

In Vivo Investigation of Perinatal PFAS Exposure through Drinking Water on Birth
Outcomes and Neurodevelopment in Offspring

by

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Thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science in the Duke Global Health Institute
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ABSTRACT

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Abstract

Pittsboro, North Carolina, has been burdened by exceptionally high concentration of per- and polyfluoroalkyl substances (PFAS) contamination in drinking water. Our previous study reported that perinatal exposure to a PFAS mixture mimicking the PFAS levels in Pittsboro, NC drinking water led to gestational hypertension and lower fetal weights in rabbits. However, there has been significant gaps in literature uncovering the toxicity and biological processes and physiological mechanisms affected by this PFAS mixture, which hindered researchers and policy makers articulate the harm of PFAS chemicals to communities like Pittsboro, NC. This study, therefore, aims to supply evidence that helps establish a causal relationship between *in utero* exposure to this PFAS mixture and birth outcomes, focusing on the neurodevelopment of offspring in rats. Various neurobehavioral tests, molecular, and pathological examinations have been accomplished to uncover physiological alterations associated with maternal PFAS mixture exposure and underlying mechanisms. Our research discovered that maternal PFAS exposure led to a significant delay in pup locomotor development in a sex-specific manner and reduced brain volume in postnatal day 1 (PND 1) pups. Transcriptome analysis identified 564 differentially expressed genes (DEGs) in cerebellum samples collected from the low-dose PFAS group. The DEGs are enriched in mitochondrial and metabolic pathways or pathogenetic ontologies suggest abnormal neurodevelopment including locomotor signals.

Dedication

This manuscript is dedicated to the locals of Pittsboro, North Carolina, and those who are, and has been affected by PFAS chemical exposure.

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1. Introduction

Perfluoroalkyl and polyfluoroalkyl substances (PFAS) are a group of organic chemical compounds widely cultivated by the industry. The presence of a fluorinated carbon chain, a signature characteristic of PFAS chemical, contributes to both the thermal and chemical stability of PFAS¹. This chemical property has been exploited to manufacture various products, such as pesticides, non-stick cookware, fire fighter foams, degradation-resistant silverware, and electronic products¹⁻². However, the stability of PFAS has also created significant environmental and health concerns.

PFAS are easily accessible through air, sediment, food, drinking water, and aquatic sources³. Researchers at the Stapleton Lab at Duke discovered that the citizens of Pittsboro, North Carolina, face a significant threat from PFAS exposure, particularly from drinking water⁴. Pittsboro is a small city with a median household income of \$44,399 and poverty rate of 7.34%⁵. In addition to financial burdens, this city has significantly suffered from the environmental burden of PFAS contamination. Exceptionally high PFAS levels were discovered from industrial effluent releases dating back to 1980. The measure far exceeded the level of 70 ng/L for PFOS and PFOA, two main types of PFAS compounds, as advised by US Environmental Protection Agency, and exceeded average exposure levels in the United States⁶⁻⁷. In the context of North Carolina PFAS contamination, the sum level of drinking water PFAS contamination at Pittsboro, which is 1076 ng/l, is 30 times the PFAS level from Durham level⁵. The PFAS contamination from water sources at Pittsboro also affects nearby regions. Wilmington was the closer city to Pittsboro; while the contamination at the region was proclaimed to be managed,

the concentration of PFAS levels at Sargent Water Treatment Plant, which is nearby Greenfield Lake, exceeded the state standard of 20.0 ppt⁵.

Various industrial companies, including 3M, Du Pont, and Chemours, were in Pittsboro, NC and released PFAS pollutants to Haws River, and companies were not held accountable as they manufactured key military tools and gears in the factories⁸. While 3M and Du Pont has publicly announced that they would discontinue PFAS usage, the fact that Du Pont has internally recognized PFAS toxicity back in 1980S and continued to use the compound for manufacturing purpose suggested that EPA needs to add more pressure to these companies and press them be responsible for restoring water quality at Pittsboro⁸. In addition, these chemical companies frequently manufactured products using PFAS compounds retired from US at other countries. Since there is a lack of coverage regarding the manufacturing process at lower-middle income countries, the environmental burden and health burden caused by these companies is critically underestimated.

Epidemiological studies have demonstrated that PFAS exposures contributed to various physiological complications, negatively affecting exposed individuals' immune function, thyroid function, and could cause various non-communicable diseases². Furthermore, PFAS exposure may affect reproductive and developmental outcomes. During early maternal pregnancy, the fetus relies entirely on trans-placental transfer of maternal thyroid hormones; while fetus entering week 12 could have the thyroid working on its own, it would not generate sufficient thyroid hormone until week 18 to 20⁹. Yet, PFAS exposure has been known to have endocrine-disrupting properties, and possible

health impact of these properties would be the impairment of adult thyroid function that could indirectly influence fetal thyroid hormone transfer¹⁰. But cohort study results were inconsistent with the developmental neurotoxicity of PFAS compounds. In Taiwan, Wang Yan et, al aimed to capture the cross-generation effect of maternal exposure to PFAS compound, where concentration of PFAS was tested from blood sample. The main exposure were from PFOA and PFOS (1.58 and 0.22 ng/mL). The data in this study showed that PFUnDA and PFNA exposure contributed to the continual deterioration of children's intelligence and risk for ADHD¹¹. However, another study from Zeyan Liew et, al at Demark did not find abnormal neurodevelopment or an increase in the frequency of ADHD in children that correlated with a maternal PFAS exposure level¹². In addition, it was clear to academia that epidemiological studies would not be sufficient to demonstrate the physiological mechanisms that underlie the health effects of PFAS compounds. Thus, the primary goal of this study was to fill the gap in the research on the developmental effect of maternal PFAS exposure, establish a causal relationship between *in utero* PFAS exposure and birth outcomes, and investigate the molecular mechanisms contributing to the potentially abnormal neurodevelopment of offspring in rats.

Bioaccumulations of PFAS could occur at various organs, and researchers have been investigating the effect of PFAS accumulation on organ functionality. While determining the exact mechanisms affecting brain through experimental evidence can be challenging, various studies, based on experimental studies, have hypothesized cellular mechanisms that could alter because of PFAS exposure. The Based on the biochemical properties of PFAS compounds, De Toni et al. has proposed that high-level exposure of

PFOS could interfere with cell membranes, mainly altering the cell membrane permeability or inducing membrane instability by increasing membrane fluidity¹³. It is possible for PFAS compound to affect membrane transporter protein, and cause bioaccumulation of PFAS or disrupt hormone transfer¹⁴. As of brain region specifically, bioaccumulation of PFAS could impact dopaminergic differentiation¹⁵. Human relies on neurotransmitter stimulation and hormone transfer to achieve more complex neuronal differentiation, and disrupted dopamine secretion could trigger stage-sensitive developmental problem in cognitive ability, motor development, and emotion control¹⁶. It is worth mentioning that alterations in cellular properties could further contribute to PFAS-relevant transgenerational pathogenesis, which affects both the livability and brain functionality of the offspring. Haimbaugh et al., through a zebrafish study, demonstrated that PFAS may trigger morphological abnormality of zebrafish after F2 generation of the study, and could further introduce disruptions in lipid pathway through epigenetic variations¹⁷. Such disruption of lipid pathway could alter intracellular processes and affect cell migration or proliferation, all critical tissue adhesion events detrimental to cerebellum development¹⁷. Since PFAS could influence dopaminergic differentiation and introduce specific epigenetic changes that affect cellular functions, we suspect that PFAS exposure could delay cerebellum development, and therefore impair motor function development as well as changes in locomotion.

It comes to researchers' awareness that the bioaccumulation of PFAS could be done through fetus development and through maternal transfer. We have investigated how maternal PFAS exposure could disrupt thyroid hormone homeostasis, which can

cause abnormal fetal brain development and neurobehavioral development in offspring. In a molecular context, delivery of thyroid hormone to the fetus's brain requires transfer across the blood–brain barrier, which involves thyroid hormone–binding proteins. PFAS competes for binding to TTR, the only binding protein detected in large amounts in cerebrospinal fluid, and potentially decreases thyroid hormone transfer to the fetus's brain¹⁸⁻²⁰. Thus, it is critical to understand how early exposure of PFAS could occur during fetus development stage, and what exact mechanistic alterations have occurred to brain development due to PFAS accumulation.

Our primary interest developed from the rat PFAS neurobehavioral study, which characterized the neurobehavioral alterations of rat offspring due to in-utero PFAS exposure. In the study, preliminary behavioral data from pup, adolescent, and adult stages of rat offspring showed that rat offspring from PFAS-exposed mothers could show differential motor function development and locomotion alterations. Thus, we developed a particular interest in learning about the cerebellum and locomotion development of younger rats. Cerebellum is a brain region that processes both sensorial information and motoric information under specific sequences to facilitate the generation of goal-directed limbic system behaviors. Through the investigation of cerebellum region, we further the insights in how maternal PFAS exposure affects rat motor development and day-to-day activity²¹. In addition, we studied the cerebellum, which has a relatively simple structure with a small number of homogenous cell types (most cells are granule neurons) that develop within spatially defined regions, which makes it an ideal choice for transcriptome and pathological analysis. Based on our current understanding of fetus–

placenta interactions, we hypothesized that in-utero PFAS exposure affects thyroid hormone homeostasis in both maternal (F0) and fetus (F1) compartments, which results in miscarriages and changes in the fetal central nervous system (CNS). Changes in the CNS lead to the compromised motor function of rats' offspring (F1).

2. Methods

2.1 Setting

The experimental study has taken place at Duke University Neurobehavioral Research Laboratory and Duke Triangle Biotechnology Lab (Durham, NC) in collaboration with Dr. Edward Levin.

2.2 Participants

Our lab has been using Sprague-Dawley rats for neurotoxicity studies. Rats are widely adopted for neurotoxicity and developmental biology studies because rats have much shorter gestation, growth period, and larger tissue organization that allows efficient data documentation. Compared with mice, rats are also more cognitive in adapting to various neurobehavioral tasks. Both male and female subjects underwent testing as neurodevelopment may vary by sex. For investigating rat reproductive outcome, the anatomy (hemochorial) of rat placenta is same to that of humans, and therefore the evaluation of rat gestational development is generalizable to human population. This study protocol was approved by the Institutional Animal Care and Use Committee of Duke University (A214-22-10).

2.3 Animal Subject and Exposure

Adult male and female Sprague-Dawley rats weighing between 200 and 250 grams (Charles Rivers Labs, Raleigh, NC, USA) were mated in animal facility room with a reversed day-night light schedule. Their offspring (n=12 control litters, 16 low-dose litters, and 11 high-dose litters) at different ages of growth were then used for behavioral

testing, neuropathological analysis, and transcriptome analysis. Similar set up were done for another pool of pregnant female adult rats (n=5 control litters, 6 low-dose litters, and 7 high-dose litters) where their fetus and placenta were collected during mid-gestation period. Animals received appropriate feeding, cage changes, and other welfare procedures and handlings by professionals as were approved by the Institutional Animal Care and Use Committee of Duke University. Upon arrival to facility, male and female rats were acclimated for seven days treated with deionized water. After the acclimation period, female rats will be exposed to one of three kinds of water: control (deionized water), low-dose PFAS mixture (concentration mimicking PFAS contaminated water from Pittsboro, NC), high-dose PFAS mixture (more concentrated mixture) (**Table 1**). Deionized water was chosen for control treatment to avoid the potential exposure effect of PFAS presence from distilled and tap water. High-dose PFAS mixture is designed to be five thousand-fold concentrated as the low-dose considering the higher PFAS metabolic rate in rats and being comparable to previous toxicology studies (Table S6). Male rats will be continually treated with deionized water. After seven days of exposure, female rats will be mated with a male, and the length of mating period was 5 days for rats that ultimately give birth of offspring for behavioral tests, and 3 days for rats undergoing mid-gestational sample collection. Drinking water consumption and gestational weight were documented (**Table S4**).

Table 1: PFAS Exposure Dosing [ng/L]

PFAS Compound	Low-dose Concentration	High-dose Concentration
PFBS	7.9	39500
PFHxS	9.6	48000
PFOS	15.4	77000
PFBA	96.1	480500
PFPA	158.4	792000
PFHxA	261.4	1307000
PFHpA	144.7	723500
PFOA	48.2	241000
PFNA	7.9	39500
PFDA	9.0	45000

2.4 Measures

2.4.1 Pre-Weaning Growth and Reflex Development Measurements

Following the birth of the pup rats, their birth to weaning development will be tracked by righting reflex test measures, and negative geotaxis test measures.

Righting reflex tests were administered on PND 2, 4, and 6. Each pup was placed on their back on a clean surface and were expected to return to prone positions. The time it took for the pup repositioning would be taken for latency assessments. Pups were allowed 30 s to complete the righting response, and a failure to complete the response after the 3rd attempt was recorded with maximum input time (30 s). The negative geotaxis tests were administered on PND 7, 9, 11, and 13. Each pup would be placed facing down a 45-degree angled metal layer and the pups were expected to reflexively turn to face up the slope. The time taken for the pup to make the reversal position and have face toward the top of the metal layer was recorded and assessed for locomotion development. Pups

were given 60s to complete the tasks, and failure to complete the test after the 3rd attempt would be recorded with maximum possible time (60 s). All pup rats would be weaned on PND 23.

2.4.2 Post-Weaning Figure-8 Locomotor Maze Test Measurements

After weaning, offspring litters completed various behavioral tests to evaluate motor function, affective response, and cognition. Post-weaning Figure-8 Locomotor Maze Tests were administered at adolescence (~PND 35) and adulthood (~PND 56) to help evaluate rat locomotor activity levels. Observations were made in the figure-8 maze, which is an enclosed locomotor chamber in the shape of a figure-8, with two additional short arms extending from the center. Each rat was placed in the apparatus, and their movement within the maze were tracked for an hour and measured by photobeams at eight evenly scattered locations within the maze. The number of photobeam breaks was analyzed across twelve, 5-min intervals to observe the rate of habituation and changes in activity level as the rats habituated to the maze environment.

2.4.3 Transcriptome Analysis

After rat pups were born, some were designated for transcriptome analysis. PND1 brain samples were collected to reflect the inherent genetic differences relevant to brain development due to differential maternal PFAS exposure. Cerebellum was surgically removed from PND 1 pup brains during necropsy and sub-merged in RNAlater and snap-frozen in liquid nitrogen and stored in -80-degree freezer for RNA extraction. PND 1 cerebellum were homogenized using FastPrep tissue homogenizer to extract RNA for

RNA-seq analysis in controls (one male and one female in each litter, n=6 litters) and low dose groups (one male and one female in each litter, n=6 litters). RNA-seq were performed at the duke sequencing and genomic technologies core, and data were analyzed by personnel from the Duke Genomic Analysis and Bioinformatics Shared Resource.

RNA-seq libraries were prepared using the commercially available KAPA Stranded mRNA-Seq Kit. All libraries are pooled in equimolar ratio and sequenced on one lane of a HiSeq 4000 at 50bp Single-Read. RNA-seq data was processed using the TrimGalore toolkit, which employs Cutadapt to trim low-quality bases and Illumina sequencing adapters from the 3' end of the reads. Reads were mapped to the Rnor 6.0 version of the rat genome and transcriptome using the STAR RNA-seq alignment tool. For genes having an overlap of at least 10 reads, gene counts were normalized, and differential expression was carried out using the DESeq2 Bioconductor package implemented for the R programming environment²². Gene set enrichment analysis was performed to identify gene ontology terms and pathways associated with altered gene expression for each of the comparisons performed.

2.4.4 Mid Gestational Necropsy

Mid gestational date (GD) was set as 15 days after mating, and the body weight and mid-gestational date were tracked for each pregnant rat (N=18). GD15 was chosen as it is relevant to the early pregnancy (beginning of the second trimester) in humans and the mature placenta was formed and fetuses' brain and body structure were developed enough for examinations. At the time of necropsy, fetus and placenta were collected,

along with the organs of the maternal rats. The liver, spleen, kidney of the maternal rats was immersed in formalin (4% Paraformaldehyde) for 48 hours, then stored in 1X PBS solution for long-term storage. The gravid uterus was collected, observed/recorded, and weighted, then placenta and fetus were harvested and weighted. The weight ratio of each fetus and corresponding placenta was calculated. Maternal weight gain was calculated by subtracting dam weight at GD15 and gravid uterus weight from maternal weight at mating.

2.4.5 Brain Volumetric and Stereological Evaluation

We adopted Developmental Neurotoxicity Study (DNS) guidelines for pathological evaluation of rat PND 10 brains from control, low, and high dose groups. PND 1 to PND 10 pup brains are relevant to the brains during third trimester of human pregnancy. And the rat cerebellum is fully developed at PND 10. Brains were collected after 50 ml PBS perfusion followed by 50 ml 4% paraformaldehyde perfusion through rat body. Brain samples were immersed in formalin (4% Paraformaldehyde) for 48 hours. We conducted volumetric measurement by creating 3d models of the brains using the artec micro 3d scanner (Artec 3D) partnered with a company named Restor3d. The scanner is an ultra-high-precision industrial desktop 3D scanner and has a max 3D point accuracy of 0.01 mm and a max 3D resolution of 0.029 mm and uses a Blue LED light to create a point cloud which can be analyzed and visualized to create a 3D brain mesh model. The brain was secured with a magnetic vice with rubber tips to prevent tissue damage (Artec 3D) after being placed in the middle of the viewfinder of the scanner. Scanning platform was rotated around with pitch and yaw being manipulated

simultaneously, moving to a new position for each frame. Total of 44 frames were taken per scan and brain orientation was changed after each scan. After scans were fixed by various orientations to create a full brain scan, volume determination was performed from watertight 3D mesh produced by “sharp fusion” using the measurement tool embedded within Artec Studio. To conduct pathological examination and stereological measurements of cell numbers from various brain compartments, we created 3D printed brain molds. Using these molds, we could trim PND 10 pup brains at a consistent distance at each trimming levels according to the DNS guideline. Luxol Fast Blue staining and hematoxylin staining were scheduled to be performed for neuron calculation.

2.5 Analysis

For pre-weaning righting reflex tests, negative geotaxis tests, post-weaning Figure-8 locomotor tests and mid-gestational sample comparisons, multivariate analysis of variance (ANOVA) was used to assess PFAS exposure and the effect of such exposure on experimental variables. Two-tailed post hoc Dunnett’s test were conducted for any tests where outcomes were significant.

Gene ontology analysis were conducted to process RNA-seq data. We first standardized the expressions of transcripts and compared the p-value adjusted by expression quantity and intensity. To highlight biological processes that can be affected by differential expressions of the transcript’s, selected genes were classified and named under specific ontology represented as a biological process, molecular functions, and cellular component. These ontologies are then scored based on the enrichment scores

generated from expression levels of genes. Volcano graphs are generated based on fold change (fc) differences between low dose and control dose rat samples. Upregulated genes are defined as genes that have greater than 1.5 fc ($\log_2 fc = 0.58$) while downregulated genes are defined as genes with expression level smaller than 0.5 fc ($\log_2 fc = -1$). Genes are considered to be differentially expressed with adjusted p-value < 0.05 .

3. Results

3.1 Growth and Development Outcome

Our results demonstrated that the negative geotaxis results showed significant variations in task completion latency. PFAS \times day was observed (**Figure 1**). Based on post hoc Dunnett's test, both low-dose and high-dose PFAS-exposed offspring had longer task completion time compared to the control offspring regardless of sexes at PND 7 ($p < 0.05$). These findings suggested delayed locomotive development. The latency overlapped on PND13, suggesting that the locomotive development delay could possibly be restored as pups gain familiarity with the tasks. No significant variations have been found from righting reflex tests latency (**Table S2**).

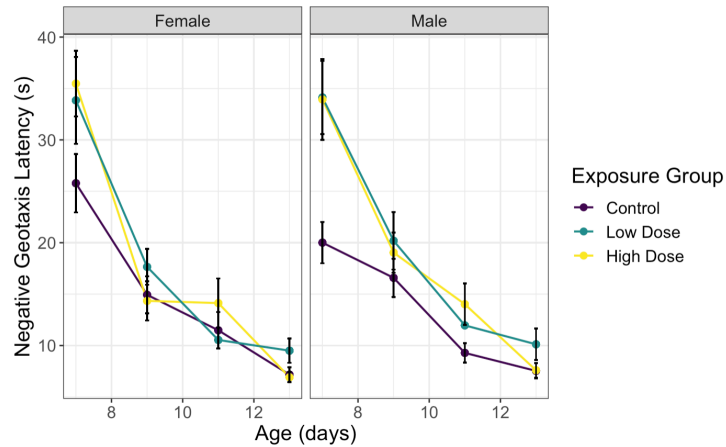


Figure 1: Negative geotaxis reflex ontogeny (PND 7, 9, 11, 13) stratified by sex and maternal exposure group. N= 12 litters (control), 16 litters (low-dose), and 11 litters (high-dose)

3.2 Behavioral Outcome

Adolescent and adult locomotion activity levels were evaluated through Figure-8 Maze tests, and significant sex-specific locomotion attenuation was observed from high dose litters. Such trend, though not significant, has also been observed from low dose rats in adolescence Figure-8 locomotion activity level comparisons (**Figure 2**). From control groups, female rats showed greater locomotive activity than male regardless of adolescence or adulthood. Such activity level difference was significantly attenuated when compared with female according to Dunnett's test ($p < 0.05$). Findings suggested strong PFAS \times sex interaction (**Table S3**).

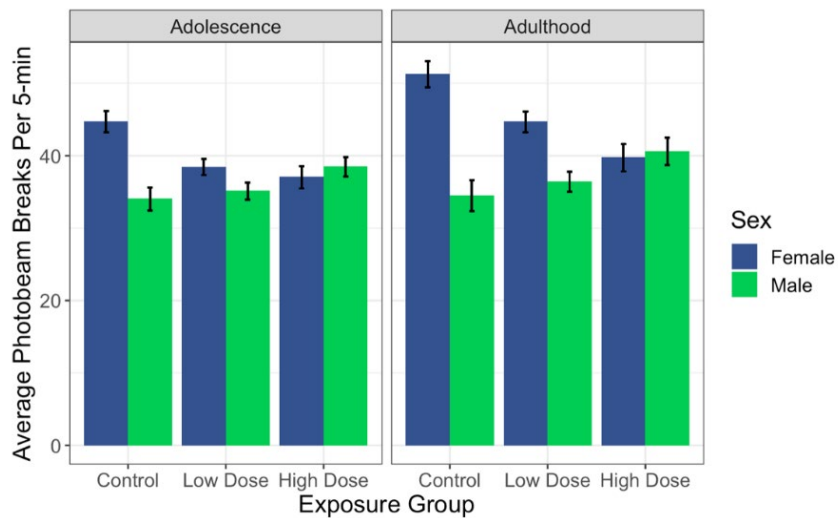


Figure 2: Figure-8 maze locomotor activity across time blocks (mean \pm standard error) in adolescent (\sim PND 35) and adult (\sim PND 56) offspring; N= 12 litters (control), 16 litters (low-dose), and 11 litters (high-dose); 1 male and 1 female per litter.

3.3 Brain Volumetric Evaluation

There were significant body weight variations among treatment groups in PND 10 pups ($p < 0.05$): control group body weight ($25.3 \pm 1.88\text{g}$), low-dose group body weight ($23.03 \pm 3.06\text{g}$), high-dose group body weight ($26.3 \pm 3.34\text{g}$). Thus, brain volume comparison was analyzed both by absolute volumetric comparison and relative volumetric comparison normalized to whole body mass of pups. Reduction of absolute brain volume was observed in low-dose group compared to control group samples ($p = 0.01$), but normalized brain volume was not significantly different among all comparisons (**Figure 3**). Further analysis is needed with integration of the litter size and sex.

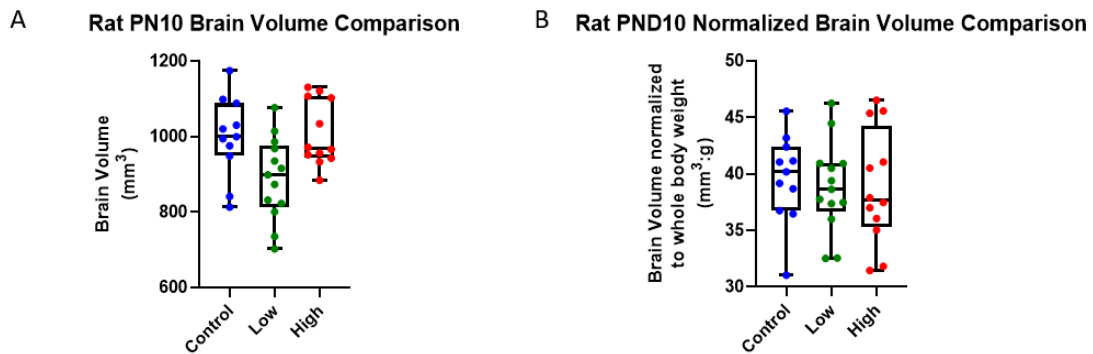


Figure 3: A. Absolute (Non-Normalized) PND10 Rat Brain Volume Comparison among Control, Low-Dose, High-Dose Groups B. PND10 Rat Brain Volume Normalized to Whole Body Weight Comparison among Control, Low-Dose, High-Dose Groups; N = 11 pups (control), 13 pups (low-dose), 12 pups (high-dose)

3.4 Transcriptome Analysis

Transcriptome analysis identified 564 differentially expressed genes (DEGs) in low-dose cerebellum samples compared to the controls (cutoff: adjusted $p < 0.05$ **Figure 4**). Top ten upregulated and ten downregulated genes were highlighted when we utilized fold change and adjusted p values as the cutoff. Sixteen of twenty genes were found to have human orthologs and are relevant to neuronal cell differentiation, regulation of intracellular processes, and tissue adhesion (**Table 2, 3**). The results are consistent with Gene Ontology analysis (**Figure 5**). Among ontologies, DEGs were enriched in non-motor seizure, dialectic seizure, atypical absence seizure, and mitochondrial relevant metabolic processes. Importantly, transcriptome analysis identified DEGs that are relevant to locomotor rhythm and locomotor behavior including *mTOR*, *Oprl1*, *Fgf14*, *Robo1*, *Raph1*, and *Rab3gap2* (**Table 4**). In our previous study, we have found that PFAS exposure significantly downregulated *Agt* gene expression in placentae. Consistent with our previous results, *Agt* gene expression was also significantly downregulated in cerebellum by PFAS exposure. Strikingly, we found *Ttr* gene expression was significantly downregulated by PFAS exposure in cerebellum. *Ttr* gene encodes for a homo-tetrameric carrier protein, which transports thyroid hormones to placenta and brain participate in cerebellum development and locomotor functions.



Figure 4: Volcano Plot of DEGs from the Comparison of Low-Dose and Control Groups; N= 3 litters (control), 3 litters (low dose); 1 males and 1 females for each litter

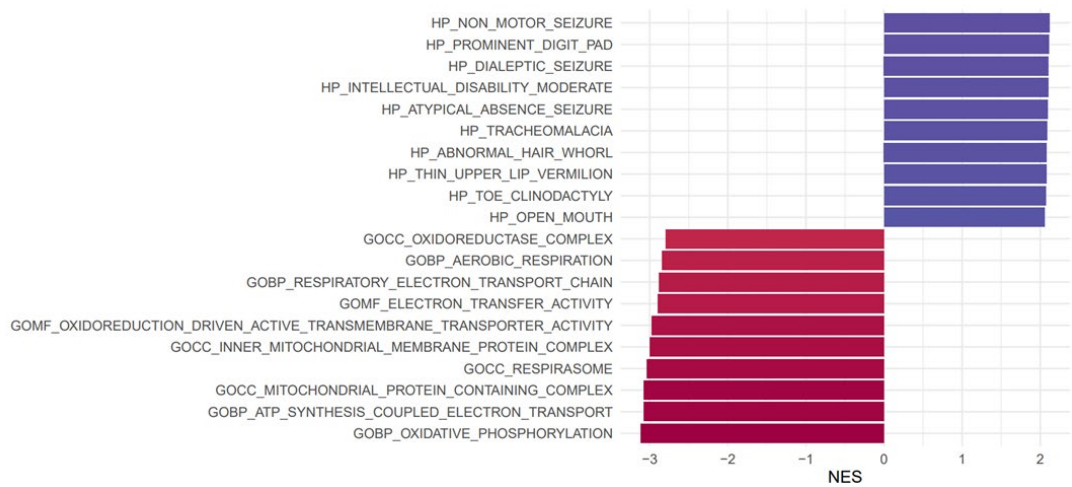


Figure 5: Low-Dose vs. Control Gene Ontology Normalized Enrichment Score (NES) reported on PND1 Cerebellum RNA samples; N= 3 litters (control), 3 litters (low dose); 1 male and 1 female for each litter

Table 2: Top Upregulated Genes with Human Orthologs

Gene ID	Adjusted p-value	Log2 Fold-Change	Functionality (based on NCBI Gene Entry²²)
Hs6st3	0.000740	0.720	Gene encodes heparan sulfate (HS) sulfotransferases necessary for sulfate pathway, which would be involved in cellular interactions for cell proliferation and differentiation, adhesion, migration.
Obscn	0.00147	0.750	Encoded protein belongs to a family of signaling proteins that includes titin and nebulin, and may have a role in the organization of myofibrils during assembly that is relevant for skeletal muscle buildup. Upregulation is relevant to autism spectrum disorder (ASD).
Kcnt2	0.00119	0.692	Translated protein regulates potassium-sodium channel activity, missense mutation could lead to overexpression that is found relevant to seizure development.
St6gal2	0.00390	0.669	Functionality has not been accurately deduced, but the gene is universally expressed from brain tissue. Polymorphisms have been found to be associated with Schizophrenia.
St8sia4	0.00220	0.667	Encoded protein facilitates the catalysis of alpha-2,8-linked sialic acid required for the synthesis of polysialic acid, a modulator of the adhesive properties of neural cell adhesion molecule (NCAM1).
Flnb	0.00369	0.639	Encoded protein belongs to a family of filamin protein family and interacts with glycoprotein Ib to repair vascular damage.
Ptchd1	0.00410	0.610	Gene encodes for a protein commonly expressed in the cerebellum region; deletion could lead to autism and intellectual disability.
Gira3	0.00767	0.608	Gene encodes for a protein that belongs to the ligand-gated ion channel protein family.
Itgb8	0.00736	0.601	Protein encoded by the gene forms a part of the integrin complexes that play a role in epithelial cell proliferation.

Table 3: Top Downregulated Genes with Human Orthologs

Gene ID	Adjusted p-value	Log2 Fold-Change	Functionality (based on NCBI Gene Entry²²)
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Errfi1	0.0105	-0.547	Upregulated during cell proliferation process where the protein product mediates cell signaling.
Mbp	0.0156	-0.517	Encodes myelin basic protein that constitute the myelin sheath of oligodendrocytes and Schwann cells in the nervous system.
Vtn	0.00767	-0.451	Gene encodes for an adhesive glycoprotein that facilitates the cell adhesion and migration process.
Gfap	0.0174	-0.448	Protein encoded is present in the central nervous system, and downregulation could cause Alexander disease, a rare disorder of astrocytes.
Laptm4b	0.0270	-0.430	Gene encodes lysosomal protein transmembrane 4 beta that is involved in the regulation of lysosomal membrane permeability and regulation of lysosome organization.
Epn3	0.0305	-0.424	Gene encodes for an extrinsic component of plasma membrane.
Fa2h	0.0357	-0.392	Gene mutation could be associated with potential spastic paraparesis that affects motor function.

Table 4: List of Selected DEG Relevant to Locomotor Development

Gene ID	Adjusted p-value	Log2 Fold-Change	Functionality (based on NCBI gene entry²²)
Agt	0.00171	-0.341	Gene encodes pre-angiotensinogen or angiotensinogen precursor and is part of the pathway that involves blood pressure regulation.
Oprl1	0.0427	0.356	Gene encodes for a receptor protein that is involved with a receptor-ligand system modulating neurobehavior, including stress responses and anxiety behavior, learning and memory, locomotor activity.
Mtor	0.0396	0.275	Gene encodes a protein that is a member of phosphatidylinositol kinase-related kinases family. The kinase is a component of mTORC1 complex, which regulates protein synthesis, cell growth and proliferation, and mTORC2, which regulates actin cytoskeleton, and promotes cell survival and cell cycle progression.
Raph1	0.00164	0.388	Gene encodes a protein that belongs to the Mig10/Rap1-interacting adaptor

			molecule/Lamellipodin family of adapter proteins, a critical type of protein involved with cell migration process. Upregulation could lead to structural changes in spine density, associated with seizure symptoms.
Rab3gap2	0.00390	0.430	The protein encoded by this gene belongs to the RAB3 protein family, members of which are involved in regulated exocytosis of neurotransmitters and hormones.
Fgf14	0.00908	0.531	Gene encodes for a protein that belongs to fibroblast growth factor (FGF) family, which is involved with cell growth process. Mutations of the gene has been found to be associated with autosomal dominant cerebral ataxia.
Robo1	0.000574	0.308	This gene encodes for an integral membrane protein that functions in axon guidance and neuronal precursor cell migration and relevant to glioma cell guidance in the developing brain.

3.5 Mid Gestational Sample Analysis

No significant variations in maternal weight and maternal weight gains have been found. The fetus-placenta weight ratio was used as a proxy of placental efficiency²⁴. No significant variations in the fetus-placenta ratio, placenta weight, fetus weight have been found (**Table S5, Figure 6**). There were higher total number of resorption sites and gaps in uterus of PFAS exposed rats, although the distribution of resorption sites per rat among three groups do not have significant variations (**Table S5**).

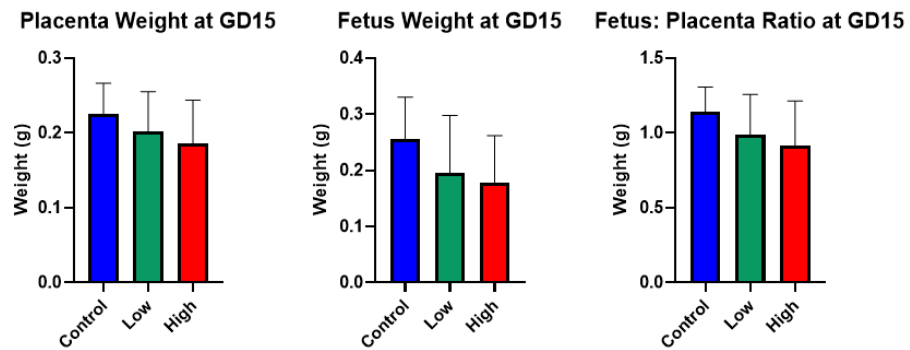


Figure 6: Comparison of Key GD15 Fetus and Placenta Measures among control, low-dose, and high-dose samples; N = 5 litters (control), 6 litters (low-dose), 7 litters (high-dose)

4. Discussion

4.1 Implications for Future Research

In our study, we found maternal exposure to PFAS mixtures via drinking water at both an environmentally relevant mixture level and a high level is associated with altered locomotor development and brain development in rat offspring.

Delays in behavioral development have been observed from negative geotaxis tests for both low- and high-dose groups, although rats eventually obtained control-level measures. Such gain of function, however, should not be seen as a healthy recovery. Rather, the impact of PFAS exposure should be evaluated by stages, and the gain of function should not undermine how other measures, like locomotion, would later show alteration at a different developmental stage. As we contextualize the locomotion variations observed from Figure-8 Maze tests, the behavioral outcomes from the F1 generation demonstrated a highly specific trend of alterations from maternal PFAS exposure and that the dose–sex interaction is more complex. The locomotion data unveil the overall activity level of rats when habituating to new environment, and the behavioral measures have been thought to be relevant to human ADHD and autism.

In addition, we should consider whether the locomotion variations from adolescence and adulthood in rats' development is consequential as predicted by the delays in motor function development. Amphibians, specifically leopard frogs, have also been extensively used to investigate the developmental consequences of PFAS exposure. Foguth et al. studied the effects of exposure to a 5-PFAS mixture, which constituted

mostly PFOS and PFHxS²⁵. After 30 days of exposure from PFAS mixture, both glutamate and 5-hydroxytryptamine (5-HT) neurotransmitter levels from dosed leopards decreased. However, such reduction did not continue in later developmental stages. Instead of a lack of two neurotransmitters, the neurotransmitter levels of glutamate and 5-HT recovered while the acetylcholine level rose significantly²⁵. Although the researchers did not suggest such an increase in acetylcholine was by any means a compensatory mechanism relevant to the recovery of glutamate and 5-HT, they did suggest neurochemical measures vary by developmental stages, and similar changes might be seen from locomotion variations and motor development.

Historically, female rats have been observed to show higher activity levels when compared with males in control settings. Yet female-specific activity level elimination has been observed from high-dose groups during both the adolescent and adult stage of rat development. The persistence of such locomotor activity elimination pattern across the lifespan of rats, therefore, is concerning. Considering the complexity of ADHD diagnosis and the extent of symptom variations among ADHD patients, potential alterations in sex differences of locomotor activity pattern could add complexity to existing rules characterizing ADHD symptoms²⁶⁻²⁷.

The mid-gestational sample collection helped cultivate insights on miscarriages as affected by PFAS exposure. However, we did not see a significant variation among fetus-placenta ratio nor fetus and placenta weight, which is consistent with the pup body weight discovered when born (**Table S1**). High total number of resorption sites from low-dose and high-dose groups were interesting results, although there were no variations in

litter sizes or pup survival rates across three dosage groups. Follow-up experiments should evaluate the PFAS presence from placenta and fetus samples and consider both the single-compound and the mixture effect of specific PFAS substance property.

Brain volumetric changes at early age tend to be more subtle, and volumetric changes of whole rat brains should be viewed in the context of more specific quantitative measures, qualitative measures, and the developmental stage of the rat²⁸⁻²⁹. We utilized an advanced technique to accurately measure the brain volume in our study. Quantitative measurement wise, brain is constituted by complex compartments, and volumetric changes of specific regions in brain should be a meaningful addition of evidence to current volumetric data. Conflicting growth pattern observed from high-dose and low-dose brain samples, furthermore, could also show different pattern of brain regression and pathogenesis development. Increment and reduction in the size of brain parenchyma, part of the brain made up by neurons and glial cells, could each represent a different direction of pathogenesis, for example. Substantial decrease in brain parenchyma sizes could suggest excessive loss of neurons and synapses that impair motor function, while subtle increase in parenchyma sizes could as well demonstrate disordered developmental changes in neuronal differentiation and disordered synaptogenesis, which can also lead to locomotion and cognitive impairment³⁰⁻³⁴. Thus, qualitative evidence through microscopic analysis would be critical to uncover tissue adhesion state and neuronal differentiation patterns, presence of lesions from compartments of interest.

The transcriptome analysis from RNA-seq presented numerous DEGs based off readings on the transcripts. Upregulated genes were categorized by three hypothetical

functionality: that discovered upregulation of genes could be compensatory for protective mechanism induction, or such upregulation could simply be functional and essential for the growth of brain at PND 10, or that such upregulation is related to pathogenesis and mechanisms that contribute to abnormal neurodevelopment. There have been precedent studies where compensatory expression of genes from brain compensated for loss of Kit gene expression from mutant mice³⁵. Thus, the upregulation of St8sia4, Itgb8 and Rab3gap2 could be protective. St8sia4 encodes the protein necessary for synapse development, and downregulation of St8sia4 typically leads to loss of synaptic connectivity in hippocampus that led to abnormal biosynthesis of polySia, a loss of function relevant to Schizophrenia³⁶. Similarly, Itgb8 encodes for the neuroepithelial cell-expressed integrin $\beta 8$ subunit that is involved with adhesion mechanisms promoting brain neurovascular development³⁷. Hs6st3, St6gal2, and Ptchd1 overexpression are unsurprising, since three genes are either commonly expressed at brain regions or are involved with pathways necessary to sustain brain activity, and their downregulation or deletion would actually be more relevant to brain pathogenesis³⁸⁻⁴⁰. The upregulation of Obscn, Kcnt2, Raph1, and mTOR are consistently relevant to autistic behavior, motor seizure, and abnormal dendritic development. Raph1 gene encodes for a family of adaptor molecules that are necessary for cell migration and regulate actin network assembly, upregulation could cause excessive actin assembly that leads to seizure-related spine density and morphology changes⁴¹⁻⁴². Obscn overexpression has been found to inhibit cell migration mechanisms that potentially lead to autism spectrum disorder, although the exact mechanisms remain unknown⁴³. Similar situation applies to Kcnt2,

where the missense mutation caused hyperactivation of *Kcnt2* variants from a patient with motor seizure⁴⁴.

The functionality of genes that are downregulated are consistent with Gene Ontology analysis, and suggested that PFAS compounds interfere with various intercellular mechanisms and metabolic activities. Among downregulated genes, research has demonstrated that TTR is an important protein that binds and transports thyroid hormones to brain cells and facilitates neuro stem cell (NSC) differentiation⁴⁵. Loss of TTR could lead to dysregulated neurogenic versus oligodendrogenic balance, and potentially lead to lack of neurogenesis that impairs motor function, lead to neurodegeneration⁴⁵. Other downregulated genes such as *Mbp*, *Fa2H*, and *Gfap* are associated with rare neuro-diseases, although the key functionality effect and mechanistic effects have not been thoroughly investigated. There are, however, genes where the protein products play key roles in mediating intracellular processes and downregulated. *Errfi1* and *Vtn* downregulation are critical in that these two genes are supposedly upregulated during the brain development process of PND 10 pups and critical for neurogenesis and cognitive development⁴⁶⁻⁴⁷. Since these genes encode for protein product necessary for cell migration and adhesion process, the downregulation of these genes could potentially decrease synaptic connectivity and tissue formation, and thus affect cerebellum formation.

The overlap of gene functionality among motor-development relevant genes that are differentially expressed and genes that are upregulated by log₂fc cutoff implied that cerebellum development could be undermined by abnormal cerebellar cell

differentiations and abnormal tissue adhesion. The differential expression of mTOR gene is significant for hypothesizing these neurodevelopment mechanisms. mTOR gene encodes for mTOR kinase that are central regulator of cellular metabolism and could enhance mRNA translation of genes involved neuronal cell proliferation⁴⁸. Various mice knockout studies have demonstrated the indispensability of mTOR expression to brain development, and deletion of mTOR could lead to structural changes and be associated with neurodegeneration⁴⁹. Mice with brain-specific mTOR deletion were found to have smaller pyramidal and Purkinje neurons from hippocampus and cerebellum.

Hyperactivation of mTOR could lead to cell overgrowth, which was experimentally demonstrated to be related to tuberous sclerosis⁵⁰. The pathogenesis was indivisible from disrupted cellular homeostasis in cerebellar Purkinje cells, where hyperactivation of mTOR led to impaired synapse elimination of climbing fibers and motor discoordination from mice, according to Sakai et al⁵⁰. Interestingly, the impairments specifically affected motor development and cell adhesion mechanisms and did not seem to affect the social behavior of mice⁴⁸. The findings are consistent with our experimental discovery that PFAS-relevant behavioral changes did not involve cognitive or memory impairments. Such consistency suggests the necessity for further evaluations in genes regulated by mTOR signaling pathway.

4.2 Study Considerations, Strengths, and Limitations

Our study highlights several strengths. First, the study adopts an environmentally relevant PFAS mixture exposure rather than duplicating the single-compound experiments. The low-dose mixture is relevant to affected population communities in

Pittsboro, whereas the high-dose mixture allows sensitive detection of physiological changes that can be altered by PFAS exposures. Therefore, the adoption of PFAS mixture not only allows the investigation of complex, practical dose-individual interactions but also mimics a real-world setting observed from Pittsboro, North Carolina. The study represents the population more effectively and provides a strong scientific message regarding the effects of PFAS exposure.

Our study also uncovers mechanistic findings that help explain neurodevelopmental assumptions made on F1 generation behaviors. Based on RNA-seq analysis and sample collections, we deduced potential signaling events and cellular mechanisms relevant to observed pathological evidence. Further pathological staining and immune-staining procedures could be combined and thus demonstrate factors relevant to motor development and locomotion variations.

There are factors for which our study could not account, one of them being the control of drinking water. Although we documented the water consumption and weight variations of rats throughout rat pregnancy, and both the net water consumption variations and pregnancy weight variations were not significant overall, individual differences in water consumption could result in differential accumulation of PFAS compounds in the body. When compared with oral gavage, however, our method did not introduce stressors that might confound the offspring measures or affect rat pregnancy. In fact, introducing external stressors to rats during pregnancy could result in pause of pregnancy.

Since the serum from rats would be taken, some may argue that it would be more statistically accurate if the samples are grouped by different PFAS level from serum samples. However, the sample size for animal studies is usually much smaller than population studies and it is not powered for linear regression model when considering the serum levels of PFAS. In addition, the serum levels of PFAS in each group are deviated significantly according to dosing groups and correlated with the doses of drinking water although we did not use oral gavage. Therefore, it is scientifically sound to perform the data analysis based on treatment groups. In addition, the bioaccumulation of PFAS compounds is not exactly a linear relationship with water consumption. For example, the level of PFOS that is relative low in the PFAS mixtures, was found to be significantly higher in serum samples. Thus, the treatment given to rats should still be considered since the correlation between the accumulation of specific PFAS compounds and the level of dosage could vary. Still, the data analysis from the study would benefit tremendously from the measurements of PFAS level from the rat serum samples.

Another limitation of our study was the difficulty we had exacting the date of mid gestation pregnancy. To assure a correct estimate of midgestational date, we left a short mating window for adult rats; therefore, the pregnancy rate was low overall for rats. Even with gestational weight measurements and the short mating window, the mid gestation date variation might still occur and potentially contribute to varied placenta–fetus ratio. Nevertheless, adult rats constituting the neurobehavioral part of the study showed no significant variations with pregnancy rate when given a sufficient mating window. In

addition, the maternal weight of pregnant rats showed no significant variations among one another.

4.3 Implications for Policy and Practice

There are various gaps in introducing effective policies and interventions to assist populations exposed to PFAS. First, there is a lack of experimental evidence that uncovers key biological processes and mechanisms regulated by PFAS compounds. As a result, it is difficult for researchers and health officials to articulate the effects of PFAS to the public. Second, accurate assessments of PFAS contamination levels and the administration responsibility of PFAS assessments have been unclear. Although various research groups and nonprofit organizations voluntarily measured PFAS concentrations from water sources, there has been limited standardizations as to how the measurement is done, which official party should be responsible for executing such standard, and how the financial payments of PFAS assessments should be done. At an administration level, officials need to not only consider the health of the affected populations but also communicate with key industrial factories about manufacturing standards and new regulations in PFAS usage.

Our findings filled the initial gap and highlighted the cross-generation effect of PFAS exposure as well as both sex-dimorphic and sex-specific physiological complications. Through the findings from dosed groups, we discovered PFAS mixture exposure could trigger health complications, even at low concentrations. The urgency of PFAS contamination and its consequences to exposed communities, therefore, should

demand public health officials to extend their action beyond informing the public about the harms of PFAS compounds.

In fact, public health and EPA officials arguably failed to introduce accountable policies because the officials underestimated the prevalence of PFAS contamination as well as its magnitude. Firefighters, for example, have been the work population constantly exposed to PFAS firefighting foam⁵¹. Yet it was not until 2022 when the EPA finally issued test orders acquiring The Chemours Company; DuPont De Nemours Inc.; and other companies manufacturing PFAS material, firefighting foam included, to conduct testing on PFAS substances for manufactured products⁵¹. More radical policies should be introduced to demand substitution of PFAS compound usage and retirement of old, classic PFAS substances such as PFOS and PFOA.

5. Conclusion

To conclude, our study demonstrated that maternal exposure to low and high doses of PFAS mixtures led to delays in motor development of F1 generation, and maternal exposure to a PFAS mixture at a high concentration caused sex-specific locomotion attenuation observed in rat offspring during adolescence and adulthood. Through transcriptome analysis of cerebellum samples, we discovered that several motor development and metabolic relevant gene ontologies were differentially regulated by maternal PFAS exposure at an environmentally relevant dose. These gene dysregulation could contribute to altered cerebellum development that are related to locomotion variations. Mid-gestation sample measures suggested that *In Utero* PFAS exposure affect both early and late pregnancy outcomes including failed implantations and increased resorption sites. Ongoing studies in Dr. Feng's lab continue to investigate the mechanisms of observed adverse fetal development upon PFAS exposure.

Appendix A

Table S1: Growth measurements

AGD (cm)				
	p-value	Control	Low Dose	High Dose
PFAS Exposure	0.224	7.05	6.98	7.11
		Male	Female	
Sex	0.0001	8.83		5.26
		Control	Low Dose	High Dose
PFAS × SEX	0.679	Male:	Male:	Male:
		Female:	Female:	Female:
PFAS × day	0.0001	-----		
		Control	Low Dose	High Dose
Measurement day		1: 3.08	1: 3.26	1: 3.15
		2: 3.46	2: 3.46	2: 3.46
		4: 4.15	4: 4.25	4: 4.12
		7: 5.23	7: 5.11	7: 5.16
		10: 6.70	10: 6.55	10: 6.60
		14: 9.03	14: 8.97	14: 8.99
		17: 11.20	17: 10.82	17: 11.40
		21: 13.58	21: 13.44	21: 14.07
Weight (g)				
	p-value	Control	Low Dose	High Dose
PFAS Exposure	0.442	27.06	26.67	27.80
		Male	Female	
Sex	0.0001	27.52		26.70
		Control	Low Dose	High Dose
PFAS × SEX	0.185	Male: 27.57	Male: 26.89	Male: 28.38
		Female: 26.56	Female: 26.45	Female: 27.22

PFAS × sex × day	0.0001	-----					
		Control		Low Dose		High Dose	
		Male	Female	Male	Female	Male	Female
Measurement day		1: 8.17	1: 7.67	1: 7.86	1: 7.67	1: 7.91	1: 7.56
		2: 9.53	2: 9.56	2: 9.48	2: 9.09	2: 9.11	2: 8.86
		4: 12.67	4: 12.14	4: 12.34	4: 11.83	4: 13.07	4: 12.63
		7: 18.90	7: 18.46	7: 18.41	7: 17.93	7: 19.43	7: 18.52
		10: 25.84	10: 25.63	10: 25.32	10: 25.05	10: 26.60	10: 25.49
		14: 39.20	14: 38.19	14: 37.89	14: 37.13	14: 40.08	14: 38.62
		17: 47.02	17: 45.75	17: 46.31	17: 45.93	17: 48.56	17: 46.94
		21: 59.19	21: 57.41	21: 61.89	21: 55.58	21: 56.93	21: 59.15

Table S2: Reflex tests

Righting reflex (s)				
	p-value	Control	Low Dose	High Dose
PFAS Exposure	0.120	9.3	11.3	8.8
		Male		Female
Sex	0.0025	8.8		11.1
		Control	Low Dose	High Dose
PFAS × SEX	0.913	Male: 7.9 Female: 10.6	Male: 10.2 Female: 12.3	Male: 7.6 Female: 10.0
PFAS × day	0.645	-----		
		Control	Low Dose	High Dose

Measurement day	0.0001	2: 14.7 4: 8.6 6: 4.6	2: 14.6 4: 12.2 6: 7.0	2: 13.5 4: 8.7 6: 4.2
Negative geotaxis (s)				
	p-value	Control	Low Dose	High Dose
PFAS Exposure	0.02	13.9	18.4	18.2
		Male	Female	
Sex	0.953	17.1	16.7	
		Control	Low Dose	High Dose
PFAS × SEX	0.332	Male: 13.1 Female: 14.7	Male: 19.1 Female: 17.6	Male: 18.6 Female: 17.7
PFAS × day	0.009	-----		
		Control	Low Dose	High Dose
Measurement day	0.0001	7: 22.3 9: 15.7 11: 10.4 13: 7.4	7: 33.0 9: 20.2 11: 11.3 13: 9.0	7: 34.7 9: 16.7 11: 14.1 13: 7.3

Table S3: Figure-8 maze measures summary; N= 12 litters (control), 16 litters (low-dose), and 11 litters (high-dose); 1 male and 1 female per litter.

Overall (Adolescent and Adult)				
		Mean beam breaks per 5-min block		
	p-value	Control	Low Dose	High Dose
PFAS Exposure	0.937	39.2	38.6	38.7
		Male	Female	
Sex	0.0006			
		Control	Low Dose	High Dose

PFAS × SEX	0.014	Male: 33.8 Female: 44.5	Male: 35.5 Female: 41.7	Male: 39.2 Female: 38.2
5-min block	0.0001	-----		
PFAS × Age × Block	0.063	-----		
Adolescent				
	p-value	Control	Low Dose	High Dose
PFAS Exposure	0.711	38.8	37.0	37.3
		Male	Female	
Sex	0.013	35.9		39.4
		Control	Low Dose	High Dose
PFAS × SEX	0.019	Male: 34.8 Female: 42.9	Male: 35.3 Female: 38.7	Male: 37.9 Female: 36.6
5-min block	0.0001	-----		
		Control	Low Dose	High Dose
PFAS × time block	0.281	1: 62.3 2: 53.1 3: 45.4 4: 43.9 5: 39.0 6: 36.6 7: 34.5 8: 33.1 9: 33.0 10: 31.8 11: 29.7 12: 23.8	1: 60.7 2: 50.9 3: 47.1 4: 38.2 5: 39.9 6: 37.3 7: 32.6 8: 34.0 9: 28.3 10: 25.2 11: 27.3 12: 22.4	1: 60.0 2: 50.6 3: 46.3 4: 41.6 5: 38.9 6: 37.6 7: 36.1 8: 29.7 9: 33.1 10: 28.8 11: 22.7 12: 21.9
Adult				
	p-value	Control	Low Dose	High Dose
PFAS Exposure		39.5	40.2	40.2

		Male	Female	
Sex		36.2	43.7	
		Control	Low Dose	High Dose
PFAS × SEX		Male: 32.8 Female: 42.9	Male: 35.3 Female: 38.7	Male: 37.8 Female: 36.6
5-min block		-----		
		Control	Low Dose	High Dose
PFAS × time block		1: 72.5 2: 58.9 3: 49.8 4: 43.8 5: 42.6 6: 40.5 7: 35.5 8: 28.0 9: 30.7 10: 25.7 11: 25.2 12: 20.3	1: 69.0 2: 58.2 3: 48.3 4: 46.3 5: 41.6 6: 36.8 7: 34.7 8: 33.8 9: 31.8 10: 29.3 11: 28.1 12: 24.7	1: 67.6 2: 53.4 3: 51.3 4: 44.0 5: 47.6 6: 41.4 7: 34.6 8: 40.2 9: 29.6 10: 27.2 11: 25.8 12: 19.2

Table S4: Maternal Drinking Water Consumption
(mean +/- standard deviation, g/kg of rat per day)

* indicates statistically significant difference in water consumption from ANOVA

	Control	Low Dose	High Dose
Cohort 1 N= 12	Week 1: 106.0 (±17.5) Week 2: 100.0 (±23.4) Week 3: 128.2 (±11.6) Week 4: 122.7 (±33.1)	Week 1: 108.5 (±37.3) Week 2: 104.8 (±25.1) Week 3: 118.7 (±24.9) Week 4: 158.6 (±29.4)	Week 1: 119.9 (±31.2) Week 2: 112.4 (±27.4) Week 3: 144.3 (±17.7) Week 4: 147.4 (±27.8)
Cohort 3 N= 12	Week 1: 122.5 (±10.2) Week 2: 98.1 (±28.0) Week 3: 151.6 (±39.1) Week 4: 156.3 (±28.6)	Week 1: 131.3 (±21.0) Week 2: 99.2 (±23.1) Week 3: 145.4 (±28.6) Week 4: 179.0 (±29.2)	Week 1: 145.1 (±35.7) Week 2: 111.6 (±23.8) Week 3: 177.8 (±41.7) Week 4: 183.2 (±27.8)
Cohort 4 N= 12	Week 1: 151.3 (±34.9) Week 2: 115.1 (±34.2) Week 3: 131.8 (±81.8) Week 4: 185.6 (±59.1)	Week 1: 146.4 (±14.6) Week 2: 109.5 (±34.0) Week 3: 114.6 (±41.8) Week 4: 156.0 (±26.2)	Week 1: 119.9 (±31.2) Week 2: 112.4 (±27.4) Week 3: 144.3 (±17.7) Week 4: 147.4 (±28.2)

Average N= 36	Week 1: 121.4 (±28.8)	Week 1: 121.6 (±32.1)	Week 1: 129.9 (±31.6)
	Week 2: 106.4 (±28.2)	Week 2: 104.4 (±28.7)	Week 2: 108.7 (±29.3)
	Week 3: 133.1 (+46.2)	Week 3: 134.7 (±32.9)	Week 3: 142.8 (±42.9)
	Week 4: 151.7 (+49.2)	Week 4: 166.5 (±28.5)	Week 4: 162.6 (±33.9)

Table S5: Mid-Gestational Sample Comparisons

Mid-Gestational Sample Measures				
	p-value	Control (n=5)	Low (n=6)	High (n=7)
Maternal Weight (mean ± standard dev)	0.68038	332.9±12.4g	326.0±13.5g	331.9±16.1g
		Control (n=5)	Low (n=6)	High (n=7)
Maternal Weight gain (mean ± standard dev)	0.9337	50.4±6.9g	53.2±17.7g	54.3±13.7g
		Control (n=5)	Low (n=6)	High (n=7)
Fetus-Placenta Ratio	0.35235	1.14±0.16	0.99±0.27	0.92±0.30
		Control (n=5)	Low (n=6)	High (n=7)
Fetus Weight (mean ± standard dev)	0.33504	0.256±0.08g	0.196±0.1g	0.179±0.08g
		Control (n=5)	Low (n=6)	High (n=7)
Placenta Weight (mean ± standard dev)	0.45668	0.226±0.07g	0.202±0.05g	0.186±0.05g
		Control (n=5)	Low (n=6)	High (n=7)
Total Resorption Sites and Gap Number		1 resorption site	9 resorption sites, 2 gaps	7 resorption sites

Table S6: Review of neurodevelopmental studies for dosing

PFAS Compound	Dose (mg/kg/day)	Dosing method & timeline	Observed effects	Source
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Neurodevelopmental Studies				
PFOS (rats)	0.1 / 0.3 / 1.0	Oral dose Pregnant females: GD 0 – PND 20	1.0 mg/kg/day: Low maternal body weight compared to control, increased motor activity and reduced habituation on PND 17 for male offspring only Maternal NOAEL: 0.3 mg/kg/day (males); >1.0 mg/kg/day (females)	Butenhoff et al. ⁵²
PFOS (rats)	0.1 / 0.4 / 1.6/ 3.2	Gavage Males: 6 weeks prior to mating-mating Females: 6 weeks prior to mating-lactation, across two generations	0.4 mg/kg/day: reductions in body weight gain and feed consumption (F0); delayed eye opening (F1 pups) 1.6 mg/kg (critical dose): 50% mortality among prenatally exposed rat pups within 4 days after delivery; delayed eye opening, air righting, surface righting, and pinna unfolding in F1 pups 3.2 mg/kg/day: decreased length of gestation, number of implantation sites, and increased numbers of dams with stillborn pups	Luebker et al. ⁵³
PFHxS (rats)	0.3 / 1 / 10	Gavage 14 days prior to mating through sacrifice (21 days of lactation for females, treatment day 42 for males)	No significant effects on functional observational battery	Butenhoff et al. ⁵⁴
PFOS (rats)	3	Gavage GD 2 to 21	No effects observed in T-maze (spatial learning and memory)	Lau et al. ⁵⁵
PFOS (mice)	6	Gavage GD 12–18	Reduced body weight on PND 4 and 8, diminished resistance during tail pull & vertical screen climb ability, diminished grip strength,	Fuentes et al. ⁵⁶

			temporarily decreased	
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