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Postnatal alcohol exposure and adolescent exercise have opposite effects on cerebellar microglia in rat

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Abstract

Developmental alcohol exposure results in altered neuroimmune function in both humans and rodents. Given the critical role for the principle neuroimmune cell, microglia, in maintaining synaptic form and function, microglial dysfunction in the cerebellum may be an important mechanism underlying the aberrant cerebellar connectivity observed in rodent models of fetal alcohol spectrum disorders. Using an established rodent model of alcohol exposure during human third-trimester fetal development, we examine the cerebellum of male and female Long Evans rats to determine the impact of early postnatal alcohol exposure on cerebellar microglia, and the potential therapeutic effects of an adolescent intervention consisting of voluntary exercise (running). All cerebelli were examined at postnatal day 42 (i.e., late adolescence), and microglia were labeled with Iba1, a microglia-specific protein. Early postnatal alcohol exposure caused an increase in microglial density throughout cerebellum and a reduction in cerebellar volume, and a reduction in the proportion of fully ramified (often called “resting state”) microglia selective to lobules 1–4. In contrast, adolescent exercise decreased microglial density throughout cerebellum and increased cerebellar volume, while activating microglia (as indicated by increases in amoeboid microglia, and reductions in fully and partially ramified microglia) selectively in lobules 1–4. These results suggest that adolescent exercise may be a suitable intervention to ameliorate alcohol-induced neuroimmune dysfunction as it alters microglia density and cerebellar volume in opposite to the effects of developmental alcohol exposure. Importantly, exercise intervention can be flexibly implemented well after the time window of vulnerability to alcohol.

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AUTHOR CONTRIBUTIONS

Z. H. Gursky, J. R. Johansson, and A. Y. Klintsova participated in the conceptualization and design of the study. Z. H. Gursky and J. R. Johansson performed preparation of biological samples. J. R. Johansson collected the data. Z. H. Gursky performed statistical analyses. Z. H. Gursky, J. R. Johansson, and A. Y. Klintsova contributed to the writing and approval of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICS APPROVAL STATEMENT

All procedures followed the animal use protocol approved by the University of Delaware Institutional Animal Care and Use Committee.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Keywords

fetal alcohol spectrum disorders; immunocytochemistry; neuroimmune; neuroplasticity; stereology

1 | INTRODUCTION

Fetal alcohol spectrum disorders (FASDs) are a range of disorders that result from alcohol consumption during pregnancy and are considered one of the leading causes of preventable mental health problems in children (Lupton, Burd, & Harwood, 2004). Symptoms of FASD vary in severity depending on the pattern of maternal drinking episodes (Maier & West, 2001) and can include physical abnormalities, as well as cognitive deficits (Hoyme, 2016). Exposure to alcohol during pregnancy can disrupt the immune system of the fetus and result in an increased risk of infections and disease in newborns that may persist throughout life (Gauthier, 2015). Studies in rodent models demonstrated that alcohol exposure in utero can lead to alterations in the neuroimmune response of offspring that persist later in adulthood (Bodnar, Hill, & Weinberg, 2016; Terasaki & Schwarz, 2016).

A key participant in the neuroimmune network is the cell type microglia, which defends the brain from harmful toxins, disposes of waste and debris, and plays a role in synaptic pruning during development (Matcovitch-Natan et al., 2016). Microglia can be characterized by their morphologies, or activation states, which change depending on the environment in which they exist (Drew, Johnson, Douglas, Phelan, & Kane, 2015). When presented with a teratogen such as alcohol, microglia shift from a resting/ ramified state with many long, thin processes and a small soma (Nimmerjahn, Kirchhoff, & Helmchen, 2005), to an activated/ amoeboid state characterized by fewer short processes and a large, circular soma (Nakajima & Kohsaka, 2001). As microglia become activated by developmental exposure to alcohol, they release cytokines that can promote inflammation and ultimately lead to neurodegeneration (Topper, Baculis, & Valenzuela, 2015). This evidence suggests that the neuroimmune system is vulnerable to the effects of alcohol early in development and could result in long-lasting changes.

Sex differences in microglia are known to manifest in the developing brain after the onset of the prenatal androgen surge, they vary by brain region and age (Schwarz, Sholar, & Bilbo, 2012). Microglial number and morphology differ between males and females during rodent development: female pups have more amoeboid microglia on the first postnatal day in several brain regions, including the hippocampus and amygdala, when compared to male rats. However, four days later situation is reversed with males display more amoeboid microglia than females in the parietal cortex, hippocampus, and amygdala (Schwarz et al., 2012). However, a few studies to date reported that cerebellum microglia does not demonstrate any sex differences in shape and quantity (reviewed in (reviewed in Bordt, Ceasrine, & Bilbo, 2020; VanRyzin, Pickett, & McCarthy, 2018).

A previous study in our lab has shown that microglia become activated in the hippocampus following a postnatal binge alcohol exposure (Boschen, Ruggiero, & Klintsova, 2016). Therefore, the current study expands upon the literature by observing the microglial response to a six-day postnatal alcohol exposure in the cerebellum. It has been well

established that the cerebellum, a region responsible for motor control (Bastian, 2006), executive function (Gottwald, Wilde, Mihajlovic, & Mehdorn, 2004; Wagner, Kim, Savall, Schnitzer, & Luo, 2017) and emotional affect (Schmahmann & Sherman, 1998), is susceptible to the damaging effects of alcohol. Following alcohol exposure during the first postnatal week in a rodent model, a moderate to high dose alcohol exposure produced significant loss of Purkinje cells, the primary output cell in the cerebellum (Goodlett, Pearlman, & Lundahl, 1998). Because microglia assist with clearance of cell debris from the brain (Matcovitch-Natan et al., 2016), it is likely that the alcohol paradigm used in this study will trigger a microglial response observable via alterations in microglia morphology and number. Cerebellar lobules I-IV and IX-X were chosen as the specific lobules of interest for this study because it has been shown previously (Goodlett, Marcussen, & West, 1990) that alcohol exposure on PD4-9 targets Purkinje cells in these lobules.

It is well established that voluntary exercise has beneficial effects on the alcohol exposed brain. For example, exercise during adulthood improved behavioral deficits in rats exposed to alcohol postnatally (Thomas, Sather, & Whinery, 2008). In our lab, it has been reported that voluntary wheel running increases neurogenesis in the dentate gyrus of animals exposed to alcohol postnatally (Helfer, Goodlett, Greenough, & Klintsova, 2009). It can also partially reverse deficits in medial prefrontal cortex layer II/III dendritic morphology in adolescent male rats (Hamilton, Criss, & Klintsova, 2015). Additionally, exercise has also been shown to mediate the microglial response associated with pathogenesis of disease models other than FASD, such as Alzheimer's (Baek & Kim, 2016) and Parkinson's (Sung, 2012). For this reason, we expected that exercise could alter the neuroimmune response measured at the adolescent time point in our FASD model.

To our knowledge, this study is the first to explore the effects of an exercise intervention during adolescence on microglial morphology and representation in the cerebellum following a postnatal alcohol exposure. It is hypothesized that our alcohol exposure paradigm will cause cell loss and volume reduction in the cerebellum and lead to an activated microglial response measured as changes in density and morphology of microglia at the PD42 time point. Furthermore, we hypothesize that voluntary exercise during adolescence may mitigate this activated microglial response.

2 | MATERIALS AND METHODS

2.1 | Animals

All procedures followed the animal use protocol approved by the University of Delaware Institutional Animal Care and Use Committee. Adult animals for breeding were obtained from Envigo (Indianapolis, IN). Long-Evans rat pups were then bred in-house at the University of Delaware. Discovery of a seminal plug was considered gestational day 0 and postnatal day 0 was considered gestational day 22. On postnatal day (PD) 3, litters were culled to eight pups. A total of 54 pups (36 males & 18 females) from 9 litters were used in this study, pseudo-randomly assigned to one of three postnatal conditions: alcohol-exposed (AE), sham-intubated (SI), or suckle-control (SC), and one of two adolescent housing conditions: social housing (SH) or wheel running (WR).

2.2 | Experimental manipulations (alcohol exposure, wheel running) & brain extraction

All animals for the current study were either generated alongside animals examined in—or are a subset of animals examined in—Helfer et al. (2009). A summary experimental timeline can be found in Figure 1, and a detailed description of the methods used for alcohol administration, wheel running intervention, and brain extraction can be found in Helfer et al. (2009). Briefly, litters were assigned to one of two conditions: suckle control (SC) litters that received no intubation or intubation litters. A split litter design was implemented for the intubated litters such that pups within these litters were randomly assigned to either sham-intubated (SI) or alcohol-exposed (AE) groups.

2.2.1 | Alcohol exposure—The intubation treatments were administered from PD4 to PD9. Milk/ethanol solution was prepared from a base milk formula according to the previously described method (West, Hamre, & Pierce, 1984) and was delivered by gastric intubation. AE pups were intubated twice daily, 2 hr apart, with a milk/ethanol solution containing 11.9% (v/v) ethanol, resulting in a total of 5.25 g/kg of ethanol delivered to each AE pup daily. All animals were weighed daily throughout treatment.

2.2.2 | Blood alcohol concentrations—To assess peak blood alcohol concentrations (BACs), blood samples were obtained from a tail clip on PD4, 90 min after the last milk/ethanol intubation. The blood was centrifuged and plasma was collected and stored at -20°C until assay. BACs were analyzed using an Analox GL-5 Alcohol Analyzer which measures oxygen consumption during oxidation of ethanol (Analox Instruments, Lunenburg, MA).

2.2.3 | Wheel running—After weaning on PD23, offspring were same-sex group-housed three per cage. On PD30, with group-housing assignments maintained, animals were randomly assigned to one of two conditions: standard cage (SH—inactive socially housed control) or voluntary exercise (WR—wheel running) condition. Wheel running animals were housed in cages attached to stainless steel running wheels and had 24-hr voluntary access to these wheels (multiple rats could run in the same wheel). The running wheels were equipped with counters that recorded each rotation of the wheel. Wheel running activity (wheel revolutions) was recorded every 24 hr, at the onset of the light cycle (9:00 AM $\pm$ 5 min). At conclusion of the study, animals were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer saline (pH 7.2) and brain tissue was stored in 30% sucrose in 4% paraformaldehyde solution. Whole cerebelli (including vermis) were divided into right and left halves and one hemisphere per brain (counterbalanced across all sexes and treatments) was sectioned parasagittally at 40 μm using a cryostat, and then, stored at -20°C in cryoprotectant solution.

2.3 | Immunohistochemistry

Every 12th serial parasagittal section was selected for immunohistochemical labeling by systematic random sampling (SRS) procedure. Antibodies against microglia-specific marker ionized calcium-binding adaptor molecule 1 (Iba1) was used to identify microglia. Briefly, sections were washed in 0.1 M of Tris buffer solution (TBS) and incubated in 2% H_2O_2 in 70% methanol for 10 min, followed by blocking in 3% normal goat serum and 1% Triton-X in 0.1 M TBS for 1 hr. Tissue was then transferred into primary antibody (Anti-Iba1

polyclonal antibody made in rabbit; Wako Chemicals; catalog # 019-19741; 1:5000) for 48 hr. Negative control sections were placed in blocking solution instead of primary antibody. Sections were incubated in secondary antibody (biotinylated anti-rabbit IgG made in goat; Vector Laboratories; catalog # BA-1000; 1:200) in washing solution (3% normal goat serum in 0.1 M TBS) for 1 hr. Following a 1 hr incubation in ABC (Vector Laboratories; catalog # PK-6100) in washing solution, the immunolabeling was visualized using nickel-enhanced diaminobenzidine. Pyronin Y (Electron Microscopy Sciences; catalog # RT 19560) was used as a counterstain, to visualize cytoarchitecture of cerebellum, and coverslips were mounted using DPX mounting medium (Electron Microscopy Sciences; catalog # RT 13512).

2.4 | Iba1 automated image analysis

We will use the word “densitometry” to refer to the percentage of Iba1⁺ pixels divided by total number of pixels within the contoured region of analyzed images. Images of entire sagittal sections of cerebellum (e.g., Figure 2a) were acquired using the “Acquire virtual tissue” function in Stereo Investigator (MBF Biosciences, Williston, VT). Densitometry analysis was completed on every 12th serial section (using systematic random sampling) using the software “ImageJ bundled with 64-bit Java 1.8.0_112” (Schneider, Rasband, & Eliceiri, 2012). This resulted in about five to nine sections analyzed per animal. For each section, lobules 1–4 and 9–10 were outlined using the “Polygon selection tool” in ImageJ (representative outlines in Figure 2b). Next, the “threshold” function was used to create a binary image (e.g., Figure 2c) such that microglia were clearly distinguishable as black pixels, but there was minimal background signal detected as black pixels or loss of Iba1 + signal (when compared to the original image by manual investigation). “Size” settings were set to 20–10,000 pixels in order to filter out any small clusters of dark signal that were unlikely to be microglia, or impractically large objects that otherwise meet the criteria for thresholding.

2.5 | Microglial territory analysis

2.5.1 | Image stack acquisition—Using Stereo Investigator, z-stack images were acquired for each tissue section at a sampling interval of every 12th serial section. First, lobules 1–4 and 9–10 were outlined using a 5× objective. Using a 40× objective, systematic random sampling (SRS) was applied to acquire 150×150 μm images every 750 × 750 μm (resulting in a 1/25 area sampling) for 30 μm thickness (25 μm probe, 2.5 μm guard zones, 1.0 μm step between images). The top plane of each z-stack was focused manually.

2.5.2 | Microglia contouring—Using Neurolucida software (MBF Bioscience), individual microglia were contoured from previously acquired SRS image stacks. Any microglial cell with intact processes and a visible cell body that did not leave the frame of the contour was analyzed. Microglia were only outlined if they came into focus within the image stack, to prevent mis-characterization of microglia that might have processes that extend outside of the systematically acquired image stacks (e.g., Figure 3a). Microglia were outlined by focusing on processes and selecting the tips of all processes connected to the cell body (as in Drew et al., 2015). The mean area of the contours for every cell analyzed within a given animal was calculated and considered the average “territory” of a microglia for that animal.

2.6 | Unbiased stereological cell quantification

Total number of microglia within cerebellar lobules 1–4 and 9–10 of every 12th serial section were counted using unbiased stereology and the optical fractionator probe (West, Slomianka, & Gundersen, 1991) in Stereo Investigator. The sampling grid was set to $400 \times 400 \mu\text{m}$, within which a $100 \times 100 \mu\text{m}$ indicated the counting frame where cell number would be quantified. Guard zones of $1 \mu\text{m}$ on either side of the $10 \mu\text{m}$ disector made sure that uneven surfaces of the mounted brain sections did not influence estimation. Microglia were counted only if they had a visible cell body with at least one visible process (of any length). Mean coefficients of error (Gundersen, $m = 1$) can be found in Table 1.

2.7 | Statistical analysis

All statistical analyses were performed using RStudio version 1.1.414 (RStudio Team, 2016) running the “tidyverse” version 1.2.0 (Wickham, 2017), “mediation” (Tingley, Yamamoto, Hirose, Keele, & Imai, 2014), “car” (Fox & Weisberg, 2011), and “lsr” (Navarro, 2015) packages. $\alpha = .050$ was used for all statistical analyses. The unit of analysis was the individual animal, as multiple animals per litter were used in some instances. After initial analysis with animals assigned to one of three postnatal treatments (SC, SI, or AE), SC and SI control groups were similar (i.e., did not statistically differ) on every neuroanatomical measure and had similar weight at every measured timepoint except one (PD42). In line with past studies in our lab and others’ (Otero, Thomas, Saski, Xia, & Kelly, 2012; Ruggiero, Boschen, Roth, & Klintsova, 2018), we combined the SC and SI control groups into a single “Control” treatment group and report these results. While combining SC and SI controls results in uneven sample sizes between AE and control groups heterogeneity of variance was maintained across all combinations of postnatal treatment-by-adolescent housing, a concern for uneven sample sizes in ANOVA. Tests to determine homogeneity of variance were chosen based on normality of underlying group distributions—that is, a Barlett test for homogeneity of variance was only used if there were no violations of normality (according to a Shapiro–Wilks test for normality) in the groups being compared, while Levene’s test for homogeneity of variance was used if none of the groups violated assumptions of normality. The tests utilized, test statistics, and p -values for normality and homogeneity of variance tests can be found in Table S1.

Between-group analyses were performed using $2 \times 2 \times 2$ factorial ANOVAs, with postnatal treatment [Control, AE], adolescent housing [SH, WR], and sex [female, male] as between-subjects factors. Although some variables demonstrated violations to assumptions of normality (see Table S1), nonparametric tests were not utilized due to the $2 \times 2 \times 2$ factorial design and a lack of suitable factorial nonparametric tests. Weights were analyzed using a repeated-measures ANOVAs at three timepoints: PD4 (immediately prior to alcohol exposure), PD9 (last day of alcohol exposure), and PD42 (at time of perfusion). Post hoc comparisons were performed as Tukey HSD tests; however, two post hoc comparisons based on observation of data (i.e., volume of lobules 1–4 and lobules 9–10) were performed as two pairs of t tests between adolescent housing conditions, within postnatal treatment group. The critical p -value for statistical significance for these t tests was Bonferroni-corrected to 0.0125 ($0.050/4$ comparisons) to correct for multiple comparisons.

To examine the dependence of changes in density of microglia on volume of cerebellum, the “mediate” function in the “mediation” package was used, as has been used by our lab in previous publications (Gursky, Spillman, & Klintsova, 2020). The output model predicted Iba1 + density from adolescent housing, postnatal treatment, and cerebellar volume, while the mediator model predicted cerebellar volume from adolescent housing and postnatal treatment. To provide replicable results, a seed for randomness was generated prior to analysis, and all analyses were bootstrapped 10,000 times. These mediations were tested for moderation by alcohol exposure (i.e., the mediations were analyzed to determine whether alcohol exposure changed the relationship between exercise and the mediating variable of cerebellar volume) using the “test.modmed” function, again bootstrapped 10,000 times. This process was performed for lobules 1–4 and again for lobules 9–10.

3 | RESULTS

3.1 | BAC on PD4 did not differ between sexes nor assigned housing conditions

Peak BAC measured on PD4 (Table 2) did not significantly differ between animals assigned to different adolescent housing conditions ($F(1,9) = 0.124$, $p = .733$, $\eta^2_{\text{partial}} = 0.026$) or sex ($F(1,9) = 0.465$, $p = .513$, $\eta^2_{\text{partial}} = .049$). There was no interaction between adolescent housing and sex ($F(1,9) = 0.002$, $p = .963$, $\eta^2_{\text{partial}} < .001$).

3.2 | Alcohol exposure led to decreased weight throughout life

There was a significant interaction between age and postnatal treatment ($F(1,46) = 31.317$, $p < .001$). Follow-up 2×2 ANOVAs indicated that AE animals weighed less than control animals at PD9 ($F(1,50) = 17.526$, $p < .001$, $\eta^2_{\text{partial}} = .250$) and PD42 ($F(1,50) = 4.218$, $p = .045$, $\eta^2_{\text{partial}} = .050$), but not prior to dosing on PD4 ($F(1,50) = 0.035$, $p = .853$, $\eta^2_{\text{partial}} < .001$). There was a main effect of sex such that males weighed significantly more than females at PD4 ($F(1,50) = 7.335$, $p = .009$, $\eta^2_{\text{partial}} = .128$) and PD42 ($F(1,50) = 49.658$, $p < .001$, $\eta^2_{\text{partial}} = .498$), but not on PD9 ($F(1,50) = 2.492$, $p = .121$, $\eta^2_{\text{partial}} = .047$).

3.3 | Postnatal alcohol exposure increases— and adolescent exercise decreases— density of microglia throughout cerebellum

AE animals displayed significantly higher density of Iba1⁺ material in lobules 1–4 ($F(1,46) = 7.434$, $p = .009$, $\eta^2_{\text{partial}} = .114$), while WR animals displayed significantly reduced density ($F(1,46) = 16.434$, $p < .001$, $\eta^2_{\text{partial}} = .251$) (Figure 2d). There was no effect of sex on density of Iba1⁺ material in lobules 1–4 ($F(1,46) = 0.213$, $p = .647$, $\eta^2_{\text{partial}} = .003$). There were not any significant interactions (p 's $> .367$).

Similar to lobules 1–4, AE increased ($F(1,40) = 6.898$, $p = .012$, $\eta^2_{\text{partial}} = .111$), and WR decreased ($F(1,40) = 11.396$, $p = .002$, $\eta^2_{\text{partial}} = .212$) density of Iba1⁺ material in lobules 9–10 (Figure 2e). Again, there was neither effect of sex ($F(1,40) = 0.462$, $p = .501$, $\eta^2_{\text{partial}} = .009$), nor were there any significant interactions (p 's $> .136$).

3.4 | Changes in cerebellar volume due to postnatal alcohol exposure and adolescent exercise reflect changes in density of microglia

AE significantly reduced volume of both lobules 1–4 ($F(1,16) = 15.074, p = .001, \eta^2_{\text{partial}} = .479$) (Figure 4a) and lobules 9–10 ($F(1,15) = 6.770, p = .020, \eta^2_{\text{partial}} = .274$) (Figure 4b). WR significantly increased the volume of both lobules 1–4 ($F(1,16) = 6.316, p = .023, \eta^2_{\text{partial}} = .297$) and lobules 9–10 ($F(1,15) = 11.133, p = .005, \eta^2_{\text{partial}} = .410$). There was neither influence of sex, nor interaction in either set of lobules (p 's $> .110$, all $\eta^2_{\text{partial}} < .145$). Despite the lack of interaction, visual inspection of the data suggests that exercise-induced increase in cerebellar volume may be quite weak in the brains of AE animals (despite nonsignificant interaction between postnatal treatment and adolescent housing, $\eta^2_{\text{partial}} = .145$). Due to this observation, four post hoc t test comparisons were run (Lobules 1–4: AE-SH vs AE-WR, and Control-SH vs Control-WR; Lobules 9–10: AE-SH vs AE-WR, and Control-SH vs Control-WR), with a Bonferroni-corrected critical p -value of .0125 (i.e., .050/4) to achieve statistical significance. These tests indicated that AE animals exposed to exercise did not differ in volume of lobules 1–4 ($t(3.29) = -0.050, p = .963$, Cohen's $d = 0.035$) or lobules 9–10 ($t(5) = 2.153, p = .084$, Cohen's $d = 1.644$) relative to socially housed AE animals. Volume of lobules 1–4 in control animals did differ due to exercise ($t(14) = 3.154, p = .007$, Cohen's $d = 1.577$), as did volume of lobules 9–10 ($t(14) = 3.102, p = .008$, Cohen's $d = 1.551$).

3.5 | Adolescent exercise reduces density of microglia throughout cerebellum independent of changes in cerebellar volume

Mediation analysis (Figure 5) revealed that the effect of wheel running on density of Iba1 + material was not mediated by changes in cerebellar volume in either lobules 1–4 ($p = .061$, 95% CI of estimated Mediation effect: -1.72 to 0.02) or lobules 9–10 ($p = .886$, 95% CI of estimated mediation effect: -1.25 to 1.04). Instead, exercise directly affected Iba1 + density in both lobules 1–4 ($p = .002$, 95% CI for estimated direct effect: -3.57 to -0.92) and lobules 9–10 ($p = .001$, 95% CI for estimated direct effect: -4.16 to -0.92). We examined the potential for alcohol exposure to moderate this relationship in both subregions of cerebellum, as visual inspection (and subsequent post hoc analysis) of the data suggest that exercise-induced increases in volume of lobules 1–4 was negligible in AE brains. Regardless, neither mediation was moderated by alcohol exposure (p 's $> .187$), suggesting that exercise decreases Iba1 + density in a highly robust manner, in a direction opposite to the effects of alcohol exposure, and despite alterations in volume of cerebellum.

3.6 | Neither postnatal alcohol exposure nor adolescent exercise changes the total number of microglia in cerebellum

AE did not alter the total number of microglia in lobules 1–4 ($F(1,16) = 1.812, p = .197, \eta^2_{\text{partial}} = .106$) (Figure 6a) or lobules 9–10 ($F(1,15) = 0.376, p = .549, \eta^2_{\text{partial}} = .020$) (Figure 6b). Similarly, there was no effect of WR in lobules 1–4 ($F(1,16) = 4.117, p = .059, \eta^2_{\text{partial}} = .240$) or lobules 9–10 ($F(1,15) = 0.789, p = .389, \eta^2_{\text{partial}} = .029$), nor were there effects of sex or interactions (p 's $> .061$).

3.7 | Adolescent exercise shifts microglia into amoeboid morphology in lobules 1–4, but not lobules 9–10

WR significantly reduced the average territory occupied by microglia in lobules 1–4 ($F(1,46) = 5.517, p = .023, \eta^2_{\text{partial}} = .115$) (Figure 3b), while it had no effect in lobules 9–10 ($F(1,40) = 3.459, p = .070, \eta^2_{\text{partial}} = .081$) (Figure 3c). There was no effect of AE on average territory occupied by microglia in either lobules 1–4 ($F(1,46) = 2.457, p = .124, \eta^2_{\text{partial}} = .065$) or lobules 9–10 ($F(1,40) = 0.032, p = .859, \eta^2_{\text{partial}} < .001$), nor were there effects of sex or interactions in either region (p 's $> .111$).

Quantifying the proportion of amoeboid (“small”), partially ramified (“medium”), and fully ramified (“large”) microglia (Figure 3d) in accordance with previous research from our lab (Boschen et al., 2016) indicated that WR increased the percentage of amoeboid microglia ($F(1,46) = 6.652, p = .013, \eta^2_{\text{partial}} = .135$) and decreased the percentage of both partially ramified ($F(1,46) = 4.415, p = .041, \eta^2_{\text{partial}} = .095$) and fully ramified microglia in lobules 1–4 ($F(1,46) = 5.009, p = .030, \eta^2_{\text{partial}} = .105$). There was no influence of AE, sex, or interactions on either amoeboid or partially ramified microglia in lobules 1–4 (p 's $> .099$).

3.8 | Postnatal alcohol exposure shifts microglia away from fully ramified morphology in lobules 1–4

AE significantly reduced the percentage of fully ramified microglia in lobules 1–4 ($F(1,46) = 7.772, p = .008, \eta^2_{\text{partial}} = .159$). There was no effect of sex, nor interactions, on percentage of fully ramified microglia in lobules 1–4 (p 's $> .272$).

4 | DISCUSSION

We have demonstrated that exposure to a voluntary exercise intervention in adolescence resulted in changes to the rat cerebellum that largely acted in opposition to the impact of early postnatal alcohol exposure. Specifically, adolescent exercise reduced the density of microglia throughout the cerebellum regardless of postnatal treatment, indicating a generally robust effect of wheel running on changes in microglia density. Neither postnatal treatment nor adolescent environment altered total number of microglia, suggesting that changes in density of microglia following exercise and alcohol exposure are caused by changes in microglial morphology rather than total number of microglia. While exercise increased cerebellar volume in control animals, this effect was not observed in alcohol exposed animals, which had decreased cerebellar volume. This indicates that adolescent exercise may work to ameliorate neuroimmune alterations following developmental alcohol exposure, but is not capable of rescuing gross neuroanatomical alterations.

Previous literature has demonstrated that alcohol exposure can result in increased proliferation, as well as increase in density of cells that are non-neuronal and non-astrocytic (Nixon & Crews, 2004). It is uncertain whether alcohol-induced increase in microglial density and exercise-induced decrease in density in our study are caused by altered microglial proliferation (e.g., more microglia needed in a smaller space), migration (e.g.,

more microglia being relocated to an area of high need), or survival (e.g., microglia are not dying in response to alcohol exposure). Microglial density increases over the first month of postnatal life (Askew et al., 2017), a process that is likely dysregulated by exposure to alcohol during this time. While the changes induced by exercise were consistent across postnatal treatments, it remains to be established whether changes in microglia density are functionally advantageous, or serve a similar role in a normal versus alcohol-exposed brain (despite a similar exercise-induced reduction in microglial density regardless of postnatal treatment).

While observed changes in microglial density and volume are consistent between the two regions of cerebellum examined, we find that the morphology of microglia is more sensitive to these manipulations in lobules 1–4 than 9–10. Such difference in microglia response was surprising as all these lobules are early-maturing and equally sensitive to alcohol exposure during PD4–9 period as indicated by significant reduction in Purkinje cell number (Marcussen, Goodlett, Mahoney, & West, 1994). Microglia changes include a general exercise-induced reduction in average territory surveyed by microglia on PD42 (as indicated by the dispersion of their processes) due to a reduction in the proportion of fully ramified microglia, and an increase in partially ramified and amoeboid microglia. These morphological changes are typically indicative of microglial activation (Drew et al., 2015). By contrast, developmental alcohol exposure only results in a reduction in the proportion of fully ramified microglia on PD42 in males, but no respective significant increases in partially ramified microglia nor amoeboid microglia. Previous literature has been limited to examining only males (McClain et al., 2011) and has demonstrated that adolescent alcohol exposure can result in long-lasting partial activation of microglia, supporting our observation that there is a reduction in fully ramified—but no significant and consistent increase in amoeboid or partially ramified—microglia. It is critical to recognize that the previously mentioned study examined adolescent alcohol exposure. In the early postnatal period of rodents, microglia primarily exist in an amoeboid-like activated state (Schwarz et al., 2012). The age at which microglia become fully ramified varies by both sex and structure. Our observation that postnatal alcohol exposure selectively impacts microglial morphology in lobules 1–4 of males supports this idea, as females have less microglia present in many brain regions during this early postnatal period (Schwarz et al., 2012).

The impact of exercise on rat cerebellar volume seems to be contingent on the age and neurological state of the rat. In models of cerebellar ataxia (Uhlendorf, Van Kummer, Yaspelkis, & Cohen, 2011) and aging (Larsen, Skalicky, & Viidik, 2000), exercise does not appear to influence cerebellar volume. In contrast, the current data indicate that exercise increases cerebellar volume when examining cerebellum in adolescence. While this suggests that age may restrict the influence of exercise on volume, exercise appears to be beneficial in preventing age-related cerebellar decline in other ways (Larsen et al., 2000). Exercise largely seems to increase brain volume, as exercise during adulthood leads to increased grey matter volume of cortex in a region-dependent manner (Sumiyoshi, Taki, Nonaka, Takeuchi, & Kawashima, 2014), consistent with our findings.

While it is not surprising that exercise is beneficial for the brain (Llorens-Martin, Torres-Aleman, & Trejo, 2008), what is less clear is why a shift toward activated microglia was

observed in cerebelli of wheel-running rats as indicated by an increase in amoeboid, and decrease in partially and fully ramified, microglia. While activation of microglia does not always result in inflammation, it can result in growth-promoting activity (Marshall et al., 2013). This leads us to believe that the activation caused by exercise may not impact the same processes that lead to a lasting reduction in fully ramified microglia following postnatal alcohol exposure. Instead, this activation of microglia could benefit the brain, as indicated by increased brain volume, while alcohol decreases brain volume through short-term inflammation (Topper et al., 2015) and drastic Purkinje cell loss (Marcussen et al., 1994; Topper et al., 2015). This activation following exercise may reduce the number of partially ramified microglia, suggesting a possible mechanism for exercise to offset neuroimmune alterations (but not gross neuroanatomical alterations) following developmental alcohol exposure.

5 | CONCLUSIONS

Our findings suggest that exercise may provide a beneficial intervention to ameliorate the alterations to neuroimmune function caused by developmental alcohol exposure. While there are some hallmark structural changes to cerebellum that are not reversed following alcohol exposure (i.e., volume reductions), exercise appears to regulate the density of microglia within both alcohol exposed and control cerebelli. While the functional implications of these neuroimmune alterations remains to be tested, the largely opposite effects between adolescent exercise and developmental alcohol exposure suggest that alcohol may be beneficial for individuals diagnosed with FASD, even when intervention happens well after the initial exposure (i.e., in adolescence).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AE	alcohol exposure/alcohol exposed (neonatal experimental group)
BAC	blood alcohol concentration
CI	confidence interval
FASD	fetal alcohol spectrum disorders
PD	postnatal day
SC	suckle control (neonatal typically developing group)

SH	socially housed (adolescent control group)
SI	Sham intubated (neonatal procedural control group)
SRS	systematic random sampling
WR	wheel running (adolescent experimental group).

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FIGURE 1.
Experimental timeline for all tissue generated and analyzed

FIGURE 2.

Postnatal alcohol exposure and adolescent exercise have opposite effects on density of microglial material in cerebellum. (a) A representative image of cerebellum (sagittal plane) of a SI-WR animal following nickel-enhanced diaminobenzidine-precipitated immunolabeling against microglial marker Iba1. Image was acquired using the “virtual tissue” function in Stereo Investigator software (MBF Bioscience, Williston, VT). All modifications (e.g., scale bar, contours) were created using Fiji (Schindelin et al., 2012). Scale bar = 1 mm. (b) The image from 2a with black dotted lines illustrating representative contours for lobules 1–4 (contour on left) and lobules 9–10 (contour on right). Scale bar = 1 mm. (c) A subset of image 2b with binary thresholding applied, representative of thresholding applied in the current study. Scale bar = 0.2 mm. (d) AE increased density of Iba1⁺ material in lobules 1–4 relative to controls. By contrast, WR decreased density of Iba1⁺ material. * p .050, ** p .010. Bar graphs represent mean + SEM. (e) Similar to the

pattern observed in lobules 1–4, AE increased density of Iba1⁺ material relative to controls in lobules 9–10—and WR decreased density of Iba1⁺ material. * p .050, ** p .010. Bar graphs represent mean + SEM

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FIGURE 3.

Adolescent exercise reduces average territory occupied by individual microglia in lobules 1–4, but not lobules 9–10. (a) A photomicrograph of Iba1 + microglia in lobules 1–4 of a SI-WR animal. Representative outlines are demonstrated on three microglia in this image; contours for the current experiment were created by focusing throughout z-depth of image stack while contours for this figure were exclusively drawn from this image plane alone. Any microglia where all processes could not be brought into focus within the systematic randomly sampled image stack, or microglia with processes extending out of the field of view (e.g., the two microglia on bottom left, white arrows) would not have been contoured for analysis. (b) Adolescent housing was the only factor that significantly influenced the average territory occupied by individual microglia in lobules 1–4. $*p < .050$. Bar graphs represent mean + SEM. (c) The average territory occupied by individual microglia in lobules 9–10 was not influenced by postnatal treatment, adolescent housing, or sex. Bar graphs

represent mean + SEM. (d) Proportion of individual microglia present in either a large “fully-ramified,” medium “partially-ramified,” or small “amoeboid” morphological state in lobules 1–4. Adolescent exercise (bottom two bars) reduced the proportion of fully and partially ramified microglia, while increasing the proportion of amoeboid microglia, relative to socially housed animals (top two bars). Early postnatal alcohol exposure (solid colored bars) decreased the proportion of fully ramified microglia relative to controls (striped bars), but did not alter the proportion of partially ramified or amoeboid microglia. Bar graphs represent mean—SEM

FIGURE 4.

Postnatal alcohol exposure decreases—while adolescent exercise increases—cerebellar volume. (a) Alcohol significantly decreased volume of lobules 1–4 compared controls. WR significantly increased volume of lobules 1–4 in control animals. * $p < .050$, ** $p < .010$. Bar graphs represent mean + SEM. (b) Alcohol significantly decreased volume of lobules 9–10 controls. WR significantly increased volume of lobules 9–10. * $p < .050$, ** $p < .010$. Bar graphs represent mean + SEM

FIGURE 5.

Effect of exercise on density of microglia in cerebellum is not mediated by changes in cerebellar volume, nor moderated by developmental alcohol exposure. (a) Adolescent exercise had a significant total effect (in parentheses) on density of Iba1 + material (“density of microglia”) in lobules 1–4. This was driven by a significant direct effect of exercise on density of Iba1 + material, and not by exercise-associated alterations in cerebellar volume, despite a significant association between exercise and volume of lobules 1–4. Neither the direct effect, nor (lack of) mediation were influenced by developmental alcohol exposure, suggesting these relationships are similar in the brains of both control and AE animals. ** $p < .010$, *** $p < .001$. n.s. $p > .050$ (nonsignificant). (b) Lobules 9–10 showed a similar relationship between exercise and density of Iba1 + material. Adolescent exercise had a significant total effect (in parentheses) on density of Iba1 + material (“density of microglia”) in lobules 9–10. This was driven by a significant direct effect of exercise on density of Iba1

+ material, and not by exercise-associated alterations in cerebellar volume, despite a significant association between exercise and volume of lobules 9–10. Neither the direct effect, nor (lack of) mediation were influenced by developmental alcohol exposure, suggesting these relationships are similar in the brains of both control and AE animals. $**p = .010$, $***p = .001$. $^{n.s.} p > .050$ (nonsignificant)

FIGURE 6.

Neither postnatal alcohol exposure nor adolescent exercise changes the total number of microglia in cerebellum. (a) Postnatal treatment, adolescent housing, or sex did not influence total number of microglia in lobules 1–4. Bar graphs represent mean + SEM. (b) Postnatal treatment, adolescent housing, or sex did not influence total number of microglia in lobules 9–10. Bar graphs represent mean + SEM

TABLE 1Gundersen coefficients of error ($m = 1$) for stereological estimates

Postnatal Treatment	Adolescent Housing	Lobules	(n)	Coefficient of Error (mean $\pm$ SEM)
SC	SH	1–4	(4)	0.085 ± 0.003
		9–10	(4)	0.143 ± 0.020
	WR	1–4	(4)	0.085 ± 0.005
		9–10	(4)	0.113 ± 0.020
SI	SH	1–4	(4)	0.090 ± 0.004
		9–10	(4)	0.140 ± 0.018
	WR	1–4	(4)	0.080 ± 0.004
		9–10	(4)	0.088 ± 0.006
AE	SH	1–4	(4)	0.090 ± 0.004
		9–10	(4)	0.100 ± 0.004
	WR	1–4	(4)	0.110 ± 0.000
		9–10	(3)	0.120 ± 0.025

TABLE 2

Weights (g) of all groups throughout the study and BACs (mg/dL) on PD4 of AE animals

Postnatal Treatment	Adolescent Housing	Sex	(n)	PD4 (mean $\pm$ SEM)	BAC on PD4 (mean $\pm$ SEM)	PD9 (mean $\pm$ SEM)	PD42 (mean $\pm$ SEM)
SC	SH	F	(3)	10.20 $\pm$ 0.21	—	18.80 $\pm$ 0.68	162.00 $\pm$ 7.57
		M	(7)	10.26 $\pm$ 0.17	—	17.64 $\pm$ 0.70	187.29 $\pm$ 6.86
	WR	F	(3)	10.17 $\pm$ 0.22	—	18.43 $\pm$ 0.73	163.67 $\pm$ 3.33
		M	(9)	10.17 $\pm$ 0.18	—	19.21 $\pm$ 0.72	198.44 $\pm$ 3.84
SI	SH	F	(3)	7.93 $\pm$ 1.50	—	15.10 $\pm$ 2.56	140.33 $\pm$ 10.73
		M	(6)	9.70 $\pm$ 0.89	—	17.08 $\pm$ 1.68	173.83 $\pm$ 9.86
	WR	F	(4)	8.95 $\pm$ 0.50	—	16.98 $\pm$ 0.80	159.25 $\pm$ 3.30
		M	(6)	10.13 $\pm$ 0.23	—	18.62 $\pm$ 0.54	192.67 $\pm$ 5.01
AE	SH	F	(2)	9.45 $\pm$ 0.55	425.0 $\pm$ 6.8	12.85 $\pm$ 1.65	143.50 $\pm$ 11.50
		M	(5)	10.34 $\pm$ 0.33	412.3 $\pm$ 13.4	16.00 $\pm$ 0.94	186.00 $\pm$ 6.64
	WR	F	(3)	8.77 $\pm$ 0.71	416.5 $\pm$ 20.9	14.23 $\pm$ 0.90	147.67 $\pm$ 2.91
		M	(3)	10.00 $\pm$ 0.30	401.9 $\pm$ 26.7	14.17 $\pm$ 1.40	175.00 $\pm$ 11.06