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Prenatal Ethanol Exposure and Postnatal Environmental Intervention Alter Dopaminergic Neuron and Microglia Morphology in the Ventral Tegmental Area During Adulthood

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Abstract

Background: Prenatal ethanol exposure (PE) impairs midbrain dopaminergic (DA) neuron function, which might contribute to various cognitive and behavioral deficits, including attention deficits and increased addiction risk, often observed in individuals with fetal alcohol spectrum disorders (FASD). Currently, the underlying mechanisms for PE-induced deficits are unclear. Prenatal ethanol exposure could lead to neuroinflammation by activating microglia, which play an important role in synaptic function. In the present study, we investigated PE effects on microglial activation and DA neuron density and morphology in the ventral tegmental area (VTA). Since postnatal environmental enrichment can reduce neuroinflammation and ameliorate several PE-induced behavioral deficits, we examined if a postnatal environmental intervention strategy using neonatal handling and post-weaning complex housing could reverse PE effects on VTA DA neurons and microglia.

Methods: Pregnant rats received 0 or 6 g/kg/day ethanol with two intragastric intubation on gestation days 8 – 20. After birth, rats were reared in the standard laboratory or enriched condition. Male adult rats (8–12 weeks old) were used for immunocytochemistry.

Results: The results showed that PE decreased VTA DA neuron body size in standardly housed rats. Moreover, there was a significant decrease in numbers of VTA microglial branches and junctions in PE rats, suggesting morphological activation of microglia and possible neuroinflammation. The PE effects on microglia were normalized by postnatal environmental intervention, which also decreased the numbers of microglial branches and junctions in control animals, possibly via reduced stress.

Conclusions: Our findings show an association between PE-induced morphological activation of microglia and impaired DA neuron morphology in the VTA. Importantly, postnatal environmental intervention rescues possible PE-induced microglial activation. These data support

that environmental intervention can be effective in ameliorating cognitive and behavioral deficits associated with VTA DA neuron dysfunctions, such as attention deficits and increased addiction risk.

Keywords

Fetal Alcohol Spectrum Disorders; Environmental Enrichment; Dopamine; Microglia; Ventral Tegmental Area

INTRODUCTION

Prenatal ethanol exposure (PE) has been shown to cause fetal alcohol spectrum disorders (FASD), which consist of various behavioral and cognitive problems (Guerri et al., 2009, Hill et al., 1989, Paley and O'Connor, 2009). Many of the behavioral and cognitive symptoms of FASD, including learning, memory, attention deficits, and increased addiction risk, are associated with dysfunctions of the mesolimbic dopamine (DA) pathway originated in the ventral tegmental area (VTA) (Geisler and Zahm, 2005, Malanga and Kosofsky, 2003, Valenzuela et al., 2012). Previous work has demonstrated that PE causes a decrease in number of VTA DA neurons firing spontaneous action potentials (Choong and Shen, 2004, Shen et al., 1999), reduced size of their cell bodies, and persistent expression of immature excitatory synapses (Shetty et al., 1993, Wang et al., 2006).

The neuronal mechanisms underlying the effects of PE on VTA DA neurons are not clear. However, a possible cause is neuroinflammation mediated by the activation of microglia (Guizzetti et al., 2014, Zhao et al., 2013). Microglia are important cells in the brain. They play a crucial role in normal neuronal development, which includes synaptic pruning and maturation (Rutherford et al., 1998, Barron, 1995, Schafer et al., 2013). When in their resting state, they exist as ramified cells with an extensive branching pattern that is key to the surveillance and maintenance of functioning synapses (Morrison and Filosa, 2013). As microglia are exposed to adverse conditions or toxins, such as ethanol exposure (Boyadjeva and Sarkar, 2010, Guizzetti et al., 2014), the cells are activated and go through a morphological transformation to gradually decrease their degree of ramification (Morrison and Filosa, 2013). Under this condition, they release many cytokines, such as tumor necrosis factor, nitric oxide, interleukin, and reactive oxygen species (ROS), which can exert neurotoxic effects on surrounding neurons (Colton et al., 1998, Lull and Block, 2010). Furthermore, activated microglia will strip synapses when they detect neuronal injury (Kettenmann et al., 2013). Given the important role of microglia in synaptic function, we hypothesize that activation of microglia in the VTA contributes to immature and altered excitatory synaptic functions of VTA DA neuron synapses following PE. The goal of the present study was to investigate if PE indeed led to persistent microglial activation during adulthood.

At the present time there are few effective intervention approaches for ameliorating the behavioral and cognitive deficits in FASD. However, there is strong evidence that supports environmental enrichment can be an effective intervention for FASD (Hannigan and Berman, 2000, Hannigan et al., 1993, Boschen et al., 2014, Boschen et al., 2017, Briones et

al., 2004, Hamilton et al., 2014). Interestingly, environmental enrichment is known to reduce microglial activation and neuroinflammation (Williamson et al., 2012). Therefore, we have also examined the effects of an environmental intervention strategy, consisting of neonatal handling and postweaning complex housing, which provide environmental enrichment, on possible PE-induced microglial activation/neuroinflammation and DA neuron morphology in the VTA.

METHODS

Prenatal Ethanol Exposure and Postnatal Rearing Conditions

The methods of breeding, prenatal treatment, and cross-fostering were reported previously (Choong and Shen, 2004). Breeding was conducted in house to strictly control for the prenatal environment. Pregnant Sprague-Dawley dams were administered two daily doses of 0 or 3 g/kg ethanol (15% w/v in 0.9% saline) via intragastric intubation between gestation day 8 through day 20. Control dams were given the same volume of sucrose solution (22.5% w/v in 0.9% saline) to equate caloric intake between control and PE animals. Dams in the control group were pair-fed with ethanol-treated dams. Dams also received thiamine injections twice a week (8 mg/kg intramuscularly) to avoid ethanol- or reduced caloric intake-induced thiamine deficiency. When possible, litters were culled to 10 pups of 5 – 8 males and 2 – 5 females on postnatal day (PD) 1. Due to limited resources and the time-consuming imaging procedures, we only used male offspring in this study. Future studies will examine PE effects on female offspring. Pups were raised in two postnatal conditions: standard housing and with environmental intervention. In the standard housing group, pups and dam were left undisturbed except for weekly cage changes before weaning (PDs 2 – 20). After weaning (on PD 21), rats were housed in pairs in standard plastic cages undisturbed except for weekly cage changes. In the environmental intervention group, dam and pups underwent a brief maternal separation (15 min) /neonatal handling procedure once daily during PDs 2 – 20. Each pup was also gently handled for one minute during this time. The purpose of maternal separation/neonatal handling was to enhance maternal behavior, as a supplemental enrichment procedure in the early developmental period (Fernández-Teruel et al., 2002, Van Praag et al., 2000, Wang et al., 2018b, Wang et al., 2018a). After weaning, 10 – 20 rats in the environmental intervention group were housed in a large wire cage (64×92×160 cm) with 30 objects/toys (pots, hideouts, ropes, wheels, ect.; Petco, San Diego, CA). Every weekday, rats were taken out of the cage for 15 min while the objects/toys were relocated in the cage to create novelty. The objects/toys were washed and replaced weekly.

Thirty rats in total were used in the present study (standardly housed: control: 8 rats from 6 litters and PE: 8 rats/7 litters; postnatal environmental intervention: control: 7 rats/5 litters and PE: 7 rats/5 litters). The number of rats and litters in each group were kept consistent throughout all experiments.

The PE treatment and postnatal environmental intervention were conducted in accordance with the guidelines of the National Institutes of Health and the American Association for Accreditation of Laboratory Animal Care. All the procedures were approved by the Institutional Animal Care and Use Committee at University at Buffalo.

Preparation of Brain Sections

Adult male rats (8–12 weeks old) were anesthetized with pentobarbital (50 mg/kg, i.p.) and transcardially perfused with ice-cold phosphate buffered saline (0.1M; 100–150 ml/rat) containing heparin (5 units/ml), followed by ice-cold paraformaldehyde (4% in 0.1M phosphate buffer; 100 ml/100 g of body weight). Brains were removed and post fixed in 4% paraformaldehyde for one hour, and then transferred to 15% sucrose/4% paraformaldehyde for 24 hours at 4°C. The midbrain areas were blocked in a brain mold (Zivic Instruments, Pittsburg, PA) and sectioned coronally at 30, 50, or 60 µm in each group using a freezing microtome with every other section collected. Thicker sections (50 and 60 µm) were used to examine DA neuron layer structure which was outside the scope of the present study.

Immunocytochemistry

Sections were first blocked with normal goat serum (Thermo Fisher Scientific, Carlsbad, CA) at 4°C for 24 hours. Sections were next placed in primary antibodies against tyrosine hydroxylase/TH (Sigma-Aldrich, St. Louis, MO; 1:1250) and ionized calcium binding adaptor molecule 1/IBA-1 (Wako Chemicals, Richmond, VA; 1:1000) at 4°C for 72 hours to label DA neurons and microglia, respectively. Lastly, sections were incubated with fluorescent secondary antibodies (Alexa Fluor 488 Goat Anti-Mouse for TH, 1:200; Alexa Fluor 594 Goat Anti-Rabbit for IBA-1, 1:200; Thermo Fisher Scientific) for 2 – 3 hours. Sections were then mounted on glass slides and coverslipped using Prolong Gold anti-fade reagent (Thermo Fisher Scientific).

Imaging and Data Acquisition

Images were taken using a Zeiss AxioImager fluorescence microscope equipped with ApoTome, Z-stacking and tiling functions (Carl Zeiss, Inc., Thornwood, NY). Images were taken in 2 × 2 tiles at 20X magnification with a 0.54 µm interval in the Z-axis between each picture throughout the thickness of the session. Prior to image analysis, one anterior (4.2 mm anterior to the interaural line) and one middle (3.4 mm anterior to the interaural line) VTA region were selected from each animal, and these regions were matched consistently among all animals to ensure the same region was used for analysis. Each image was then processed in Zen (Carl Zeiss, Inc.) and Fiji/Image J using manual thresholding and various plugins (Schindelin et al., 2012). All images were blindly examined and analyzed by two people.

Quantification of cell body size for DA neurons and cell density for DA neurons and microglia.

We found DA neurons were organized in layers separated by DA neuron free areas roughly parallel to the coronal cutting plane. Six continuous images along the z-stack showing a complete single layer of VTA DA neurons were selected for image analysis. The brightness and contrast were kept consistent between images in Zen before the images were exported into Fiji. The 6 individual images were compiled into a single 2-D projection image using the z-projection function. The projection image was then spilt into green (DA) and red (microglia) channels (Fig.1A). To analyze DA neuron density, a region of interest (ROI) was isolated in an area (78002.665 µm²) with a high concentration of DA neurons in the anterior and middle VTA. The same ROI was also used to analyze microglia density. In this study,

cell density refers to cell number within the ROI. The position of the ROI was matched consistently from animal to animal (Fig.1C). A mask of the cell bodies was created after segmentation to overlay on top of the original image using Fiji. Cell density was examined again visually to ensure all DA neuron and microglia cell bodies were counted correctly. Only DA neurons showing clear nucleus were included. In the present study, we did not use the stereology approach to count cells. The approach we utilized is, therefore, considered a semi-quantification method, which cannot provide information regarding absolute cell density throughout the VTA. However, we believe the imaging methods, including the 3-D quantification approach, allowed us to limit biases and were sufficient for group comparisons to detect major differences.

Quantification of microglia body size and branching pattern.

We utilized 3-D analysis to more accurately analyze the cell body size and branching pattern of microglia. We divided each of the anterior or middle VTA section into 4 quadrants. Two individual microglia from each quadrant were randomly selected. A ROI was defined for each cell making sure that the branches were not truncated in the 3-D stack. A total of 16 microglia were sampled and analyzed per animal. Because microglia cell bodies have irregular shapes, we used a 3-D methodology for the analysis of their sizes. The 3-D methodology was also used for more accurate branching pattern analysis. For cell body size analysis, a single microglia was analyzed using the segmentation editor in Fiji, which allows manual 3-D delineation of the microglia cell body border through the Z-stack feature. This was achieved by using the brush tool to scroll through the entire z-stack to trace the cell body area, followed by using the threshold to refine the traced area. The 3D-interpolation function was selected to yield the cell body size. The same ROI containing the 3-D stack of the same microglia was used for branching pattern analysis. After background adjustment, the image was first skeletonized using the “skeleton” plugin, followed by the function of “analyze skeleton” to obtain the branching information including number of branches, number of junctions, which were points of arborization, and average branch length.

Statistical Analysis

Data were analyzed with SAS 9.4 (SAS Institute Inc., Cary, NC), OriginPro 2019 (OriginLab Co., Northampton, MA), and Statistica 7 (Tibco Software Inc., Palo Alto, CA). Two-way ANOVA (prenatal treatment: control vs. PE; postnatal rearing condition: standard housing vs postnatal environmental intervention) with litter as a nested factor was used in analyses of all the dependent variables. Planned comparisons were performed after ANOVA for pairwise comparisons. The Pearson product-moment correlation coefficient test was applied to compute correlation coefficients. The Shapiro-Wilk and Anderson-Darling tests were utilized to assess whether the dependent variables have normal distributions (Razali and Wah, 2011). Data are presented as Mean $\pm$ SEM in the text and figures. $P < 0.05$ was considered significant.

RESULTS

Prenatal ethanol exposure and postnatal environmental intervention had no effects on VTA DA neuron density in adult rats.

Dopaminergic neurons were analyzed in the anterior and middle VTA for cell density. There were no effects of PE or postnatal environmental intervention on DA neuron density (2-way ANOVA with litter as a nested factor; no litter effects were observed; Fig. 2). In the standard housing condition, the DA neuron density in control and PE rats was 32.4 ± 3.1 cells ($n = 8$ rats/6 litters) and 33.1 ± 2.7 cells ($n = 8$ rats/7 litters), respectively in the anterior VTA, and 33.5 ± 2.3 cells and 30.9 ± 1.8 cells, respectively in the middle VTA. In the environmental intervention condition, the DA neuron density in control and PE rats was 33.6 ± 3.4 cells ($n = 7$ rats/5 litters) and 36.6 ± 3.1 cells ($n = 7$ rats/5 litters), respectively in the anterior VTA, and 31.0 ± 2.9 cells and 33.0 ± 4.2 cells, respectively in the middle VTA.

Prenatal ethanol exposure decreased VTA DA neuron body size in adult rats.

As for DA neuron body size, we found it did not fit normal distribution within any subgroup (Shapiro-Wilk test: $p < 0.001$; Anderson-Darling test: $p < 0.01$ for all the subgroups). The distributions were skewed to the right with more DA neurons having smaller cell bodies than larger cell bodies. However, it has been shown recently that ANOVA is robust even if the normality assumption is violated (Blanca et al., 2017). Therefore, we still applied 2-way ANOVA with litter as a nested factor.

The major finding was that, in standardly housed animals, there was a significant decrease in DA neuron body size in PE rats in both the anterior and middle VTA. In the anterior VTA, a 2-way ANOVA with litter as a nested factor showed a main effect of postnatal rearing ($F_{1,992} = 9.68$, $p < 0.01$; there was also a litter effect, $F_{19,992} = 3.14$, $p < 0.001$). In standardly housed rats, we observed a reduction in VTA DA neuron body size in PE rats, compared with controls (planned comparison: $p < 0.05$; control: $253.1 \pm 8.0 \mu\text{m}^2$, $n = 259$ cells from 8 rats/6 litters; PE: $231.2 \pm 7.6 \mu\text{m}^2$, $n = 265$ cells from 8 rats/7 litters; Fig. 3). In contrast, in rats with postnatal environmental intervention, no differences were observed between control and PE rats (planned comparison; control: $224.2 \pm 7.6 \mu\text{m}^2$, $n = 235$ cells from 7 rats/5 litters; PE: $219.5 \pm 7.2 \mu\text{m}^2$, $n = 256$ cells from 7 rats/5 litters; Fig. 3). Furthermore, in control rats, a reduction in DA neuron body size was observed in rats with postnatal environmental intervention, compared with rats reared in the standard condition (planned comparison: $p < 0.01$).

In the middle VTA, a nested 2-way ANOVA revealed a significant main effect of prenatal treatment ($F_{1,940} = 12.26$, $p < 0.001$; a litter effect was also observed, $F_{19,940} = 2.64$, $p < 0.001$). In standardly housed PE rats, a reduction in DA neuron body size was observed, compared with controls (planned comparison: $p < 0.05$; control: $237.1 \pm 8.1 \mu\text{m}^2$, $n = 268$ cells; PE: $217.4 \pm 6.7 \mu\text{m}^2$, $n = 247$ cells; Fig. 3). A similar effect was observed in rats with postnatal environmental intervention (planned comparison: $p < 0.05$; control: $237.2 \pm 8.4 \mu\text{m}^2$, $n = 217$ cells; PE: $208.3 \pm 7.3 \mu\text{m}^2$, $n = 231$ cells; Fig. 3). These results are consistent with our previous electrophysiology observation that DA neurons in PE animals had a higher average input resistance than in control animals (both reared in the standard condition),

which means that PE animals had a lower average DA neuron body size than control animals (Wang et al., 2006).

Prenatal ethanol exposure and postnatal environmental intervention had no effects on VTA microglia density in adult rats

We analyzed microglia density in the VTA. There were no PE effects or postnatal environmental intervention effects (2-way ANOVA with litter as a nested factor) in the anterior VTA (in standardly housed rats, control: 19.5 ± 2.9 cells, $n=8$ rats/6 litters; PE: 16.1 ± 2.3 cells, $n = 8$ rats/7 litters; in rats with postnatal environmental intervention, control: 18.1 ± 3.3 cells, $n = 7$ rats/5 litters; PE: 17.1 ± 2.5 cells, $n = 7$ rats/5 litters; Fig. 4A) or middle VTA (in standardly housed rats, control: 20.1 ± 2.7 cells; PE: 18.1 ± 1.8 cells; in rats with postnatal environmental intervention, control 21.1 ± 4.2 cells; PE: and 19.3 ± 2.1 cells; Fig. 4A).

Prenatal ethanol exposure and postnatal environmental intervention had no effects on VTA microglia body size in adult rats

Due to the irregular morphology of microglia cell body, we analyzed microglia body size using 3-D segmentation from 8 cells in the anterior VTA and 8 cells in middle VTA per animal. The same cells were used in the morphology analyses described below.

We did not observe group differences in microglia body size between rats with different prenatal treatments or in different postnatal rearing conditions (2-way ANOVA with litter as a nested factor) in the anterior VTA (standardly housed rats, control: $305.5 \pm 15.2 \mu\text{m}^3$, $n = 64$ cells from 8 rats/6 litters; PE: $317.6 \pm 13.7 \mu\text{m}^3$, $n = 64$ cells from 8 rats/7 litters; rats with postnatal environmental intervention, control: $329.8 \pm 15.6 \mu\text{m}^3$, $n = 56$ cells from 7 rats/5 litters; PE: $330.8 \pm 16.3 \mu\text{m}^3$, $n = 56$ cells from 7 rats/5 litters; Fig. 4B) or middle VTA (standardly housed rats, control: $356.9 \pm 14.5 \mu\text{m}^3$, $n = 64$ cells; PE: $347.8 \pm 16.4 \mu\text{m}^3$, $n = 64$ cells; rats with postnatal environmental intervention, control: $355.6 \pm 16.3 \mu\text{m}^3$, $n = 56$ cells; PE: $330.9 \pm 13.5 \mu\text{m}^3$, $n = 56$ cells; Fig. 4B). Significant litter effects were observed in both the analyses for the anterior VTA ($F_{19,217} = 1.93$, $p < 0.05$) and the middle VTA ($F_{19,217} = 3.22$, $p < 0.001$).

Prenatal ethanol exposure reduced microglia branch numbers and junction numbers without changing branch length in adult rats, effects rescued by postnatal environmental intervention.

We found that PE significantly decreased branch numbers of microglia in animals housed in the standard condition in both the anterior and middle VTA (Fig. 5B). In the anterior VTA, a 2-way ANOVA with litter as a nested factor showed a significant interaction effect between prenatal treatment and postnatal rearing condition ($F_{1,217} = 19.58$, $p < 0.001$; a litter effect was also observed, $F_{19,217} = 4.05$, $p < 0.001$). In standardly housed rats, reduced branch numbers were observed in PE rats, compared with controls (planned comparison: $p < 0.001$; control: 263.7 ± 24.7 branches, $n = 64$ cells from 8 rats/6 litters; PE: 117.8 ± 9.1 branches, $n = 64$ cells from 8 rats/7 litters; Fig. 5B). In rats with postnatal environmental intervention, there were no differences between control and PE rats (planned comparison; control: 218.2 ± 24.8 branches, $n = 56$ cells from 7 rats/5 litters; PE: 229.2 ± 21.4 branches, $n = 56$ cells from

7 rats/5 litters; Fig. 5B). The results also showed that postnatal environmental intervention reversed the reduction in branch number observed in standardly housed PE rats (planned comparison: $p < 0.001$; Fig. 5B). Lastly, we observed that in control animals, postnatal environmental intervention, relative to the standard rearing condition, led to a decrease in branch number (planned comparison: $p < 0.05$; Fig. 5B).

A similar pattern in microglial branch numbers was also observed in the middle VTA. A nested 2-way ANOVA showed an interaction effect between prenatal treatment and postnatal environmental intervention ($F_{1,217} = 31.66$, $p < 0.001$; a litter effect was also observed, $F_{19,217} = 4.76$, $p < 0.001$). A reduction in branch number was observed in standardly housed PE rats compared with controls (planned comparison: $p < 0.001$; control: 243.6 ± 17.9 branches, $n = 64$ cells; PE: 130.2 ± 12.1 branches, $n = 64$ cells; Fig. 5B). In contrast, an increase in branch number was observed in PE animals with postnatal environmental intervention, compared with their control counterparts (planned comparison: $p < 0.05$; control: 187.7 ± 19.1 branches, $n = 56$ cells; PE: 233.5 ± 23.1 branches, $n = 56$ cells; Fig. 5B). Postnatal environmental intervention reversed the reduction in branch number observed in standardly housed PE animals (planned comparison: $p < 0.001$; Fig. 5B). Lastly, we observed that in control animals, postnatal environmental intervention, relative to the standard rearing condition, led to a decrease in branch number (planned comparison: $p < 0.001$; Fig. 5B).

Similar results were observed in microglial junction numbers. In the anterior VTA, a 2-way ANOVA with litter as a nested factor produced a significant interaction effect between prenatal treatment and postnatal rearing condition ($F_{1,217} = 20.63$, $p < 0.001$; a litter effect was also observed, $F_{19,217} = 4.37$, $p < 0.001$; Fig. 5C). In standardly housed rats, reduced junction numbers were observed in PE rats, compared with controls (planned comparison: $p < 0.001$; control: 119.8 ± 10.5 junctions, $n = 64$ cells from 8 animals/6 litters; PE: 53.0 ± 3.8 junctions, $n = 64$ cells from 8 animals/7 litters; Fig. 5C). In rats with postnatal environmental intervention, there were no differences between control and PE rats (planned comparison; control: 101.6 ± 11.2 junctions, $n = 56$ cells from 7 animals/5 litters; PE: 95.0 ± 7.4 junctions, $n = 56$ cells from 7 animals/5 litters; Fig. 5C). The results also showed a normalization of junction numbers in PE rats with postnatal environmental intervention, relative to PE rats reared in the standard condition (planned comparison: $p < 0.001$; Fig. 5C). In addition, in control animals, decreased junction numbers were observed in those with postnatal environmental intervention, compared with their standardly housed counterparts (planned comparison: $p < 0.01$; Fig. 5C).

In the middle VTA, a nested 2-way ANOVA revealed an interaction effect between prenatal treatment and postnatal rearing condition ($F_{1,217} = 37.00$, $p < 0.001$, with a litter effect, $F_{19,217} = 5.17$, $p < 0.001$). In the standardly housed rats, reduced junction number was observed in PE rats compared with controls (planned comparison: $p < 0.001$; control: 112.4 ± 8.0 junctions, $n = 64$ cells; PE: 58.3 ± 4.4 junctions, $n = 64$ cells; Fig. 5C). In contrast, in rats with postnatal environmental intervention, increased junction numbers were observed in PE rats compared with controls (planned comparison: $p < 0.05$; control: 85.2 ± 8.8 junctions, $n = 56$ cells; PE: 99.4 ± 8.5 junctions, $n = 56$ cells; Fig. 5C). Similar to that observed in branch number, postnatal environmental intervention reversed the reduction in junction

number observed in standardly housed PE animals (planned comparison: $p < 0.001$; Fig. 5C). Lastly, in control animals, postnatal environmental intervention, relative to the standard rearing condition, led to a decrease in junction number (planned comparison: $p < 0.001$; Fig. 5C).

The average branch length of microglia did not differ between standardly housed control and PE rats in either the anterior or middle VTA (Fig. 5D). In the anterior VTA, 2-way ANOVA with litter as a nested factor revealed an interaction effect between prenatal treatment and postnatal rearing condition ($F_{1,217} = 4.09$, $p < 0.05$ with a litter effect, $F_{19,217} = 1.85$, $p < 0.05$). However, no group differences were observed in standardly housed control and PE rats (planned comparison; control: $3.18 \pm 0.06 \mu\text{m}$, $n = 64$ cells from 8 animals/6 litters; PE: $3.36 \pm 0.09 \mu\text{m}$, $n = 64$ cells from 8 animals/7 litters; Fig. 5D) or between control and PE rats with postnatal environmental intervention (planned comparison; control: $3.38 \pm 0.08 \mu\text{m}$, $n = 56$ cells from 7 animals/5 litters; PE: $3.23 \pm 0.08 \mu\text{m}$, $n = 56$ cells from 7 animals/5 litters; Fig. 5D).

In the middle VTA, a 2-way ANOVA with litter as a nested factor revealed an interaction effect between prenatal treatment and postnatal rearing condition ($F_{1,217} = 12.16$, $p < 0.001$ with a litter effect, $F_{19,217} = 1.84$, $p < 0.05$). However, in the standardly housed rats, no group differences between control and PE rats were observed (control: $3.17 \pm 0.06 \mu\text{m}$, $n = 64$ cells; PE: $3.33 \pm 0.09 \mu\text{m}$, $n = 64$ cells; Fig. 5D). In contrast, in rats with postnatal environmental intervention, shorter branch length was observed in PE rats compared with controls (planned comparison: $p < 0.001$; control: $3.50 \pm 0.11 \mu\text{m}$, $n = 56$ cells; PE: $3.11 \pm 0.07 \mu\text{m}$, $n = 56$ cells; Fig. 5D). In addition, postnatal environmental intervention led to longer average branch length in microglia in control rats when compared with standardly housed controls (planned comparison: $p < 0.001$; Fig. 5D).

There was a strong and significant correlation between number of branches and number of junctions in the anterior (Pearson product-moment correlation coefficient, $r = 0.95$, $n = 240$ cells, $p < 0.001$) and middle VTA ($r = 0.94$, $n = 240$ cells, $p < 0.001$). In addition, negative correlations were observed between number of branches and average branch length in the anterior ($r = -0.45$, $n = 240$ cells, $p < 0.001$) and middle VTA ($r = -0.40$, $n = 240$ cells, $p < 0.001$), indicating that the average branch length was longer in a microglia with fewer branches. These observations suggest that changes in microglial branching pattern are mainly due to an increase or decrease in branch number, not in average branch length.

DISCUSSION

In the present study, we have shown that PE leads to a significant decrease in VTA DA neuron body size without altering their density in adult animals. Furthermore, PE leads to reduced microglial branch and junction numbers without influencing microglia body size or density in the VTA in adult animals. Postnatal environmental intervention exerts no clear effects on VTA DA neuron or microglia density or cell body size in control or PE animals. However, it reverses the reduction in microglial branch and junction numbers in PE animals. These observations emphasize that PE can lead to persistent changes in the morphology of VTA DA neurons and microglia into adulthood.

The reduction in cell body size of VTA DA neurons without changes in VTA DA neuron density in adult PE animals is consistent with the observations from previous anatomical and electrophysiological studies showing that PE does not lead to loss of VTA DA neurons, but leads to reduced dendritic branching and increased input resistance indicative of smaller cell bodies (Choong and Shen, 2004, Shen et al., 1999, Shetty et al., 1993, Wang et al., 2006). On the other hand, we have not observed changes in cell body size in VTA microglia. In the present study, we analyzed cell body size by pooling all cells in a specific group. This might not be the best approach. A nested design ANOVA with cells nested under each rat would be an ideal (Galbraith et al., 2010). However, we did not use this approach based on following reasons. First, we were already using nested design to analyze the litter effect. Second, a substantially larger group size (50) (Aarts et al., 2014) is required for a sufficient statistical power even for a moderate effect size if we use ANOVA with cells nested under each rat. Because we have indeed shown reduced VTA DA neuron body size in PE rats, which are consistent with the findings from our previous electrophysiological studies, we believe the statistical approach used to analyze cell body size is appropriate.

To the best of our knowledge, the present study represents the first opportunity to describe persistent PE effects on microglia in the VTA during adulthood. Our observation that VTA microglia have reduced branch and junction numbers after PE supports the possibility that PE causes microglial activation and neuroinflammation. It is well documented that microglia experience morphological activation – a gradual morphological change when activated in the state of neuroinflammation caused by systemic toxins or brain injuries (Karperien et al., 2013, Morrison and Filosa, 2013). Microglia go through stages of morphological activation. In the first stage, microglia retract their processes. In the second “motility” stage, microglia can go through several phases of extending and retracting their processes while the cell body increases in size. Finally, the third stage, called the “locomotory” stage, involves translocation of the microglia. Since we have not observed enlarged cell bodies, but only reduced branching in PE animals, we posit that microglia in the VTA resemble the first stage of activation (Stence et al., 2001). These observations are consistent with previous studies showing acute and chronic ethanol exposure persistently activate microglia (Cruz et al., 2017, McClain et al., 2011). At the present time, there are a limited number of studies investigating the effects of prenatal or perinatal ethanol exposure on microglia. One study investigating PE during the 2nd trimester shows that activated microglia and the secretion of proinflammatory cytokines are associated with cell death and structural changes in the neocortex in fetal and neonatal mice. This effect is ameliorated by the administration of PPAR γ agonist pioglitazone, which reduces microglial activation and neuroinflammation (Komada et al., 2017). It has also been demonstrated that the 3rd trimester binge-drinking pattern of ethanol exposure leads to microglial activation in the cortex, hippocampus, and cerebellum (Topper et al., 2015, Boschen et al., 2016, Kane et al., 2011). Such an effect is also accompanied with secretion of pro-inflammatory cytokines (Kane et al., 2011). These effects can be rescued by the administration of pioglitazone (Drew et al., 2015). So far, only one study has examined possible persistent effects of PE on microglial activation, which reports that microglia display resting morphology during adolescence following neonatal ethanol exposure (Topper et al., 2015).

Over the last few years, great progress has been made in understanding the function of microglia. Not only are microglia responsible for the innate immune response and apoptosis, they also play an important role in surveying and maintaining synaptic function in normal conditions as well as in synaptic maturation and pruning during development (Barron, 1995, Bilimoria and Stevens, 2015, Harry, 2013, Hoshiko et al., 2012). Microglia's involvement in synaptic maturation is supported by the observation that morphological maturation of microglia coincides with synaptic maturation (Dalmau et al., 1998). A disruption of microglial function or the signaling between microglia and neurons during early development has been shown to delay maturation of synapses, resulting in an excess of immature synapses (Paolicelli et al., 2011). The pruning process is largely mediated via elimination of less active presynaptic terminals through trophocytosis (synaptic nibbling), i.e., ramified and yet activated microglia remove the presynaptic terminals only by 250 nm (Otto, 2018, Weinhard et al., 2018). Interestingly, findings from our previous studies show that PE persistently leads to more excitatory synapses onto VTA DA neurons, a large proportion of which are immature synapses (Hausknecht et al., 2015). Specifically, in PE animals, immature calcium-permeable AMPA receptors do not switch to the mature form of calcium impermeable AMPA receptors even in rats in their young adulthood (12 weeks old). The persistent presence of calcium-permeable AMPARs significantly increases the excitatory synaptic strength. It also leads to a unique form of anti-Hebbian long-term potentiation, which further promotes synaptic strengthening. Together, these effects are similar to those observed after chronic drug intake and have been proposed as critical cellular mechanisms for drug addiction (Bellone and Luscher, 2006, Argilli et al., 2008). Therefore, we have proposed that these PE-induced immature excitatory synapses onto VTA DA neurons contribute to increased addiction risk in FASD, observed in both clinical and animal studies (Hausknecht et al., 2015). Based on the observation from the present study that PE leads to reduced branching pattern of microglia possibly indicative of activated microglia, we posit that this effect may contribute to the persistent presence of immature excitatory synapses onto VTA DA neurons. It is also tempting to speculate that reduced branching in microglia in PE animals could lead to insufficient surveillance and pruning of synapses by microglia, which result in immature excitatory synapses in VTA DA neurons. This notion that microglial activation leads to failure of synaptic maturation is supported by the observation that peripheral colonic inflammation, known to activate microglia in the brain, leads to an increase in immature calcium-permeable AMPARs and excitatory synaptic strength, as well as altered excitatory synaptic plasticity. In addition, treatment with minocycline, which reduces microglial activation and exerts anti-neuroinflammatory effects, rescues these effects (Riazi et al., 2015), further supporting activated microglia are responsible for the expression of immature calcium-permeable AMPARs.

Another major finding in the present study is that the reduced branching pattern of microglia in PE animals is normalized by postnatal environmental intervention. This is indicated by a reversal of reduced number of branches and junction points in microglia to near control levels (Fig. 5). Although previous studies have already shown that postnatal environmental enrichment is neuroprotective and can ameliorate cognitive and behavioral deficits after PE (Hannigan and Berman, 2000, Hannigan et al., 1993, Boschen et al., 2014, Boschen et al., 2017), the basic mechanism is not clear. Our results suggest a potential mechanism

underlying the beneficial effects of postnatal environmental intervention on PE-induced deficits is via the reduction in microglial activation-induced neuroinflammation. Indeed, environmental enrichment has been found to prevent and/or reduce neuroinflammation caused by various reasons, such as influenza (Jurgens and Johnson, 2012), traumatic brain injury (Benn et al., 2010), and amyloid beta (Xu et al., 2016, Ryan and Nolan, 2016). Environmental enrichment also reverses persistent neuroinflammation caused by adverse perinatal factors such as prenatal gram negative endotoxin Lipopolysaccharide (LPS) exposure or neonatal hypoxia. Such an effect is observed when environmental enrichment occurs during late adolescence to young adulthood long after prenatal LPS treatment (Kentner et al., 2016). In the present study, our environmental intervention paradigm takes place right after birth with the neonatal handling procedure, which facilitates maternal behavior (Pryce et al., 2001), followed by enriched housing after weaning, consisting of social interaction, exercise, and sensory stimulation, until animals are sacrificed during adulthood. The goal is to start intervention as early as possible and to maximize the intervention effects. Furthermore, the combined method could exert additive beneficial effects (Fernández-Teruel et al., 2002, Escorihuela et al., 1994, Escorihuela et al., 1995, Pham et al., 1999). Importantly, using this approach, we have demonstrated that increased addiction risk after PE can be reverted (Wang et al., 2018b). At the present time, it is not clear how neonatal handling and enriched housing proportionally contribute to the neuroinflammation-reducing effects. Future studies are required to tease apart the timing and types of postnatal enrichment in reducing neuroinflammation after PE.

One interesting finding in the present study is that environment intervention slightly reduces microglial branching in control animals, an effect opposite to that observed in PE rats. At a glance, it seems at odds with the notion that environmental intervention can reduce microglial activation, which should be associated with reduced branching in microglia. However, it has been reported that, under chronic mild stress, microglia can be in a state of hyper-ramification, displaying increased branching and lengthening of processes (Hinwood et al., 2013, Walker et al., 2013). It has been shown that standardly housed rats are more stressed than rats in enriched housing (van der Harst et al., 2003). The hyper-ramified microglia are slightly activated microglia, which can secrete proinflammatory cytokines and are associated with cognitive deficits that can be reversed by inhibiting microglial activation (Hinwood et al., 2013, Crews and Vetreno, 2016). Microglia in the VTA have been shown to respond to chronic stress (Tynan et al., 2010). It is possible that the microglia in the VTA in control rats are in the state of hyper-ramification and environmental intervention normalizes this state. This possibility emphasizes that the function of microglia is complex and sensitive to environmental changes, which is consistent with recent observations and the notion that microglial responses/functional states are diverse and the relationship between the functional state of microglia and morphology is not always linear. Microglia with highly ramified morphology can also be in an activated state (Dorman and Molofsky, 2019, Kentner et al., 2016, Ransohoff, 2016). Therefore, understanding how morphological changes are related to the complex functions of microglia and how morphological and functional changes influence the role of microglia in modulating synaptic functions will be an important future research area.

There is evidence in clinical research suggesting that postnatal environmental complexity could influence the outcome of PE. Specifically, it has been reported that deficits in executive function in FASD are directly influenced by the individual's environment (Nash et al., 2015). More positive and complex environments including younger caregivers and a larger family (Coles et al., 2009) have been shown to ameliorate the deficits associated with PE, including a lack of behavioral regulation, emotional control, and language production (Doyle et al., 2018, Coles et al., 2009). In addition, a longitudinal study shows that early diagnosis/interventions, as well as stable and nurturing family environments, correlate with significant improvements in both academic and behavioral assessments (Streissguth et al., 2004). These clinical studies in general show that enriched and positive environments are effective in ameliorating cognitive deficits caused by PE.

Taken together, our study shows that PE leads to persistent effects in the VTA during adulthood, including decreased cell body size of DA neurons and possible morphological activation of microglia. The latter effect can be reversed by postnatal environmental intervention comprised of neonatal handling and complex housing after weaning. An additional finding is the observation of potential hyper-ramification of VTA microglia in control animals without environmental intervention. The PE-induced microglial activation could contribute to persistently immature excitatory synapses onto VTA DA neurons that mediate increased addiction risk during adulthood, which was previously observed. Therefore, environmental intervention might serve as an effective intervention approach to ameliorate the long-term behavioral and cognitive deficits observed in children with FASD.

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REFERENCES

- Aarts E, Verhage M, Veenliet JV, Dolan CV, Van Der Sluis S (2014) A solution to dependency: using multilevel analysis to accommodate nested data. *Nature neuroscience* 17:491. [PubMed: 24671065]
- Argilli E, Sibley DR, Malenka RC, England PM, Bonci A (2008) Mechanism and time course of cocaine-induced long-term potentiation in the ventral tegmental area. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 28:9092–9100. [PubMed: 18784289]
- Barron KD (1995) The microglial cell. A historical review. *J Neurol Sci* 134 Suppl:57–68.
- Bellone C, Luscher C (2006) Cocaine triggered AMPA receptor redistribution is reversed in vivo by mGluR-dependent long-term depression. *Nature neuroscience* 9:636–641. [PubMed: 16582902]
- Benn CL, Luthi-Carter R, Kuhn A, Sadri-Vakili G, Blankson KL, Dalai SC, Goldstein DR, Spires TL, Pritchard J, Olson JM, van Dellen A, Hannan AJ, Cha JH (2010) Environmental enrichment reduces neuronal intranuclear inclusion load but has no effect on messenger RNA expression in a mouse model of Huntington disease. *Journal of neuropathology and experimental neurology* 69:817–827. [PubMed: 20613636]
- Bilimoria PM, Stevens B (2015) Microglia function during brain development: New insights from animal models. *Brain research* 1617:7–17. [PubMed: 25463024]
- Blanca M, Alarcón R, Arnau J, Bono R, Bendayan R (2017) Non-normal data: Is ANOVA still a valid option? *Psicothema* 29:552–557. [PubMed: 29048317]
- Boschen KE, Hamilton GF, Delorme JE, Klintsova AY (2014) Activity and social behavior in a complex environment in rats neonatally exposed to alcohol. *Alcohol* 48:533–541. [PubMed: 25150044]

- Boschen KE, McKeown SE, Roth TL, Klintsova AY (2017) Impact of exercise and a complex environment on hippocampal dendritic morphology, Bdnf gene expression, and DNA methylation in male rat pups neonatally exposed to alcohol. *Developmental neurobiology* 77:708–725. [PubMed: 27597545]
- Boschen KE, Ruggiero MJ, Klintsova AY (2016) Neonatal binge alcohol exposure increases microglial activation in the developing rat hippocampus. *Neuroscience* 324:355–366. [PubMed: 26996510]
- Boyadjieva NI, Sarkar DK (2010) Role of microglia in ethanol's apoptotic action on hypothalamic neuronal cells in primary cultures. *Alcoholism, clinical and experimental research* 34:1835–1842.
- Briones TL, Klintsova AY, Greenough WT (2004) Stability of synaptic plasticity in the adult rat visual cortex induced by complex environment exposure. *Brain research* 1018:130–135. [PubMed: 15262214]
- Choong K, Shen R (2004) Prenatal ethanol exposure alters the postnatal development of the spontaneous electrical activity of dopamine neurons in the ventral tegmental area. *Neuroscience* 126:1083–1091. [PubMed: 15207341]
- Coles CD, Kable JA, Taddeo E (2009) Math performance and behavior problems in children affected by prenatal alcohol exposure: intervention and follow-up. *J Dev Behav Pediatr* 30:7–15. [PubMed: 19194327]
- Colton CA, Snell-Callanan J, Chernyshev ON (1998) Ethanol induced changes in superoxide anion and nitric oxide in cultured microglia. *Alcoholism, clinical and experimental research* 22:710–716.
- Crews FT, Vetreno RP (2016) Mechanisms of neuroimmune gene induction in alcoholism. *Psychopharmacology* 233:1543–1557. [PubMed: 25787746]
- Cruz C, Meireles M, Silva SM (2017) Chronic ethanol intake induces partial microglial activation that is not reversed by long-term ethanol withdrawal in the rat hippocampal formation. *Neurotoxicology* 60:107–115. [PubMed: 28408342]
- Dalmau I, Finsen B, Zimmer J, Gonzalez B, Castellano B (1998) Development of microglia in the postnatal rat hippocampus. *Hippocampus* 8:458–474. [PubMed: 9825958]
- Dorman LC, Molofsky AV (2019) Demystifying Microglia: And Now the Work Begins. *Immunity* 50:11–13. [PubMed: 30650371]
- Doyle LR, Moore EM, Coles CD, Kable JA, Sowell ER, Wozniak JR, Jones KL, Riley EP, Mattson SN, Cifas (2018) Executive Functioning Correlates With Communication Ability in Youth With Histories of Heavy Prenatal Alcohol Exposure. *J Int Neuropsychol Soc* 24:1026–1037. [PubMed: 30322415]
- Drew PD, Johnson JW, Douglas JC, Phelan KD, Kane CJ (2015) Pioglitazone blocks ethanol induction of microglial activation and immune responses in the hippocampus, cerebellum, and cerebral cortex in a mouse model of fetal alcohol spectrum disorders. *Alcoholism, clinical and experimental research* 39:445–454.
- Escorihuela RM, Tobena A, Fernández-Teruel A (1994) Environmental enrichment reverses the detrimental action of early inconsistent stimulation and increases the beneficial effects of postnatal handling on shuttlebox learning in adult rats. *Behavioural brain research* 61:169–173. [PubMed: 8037864]
- Escorihuela RM, Tobena A, Fernández-Teruel A (1995) Environmental enrichment and postnatal handling prevent spatial learning deficits in aged hypoemotional (Roman high-avoidance) and hyperemotional (Roman low-avoidance) rats. *Learning & memory* 2:40–48. [PubMed: 10467565]
- Fernández-Teruel A, Giménez-Llort L, Escorihuela RM, Gil L, Aguilar R, Steimer T, Tobena A (2002) Early-life handling stimulation and environmental enrichment: are some of their effects mediated by similar neural mechanisms? *Pharmacology Biochemistry and Behavior* 73:233–245.
- Galbraith S, Daniel JA, Vissel B (2010) A study of clustered data and approaches to its analysis. *Journal of Neuroscience* 30:10601–10608. [PubMed: 20702692]
- Geisler S, Zahm DS (2005) Afferents of the ventral tegmental area in the rat-anatomical substratum for integrative functions. *The Journal of comparative neurology* 490:270–294. [PubMed: 16082674]
- Guerri C, Bazinet A, Riley EP (2009) Foetal Alcohol Spectrum Disorders and alterations in brain and behaviour. *Alcohol and alcoholism* 44:108–114. [PubMed: 19147799]
- Guizzetti M, Zhang X, Goeke C, Gavin DP (2014) Glia and neurodevelopment: focus on fetal alcohol spectrum disorders. *Front Pediatr* 2:123. [PubMed: 25426477]

- Hamilton GF, Jablonski SA, Schiffino FL, St Cyr SA, Stanton ME, Klintsova AY (2014) Exercise and environment as an intervention for neonatal alcohol effects on hippocampal adult neurogenesis and learning. *Neuroscience* 265:274–290. [PubMed: 24513389]
- Hannigan JH, Berman RF (2000) Amelioration of fetal alcohol-related neurodevelopmental disorders in rats: exploring pharmacological and environmental treatments. *Neurotoxicology and teratology* 22:103–111. [PubMed: 10642119]
- Hannigan JH, Berman RF, Zajac CS (1993) Environmental enrichment and the behavioral effects of prenatal exposure to alcohol in rats. *Neurotoxicology and teratology* 15:261–266. [PubMed: 8413080]
- Harry GJ (2013) Microglia during development and aging. *Pharmacology & therapeutics* 139:313–326. [PubMed: 23644076]
- Hausknecht K, Haj-Dahmane S, Shen YL, Vezina P, Dlugos C, Shen RY (2015) Excitatory Synaptic Function and Plasticity is Persistently Altered in Ventral Tegmental Area Dopamine Neurons after Prenatal Ethanol Exposure. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*.
- Hill RM, Hegemier S, Tennyson LM (1989) The fetal alcohol syndrome: a multihandicapped child. *Neurotoxicology* 10:585–595. [PubMed: 2696901]
- Hinwood M, Tynan RJ, Charnley JL, Beynon SB, Day TA, Walker FR (2013) Chronic stress induced remodeling of the prefrontal cortex: structural re-organization of microglia and the inhibitory effect of minocycline. *Cerebral cortex* 23:1784–1797. [PubMed: 22710611]
- Hoshiko M, Arnoux I, Avignone E, Yamamoto N, Audinat E (2012) Deficiency of the microglial receptor CX3CR1 impairs postnatal functional development of thalamocortical synapses in the barrel cortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 32:15106–15111.
- van der Harst JE, Bears AM, Spruijt BM. (2003) Standard housed rats are more sensitive to reward than enriched housed rats as reflected by their anticipatory behaviour. *Behavioural brain research* 142:151–156. [PubMed: 12798276]
- Jurgens HA, Johnson RW (2012) Environmental enrichment attenuates hippocampal neuroinflammation and improves cognitive function during influenza infection. *Brain, behavior, and immunity* 26:1006–1016.
- Kane CJ, Phelan KD, Han L, Smith RR, Xie J, Douglas JC, Drew PD (2011) Protection of neurons and microglia against ethanol in a mouse model of fetal alcohol spectrum disorders by peroxisome proliferator-activated receptor-gamma agonists. *Brain, behavior, and immunity* 25 Suppl 1:S137–145.
- Karperien A, Ahammer H, Jelinek HF (2013) Quantitating the subtleties of microglial morphology with fractal analysis. *Frontiers in cellular neuroscience* 7:3. [PubMed: 23386810]
- Kentner AC, Khoury A, Lima Queiroz E, MacRae M (2016) Environmental enrichment rescues the effects of early life inflammation on markers of synaptic transmission and plasticity. *Brain, behavior, and immunity* 57:151–160.
- Kettenmann H, Kirchhoff F, Verkhratsky A (2013) Microglia: new roles for the synaptic stripper. *Neuron* 77:10–18. [PubMed: 23312512]
- Komada M, Hara N, Kawachi S, Kawachi K, Kagawa N, Nagao T, Ikeda Y (2017) Mechanisms underlying neuro-inflammation and neurodevelopmental toxicity in the mouse neocortex following prenatal exposure to ethanol. *Sci Rep* 7:4934. [PubMed: 28694481]
- Lull ME, Block ML (2010) Microglial activation and chronic neurodegeneration. *Neurotherapeutics* 7:354–365. [PubMed: 20880500]
- Malanga CJ, Kosofsky BE (2003) Does drug abuse beget drug abuse? Behavioral analysis of addiction liability in animal models of prenatal drug exposure. *Brain Res Dev Brain Res* 147:47–57. [PubMed: 14741750]
- McClain JA, Morris SA, Deeny MA, Marshall SA, Hayes DM, Kiser ZM, Nixon K (2011) Adolescent binge alcohol exposure induces long-lasting partial activation of microglia. *Brain, behavior, and immunity* 25 Suppl 1:S120–128.
- Morrison HW, Filosa JA (2013) A quantitative spatiotemporal analysis of microglia morphology during ischemic stroke and reperfusion. *Journal of neuroinflammation* 10:4. [PubMed: 23311642]

- Nash K, Stevens S, Greenbaum R, Weiner J, Koren G, Rovet J (2015) Improving executive functioning in children with fetal alcohol spectrum disorders. *Child Neuropsychol* 21:191–209. [PubMed: 25010354]
- Otto G (2018) Synaptic nibbling. *Nature reviews. Neuroscience* 19:322.
- Paley B, O'Connor MJ (2009) Intervention for individuals with fetal alcohol spectrum disorders: treatment approaches and case management. *Dev Disabil Res Rev* 15:258–267. [PubMed: 19731383]
- Paolicelli RC, Bolas G, Pagani F, Maggi L, Scianni M, Panzanelli P, Giustetto M, Ferreira TA, Guiducci E, Dumas L, Ragozzino D, Gross CT (2011) Synaptic pruning by microglia is necessary for normal brain development. *Science* 333:1456–1458. [PubMed: 21778362]
- Pham TM, Söderström S, Winblad B, Mohammed AH (1999) Effects of environmental enrichment on cognitive function and hippocampal NGF in the non-handled rats. *Behavioural brain research* 103:63–70. [PubMed: 10475165]
- Pryce CR, Bettschen D, Feldon J (2001) Comparison of the effects of early handling and early deprivation on maternal care in the rat. *Developmental psychobiology* 38:239–251. [PubMed: 11319730]
- Ransohoff RM (2016) A polarizing question: do M1 and M2 microglia exist? *Nature neuroscience* 19:987–991. [PubMed: 27459405]
- Razali NM, Wah YB (2011) Power comparisons of shapiro-wilk, kolmogorov-smirnov, lilliefors and anderson-darling tests. *Journal of statistical modeling and analytics* 2:21–33.
- Riazi K, Galic MA, Kentner AC, Reid AY, Sharkey KA, Pittman QJ (2015) Microglia-dependent alteration of glutamatergic synaptic transmission and plasticity in the hippocampus during peripheral inflammation. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 35:4942–4952.
- Rutherford LC, Nelson SB, Turrigiano GG (1998) BDNF has opposite effects on the quantal amplitude of pyramidal neuron and interneuron excitatory synapses. *Neuron* 21:521–530. [PubMed: 9768839]
- Ryan SM, Nolan YM (2016) Neuroinflammation negatively affects adult hippocampal neurogenesis and cognition: can exercise compensate? *Neuroscience and biobehavioral reviews* 61:121–131. [PubMed: 26695382]
- Schafer DP, Lehrman EK, Stevens B (2013) The “quad-partite” synapse: microglia-synapse interactions in the developing and mature CNS. *Glia* 61:24–36. [PubMed: 22829357]
- Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9:676–682. [PubMed: 22743772]
- Shen RY, Hannigan JH, Kapatos G (1999) Prenatal ethanol reduces the activity of adult midbrain dopamine neurons. *Alcoholism: Clinical & Experimental Research* 23:1801–1807.
- Shetty AK, Burrows RC, Phillips DE (1993) Alterations in neuronal development in the substantia nigra pars compacta following in utero ethanol exposure: immunohistochemical and Golgi studies. *Neuroscience* 52:311–322. [PubMed: 8095703]
- Stence N, Waite M, Dailey ME (2001) Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. *Glia* 33:256–266. [PubMed: 11241743]
- Streissguth AP, Bookstein FL, Barr HM, Sampson PD, O'Malley K, Young JK (2004) Risk factors for adverse life outcomes in fetal alcohol syndrome and fetal alcohol effects. *J Dev Behav Pediatr* 25:228–238. [PubMed: 15308923]
- Topper LA, Baculis BC, Valenzuela CF (2015) Exposure of neonatal rats to alcohol has differential effects on neuroinflammation and neuronal survival in the cerebellum and hippocampus. *Journal of neuroinflammation* 12:160. [PubMed: 26337952]
- Tynan RJ, Naicker S, Hinwood M, Nalivaiko E, Buller KM, Pow DV, Day TA, Walker FR (2010) Chronic stress alters the density and morphology of microglia in a subset of stress-responsive brain regions. *Brain, behavior, and immunity* 24:1058–1068.
- Valenzuela CF, Morton RA, Diaz MR, Topper L (2012) Does moderate drinking harm the fetal brain? Insights from animal models. *Trends in neurosciences* 35:284–292. [PubMed: 22402065]

- Author Manuscript
- Author Manuscript
- Author Manuscript
- Author Manuscript
- Van Praag H, Kempermann G, Gage FH (2000) Neural consequences of environmental enrichment. *Nature Reviews Neuroscience* 1:191. [PubMed: 11257907]
- Walker FR, Nilsson M, Jones K (2013) Acute and chronic stress-induced disturbances of microglial plasticity, phenotype and function. *Curr Drug Targets* 14:1262–1276. [PubMed: 24020974]
- Wang J, Haj-Dahmane S, Shen RY (2006) Effects of prenatal ethanol exposure on the excitability of ventral tegmental area dopamine neurons in vitro. *The Journal of pharmacology and experimental therapeutics* 319:857–863. [PubMed: 16905687]
- Wang R, Hausknecht KA, Haj-Dahmane S, Shen R- Y, Richards JB (2018a) Decreased environmental complexity during development impairs habituation of reinforcer effectiveness of sensory stimuli. *Behavioural brain research* 337:53–60. [PubMed: 28943426]
- Wang R, Hausknecht KA, Shen YL, Haj-Dahmane S, Vezina P, Shen RY (2018b) Environmental enrichment reverses increased addiction risk caused by prenatal ethanol exposure. *Drug Alcohol Depend* 191:343–347. [PubMed: 30176547]
- Weinhard L, di Bartolomei G, Bolasco G, Machado P, Schieber NL, Neniskyte U, Exiga M, Vadasiute A, Raggioli A, Schertel A, Schwab Y, Gross CT (2018) Microglia remodel synapses by presynaptic trogocytosis and spine head filopodia induction. *Nature communications* 9:1228.
- Williamson LL, Chao A, Bilbo SD (2012) Environmental enrichment alters glial antigen expression and neuroimmune function in the adult rat hippocampus. *Brain, behavior, and immunity* 26:500–510.
- Xu H, Gelyana E, Rajsombath M, Yang T, Li S, Selkoe D (2016) Environmental Enrichment Potently Prevents Microglia-Mediated Neuroinflammation by Human Amyloid beta-Protein Oligomers. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 36:9041–9056.
- Zhao YN, Wang F, Fan YX, Ping GF, Yang JY, Wu CF (2013) Activated microglia are implicated in cognitive deficits, neuronal death, and successful recovery following intermittent ethanol exposure. *Behavioural brain research* 236:270–282. [PubMed: 22985845]

Figure 1. Quantifying ventral tegmental area (VTA) cells after prenatal ethanol exposure (PE). Representative sections were obtained from 8–12-weeks-old male rats after PE and stained for tyrosine hydroxylase (TH; dopamine/DA neuron marker) and ionized calcium binding adaptor molecule 1 (IBA-1; microglia marker) in the anterior VTA (A) and middle VTA (B). The region of interest (ROI) for counting DA neurons and microglia is shown in (C) from the anterior VTA (area of the ROI: 78002.665 μm^2). All the images in this figure are derived from a 2D projection of 6 Z-stack sections (0.54 μm between sections). The projection images were used to quantify the density of DA neurons and microglia with Fiji. This method was also used to measure DA cell body size in the VTA.

Figure 2. Prenatal ethanol exposure (PE) and postnatal environmental intervention effects on dopamine (DA) neuron density in the ventral tegmental area (VTA).

The results show that PE and postnatal environmental intervention did not exert any effects on DA neuron density in the anterior or middle VTA. Neuron density is measured by number of neurons within the region of interest. Data are presented as Mean $\pm$ SEM.

Figure 3. Prenatal ethanol exposure (PE) and postnatal environmental intervention effects on ventral tegmental area (VTA) dopamine (DA) neuron body size.

The results showed that PE led to a significant decrease in DA cell body size in standardly housed animals in both the anterior and middle VTA. This effect was not reversed by postnatal environmental intervention.. Data are presented as Mean $\pm$ SEM. *: $p < 0.05$, control vs. PE in the same rearing condition; ##: $p < 0.01$, standard vs. enriched controls

Figure 4. Prenatal ethanol exposure (PE) and postnatal environmental intervention effects on ventral tegmental area (VTA) microglia density and body size.

(A) Microglia density. There were no differences in microglia density between control and PE rats in either the anterior or middle VTA. Postnatal environmental intervention did not exert any effects on microglia density in either the anterior or the middle VTA. Microglia density is measured by number of microglia within the region of interest. (B) Microglia body size. There were no differences in microglia body size between control and PE rats in either the anterior or the middle VTA. Postnatal environmental intervention did not have any effects on microglia body size. Data are presented as Mean $\pm$ SEM.

Figure 5. Prenatal ethanol exposure (PE) and postnatal environmental intervention effects on ventral tegmental area (VTA) microglia branching patterns.

(A) Representative 2-D projection images from 3-D Z-stack images of VTA microglia. The quantification of microglia branching patterns was achieved using the 3-D skeleton module in Fiji. (B), (C), & (D) Microglial branch number, junction number, and average branch length, respectively. In standardly housed animals, PE decreased the number of branches and number of junctions while the branch length was not altered in both the anterior and middle VTA. Postnatal environmental intervention reversed the PE-induced reductions in numbers of microglial branches and junctions observed in standardly housed rats. In addition, in

control animals, small but significant decreases in numbers of branches and junctions in the anterior and middle VTA were observed in rats with postnatal environmental intervention, relative to their standardly housed counterparts. Data are presented as Mean $\pm$ SEM. *: $p < 0.05$; ***: $p < 0.001$, control vs. PE rats in the same rearing condition. #: $p < 0.05$; ##: $p < 0.01$; ###: $p < 0.001$, standardly housed vs. rats undergoing environmental intervention with the same prenatal treatment.

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