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Enhanced Cerebellar Myelination with Concomitant Iron Elevation and Ultrastructural Irregularities following Prenatal Exposure to Ambient Particulate Matter in the Mouse

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Abstract

Accumulating evidence indicates the developing central nervous system (CNS) is a target of air pollution toxicity. Epidemiological reports increasingly demonstrate that exposure to the particulate matter (PM) fraction of air pollution during neurodevelopment is associated with an increased risk of neurodevelopmental disorders (NDDs) such as autism spectrum disorder (ASD). These observations are supported by animal studies demonstrating prenatal exposure to concentrated ambient PM induces neuropathologies characteristic of ASD, including ventriculomegaly and aberrant corpus callosum (CC) myelination. Given the role of the CC and cerebellum in ASD etiology, this study tested whether prenatal exposure to concentrated ambient particles (CAPs) produced pathological features in offspring CC and cerebella consistent with ASD. Analysis of cerebellar myelin density revealed male-specific hypermyelination in CAPs-exposed offspring at postnatal days (PNDs) 11-15 without alteration of cerebellar area. Atomic absorption spectroscopy (AAS) revealed elevated iron (Fe) in the cerebellum of CAPs-exposed female offspring at PNDs 11-15, which connects with previously observed elevated Fe in the female CC. The presence of Fe inclusions, along with aluminum (Al) and silicon (Si) inclusions, were confirmed at nanoscale resolution in the CC along with ultrastructural myelin sheath damage. Further, RNAseq and gene ontology (GO) enrichment analyses revealed cerebellar gene expression was significantly affected by sex and prenatal CAPs exposure with significant enrichment in inflammation and transmembrane transport processes that could underlie observed myelin and metal pathologies. Overall, this study highlights the ability of PM exposure to disrupt

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myelinogenesis and elucidates novel molecular targets of PM-induced developmental neurotoxicity.

Keywords

Autism; air pollution; particulate matter (PM); UFP; hypermyelination; cerebellum; corpus callosum; iron

Introduction

Accumulating epidemiological evidence indicates that the developing brain is a target of air pollution toxicity. Exposure to air pollution during critical neurodevelopmental periods is associated with increased risk of autism spectrum disorder (ASD), a heterogeneous disorder that classically presents with repetitive behaviors and social impairments (Blatt 2012). The hypothesized underlying mechanisms driving ASD pathophysiology are numerous, with heritable genetic polymorphisms being a prominent contributing factor (Ginsberg et al. 2012). However, genetics cannot explain the entirety of ASD cases, nor the recent increase in ASD incidence, leading researchers to examine the specific contribution of environmental factors in the etiology of ASD and related disorders. Among these contributing environmental factors are endocrine disrupting chemicals (EDCs; e.g. phthalates, bisphenol-A), inorganic metals, prenatal infection (i.e. maternal immune activation), and various components of air pollution (reviewed in Kalkbrenner et al. 2014).

Air pollution is a complex, heterogeneous mixture consisting primarily of particulate matter (PM), combustion-derived polycyclic aromatic hydrocarbons (PAHs), reactive gases (CO_2 , CO , NO_x , SO_x , O_3), byproducts of industrial manufacturing (e.g. polychlorinated biphenyls; PCBs) and metals (Lonneman et al. 1974; Dergham et al. 2015; Settimo and Viviano 2015). PM is classified by size, which ranges from larger, coarse PM ($\geq 10 \mu\text{m}$ in diameter; PM_{10}), fine PM ($\leq 2.5 \mu\text{m}$ in diameter; $\text{PM}_{2.5}$), and ultrafine particles ($\leq 100 \text{ nm}$ in diameter; UFPs). Levels of PM_{10} and $\text{PM}_{2.5}$ are regulated by the United States Environmental Protection Agency (EPA) as criteria pollutants in the National Ambient Air Quality Standards (NAAQS). Indeed, air quality has generally improved in the United States since the implementation of PM_{10} and $\text{PM}_{2.5}$ regulations (Hopke et al. 2018). However, UFPs are not subject to specific federal regulation and thus remain a concern, as these nanoscale particles comprise a significant proportion of PM and are associated with significant morbidity and mortality (Chen et al. 2016). UFPs are putatively the most toxic component of PM given their large surface area to mass ratio, allowing the adsorption of additional toxicants such as metals and combustion-derived organic and inorganic compounds (Oberdorster 2000). As such, UFPs present a mechanism by which exposure to multiple toxicants can occur simultaneously and potentially to a greater extent than PM_{10} or $\text{PM}_{2.5}$.

In recent years, several epidemiological studies have investigated whether PM exposure during pregnancy and early life are associated with ASD (Costa et al. 2015; Kalkbrenner et al. 2015). Close proximity to major highways, used as a proxy for $\text{PM}_{2.5}$ exposure, was found to be a risk factor for ASD in Los Angeles (Becerra et al. 2013). Similarly, exposure to $\text{PM}_{2.5}$ during both prenatal and postnatal periods was associated with increased risk of

ASD in Pennsylvania (Talbot et al. 2015). Ambient metals present in air pollution, including lead (Pb), manganese (Mn), and mercury (Hg), were associated with increased ASD risk (Roberts et al. 2013; Yassa 2014). Children with ASD were reported to have elevated levels of several metal species in hair (Skalny, Simashkova, Klyushnik, et al. 2017), blood (Vergani et al. 2011), and serum (Skalny, Simashkova, Skalnaya, et al. 2017), including iron (Fe), aluminum (Al), silicon (Si), zinc (Zn), manganese (Mn), copper (Cu), cadmium (Cd), and arsenic (As). Despite the significant body of epidemiological evidence associating PM and metal components of air pollution with adverse neurodevelopmental outcome, there is a paucity of *in vivo* toxicology studies providing biological plausibility and mechanistic consensus as to how air pollution contributes to the etiology of NDDs.

The cerebellum appears to be particularly involved ASD etiology (Becker and Stoodley 2013; Fatemi 2013). The cerebellum is heavily involved in control of motor activity and coordination, as well as higher-order cognitive functions (Koziol et al. 2014). In the context of ASD, motor impairments, including repetitive movement, are a cardinal ASD feature (Fournier et al. 2010). Imaging studies have reported cerebellar hypoplasia and white matter (WM) overgrowth, i.e. excess myelin or hypermyelination, in individuals with ASD (Ben Bashat et al. 2007; Wolff et al. 2012; Abdel Razek et al. 2014). In fact, cerebellar WM overgrowth has been directly associated with increased incidence of repetitive behaviors and social impairments (Catani et al. 2008; Cheung et al. 2009).

In addition to the cerebellum, the CC appears to play a role in ASD etiology, as WM alterations have also been documented in individuals with ASD (Hardan et al. 2009; Travers et al. 2015; Wolff et al. 2015). As the brain's major WM tract, the CC facilitates interhemispheric signaling. Disruption of CC myelin formation contributes to interhemispheric disconnectivity, a phenotype posited to underlie ASD and has been associated with poor neurological outcome. WM alterations are also observed in other neurological disorders, including major depression and schizophrenia (Fields 2008). Like ASD, these disorders share an epidemiological association with air pollution exposure (Pedersen et al. 2004; Kelly et al. 2010; Lim et al. 2012; Szyszkowicz et al. 2016). In considering the relationship of metals and WM, dysregulation of metal homeostasis can either increase or decrease myelination depending on the metal species. Elemental iron (Fe) is critical for oligodendrocyte maturation, myelination, and WM tract development (Connor and Menzies 1996; Todorich et al. 2009; Badaracco et al. 2010). This is underscored in models of Fe deficiency, in which myelin loss or underdevelopment is commonly observed (Lozoff and Georgieff 2006; Amin et al. 2013). We have previously shown that gestational CAPs exposure produced elevated ferric iron (Fe^{3+}) inclusions in the CC with concurrent hypermyelination in offspring of both sexes. Disruption of other metal species within the brain has been shown to negatively affect developmental myelination or induce demyelination. This is perhaps best evidenced by the experimental use of cuprizone, a commonly-used demyelinating agent that functions as a copper (Cu) chelator. Fe and Cu are present in the white matter of the cerebellum (Popescu et al. 2009), and perturbation of these metals could lead to alterations of WM tract development and downstream impairment of cerebral-cerebellar circuitry.

Given the links between early-life exposure to PM/metals to cerebellar disruption of WM development and the significant functional and neurobehavioral consequences of such toxicity, it is imperative to understand how early-life exposure to air pollutants adversely affect cerebellar development. Given this significant role of the cerebellum in the etiology of ASD and other NDDs, this study aimed to determine if exposure to concentrated ambient particles (CAPs) during gestation results in altered WM and metal homeostasis in the offspring cerebellum. Additionally, this study sought to elucidate if prenatal CAPs exposure resulted in changes in cerebellar gene expression could explain the observed histological phenotypes and provide molecular targets for future mechanistic studies.

Materials and methods

Animals and exposures

This study is an extension of prior work and utilized brain tissue from previously performed air pollution exposures (Blum et al. 2017; Klocke et al. 2017). Briefly, 8-10 week-old male and female B6C3F1 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and paired as described previously (Blum et al. 2017). Females were removed from the pairing upon discovery of a vaginal plug, designated gestational day 0.5 (GD0.5). Pregnant dams (N=12) were then placed into compartmentalized exposure chambers of the Versatile Aerosol Concentration Enrichment System (VACES; New York University Department of Environmental Medicine, Sterling Forest, NY; Maciejczyk et al. 2005). Using the VACES, dams (N=16) were exposed via whole-body inhalation to CAPs or HEPA-filtered air (Air; control) for 6 hrs daily from GD0.5-GD16.5 during the hours of 0800-1400. Dams had *ad libitum* access to food and water except during exposures and were maintained in ambient air upon conclusion of exposures (GD16.5) through birth and the postnatal period. Offspring sex was determined by visual examination.

Mass concentration and elemental composition of CAPs exposures was determined as described previously (Blum et al. 2017; Klocke et al. 2017). X-ray fluorescence spectroscopy (XRF) was used to determine the elemental composition of particles captured on Teflon filters within exposure chambers using an ARL QUANT'X EDXRF Analyzer (ThermoFisher Scientific, Waltham, Massachusetts). As mentioned, the CAPs exposures utilized in this study are an extension of previous studies by this group focusing on the neurodevelopmental effects of prenatal CAPs (Klocke et al. 2017; Klocke et al. 2018). Thus, the concentration, particle size distribution, and composition metrics are identical across the studies and the endpoints examined in each study are comparable. The particle size distribution of CAPs produced by the VACES includes PM_{2.5} and UFP (Maciejczyk et al. 2005). CAPs exposure metrics are summarized in the Results. N=Mice used in this study were treated humanely with regard to alleviation of suffering and all study protocols were approved by the New York University Institutional Animal Care and Use Committee. For all analyses, the litter was the unit of statistical measure.

Myelin immunohistochemistry

Immunohistochemical detection of myelin basic protein (MBP) was performed as described previously (Klocke et al. 2017). Briefly, whole brains (N=6-11/sex/exposure group; total

N=33) were extracted at PNDs 11-15 following rapid decapitation and fixed in 4% paraformaldehyde (PFA) for 24 hrs and post-fixed in 30% sucrose. Post-fixed brains were sectioned sagittally at 40 μ m in a 6-series on a freezing-sliding microtome into cryoprotectant and stored at -20° C until staining. Free-floating sections were washed to remove cryoprotectant and quench endogenous peroxidases, then blocked in 10% normal goat serum (NGS) followed by overnight incubation in a solution containing primary antibody against MBP (1:1000; Millipore, Billerica, MA) and 1% NGS. A single well of tissue incubated overnight in 1% NGS without primary antibody served as a negative control. Tissue was incubated with biotinylated secondary antibody and Vectastain ABC solution (Vector Labs, Burlingame, CA) the following day. Chromogenic antibody detection was then performed using metal-enhanced 3,3'-diaminobenzidinetetrahydrochloride (DAB; Sigma Aldrich, St. Louis, MO). Tissue sections were mounted onto Superfrost Plus slides (VWR, Radnor, PA) and coverslipped with Cytoseal 60 (Thermo Fisher Scientific, Waltham, MA).

Histological detection of ferric iron

Cerebellar ferric iron (Fe^{3+}) was visualized histologically using Perl's Prussian Blue Stain as described previously (Klocke et al. 2017). Briefly, tissue sections from gestationally-exposed offspring brains (N=6-11/sex/exposure group; total N=33) were mounted onto pig gelatin-subbed slides and hydrated to 100% ethanol prior to immersion in 5% potassium ferrocyanide [$\text{K}_4\text{Fe}(\text{CN})_6$; J.T. Baker, Phillipsburg, NJ]. Tissue was then immersed in a solution of 5% potassium ferrocyanide and 20% hydrochloric acid, washed, and counterstained with neutral red to visualize nuclei and neuroanatomical structures. Tissue was differentiated in acetate-buffered ethanol (70% and 95%), dehydrated, and cleared using Histoclear (National Diagnostics, Atlanta, GA). Slides were allowed to dry prior to coverslipping with Permount (Thermo Fisher Scientific).

Imaging and histological analyses

Slide-mounted tissue sections were visualized on an Olympus BX41 microscope (Olympus America, Inc., Central Valley, PA) fitted with an MBF CX9000 camera (MBF, Villiston, VT). All histological analyses were performed by a blinded experimenter.

For analysis of MBP density, montage micrographs were captured at 20X magnification using the virtual tissue tool within Neurolucida (MBF). The density of MBP staining with respect to cerebellar area was determined using optical thresholding in ImageJ (Klocke et al. 2017). Visible Fe inclusions were quantified within the cerebellum at 40X magnification using Neurolucida software. For both analyses, a neuroanatomical atlas was utilized to ensure homologous tissue sections were analyzed between animals. Three sequential tissue sections were analyzed per animal for evaluation of Perl's staining and 5 sequential sections were evaluated for MBP density (approximate adult-equivalent bregma range: 0.12 to 1.68 mm; Franklin and Paxinos 2007).

Whole-cerebellum analysis of iron content

At the time of euthanasia, a subset of animals (N=3-6 offspring per sex per exposure; total N=18) had the cerebellum dissected using a dedicated set of instruments and tissue was

snap-frozen in plastic microcentrifuge tubes on dry ice. There was no difference in cerebellar wet weight between exposure groups (data not shown). Atomic absorption spectroscopy (AAS) was performed to quantify total Fe content in whole cerebellum as previously described (Elder et al. 2006). Briefly, frozen cerebella were thawed and then wet ashed using ultrapure 70% nitric acid (HNO₃; Baseline, SeaStar Chemicals Inc., Sidney, British Columbia, Canada). Tissue residue was resuspended/diluted in 2% HNO₃ followed by graphite furnace AAS analysis. Cerebellar Fe concentrations were determined by comparison to a reference standard (Standard Reference Material 1577c from bovine liver; National Institute of Standards and Technology, Gaithersburg, MD).

Characterization of ultrastructural myelin and inorganic inclusions within the corpus callosum

Offspring mice were euthanized by rapid decapitation at PND12 (N=1/sex/exposure group; total N=4). Brains were extracted and placed in filtered 4% PFA for initial tissue fixation. The CC region was carefully dissected from the right hemisphere and post-fixed in 2.5% glutaraldehyde in 0.1 M Millonig's sodium phosphate buffer overnight at 4°C and rinsed in the same buffer prior to second post-fixation in combined 1.5% iron monoisocyanide (FeCN) and 1% osmium tetroxide (OsO₄) in Millonig's buffer for 2 hrs in the dark. Slices were rinsed 3 times in distilled water, dehydrated in a graded series of ethanol to 100% ethanol, transitioned into 50% ethanol/50% propylene oxide (PO), 100% PO, 100% PO/EPON-Araldite epoxy resin, and finally 100% EPON-Araldite epoxy overnight. The following day, slices were embedded in fresh epoxy and placed into molds for polymerization overnight at 60°C. Tissue was thin-sectioned at 70 nm onto nickel formvar/carbon coated slot grids (Ted Pella Inc., Redding, CA) using a diamond knife and Boeckler PT-XL ultramicrotome (Boeckeler Instruments, Inc., Tuscon, AZ). The grids were stained using aqueous uranyl acetate and lead citrate prior to imaging of the ultrastructures using a Hitachi 7650 transmission electron microscope (Hitachi, Tokyo, Japan) with an attached Gatan Erlangshen 11-megapixel digital camera and Gatan Digital-Micrograph software (Gatan, Inc., Pleasanton, CA).

High-resolution scanning/transmission electron microscopy (S/TEM) coupled with spectroscopic elemental mapping was performed using a JEOL 2100F field emission S/TEM operated at 200 kV with an analytic pole piece. High-resolution images were recorded with a Gatan Ultrascan 4k × 4k CCD camera and data analysis and processing used Gatan Digital Micrograph software (Gatan, Inc.). S/TEM imaging and energy dispersive spectroscopic analysis (EDS) were performed with a GATAN high angle annular dark field detector (HAADF), Digiscan II, Gatan 2000 Image Filter (GIF), and an Oxford Aztec EDS system (Oxford Instruments, Oxfordshire, United Kingdom), respectively. AztecTEM software (Oxford Instruments) was used to acquire and process EDS data including drift correction. All S/TEM images were acquired using an analytical probe with 0.17 nm. An FEI Talos F200X was used for fast EDS mapping with a high degree of sensitivity due to the wrap-around style EDS detector mounted in the objective lens. Maps generally took 1-2 mins to acquire with sufficient sensitivity to detect elemental concentrations in < 5 nm size particles without causing destruction of the tissue due to prolonged electron-beam interaction. Tissue for these analyses was analyzed by a blinded experimenter.

mRNA isolation, sequencing, and processing

PNDs 11-13 were selected for RNAseq analysis to capture transient gene expression changes due to prenatal CAPs exposure during the onset of cerebellar myelination (Foran and Peterson 1992). A subset of offspring brains extracted at PNDs 11-13 had the cerebellum dissected (N=3/sex/exposure group; total N=12). Cerebellar tissue was snap-frozen on dry ice and stored at -80°C until processing. RNA was isolated from the cerebellum (average mass = 80.3 ± 8.5 mg) using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA) per manufacturer's instructions. Briefly, tissue was mechanically disrupted to lyse and homogenize cells. Chloroform was added and incubated at room temperature ($22-25^{\circ}\text{C}$) prior to centrifugation to fully separate phases. The aqueous phase was retained and mixed with 70% ethanol. The cell lysate/chloroform mixture was added to a spin column and centrifuged at room temperature to bind total RNA to the column. The column was washed with manufacturer-supplied buffer 3 times with centrifugation between each wash followed by elution in RNase-free H_2O .

Total RNA concentration was verified with the NanopDrop1000 spectrophotometer (NanoDrop, Wilmington, DE) and quality assessed with the Agilent Bioanalyzer (Agilent, Santa Clara, CA). The TruSeq Stranded mRNA Sample Preparation Kit (Illumina, San Diego, CA) was used for next-generation sequencing library construction per manufacturer's protocols. Briefly, mRNA was purified from 200ng total RNA with oligo-dT magnetic beads and fragmented. First-strand cDNA synthesis was performed with random hexamer priming followed by second-strand cDNA synthesis using dUTP incorporation for strand marking. End repair and 3' adenylation was then performed on the double stranded cDNA. Illumina adaptors were ligated to both ends of the cDNA, purified by gel electrophoresis, and amplified with PCR primers specific to the adaptor sequences to generate cDNA amplicons of approximately 200-500 bp in size. The amplified libraries were hybridized to the Illumina single end flow cell and amplified using the cBot (Illumina, San Diego, CA). Single end reads of 100 nt were generated for each sample. Raw reads generated from the Illumina HiSeq2500 sequencer are de-multiplexed using associated software (configurebcl2fastq.pl version 1.8.4). Quality filtering and adapter removal were performed using Trimmomatic (version 0.32).

Differential gene expression and gene ontology enrichment analysis

Differential gene expression analysis was performed within the R computing environment (version 3.3.3) via R-Studio (version 1.0.136; R Development Core Team, 2005). DESeq2 was used to perform data normalization and differential expression analysis with an adjusted p -value threshold of 0.05. Due to differing read counts per sample, count data was filtered to remove counts of 0 or 1 and underwent variance-stabilizing normalization. Count data were then log-transformed prior to principle component analysis (PCA) and hierarchical clustering analysis. Data were then normalized in a manner similar to previously mentioned variance-stabilization. This normalization method models the variance to minimize sample values for lowly expressed genes with high variance.

PCA and hierarchical clustering indicated that there was an effect of sex driving differences in the count data, thus sex was included as a factor in subsequent modeling. Hierarchical

clustering revealed that expression levels in PND13 animals were significantly different from PNDs 11-12, and therefore PND13 animals were excluded from subsequent analyses (N=1 excluded animal per sex from the CAPs exposure group; in total N=2 exclusions). To determine the effects of gestational CAPs exposure while controlling for sex, the data were first normalized and counts were log-transformed as a pre-processing step. R/Bioconductor package DESeq2 (Love et al. 2014) was then used to calculate the log fold-change in gene expression resulting from gestational CAPs exposure and perform the differential gene expression analysis. The statistical model was designed to test the effect of CAPs exposure compared to Air controls in a manner similar to ANOVA, controlling for sex and false discovery rate (FDR), with an adjusted p -value threshold of 0.05. (See Supplementary Data).

Pathway and ontology enrichment analyses were performed using R/Bioconductor (Huber et al. 2015). The GAGE package (Generally Applicable Gene-set Enrichment for Pathway Analysis; Luo et al. 2009) was used to perform pathway analysis on DESeq2 results that had been previously controlled for sex. Briefly, the pathways were mapped according to level of enrichment (i.e., $\log_2$ FoldChange via DESeq2 analysis) to the Kyoto Encyclopedia of Genes and Genomes mapper (KEGG mapper; Kanehisa and Goto 2000; Kanehisa et al. 2016; Kanehisa et al. 2017). Subsequently, gene ontology (GO) analysis was performed by mapping the same data to GO terms. For all pathway and GO enrichment analyses, an adjusted p -value threshold 0.05 was considered statistically significant.

Statistical analysis for non-sequencing endpoints

Statistical analyses for MBP, histological Fe, and AAS were performed using JMP Pro 12.2. Linear MBP density (reported by sequential tissue section; Figure 1a) was analyzed using a repeated measures ANOVA with exposure group, sex, and PND as fully factorial, between-group factors and tissue section as a continuous, within-group factor. Averaged MBP density, histological Fe, and Fe measured via AAS were analyzed separately by sex via one-factor ANOVA with exposure group as a between-group factor. ANOVA results are presented with F statistics accompanied by degrees of freedom followed by the p -value. P -values 0.05 were considered statistically significant.

Results

CAPs exposure metrics

As reported in greater detail in Klocke et al. (2017) and Blum et al. (2017), the concentration of CAPs experienced by the pregnant dams averaged $92.69 \mu\text{g}/\text{m}^3$ (daily range: 32.95 - $184.431 \mu\text{g}/\text{m}^3$) over the course of gestation and these levels did not induce overt reproductive toxicity to the dams or offspring. Air control dams experienced an average particle concentration of $3.52 \pm 0.87 \mu\text{g}/\text{m}^3$.

XRF analysis revealed that CAPs-exposed dams experienced elevated atmospheric concentrations of the following elemental species: sodium (Na; 57.5-fold increase vs. Air), magnesium (Mg; 23.4-fold increase), sulfur (S; 675.0-fold increase), potassium (K; 125.2-fold increase), calcium (Ca; 25.0-fold increase), Mn (101.8-fold increase), Fe (376.3-fold

increase), nickel (Ni; 307.5-fold increase), Cu (40.8-fold increase), zinc (Zn; 31.5-fold increase), and strontium (Sr; 59.0-fold increase; Klocke et al. 2017).

Cerebellar myelin density

MBP density was analyzed in the cerebellum at PNDs 11-15. There was a statistically significant effect of CAPs resulting in increased cerebellar MBP density in males ($F_{3,11} = 6.02$, $p = 0.011$), but not females, when analyzed across the cerebellum (Figure 1a-b). There was a significant effect of PND in both males ($F_{1,11} = 7.74$, $p = 0.018$) and females ($F_{1,14} = 5.81$, $p = 0.03$) that was driven by lower MBP density among PND11 offspring. There was an interaction effect between exposure and PND in CAPs-exposed females ($F_{4,11} = 7.95$, $p = 0.0029$). When MBP density is collapsed and averaged across sections, the significant elevation observed in CAPs-exposed males remained ($F_{1,13} = 3.31$, $p = 0.026$; Figure 1c). There was no significant effect of prenatal CAPs exposure on average MBP density in the female offspring cerebellum.

Cerebellar Fe content

Ferric (Fe^{3+}) Fe inclusions were visualized by Perl's Prussian blue staining and quantified in the cerebellum of offspring exposed prenatally to CAPs or Air. There was a statistically significant elevation in cerebellar Fe inclusions due to prenatal CAPs exposure across three sequential tissue sections in females ($F_{1,17} = 10.12$, $p = 0.0058$) and males ($F_{1,14} = 12.31$, $p = 0.0039$; Figure 2a). Sex and PND did not have a significant effect on this endpoint and no interaction effects were observed. When normalized to cerebellar area, the number of Fe inclusions per mm^2 remained significantly elevated in CAPs-exposed females ($F_{1,17} = 10.91$, $p = 0.0045$) and males ($F_{1,14} = 11.91$, $p = 0.0043$; Figure 2a).

The concentration and total amount (ng) of all Fe were determined by AAS. Fe levels were significantly elevated in CAPs-exposed females ([Fe] concentration in $\mu\text{g/g}$: $F_{1,7} = 11.95$, $p = 0.014$; total ng Fe: $F_{1,7} = 20.14$, $p = 0.0042$; Figure 2b). In males, neither total ng Fe nor [Fe] concentration were significantly affected by prenatal CAPs exposure.

Qualitative characterization of ultrastructural myelin and inorganic inclusions in the CC at high-resolution

Tissue from the CC midbody of Air and CAPs-exposed offspring was prepared for exploratory TEM and S/TEM and assessed for changes in myelin sheath thickness and the presence of nanoscale inorganic inclusions. Prenatal CAPs exposure increased myelin sheath thickness in both male and female offspring when visualized by TEM (Figure S1). The effect of prenatal CAPs on axon caliber (i.e. diameter) was sex-specific, with CAPs-exposed males having increased axon caliber than Air males, and CAPs-exposed females having lower axon caliber than Air females (Figure S1b). When myelin sheath thickness was normalized to axon caliber, the resulting g -ratio of prenatally-exposed CAPs offspring was lower than Air control offspring, indicative of enhanced and/or accelerated myelination (Figure S1b). Axon caliber was positively correlated myelin g -ratio and myelin sheath thickness in all exposure groups, although prenatal CAPs exposure affected this relationship (Figure S1c-d, e-f). Prenatal CAPs exposure also appeared to induce focal myelin sheath damage in the CC, characterized by redundant loops and whorl-like pathology (Figure S2a-

b). A greater number of axons were myelinated in CAPs-exposed offspring compared to Air controls (Figure S2c), and CAPs-exposed female offspring had an increase in the number of CC axons compared to a decrease in axons in CAPs-exposed male offspring (Figure S2d).

At high resolution, OsO₄ nanoparticles (~1-2 nm) from TEM preparation were observed as 5-7 nm agglomerates (Figure 3a). In some fields, multiple agglomerates approached 20 nm in diameter on tissue surfaces. Os nanoparticles (Figure 3a) appeared as ultrafine, electron-dense spots in the S/TEM images. The unique dispersion of the OsO₄ nanoparticles inside the tissue provided excellent contrast during S/TEM ultrastructural examination. The OsO₄ nanoparticles are significantly (~1 order of magnitude) smaller than non-Os inorganic inclusions and were observed to preferentially adhere to regions of myelin damage (Figure 3a, arrows).

S/TEM allowed in-depth assessment of myelin ultrastructure and confirmed the myelin damage and/or dysregulated compaction observed in TEM (Figure 3b-c, Figure S2). Myelin sheath damage consisted of single or multiple interconnected crater openings of varying size and circumference (Figure 3c). This pathology was more common in CAPs-exposed offspring than Air offspring (Figure S2). Unexpectedly, S/TEM analysis revealed the presence of inorganic, electron-dense inclusions in the CC midbody in both CAPs-exposed offspring and Air offspring (Figures 3b). Using nanoscale resolution (1 nm beam size), S/TEM combined with EDS mapping revealed the inclusions were composed of Fe, Si, and Al in the oxide form (Figure 3d-e). The size of identified inclusions was variable, with some larger agglomerates measuring just under 200 nm in diameter, and others much smaller, around 10-20 nm. The largest observed agglomerates were composites of several crystallites rather than large, single crystals. The shapes of identified agglomerates were variable, with some agglomerates appearing spherical and others appearing more irregular (Figure 4). Evidence of cellular uptake is shown in Figure 4c-d (CAPs female) via the enhanced brightness of subcellular compartments in this region. EDS analysis of this specific area revealed the subcellular compartment to be Fe-rich, suggesting elevated Fe uptake.

Differential gene expression in the cerebellum

The effect of prenatal CAPs exposure on the cerebellar transcriptome was assessed utilizing RNAseq. As described in the Methods, PND13 animals were excluded from gene expression analysis because the level of mRNA expression in these animals was significantly different from PNDs 11-12 (Figure S3a). Hierarchical clustering and variance stabilization performed on PND11 and 12 data indicated that there were no significant differences in overall gene expression level and variance (Figure S3a-b). PCA analysis was performed to confirm this observation (Figure S3c). Prenatal CAPs exposure significantly affected total cerebellar mRNA expression (Figure S3e). Comparison of normalized and log-transformed counts revealed significantly differentially expressed genes as a result of prenatal CAPs exposure (Figure S3d and Figure 5).

The effect of prenatal CAPs exposure on log-normalized counts of significantly differentially expressed genes, controlling for sex, are shown in Figure 5a with corresponding log-normalized fold change compared to Air-exposed controls indicated in Figure 5b. The significance level (adjusted *p*-value) of these genes are represented

graphically in Figure 5c. In order of statistical significance, the transcripts that were affected were: predicted gene 5148 (*Gm5148*; upregulated); SRY (sex determining region Y)-box 2 overlapping transcript (*Sox2ot*; upregulated), proprotein convertase subtilisin/kexin type 9 (*Pcsk9*; upregulated), exosome component 9 (*Exosc9*; upregulated), F-actin-capping protein subunit alpha-1 (*Capza1*; upregulated), zinc finger homeobox 2, opposite strand (*Zfx2os*; downregulated), growth arrest-specific 5 (*Gas5*; downregulated), potassium large conductance calcium-activated channel, subfamily M, beta member 2 (*Kcnmb2*; downregulated), solute carrier family 39 (*Slc39a2*; downregulated), polyadenylate-binding protein 2 (*Pabpn1*; downregulated), processed pseudogene (*RP23-111M12.3*; upregulated), RIKEN cDNA 1500004A13 gene (*1500004A13Rik*; upregulated), olfactomedin-like 2B (*Olfml2b*; upregulated), ribosomal protein L21 (*Rpl21*; upregulated), and Fc receptor-like S, scavenger receptor (*Fcrls*; upregulated). These genes along with any known neurological or neuropathological associations are reported in Supplementary Table S1.

Gene ontology enrichment analysis

Differential mRNA expression data was used to inform gene ontology enrichment analysis. Ontology enrichment analysis was performed in R using GAGE to map differential expression results to the KEGG GO database. Significantly up- and downregulated GO processes ($p\text{-adj.} \leq 0.05$) are reported in **Supplementary Table S2**. Notable enriched processes (in order of statistical significance) include drug transmembrane transport (GO:0006855), inflammatory response (GO:0006954), regulation of BMP signaling pathway (GO:0030510), G-protein coupled receptor signaling pathway, coupled to cyclic nucleotide second messenger (GO:0007187), purine ribonucleotide biosynthetic process (GO:0009152), and autonomic nervous system development (GO:0048483). Non-enriched gene ontologies of note include protein ubiquitination (GO:0016567), protein modification by small protein conjugation or removal (GO:0070647), protein modification by small protein conjugation (GO:0032446), and small GTPase mediated signal transduction (GO:0007264).

Discussion

This study provides *in vivo* evidence that prenatal exposure to CAPs targets the developing offspring cerebellum in a sex-specific manner and provides mechanistic support for potential processes and molecular targets that may underlie CAPs-induced neurodevelopmental pathologies.

Prenatal CAPs exposure induced male-specific hypermyelination with concomitant elevation of Fe^{3+} inclusions. Elevated Fe was also present in CAPs-exposed female cerebella, although unlike CAPs-exposed males, female cerebella had elevation of total Fe (vs. Fe^{3+} alone), indicative of an overall perturbation of Fe homeostasis. Fe is a critical component of myelination (Connor and Menzies 1996; Todorich et al. 2009) and it is possible that the elevation of cerebellar Fe may contribute to hypermyelination in CAPs-exposed male offspring. Regarding the sex-specific effect of prenatal CAPs on cerebellar Fe, regulation of Fe uptake and storage is known to be sex-specific in human infants (Tamura et al. 1999; Domellof et al. 2002) and rodents (Kong et al. 2014; Duck et al. 2018). Fe uptake and storage appear to be more robust in females compared to males, which is directly opposite of

the phenotype we previously observed in the CC of control offspring, in which filtered Air control females had lower CC Fe than Air males (Klocke et al. 2017). Sex differences may underlie CAPs-induced Fe alterations on a brain region-specific basis, evidenced by the sex-specificity in the enhanced response to CAPs exposure in female offspring. The lack of sex-specificity of cerebellar Fe in Air controls suggests that altered cerebellar Fe is a direct result of the animals attempting to handle and/or ameliorate the effects of CAPs exposure and indicates that this process is under the control of sex-specific regulatory mechanisms. Indeed, male CAPs-exposed offspring experienced an elevation in Fe³⁺ inclusions, but not an elevation in total Fe relative to cerebellar mass (i.e., cerebellar Fe concentration). This sex-specific elevation of Fe in CAPs-exposed male offspring may be restricted to the ferric (Fe³⁺) species alone. The lack of elevation of cerebellar [Fe] concentration in CAPs-exposed males may simply be due the presence of Fe in other valence states, indicative of sex-specific dysregulation of Fe species between CAPs-exposed male and female offspring. Future studies should investigate the sex-specificity of PM and metals on sex hormone pathways, as disruption of these pathways could affect neurodevelopmental processes dependent on sex hormones, including the expansion/activation of microglia and astrocytes (Schwarz and Bilbo 2012; Lenz and McCarthy 2015), as well as oligodendrocyte proliferation and differentiation (Marin-Husstege et al. 2004; Cerghet et al. 2006).

Disruption of myelinogenesis during development has been repeatedly identified in our previous work, both in prenatal and postnatal CAPs exposures. However, these effects have been shown to be timing-, sex-, and region-specific. For prenatal exposures, tissue collection occurred across a period of significant postnatal neurodevelopment (PNDs 11-15 for histological analyses and PNDs 11-12 for sequencing). This period encompasses peak cerebellar myelination (Smith 1973; Verity and Campagnoni 1988). This effect of sex differs from a previous study in which the same gestational CAPs exposures produced hypermyelination of the corpus callosum (CC) in both female and male offspring, suggesting the sex-specificity of effects are brain region-specific (Klocke et al. 2017). This is in line with the weight of evidence on sex-specific neurodevelopment in rodents, in which mechanisms are highly region-specific (McCarthy et al. 2017). It is possible that excess cerebellar WM reflects accelerated or premature myelination rather than a persistent, pathological excess of myelin. Postnatal brain overgrowth is a feature observed in children with ASD (Courchesne 2004; Ben Bashat et al. 2007; Hazlett et al. 2011). In contrast, the current study showed no effect of prenatal CAPs exposure on cerebellar area, similar to a previous *in vivo* study where prenatal CAPs had no effect on cortical thickness (Klocke et al. 2017). These findings differ from the epidemiological literature in that early WM overgrowth of the CC was persistent through PNDs 57-60 (Klocke et al. 2018), whereas this phenotype is typically transient in ASD (Courchesne 2004).

Exposure to CAPs during the postnatal period showed a differential susceptibility but continued targeting of myelination. Mice exposed to CAPs on PNDs 4-7 and 10-13 (human 3rd trimester equivalent) produced a male-specific loss of CC myelin (Allen et al. 2017). While that study did not examine cerebellar myelin, it highlights the ability of PM to induce region-specific WM alterations and underscores the importance of examining the effect of pollutant exposures during various critical neurodevelopmental periods. In the context of the current study, postnatal cerebellar development is sexually dimorphic (Tiemeier et al. 2010)

and it is possible that differential vulnerability of the developing brain to CAPs exposure contributes to the observed male-specific phenotypes. Regional differences in the directionality and sexual specificity of gestational CAPs exposure on myelin pathology could be attributed to the timing, composition, and/or concentration of CAPs exposures.

Myelin sheath thickness was increased in CAPs-exposed offspring with a corresponding reduction in myelin sheath integrity. This phenotype is particularly interesting given that, while WM overgrowth may be characteristic of NDDs, it is possible that the integrity of the myelin is compromised due to aberrant formation or repair processes. Disorganized and damaged myelin can activate microglia, which can perpetuate myelin damage via the release of soluble inflammatory mediators (Fan et al. 2005). The presence of inorganic inclusions could also enhance microglial activation and further contribute to myelin damage. This is especially true in the case of Fe, as a complex functional relationship exists between myelinating oligodendrocytes, microglia, and Fe (Zhang et al. 2006). Microglia are immune scavengers of the CNS and the presence of extracellular Fe could lead to activation and ROS release as they sequester excess Fe (Wang Y et al. 2011), a process which could damage myelin sheaths. Further, the presence of extracellular Fe itself could be detrimental to myelin sheaths as Fe readily undergoes the Fenton reaction, which results in the production of membrane-damaging free radicals (Mehlhase et al. 2006). GO analysis revealed a significant CAPs-induced enrichment in inflammatory processes that are supportive of microglial activation in response to Fe deposition and myelin damage. Microglial activation was previously observed in the CC of CAPs-exposed offspring that positively correlated with the number of Fe inclusions. While the current study did not assess cerebellar microglial activation, given the presence of hypermyelination, Fe inclusions, and inflammatory process enrichment it is possible that this region is also experiencing aberrant microglial activation in CAPs-exposed animals. Additionally, it is possible that astrocytes are contributing to the elevation of inflammatory processes given they also regulate Fe and provide metabolic support for myelinating oligodendrocytes (Clemente et al. 2013). Future studies should assess the role, if any, of astrogliosis within the scope of PM- and metal-induced developmental neurotoxicity.

At the ultrastructural level, agglomerates of Fe, Al, and Si oxides were observed in the midbody CC of CAPs- and Air-exposed offspring. As the Air-exposed group experienced a very low level of PM exposure, it is imperative to consider the possibility that even minimal levels of PM can dysregulate CNS metal uptake and homeostasis. While S/TEM analyses were not quantitative, the elevated levels of Fe inclusions as determined by Perl's staining suggest that CAPs-exposed offspring may have increased metal deposition compared to Air controls. Further, the observation of myelin sheath damage in high-resolution TEM imaging raises the question of the causal mechanism of such damage and whether micro- and nanoscale metal inclusions play a role or if they are indirectly related. The preferential targeting of Os nanoparticles to areas of unsaturated hydrocarbons is a documented phenomenon and it is hypothesized that OsO₄ nanoparticles adhere to charged regions (Komissarchik and Korolev 1981). Uncompacted myelin sheaths observed in TEM analyses could be the result of incomplete myelination, as PND12 is a period of ongoing myelination in both the CC and the cerebellum, or it could be indicative of damage. S/TEM analysis of cross-sectional CC myelin revealed holes/pitting, within the myelin sheaths which is

supportive of CAPs-induced myelin damage. Preliminary quantitation demonstrated that this phenotype was significantly more prevalent in CAPs-exposed offspring than Air controls. Given the uncompacted, damaged nature of the myelin sheaths in combination with targeting of Os nanoparticles to these regions suggests prenatal CAPs exposure induces an accelerated yet aberrant myelination program. The metal inclusions, especially the Al and Fe inclusions, may also be inducing free radicals production that may contribute to this pathology via lipid peroxidation of nascent myelin sheaths (Verstraeten et al. 1997). One additional possibility is that thicker myelin in CAPs-exposed animals is due to ongoing repair processes. As such, the uneven brightness of nanoscale inclusions, especially near particle edges, indicates increased porosity of the particle and suggests partial dissolution and ongoing clearance mechanisms are occurring (Brown et al. 2014; Graham et al. 2014; Graham et al. 2017).

The key role myelination plays in the etiology of AP-associated NDDs in humans impelled us to investigate the genome-wide transcriptional profile in the cerebellum. The transcriptional profile at PNDs 11-12 provided a snapshot which may elucidate yet unknown pathways critical for understanding the mechanisms of brain myelination. Potential alterations in gene expression mechanisms driving CAPs-induced hypermyelination were elucidated using RNAseq. However, RNAseq and GO enrichment analyses were not immediately indicative of gene expression changes that would directly contribute to hypermyelination and elevated Fe in the cerebellum. More specifically, increased MBP density and Fe would suggest upregulation of genes that directly contribute to these phenotypes, such as upregulation of the myelin basic protein gene (*Mbp*), transferrin (*Tf*), or ferritin (*Fth1*), for example. None of these genes nor any other genes related to myelin or metal ion transport/storage were significantly differentially expressed, with the exception of *Slc39a2*.

Slc39a2 encodes zinc (Zn) transporter ZIP2 and is hypothesized to transport Fe (Peters et al. 2007). Deng et al. (2009) observed increased cellular Fe uptake and oxidative stress following exposure to Zn *in vitro*. However, in this respiratory model, exposure to Zn resulted in upregulation of divalent metal transporter-1 (DMT1) but no change in ZIP2 expression (Deng et al. 2009). It is possible that interactions between Zn and Fe, including activation of *Slc39a2*, along with other metal ion transporters, contributed to the elevation of cerebellar Fe. In considering CAPs exposure composition, there were a number of metal species detected on filters in the exposure chambers in addition to Fe and Zn, including sodium (Na), magnesium (Mg), potassium (K), calcium (Ca), manganese (Mn), nickel (Ni), copper (Cu), and strontium (Sr; Klocke et al. 2017). CAPs-exposed dams in the current study experienced an atmospheric concentration of 1430 ng/m³ Fe (376-fold increase vs. Air controls) and 34.6 ng/m³ Zn (a 31-fold increase vs. Air controls; Klocke et al. 2017). While the current study does not investigate the effects on the maternal respiratory system, the upregulation of *Slc39a2* in response to these levels of Fe and Zn suggests that maternal inhalation of metals can perturb the complex regulatory relationship of Fe homeostasis in the fetal brain. Both Fe and Zn are essential trace elements utilized by all organs and tissues, therefore this finding has significant implications in the context of development given that disruption of metal homeostasis, regardless of directionality, negatively affects neurodevelopmental processes and is associated with increased risk of cognitive deficits and NDDs (Caito and Aschner 2015). While the current study only investigated the presence of

Fe in the cerebellum, the pregnant dams in the CAPs chambers were exposed to a mixture of metals and other pollution components (e.g. carbon particles and adsorbed PAHs, gases, etc.), thus the potential for mixture-specific effects cannot be excluded. The upregulation of *Slc39a2* in CAPs-exposed offspring may be indicative of Zn alterations with secondary effects on Fe. Metal dysregulation has been posited as a contributing factor in the etiology of NDDs, including ASD (Vergani et al. 2011; Arora et al. 2017), as well as neurodegenerative disease (Zatta et al. 2003; Levesque et al. 2011; Li et al. 2017).

Transcriptional analysis of whole cerebellum uncovered a variety of other target genes differentially expressed due to prenatal CAPs exposure that are also implicated in the etiology of neurodevelopmental or neurodegenerative diseases, including *Sox2ot*, *Exosc9*, *Kcnmb2*, *Papbn1*, *Olfml2b*, and *Rpl21* (see Supplementary Table S1). Of note, RNA processing appears to be a target of prenatal CAPs exposure, as suggested by the differential expression of *Exosc9*, part of the exosome complex responsible for RNA degradation, and *Rpl21*, a component of the 60S ribosomal subunit. Alterations in RNA degradation or translation could change protein levels in the cell, a potentially critical effect in a developmental context depending on the specific proteins affected. The current study was not designed to test if RNA processing is affected by prenatal CAPs exposure, but this endpoint should be a consideration of future research. In fact, small variations in RNA stability have been proposed to act synergistically with environmental exposure as contributing factors in ASD etiology (Poliakov et al. 2014).

The significant upregulation of *Sox2ot* may be indicative of alterations in neural stem cell (NSC) pluripotency. *Sox2ot* is a long noncoding RNA (lncRNA) in which the *Sox2* gene is present as an intron (Amaral et al. 2009). *Sox2* is a critical factor involved in vertebrate embryonic development and functions to maintain stem cell pluripotency. Information regarding the function and spatiotemporal expression patterns of *Sox2ot* is limited, but it appears to act as an enhancer for *Sox2* and follows similar expression patterns, i.e. enriched in embryonic stem cells and downregulated upon the onset of differentiation. Amaral et al. (2009) showed that *Sox2ot* is highly expressed in neural tissues during prenatal development. This group and others have also hypothesized a role for *Sox2ot* in adult neurogenesis as there is low-level *Sox2ot* expression in neurogenic regions of the adult brain in mice and humans (Mercer et al. 2008; Arisi et al. 2011).

Interestingly, *Sox2ot* expression appears to be regulated by the androgen receptor (AR) and contains three androgen response elements (AREs) upstream of the *Sox2* locus (Tosetti et al. 2017). This study also showed that selective AR inhibition attenuated *Sox2ot* expression in embryonic mouse brain at GD12.5 and in cultured neurospheres. Given that AR is involved in embryonic and adult neurogenesis, its regulatory role in the context of *Sox2ot/Sox2* expression may underlie sex differences observed in cerebellar myelination. Further, we previously observed that prenatal CAPs exposure persistently enhances oligodendrocyte precursor cell (OPC) proliferation in the female CC (Klocke et al. 2018). Therefore, it is possible that prenatal CAPs exposure is shifting the trajectory of NSC expansion via *Sox2ot/Sox2* in a manner that is both sex-specific and persistent. Other NSC transcription factors are likely involved and differentially regulated due to prenatal CAPs and should be examined in

future studies. However, these data present a novel mechanism by which developmental air pollution toxicity may occur as a potential starting point for future studies.

A limitation of the current study is that the experimental design did not allow sufficient animal numbers for validation of differential gene expression via PCR or Western blot. RNAseq and GO analyses were already limited by small sample size, therefore variance in gene count was stabilized and tested, and subsequent differential expression and GO analyses were carefully controlled for sex and PND. Despite these efforts, the small sample size remains a limiting factor in the context of interpretation and scope. There are a variety of factors that could contribute to the lack of additional significant changes in gene expression directly related to myelin and metal homeostasis, including the fact that RNAseq analysis was performed on whole cerebellum without isolation of specific cell types (i.e. neurons, oligodendrocytes, microglia, astrocytes, etc.). Additionally, future studies should investigate transcriptional profiles separately by sex, as myelination, metal homeostasis and NDDs associated with air pollution all show a significant sex-bias. Future prenatal studies should also prioritize neurobehavioral outcomes and assess transcriptional profiles into adulthood to help translate this research to NDDs.

This study did not examine functional neurobehavioral consequences of gestational CAPs exposure. Based on the histological phenotypes in this study and in previous work (Klocke et al. 2017; Klocke et al. 2018), the authors hypothesize that elevation in cerebellar and CC myelin may be reflected in altered motor and coordination behaviors. Both the cerebellum and CC are involved in motor coordination (Geffen et al. 1994; Groiss and Ugawa 2013), although more recent studies implicate these regions in non-motor, i.e., cognitive, functions that may be disrupted in ASD (Schulte and Muller-Oehring 2010; Fatemi et al. 2012; Becker and Stoodley 2013; Koziol et al. 2014; Wang SS et al. 2014). Previous studies investigating postnatal CAPs exposure (human 3rd trimester equivalent) observed male-specific behavioral changes, including increased impulsivity and altered short-term memory (Allen et al. 2013; Allen et al. 2014). Because of the vastly different developmental processes occurring in the postnatal period compared to the gestational period in rodents, the behavioral phenotypes resulting from postnatal CAPs exposure likely do not extend to prenatal CAPs exposure. Rather, it is likely that gestational exposure produces an altogether different behavioral phenotype, especially considering prenatal vs. postnatal CAPs had the opposite effect on myelin (hyper- vs. hypomyelination, respectively). While specific critical windows remain elusive, epidemiological studies have identified associations between pollutant exposure during each trimester of pregnancy and NDD risk (Volk et al. 2013; Kalkbrenner et al. 2015; Talbott et al. 2015). Another important consideration is the spatiotemporal heterogeneity of particulate matter composition (van Donkelaar et al. 2010) and the duration of exposure, both of which could underlie differences in WM density and other phenotypes observed in animal models compared to human imaging studies.

In conclusion, this study provides *in vivo* support of a growing literature that associates gestational air pollution exposure to adverse neurodevelopmental outcomes. Due to the role of the cerebellum in ASD etiology, effects of prenatal CAPs exposure on cerebellar myelin, Fe status, and gene expression were assessed. This study highlights the sex- and region-specificity of hypermyelination as well as dysregulation of cerebellar Fe, both of which are

phenotypes reminiscent of those observed in ASD. Potential molecular mechanisms were identified using differential gene expression and GO analyses to elucidate potential mechanisms and molecular targets for future study. Overall, this study underscores a need to revisit PM and UFP regulation and recommendations for pregnant women with regard to limiting air pollution exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Gestational CAPs exposure elevates cerebellar MBP density in male offspring at PNDs 11-15.

Tissue was stained for MBP to assess myelin density relative to cerebellar area. **A)** Representative micrographs of cerebellar MBP immunostaining at 4X magnification. Scale bar represents 500 μ m. **B)** In males, analysis of cerebellar MBP density revealed a significant main effect of CAPs exposure. There was no effect of gestational CAPs exposure on cerebellar MBP density in females. Data represent mean $\pm$ SEM of 5 serial tissue sections (N=6-11/sex/exposure) analyzed by repeated measures ANOVA with exposure (CAPs or Air), sex, and PND as fully factorial, between-group factors and tissue section as a

continuous, within-group factor. **C)** Representation of approximate locations tissue sections analyzed with respect to midline bregma. **D)** MBP staining density averaged across tissue sections revealed a significant increase in CAPs-exposed males. Statistical outcome: * p 0.05.

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Figure 2: Gestational exposure to CAPs increases cerebellar iron content in both females and males at PNDs 11-15.

Tissue was stained with Perl's Prussian blue for Fe and counterstained with neutral red. All Fe inclusions visible at 10X magnification were quantified across 3 sequential tissue sections. **A)** Representative image of staining in a CAPs-exposed male at 20X magnification. Arrows indicate locations of Fe³⁺ inclusions (i.e., blue staining). Scale bar represents 100 μm. **B)** Gestational CAPs exposure significantly increased the number of cerebellar Fe inclusions as well as the density relative to cerebellar area. Data represent mean ± SEM of 3 serial sections analyzed by one-way ANOVA (N=6-11/sex/exposure). **C)** Fe elevations observed in histological analyses were confirmed using AAS on flash-frozen cerebellum. Fe

was significantly increased in CAPs-exposed females. Although CAPs-exposed males experienced elevated Fe levels, the effect was not significant. The concentration of cerebellar Fe relative to cerebellar wet weight (in g) was significantly elevated in CAPs-exposed females. Data represent mean $\pm$ SEM of N=6-11/sex/exposure analyzed by one-way ANOVA. Statistical outcome: * p 0.05, ** p 0.01.

Figure 3: Ultra high-resolution analysis of CC myelin revealed focal myelin sheath damage and elemental agglomerate formation following prenatal CAPs exposure.

Tissue was prepared for S/TEM and assessed for ultrastructural alterations and particle deposition. **A)** Representative micrograph of Os nanoparticles used in EM tissue preparation and staining. Groups of Os nanoparticles (arrows) form 5-20 nm clusters and are present on all micrographs as a result of the tissue staining and appear to preferentially target areas of myelin sheath damage. Scale bar represents 20 nm. **B)** Evidence of focal myelin sheath damage and particle deposition in an Air-exposed female. Air-exposed animals were exposed to a very low level of CAPs ($3.52 \pm 0.87 \mu\text{g}/\text{m}^3$) as a result of incomplete particle filtration by the VACES system. Arrows indicate areas of myelin sheath damage and circles indicate regions of particle deposition, identified by bright appearance relative to surrounding tissue. Scale bar represents 1 µm. **C)** Examples of focal myelin sheath damage in CAPs-exposed male and female samples, indicated by arrows. OsO₄ nanoparticles from EM tissue preparation are consistently seen preferentially adhering to sites of myelin damage. **D)** Select CC regions are illustrated in S/TEM with nanoparticle uptake. Corresponding EDS mapping over select regions with nanoparticles: Fe (red), Si (green), and Al (blue) over select regions confirm elemental compositions of inorganic inclusions. All observed nanoparticles are oxides (oxygen maps not shown). Scale bars represent 0.02 µm to 0.05 µm as denoted. **E)** Representative EDS spectra corresponding to micrographs in

D were obtained using a Talos F200X. Elemental analysis and identification of dominant peaks in the spectra are included. The Cu peaks are from the copper mesh grid and are not native to the sample; the Os peak is due to the OsO₄ staining in all spectra.

Figure 4: Non-Os inorganic inclusions observed in the CC.

Inclusions are observed in the midbody CC in both the CAPs-exposed group and Air controls, indicating that low levels of PM exposure can lead to inclusion formation. Inorganic inclusions have a bright appearance compared to surrounding tissue due to high electron densities. Organic molecules appear dark due to relatively low electron density. Boxes indicate area of detail on bottom panel. **A-B)** Representative micrograph of an Fe-containing particle inclusion in an Air female. The inclusion presented with an irregular shape and blurry halo around the inclusion, suggesting partial dissolution and/or clearance of the particle. The stippled appearance of the inclusion in is due to damage from the electron beam during analysis. Scale bars represent 0.2 μm in **A, C, E, and G**, and 50 nm in **B, D, F, and H**. **C-D)** Evidence of cellular Fe uptake in a CAPs-exposed female. It is unclear whether this Fe originated in PM exposures or another intracellular source. Scale bars represent 1 μm and 0.2 μm . **E-F)** A particle inclusion is present in an Air-treated male. The particle is irregularly shaped and shows evidence of partial dissolution based on the blurred particle edges. Scale bars represent 1 μm and 100 nm. **G-H)** An irregularly shaped particle observed in a CAPs-treated male. There is evidence of partial dissolution, indicative of ongoing clearance. Scale bars represent 1 μm and 0.1 μm .

Figure 5: Gestational CAPs exposure significantly alters gene expression in males and females at PNDs 11-12.

Frozen cerebellum tissue from PNDs 11-12 was processed for RNAseq. PND did not have a significant effect on gene expression and therefore both PND11 and PND12 are included in analyses. **A)** DESeq2 analysis revealed a significant effect of gestational CAPs exposure on the expression log-transformed counts of the indicated genes in the cerebellum when sex and exposure are included as factors. **B)** Log-transformed fold change of each significantly differentially expressed gene of gestationally exposed CAPs offspring vs. Air-exposed offspring. **C)** Adjusted p -value of each gene with significant differential expression (p -adj. 0.05). Data represent $N=3$ /sex/exposure. Statistical outcome: p -value of log-normalized data adjusted for sex, PND, and FDR = 0.05.

Table 1:

Enriched and non-enriched gene ontologies as a result of prenatal CAPs exposure.

GO Term	Enriched processes	Set size	Stat mean	<i>p</i> -adj.
GO:0051707	Response to other organism	357	1.870	0.031
GO:0043900	Regulation of multi-organism process	132	1.743	0.041
GO:0009607	Response to biotic stimulus	383	1.736	0.042
GO:0007188	Adenylate cyclase-modulating G-protein coupled receptor signaling pathway	86	1.740	0.042
GO:0006855	Drug transmembrane transport	12	1.821	0.042
GO:0060218	Hematopoietic stem cell differentiation	10	1.826	0.045
GO:0006954	Inflammatory response	329	1.691	0.046
GO:0030510	Regulation of BMP signaling pathway	61	1.701	0.046
GO:0060456	Positive regulation of digestive system process	10	1.780	0.047
GO:0007187	G-protein coupled receptor signaling pathway, coupled to cyclic nucleotide second messenger	96	1.684	0.047
GO:0046632	Alpha-beta T cell differentiation	60	1.662	0.050
GO:0009152	Purine ribonucleotide biosynthetic process	164	1.651	0.050
GO:0048483	Autonomic nervous system development	36	1.671	0.050
GO:0051240	Positive regulation of multicellular organismal process	486	1.645	0.050
GO Term	Non-enriched processes	Set size	Stat mean	<i>p</i> -adj.
GO:0016567	Protein ubiquitination	357	-1.935	0.027
GO:0001541	Ovarian follicle development	47	-1.961	0.027
GO:0070647	Protein modification by small protein conjugation or removal	451	-1.867	0.031
GO:0060048	Cardiac muscle contraction	51	-1.821	0.036
GO:0032446	Protein modification by small protein conjugation	387	-1.788	0.037
GO:0060113	Inner ear receptor cell differentiation	46	-1.717	0.045
GO:0007264	Small GTPase mediated signal transduction	410	-1.652	0.050

Stat mean: magnitude of gene set enrichment in CAPs-exposed offspring compared to Air controls; set size: number of genes in the ontology enrichment set test; *p*-adj.: *p*-value of log-normalized differential expression data adjusted for exposure, sex, and FDR.