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# Perinatal exposure to PFOS and sustained high-fat diet promote neurodevelopmental disorders via genomic reprogramming of pathways associated with neuromotor development

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## ABSTRACT

Perfluorooctanesulfonic acid (PFOS) is a neurotoxic widespread organic contaminant which affects several brain functions including memory, motor coordination and social activity. PFOS has the ability to traverse the placenta and the blood brain barrier (BBB) and cause weight gain in female mice. It's also known that obesity and consumption of a high fat diet have negative effects on the brain, impairs cognition and increases the risk for the development of dementia. The combination effect of developmental exposure to PFOS and the intake of a high-fat diet (HFD) has not been explored. This study investigates the effect of PFOS and /or HFD on weight gain, behavior and transcriptomic and proteomic analysis of adult brain mice. We found that female mice exposed to PFOS alone showed an increase in weight, while HFD expectedly increased body weight. The combination of HFD and PFOS exacerbated generalized behavior such as time spent in the center and rearing, while PFOS alone impacted the distance travelled. These results suggest that PFOS exposure may promote hyperactivity. The combination of PFOS and HFD alter social behavior such as rearing and withdrawal. Although HFD interfered with memory retrieval, biomarkers of dementia did not change except for total Tau and phosphorylated Tau. Tau was impacted by either or both PFOS exposure and HFD. Consistent with behavioral observations, global cerebral transcriptomic analysis showed that PFOS exposure affects calcium signaling, MAPK pathways, ion transmembrane transport, and developmental processes. The combination of HFD with PFOS enhances the effect of PFOS in the brain and affects pathways related to ER stress, axon guidance and extension, and neural migration. Proteomic analysis showed that HFD enhances the impact of PFOS on inflammatory pathways, regulation of cell migration and proliferation, and MAPK signaling pathways. Overall, these data show that PFOS combined with HFD may reprogram the genome and modulate neuromotor development and may promote symptoms linked to attention deficit-hyperactivity disorders (ADHD) and autism spectrum disorders (ASD). Future work will be needed to confirm these connections.

## 1. Introduction

Persistent organic pollutants (POPs) are hazardous chemicals that

are widely utilized in industry causing an environmental contamination and a continuous exposure and bioaccumulation in human and wildlife (Wang et al., 2019). Poly and perfluoroalkyl substances (PFAS) are a

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class of emerging POPs with high food chain bioaccumulation and with potential neurotoxic effects (Ninomiya et al., 2022). Elevated PFAS concentrations are detected in high calorie foods, in fast food containers, and in packaging of ready-made foods. Early exposure to these exogenous substances alter several endocrine system functions and lead to health problem in later life (Panikkar et al., 2019; McGlinchey et al., 2020; Sinisalu et al., 2020; Goodrich et al., 2023).

Perfluorooctanesulfonic acid (PFOS) is the most employed PFAS. PFOS has a serum half life reaching 5.4 years and has high affinity to plasma albumin (S.-C. Chang et al., 2012; Mondal et al., 2012; Forsthuber et al., 2020). Additionally, PFOS can pass through the blood brain barrier (BBB) (Lau et al., 2006, 2007). PFOS has a wide range of toxicities including hepatotoxicity, reproductive impairment, carcinogenicity and DNA methylation (Cui et al., 2009; Han and Fang, 2010; E. T. Chang et al., 2014; Robinson et al., 2021). PFOS is neurotoxic and has been proven to affect several brain functions including memory, motor coordination and social activity (Harris et al., 2018; Vuong et al., 2019; Nesan and Kurrasch, 2020; Skogheim et al., 2021). By causing the release of inflammatory factors, PFOS can induce neural damage and cholinergic alterations which affect behavior and learning (Fuentes et al., 2007; Wang et al., 2015). PFOS levels in various environmental media have been reported by Vedagiri et al. (Vedagiri et al., 2018). It is often found in different environmental media without regard to particular site of contamination (soil, water and air). The level of perfluorooctane sulfonate (PFOS), in air, water, sediment, fish, and others media varied between below and above the health-based threshold (Gewurtz et al., 2013; Vedagiri et al., 2018).

A diet high in fat can cause metabolic disturbances, as well as neuronal and cognitive alterations which may increase the risk of dementia (Luchsinger et al., 2002; Freeman et al., 2014). In transgenic mice, HFD overtime exacerbates behavioral disorders and cognitive deficits (Xiong et al., 2022). Moreover, HFD increases Tau expression and phosphorylation in the brain of mice independent of peripheral metabolic status (Bhat and Thirumangalakudi, 2013; Takalo et al., 2014). A diet high in fat/high cholesterol (HFC) results in a loss of working memory in mice correlated with neuroinflammatory changes (Thirumangalakudi et al., 2008).

Our previous work demonstrated that early-life exposure to PFOS significantly increased body weight and elevated biomarkers associated with Alzheimer's disease (AD) such as amyloid precursor protein (APP), phosphorylated Tau as well as caused behavioral abnormalities in exposed mice (Basaly et al., 2021). Other work demonstrates that gestational or lactational PFOS exposure damages neuromotor development and can play a role in the emergence of Attention-Deficit / Hyperactivity Disorder (ADHD) and Autism Spectrum Disorder (ASD) (Butenhoff et al., 2009; Shin et al., 2020; Skogheim et al., 2021). Furthermore, a prospective study reported that higher baseline plasma PFAS concentrations were linked to more weight gain, particularly in women with high risk of developing metabolic disorders such as diabetes (G. Liu et al., 2018). Moreover, studies show that increased high fat diet consumption and obesity increase the risk of developing dementia (Freeman et al., 2014).

PFOS and HFD both have separate known impact on the nervous system. In addition to the potential of combinatorial effects due to the prevalence of high fat consumption among populations, a diet high in fat would be expected to alter the distribution and bioavailability of PFOS in the body. Given that there is a link between HFD, obesity, and dementia, it is imperative to clarify the relationship between early exposure to PFOS, weight gain-metabolic disorder and neurodegenerative or neurodevelopmental disorders.

The primary goal of this research is to investigate the combinatorial effects of diet and PFOS exposure on weight gain, behavioral abnormalities associated with ADHD and ASD as well as dementia. We also aim to measure the latent effect on key biomarkers of dementia in the brain of mice one year after developmental exposure to PFOS and/or a high fat diet. Further, we conducted global cerebral transcriptomic

profiling and disease specific proteomic panels screening to identify novel transcriptomic and proteomic signatures and biomarkers of neurodegeneration and neurodevelopmental disorders.

## 2. Materials and methods

### 2.1. PFOS animal exposure

Charles Rivers Laboratories (Wilmington, MA) provided pregnant CD-1 mice which arrived at the Comparative Biology Resource Center (CBRC) of the University of Rhode Island (URI) on gestation day 1. For each exposure group, an average of three dams with their litters were used (total 12 dams). Each litter belonging to each dam consisted of 6 to 8 pups. After weaning, each mouse was tracked and identified with its dam. For each experiment we took mice related to each dam at random to reach a minimum of ( $n = 6-9$ ). Mice were checked for weight and accommodated separately in an assigned space with a controlled temperature (20–26 °C), humidity (30–70%) and light/dark cycle (12:12). A high fat diet (60% kcal diet Cat# D12492, Research Diet, Inc New Brunswick, NJ) or standard chow diet (SD, Teklad Extrude global diet, 2020X) was provided ad-libitum. Dams were randomly allocated to four groups and exposed to either vehicle containing 0.5% Tween-20 in deionized water or 1 mg/kg/day PFOS (Sigma Aldrich, MO) by oral gavage of the dams during gestation to the end of lactation PND20 (Fig. 1). Pups were weaned on PND20 and maintained on either a control or HFD and euthanized on PND365. The behavior studies are conducted over period of weeks which includes acquisition trials followed by retention trials. These were done between PND330 and 365 (Fig. 1). All biomarker studies were conducted on tissues extracted on that last date. As to PFOS exposure, it was given directly to the dam from gestation day one until weaning on PND20 of the pups. The pups were getting their PFOS exposure indirectly through the dam (gestational, lactational). The level of PFOS exposure in this study was between the low adverse event level (LOAEL) dose which is 3.0 mg/kg/day and the no adverse event level (NOAEL) dose which is 0.3 mg/kg/day as per the United states Environmental Protection Agency (EPA) (Wan et al., 2014). To ensure equal lactation and reduce animal effects, neonates were randomly allocated to dams with identical treatment groups. To determine the dose, dams were checked for weight every 3–4 days. Mice were euthanized on PND360 under isoflurane. To collect the serum, whole blood left at room temperature for at least 15 min and then centrifuged at 1500 xg for 10 min at 4 °C. Brain tissues were extracted and kept at – 80 °C. The URI CBRC staff tracked the animals during the study and the Institutional Animal Care and Use Committee (IACUC) of URI approved the study protocols.

### 2.2. Barnes maze

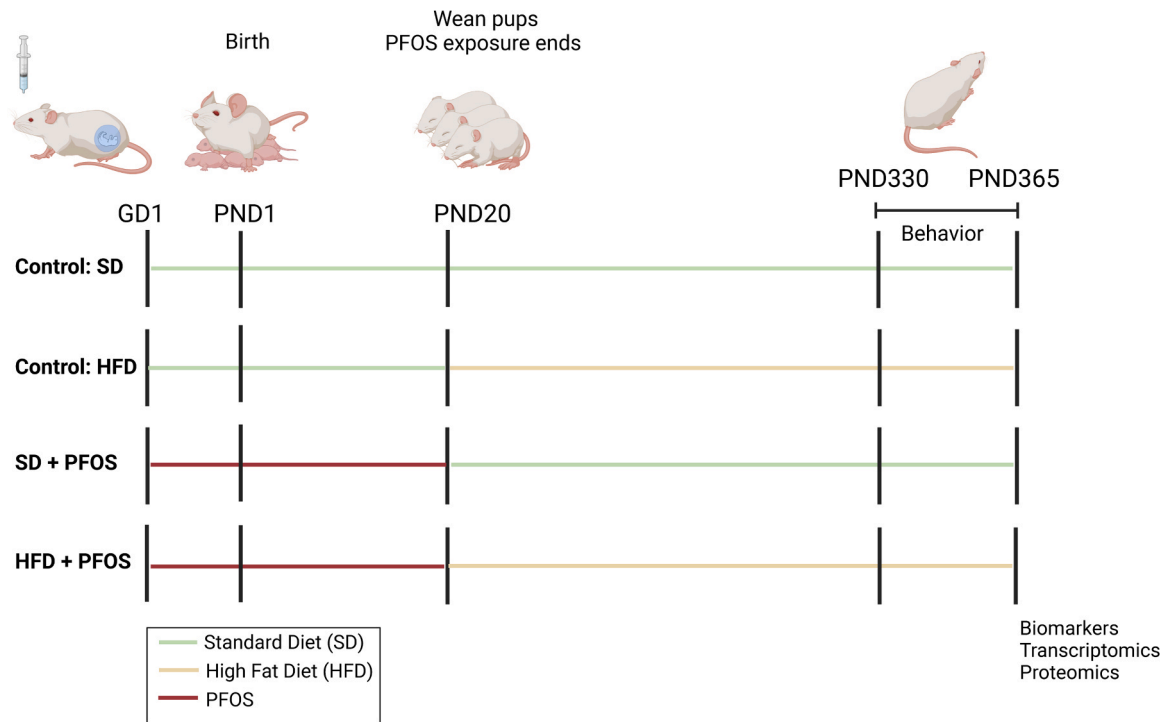
Barnes maze testing first was performed as described previously (Basaly et al., 2021). During each trial, the time it took to escape was recorded. For the probe trial, an imitation of the hole was used in place of the escape box. The memory retention test was conducted two days after the final acquisition trial, involving keeping track of the rodent's overall percentage of time spent in the target quadrant over the course of a five-minute test.

### 2.3. Open field

To evaluate the general motor activity, anxiety-like behavior and center activity, the open field test was done as described previously (Basaly et al., 2021). The number of times reared, the distance traveled and the time in the center were recorded and analyzed.

### 2.4. Western blot of the extracted protein

The brain cortex tissues from female mice were lysed using lysis



**Fig. 1.** PFOS exposure scheme for timed-pregnant CD-1 mice. Pregnant mice (GD1-GD18 or 19) and lactation until day 20 post-natal (PND20) were administered PFOS orally. Pups (males and females together) were assigned an average of 6–8 to each dam-exposure group and weaned pups separated by sex were kept two to three per cage. Full details in method section.

**Table 1**  
Primary antibodies used in Western blot analysis.

Antibody	Company	Isotype/ Source	Dilution
Anti-ApoE	Thermo Scientific, MA, USA	IgG/Rabbit	1:1000
Tau (Tau46)	Cell Signaling Technology, MA, USA	Mouse IgG1	1:1000
Phospho-Tau (Ser404) (D2Z4G)	Cell Signaling Technology, MA, USA	Rabbit IgG	1:1000
Phospho-Tau (Thr181) (D9F4G)	Cell Signaling Technology, MA, USA	Rabbit IgG	1:1000
CDK5 (D1F7M)	Cell Signaling Technology, MA, USA	Rabbit IgG	1:1000
APP (E8B3O) XP®	Cell Signaling Technology, MA, USA	Rabbit IgG	1:1000
GSK3b	Cell Signaling Technology, MA, USA	Rabbit IgG	1:1000
GAPDH (D16H11) XP®	Cell Signaling Technology, MA, USA	Rabbit IgG	1:1000
Anti-mouse IgG-HRP linked antibody	Cell Signaling Technology, MA, USA	Horse IgG	1:20000
Anti-rabbit IgG-HRP linked antibody	Cell Signaling Technology, MA, USA	Goat IgG	1:20000

buffer ( RIPA, Sigma Aldrich, MO, USA) with protease inhibitor (Thermo Scientific, MA, USA) according to the established protocol (Bihagi et al., 2018). A micro BCA protein assay (Thermos Scientific, MA, USA) was used to determine protein concentration and 20 µg of protein were analyzed using SDS-PAGE on 4–12% NuPAGE Tris Cell precast gels (Novex life technologies) and transferred onto nitrocellulose membranes using BioRad mini protean tetra cell system (BioRad, USA) for Western blotting. The membrane was blocked in 5% skimmed milk resuspended in Tris-Buffer supplemented with 0.05% Tween-20 and incubated with primary antibodies (Table 1). Secondary antibodies coupled to HRP (anti rabbit or anti mouse, Cell Signaling Technology®, MA, USA) were then used for detection. Images were taken using BioRad ChemiDoc MP Imaging System. ImageJ software was used to quantify the bands which were normalized against GAPDH.

2.5. RNA isolation and RT-qPCR

Using miRNeasy mini kit (cat. 217004, Qiagen, CA, USA), total RNA from male and female mice groups was extracted from the cerebral cortex in accordance with the manufacturer’s instruction. Nanodrop (Thermo Scientific, DE, USA) was used to check the integrity and the concentration of the obtained RNA. Complementary DNA was synthesized using 1.5 µg of RNA and the Reverse Transcription kit (High-Capacity cDNA, Applied Biosystems, CA). The qPCR reactions were done using the RNA extracted from female mice with Applied Biosystem 7500 fast Real time PCR system and the SYBER green PCR master mix (Applied Biosystem, CA). The list of the sense and antisense primers is shown in Table 2. The  $2^{-\Delta\Delta CT}$  method was used to report the expression of mRNA with GAPDH as intrinsic control.

**Table 2**  
List of forward and reverse primers that were used for qPCR analysis.

Gene	Sense Strand	Anti-Sense Strand
Mouse TAU	5-CCTGAGCAAAGTGACCTCC AAG-3	5-CAAGGAGCCAATCTTCGACTGG-3
Mouse GAPDH	5-TGGTGA AGCAGGCATCTGAG-3	5-TGCTGTGTG AAGTCGCAGGAG-3

## 2.6. RNA seq analysis

Cerebral cortex tissues were used for total RNA isolation as mentioned above. The quality and concentration of the RNA were subsequently checked by NanoDrop spectrophotometer (Cat. ND-ONEC-W, ThermoFisher Scientific, Waltham, MA). For the below steps, a mixed sample of male and female was used. The iScript cDNA synthesis kit (cat. 1708891, BioRad, Hercules, CA) was used to generate the cDNA from 0.5 µg of RNA. The library was prepared using TruSeq Stranded mRNA kit (Cat. 20020594) from illumina adhering to the manufacturer's guidelines and mRNA molecules were purified and then fragmented. The cleaved RNA was used for synthesis of the cDNA using random priming during first and second strand synthesis. The DNA was amplified after barcode DNA adapters ligation to both ends. Libraries that passed quality control and sequenced at least 20 million paired end reads (2×75bp) per sample on an Illumina HiSeq4000. The sequences are trimmed, aligned and quantified using the Galaxy platform starting with the fastq files (Afgan et al., 2018). The trimming of the end reads with cutAdapt (Martin, 2011) using default parameter settings. The alignment of the reads was done with HiSAT2 (Kim, Langmead, and Salzberg, 2015) to mouse reference genome GRCm38/mm10 with default parameter settings. We employed MetaboAnalyst 5.0 for differential expression analysis (Pang et al., 2021), genes were referred to as differentially expressed with p values < 0.1 and fold change of 1.3. Pathway analysis was determined by String software (von Mering et al., 2003). For clustering the enriched pathways, we used David application (Sherman et al., 2022). iDEP.96 was used to identify the enriched pathways by biclustering application (Ge, Son, and Yao, 2018). The method analyzes DEGs using the biclustering method (biclust R

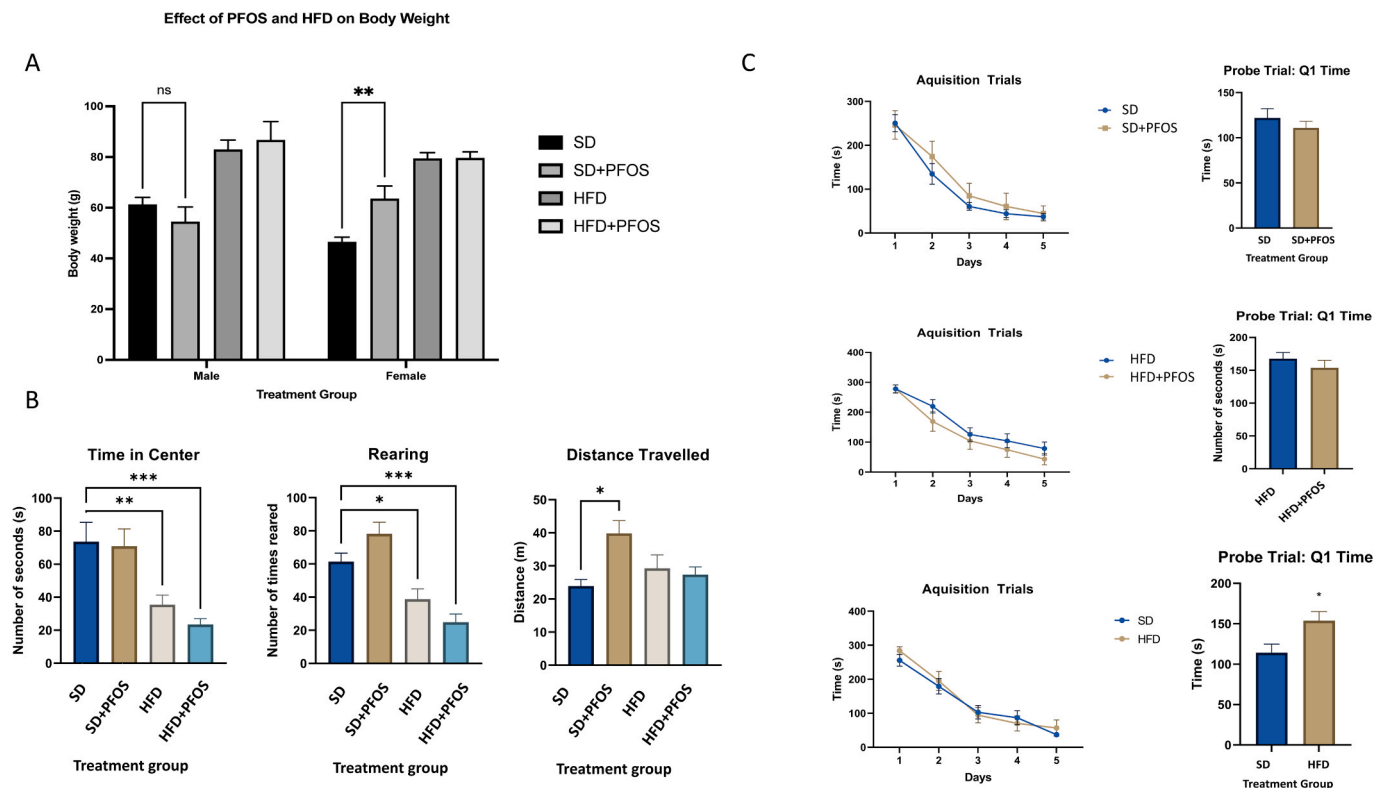
package). This is different from traditional clustering methods where correlation is defined across all samples.

## 2.7. Olink proteomic assays

The Olink Target 96 Mouse Exploratory (92) panel was used to perform the Olink experiment at Olink certified proteomics core facility of our institute. A mixed sample of male and female was analyzed with the Olink proximity extension assays (PEA), 92-plex immunoassay (Uppsala, Sweden). Quality control and data normalization were done by Normalized Protein eXpression (NPX) software. All runs were validated by Olink, Uppsala support team. Starting with the pre-processed NPX data, protein differential expression analysis was performed using a metaboanalyst tool with t-test comparison (Pang et al., 2021). Proteins p-values < 0.05 were called differentially expressed.

## 2.8. Statistical and Bioinformatic analysis

The statistical analysis was done utilizing GraphPad Prism 8.0 computer software (La Jolla, CA, USA). The mean and standard error of the mean (SEM) were used to represent the results of the different exposed groups compared to the control. One-way analysis of variance (ANOVA) or a parametric two tailed unpaired t test with Welch's correction was investigated taken in account that p are significant. The acquisition trials results were analyzed using two-way ANOVA, with a p-value of 0.05 considered statistically significant.



**Fig. 2.** Body and behavioral changes in control and PFOS- and HFD-exposed mice. (A) Body weight. Body weights of each sex were measured daily in each exposure group and mice were sacrificed on PND365. Results are expressed as mean  $\pm$  SEM using a one-way ANOVA; \* $p$  < 0.05, was considered significant. On PND 330–365, behavior, learning, and memory retention were evaluated on animal groups of both males and females combined ( $n$  = 6–9). (B) For the open field test, the time spent in the center, the time of rearing and the distance traveled were recorded. Results are expressed as mean  $\pm$  SEM and using a one-way ANOVA test to determine the statistical significance. (C) Barnes maze tests of male and female mice to compare the effect between the different mice groups for cognitive impairment or locomotor differences by acquisition trials and probe trials. The probe trials were analyzed using T-test with welch's correction and the acquisition trials were analyzed using two-way ANOVA.



### 3. Results

#### 3.1. Effects on body weight and behavior of developmental PFOS exposure

While male mice exposed to PFOS did not noticeably change in body weight, female mice that were exposed to PFOS during development had significantly gained weight in middle age (Fig. 2A). In open field test analyses, PFOS exposed mice exhibited changes in distance travelled (Fig. 2B). PFOS alone significantly increased the distances travelled by mice, and the addition of HFD alleviated this effect (Fig. 2B). HFD significantly reduced time spent in center and rearing (Fig. 2B). The combination of HFD and PFOS exposure appears to exacerbate this effect (Fig. 2B). Barnes Maze analysis did not show any significant cognitive changes in the presence of PFOS; however, exposure to HFD alone resulted in memory loss (Fig. 2C).

#### 3.2. Effects on the expression of total Tau and phosphorylated Tau (pTau) in female mice

While we examined tissues derived from both male and females, we only found consistent significant results and trends in female mice. Western blot analyses showed significant differences in total Tau and pTau (S404 and T181) between the control SD and other groups (SD + PFOS, HFD, and HFD + PFOS groups) (Fig. 3A and B). A strong trend in increased pTau (T181) was also observed (Fig. 3C). The ratio of pTau to total Tau showed no significant differences between groups (Sup-Fig. 1). Tau mRNA levels measured by qPCR revealed a trend of increasing Tau mRNA levels in the HFD + PFOS group compared to other groups (Fig. 3D).

#### 3.3. Effects on protein levels of AD biomarkers (APP, kinases, and total ApoE) in female mice

We looked at the impact of PFOS exposure on AD biomarkers associated with the amyloidogenic pathway. APP protein levels did not show any significant alterations within the different groups (Fig. 4A). Likewise, there was no significant change in CDK5 protein expression

(Fig. 4B). Western blot analysis also showed no significant change in total ApoE protein levels in the cortices of exposed mice compared to the control group (Fig. 4C). Antibodies directed against GSK3 $\beta$  showed no significant change in the protein expression levels between the groups (Fig. 4D).

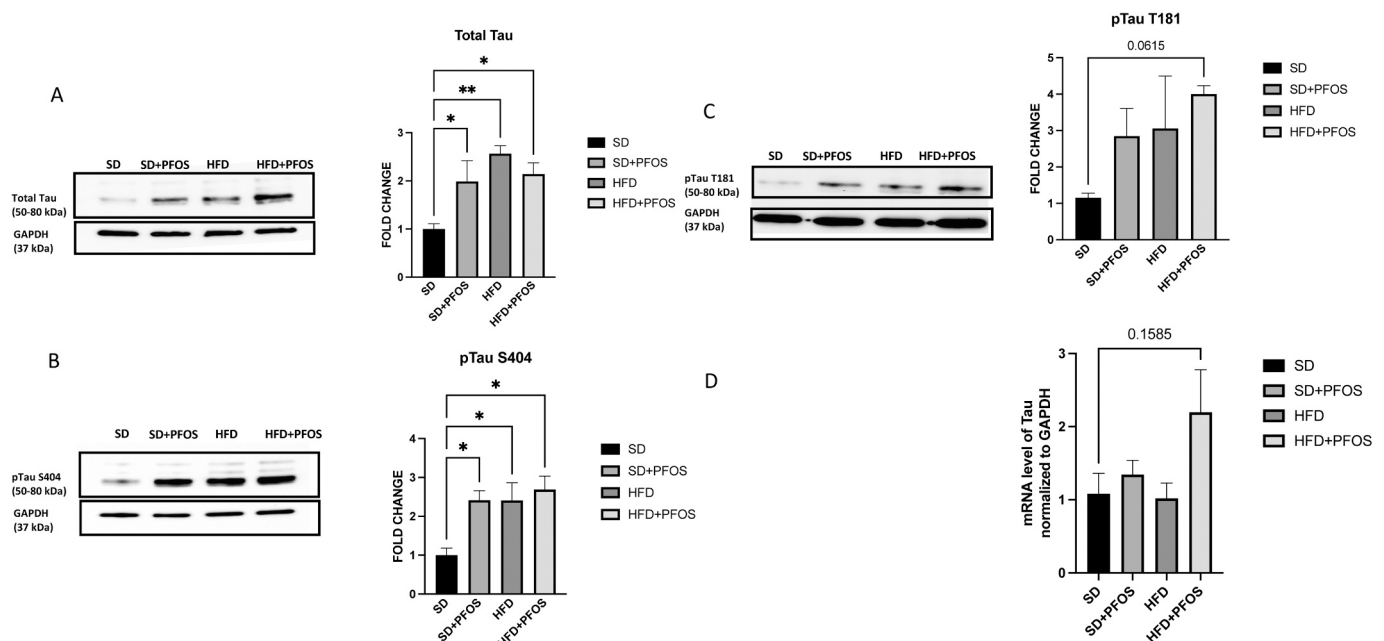
#### 3.4. Gene expression and transcriptomic analysis

Whole brain tissue from the four different groups of both male and female mice were collected after euthanasia and RNA were extracted from cerebral cortices for transcriptomic analysis. The sequences were analyzed using Metaboanalyst 5.0 (Pang et al., 2021). Principal component analysis (PCA) revealed a consistent outlier of two samples (sample 4 from SD group and sample 7 from HFD group) which were excluded from further analysis. The principal component 1 showed 53.6% and the second 14.3% of variance (Sup-Fig. 2A).

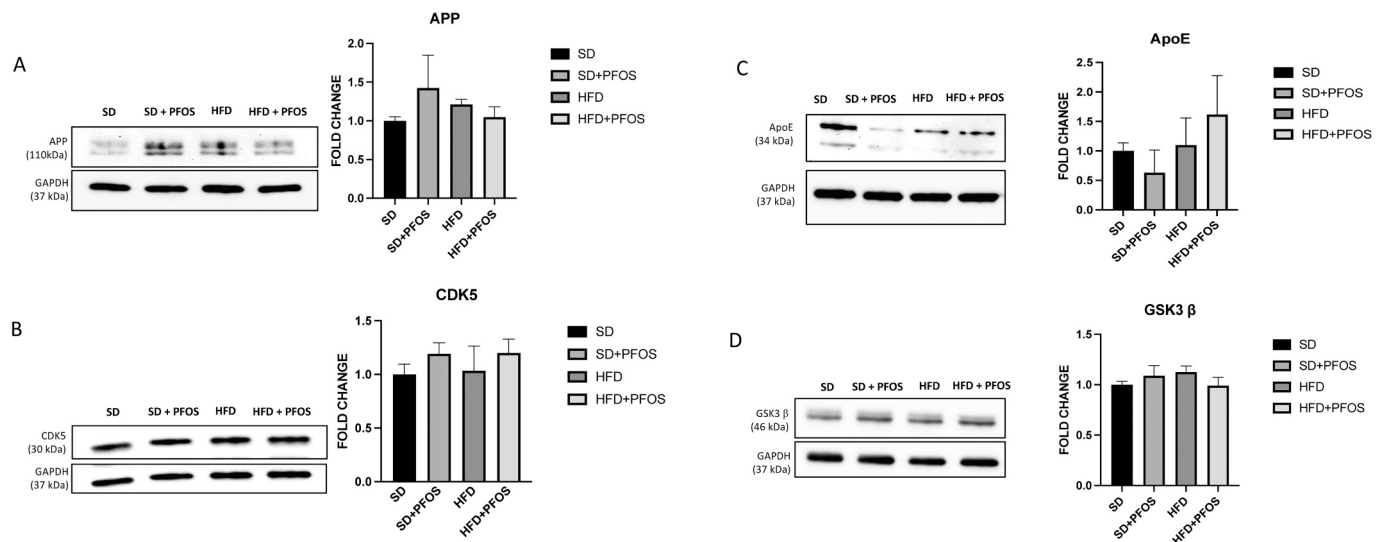
The volcano plot generated from the comparison between the groups of SD vs PFOS revealed 107 significant differentially expressed genes (DEG) (67 downregulated and 40 upregulated with (Fig. 5). DEG generated from the comparison between SD vs HFD+PFOS revealed different profiles with 70 DEGs with more upregulated DEGs (only 9 genes downregulated and 61 upregulated). The comparison between HFD vs HFD+PFOS revealed a low number of DEG (7 downregulated and 13 upregulated). SD vs HFD comparison showed 27 DEGs with 21 upregulated and 6 downregulated. Venn diagrams resulting from the four comparisons showed only 15 shared DEGs between SD vs PFOS and SD vs HFD + PFOS. Only 2 shared DEG between HFD vs HFD+PFOS and SD vs HFD+PFOS (Sup-Fig. 2B).

#### 3.5. The functional contribution of DEGs

The comparison of SD vs SD + PFOS revealed different enriched pathways related to neuronal projection, somatodendritic compartment, synapse, presynaptic and postsynaptic compartments (S-Table 1A-B). Enriched pathways related to nervous system development, regulation of cellular and molecular function, and synaptic signaling were found. The pathways related to the behavior, learning, and calcium-ion regulated exocytosis were revealed in the analysis (S-Table 1A-B).



**Fig. 3.** Protein and mRNA Tau levels at PND365 in the cortex of female CD-1 mice developmentally exposed to PFOS. (A-C) Blots of representative samples ( $n = 4$ ) and quantification of Total Tau (A), pTau S404 (B) and pTau T181 (C) protein levels with GAPDH normalization. (D) Tau mRNA levels normalized to GAPDH. Results are expressed as mean  $\pm$  S.E.M using multiple comparisons ordinary to the one-way ANOVA. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  was considered significant.



**Fig. 4.** Biomarkers of dementia measured on PND365 in the cortex of female CD-1 mice developmentally exposed to PFOS. Blots of representative samples ( $n = 4$ ) and quantification of protein levels normalized to GAPDH. (A) APP, (B) CDK5, (C) ApoE, (D) GSK3 $\beta$ . Results expressed as mean of fold change  $\pm$  SEM using a one-way ANOVA.

The different enriched pathways detected were grouped into 6 clusters according to their enrichment score (S-Table 2A) and plotted in histogram based on their fold enrichment and gene count (Fig. 6). The first enriched cluster is related to the calcium signaling pathway and calmodulin binding. The second cluster is related to MAPK and Ras signaling pathways. The third enriched cluster is related to regulation of ion and sodium transmembrane transport. The remaining clusters were related to DNA binding for positive transcription from RNA polymerase II promoter and to phosphorylation.

The comparison of SD vs HFD+PFOS revealed a higher number of pathways involved in the cell surface, neuron projection, cytoplasm, plasma membrane, and endoplasmic reticulum (ER). The pathways are related to abnormal neuron and cell differentiation, and abnormal neuron morphology (S-Table 1C). GO-enriched pathways revealed the regulation of cellular morphogenesis and processes involved in the differentiation of neurons and regulation of axon genesis. GO pathways related to behavior learning, growth regulation, and potassium ion transport were also impacted (S-Table 1D). The different enriched pathways detected are clustered in mainly six clusters using DAVID according to their enrichment score (S-Table 2B). Cluster 1 included positive regulation of cell migration, negative regulation of axon extension and guidance, negative regulation of axon extension involved in axon guidance, and semaphorin-plexin signaling pathways. Cluster 2 included pathways involved in protein processing in the ER. In cluster 3, pathways related to ion transport and potassium voltage-gated ion channel transmembrane activity were revealed. Cluster 4 included PI3K-Akt, Ras and MAPK signaling pathways. Clusters 5 and 6 included pathways for protein kinase activity and phosphorylation, and the regulation of transcription from RNA polymerase II promoter, respectively (Fig. 6 and S-Table 2B).

Due to the low number of DEGs between HFD vs HFD + PFOS and SD vs HFD, no enriched pathways were determined. However, HFD vs HFD + PFOS revealed DEGs that play a role in the ER, cytosol, and cytoplasmic compartments.

To detect groups of genes that are correlated among a subset of samples, we used iDEP 96. The most enriched genes, cluster 1, represents 872 genes that are correlated across the 20 samples. The pathways detected revealed neuropeptide signaling, cell-cell signaling, organ morphogenesis, development, behavior, ion and cation homeostasis (Table 3). KEGG pathways detected are mostly related to neuroactive ligand-receptor interaction and calcium signaling pathways. A significant number of genes related to cell adhesion molecules, cAMP signaling

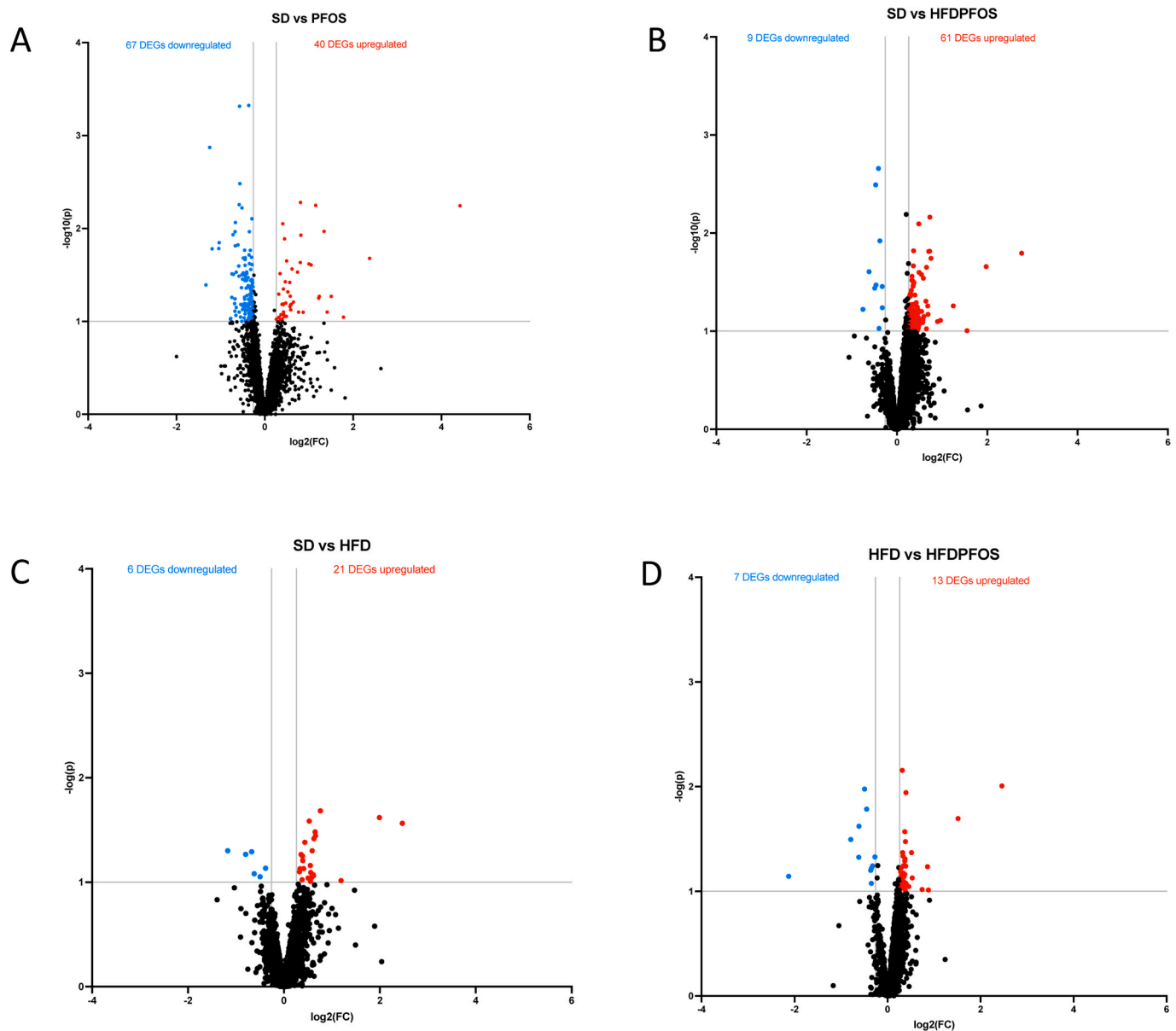
pathways and autoimmune thyroid disease were also found. The GO molecular function pathways are mostly G protein-coupled peptide receptor activity, DNA-binding transcription factor activity and ligand-gated ion channel activity.

### 3.6. Differential Expressed proteins determined from Olink analysis

Olink analysis was done on tissues derived from both male and female mice. Principal component analysis (PCA) revealed a consistent outlier of one sample from the HFD + PFOS group which was excluded from further analysis. The principal component showed 27% and the second 18.4% of variance (Sup-Fig. 3A). The differentially expressed proteins (DEP) are summarized in the Table 3 and Sup-Fig. 3B). Out of a panel of 92 protein biomarkers, 5 proteins were significantly differentially expressed between SD vs SD + PFOS groups (S-Table 3A). The comparison between SD vs HFD + PFOS revealed 14 DEPs with 2 in common with the SD vs SD + PFOS comparison. HFD vs HFD + PFOS revealed 9 DEPs where 3 are common with SD vs HFD + PFOS. Ten proteins were differentially expressed between SD vs HFD. NTF3 protein (Neurotrophin-3) that controls survival and differentiation of mammalian neurons and promotes the survival of visceral and proprioceptive sensory neurons was detected both in SD vs SD + PFOS and SD vs HFD + PFOS. LPL (lipoprotein lipase) were found in the three comparisons (SD vs HFD, SD vs SD + PFOS and SD vs HFD + PFOS) (Sup Fig. 3C).

### 3.7. The impact of DEPs on biological functions

The functional contribution of DEPs determined by the analysis of GO and KEGG pathways is summarized in supplementary S-Table 3 (B-E) and the pathway histogram analysis in Fig. 7. DEPs from SD vs SD + PFOS are related to inflammatory pathways (inflammatory response, cytokine-cytokine receptor interaction) and regulation of peptidyl-tyrosine phosphorylation. A higher number of pathways was revealed in SD vs HFD + PFOS and clustered in 4 main clusters (S-Table 3C). The pathways are mainly related to the regulation of gene expression, positive regulation of cell migration, inflammatory response, cytokine-cytokine receptor interaction and chemotaxis, positive regulation of cell migration and proliferation. The implication of MAPK signaling pathway, positive regulation of ERK1 and ERK2 cascade and PI3K-Akt signaling pathway were revealed. The DEPs from SD vs HFD comparison contribute mainly to inflammatory response with positive response to chemokine and interleukins implicated in JAK-STAT and ERK1 and



**Fig. 5.** Differential gene expression on PND365 of both male and female mice combined. (A-D) Volcano plot showing differentially expressed genes (DEG) for (A) between SD vs PFOS, (B) between SD vs HFD +PFOS, (C) for HFD vs HFD +PFOS and (D) for HFD vs HFD+PFOS. The up-regulated genes (red), and the down-regulated genes (blue) with a fold change of 1.3, and with  $P < 0.1$ , ( $n = 4-9$ ).

ERK2 cascades. The HFD vs HFD + PFOS DEPs contribute also to inflammatory response, pathways of regulation of cell proliferation, dendrite regeneration and axon guidance with Jak-STAT signaling. The enriched protein sets with bicluster tool of iDEP (Table 3) revealed that 92 DEPs correlated across the 30 samples confirming the functional contribution of the DEPs where pathways related to cellular response to chemical and growth factor stimuli, cell differentiation, cell surface receptor signaling, and positive regulation of protein phosphorylation pathways were revealed (Table 3). KEGG analysis using biclustering revealed MAPK signaling pathways and cytokine-cytokine receptor interaction. Pathways related to cancer and lipid atherosclerosis were also found in KEGG with a significant number of genes (Table 3).

#### 4. Discussion

It has been documented that PFOS has a developmental impact on brain function, behavior, and memory (Long et al., 2013; Wang et al., 2015; Sun et al., 2019; Ninomiya et al., 2022). Some studies suggested

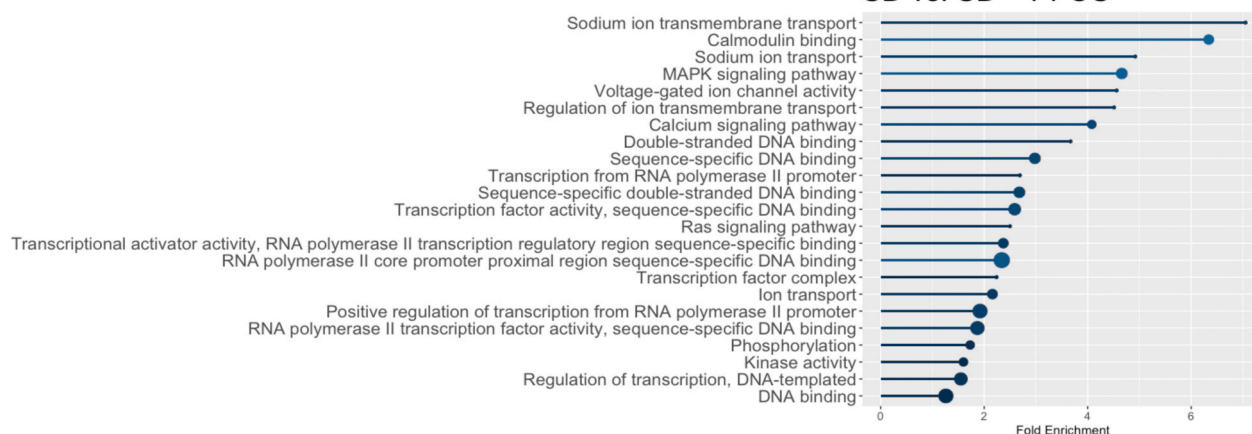
that PFOS exposure during development increased the level of Tau in mice (Johansson et al., 2009) and increased AD pathological indicators in rats (Zhang et al., 2016).

We explored in the present work the combinatorial impact of PFOS exposure and a HFD on brain function by analyzing changes in behavioral outcomes. We also performed transcriptomic analysis, proteomic profiling, and measurements of AD biomarkers. Total body and brain weights were assessed on adult mice that were developmentally exposed to PFOS and/or sustained on a HFD. A year after the end of PFOS exposure, female mice exhibited increased body weight (Fig. 2A). This suggested that developmental PFOS exposure can result in the reprogramming of metabolic pathways that may lead to weight gain later life. These results are consistent with recent data showing that PFOS may disrupt human body weight regulation and metabolism (Maisonnet et al., 2012; G. Liu et al., 2018; Christou et al., 2021); however, it is not clear why females were selectively affected.

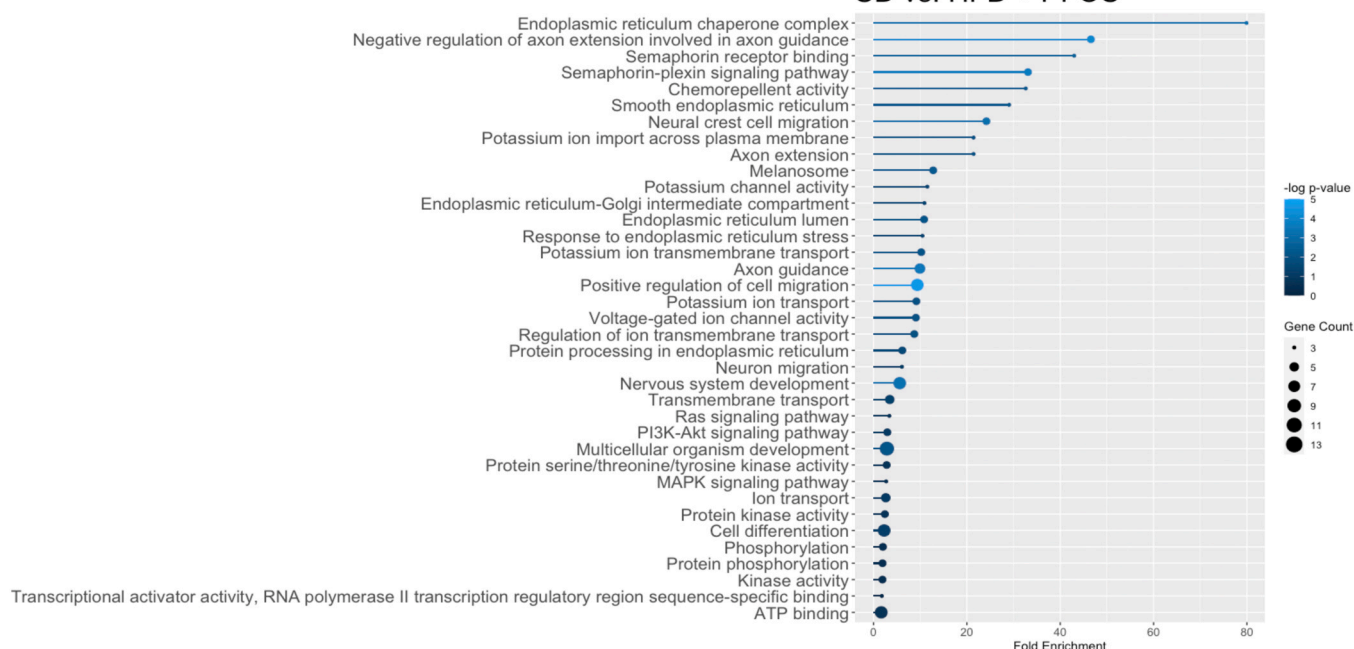
In the open field behavioral test of both male and female mice combined, PFOS-exposed mice exhibited changes in distance travelled



## SD vs. SD + PFOS



## SD vs. HFD + PFOS



**Fig. 6.** Histogram of the enriched GO and KEGG pathways of DEGs. Y-axis, pathway categories; X-axis, statistical significance of the enrichment. With p value and Gene count mentioned in color and oval dots. ‘Log p-value’ is the log 10 of the p-value. ‘Gene count’ is the number of genes enriched in a pathway.

(Fig. 2B). HFD significantly reduced time spent in center and rearing (Fig. 2B). The combination of HFD and PFOS exposure appears to exacerbate these effects (Fig. 2B). The HFD alone or in combination with PFOS significantly decreased rearing and time in center (Fig. 2B). These results confirm that a HFD affects behavior and aggravates cognitive tasks in mice but appears to be a significant contributor to neuromotor modulation and thus maybe a contributing environmental risk factor for the development of ASD and ADHD (Skogheim et al., 2021).

Our current study supports previous findings that PFOS and HFD increase Tau protein expression and phosphorylation in different regions in the brain of female mice (Fig. 3) (Johansson et al., 2009; Zhang et al., 2016; Basaly et al., 2021). The protein tau has been shown to play a key role in cognitive decline in dementia patients (Bejanin et al., 2017). Although PFOS alone does not impact memory functions in these mice, we expect that it may do so if the mice were aged further. It is important to note that in this experiment there was no significant change in biomarkers associated with the amyloid pathway.

The functional contribution of the DEGs revealed by comparing the transcriptomic profiles of SD vs PFOS groups displayed high enrichment

of calcium signaling pathways that may subsequently result in the disruption of MAPK signaling pathways (Fig. 6 and Table 3). The effects of PFOS on calcium signaling have been reported before (Christou et al., 2020; Lee et al., 2021; Min et al., 2023). Both calcium and MAPK signaling dysregulation are associated with weight gain, which may explain the observed changes in body weight in female mice (Fig. 2A) (Song et al., 2019; Gao et al., 2021); however, it is not clear why male mice did not gain weight. MAPK signaling also plays a role in learning, memory and behavior (Jeanneteau and Deinhardt, 2011; Thomas and Haganir, 2004). SD vs PFOS comparison also revealed pathways related to voltage-gated ion channels and regulation of ion transport (Fig. 6) which supports the idea that PFOS may change membrane surface potential as one of the mechanisms that may lead to neurotoxicity (Harada et al., 2005). The combination of HFD with PFOS seems to increase the number of DEGs and enhances the effect of PFOS in the brain by drawing new pathways related to ER stress, axon guidance and extension, and neural migration (Fig. 6), which aligns with the observed alterations in Tau protein expression and phosphorylation (Fig. 3). These pathways are consistent with a reprogramming that could lead to disturbed neurodevelopment, neuromotor disorders, and behavioral changes as

**Table 3**  
GO and KEGG pathways generated from DEGs and DEP using iDEP biclustering application.

Pathways generated from DEGs using iDEP biclustering			Pathways generated from DEPs using iDEP biclustering		
GO					
adj.Pval	Genes	Pathways	adj.Pval	Genes	Pathways
1.40E-12	26	Neuropeptide signaling pathway	3.10E-23	52	Cell surface receptor signaling pathway
3.10E-12	87	Animal organ morphogenesis	1.40E-19	52	Cellular response to chemical stimulus
9.80E-11	246	System development	2.20E-19	47	Cellular response to organic substance
3.30E-10	195	Animal organ development	2.20E-16	59	System development
3.60E-10	157	Anatomical structure morphogenesis	3.50E-16	50	Response to organic substance
3.60E-10	64	Behavior	3.50E-16	44	Regulation of developmental process
4.10E-10	69	Ion homeostasis	9.60E-16	17	Positive regulation of peptidyl-tyrosine phosphorylation
4.60E-10	103	Cell-cell signaling	2.70E-15	25	Cellular response to growth factor stimulus
5.00E-10	44	Regulation of cytosolic calcium ion concentration	3.60E-15	26	Positive regulation of protein phosphorylation
1.40E-09	24	Feeding behavior	4.90E-15	50	Regulation of response to stimulus
1.40E-09	40	Positive regulation of cytosolic calcium ion concentration	5.10E-15	25	Response to growth factor
5.00E-09	63	Cation homeostasis	1.90E-14	43	Regulation of multicellular organismal process
5.80E-09	195	Regulation of biological quality	2.50E-14	46	Regulation of cell communication
8.40E-09	63	Inorganic ion homeostasis	2.50E-14	46	Regulation of signaling
8.40E-09	152	Regulation of multicellular organismal process	2.50E-14	53	Cell differentiation
KEGG					
9.90E-25	63	Neuroactive ligand-receptor interaction	5.50E-23	24	Cytokine-cytokine receptor interaction
4.00E-04	23	Calcium signaling pathway	6.90E-14	12	Rheumatoid arthritis
1.30E-03	14	Staphylococcus aureus infection	1.40E-12	12	TNF signaling pathway
1.50E-03	9	Allograft rejection	1.70E-12	10	Inflammatory bowel disease
1.50E-03	9	Graft-versus-host disease	2.30E-12	11	IL-17 signaling pathway
2.00E-03	10	Autoimmune thyroid disease	1.80E-11	15	MAPK signaling pathway
2.40E-03	19	CAMP signaling pathway	2.30E-09	9	Viral protein interaction with cytokine and cytokine receptor
2.60E-03	9	Type I diabetes mellitus	4.70E-09	9	AGE-RAGE signaling pathway in diabetic complications
2.60E-03	16	Phagosome	5.00E-09	9	Chagas disease
4.10E-03	15	Cell adhesion molecules	6.50E-09	16	Pathways in cancer
			2.80E-08	7	Malaria
			3.90E-08	8	Hematopoietic cell lineage
			5.00E-08	9	Alcoholic liver disease
			9.50E-08	8	Amoebiasis
			1.50E-07	10	Lipid and atherosclerosis

evidenced by the open field test results (Fig. 2B). Moreover, the dysregulation of ion channel activity, immune system dysfunction, and increased Tau phosphorylation have been linked to ASD (Aluko et al., 2021; Gąsowska-Dobrowolska et al., 2021; Pourtavakoli and Ghafouri-Fard, 2022; Robinson-Agramonte et al., 2022). Furthermore, modifications in rearing as repetitive behavior and distance travelled as hyperactivity behaviors were also previously considered as ASD behavior markers in mice (Choi et al., 2015; Hisaoka et al., 2018). Further studies are needed to examine the link between PFOS and specific ASD and ADHD biomarkers.

The proteomic analysis of brain tissue was derived from both male and female mice. Olink Target 96 Mouse exploratory panel was used and it includes protein biomarkers involved in critical biological processes like cellular regulation, development, signal transduction, and stress responses revealed that PFOS has immunomodulatory potential by affecting cell surface receptor signaling pathways and cytokine-cytokine receptor interaction (Fig. 7). The pro-inflammatory pathways like TNF signaling and IL-17 signaling pathways were also altered confirming that PFOS affects the immune system as previously reported (Ehrlich et al., 2023). MAPK signaling pathway which was revealed in both transcriptomic and proteomic analyses agrees with previous reports that PFOS has an effect on developmental processes and subsequently leads to modifications of learning and behavior (Fang et al., 2009). A large number of genes implicated in the cellular response to chemical stimulus were detected. It is also important to mention that fatty acid metabolism has been proposed to be an essential factor affecting inflammation and cancers (Attané et al., 2020; P. Liu et al., 2021; Xu et al., 2021). The results obtained herein suggest that PFOS along with HFD exposure had a synergistic effect and deregulated a panel of pathways related to neuropeptide signaling, cell differentiation and development, proinflammatory pathways and ion homeostasis. The calcium signaling

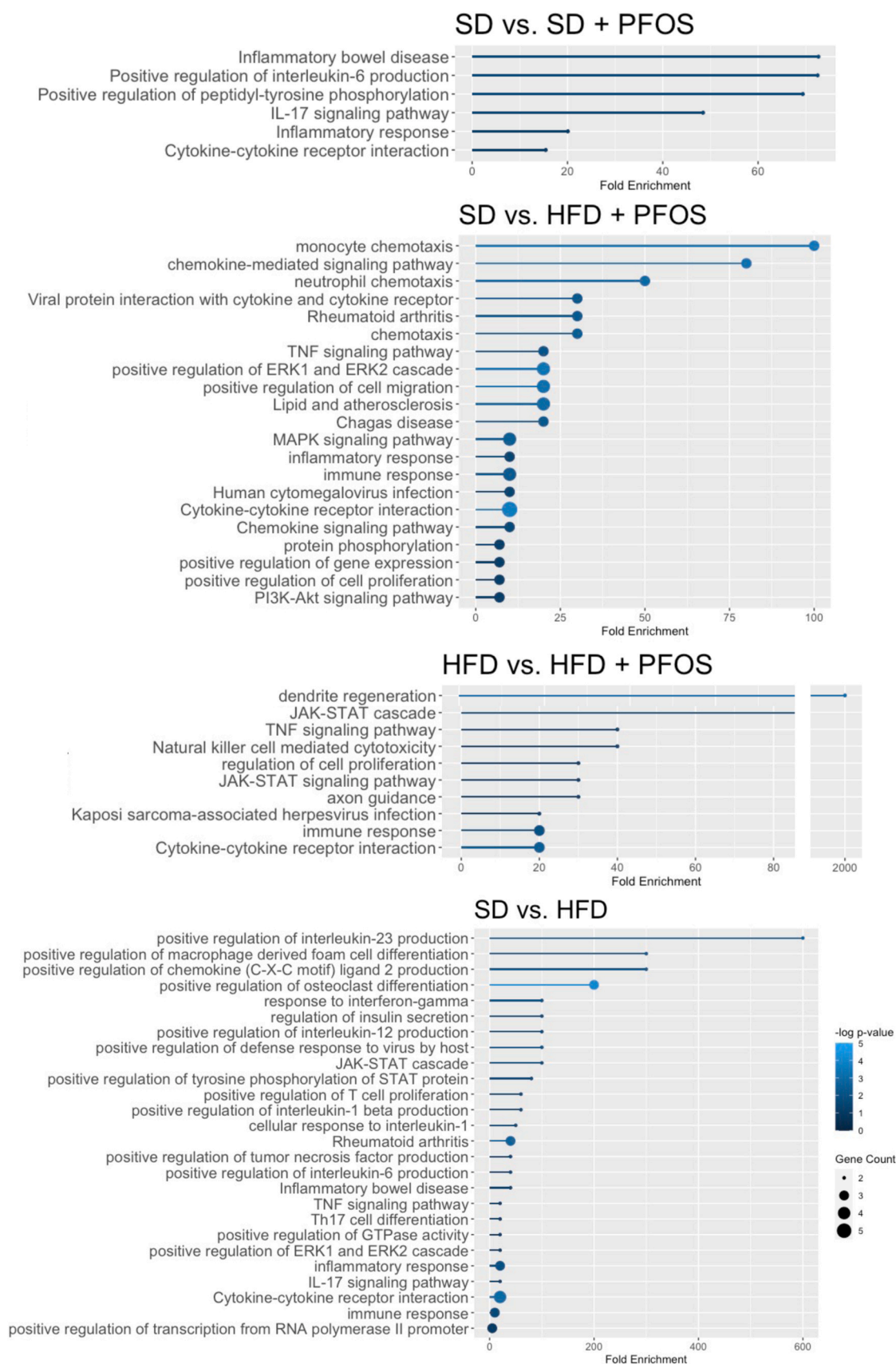
pathway is strongly affected which directly affects the downstream MAPK pathway responsible for learning, memory, and behavior.

In summary, exposure of PFOS +/- HFD promoted repetitive behavior and hyperactivity as well as social withdrawal. There is significant effect on protein biomarkers such as Tau (in female mice) and signaling pathways associated with neuromotor functions such as calcium, MAPK signaling and developmental process. These behavioral, biochemical, transcriptomic and proteomic profiles suggest that PFOS augmented by HFD may be risk factors for ASD and ADHD as well as dementia. Further studies are needed to delineate the mechanisms involved and to define the risks.

It is important to note that this work is not without limitations. There may be sex differences in the effects of PFOS and HFD, however, this study in large part combined both sexes for behavior and all global transcriptomic and proteomic analysis, while, western blots studies only detected significant changes in female mice. Additionally, it's possible that the Barnes maze was not sensitive enough to detect learning and memory impairment in this model thus other cognitive tests should be used in the future. Lastly, probing specific markers of ASD and ADHD would provide strong evidence for the link between PFOS, HFD and those disorders. Future studies should focus more specifically on those pathways and biomarkers.

**CRedit authorship contribution statement**

**Sondhi Anya:** Methodology. **Slitt Angela L.:** Methodology. **Bihaqi Syed Waseem:** Investigation. **Marques Emily:** Methodology. **Modaresi Sayed Mohamad Sadegh:** Formal analysis. **Ouararhni Khalid:** Methodology. **Abedsselem Houari:** Methodology. **Hill Jaunetta:** Methodology, Formal analysis, Conceptualization. **Shalaby Karim E:** Formal analysis. **Zawia Nasser H.:** Writing – review & editing, Supervision,



**Fig. 7.** Histogram of the enriched GO and KEGG pathways of DEPs derived from tissues obtained from both male and female mice. Y-axis, pathway categories; X-axis, statistical significance of the enrichment. With p value and Gene count mentioned in color and oval dots.



Methodology, Investigation, Funding acquisition, Conceptualization. **HMILA Issam:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. Nasser Zawia reports financial support was provided by Qatar Biomedical Research Institute. Nasser Zawia reports a relationship with National Institutes of Health that includes: funding grants.

## Data availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2024.116070](https://doi.org/10.1016/j.ecoenv.2024.116070).

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