Published in final edited form as:

Synapse. 2015 August ; 69(8): 405–415. doi:10.1002/syn.21827.

Voluntary Exercise Partially Reverses Neonatal Alcohol-Induced Deficits in mPFC Layer II/III Dendritic Morphology of Male Adolescent Rats

G.F. Hamilton[§], K.J. Criss, and A.Y. Klintsova

Department of Psychological and Brain Sciences, Univ. of Delaware, Newark, DE 19716, USA

Abstract

Developmental alcohol exposure in humans can produce a wide range of deficits collectively referred to as Fetal Alcohol Spectrum Disorders (FASD). FASD-related impairments in executive functioning later in life suggest long-term damage to the prefrontal cortex (PFC). In rodent neonates, moderate to high levels of alcohol exposure decreased frontal lobe brain size and altered medial PFC pyramidal neuron dendritic morphology. Previous research in our lab demonstrated that neonatal alcohol exposure decreased basilar dendritic complexity but did not affect spine density in Layer II/III pyramidal neurons in 26–30 day old rats. The current study adds to the literature by evaluating the effect of neonatal alcohol exposure on mPFC Layer II/III basilar dendritic morphology in adolescent male rats. Additionally, it examines the potential for voluntary exercise to mitigate alcohol-induced deficits on mPFC dendritic complexity. An animal model of binge drinking during the third trimester of pregnancy was used. Rats were intubated with alcohol (alcohol-exposed, AE; 5.25g/kg/day) on postnatal day (PD) 4–9; two control groups were included (suckle control and sham-intubated). Rats were anesthetized and perfused with heparinized saline solution on PD 42, and brains were processed for Golgi-Cox staining. Developmental alcohol exposure decreased spine density and dendritic complexity of basilar dendrites of Layer II/III neurons in the medial PFC (mPFC) compared to dendrites of control animals. Voluntary exercise increased spine density and dendritic length in AE animals resulting in elimination of the differences between AE and SH rats. Thus, voluntary exercise during early adolescence selectively rescued alcohol-induced morphological deficits in the mPFC.

Graphical Abstract

*Correspondence to: Anna Y. Klintsova, Department of Psychological and Brain Sciences, University of Delaware, 108 Wolf Hall, Newark, DE, 19716, USA, Phone: (302) 831-2647, Fax: (302) 831-3645, klintsov@udel.edu.

[§]Currently at Beckman Institute, University of Illinois at Urbana-Champaign

The authors have no conflict of interest.

Keywords

Golgi; plasticity; wheel running; fetal alcohol spectrum disorders; intervention

INTRODUCTION

The number of children diagnosed with Fetal Alcohol Spectrum Disorders (FASD) has not decreased since 1981, and its estimated occurrence is as high as 2–5% of all live births in the United States (May et al., 2009; Morleo et al., 2011; Sampson et al., 1997). Additionally, despite the known dangers of alcohol consumption during pregnancy, a study conducted by the Centers for Disease Control and Prevention between 1995 and 2001 found the average annual percentage of any alcohol use among pregnant women to be 12.2% with 1.9% of women engaging in binge drinking, which was defined as four or more glasses at one time (Denny et al., 2009). Thus, the need for therapeutic interventions for children born with FASD is necessary and long overdue.

Individuals with FASD exhibit a wide range of deficits including, but not limited to, learning and memory impairments as well as behavioral disabilities consistent with those seen in patients with frontal lobe damage (i.e. executive functioning impairments; Connor et al., 2000; Rasmussen, 2005). Furthermore, neuroanatomical abnormalities resulting from developmental alcohol exposure, such as decreased frontal lobe size (Rasmussen, 2005; Sowell et al., 2002; Wass et al., 2001), may underlie FASD-related behavioral and learning deficits. Given that dendritic abnormalities are the most consistent anatomical correlates of mental retardation (Kaufmann and Moser, 2000); they likely play a role in said neurobehavioral and gross neuroanatomical deficits. In fact, reductions in dendritic spine density and a predominance of immature spines were evident in the brain of a four-month-old child born to a chronic alcoholic mother, as well as abnormal maturation of nerve cells (Ferrer and Galofre, 1987).

Rodent models of FASD have examined the impact of alcohol exposure on the developing brain. Brain regions such as the cerebellum, hippocampal dentate gyrus and the prefrontal cortex (PFC) appear especially sensitive to alcohol exposure during the brain growth spurt—a period of neurodevelopment that occurs in the third trimester in humans and the first two postnatal weeks in rodents (Dobbing and Sands, 1979; Olney et al., 2002). In rodents, gestational alcohol exposure resulted in significant reduction (44%) in brain weight, along with decreased cell packing and neurodegeneration (mostly pyknotic pyramidal neurons, with collapsed cell bodies, obliterated nuclei and nucleoli) in Layer V of the cerebral cortex when measured in adolescence ((postnatal day (PD) 42) Fakoya and Caxton-Martins, 2006). Administration of a single binge-like exposure to alcohol on different days throughout neonatal development demonstrated a peak in the vulnerability of brain regions, including the frontal cortex, to alcohol on PD 7, as measured by levels of apoptosis (Ikonomidou et al., 2000). PD 7 alcohol exposure also decreased levels of frontal cortical parvalbumin immunoreactive interneurons when measured in adulthood (PD82) (Coleman et al., 2012). Further, alcohol exposure by inhalation during the third trimester-equivalent resulted in simplified dendritic branching in basilar dendrites of Layer III neurons of the somatosensory

cortex in adult rats (Granato et al., 2003). Additionally, previous work in our lab demonstrated that a postnatal binge exposure to alcohol reduced Layer III spine density on apical dendrites in mPFC pyramidal neurons (Whitcher and Klintsova, 2008) and decreased dendritic complexity in the proximal basilar dendrites of Layer II/III mPFC neurons (Hamilton et al., 2010). Together, these data suggest that neonatal alcohol exposure may alter the connectivity of mPFC pyramidal neurons, which likely plays a role in FASD behavioral deficits.

Exercise has consistently been shown to enhance brain plasticity. In humans, aerobic exercise increases prefrontal cortical and grey matter volume (de Lange et al., 2008; Erickson and Kramer, 2009; Floel et al., 2010). Further, in rodent models of FASD, an exercise intervention has proven time and again to be a successful therapeutic tool when targeting alcohol-induced deficits in other brain regions (for review see Klintsova et al., 2012). The literature examining the impact of exercise on the PFC in rodent models of FASD, however, is scarce. Recent work in the field has demonstrated increased levels of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) (Aksu et al., 2012; Hopkins et al., 2011; Uysal et al., 2011), in the cortex, suggesting a global enhancement in cellular plasticity. Together, these data suggest the mPFC may be susceptible to the beneficial influence of voluntary exercise; however, this influence may be different than that seen in the hippocampus.

The current study provides an important addition to the literature by first determining whether a third trimester binge alcohol exposure has a long-lasting detrimental influence on the dendritic organization of mPFC Layer II/III pyramidal neurons (as measured on PD 42). Second, it addresses the role of voluntary exercise (WR) as a therapeutic tool for FASD-related mPFC morphological damage. To the authors' knowledge, it is the first study to explore the possible beneficial effect of exercise on alcohol-induced damage to the dendritic complexity of mPFC Layer II/III pyramidal neurons.

MATERIALS AND METHODS

Subjects

All procedures were done in accordance with the University of Delaware Institutional Animal Care and Use Committee. Subjects were male Long Evans offspring from timed pregnancies bred at the University of Delaware animal breeding colony. Seven litters were culled to eight pups each—six males and two females when possible—on PD 3. In two litters, only five males were born, resulting in a total number of forty male Long-Evans rat pups. Additionally, three male rats died due to improper intubations, and the tissue from seven male animals was unusable due to poor staining quality and/or sliding of the tissue when focusing at 1000× magnification (100× objective). This resulted in a final N of thirty male rats for this study allocated as follows: suckle control (SC)/social housed (SH), n=4; sham-intubated (SI)/SH, n=5; Alcohol-exposed (AE)/SH, n=5; SC/wheel running (WR), n=6; SI/WR, n=5; AE/WR, n=5. A maximum of three males per litter were assigned to each condition. Previous work from our laboratory has demonstrated no litter effect, therefore, we chose to utilize litters efficiently. Only males were used in this study as it was a follow-up to previous studies, which only looked at males and also, we first wanted to establish that

alcohol would impact dendritic complexity at this age in males before adding another variable given the time it takes to properly collect data for one animal. On PD9, all pups received a single ear punch to further distinguish between litters (even numbered litters received a right ear punch, odd numbered litters a left ear punch). Pups remained housed with their dams and littermates under standard conditions in polypropylene cages measuring 21 cm high $\times$ 45 cm long $\times$ 24 cm wide until weaning on PD 23 when animals were placed in social housing cages (17 cm high $\times$ 145 cm long $\times$ 24 cm). Social housing consisted of three rats, one of each postnatal treatment, to a cage whenever possible. On PD 30 cages were then replaced with larger ones (21 $\times$ 45 $\times$ 24 cm) to accommodate the growing rats. Rats were given access to food *ad libitum* for the duration of the study. The housing facility was maintained on a 12:12-hr light-dark cycle, with lights on at 0900hr.

Alcohol Exposure

Administration of alcohol was similar to previous studies from the Klintsova lab (Hamilton et al., 2010; Whitcher and Klintsova, 2008). On PD 3, following culling, pups were randomly assigned to one of three dosing conditions: SC, SI or AE. From PD 4–9, AE animals received three intragastric intubations two hours apart. A daily dose of 5.25g/kg of alcohol (11.9% solution) was divided between the first two intubations each day. A third intubation of milk (no ethanol) was administered 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 to compensate for reduced milk intake by the pups from the dam. To control for the stress of the dosing procedure, SI pups were intubated alongside the AE animals. The dosing tube was removed after approximately ten to fifteen seconds without the infusion of any solution. In addition, a second control group, SC animals, was derived from separate litters than the other two conditions. SC animals were removed from the dams briefly each day and weighed during the dosing period but were otherwise left undisturbed.

Blood Alcohol Concentrations

Blood samples were collected from the tail vein by clipping the tail end of AE and SI pups 90 minutes after the second alcohol dose on PD 4. Samples from AE animals were centrifuged (15000 rpm for 15 mins) and then the plasma was collected and frozen (-20°C) for future analysis using an Analox GL5 Alcohol Analyzer (Analox Instruments, Boston, MA). Samples from SI animals were discarded after collection.

Wheel Running

Similar to previous studies from our lab (Helfer et al., 2009) and other labs (Eadie et al., 2005; van Praag et al., 1999), animals were housed in cages (21 $\times$ 45 $\times$ 24 cm) attached with stainless steel running wheels to allow voluntary access to exercise. Specifically in this study, half of the animals from each postnatal condition were pseudorandomly assigned to cages with attached stainless steel running wheels, allowing them to have 24-hour access to voluntary exercise beginning at 9:00am on PD 30 and ending at 9:00am on PD 42. Each cage contained one animal from each of the three postnatal treatments (AE, SI, and SC) since previous studies have shown that there is no difference in running activity between the animals from these three groups (Helfer et al., 2009). Running wheels were equipped with

counters, which allowed for record keeping of running activity. Each morning at the onset of the light cycle (9:00am), the number of wheel rotations was recorded in order to determine running distance across days, as well as across the entire exposure. Every third day, the bedding was replaced in the cages without the removal of the rats. Sedentary animals from all three postnatal treatments remained in social housing (SH), which consisted of polypropylene (21×45 × 24 cm) cages throughout the duration of the experiment.

Golgi-Cox Staining

The impregnation procedure was based on methods developed by Gibb and Kolb (1998) and has been used previously in the Klintsova lab (Hamilton et al., 2010; Whitcher and Klintsova, 2008). On PD 42, animals were deeply anesthetized and perfused through the aorta with 0.9% saline. Brains were placed in Golgi-Cox solution (1% potassium dichromate/1% mercuric chloride/1% potassium chromate in distilled water) and left in the dark for 2 weeks as in the original protocol (Gibb and Kolb, 1998). For two days prior to sectioning of the tissue, the solution was replaced with 30% sucrose in 0.9% saline each day. Coronal 200µm thick sections were obtained with a vibratome and mounted on gelatinized double subbed slides. After being kept in cool, high humidity environment overnight, slides were dipped in distilled water for 1 minute followed by a 30 minute immersion in 2:1 ammonium hydroxide: distilled water solution in the dark. Slides were then rinsed in distilled water for 1 minute and placed in a 1:1 Kodak rapid fixer: distilled water solution for 30 minutes in the dark. Again, the slides were washed in distilled water and then run through a series of alcohol solutions (70%, 95%, 100%) to dehydrate the tissue. Finally, the slides were placed in 1:1 100% ethanol: histoclear solution followed by histoclear for 15 minutes each. Immediately following this procedure, the slides were coverslipped using Permount (Fisher Scientific) and stored in a cool dark place. Figure 1 shows a representative Golgi-impregnated cell from an Alcohol-SH animal.

Dendritic Analysis

Sholl Analysis—Tissue was coded to ensure that the experimenter remained blind to animal condition throughout the analysis procedure. The area of interest began at Bregma 3.7 and extended through the next seven posterior sections in rostral-caudal direction, a total of eight sections per animal. Coded tissue was traced and measured using a computer-based neuron tracing system (NeuroLucida-Version 8.10.1; MBF Bioscience, Williston, VT). For each section, the mPFC was first viewed at the lowest magnification (5× objective) and outlined on the image projected onto the computer screen. Cells that demonstrated characteristic pyramidal-shaped cell body, were located between 300–500µm from the cortical surface within Zilles Cg1 or Cg3 region (Zilles, 1985) and whose basilar branches were fully impregnated, unobstructed, and contained within the section being observed were selected for analysis (selection occurred at 10×). The Line Measure Tool in NeuroLucida software was used to ensure that selected neurons were at appropriate depth from the cortical surface. Layer II/III neurons were then traced using a 100×/1.30 N.A. oil lens. While tracing the neuron, the software automatically assigned the branching order beginning with the first bifurcation of the dendrite. A Sholl analysis was performed in order to determine dendritic complexity and their extension. The total length of dendrites, the number of bifurcations and the number of endings were also analyzed for each neuron. Seven cells

were analyzed per animal, and the mean of all neurons in an individual animal was used to perform statistics.

Spine Analysis—Spine analysis was performed on previously traced dendritic trees for Order 2 (which are located proximal to the cell body) and Order 4 branches (more distal). Only branches greater than 20µm in length were analyzed. Estimation of spine density consisted of marking all the spines on the selected dendritic branches for each cell and then calculating the number of spines per 10µm. In addition, the first ten spines of each branch were classified as immature or mature based on the characteristics outlined by Irwin and colleagues (Irwin et al., 2002). The total density of mature versus immature spines was then calculated for each animal. Further, at the site of each spine included in analysis, the dendritic width was measured to ensure that the thickness of dendritic branches did not differ between any of the three animal groups. No significant differences existed between groups in the width of dendrites used for spine phenotypes.

Data Analysis

Body weights were analyzed using a repeated-measure ANOVA with Treatment (Alcohol, SI, SC) as the between subjects factor and Postnatal Day (PD 4, 9, 30 and 42) as a within subjects factor. Weights from the neonatal period (PD 4 and 9) versus adolescence (PD 30 and 42) were analyzed separately.

To determine the impact of developmental alcohol exposure on Layer II/III mPFC basilar dendrites, initially ANOVAs were performed that included all three of the treatment groups. However, no significant differences between the two control groups were evident. Therefore, in accordance with previously published work, further analyses were performed with the two control groups (SC and SI) combined (Otero et al., 2012). To analyze dendritic complexity, initially, a Sholl Ring $\times$ Treatment $\times$ Intervention repeated measure ANOVA was performed. Then, given our a priori hypothesis that differences between the treatment groups in SH but not WR conditions, two-way ANOVAs were performed. Further, two-way ANOVAs were used to evaluate the effects of Treatment (Control, Alcohol) and Intervention (SH, WR) on total dendritic length, spine density and the ratio of mature:immature spine phenotypes of basilar dendrites. The SPSS (Version 20, IBM SPSS Statistics) statistical package was used for all analyses. The level of significance was set at $p < 0.05$ for all tests. The data in the text and figures are presented as mean $\pm$ SEM.

RESULTS

Weights

Animal weights were recorded every day during alcohol administration (PD 4 through PD 9) and at the onset and end of WR (PD 30 and 42). All animals continued to gain weight throughout treatments as summarized in Table 1. The influence of postnatal alcohol exposure on body weights was determined using a repeated measures ANOVA with Treatment (SC, SI, AE) as the between subjects factor and Day (4, 9, 30, 42) as a within subjects factor, followed by post hoc analysis (Tukey's test). 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 (PD 30, 42) were analyzed separately. Analysis of neonatal body weights revealed a Day $\times$ Treatment interaction [$F_{(1,26)}=25.540$, $p < 0.01$] as well as a main effect of Day [$F_{(1,26)}=1390.40$, $p < 0.01$]. Interestingly, no main effect of Treatment was present. A univariate ANOVA on PD 4 weights revealed no main effect of Treatment, while the same type of analysis on PD 9 weights revealed a significant main effect of Treatment [$F_{(1,26)}=8.662$, $p < 0.01$]. Posthoc tests demonstrated that AE animals weighed significantly less than both SI ($p < .01$) and SC ($p < .01$), while no difference between body weights of SI and SC animals was evident. This suggests a powerful effect of alcohol in stunting developmental growth. Analysis of adolescent body weights (PD 30 and 42) revealed a main effect of Day [$F_{(1, 26)}=4581.30$, $p < 0.01$], but neither a Day $\times$ Treatment interaction nor a main effect of Treatment, suggesting that Alcohol animals recovered from the reduction in body weight that resulted from neonatal alcohol exposure. In fact, similar decreases in body weights of AE animals have been reported using this model of alcohol exposure; however, given the addition of the current results to what we have previously reported, it appears that the influence of alcohol on body weight is temporally limited. As, when assessed on PD 30, there is no longer a difference in body weight between AE, SC or SI animals (Boschen et al., 2014; Hamilton et al., 2012). Still, the role of under-nutrition should not be discounted when interpreting the results of the current study.

Blood Alcohol Concentrations and Running Activity

Blood alcohol concentrations (BAC) were measured in samples obtained from each Alcohol animal 1.5 hours after the second ethanol dose on PD 4. BAC's ranged from 342.2 through 471.2 mg/dl with an average BAC of 400.1 ± 12.4 mg/dl. These BACs are similar to previously published studies using the same alcohol dose and delivery approach (Hamilton et al., 2012; Hamilton et al., 2011). WR activity was recorded each morning at 9:00am. Running distance was determined by using each cage as an individual unit. It ranged from 1.23 miles/day to 3.35 miles/day. The average running distance in this study was 2.49 ± 0.29 miles/day.

Dendritic Complexity

In order to assess the influence of postnatal alcohol exposure on cell morphology, a repeated measures ANOVA with Treatment (Control, Alcohol) as the between subjects factor and Ring (20 μ m – 300 μ m) as a within subjects factor was performed, followed by post hoc analysis (Tukey's test) was performed. Results demonstrated a significant main effect of Treatment [$F_{(1,26)}= 8.596$, $p < 0.01$] on dendritic complexity, in which the dendrites of Alcohol animals were significantly less complex than those of Control animals ($p < 0.05$). Still, neither a main effect of Intervention nor a Treatment $\times$ Intervention interaction was found. Given our *a priori* hypothesis that we would expect to see a difference in Treatment in the SH animals but not in the WR animals, separate repeated measure ANOVAs were performed for both Intervention groups. In the SH condition, there was a main effect of Treatment [$F_{(1,12)}=7.147$, $p = 0.020$]. Post hoc tests revealed significant differences between treatment groups at 40 μ m ($p < 0.05$), 60 μ m ($p < 0.05$) and 80 μ m ($p < 0.05$) from the cell soma, wherein dendrites of Alcohol-SH animals had fewer intersections with the Sholl rings than did those of Control-SH animals (Figure 2a). No such effect of Treatment was

evident in the data from WR animals [$F(1,14)=2.925$, $p = 0.11$] (Figure 2b). Additionally, no overall effect of WR on dendritic complexity was evident in the pyramidal neurons of Control (Figure 2c) and Alcohol (Figure 2d) animals. The impact of Alcohol on dendritic complexity is illustrated in Figure 3, which depicts Neurolucida tracings of cells from both an Alcohol-SH and a Control-SH rat. These data indicate a negative impact of neonatal alcohol on dendritic morphology that is not rescued by adolescent exposure to voluntary exercise.

Dendritic Length

Dendritic length is another parameter that was assessed to determine the long-term impact of both neonatal alcohol exposure (Treatment) and voluntary exercise (Intervention) on dendritic complexity. A two-way ANOVA revealed a lack of Treatment $\times$ Intervention interaction, while a main effect of Treatment [$F_{(1,26)}=5.184$, $p = 0.031$] and a trend for an effect of Intervention [$F_{(1,26)}=3.232$, $p = 0.084$] were present. Still, given our *a priori* hypothesis that dendritic length would be decreased in the Alcohol-SH animals compared to the Control-SH animals but that all WR animals (both Alcohol and Control groups) would have similar total dendritic length, one-way ANOVAs were performed separately for the data from SH animals and WR animals. Alcohol-SH animals had significantly less total dendritic length than did Control-SH animals ($1348.94 \pm 168.16 \mu\text{m}$ versus $1560.81 \pm 116.73 \mu\text{m}$), [$F_{(1,12)}=7.795$, $p = 0.016$]. In comparison, neither a significant effect of Treatment was seen in the WR animals (Figure 4a), nor was there a significant effect of Intervention in the alcohol animals. Overall, these data indicate the negative impact of neonatal alcohol exposure on total dendritic length.

Dendritic Bifurcations

In addition, the influence of neonatal alcohol exposure (Treatment) and voluntary exercise (Intervention) on the number of bifurcations in the dendritic tree was examined. Results indicate a main effect of Treatment [$F_{(1,26)}=4.816$, $p = 0.037$], as the basilar dendritic trees of Alcohol animals have significantly fewer bifurcations than do those of Control animals (Figure 4b). Neither a main effect of Intervention nor a Treatment $\times$ Intervention interaction was detected. Still, given our *a priori* hypothesis that the number of bifurcations would be decreased in the Alcohol-SH animals compared to the Control-SH animals but that all WR animals (both Alcohol and Control groups) would have similar numbers of bifurcations, one-way ANOVAs were performed separately for the data from SH animals and WR animals. In accordance with our original hypothesis, separate ANOVAs exploring the influence of Treatment in the distinct Intervention groups were performed. A trend for an effect of Treatment was seen in the SH group [$F_{(1,12)}=3.615$, $p = 0.082$], while no difference between groups was evident in the WR animals. Still, WR did not appear to fully rescue alcohol-induced deficits in this measure of plasticity, as no difference between the Alcohol-SH and Alcohol-WR group was found.

Dendritic Endpoints

The influence of neonatal alcohol exposure (Treatment) and voluntary exercise (Intervention) on the total number of dendritic endpoints was explored. Results indicated a

main effect of Treatment, wherein alcohol exposure resulted in a decreased number of dendritic endpoints in cells [$F_{(1,26)}=4.922$, $p = 0.035$], but neither a main effect of Intervention nor an interaction between the two variables was evident. As our *a priori* hypothesis stated that the number of endpoints would be decreased in the Alcohol-SH animals but not the Control-SH animals and that no difference would be seen in the WR animals (both Alcohol and Control groups). Therefore, univariate ANOVAs were performed separately for the data from SH animals and WR animals. A one-way ANOVA exploring the influence of alcohol in the SH animals demonstrated a significant decrease in the number of end points [$F_{(1,12)}=4.905$, $p = 0.047$] in the Alcohol-SH animals compared to the Control-SH (14.68 ± 0.79 versus 16.84 ± 0.58). Interestingly, WR did not seem to rescue alcohol-induced deficits in this measure of dendritic complexity (AE-SH vs AE-WR: $p > 0.05$).

Dendritic Spine Density

In order to examine the influence of neonatal alcohol exposure (Treatment) and voluntary exercise (Intervention) on dendritic spine plasticity, spine density was calculated per $10\mu\text{m}$ of dendritic length in two different locations on dendritic tree. For Order 2 Spine density, a two-way ANOVA exploring Treatment $\times$ Intervention revealed no significant Treatment $\times$ Intervention interaction; however, a main effect of Treatment was found, wherein Alcohol animals had fewer Order 2 dendritic spines than did Control animals [$F_{(1,25)} = 6.890$, $p = 0.015$]. A trend for an effect of Intervention was evident, in which animals exposed to WR exhibited a higher density of spines than did SH animals [$F_{(1,25)} = 4.136$, $p = 0.053$]. A separate one-way univariate ANOVA was next performed to examine the sole influence of Treatment on Order 2 spine density. Results indicate a significant difference between Order 2 spine density of Alcohol animals compared to Controls [$F_{(1,27)}=6.359$, $p = 0.018$], wherein Alcohol animals have significantly fewer spines than do Controls (9.18 ± 0.64 versus 10.60 ± 0.23 , respectively). In addition, the results of a one-way ANOVA revealed a significant difference between Order 2 spine density of Control-SH animals compared to that of the Alcohol-SH group ($p < 0.05$; 10.40 ± 0.34 versus 8.28 ± 0.90 , respectively). No such effect was found in the WR animals (Figure 5a).

For Order 4 branches, a two-way ANOVA of Treatment $\times$ Intervention revealed a main effect of Treatment [$F_{(1,25)} = 8.201$, $p < 0.01$] but neither a main effect of Intervention nor a Treatment $\times$ Intervention interaction (Figure 5b). A one-way ANOVA examining the impact of Treatment on Order 4 spine density showed a significant difference in Order 4 spine density across Treatment [$F_{(1,27)} = 8.720$, $p < 0.01$].

Dendritic Spine Phenotypes

The first ten spines of one Order 2 and one Order 4 branch were classified based on characteristics previously outlined (Irwin et al., 2002; McKinney et al., 2005). For Order 2 branches, a two-way univariate ANOVA of Treatment $\times$ Intervention revealed no main effect of either parameter. Similar outcomes were seen for Order 4 branches. Interestingly, a main effect of Order was found for both WR [$F_{(1,28)} = 16.071$, $p < 0.01$] and SH [$F_{(1,26)} = 44.257$, $p < 0.01$] animals, such that there was a significantly higher percentage of immature spines found on Order 4 compared to Order 2 branches in both SH (Figure 6a) and WR animals (Figure 6b).

DISCUSSION

The results of our study demonstrate the long-lasting detrimental influence of a binge exposure to alcohol during the third trimester-equivalent on the basilar dendritic morphology of Layer II/III mPFC pyramidal neurons. Neonatal alcohol exposure decreased complexity of the dendritic tree proximal to the soma as well as total dendritic length, the number of end points and spine density in both second and fourth order branches. In addition, the results of our study are the first to demonstrate that voluntary exercise may partially rescue developmental alcohol-related mPFC deficits, as running in the wheels mitigated the effects of neonatal alcohol exposure in some, but not all, parameters of dendritic complexity. For example, voluntary exercise ameliorated alcohol-induced deficits on dendritic length, number of endpoints and spine density, but had no influence on dendritic complexity as measured by the Sholl Analysis.

Previous work addressing the long-term influence of neonatal alcohol exposure on dendritic complexity in the mPFC is sparse. In a study by Granato and Van Pelt (2003), ethanol was administered via inhalation (95% v/v injected at a rate of 2.5 ml/min) to neonatal pups on PD 2–6 for 3 hours a day. Using a computational dendritic growth model, this study determined that neonatal alcohol exposure affects mainly the branching of the outgrowing neurites in Layer II/III associative pyramidal neurons. This was followed by a study wherein Granato and colleagues (2003) examined the long-term influence of neonatal alcohol exposure on the labeling and complexity of Layer II/III associative pyramidal neurons in the somatosensory cortex of PD 90 rats. Neonatal alcohol exposure produced a robust simplification of dendritic branching, as evidenced by a reduced number of dendritic end points as well as an overall significant decrease in the total length of basal dendrites. Interestingly, while neonatal alcohol exposure increased dendritic complexity in Layer V neurons (Granato et al., 2003) it did not influence dendritic complexity in apical dendrites of Layer II/III mPFC neurons, but rather it decreased spine density on these branches (Whitcher and Klintsova, 2008). In addition, administration of alcohol throughout all three trimesters (dams received 4.5 g/kg/day in drinking water during gestation, PD2–10 pups were intubated with 3.0g/kg/day) significantly reduced both basilar and apical spine density in PD 90 rats, but had no long-term effect on branching patterns or overall length (Lawrence et al., 2012). Previous work from our lab exploring the influence of a third trimester-equivalent binge alcohol-exposure (PD 4–9 5.25 g/kg/day) on dendritic morphology in preadolescent rats (PD 26–30) demonstrated decreased complexity in the proximal dendrites of Layer II/III mPFC neurons (Hamilton et al., 2010). The results of the current study add to this growing literature by showing that alterations in dendritic morphology resulting from a third trimester-equivalent binge alcohol-exposure persist at PD 42; however, deficits were evident in different measures of dendritic integrity (i.e. spine density) than found in previous work. Together, these data, along with the current literature, suggest a time window in which ethanol exposure induces damage to specific neuronal regions (i.e. apical vs. basal) and measures of dendritic integrity.

Additionally, this study demonstrates a decrease in spine density in adolescent rats exposed to alcohol neonatally. Previous studies in our lab showed that a similar exposure to alcohol did not affect spine density of basilar dendrites but did cause an increase in the number of

Author Manuscript

mature spines in preadolescent rats (Hamilton et al., 2010; Whitcher and Klintsova, 2008). The decrease observed in the present study can be explained by the fact that, following an initial increase, spine density decreases naturally due to spine pruning after the fourth week of postnatal life in rodents (Yuste, 2010). Therefore, neonatal alcohol exposure may accelerate this natural pruning, similar to how alcohol exposure has been shown to increase the incidence of death of neocortical cells both *in vitro* and *in vivo* (Ikonomidou et al., 2000; Jacobs and Miller, 2001). Decreases in spine density resulting from perinatal alcohol exposure also have been shown at PD 90 (Lawrence et al., 2012), which suggests that this effect is long-lasting and is perhaps triggered during the adolescent period. The natural pruning of spines that occurs during adolescence sculpts the brain circuitry so that it matches the demands asked of that particular cortical area (Yuste, 2010). Thus, alterations during this very sensitive time window could have long-lasting implications, such as FASD-related behavioral deficits.

Author Manuscript

This study, to the authors' knowledge, is the first to examine the role of voluntary exercise as a therapeutic intervention that could alleviate neonatal alcohol-induced morphological deficits in the mPFC. Exercise has consistently been shown to enhance brain plasticity, and there is growing support suggesting that neurotrophic factors mediate these beneficial changes. In particular, the neurotrophic factor BDNF, which promotes the differentiation, neurite extension and survival of a variety of neuronal populations, is highly influenced by exercise (Neeper et al., 1995; Vaynman and Gomez-Pinilla, 2005). Physical activity increases BDNF levels in brain regions known to be especially susceptible to developmental alcohol exposure: the cerebellum, cortex and hippocampus (Cotman and Berchtold, 2002; Vaynman and Gomez-Pinilla, 2005). Therefore, it is possible that exercise-induced increases in neurotrophic factors could have a significant positive impact on areas damaged during developmental alcohol exposure. In fact, previous work illustrates that running mitigated developmental alcohol-induced deficits in levels of not only both hippocampal BDNF and adult neurogenesis (Boehme et al., 2011) but also c-Fos expression in the hippocampus (Sim et al., 2008). Voluntary exercise has also been shown to rescue hippocampal-dependent behavioral deficits that result from developmental alcohol exposure (Thomas et al., 2008). Although the current study did not examine BDNF levels, it did provides novel information regarding the beneficial influence of voluntary exercise on prefrontal dendritic plasticity, which is modulated by BDNF, in the prefrontal cortex in developmental alcohol-exposed animals.

Author Manuscript

Our study provides evidence that voluntary exercise may be a suitable therapeutic intervention to mitigate the mPFC neuroanatomical deficits associated with FASD. Specifically, in this study we observed long-term alterations in dendritic complexity, dendritic length, number of dendritic endpoints and spine density as a result of a third trimester binge-like alcohol exposure. Access to a running wheel for twelve days during adolescence was selective in its influence, as it rescued dendritic length, number of endpoints in the dendritic tree, spine density, but not complexity in Layer II/III pyramidal neurons in mPFC. Limited work has examined the detrimental influence of fetal alcohol exposure in mPFC morphology and function. Thus, future studies should focus on determining how developmental alcohol exposure impacts the mPFC. Further, this is one of

the, if not the first, studies to examine the therapeutic role of exercise on alcohol-induced mPFC abnormalities. More research should focus on this understudied area, as it is possible that an exercise therapy could be beneficial in lessening mPFC behavioral and neuroanatomical deficits of FASD children.

ACKNOWLEDGEMENTS

This study was supported by the NIH AA009838 to AYK.

REFERENCES

- Aksu I, Baykara B, Ozbal S, Cetin F, Sisman AR, Dayi A, Gencoglu C, Tas A, Buyuk E, Gonenc-Arda S, Uysal N. Maternal treadmill exercise during pregnancy decreases anxiety and increases prefrontal cortex VEGF and BDNF levels of rat pups in early and late periods of life. *Neuroscience letters*. 2012; 516(2):221–225. [PubMed: 22503727]
- 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. *The European journal of neuroscience*. 2011; 33(10):1799–1811. [PubMed: 21535455]
- Boschen KE, Hamilton GF, Delorme JE, Klintsova AY. Activity and social behavior in a complex environment in rats neonatally exposed to alcohol. *Alcohol*. 2014; 48(6):533–541. [PubMed: 25150044]
- Coleman LG Jr, Oguz I, Lee J, Styner M, Crews FT. Postnatal day 7 ethanol treatment causes persistent reductions in adult mouse brain volume and cortical neurons with sex specific effects on neurogenesis. *Alcohol*. 2012; 46(6):603–612. [PubMed: 22572057]
- Connor PD, Sampson PD, Bookstein FL, Barr HM, Streissguth AP. Direct and indirect effects of prenatal alcohol damage on executive function. *Developmental neuropsychology*. 2000; 18(3):331–354. [PubMed: 11385829]
- Cotman CW, Berchtold NC. Exercise: a behavioral intervention to enhance brain health and plasticity. *Trends in neurosciences*. 2002; 25(6):295–301. [PubMed: 12086747]
- de Lange FP, Koers A, Kalkman JS, Bleijenberg G, Hagoort P, van der Meer JW, Toni I. Increase in prefrontal cortical volume following cognitive behavioural therapy in patients with chronic fatigue syndrome. *Brain : a journal of neurology*. 2008; 131(Pt 8):2172–2180. [PubMed: 18587150]
- Denny CH, Floyd RL, Tsai J, Green PP. Alcohol Use among Women of Childbearing Age - United States, 2001–2005. *Alcohol Clin Exp Res*. 2009; 33(6):59a–59a.
- Dobbing J, Sands J. Comparative aspects of the brain growth spurt. *Early human development*. 1979; 3(1):79–83. [PubMed: 118862]
- Eadie BD, Redila VA, Christie BR. Voluntary exercise alters the cytoarchitecture of the adult dentate gyrus by increasing cellular proliferation, dendritic complexity, and spine density. *The Journal of comparative neurology*. 2005; 486(1):39–47. [PubMed: 15834963]
- Erickson KI, Kramer AF. Aerobic exercise effects on cognitive and neural plasticity in older adults. *British journal of sports medicine*. 2009; 43(1):22–24. [PubMed: 18927158]
- Fakoya FA, Caxton-Martins EA. Neocortical neurodegeneration in young adult Wistar rats prenatally exposed to ethanol. *Neurotoxicology and teratology*. 2006; 28(2):229–237. [PubMed: 16503114]
- Ferrer I, Galofre E. Dendritic spine anomalies in fetal alcohol syndrome. *Neuropediatrics*. 1987; 18(3):161–163. [PubMed: 3683757]
- Floel A, Ruscheweyh R, Kruger K, Willemer C, Winter B, Volker K, Lohmann H, Zitzmann M, Mooren F, Breitenstein C, Knecht S. Physical activity and memory functions: Are neurotrophins and cerebral gray matter volume the missing link? *NeuroImage*. 2010; 49(3):2756–2763. [PubMed: 19853041]
- Gibb R, Kolb B. A method for vibratome sectioning of Golgi-Cox stained whole rat brain. *Journal of neuroscience methods*. 1998; 79(1):1–4. [PubMed: 9531453]

- Granato A, Di Rocco F, Zumbo A, Toesca A, Giannetti S. Organization of cortico-cortical associative projections in rats exposed to ethanol during early postnatal life. *Brain research bulletin*. 2003; 60(4):339–344. [PubMed: 12781322]
- Granato A, Van Pelt J. Effects of early ethanol exposure on dendrite growth of cortical pyramidal neurons: inferences from a computational model. *Brain research Developmental brain research*. 2003; 142(2):223–227. [PubMed: 12711375]
- Hamilton GF, Boschen KE, Goodlett CR, Greenough WT, Klintsova AY. 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. *Alcoholism, clinical and experimental research*. 2012; 36(7):1196–1204.
- 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]
- Hamilton GF, Whitcher LT, Klintsova AY. Postnatal binge-like alcohol exposure decreases dendritic complexity while increasing the density of mature spines in mPFC Layer II/III pyramidal neurons. *Synapse*. 2010; 64(2):127–135. [PubMed: 19771589]
- 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*. 2009; 1294:1–11. [PubMed: 19647724]
- Hopkins ME, Nitecki R, Bucci DJ. Physical exercise during adolescence versus adulthood: differential effects on object recognition memory and brain-derived neurotrophic factor levels. *Neuroscience*. 2011; 194:84–94. [PubMed: 21839807]
- Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovskaya V, Horster F, Tenkova T, Dikranian K, Olney JW. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science*. 2000; 287(5455):1056–1060. [PubMed: 10669420]
- Irwin SA, Idupulapati M, Gilbert ME, Harris JB, Chakravarti AB, Rogers EJ, Crisostomo RA, Larsen BP, Mehta A, Alcantara CJ, Patel B, Swain RA, Weiler IJ, Oostra BA, Greenough WT. Dendritic spine and dendritic field characteristics of layer V pyramidal neurons in the visual cortex of fragile-X knockout mice. *American journal of medical genetics*. 2002; 111(2):140–146. [PubMed: 12210340]
- Jacobs JS, Miller MW. Proliferation and death of cultured fetal neocortical neurons: effects of ethanol on the dynamics of cell growth. *Journal of neurocytology*. 2001; 30(5):391–401. [PubMed: 11951050]
- Kaufmann WE, Moser HW. Dendritic anomalies in disorders associated with mental retardation. *Cereb Cortex*. 2000; 10(10):981–991. [PubMed: 11007549]
- Klintsova AY, Hamilton GF, Boschen KE. Long-term consequences of developmental alcohol exposure on brain structure and function: therapeutic benefits of physical activity. *Brain sciences*. 2012; 3(1):1–38. [PubMed: 24961305]
- Lawrence RC, Otero NK, Kelly SJ. Selective effects of perinatal ethanol exposure in medial prefrontal cortex and nucleus accumbens. *Neurotoxicology and teratology*. 2012; 34(1):128–135. [PubMed: 21871563]
- May PA, Gossage JP, Kalberg WO, Robinson LK, Buckley D, Manning M, Hoyme HE. Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. *Developmental disabilities research reviews*. 2009; 15(3):176–192. [PubMed: 19731384]
- McKinney BC, Grossman AW, Elisseou NM, Greenough WT. Dendritic spine abnormalities in the occipital cortex of C57BL/6 Fmr1 knockout mice. *American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics*. 2005; 136B(1):98–102.
- Morleo M, Woolfall K, Dedman D, Mukherjee R, Bellis MA, Cook PA. Under-reporting of foetal alcohol spectrum disorders: an analysis of hospital episode statistics. *BMC pediatrics*. 2011; 11:14. [PubMed: 21303524]
- Neeper SA, Gomez-Pinilla F, Choi J, Cotman C. Exercise and brain neurotrophins. *Nature*. 1995; 373(6510):6109.

- Olney JW, Wozniak DF, Farber NB, Jevtovic-Todorovic V, Bittigau P, Ikonomidou C. The enigma of fetal alcohol neurotoxicity. *Annals of medicine*. 2002; 34(2):109–119. [PubMed: 12108574]
- Otero NK, 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(10):1701–1709.
- Rasmussen C. Executive functioning and working memory in fetal alcohol spectrum disorder. *Alcoholism, clinical and experimental research*. 2005; 29(8):1359–1367.
- Sampson PD, Streissguth AP, Bookstein FL, Little RE, Clarren SK, Dehaene P, Hanson JW, Graham JM Jr. Incidence of fetal alcohol syndrome and prevalence of alcohol-related neurodevelopmental disorder. *Teratology*. 1997; 56(5):317–326. [PubMed: 9451756]
- Sim YJ, Kim H, Shin MS, Chang HK, Shin MC, Ko IG, Kim KJ, Kim TS, Kim BK, Rhim YT, Kim S, Park HY, Yi JW, Lee SJ, Kim CJ. Effect of postnatal treadmill exercise on c-Fos expression in the hippocampus of rat pups born from the alcohol-intoxicated mothers. *Brain & development*. 2008; 30(2):118–125. [PubMed: 17723286]
- Sowell ER, Thompson PM, Mattson SN, Tessner KD, Jernigan TL, Riley EP, Toga AW. Regional brain shape abnormalities persist into adolescence after heavy prenatal alcohol exposure. *Cereb Cortex*. 2002; 12(8):856–865. [PubMed: 12122034]
- Thomas JD, Sather TM, Whinery LA. Voluntary exercise influences behavioral development in rats exposed to alcohol during the neonatal brain growth spurt. *Behavioral neuroscience*. 2008; 122(6):1264–1273. [PubMed: 19045946]
- Uysal N, Sisman AR, Dayi A, Aksu I, Cetin F, Gencoglu C, Tas A, Buyuk E. Maternal exercise decreases maternal deprivation induced anxiety of pups and correlates to increased prefrontal cortex BDNF and VEGF. *Neuroscience letters*. 2011; 505(3):273–278. [PubMed: 22044872]
- van Praag H, Christie BR, Sejnowski TJ, Gage FH. Running enhances neurogenesis, learning, and long-term potentiation in mice. *Proceedings of the National Academy of Sciences of the United States of America*. 1999; 96(23):13427–13431. [PubMed: 10557337]
- Vaynman S, Gomez-Pinilla F. License to run: exercise impacts functional plasticity in the intact and injured central nervous system by using neurotrophins. *Neurorehabilitation and neural repair*. 2005; 19(4):283–295. [PubMed: 16263961]
- Wass TS, Persutte WH, Hobbins JC. The impact of prenatal alcohol exposure on frontal cortex development in utero. *American journal of obstetrics and gynecology*. 2001; 185(3):737–742. [PubMed: 11568807]
- Whitcher LT, Klintsova AY. Postnatal binge-like alcohol exposure reduces spine density without affecting dendritic morphology in rat mPFC. *Synapse*. 2008; 62(8):566–573. [PubMed: 18512209]
- Yuste, R. Dendritic spines. Vol. xiv. Cambridge, Mass: MIT Press; 2010. p. 264
- Zilles, KJ. The cortex of the rat : a stereotaxic atlas. Vol. v. Berlin ; New York: Springer-Verlag; 1985. p. 121

Figure 1. Golgi-impregnated mPFC Layer II/III pyramidal neuron

a) Fully impregnated mPFC Layer II/III pyramidal neuron of an Alcohol-SH animal. Scale bar = 20 μm . (b, c) high magnification images of spines on order 3 (b) and order 4 (c) basilar dendritic branches. Note the different shapes of spines. Scale bar = 10 μm .

Figure 2. WR Has No Effect on mPFC Dendritic Complexity While Postnatal Alcohol Decreases It

Each graph depicts the number of dendritic tree intersections with Sholl radii in each housing and postnatal condition. (a) A significant decrease in dendritic complexity was evident in AE-SH animals when compared to Control-SH animals. (b) No difference in dendritic complexity was evident in WR animals. As such, twelve days of exposure to voluntary wheel running does not significantly increase the number of intersections in either Control (c) or Alcohol (d) rats. (WR – wheel runner; SH – social housing; * – $p < .05$). Values indicate means $\pm$ S.E.M.

Figure 3. Neurolucida Tracings of Representative Neurons across Conditions

Sample tracing of the basal tree from a Layer II/III mPFC neuron in a (a) Control-SH and (b) Alcohol-SH animal. Note, the cell from an Alcohol animal exhibits a significantly less complex dendritic tree than does that of a Control. (SH—Social housing).

Figure 4. Alcohol-Induced mPFC Dendritic Abnormalities

PD 4–9 binge like alcohol exposure results in decreased dendritic length (a) and decreased number of endpoints (b) in Layer III mPFC pyramidal neurons of adolescent rats. (WR – wheel runner; SH – standard housing; * – $p < 0.05$; # – $p < 0.01$). Values indicate means $\pm$ S.E.M.

Figure 5. Neonatal Alcohol Exposure Decreases Spine Density

(a) Alcohol rats have significantly fewer spines on Order 2 branches than do Control rats ($p < 0.05$). WR negates this effect. (b) Neither Treatment nor Intervention altered spine density in Order 4 branches (WR – wheel runner; SH – social housing; * – $p < 0.05$). Values indicate means $\pm$ S.E.M

Figure 6. Distal Branches Have More Immature Spines

No difference across Treatment was seen in the percentage of immature spines in either the SH (a) or the WR (b) groups. However, both demonstrate that distal branches have significantly more immature spines when compared to proximal branches. (WR – wheel runner; SH – social housing; # – $p < 0.01$). Values indicate means $\pm$ S.E.M

Table 1

Body Weights and BACs

Condition	Weights (g)				BACs (mg/dl)
	PD4	PD9	PD30	PD42	
SC	10.39 ± 0.09	19.92 ± 0.43	100.56 ± 1.41	181.89 ± 2.63	
SI	10.59 ± 0.30	19.45 ± 0.66	104.30 ± 2.57	185.90 ± 4.16	
AE	11.18 ± 0.26	17.14 ± 0.36**	101.20 ± 1.63	182.80 ± 2.69	400.10±12.40

Average body weights (grams ± SEM) are presented for suckle control (SC), sham-intubated (SI) and alcohol-exposed (AE) animals. Data are reported across the first and last day of the dosing procedure (PD 4 and PD 9) as well as the first and last day of wheel-running (WR) exposure (PD 30 and PD 42). Significant differences between groups are represented with an **. BACs were taken from blood samples collected 90 minutes following the second dose (reported in mg/dl).

** -- $p < 0.01$