 1997, Murawski and Stanton, 2010). The current report examined a possible link between developmental alcohol exposure, hippocampal adult neurogenesis and behavioral performance in a hippocampus-dependent trace eyeblink conditioning paradigm. In the standard housing condition (SH/SH), neonatal alcohol impaired CR production and amplitude relative to their SC and SI control groups. In contrast, AE animals from the WR/EC intervention condition, displayed comparable performance to controls, indicating a mitigation of the alcohol-induced impairment following wheel running and environmental complexity.

It is possible that cerebellar damage may contribute to the alcohol-related deficits found in trace eyeblink conditioning. The sensitivity of the cerebellum to developmental alcohol exposure, particularly from PD 4–9, has been well documented in studies involving delay

eyeblink conditioning (Stanton and Goodlett, 1998, Goodlett et al., 2000, Green et al., 2000, Tran et al., 2007, Brown et al., 2008, Brown et al., 2009). Alterations in single-unit interpositus nuclei activity and cerebellar deep nuclei cell loss also correlate with weakened conditioning in alcohol-exposed animals (Green et al., 2002). However, similar to the current report, PD4–9 alcohol exposure also impairs hippocampus-dependent trace eyeblink conditioning (Thomas and Tran, 2012, Murawski et al., 2013). Recently, Thomas and Tran (Thomas and Tran, 2012) reported that choline administration attenuated the alcohol-related deficits in trace, but not delay eyeblink conditioning. This may reflect altered cholinergic signaling within the hippocampus (Monk et al., 2012) or hypermethylation in the hippocampus and prefrontal cortex, which was significantly reduced after choline supplementation (Otero et al., 2012). Trace eyeblink conditioning increases the survival of newly generated cells (Gould et al., 1999, Shors et al., 2001) in a manner that correlates with asymptotic performance (Dalla et al., 2007, Dalla et al., 2009). These reports, together with the present findings, suggest a possible link between developmental alcohol exposure, impaired adult neurogenesis and trace eyeblink conditioning. However, it is also possible that other brain regions (e.g., medial prefrontal cortex) or neural processes could contribute to the alcohol-induced behavioral deficits and intervention effects reported here.

Results from Experiment 2b indicate that alcohol impairs the CPFE in normally reared (SH/SH) rats. The conditioned fear demonstrated by SC and SI controls given prior exposure to the shock context, but not AE animals from the same exposure condition, replicates previous findings from this lab (Hamilton et al., 2011). Similar to trace eyeblink conditioning (Experiment 2a), the WR/EC intervention reversed this impairment. Attenuation of the alcohol deficit in animals given access to a running wheel followed by environmental complexity may be due, in part, to enhanced hippocampal neurogenesis in these animals. Previous reports linking neurogenesis to fear conditioning have reported inconsistent results (Shors et al., 2002, Saxe et al., 2006, Winocur et al., 2006), suggesting neurogenesis may be related to the formation of some but not all types of hippocampus-dependent memory. However, all of these studies do reliably indicate that disrupting neurogenesis does not alter performance in various hippocampal-independent tasks such as cued or delay fear conditioning. Indeed, we have reported that neither neonatal alcohol (Murawski & Stanton, 2010; Schreiber et al., 2013) nor our WR/EC intervention has any effect on delay fear conditioning (Schreiber et al., 2013). This is important because it indicates that impairment and rescue of the CPFE in the present study is specific to context conditioning and does not reflect sensory, motor, or motivational “performance effects” of alcohol treatment or our behavioral intervention. Drew et al (Drew et al., 2010) report impairment in contextual fear conditioning following irradiation treatment in mice when a single-trial procedure was used but not during multiple-trial procedures. Interestingly, the CPFE paradigm used in the current report also involves a single-trial conditioning phase, supporting the possible importance of trial number in mapping of neurogenesis onto contextual fear conditioning. We have recently reported developmental alcohol exposure impairs context and trace conditioning in adult rats (Schreiber et al., 2013). In contrast to the present findings, WR/EC did not reverse alcohol-induced deficits in either trace- or contextual-fear conditioning. Consistent with Drew et al (Drew et al., 2010), contextual fear conditioning in this study involved multiple shocks during prolonged exposure to the

context and was likely not dependent on adult granule cell neurogenesis. These findings highlight the variation in the ability of WR/EC to rescue alcohol-induced deficits in various hippocampus-dependent tasks. As acknowledged previously, there are many possible mechanisms, apart from adult neurogenesis, that may contribute both to memory deficits produced by neonatal alcohol; as well as to the rescue of these deficits by WR/EC. A more complete understanding of these possibilities requires further research.

The issues of long-term neonatal alcohol effects on brain plasticity and possibilities of its enhancement in response to exercise and environmental stimulation are very important as they may serve as a foundation for successful therapeutic intervention(s) for children with FASD. Our data indicates that a “super-intervention” in the form of exercise followed by complex environmental exposure is sufficient to successfully enhance brain plasticity and recovery of behavioral function.

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Abbreviations

AE	alcohol exposed
BAC	blood alcohol concentration
BrdU	BromodeoxyUridine
CMAs	conditioned response maximal amplitudes
CPFE	context preexposure facilitation effect
CR	Conditioned response
CS	Conditioned stimulus
EC	Environmental complexity
EMG	electromyogram
FASD	Fetal alcohol spectrum disorder
No-Pre	Preexposure to alternate context
PD	Postnatal day
Pre	Preexposure to context
SC	suckle control
SH	social housing
SI	sham intubated
SMAs	Startle response maximal amplitudes
SRs	Startle responses

UMAs	Unconditioned response maximal amplitudes
UR	Unconditioned response
US	Unconditioned stimulus
WR	Wheel running

References

Abel E. Fetal Alcohol Syndrome: The American paradox. Alcohol and Alcoholism. 1998; 33:195–201. [PubMed: 9632044]

Adlard PA, Perreau VM, Engesser-Cesar C, Cotman CW. The timecourse of induction of brain-derived neurotrophic factor mRNA and protein in the rat hippocampus following voluntary exercise. Neuroscience Letters. 2004; 363:43–48. [PubMed: 15157993]

Angelucci F, De Bartolo P, Gelfo F, Foti F, Cutuli D, Bossu P, Caltagirone C, Petrosini L. Increased concentrations of nerve growth factor and brain-derived neurotrophic factor in the rat cerebellum after exposure to environmental enrichment. Cerebellum. 2009; 8:499–506. [PubMed: 19688409]

Archibald S, Fennema-Notestine C, Gamst A, Riley E, Mattson S, Jernigan T. Brain dysmorphology in individuals with severe prenatal alcohol exposure. Developmental Medicine and Child Neurology. 2001; 43:148–154. [PubMed: 11263683]

Autti-Ramo I, Autti T, Korkman M, Kettunen S, Salonen O, Valanne L. MRI findings in children with school problems who had been exposed prenatally to alcohol. Dev Med Child Neurol. 2002; 44:98–106. [PubMed: 11848116]

Beauquis J, Roig P, De Nicola AF, Saravia F. Short-Term Environmental Enrichment Enhances Adult Neurogenesis, Vascular Network and Dendritic Complexity in the Hippocampus of Type 1 Diabetic Mice. PLoS ONE. 2010; 5:e13993. [PubMed: 21085588]

Berman R, Hannigan J. Effects of prenatal alcohol exposure on the hippocampus: spatial behavior, electrophysiology and neuroanatomy. Hippocampus. 2000; 10:94–110. [PubMed: 10706221]

Boehme F, Gil-Mohapel J, Cox A, Patten A, Giles E, Brocardo PS, Christie BR. Voluntary exercise induces adult hippocampal neurogenesis and BDNF expression in a rodent model of fetal alcohol spectrum disorders. European Journal of Neuroscience. 2011; 33:1799–1811. [PubMed: 21535455]

Brown J, Cooper-Kuhn CM, Kempermann G, Van Praag H, Winkler J, Gage FH, Kuhn HG. Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. European Journal of Neuroscience. 2003; 17:2042–2046. [PubMed: 12786970]

Brown KL, Burman MA, Duong HB, Stanton ME. Neonatal binge alcohol exposure produces dose dependent deficits in interstimulus interval discrimination eyeblink conditioning in juvenile rats. Brain Research. 2009; 1248:162–175. [PubMed: 19007754]

Brown KL, Calizo LH, Stanton ME. Dose-Dependent Deficits in Dual Interstimulus Interval Classical Eyeblink Conditioning Tasks Following Neonatal Binge Alcohol Exposure in Rats. Alcoholism: Clinical and Experimental Research. 2008; 32:277–293.

Choi I, Allan AM, Cunningham LA. Moderate Fetal Alcohol Exposure Impairs the Neurogenic Response to an Enriched Environment in Adult Mice. Alcoholism: Clinical & Experimental Research. 2005; 29:2053–2062.

Christie BR, Swann SE, Fox CJ, Froc D, Lieblich SE, Redila V, Webber A. Voluntary exercise rescues deficits in spatial memory and long-term potentiation in prenatal ethanol-exposed male rats. European Journal of Neuroscience. 2005; 21:1719–1726. [PubMed: 15845099]

Clark P, Kohman R, Miller D, Bhattachary aT, Brzezinska W, Rhodes J. Genetic influences on exercise-induced adult hippocampal neurogenesis across 12 divergent mouse strains. Genes Brain Behav. 2011; 10:345–353. [PubMed: 21223504]

Crews F, Nixon K. Alcohol, neural stem cells, and adult neurogenesis. Alcohol Res Health. 2003; 27:197–204. [PubMed: 15303631]

Crocker N, Vaurio L, Riley E, Mattson S. Comparison of verbal learning and memory in children with heavy prenatal alcohol exposure or attention-deficit/hyperactivity disorder. Alcohol Clin Exp Res. 2011; 35:1114–1121. [PubMed: 21410480]

Dalla C, Bangasser D, Edgecomb C, Shors T. Neurogenesis and learning: Acquisition and asymptotic performance predict how many new cells survive in the hippocampus. Neurobiology of Learning and Memory. 2007; 88:143–148. [PubMed: 17412617]

Dalla C, Papachristos EB, Whetstone AS, Shors TJ. Female rats learn trace memories better than male rats and consequently retain a greater proportion of new neurons in their hippocampi. Proceedings of the National Academy of Sciences. 2009; 106:2927–2932.

Deisseroth K, Singla S, Toda H, Monje M, Palmer TD, Malenka RC. Excitation-Neurogenesis Coupling in Adult Neural Stem/Progenitor Cells. Neuron. 2004; 42:535–552. [PubMed: 15157417]

Denny C, Tsai J, Floyd R, PP G. Alcohol Use Among Pregnant and Nonpregnant Women of Childbearing Age --- United States, 1991—2005. Div of Birth Defects and Developmental Disabilities, National Center on Birth Defects and Developmental Disabilities, CDC 5. 2009; 58:529–532.

Drew M, Denny C, Hen R. Arrest of adult hippocampal neurogenesis in mice impairs single-but not multiple-trial contextual fear conditioning. Behav Neurosci. 2010; 124:446–454. [PubMed: 20695644]

Dupret D, Fabre A, Döbrössy MtDn, Panatier A, Rodríguez JJ, Lamarque Sp, Lemaire V, Oliet SHR, Piazza P-V, Abrous DN. Spatial Learning Depends on Both the Addition and Removal of New Hippocampal Neurons. PLoS Biol. 2007; 5:e214. [PubMed: 17683201]

During M, Cao L. VEGF, a mediator of the effect of experience on hippocampal neurogenesis. Curr Alzheimer Res. 2006; 3:29–33. [PubMed: 16472200]

Emsley JG, Mitchell BD, Kempermann G, Macklis JD. Adult neurogenesis and repair of the adult CNS with neural progenitors, precursors, and stem cells. Prog Neurobiol. 2005; 75:321–341. [PubMed: 15913880]

Ethen M, Ramadhani T, Scheuerle A. Alcohol consumption by women before and during pregnancy. Maternal Child Health Journal. 2009; 13:274–285. [PubMed: 18317893]

Fabel K, Fabel K, Tam B, Kaufer D, Baiker A, Simmons N, Kuo CJ, Palmer TD. VEGF is necessary for exercise-induced adult hippocampal neurogenesis. European Journal of Neuroscience. 2003; 18:2803–2812. [PubMed: 14656329]

Gil-Mohapel J, Simpson JM, Ghilan M, Christie BR. Neurogenesis in Huntington's disease: Can studying adult neurogenesis lead to the development of new therapeutic strategies? Brain Research. 2011; 1406:84–105. [PubMed: 21742312]

González-Burgos I, Alejandre-Gómez M, Olvera-Cortés M, Pérez-Vega M, Evans S, Feria-Velasco A. Prenatal-through-postnatal exposure to moderate levels of ethanol leads to damage on the hippocampal CA1 field of juvenile rats: a stereology and Golgi study. Neurosci Res. 2006; 56:400–408. [PubMed: 16978724]

Goodlett, C.; Stanton, M.; Steinmetz, J. Alcohol-induced damage to the developing brain: functional approaches using classical eyeblink conditioning. In: Woodruff-Pak, D.; Steinmetz, J., editors. Eyeblink Classical Conditioning, vol. 2, Animal Models. Boston: Kluwer Academic Publishers; 2000. p. 135-153.

Goodlett CR, Johnson TB. Neonatal binge ethanol exposure using intubation: timing and dose effects on place learning. Neurotoxicol Teratol. 1997; 19:435–446. [PubMed: 9392779]

Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ. Learning enhances adult neurogenesis in the hippocampal formation. Nature Neuroscience. 1999; 2:260–265.

Green J, Rogers R, Goodlett C, Steinmetz J. Impairment in eyeblink classical conditioning in adult rats exposed to ethanol as neonates. Alcohol Clin Exp Res. 2000; 24:438–447. [PubMed: 10798579]

Green JT, Johnson TB, Goodlett CR, Steinmetz JE. Eyeblink Classical Conditioning and Interpositus Nucleus Activity Are Disrupted in Adult Rats Exposed to Ethanol as Neonates. Learning & Memory. 2002; 9:304–320. [PubMed: 12359839]

Hamilton G, Boschen K, Goodlett C, Greenough W, Klintsova A. Housing in environmental complexity following wheel running augments survival of newly generated hippocampal neurons

in a rat model of binge alcohol exposure during the third trimester equivalent. Alcohol Clin Exp Res. 2012; 36:1196–1204. [PubMed: 22324755]

Hamilton GF, Murawski NJ, St. Cyr SA, Jablonski SA, Schiffino FL, Stanton ME, Klintsova AY. Neonatal alcohol exposure disrupts hippocampal neurogenesis and contextual fear conditioning in adult rats. Brain Research. 2011; 1412:88–101. [PubMed: 21816390]

Hannigan J, Berman R, Zajac C. Environmental enrichment and the behavioral effects of prenatal exposure to alcohol in rats. Neurotoxicol Teratol. 1993; 15:261–266. [PubMed: 8413080]

Helfer J, Calizo L, Dong W, Goodlett C, Greenough W, Klintsova A. Binge-like postnatal alcohol exposure triggers cortical gliogenesis in adolescent rats. The Journal of Comparative Neurology. 2009a; 514:259–271. [PubMed: 19296475]

Helfer JL, Goodlett CR, Greenough WT, Klintsova AY. The effects of exercise on adolescent hippocampal neurogenesis in a rat model of binge alcohol exposure during the brain growth spurt. Brain Research. 2009b; 1294:1–11. [PubMed: 19647724]

Holmes M, Galea L, Mistlberger R, Kempermann G. Adult hippocampal neurogenesis and voluntary running activity: Circadian and dose-dependent effects. Journal of Neuroscience Research. 2004; 76:216–222. [PubMed: 15048919]

Ieraci A, Herrera D. Single alcohol exposure in early life damages hippocampal stem/progenitor cells and reduces adult neurogenesis. Neurobiology of Disease. 2007; 26:597–605. [PubMed: 17490887]

Ivkovich D, Paczkowski CM, Stanton ME. Ontogeny of delay versus trace eyeblink conditioning in the rat. Developmental Psychobiology. 2000; 36:148–160. [PubMed: 10689285]

Ivkovich D, Stanton ME. Effects of Early Hippocampal Lesions on Trace, Delay, and Long-Delay Eyeblink Conditioning in Developing Rats. Neurobiology of Learning and Memory. 2001; 76:426–446. [PubMed: 11726246]

Kelly S, Lawrence C. Intragastric intubation of alcohol during the perinatal period. Methods Mol Biol. 2008; 447:101–110. [PubMed: 18369914]

Kempermann G, Gage FH. Genetic influence on phenotypic differentiation in adult hippocampal neurogenesis. Developmental Brain Research. 2002; 134:1–12. [PubMed: 11947932]

Klintsova AY, Helfer JL, Calizo LH, Dong WK, Goodlett CR, Greenough WT. Persistent Impairment of Hippocampal Neurogenesis in Young Adult Rats Following Early Postnatal Alcohol Exposure. Alcoholism: Clinical and Experimental Research. 2007; 31:2073–2082.

Kobilo T, Liu QR, Gandhi K, Mughal M, Shaham Y, van Praag H. Running is the neurogenic and neurotrophic stimulus in environmental enrichment. Learning & memory (Cold Spring Harbor, NY). 2011; 18:605–609.

Kooistra L, Crawford S, Gibbard B, Ramage B, Kaplan B. Differentiating attention deficits in children with fetal alcohol spectrum disorder or attention-deficit-hyperactivity disorder. Dev Med Child Neurol. 2010; 52:205–211. [PubMed: 19549201]

Livy DJ, Miller EK, Maier SE, West JR. Fetal alcohol exposure and temporal vulnerability: effects of binge-like alcohol exposure on the developing rat hippocampus. Neurotoxicology and Teratology. 2003; 25:447–458. [PubMed: 12798962]

Marrone DF, Ramirez-Amaya V, Barnes CA. Neurons generated in senescence maintain capacity for functional integration. Hippocampus. 2012; 22:1134–1142. [PubMed: 21695743]

Mattson S, Crocker N, TT N. Fetal alcohol spectrum disorders: neuropsychological and behavioral features. Neuropsychol Rev. 2011; 21:81–101. [PubMed: 21503685]

Mattson S, Riley E, Sowell E, Jernigan T, Sobel T, Jones K. A decrease in the size of the basal ganglia in children with fetal alcohol syndrome. Alcoholism: Clinical and Experimental Research. 1996; 20:1088–1093.

Mattson SN, Schoenfeld AM, Riley EP. Teratogenic effects of alcohol on brain and behavior. Alcohol Research and Health. 2001; 25:185–191. [PubMed: 11810956]

McClain JA, Hayes DM, Morris SA, Nixon K. Adolescent binge alcohol exposure alters hippocampal progenitor cell proliferation in rats: Effects on cell cycle kinetics. The Journal of Comparative Neurology. 2011; 519:2697–2710. [PubMed: 21484803]

Miller M. Generation of neurons in the rat dentate gyrus and hippocampus: effects of prenatal and postnatal treatment with ethanol. Alcoholism: Clinical and Experimental Research. 1995; 19:1500–1509.

Mirescu C, Peters JD, Gould E. Early life experience alters response of adult neurogenesis to stress. Nat Neurosci. 2004; 7:841–846. [PubMed: 15273691]

Monk B, Leslie F, Thomas J. The effects of perinatal choline supplementation on hippocampal cholinergic development in rats exposed to alcohol during the brain growth spurt. Hippocampus. 2012; 22:1750–1757. [PubMed: 22431326]

Murawski N, Stanton M. Variants of contextual fear conditioning are differentially impaired in the juvenile rat by binge ethanol exposure on postnatal days 4–9. Behav Brain Res. 2010; 212:133–142. [PubMed: 20385174]

Murawski NJ, Brown KL, Stanton ME. Interstimulus interval (ISI) discrimination of the conditioned eyeblink response in a rodent model of autism. Behavioural Brain Research. 2009; 196:297–303. [PubMed: 18940202]

Murawski NJ, Jablonski SA, Brown KL, Stanton ME. Effects of neonatal alcohol dose and exposure window on long delay and trace eyeblink conditioning in juvenile rats. Behavioural Brain Research. 2013; 236:307–318. [PubMed: 22947903]

Murawski NJ, Klintsova AY, Stanton ME. Neonatal alcohol exposure and the hippocampus in developing male rats: effects on behaviorally induced CA1 c-Fos expression, CA1 pyramidal cell number, and contextual fear conditioning. Neuroscience. 2012; 206:89–99. [PubMed: 22285885]

Mustroph M, Chen S, Desai S, Cay E, Deyoung E, Rhodes J. Aerobic exercise is the critical variable in an enriched environment that increases hippocampal neurogenesis and water maze learning in male C57BL/6J mice. Neuroscience. 2012; 219:62–71. [PubMed: 22698691]

Olson AK, Eadie B, Ernst C, Christie BR. Environmental enrichment and voluntary exercise massively increase neurogenesis in the adult hippocampus via dissociable pathways. Hippocampus. 2006; 16:250–260. [PubMed: 16411242]

Otero NKH, Thomas JD, Saski CA, Xia X, Kelly SJ. Choline Supplementation and DNA Methylation in the Hippocampus and Prefrontal Cortex of Rats Exposed to Alcohol During Development. Alcoholism: Clinical and Experimental Research. 2012; 36:1701–1709.

Redila V, Olson A, Swann S, Mohades G, Webber A, Weinberg J, Christie B. Hippocampal cell proliferation is reduced following prenatal ethanol exposure but can be rescued with voluntary exercise. Hippocampus. 2006a; 16:305–311. [PubMed: 16425237]

Redila VA, Olson AK, Swann SE, Mohades G, Webber AJ, Weinberg J, Christie BR. Hippocampal cell proliferation is reduced following prenatal ethanol exposure but can be rescued with voluntary exercise. Hippocampus. 2006b; 16:305–311. [PubMed: 16425237]

Riley E, Mattson S, Sowell E, Jernigan T, Sobel D, Jones K. Abnormalities of the corpus callosum in children prenatally exposed to alcohol. Alcoholism: Clinical and Experimental Research. 1995; 19:1198–1202.

Rizzi S, Bianchi P, Guidi S, Ciani E, Bartesaghi R. Impact of environmental enrichment on neurogenesis in the dentate gyrus during the early postnatal period. Brain Res. 2011; 1415:23–33. [PubMed: 21885039]

Saxe M, Battaglia F, Wang J, Malleret G, David D, Monckton J, Garcia A, Sofroniew M, Kandel E, Santarelli L, Hen R, Drew M. Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. PNAS. 2006; 103:17501–17506. [PubMed: 17088541]

Schreiber WB, St. Cyr SA, Jablonski SA, Hunt PS, Klintsova AY, Stanton ME. Effects of exercise and environmental complexity on deficits in trace and contextual fear conditioning produced by neonatal alcohol exposure in rats. Developmental Psychobiology. 2013; 55:483–495. [PubMed: 22644967]

Shors T, Townsend D, Zhao M, Kozorovitskiy Y, Gould E. Neurogenesis may relate to some but not all types of hippocampal-dependent learning. Hippocampus. 2002; 12:578–584. [PubMed: 12440573]

Shors TJ, Miesegaes G, Beylin A, Zhao M, Rydel T, Gould E. Neurogenesis in the adult is involved in the formation of trace memories. Nature. 2001; 410:372–376. [PubMed: 11268214]

Skelton R. Bilateral cerebellar lesions disrupt conditioned eyelid responses in unrestrained rats. Behav Neurosci. 1988; 102:586–590. [PubMed: 3166733]

Snyder JS, Choe JS, Clifford MA, Jeurling SI, Hurley P, Brown A, Kamhi JF, Cameron HA. Adult-Born Hippocampal Neurons Are More Numerous, Faster Maturing, and More Involved in Behavior in Rats than in Mice. The Journal of Neuroscience. 2009; 29:14484–14495. [PubMed: 19923282]

Stanton M, Goodlett C. Neonatal ethanol exposure impairs eyeblink conditioning in weanling rats. Alcohol Clin Exp Res. 1998; 22:270–275. [PubMed: 9514318]

Steiner B, Kronenberg G, Jessberger S, Brandt M, Reuter K, Kempermann G. Differential regulation of gliogenesis in the context of adult hippocampal neurogenesis in mice. Glia. 2004; 46:41–52. [PubMed: 14999812]

Tanapat P, Hastings NB, Reeves AJ, Gould E. Estrogen Stimulates a Transient Increase in the Number of New Neurons in the Dentate Gyrus of the Adult Female Rat. J Neurosci. 1999; 19:5792–5801. [PubMed: 10407020]

Thomas JD, Tran TD. Choline supplementation mitigates trace, but not delay, eyeblink conditioning deficits in rats exposed to alcohol during development. Hippocampus. 2012; 22:619–630. [PubMed: 21542051]

Tran T, Stanton M, Goodlett C. Binge-like ethanol exposure during the early postnatal period impairs eyeblink conditioning at short and long CS-US intervals in rats. Dev Psychobiol. 2007; 49:589–605. [PubMed: 17680607]

van Praag H, Christie BR, Sejnowski TJ, Gage FH. Running enhances neurogenesis, learning, and long-term potentiation in mice. PNAS. 1999; 96:13427–13431. [PubMed: 10557337]

Winocur G, Wojtowicz J, Sekeres M, Snyder J, Wang S. Inhibition of neurogenesis interferes with hippocampus-dependent memory function. Hippocampus. 2006; 16:296–304. [PubMed: 16411241]

Wozniak DF, Hartman RE, Boyle MP, Vogt SK, Brooks AR, Tenkova T, Young C, Olney JW, Muglia LJ. Apoptotic neurodegeneration induced by ethanol in neonatal mice is associated with profound learning/memory deficits in juveniles followed by progressive functional recovery in adults. Neurobiology of Disease. 2004; 17:403–414. [PubMed: 15571976]

HIGHLIGHTS

- Neonatal alcohol impairs learning in adulthood, permanently damages the hippocampus
- ”Super-intervention” (exercise followed by complex environment) was studied
- In neonatally alcohol-exposed rats “super-intervention” increased new neuron survival
- “Super-intervention” reversed learning deficits in alcohol-exposed rats

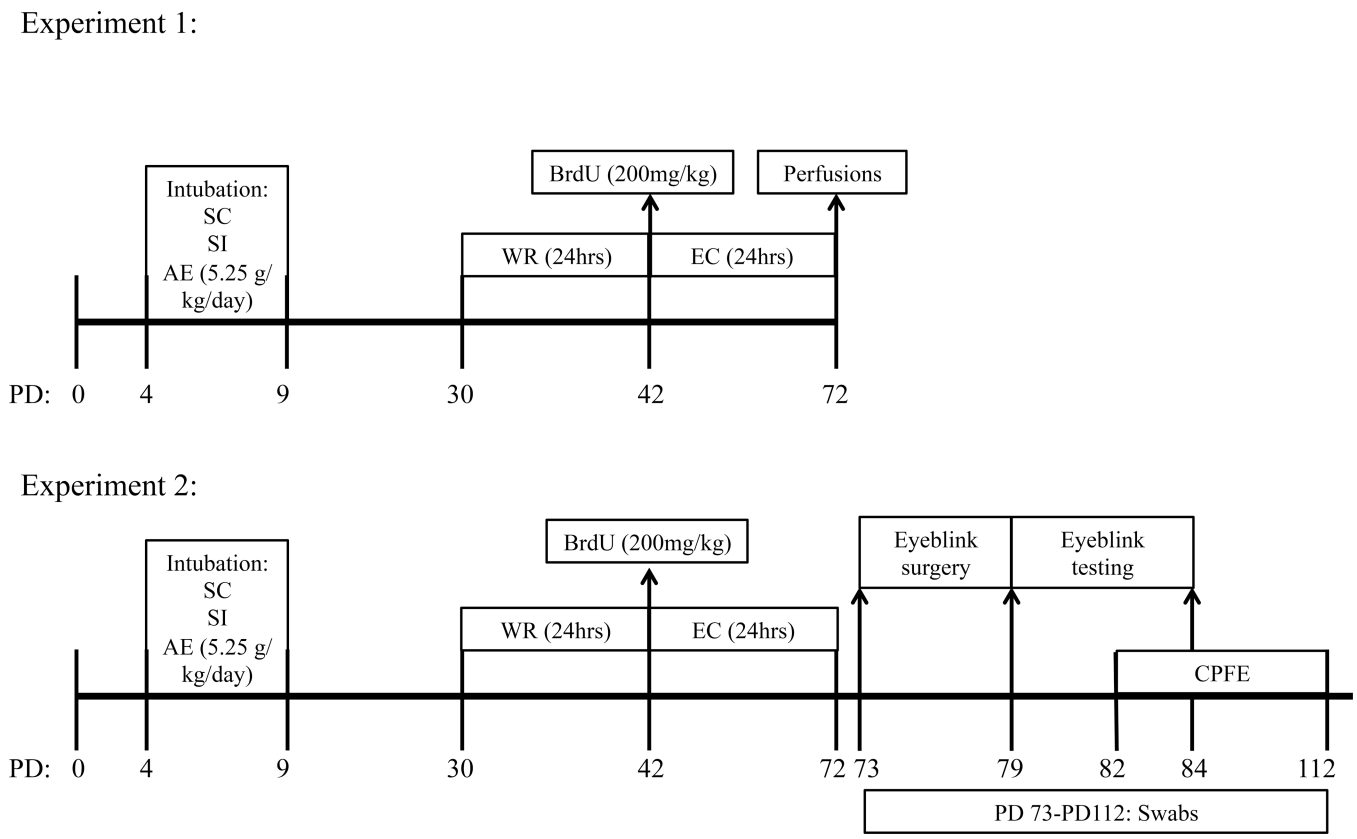

Figure 1. Timeline for Experiments 1 and 2

Schematic drawing of the experimental design and BrdU injection protocol. Between PD4 and 9 rat pups received daily two intubations of ethanol/milk mix (Alcohol Exposed, AE; 5.25 g/kg/day), were sham-intubated (SI) or remained with their mother (suckle controls, SC). Pups were weaned on PD23 and were housed in cages with same-sex littermates. On PD30 half of the pups were housed in cages with running wheels attached (WR), while the other half remained in social housing condition (SH). All animals were injected with 200 mg/kg BrdU in the morning on PD42. WR rats were immediately transferred to complex environment cages (EC) while SH rats remained in their cages for 30 more days. All animals were sacrificed for neuroanatomical study (Experiment 1) on PD72. For behavioral experiments, after completion of WR/EC exposure rats underwent eyeblink surgery between PD72–79 and were tested for trace eyeblink conditioning between PD79 and 84 and for CPFE – between PD82–112.

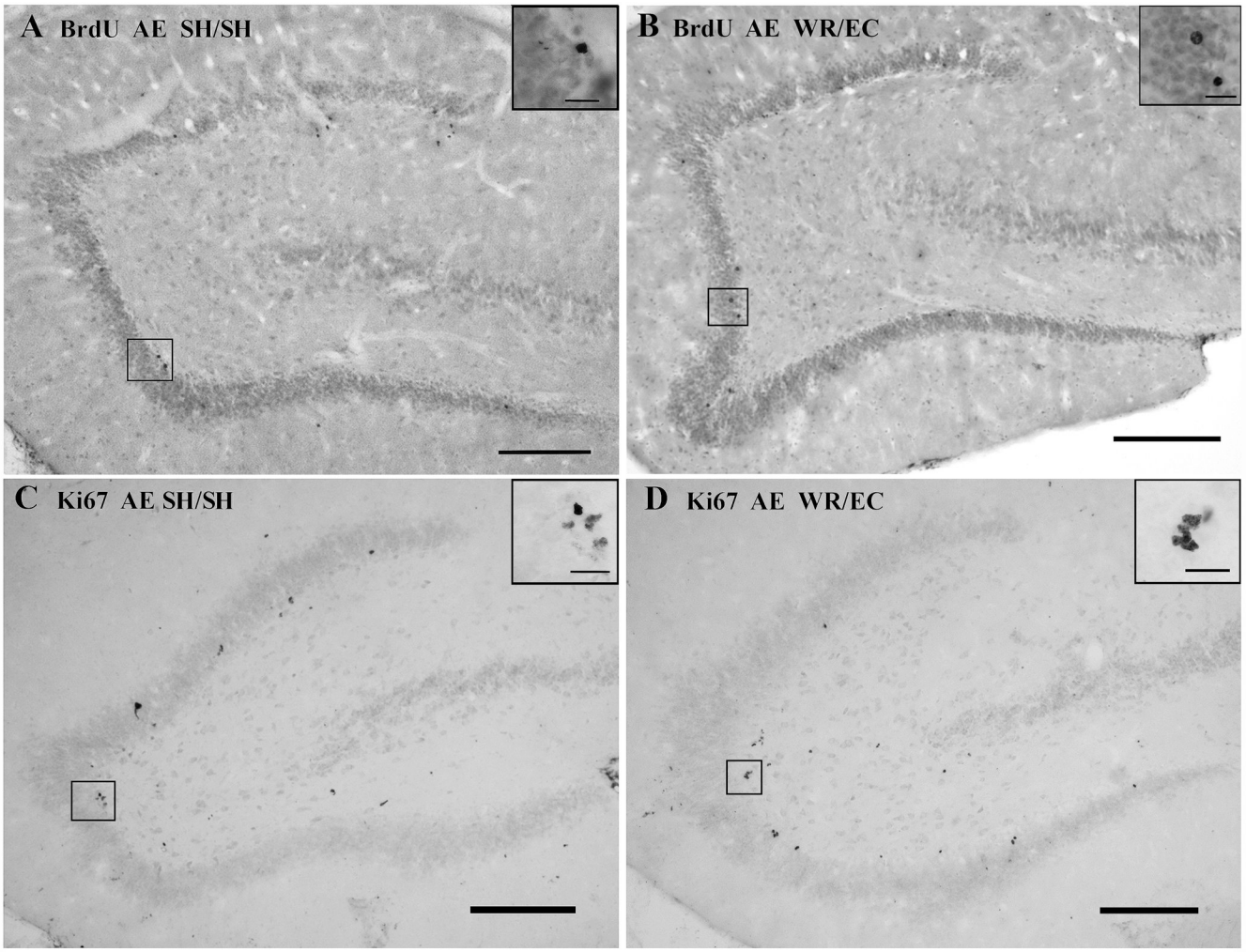

Figure 2. BrdU and Ki67 immunoreactivity in hippocampal dentate gyrus from SH/SH and WR/EC rats

The number of BrdU-positive cells in hippocampal dentate gyrus (DG) granule cell layer is increased after exposure to exercise (running wheel, WR) for 12 days followed by thirty days of living in complex environment (EC) (**A vs B**). Progenitor cell proliferation (demonstrated by Ki67 immunocytochemistry) remains at the same level after exposure to these conditions (**C and D**). Photomicrographs of BrdU and Ki67 immunostaining in the DG of alcohol-exposed (AE) rats from (**A**) standard housing condition (SH/SH), and from (**B**) rats in "super-intervention" condition (WR/EC). Scale bars, 250 μm for low magnification images and 25 μm for inserts.

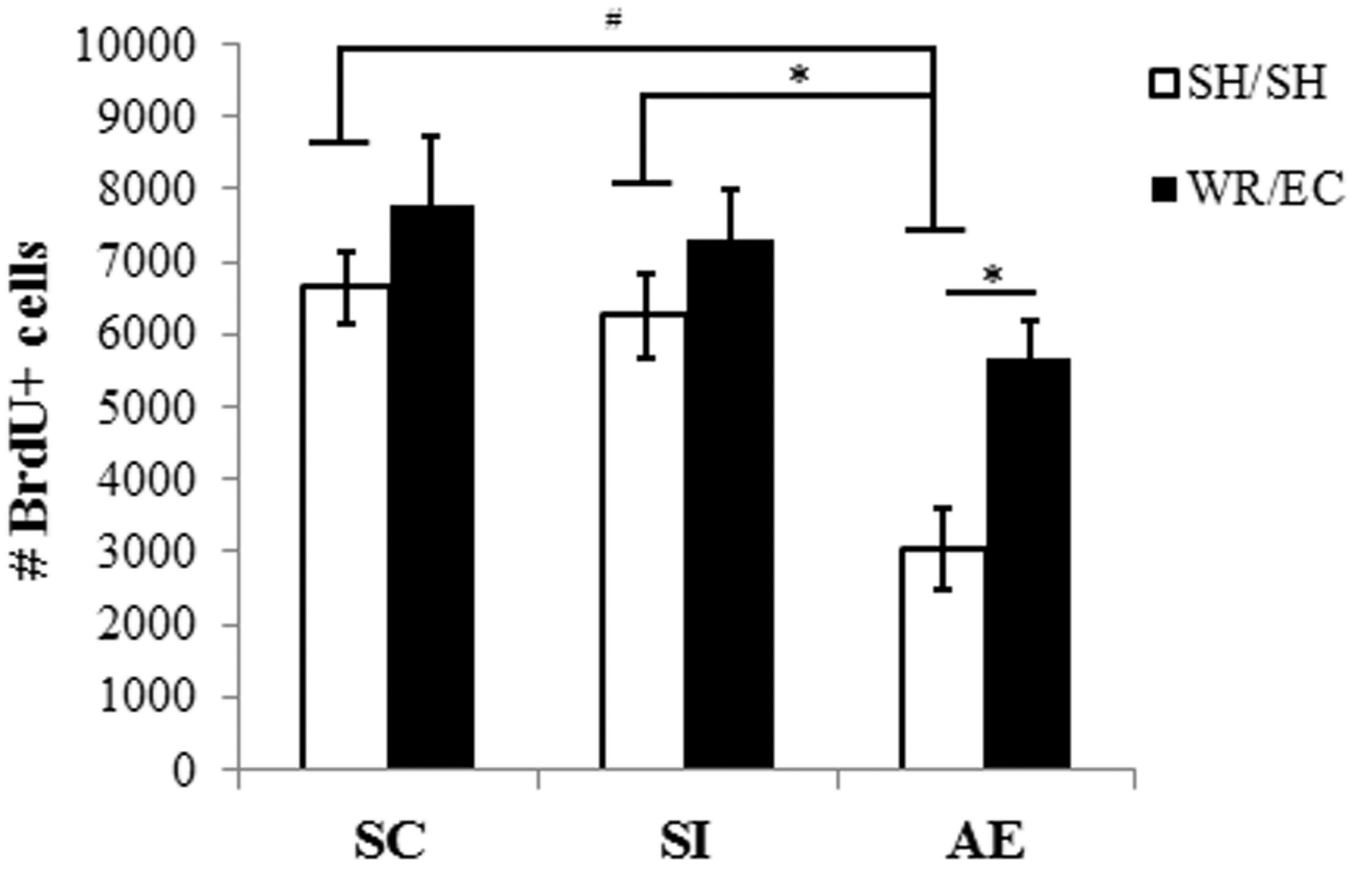

Figure 3. Total number of BrdU+ cells in males on PD72 (31 days post BrdU injection)

In the SH/SH animals, the number of BrdU+ cells is significantly decreased in AE animals compared to both SC and SI. In the WR/EC animals, there was no effect of postnatal condition on the number of surviving BrdU+ cells (AE-WR/EC n=7; SI-WR/EC, n=6; SC-WR/EC, n=7; AE-SH/SH, n=6; SI-SH/SH n=8; and SC-SH/SH n=13) (SC, suckle control; SI, sham intubated; AE, alcohol exposed; SH/SH, standard house/standard house; WR/EC, wheel running/environmental complexity). * = $p < 0.05$; # = $p < 0.01$. Values indicate means ± SEM.

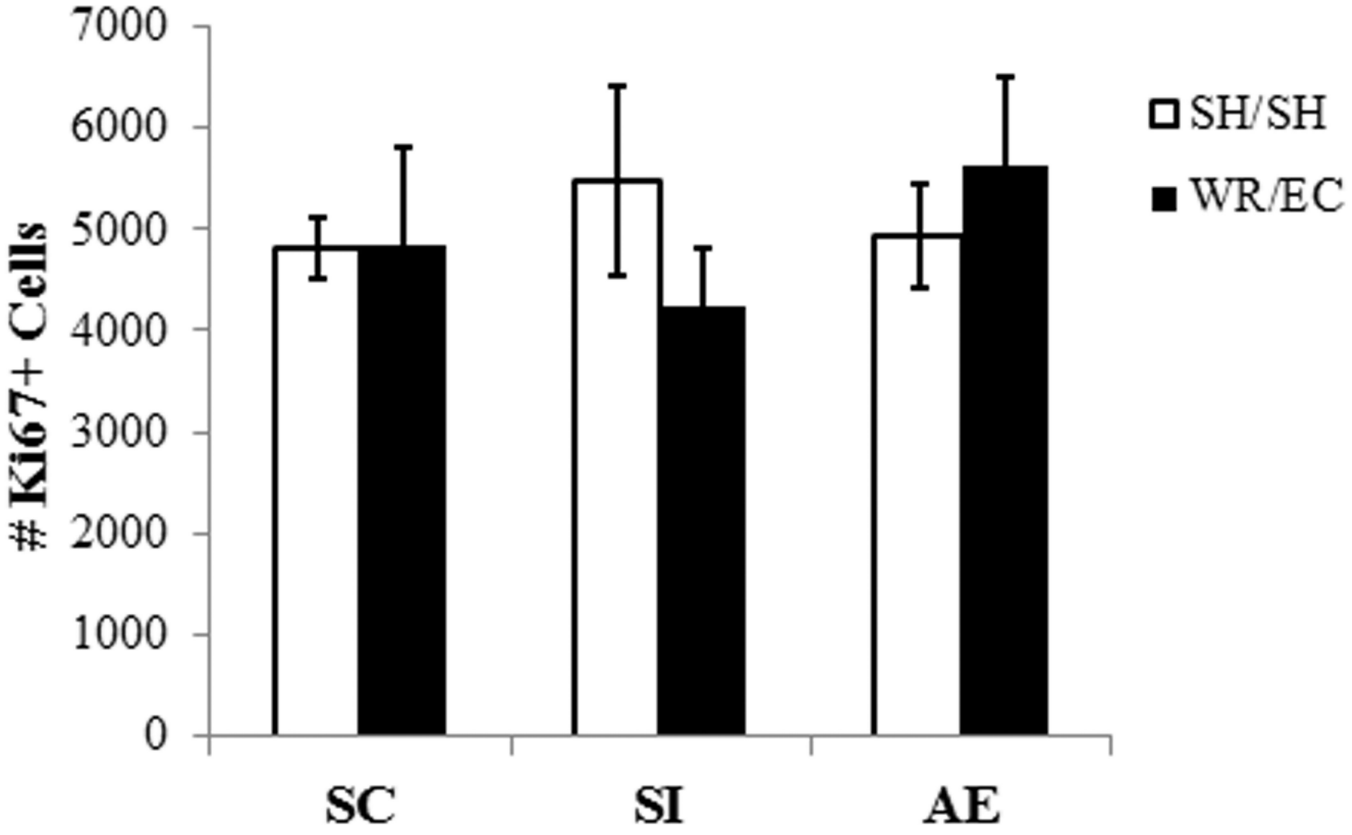

Figure 4. Total number of Ki67+ cells in males on PD72

Neither prenatal alcohol exposure nor exposure to WR/EC affect the number of proliferating cells on PD72. (AE-WR/EC, n=7; SI-WR/EC, n=7; SC-WR/EC, n=8; AE-SH/SH, n=8; SI-SH/SH, n=11; SC-SH/SH, n=15) (SC, suckle control; SI, sham intubated; AE, alcohol exposed; SH/SH, standard house/standard house; WR/EC, wheel running/environmental complexity).

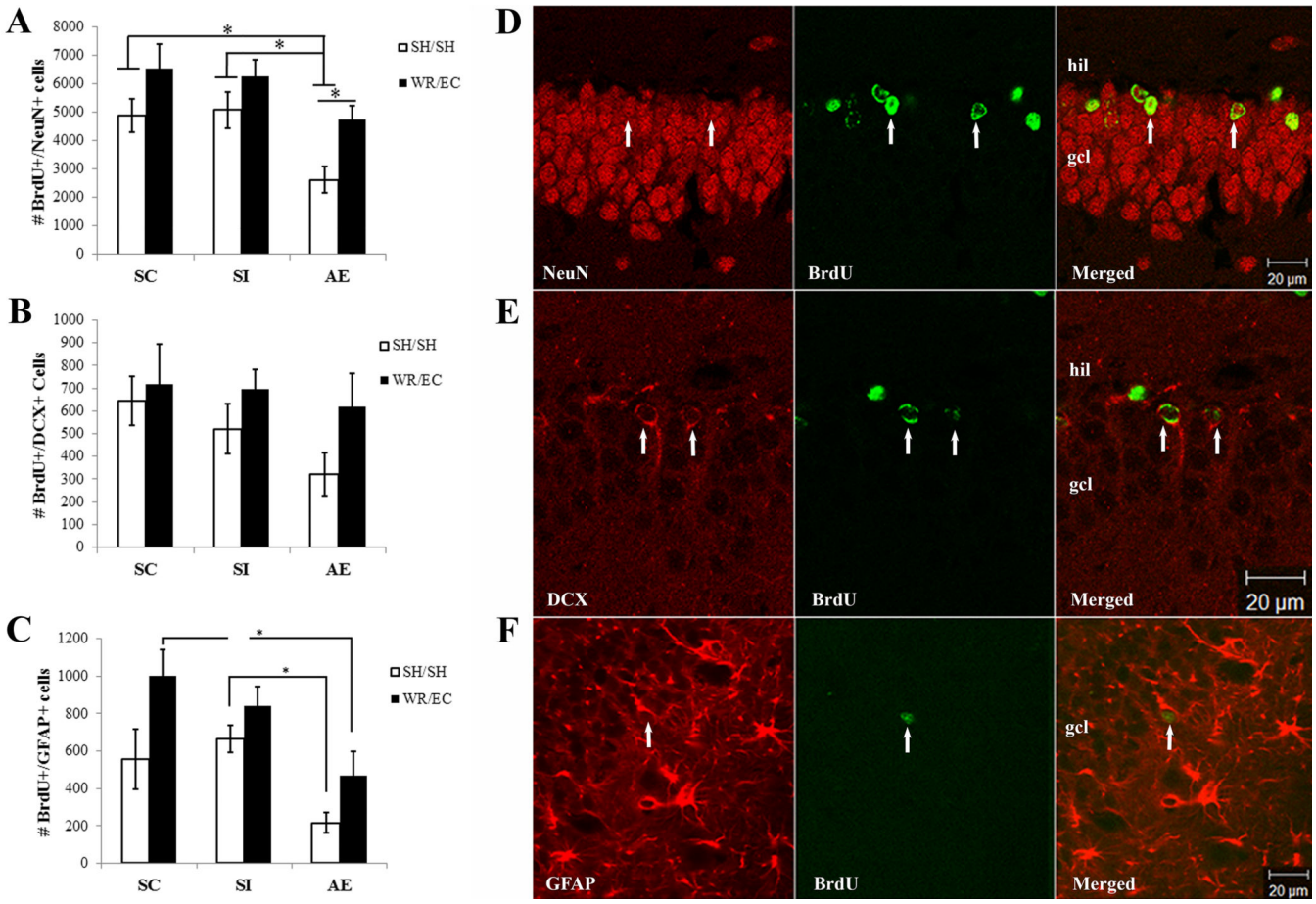

Figure 5. Phenotype of Bromodeoxyuridine (BrdU)+ cells

(**A**) Phenotyping of mature neurons using the neuronal marker NeuN. In the SH/SH animals, the number of double-labeled cells (BrdU+/NeuN+) is significantly decreased in the AE animals compared to the SI and SC. However, in the AE animals, exposure to WR/EC results in a significant increase in the number of double-labeled cells compared to the AE SH/SH animals. (SC-SH/SH, n=7; SI-SH/SH, n=6; AE-SH/SH, n=6; SC-WR/EC, n=6; SI-WR/EC, n=6; AE-WR/EC, n=6) (**B**) Phenotyping of immature neurons using the immature neuronal marker DCX. Neither prenatal alcohol exposure nor exposure to WR/EC affect the number of double labeled cells (BrdU+/DCX+) (SC-SH/SH, n=5; SI-SH/SH, n=5; AE-SH/SH, n=4; SC-WR/EC, n=5; SI-WR/ EC, n=5; AE-WR/EC, n=5) (**C**) Phenotyping of astrocytes using the astrocyte marker GFAP. In both Adult Housing conditions, the number of double labeled cells was significantly decreased in the AE animals compared to controls. (**D**) Confocal images of immunostaining for neuronal marker NeuN (red) and BrdU (green), arrows point to mature neurons (NeuN +) co-labeled with proliferation marker 4 wks after BrdU injection (**E**) Confocal images of immunostaining for DCX (red) and BrdU (green), arrows point to immature neurons (DCX+) co-labeled with BrdU (**F**) Confocal images of immunostaining for GFAP (red) and BrdU (green), arrows point to GFAP+ cell co-labeled with BrdU. (SC-SH/SH, n=5; SI-SH/SH, n=6; AE-SH/SH, n=4; SC-WR/EC, n=6; SI-WR/EC, n=5; AE-WR/EC, n=5) (SC, suckle control; SI, sham intubated; AE, alcohol exposed; SH/SH, standard house/standard house; WR/EC, wheel running/environmental complexity).

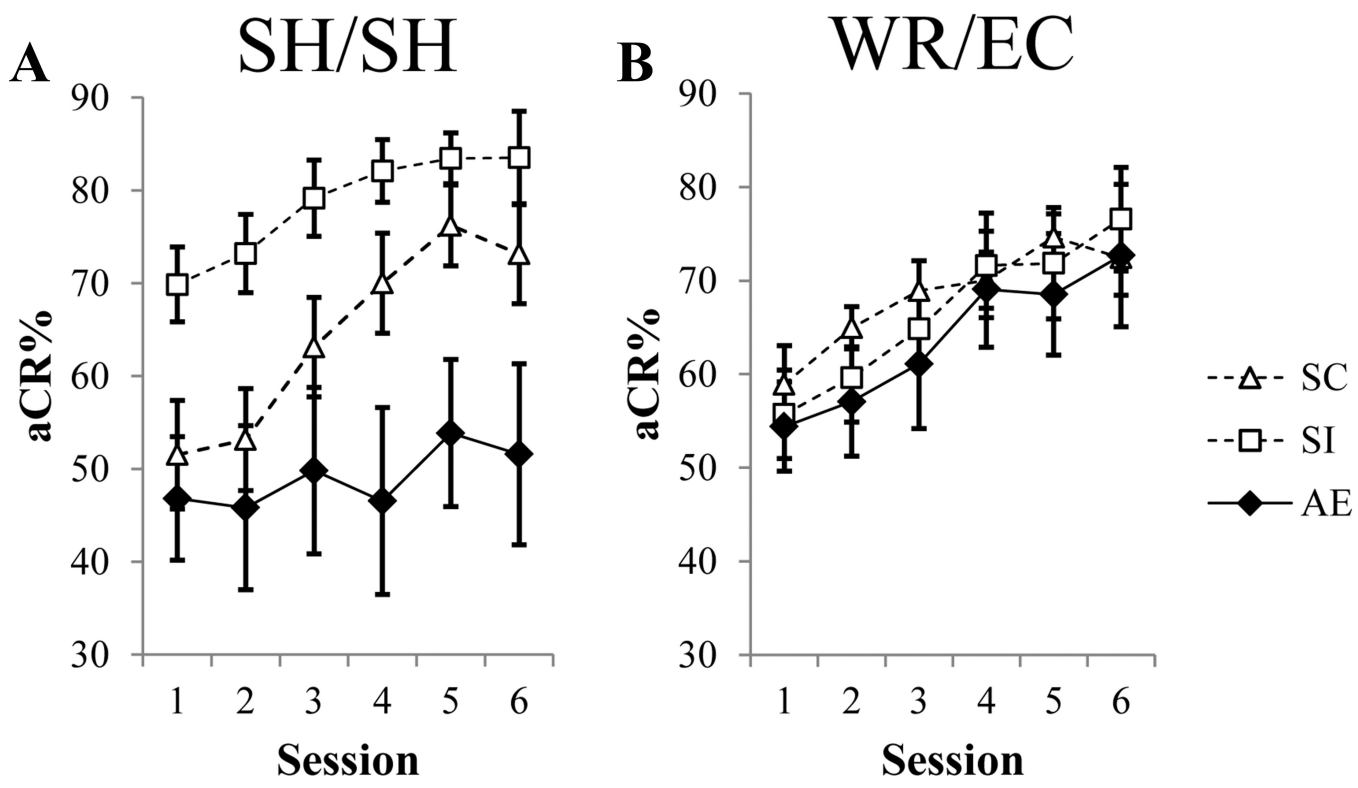

Figure 6. Trace eyeblink conditioning – Conditioned Responses (CR)

Mean (±S.E.) CR percentage for the social control condition (SH/SH; 6A) and Intervention condition (WR/EC; 6B) as a function of 6, 100-trial sessions. AE-WR/EC rats show enhanced CR acquisition relative to AE-SH/SH controls. “Adaptive” measure represents only the last 200 ms of the long CS trial. (SC-SH/SH, n=13; SI-SH/SH, n=15; AE-SH/SH, n= 8; SC-WR/EC, n=10; SI-WR/EC, n=9; AE-WR/EC, n=7).

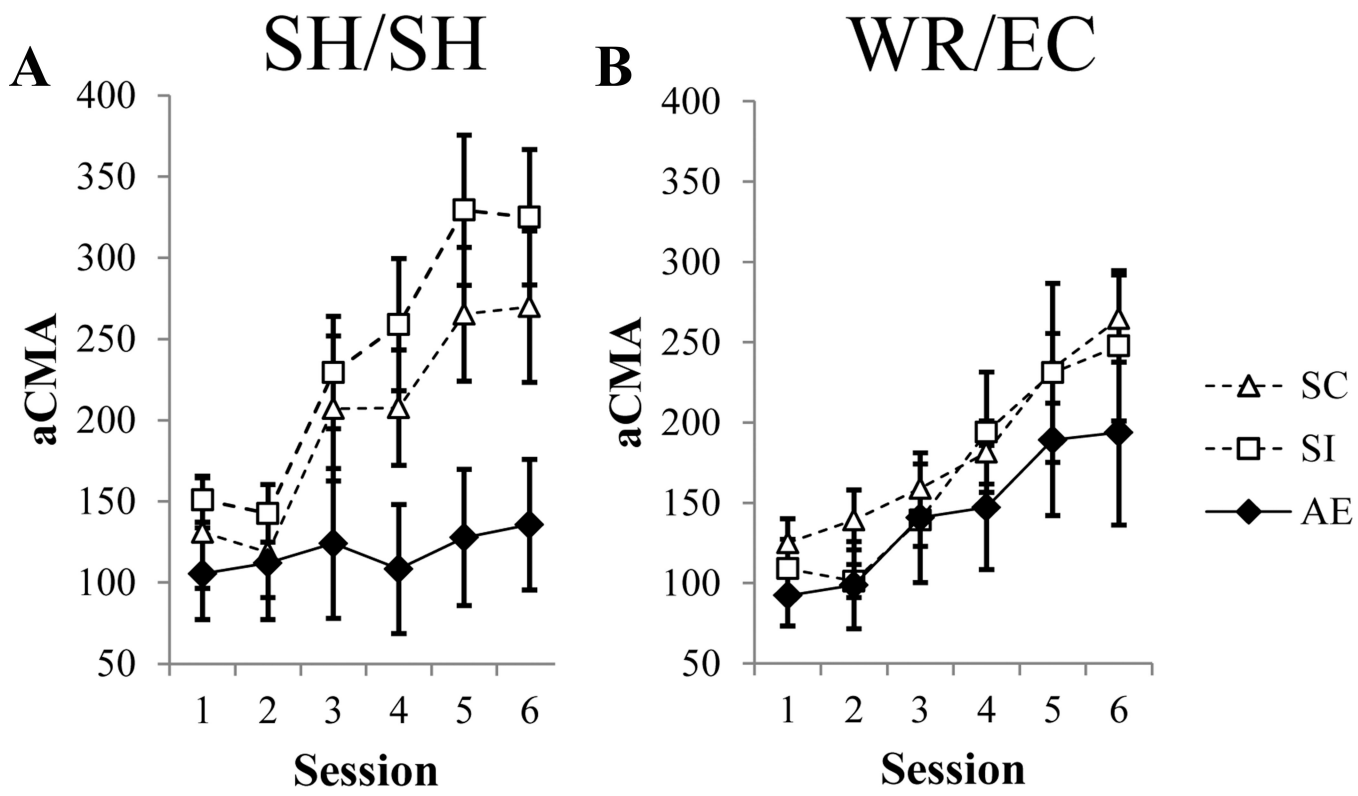

Figure 7. Trace eyeblink conditioning – Conditioned Response Maximum Amplitude (CMA)

Mean (±S.E.) CMA for the social control condition (SH/SH; 7A) and Intervention condition (WR/EC; 7B) as a function of 6, 100-trial sessions. AE-WR/EC rats show enhanced CR amplitudes relative to AE-SH/SH controls. “Adaptive” measure represents only the last 200 ms of the long CS trial. (SC-SH/SH, n=13; SI-SH/SH, n=15; AE-SH/SH, n= 8; SC-WR/EC, n=10; SI-WR/EC, n=9; AE-WR/EC, n=7).

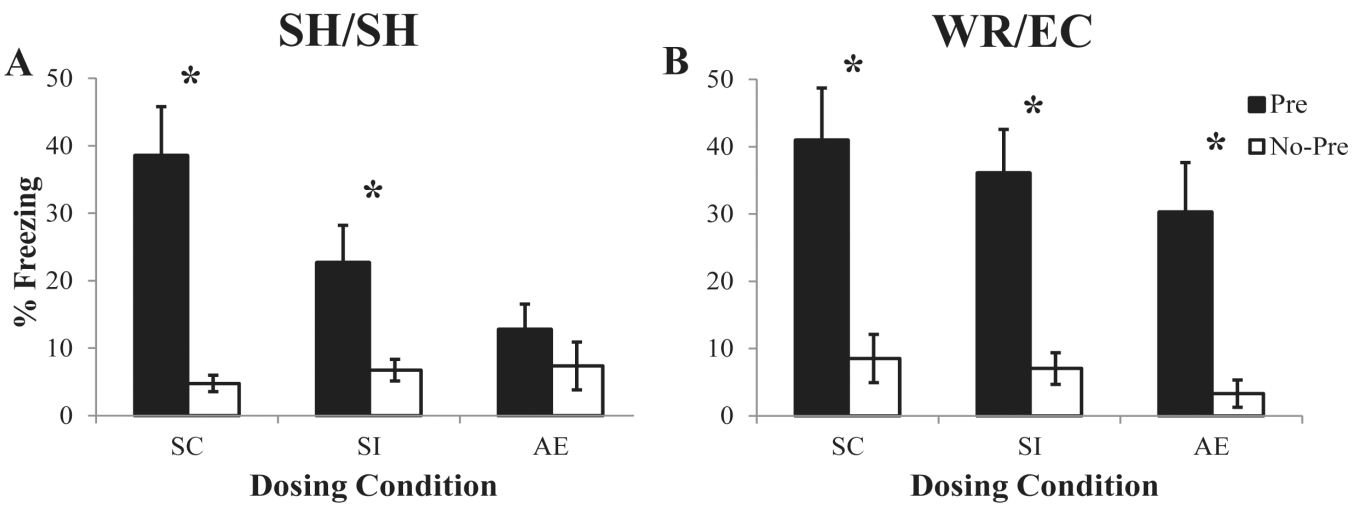

Figure 8. Contextual Fear Conditioning

Mean (±S.E.) percent freezing throughout the 5-min testing session in the CPFE for the social control condition (SH/SH; 8A) and Intervention condition (WR/EC; 8B). AE-WR/EC rats, but not AE-SH/SH rats show comparable conditioned freezing relative to controls. (SC-SH/SH-Pre, n= 8M, 8F; SC-SH/SH-NoPre, n= 7M, 8F; SI-SH/SH-Pre, n= 8M, 7F; SI-SH/SH-NoPre, n= 7M, 6F; AE-SH/SH-Pre, n=5M, 8F; AE-SH/SH-NoPre, n= 5M, 6F; SC-WR/EC-Pre, n= 7M, 7F; SC-WR/EC-NoPre, n= 6M, 6F; SI-WR/EC-Pre, n= 8M, 8F; SI-WR/EC-NoPre, n= 6M, 7F; AE-WR/EC-Pre, n= 8M, 6F; AE-WR/EC-NoPre, n= 2M, 6F).

Table 1

BACs and Body Weights

Body Weights (g)						**BACs**
Experiment 1						
Group	PD4 (g)	PD9 (g)	PD30 (g)	PD42 (g)	PD72 (g)	(mg/dl)
SC	10.4 ± 0.2	19.3 ± 0.4	100.5 ± 2.2	186.1 ± 3.3	371.0 ± 7.7	
SI	10.6 ± 0.2	19.6 ± 0.4	98.2 ± 1.7	182.3 ± 2.9	367.4 ± 5.7	
AE	10.4 ± 0.2	16.0 ± 0.5	91.4 ± 2.1	173.1 ± 3.3	361.0 ± 6.0	409.06 ± 13.96
Experiment 2A						
Group	PD4 (g)	PD9 (g)	PD30 (g)	PD42 (g)	PD72 (g)	(mg/dl)
SC	10.6 ± 0.3	19.0 ± 0.4	100.7 ± 2.2	184.5 ± 2.8	380.5 ± 5.1	
SI	10.3 ± 0.2	18.9 ± 0.4	98.8 ± 2.2	181.6 ± 3.8	376.2 ± 7.5	
AE	10.6 ± 0.2	15.3 ± 0.5	93.3 ± 2.1	175.3 ± 3.5	362.9 ± 6.1	406.3 ± 14.7
Experiment 2B						
Group	PD4 (g)	PD9 (g)	PD30 (g)	PD42 (g)	PD72 (g)	(mg/dl)
Male SC	10.9 ± 0.3	19.4 ± 0.5	101.5 ± 2.4	186.0 ± 3.2	380.6 ± 5.1	
Male SI	10.4 ± 0.3	18.9 ± 0.4	99.0 ± 2.0	181.5 ± 3.2	374.2 ± 5.8	
Male AE	10.5 ± 0.2	15.3 ± 0.4	91.8 ± 1.6	168.9 ± 3.7	354.2 ± 6.2	418.6 ± 11.6
Female SC	9.9 ± 0.2	17.7 ± 0.4	89.9 ± 2.2	149.3 ± 2.7	238.0 ± 4.4	
Female SI	10.1 ± 0.2	18.6 ± 0.4	92.5 ± 1.7	151.7 ± 2.5	238.2 ± 3.3	
Female AE	10.3 ± 0.2	15.1 ± 0.5	85.6 ± 1.9	144.0 ± 2.9	237.9 ± 6.9	433.1 ± 10.6