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NEONATAL ALCOHOL EXPOSURE REDUCES NUMBER OF PARVALBUMIN-POSITIVE INTERNEURONS IN THE MEDIAL PREFRONTAL CORTEX AND IMPAIRS PASSIVE AVOIDANCE ACQUISITION IN MICE DEFICITS NOT RESCUED FROM EXERCISE

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Abstract—Developmental alcohol exposure causes a host of cognitive and neuroanatomical abnormalities, one of which is impaired executive functioning resulting from medial prefrontal cortex (mPFC) damage. This study determined whether third-trimester equivalent alcohol exposure reduced the number of mPFC GABAergic parvalbumin-positive (PV+) interneurons, hypothesized to play an important role in local inhibition of the mPFC. The impact on passive avoidance learning and the therapeutic role of aerobic exercise in adulthood was also explored. Male C57BL/6J mice received either saline or 5 g/kg ethanol (two doses, two hours apart) on PD 5, 7, and 9. On PD 35, animals received a running wheel or remained sedentary for 48 days before behavioral testing and perfusion on PD 83. The number of PV+ interneurons was stereologically measured in three separate mPFC subregions: infralimbic, prelimbic and anterior cingulate cortices (ACC). Neonatal alcohol exposure decreased number of PV+ interneurons and volume of the ACC, but the other regions of the mPFC were spared. Alcohol impaired acquisition, but not retrieval of passive avoidance, and had no effect on motor performance on the rotarod. Exercise had no impact on PV+ cell number, mPFC volume, or acquisition of passive avoidance, but enhanced retrieval in both control and alcohol-exposed groups, and enhanced rotarod performance in the control mice. Results support the hypothesis that part of the behavioral deficits associated with developmental alcohol exposure are due to reduced PV+ interneurons in the ACC, but unfortunately exercise does not appear to be able to reverse any of these deficits.

Key words: anterior cingulate cortex, executive function, inhibition, fear, running, fetal alcohol spectrum disorders.

INTRODUCTION

Developmental alcohol exposure in humans produces a wide range of deficits collectively referred to as Fetal Alcohol Spectrum Disorders (FASD). FASD occurs in approximately 2–5% of all live births and, despite growing awareness of this disease; the rate continues to increase (May et al., 2009). Patients with FASD exhibit neurobehavioral deficits, which include physical, cognitive, learning and behavioral disabilities. These effects are long lasting and have possible lifelong implications (Calhoun et al., 2006). Third trimester alcohol exposure is associated with deficits in executive functioning, planning, organized search, inhibition, working memory, and flexible thinking (Connor et al., 2000; Rasmussen, 2005). These behaviors are highly associated with brain regions that include, but are not limited to, the prefrontal cortex (PFC) (Mattson et al., 2001, 2011; Kooistra et al., 2010). Furthermore, maternal alcohol consumption reduces frontal lobe size and increases gray matter asymmetry between frontal lobes (Wass et al., 2001; Sowell et al., 2002; Rasmussen, 2005). Thus, FASD-related impairments in cognition and learning persist into adulthood and are accompanied by structural changes in the PFC.

The PFC is the last cortical structure to fully develop. Beginning with the generation of neurons *in utero*, development is not fully complete until late adolescence. Of note, during the third trimester, the PFC, along with other brain regions, goes through a transient period of mass neurodevelopment and rapid growth. This is referred to as the brain growth spurt. The equivalent of the brain growth spurt occurs over the first ten postnatal days in rodents (Dobbing and Sands, 1979). During this time period many essential features of the PFC are developing. In humans, it is during the third trimester that the formation of the six-layered pattern of the cortex occurs (Mrzljak et al., 1990). In rodents, the medial PFC (mPFC) laminar architecture is completed after birth. By postnatal day (PD) 6, thalamic fibers reach their layer of termination (Layer III) and it is not until PD 10 that all cortical layers can be recognized in the mPFC. The rodent mPFC consists of infralimbic (IL), prelimbic (PL) and anterior cingulate (ACC) subregions. Each subregion is made up of a different architectonic organization and laminar differentiation (Van Eden and Uylings, 1985) and these anatomical distinctions suggest functional differences. For example,

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Abbreviations: ACC, anterior cingulate cortex; FASD, fetal alcohol spectrum disorders; IL, infralimbic cortex; mPFC, medial prefrontal cortex; PBS, phosphate-buffering solution; PD, postnatal day; PL, prelimbic cortex; PV+, parvalbumin-positive.

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each subregion has been associated with fear-associated learning; however, their roles appear slightly unique. The IL and PL subregions are more highly associated with either fear suppression or expression, respectively (*for review see* Giustino and Maren, 2015), while the ACC is associated with fear acquisition (Hamner et al., 1999; Bissiere et al., 2008; Etkin et al., 2011). Therefore, it is it is possible that alcohol exposure during the brain growth spurt, could differentially impact the mPFC subregions both anatomically and functionally.

Alcohol exposure during this period of rapid neurodevelopment leads to mPFC anatomical abnormalities. Not only does maternal alcohol consumption result in a smaller frontal lobe size in the offspring (Fakoya and Caxton-Martins, 2006), but also it changes the connectivity. For example, human literature demonstrates that maternal alcohol consumption alters mPFC pyramidal cell spine density and morphology (Ferrer and Galofre, 1987). Further rodent studies show third trimester equivalent alcohol exposure produces simplified dendritic organization and decreased spine density of Layer II/III mPFC pyramidal cells (Granato and Van Pelt, 2003; Whitcher and Klintsova, 2008; Hamilton et al., 2010). Ethanol is a known NMDA antagonist, and research shows that PD 7 treatment of a NMDA antagonist not only significantly reduced the number of Layer V pyramidal neurons, but it also halved the number of parvalbumin-positive (PV+) interneurons in the frontal cortex, when measured in adulthood (Coleman et al., 2009). Prenatal alcohol exposure also produced long-term anomalies in striatal PV+ interneurons, mainly represented by the shrinkage of their dendritic tree (De Giorgio et al., 2012). In the mPFC, PV+ interneurons primarily receive input from the mediodorsal nucleus of the thalamus (Rotaru et al., 2005) and project primarily on the proximal basilar dendrites of pyramidal neurons (Shibata, 1993; Rotaru et al., 2005). It is through this strong indirect inhibitory influence that the mediodorsal nucleus of the thalamus is able to gate pyramidal cell excitability to other inputs (Floresco and Grace, 2003; Kuroda et al., 2004; Rotaru et al., 2005). Therefore, alterations in PFC connectivity, perhaps specifically through altered GABAergic inhibition, may mitigate certain FASD-associated behavioral deficits.

Given the continuously increasing number of FASD patients who are undoubtedly suffering from behavioral deficits, it is essential to develop therapeutic interventions. Consistently, exercise participation has proven to improve brain health and function. In humans, participation in physical activity not only improves learning and memory (Colcombe et al., 2004) but is also argued to slow the progress of neurodegenerative diseases such as Alzheimer's disease (Nelson and Tabet, 2015). Further, work with rodents has demonstrated increased levels of neurotrophic factors such as BDNF and VEGF in the cortex following voluntary exercise (Hopkins et al., 2011; Uysal et al., 2011; Aksu et al., 2012). These factors promote the differentiation, neurite extension, and survival of a variety of neuronal populations (Cotman and Berchtold, 2002). In particular, Marty and colleagues (1997) argue that because both the synthesis and secretion of BDNF by pyramidal neurons are regulated by neuronal activity, BDNF may mediate activity dependent interactions between interneurons and their target cells. In fact, voluntary exercise has been shown to enhance cellular plasticity in the PFC of corticosterone-treated (Ekstrand et al., 2008) and methamphetamine-treated (Mandyam et al., 2007) male adult rats. Further, voluntary exercise has been shown to rescue decreases in spine density and eliminate deficits in dendritic complexity in mPFC pyramidal cells of rats exposed to a third trimester equivalent alcohol exposure (Hamilton et al., 2015). Therefore, it is possible that by promoting neuroplasticity, exercise later in life could facilitate cell survival in the alcohol-exposed brain.

Although it is known that neonatal alcohol exposure leads to mPFC abnormalities such as altered pyramidal cell morphology (i.e. reduced spine density and simplified dendritic organization), to the best of our knowledge no previous study has specifically investigated the impact of neonatal alcohol exposure on the number of mPFC PV+ interneurons. Therefore, the goal of this study was first to establish the effects of neonatal alcohol exposure on the number of PV+ interneurons in distinct mPFC subregions. Second, given the unique contributions of each subregion to fear-associated learning, this study examined the impact of neonatal alcohol exposure on the passive avoidance task. Third, we sought to determine the extent to which wheel running could mitigate any observed deficits. To this regard, rotarod performance served as a positive control for the effects of alcohol and exercise, given that neonatal alcohol exposure has been known to influence motor performance (Goodlett et al., 1991; Klintsova et al., 2000) and exercise is known to mitigate cerebellar damage (Cotman and Berchtold, 2002; Hamilton et al., 2016). Given the role of PV+ neurons in local inhibition and the crucial role of the mPFC in fear-associated learning, we hypothesized that neonatal alcohol exposure may reduce PV+ interneurons in mPFC subregions and that these neuroanatomical abnormalities would be associated with impaired passive avoidance performance. Further, we hypothesized that wheel running would mitigate some of the alcohol-induced behavioral and neuroanatomical deficits, given its proven ability to improve brain health and function.

EXPERIMENTAL PROCEDURES

Subjects

The mice used in this study were the control (non-transgenic litter-mates) produced by crossing two hemizygous transgenic Nestin-TK mice (Yu et al., 2008). The transgenes were inserted in C57BL/6J mouse zygotes for an unrelated study. Genotyping confirmed the mice used in this study did not carry the transgene, hence they were plain C57BL/6J. A total of 37 male mice were used. Only males were used in this study, as we first wanted to establish that this alcohol model would impact the number of PV+ interneurons without the complication induced by the estrus cycle. It has been shown that

female rats have a significantly higher number of PV+ interneurons in the prefrontal cortex than do male rats (Smiley et al., 2015; Wischhof et al., 2015). Therefore, it is possible that females would respond differently from males. All mice were bred in house at the Beckman Institute. Individual mice from each litter were assigned to different groups, to account for litter effects. Throughout the study, mice were housed in standard polycarbonate shoebox cages (29 cm × 19 cm × 13 cm; L × W × H) with corncob bedding (Teklad 7012; Harlan Teklad, Madison, WI, USA) and nesting material. Rooms were controlled for temperature (21 °C ± 1 °C) and photo-period (12:12 D:L; lights off at 9:00 am CST and on at 9:00 pm) with food and water provided *ad libitum*. The Beckman Institute Animal Facility is AAALAC approved. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and adhered to NIH guidelines.

Neonatal treatment

On PD 5, 7, and 9, whole litters were assigned to one of two treatment groups: saline or ethanol solution. Twenty ethanol-treated pups were injected subcutaneously twice daily, two hours apart (9:00 AM and 11:00 AM) with 2.5 g/kg ethanol, amounting to a total of 5 mg/kg per day. The ethanol was delivered at a concentration of 20% (Decon Laboratories, Inc., King of Prussia, PA, USA) in sterile saline. This dose was determined based on the literature (Ikonomidou et al., 2000; Wozniak et al., 2004; Ieraci and Herrera, 2007; Lantz et al., 2012; Hamilton et al., 2016). Seventeen pups served as control animals by receiving saline injections. On PD 21, all animals were weaned into groups of four. Blood samples were not collected from animals, but previous work demonstrates that this concentration of alcohol is enough to produce a toxic level, above 200 mg/dl for four consecutive hours or more (Ikonomidou et al., 2000; Hamilton et al., 2016).

5′bromo-5′-deoxyuridine (BrdU) injections

On PD 35–44, all mice received daily intraperitoneal injections of 50 mg/kg 5-Bromo-2′-Deoxyuridine in order to label newly dividing cells in the dentate gyrus of the hippocampus (data not included).

Exercise intervention

Beginning on PD 35, all mice were placed in single housing where they either had access to or did not have access to running wheels. This method was chosen in order to obtain individual running levels. Therefore, final group numbers in each treatment condition were: saline sedentary: $n = 8$; saline runner; $n = 9$; alcohol sedentary, $n = 10$; alcohol runner: $n = 10$. Runners were placed in cages (36 cm × 20 cm × 14 cm; L × W × H) with a 23 cm diameter wheel mounted to the cage top (Respironics, Bend, OR, USA) while sedentary animals remained in standard polycarbonate boxes without running wheels. Wheel rotations were monitored continuously in one-minute increments via magnetic switches interfaced to a computer running VitalView software (Respironics, Bend, OR, USA). Sedentary mice were not kept in cages with locked wheels, as mice will climb on top of locked wheels and this would introduce additional activity in the sedentary group (Koteja et al., 1999). Animals remained in their designated housing throughout the behavioral testing except when temporarily moved into behavioral testing apparatuses. Similar to previous studies from our lab, mice were given at least 30-day access to the exercise intervention prior to any behavioral testing. As such, animals began behavioral testing on PD 79. Animals remained on wheels until time of perfusion.

Rotarod

Children with FASD typically have poor motor coordination. As such, mice were tested on the rotarod task to establish whether a PD 5, 7, and 9 alcohol exposure would impair motor coordination. Further, we hoped to determine whether exercise could ameliorate any alcohol-induced deficit. The rotarod task was conducted on PD 79 and PD 80 to measure motor coordination. The apparatus was a horizontal rod positioned above a box (AccuRotor Rota Rod Tall Unit, 63 cm fall height, 8 cm diameter rotating dowel; Accuscan, Columbus, OH). This rod rotated at a constantly accelerating rate of 60 rpm/minute. The rod was divided into four small, even compartments by disks. Mice were placed in their own compartments and motor coordination was assessed by latency for the animal to fall from the rod. The response variable that was statistically analyzed was average latency to fall across all four trials on each of the 2 days.

Passive avoidance

Passive avoidance testing occurred on PD 81- PD 82, one hour following lights off. On Day 1 (PD 81) animals were placed in a passive avoidance chamber (San Diego Instruments, San Diego, CA, USA) that consisted of two compartments, both with a metal grid flooring. The compartments, one of which was lit while the other was unlit, were separated by a guillotine door. At the beginning of each trial, animals were placed in the lit compartment and the guillotine door was raised. Mice have a natural aversion to light and were thus motivated to flee from the lit chamber through the open door and into the unlit compartment. When animals crossed fully into the unlit compartment, the guillotine door closed and a mild foot shock (0.5 mV) was delivered via the metal grid floor of the compartment. The door remained closed for a ten second post conditioned stimulus period, after which it opened and the house light was turned on in the compartment containing the mouse (previously unlit), while the other compartment went dark. This counterbalancing ensured that the animal was conditioned to the light/dark cues and not a particular side of the chamber. The animal repeated this task until it learned to remain in the lit compartment for 120 consecutive seconds. The time it took for the animal to reach criterion was determined for each

subject (latency to reach criterion). On Day 2 (PD 82), animals were tested for task retention. They were placed into the lit compartment and the time it took for them to cross over to the unlit compartment was recorded (latency to cross).

Tissue preparation and immunohistochemistry

Perfusions. Perfusions occurred on PD 83. All mice were euthanized via transcardial injection of 4% paraformaldehyde in phosphate buffer solution (PBS; 0.287% sodium phosphate monobasic anhydrous, 1.102% sodium phosphate dibasic anhydrous, 0.9% sodium chloride in water). Brains were placed in 4% paraformaldehyde for two days and subsequently transferred to a 30% sucrose in PBS. Brains were sectioned into 40-μm coronal sections on a cryostat and then immediately stored in cryoprotectant (30% ethylene glycol, 25% glycerin, 45% PBS) at −20 °C.

Immunohistochemistry. Using a 1-in-5 series the mPFC was stained for PV+ interneurons using the primary antibody: anti-PV in rat. Tissue sections were first rinsed in PBS, followed by a 30-min immersion in 0.6% hydrogen peroxide. Sections were subsequently washed with PBS again and then exposed to a solution of 0.3% Triton-X and 3% goat serum in PBS for 30 min (PBS-X plus). Next, tissue sections were incubated with primary antibody anti PV in mouse (1:2000; AbD Serotec, Raleigh, NC, USA) in PBS-X plus for 24 h at approximately 4 °C. After washing with PBS-X plus, sections were treated with goat anti-mouse secondary antibody (1:250) in PBS-X plus for 90 min at room temperature. Sections were then treated with the ABC system (Vector, Burlingame, CA, USA), washed in PBS-X, and stained using a diaminobenzidine kit. Tissue sections were mounted on microscope slides, left to dry overnight, dehydrated and stained using Methylene Azure and subsequently cover slipped.

Unbiased stereology

Quantification of PV+ interneurons was performed in accordance with an unbiased stereology approach. A series of sections (every 5th section, 7–9 sections per animal) throughout the entire mPFC was used for each staining batch (Markham et al., 2007). All cell counts were made on coded slides by an investigator blinded to the treatment conditions. Tracing of the mPFC was conducted for every tissue section by referencing the Allen Mouse Brain Atlas, for the location of the IL, PL and ACC subregions (Markham et al., 2007; Willing and Juraska, 2015). Each subregion was outlined on the digitized image on each section using StereoInvestigator software (Fig. 1). Counts were made in an unbiased manner within a known volume of each mPFC subregion using the optical fractionator workflow (StereoInvestigator, MicroBrightField 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 14 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. Average section thickness was determined and used to estimate both the total volume of each mPFC subregion and also the total number of PV+ interneurons in each mPFC subregion. For each animal, the coefficient of error (CE) was used to determine the precision of PV+ cell number estimates in a set of sections (Gundersen et al., 1999; Slomianka and West, 2005). A CE of less than 0.10 was considered adequate. (Gundersen et al., 1999). Actual CEs ranged between 0.05 and 0.08.

Statistical analysis

Data were analyzed using SPSS (version 22) statistical software. For all analyses, $p < 0.05$ was considered statistically significant. The following variables were analyzed using a repeated measures analysis with Day as the within measure and Postnatal Treatment (alcohol vs. saline) and Intervention (runner vs. sedentary) as the between factors: body weights (g), latency to fall from the rotarod (s). The following variables were analyzed using a two-way analysis of variance (ANOVA), with Postnatal Treatment (alcohol vs. saline) and Intervention (runner vs. sedentary) as the two factors: brain weights (g), total number of PV+ cells, average distance run on wheels, latency to reach criterion, latency to cross in passive avoidance (s). Post-hoc analyses (LSD) were used to characterize treatment and interaction effects, when statistically significant ($p < 0.05$).

RESULTS

Body weights

Body weights for all animals are summarized in Table 1. All animals continued to gain weight throughout the experiment. Neonatal body weights (PD 5, 7, and 9) versus body weights from later in life (PD 21, 35, 44 and 83) were analyzed separately. To analyze neonatal body weights, a repeated measures ANOVA with Postnatal Treatment as an independent variable was used. Results show a main effect of Day ($F_{(1,360)} = 3122.31$, $p < 0.001$), with all animals gaining weight each day. A Postnatal Treatment × Day interaction ($F_{(1,360)} = 131.14$, $p < 0.001$) and a main effect of Postnatal Treatment ($F_{(1,360)} = 82.75$, $p < 0.001$) were also found. These results indicate that alcohol-exposed animals gained less weight between each weighing period when compared to their saline-exposed counterparts. This was evident on PD 5 ($p < 0.05$), PD 7 ($p < 0.001$) and PD 9 ($p < 0.001$). It should be noted that on PD 5, animals had not yet been injected with either ethanol or saline at the time of weighing, which indicates a preexisting weight difference between these randomly assigned groups.

Consistently, to analyze body weights from later in life (PD 21, 35, 44 and 83), a repeated measures ANOVA

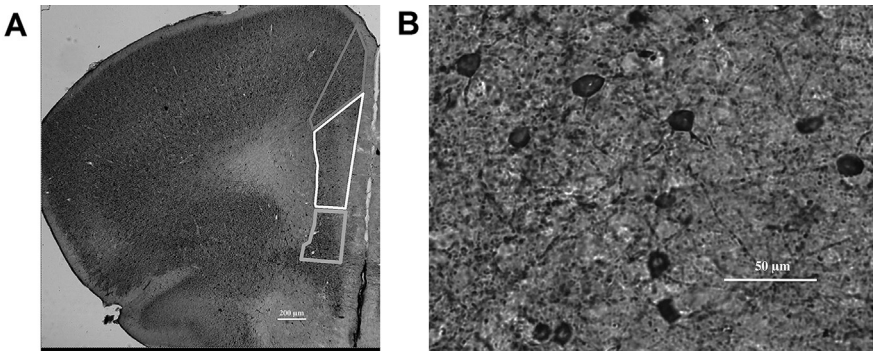


Fig. 1. mPFC subregion identification and staining. (A) Methylene blue stained coronal mPFC section (2.5× magnification) with example tracings of the different subregions (top: anterior cingulate (ACC); middle: prelimbic (PL); bottom: infralimbic (IL)). (B) mPFC PV/DAB labeled GABAergic interneurons (20× magnification).

with Postnatal Treatment as an independent variable was performed. The results of which revealed not only a significant main effect of Day ($F_{(1,22)}$ = 2753.23, $p < 0.001$) but also a main effect of Postnatal Treatment ($F_{(1,22)}$ = 8.23, $p < 0.01$), suggesting a long-term detrimental impact of neonatal alcohol exposure on body weight. However, although alcohol-exposed animals exhibited lower body weights than their saline-exposed counterparts during the juvenile and adolescent time period (PD 21: $p = 0.001$; PD 35: $p < 0.05$; PD 45: $p < 0.05$), this effect was no longer significant when measured in adulthood (PD 83). Together these data indicate that neonatal alcohol exposure causes a transient reduction in body weight for male mice.

Brain weights

Brain weights were recorded following perfusion in order to assess whether PD 5, 7, and 9 alcohol exposure had any long-term effects on brain weight. A univariate ANOVA revealed a main effect of Postnatal Treatment ($F_{(1,35)}$ = 7.75, $p < 0.01$). More specifically, these results demonstrated that mice exposed to neonatal alcohol exposure had significantly smaller brains when compared to their saline-exposed littermates (Fig. 2a). Similarly, a decrease in the calculated brain/body weights ratios for both Treatment groups was also found (Saline: 0.0192 g ± 0.0007 g, Alcohol: 0.0175 g ± 0.0004 g). Further, an ANCOVA with PD 83 body mass as a covariate revealed that not only was body mass significantly correlated with brain weight ($F_{(1,34)}$ = 6.51, $p < 0.05$) but that the effect of Treatment was also significant ($F_{(1,34)}$ = 5,11, $p < 0.05$). Together, these data demonstrate that PD 5, 7 and 9 alcohol exposure has a significantly damaging impact on brain development that is still evident in adulthood and that this impact is not due to simply lower body weights (Fig. 2b).

Wheel running

Mice exposed to neonatal alcohol exposure run significantly less than their saline-exposed counterparts ($F_{(1,17)}$ = 6.222, $p = 0.023$; Fig. 3a). This effect was persistent throughout the entirety of the intervention period (Fig. 3b). This suggests neonatal alcohol exposure may suppress motivation for physical activity into adulthood, as suggested by the decreased running levels.

Rotarod

A repeated measures ANOVA revealed a main effect of Day ($F_{(1,33)}$ = 22.828, $p < 0.001$), a main effect of Intervention ($F_{(1,33)}$ = 8.102, $p < 0.01$) and a Day x Postnatal Treatment x Intervention interaction ($F_{(1,33)}$ = 4.097, $p = 0.05$). Regardless of Postnatal Treatment, runners stayed significantly longer on the rod than their sedentary counterparts. However, it appears that although Saline Runners showed a significant enhancement from Day 1 to Day 2 ($F_{(1,10)}$ = 11.080, $p < 0.01$), Alcohol Runners failed to exhibit this same benefit from previous running experience (Fig. 4). These data suggest that although neonatal alcohol exposure does not negatively impact rotarod performance, it does limit the ability of exercise to enhance performance on this task.

Passive avoidance

Day 1: A PD 5, 7, and 9 alcohol exposure resulted in long-term behavioral deficits on the passive avoidance task, regardless of whether or not mice had access to a running wheel. Alcohol-exposed mice took a significantly longer time to reach criterion on the task than did their saline-exposed littermates. Specifically there was a main effect of Postnatal Treatment ($F_{(1,33)}$ = 6.315, $p < 0.05$; Fig. 5a).

Day 2: In contrast, Postnatal Treatment had no impact on Day 2 performance. Rather, runners showed a longer

Table 1. Effect of postnatal alcohol exposure on body weight

	PD 5	PD 7	PD 9	PD 21	PD 35	PD 45	PD 83
Saline	3.09 ± 0.04	4.13 ± 0.05	5.02 ± 0.06	10.24 ± 0.30	16.17 ± 0.52	17.28 ± 0.41	23.56 ± 0.43
Alcohol	2.97 ± 0.03*	3.52 ± 0.03**	4.24 ± 0.05**	8.69 ± 0.27**	14.90 ± 0.39*	16.10 ± 0.29*	22.76 ± 0.32

PD, postnatal day. The weights are reported as group means ± SEM.
* $p < 0.05$.
** $p < 0.001$.

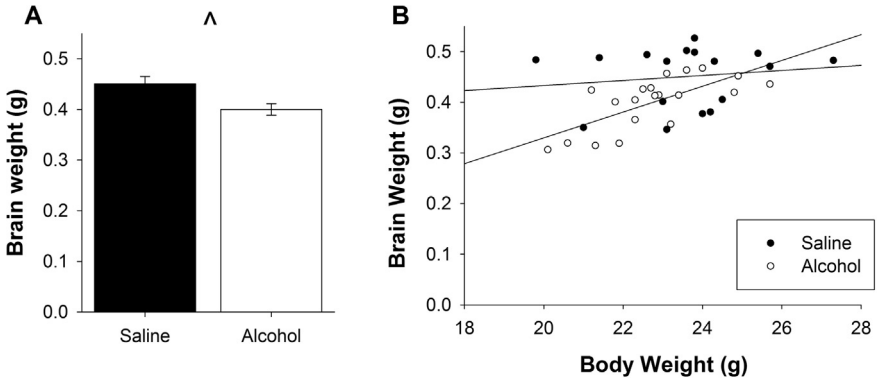


Fig. 2. Persistent impact of PD 5, 7, and 9 alcohol exposure evident in brain weight as adults. (A) When measured in adulthood, the brains of mice exposed to a PD 5, 7, and 9 alcohol exposure are significantly smaller than those of their saline-exposed mice littermates. (B) The negative impact of PD 5, 7, and 9 alcohol exposure on brain weight is still evident even when animals are matched for body size. All values represent mean ± SEM. $^{*}p < 0.05$.

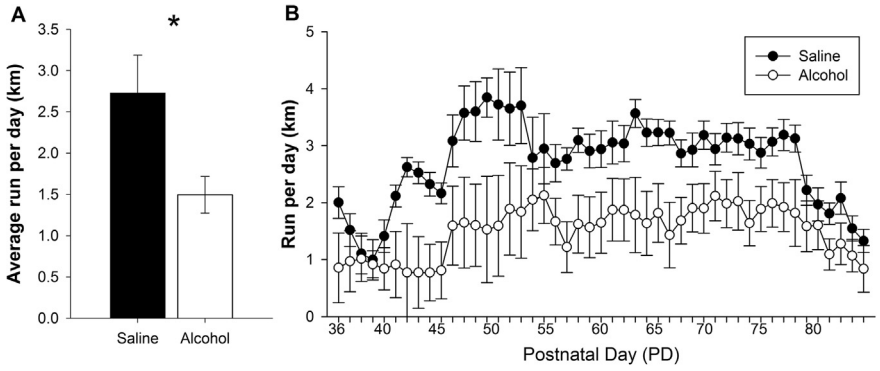


Fig. 3. Wheel Running Levels. (A) Data demonstrate the average running distance (km) per 24 h of alcohol- and saline-exposed mice. Animals exposed to a PD 5, 7, and 9 alcohol exposure ran significantly less than their saline-exposed littermates. (B) Daily running distance (km) of alcohol-exposed and saline-exposed mice throughout the entirety of the intervention. All values represent mean ± SEM. $^{*}p < 0.05$.

latency to cross over into the dark chamber when compared to their sedentary counterparts, indicating a better task retention (Fig. 5b). Specifically, there was a significant main effect of Intervention ($F_{(1,31)} = 8.433$, $p < 0.01$). Therefore, it appears that a PD 5, 7, and 9 alcohol exposure is sufficient to impact acquisition but not retention of the passive avoidance task, while an exercise intervention is sufficient to enhance retention but not acquisition of the task.

Medial prefrontal cortex (mPFC) volume

Neither a PD 5, 7, and 9 alcohol exposure, nor an exercise intervention impacted the overall volume of the mPFC. Specifically, no significant differences were found between the mPFC volumes of alcohol-exposed versus saline-exposed mice regardless of exercise intervention (Fig. 6a). Interestingly, PD 5, 7, and 9 alcohol exposure did significantly reduce the volume of the ACC dorsal mPFC subregion ($F_{(1,20)} = 7.081$ $p < 0.05$; Fig. 6b); while a lack of significant impact of PD 5, 7, and 9 alcohol exposure on the volume of the combined prelimbic and infralimbic (PL/IL) ventral mPFC subregion was found (Fig. 6c). No significant impact of exercise intervention was present in any subregion. These results suggest that PD 5, 7, and 9 alcohol exposure has a persistent effect that specifically targets the ACC.

Parvalbumin-positive (PV+) cell counts

Despite having no impact on mPFC volume, PD 5, 7, and 9 alcohol exposure significantly reduced the number of mPFC PV+ GABAergic interneurons ($F_{(1,20)} = 8.974$ $p < 0.01$), while neither an impact of Intervention ($F_{(1,20)} = 0.063$ $p = 0.80$) nor a Postnatal Treatment x Intervention interaction ($F_{(1,20)} = 2.716$ $p = 0.12$) was found. When data were collapsed across the Intervention, a main effect of Postnatal Treatment was still evident, such that there was a significant reduction in the total number of mPFC PV+ GABAergic interneurons ($F_{(1,22)} = 8.667$ $p < 0.01$; Fig. 7a). This effect was particularly prevalent in the ACC with brains of alcohol-exposed animals mice having significantly fewer PV+ cells than those of their saline counterparts ($F_{(1,22)} = 7.755$ $p < 0.05$; Fig. 7b). No impact of PD 5, 7, and 9 alcohol exposure was seen in the PL/IL subregions (Fig. 7c). Together, this suggests that PD 5, 7, and 9 alcohol exposure has a long-lasting impact, specifically on the ACC.

DISCUSSION

This study is the first to demonstrate that a third-trimester alcohol exposure reduces both the volume and the number of PV+ interneurons in the mPFC, an effect that is most profound in the ACC. PV+ interneurons provide a crucial inhibitory tone on mPFC pyramidal neurons, potentially gating their communication to other brain regions involved in higher cognitive processing. A loss of inhibitory tone, through a decreased number of PV+ interneurons, could result in an overactive mPFC. Thus, these novel findings could help explain alcohol-induced deficits in mPFC-associated behaviors such as inhibitory avoidance and fear-associated learning. Overall, these data are important because they illustrate the unique vulnerability of the PV+ cell population in the ACC to a third-trimester equivalent alcohol exposure and implicate the role of the ACC in FASD-associated behavioral impairments.

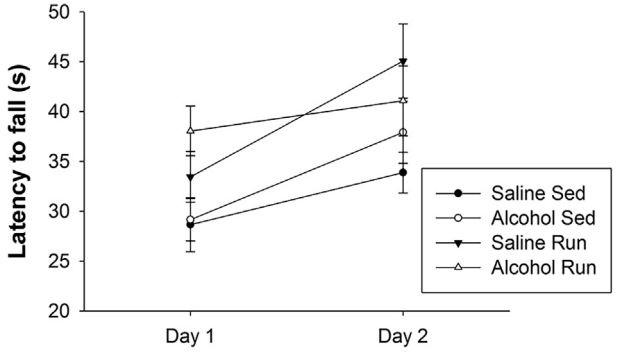


Fig. 4. Neonatal alcohol exposure has no impact on Rotarod performance, while previous exposure to exercise enhances performance. PD 5, 7, and 9 alcohol exposure fails to impact Rotarod performance in adulthood on both Days of Rotarod testing. Running wheel access improved Rotarod performance in both saline and alcohol-exposed mice on Day 1. On Day 2, Alcohol Runners failed to show behavioral improvements on the task, while Saline Runners showed a significant improvement across days. All values represent mean ± SEM. $^{*}p < 0.05$.

Although a few studies suggest neonatal alcohol exposure can either increase or have no impact on the number of PV+ interneurons (Mitchell et al., 2000; Skorput and Yeh, 2016), the current data add to a growing literature that shows a negative influence of neonatal alcohol exposure on PV+ interneurons (Moore et al., 1998; Coleman et al., 2009; Smiley et al., 2015). PD 7 ethanol treatment has been shown to result in over a 30% reduction in the number of PV+ interneurons in the cerebral cortex when measured in adulthood (Smiley et al., 2015) and rat pups born to dams fed an ethanol-containing liquid diet throughout gestation exhibit 45% fewer PV+ interneurons in the ACC in the absence of changes in structure volume on PD 60 (Moore et al., 1998). It is well acknowledged that PV+ interneurons have a strong influence over the output of mPFC pyramidal cells (Rotaru et al., 2005; Woo and Lu, 2006). Therefore, perhaps PD 5, 7, and 9 alcohol exposure impacts the role of PV+ interneurons, via population reduction, most notably in the ACC.

Alterations to the number of PV+ interneurons can dysregulate the information that is transmitted from the mediodorsal nucleus of the thalamus to mPFC pyramidal cells (Kuroda et al., 2004; Rotaru et al., 2005) and as such can affect mPFC networks. For example, research shows that by increasing the activity of PV+ fast spiking intervals, reward-related behavioral flexibility may be enhanced (Sparta et al., 2014). Moreover, Plaur null mice (decreased GABAergic interneurons in frontal and parietal regions) demonstrate impaired medial frontal cortical function in extinction of cued fear conditioning and the inability to form attentional sets (Bissonette et al., 2015). Therefore, fewer PV+ interneurons could result in less inhibition on the pyramidal cells and lead to an overexcited mPFC. In fact, methylazoxymethanol acetate-treated rats display a decreased density of PV+ cells throughout the mPFC and this is correlated with impaired behavioral expression of latent inhibition (Lodge et al., 2009). Therefore, by reducing numbers of PV+ interneurons in the ACC, PD 5, 7, and 9 alcohol exposure may likely result in altered ACC function.

The ACC is known to be involved in many behaviors, most notably inhibitory avoidance and fear-associated learning. Specifically, the ACC appears to be recruited during aversive conditions (Albrechet-Souza et al., 2009) and plays a key role in the acquisition of fear (Rotaru et al., 2005; Bissiere et al., 2008; Kooistra et al., 2010). Therefore, damage to the ACC would undoubtedly impact fear-conditioning performance. In fact, intra-ACC injections of muscimol have been shown to decrease freezing responses (Almada et al., 2015) while lesions to the ACC prior to training impair fear acquisition, while leaving fear expression intact (Bissiere et al., 2008). Further, rabbits that received ACC lesions exhibit delayed acquisition of discriminative avoidance learning (Gabriel et al., 1991). The passive avoidance task is a variety of fear conditioning that also requires the animal to demonstrate apt inhibitory avoidance performance. Therefore this is a behavioral task that most likely requires the proper functioning of the ACC. It is possible that alcohol-induced reductions in the number of PV+ interneurons in the ACC may be associated with the impaired passive avoidance performance seen in alcohol-exposed mice.

Behavioral deficits on the passive avoidance task could have been partially mediated by the loss and therefore function of PV+ cells in the anterior cingulate network. Our results demonstrate that a PD 5, 7, and 9 alcohol exposure impaired acquisition but not retrieval of passive avoidance. Acquisition of this task requires the animal to learn to ignore its innate response to move to the

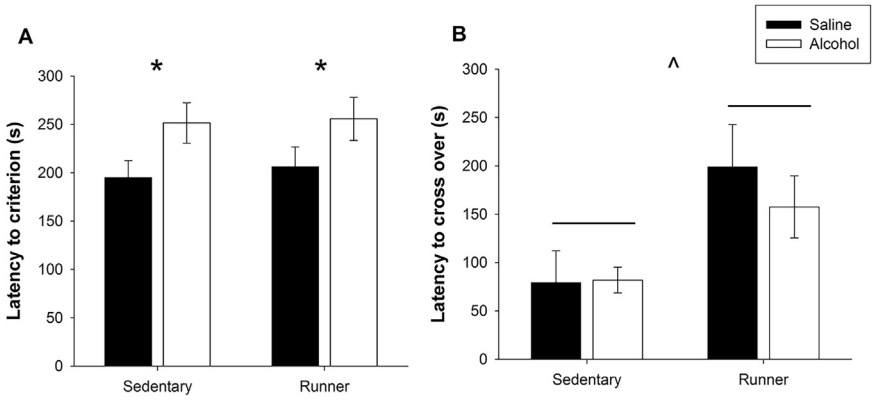
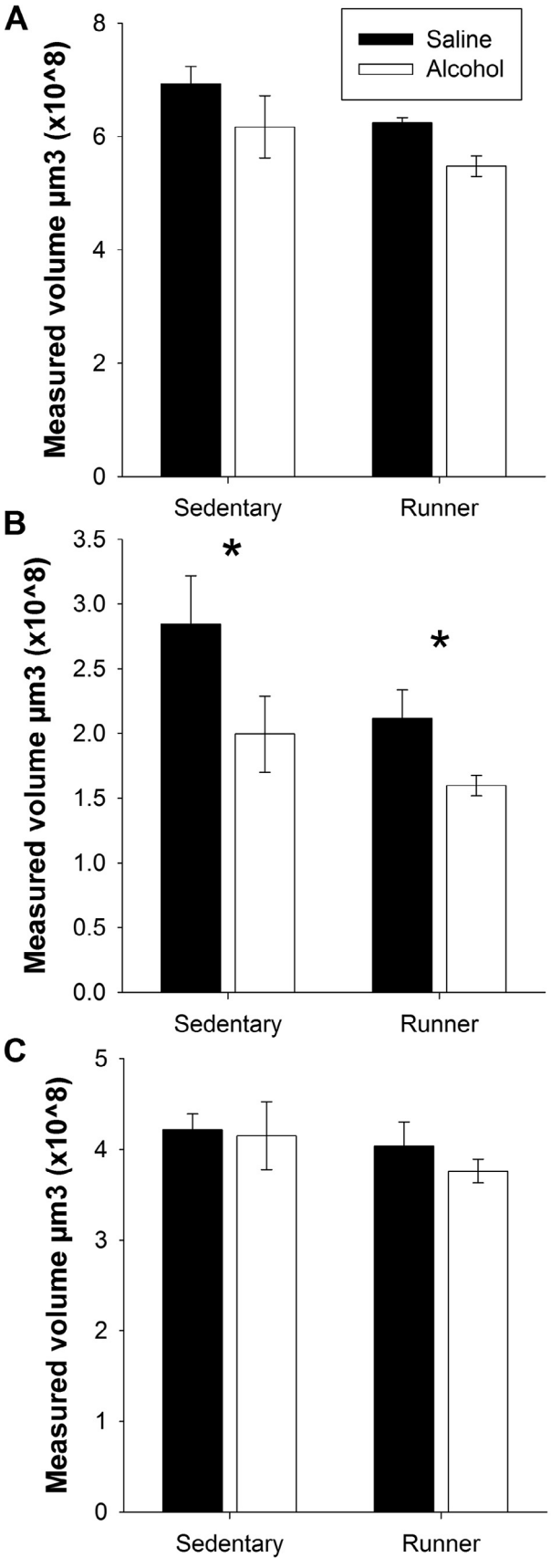


Fig. 5. PD 5, 7, and 9 alcohol exposure impairs passive avoidance performance. (A) Latency to reach criterion on Day 1. When tested in adulthood, mice exposed to a PD 5, 7, and 9 alcohol exposure required a significantly longer time to reach criterion compared to their saline-exposed littermates, regardless of Intervention. (B) Latency to cross over into the dark chamber on Day 2. Mice given access to a running wheel demonstrate significantly longer latencies to cross over than their sedentary littermates, regardless of Postnatal Treatment condition. All values represent mean ± SEM. $^{*}p < 0.05$, $^{\wedge}p < 0.001$.



dark side of the box and instead stay in the light side. Therefore, this component of the task requires behavioral flexibility and proper inhibitory control, two highly PFC-associated behavioral traits, in order to be properly executed. And in fact, we saw that alcohol-exposed animals required a significantly longer time to learn this task than did their saline counterparts (Fig. 5a). Bilateral electrolytic lesion of the mediodorsal nucleus in PD 4 rat pups also produced delays in acquisition during the passive avoidance test when tested in young adulthood (Ouhaz et al., 2015). PV+ interneurons mediate mediodorsal nucleus-driven feed-forward inhibition in the dorsal ACC (Delevich et al., 2015). In fact, Delevich and colleagues (2015) suggested that PV+ cells limit the time window during which pyramidal neurons are capable of integrating excitatory inputs. Therefore, PD 5, 7 and 9 alcohol exposure may impact passive avoidance acquisition by altering the mediodorsal-ACC circuit.

It is not completely clear why the ACC region was selectively sensitive to the PD 5, 7 and 9 alcohol exposure. It is possible that the mediodorsal-ACC circuit, which is still developing during this time window, is preferentially affected. It is also likely that the IL and PL are indeed sensitive to alcohol exposure; but that this is just not evident with the measures we chose. Still the ACC has shown a selective sensitivity to other neonatal insults. For example, in the Degus rodent, stress exposure during the first three postnatal weeks has been shown to produce a late developmental time window between puberty and adulthood in the density of interneurons in the ACC but not the PL or IL (Helmeke et al., 2008). Thus, more research is needed in understanding the selective sensitivity of the ACC to neonatal alcohol exposure.

Interestingly, PD 5, 7 and 9 alcohol exposure did not significantly impair rotarod performance. This was a little surprising given that motor deficits are well described in the alcohol literature (Goodlett et al., 1991; Klintsova et al., 2000). Still, alcohol-exposed animals did exhibit a lack of benefit from the exercise intervention. This perhaps suggests that there were still some motor deficits present. Moreover, recently Fish and colleagues (2016) failed to see any impact of an acute gestational alcohol exposure on the rotarod performance of C57BL/6J males. Therefore, it is possible that the impact of neonatal alcohol exposure on motor performance is a little less reliable in mice than it is in rats.

Exercise did not prove to be an effective intervention for treating alcohol-induced mPFC deficits. It had no impact on the number of PV+ interneurons and it did not rescue behavioral deficits on Day 1 (acquisition) of the passive avoidance task. Previously, we have shown that exercise can both increase levels of adult

Fig. 6. PD 5, 7, and 9 administration has long-term impact on not the overall volume, but rather distinct mPFC subregions. (A) Neither PD 5, 7, and 9 alcohol exposure nor exercise impacts mPFC volume. (B) PD 5, 7, and 9 alcohol exposure significantly reduces the volume of the ACC of the mPFC. Exercise has no impact on ACC volume. (C) Neither PD 5, 7, and 9 alcohol exposure nor exercise impacts the volume of the PL/IL. All values represent mean ± SEM. $^{*}p < 0.05$.

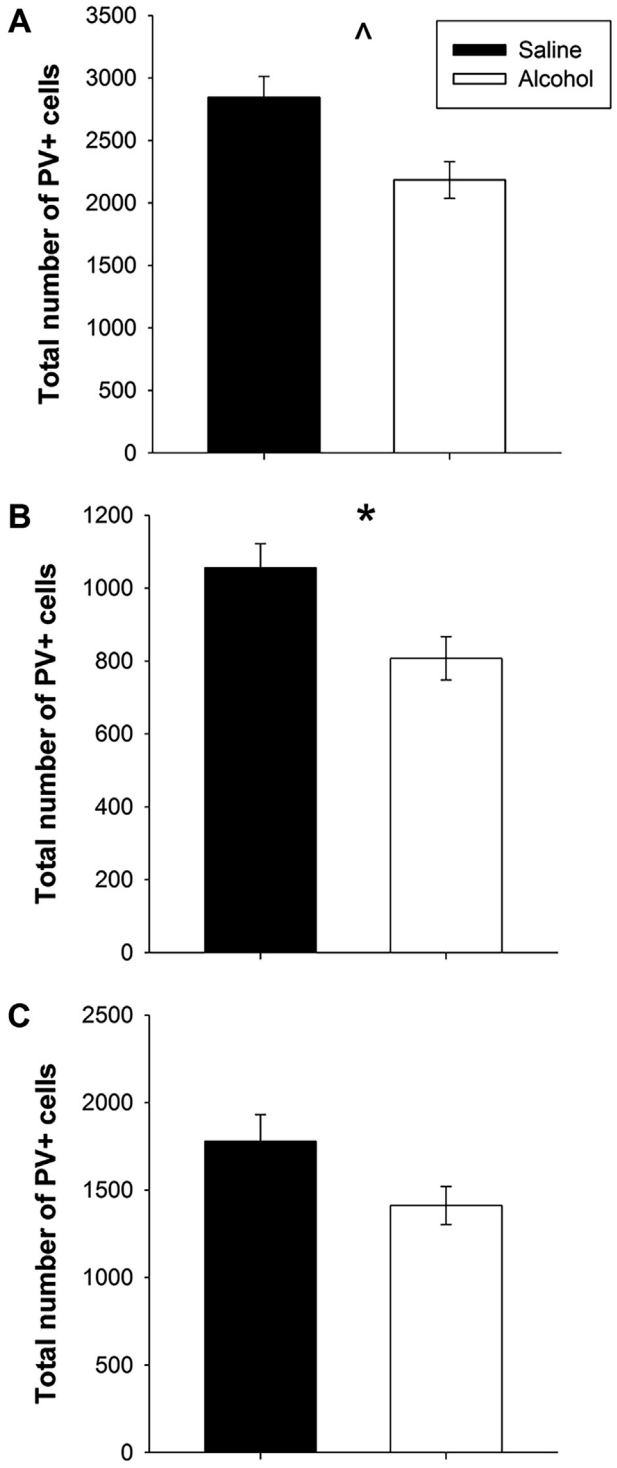


Fig. 7. PD 5, 7, and 9 administration has long-term impact on the number of mPFC PV+ interneurons. (A) PD 5, 7, and 9 alcohol exposure significantly reduces the number of GABAergic PV+ interneurons in the mPFC. Exercise has no impact on number of PV+ interneurons. (B) PD 5, 7, and 9 alcohol exposure significantly reduces the number of PV+ interneurons in the ACC. No effect of exercise. (C) Neither PD 5, 7, and 9 alcohol exposure nor exercise impacts the number of PV+ GABAergic interneurons in the PL/IL. All values represent mean ± SEM. $^{*}p < 0.05$, $^{\wedge}p < 0.001$.

hippocampal neurogenesis and enhance behavioral performance on Day 2 (retrieval). However, neither of these measures are impacted by PD 5, 7, and 9 alcohol exposure (Hamilton et al., 2016). Similarly, our current results show that although motor performance was not impacted by a PD 5, 7, and 9 alcohol exposure, alcohol-exposed mice did exhibit a limited benefit of the exercise intervention. This may be due to the fact that alcohol-exposed animals ran significantly less than their saline counterparts. Therefore, these results simply demonstrate the impact of exercise rather than judge its merit as an intervention. Exercise alone has been shown to increase the number of PV+ interneurons in the hippocampus. However, the hippocampus is a neurogenic region and it is likely a lot more susceptible to the benefits of exercise as there is a large literature that shows exercise increases levels of hippocampal plasticity (*for review see* Trivino-Paredes et al., 2016). Another commonly utilized intervention that sometimes includes aerobic exercise is environmental enrichment. Access to an enriched environment has been shown to increase the number of PV+ interneurons in the basolateral amygdala (Urakawa et al., 2013) and also rescue impaired freezing responses as well as developmental delays in PV+ interneurons in the PFC and hippocampus of animals exposed to a PD 6–8 NMDA antagonist (sevofluorance) exposure (Ji et al., 2016). It is possible that the running alone may not be sufficient to consistently rescue alcohol-related deficits in the mPFC and that instead more complex interventions, including environmental enrichment components may be necessary.

Finally, there are a few important limitations that must be acknowledged within this study. The first is the use of single housing. This housing option was required in order to record individual running levels. We acknowledge that single housing has been found to induce stress responses in both rats (Chappell et al., 2013) and mice (Berry et al., 2012). Single housing has been linked to not only increased locomotor activity in several tests of exploration but also reduced anxiety-like behaviors in exploration (Haupt and Schaefers, 2010). However, a growing literature suggests that single housing may not be as stressful for mice as it is for rats. For example, a recent study by Lopez and Laber (2015) found that as long as mice were provided with nesting material during adolescence, they showed no difference in voluntary ethanol intake when compared to group-housed animals. In addition, studies have found no significant differences in stress marks of mice raised in single housing versus mice raised in group housing (Hunt and Hambly, 2006; Arndt et al., 2009). Rather, one study showed that group housing significantly increased corticosterone levels in male mice when compared to those housed singly (Kamakura and Leppaluoto, 2016). Regardless of the impact single housing may have had, all the groups were singly housed in our study, hence single housing was a controlled variable. Second, it is important to note that the authors chose to focus solely on PV+ interneurons. This was done because we were primarily interested in the crucial inhibitory role PV+ interneurons have on mPFC pyramidal neurons. However, it is very likely that neonatal alcohol exposure impacts other classes of interneurons. In fact, it was recently demonstrated that a PD 7 alcohol exposure

leads to long-term reductions in not only the number of PV+ (34%) but also the number of calretinin-positive (32%) neurons in the cerebral cortex when measured in adulthood (Smiley et al., 2015). Further, gestational alcohol exposure has been shown to cause alterations in the immunoreaction of calbindin-D28k, calretinin and PV in neurons of the cerebellar cortex of ten-day-old rat pups (Wierzba-Bobrowicz et al., 2011). Therefore, future research should explore this question of how PD 5, 7, and 9 alcohol exposure impacts other interneuron classes. This would provide a better understanding of how neonatal alcohol exposure impacts inhibitory connectivity in the mPFC. Lastly, we chose to focus on the impact of neonatal alcohol exposure on PV+ populations in specific mPFC subregions rather than the layer-specific distribution of PV+ cells. This was because we hypothesized there would be region-specific decreases in the number of PV+ interneurons and that they would coincide with functional deficits on the passive avoidance task. However, we must still acknowledge that interneurons generated from the caudal ganglionic eminence are still migrating to their layer II/III adult location during the first postnatal week (Miyoshi and Fishell, 2011) and that the PD 5, 7 and 9 alcohol exposure likely impacted this migration pattern. On the other hand, it has been suggested that PV+ interneurons originate primarily within the medial ganglionic eminence (Xu et al., 2004) and therefore have finished migrating (Miyoshi and Fishell, 2011). Future research should examine the impact of a PD 5, 7, and 9 alcohol exposure on the layer-specific distribution of ont only PV+ interneurons but other interneuron classes as well. In doing so, it would provide us with a better understanding of how alcohol exposure impacts the migrational pattern of developing interneurons.

CONCLUSION

In summary, this study found for the first time that a PD 5, 7, and 9 alcohol exposure significantly reduces the number of PV+ interneurons in a specific subregion of the mPFC, the ACC. PV+ interneurons crucially regulate the activation of the mPFC, and the ACC plays an important role in behavioral inhibition and fear-associated learning. The current data suggest that impaired circuitry in the ACC may be associated with impaired performance on the Passive avoidance task, specifically during the acquisition stage. This possible association between brain region and functional deficit in the alcohol-exposed brain could be useful for developing more targeted interventions in order to mitigate alcohol-induced mPFC damage.

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