Transcriptomics effects of per- and polyfluorinated alkyl substances in differentiated neuronal cells

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Abstract:

Per- and polyfluorinated alkyl substances (PFAS) are ubiquitous in most environments, accumulate in several tissues, and can adversely affect human health. PFAS have been implicated in neurodegenerative and behavioral disorders. However, the mechanisms through which PFAS accumulation in neurons affects biological function remain unknown. In this study, SH-SY5Y neuroblastoma cells were used to investigate how perfluorooctanoic acid (PFOA), perfluorooctansulfonic acid (PFOS), perfluorodecanoic acid (PFDA) perfluorodecanesulfonic acid (PFDS), 8:2 fluorotelomer sulfate (8:2 FTS) and 8:2 fluorotelomer alcohol (8:2 FTOH) exposures influence neuronal health. After a 30 μM-, 24-hour exposure, cells accumulated up to 800 ng PFAS/mg protein. Transcriptomics analysis of control and PFAS-exposed cells revealed 721 genes were differentially expressed across six treatments ($p_{adj} < 0.05$). Eleven of these differentially expressed genes were observed for all treatments, suggesting that these genes are potential markers for neuronal PFAS exposure. In PFOA-treated cells, we observed multiple downregulated genes are enriched for functions related to synaptic growth and neural function. In contrast, upregulated genes in PFOS, PFDS, FTS, and FTOH-treated cells showed enrichment in functions related to response to hypoxia and amino acid metabolism. Overall, our results highlight specific biological processes that potentially underlie negative effects of PFAS on neuronal health.

Keywords: PFAS, Biomarker, RNA-seq, Neuronal differentiation, neurotoxicity

Synopsis:

Per- and poly fluorinated alkyl substances (PFAS) have been shown to bioaccumulate in human tissues and affect health. This study aims to provide insight into the specific biological processes by which PFAS exposure affects neuronal cells.

INTRODUCTION

Per- and poly fluorinated alkyl substances (PFAS) are a class of chemical contaminants of global concern. PFAS has been commonly studied in recent years; however, the environmental burden and human health impact of these compounds are still not fully understood. A recent study in the United States (US) that showed detectable levels of PFAS in serum samples from most participants (97%) aged 12-80 years old., highlighted the ubiquitous presence of these compounds in the environment and the risk of human exposure.[1] Monitoring efforts in the past few decades resulted in some positive trends towards phasing out certain legacy PFAS, such as perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS).[2, 3] However, the US Environmental Protection Agency (EPA) reports that tens of thousands of tons of PFAS-containing materials are still produced globally each year.[4] These include linear or branched alkyl short-chain PFAS, which are fully or partially fluorinated with ether, alcohol, sulfates, or carboxylate headgroups, to give a few examples.[5]

PFAS exposure is particular relevant to epidemiology of humans because of these molecules' inherent chemical stability[6, 7] and fat solubility[8]. A study in mice found that radiolabeled PFOA, perfluorohexanoic acid (PFHxA) and perfluorobutanoic acid (PFBA), were detected four hours after administration in every organ, including the brain.[9] Further, the extended serum half-lives of PFAS (months to a year for short chain[10] – up to 5 years for long chains[11]) lead to bioaccumulation of PFAS in humans after exposure. While, certain professions are at an increased risk for PFAS exposure including firefighters, industrial workers, ski board waxers, general communities are also at risk from volatile PFAS, and the ones found in our water sources, food packing and cosmetics.[12] For example, autopsy tissue from 20 donors in Spain identified PFAS of various chain lengths and headgroups in the lung, kidney, liver, brain, and bone.[13] PFAS are also routinely detected in human blood samples and in breast milk, resulting in early exposure of infants.[14]

PFAS affect endocrine system[15, 16], disrupting menstrual cycle, decreasing sperm mobility, and lowering the age of onset puberty.[17] The liver accumulation of PFAS is common, and hepatoxicity has been observed in human studies.[18, 19] The exact mechanism of liver toxicity is unknown but PFOS/PFOA exposures have been shown to trigger proliferator activated receptor alpha (PPAR-α).[20, 21] Certain cancers such as liver, prostrate or testicular cancer have been linked to PFAS mediated PPAR-α disruption and epigenetic changes.[22, 23]

PFAS is also detected in brain tissue and cerebrospinal fluid.[24, 25] Perfluorocarboxylates and perfluorosulfates with 6-14 carbon chains were detected in rodents, fish, and bears, with the longer chain PFAS generally occurring at higher concentrations.[26] Further, PFHxA and PFBA have been detected in brain tissues up to 300 ng/g[13]. A recent study found PFOA, perfluorohexane sulfonic acid (PFHxS), PFHxA in various parts of the male brain, with levels correlated strongly with PFAS levels in respective blood samples from these individuals. Surprisingly, the same study showed that other types of PFAS (i.e., PFOS) were detected at low levels in the blood and not in the brain, indicating selective accumulation of different PFAS in the brain.[27] Previous studies have suggested that PFAS transport into the brain across the blood-brain barrier (BBB) could be mediated by lipid transporters[28], other organic anionic transporter,[29] and the disruption of the BBB[30]. Multiple potential routes of exposure, evidence of PFAS accumulation in the brain, and the myriad PFAS

species prevalent in the environment could suggest a high risk of PFAS exposure that is underreported. Furthermore, the biomedical effects of these chemicals on human brains remain understudied.

Examples of suspected neurotoxic impacts of PFAS in humans extend to behavioral and neurodegenerative disorders. Studies have linked PFAS neurotoxicity to changes in neurotransmitter levels and disruption of synaptic homeostasis, which in turn, can serve as risk factors for neurodegenerative diseases, such as Alzheimer's or Parkinson's disease.[36, 37] Further, infant exposure of PFAS during pregnancy and childhood through the mothers' blood and breast milk has been linked to dysregulations in childhood physiology and behavior.[38, 39] PFOS, PFHxS, and perfluorononanoic acid (PFNA) have been linked to several symptoms of attention deficit hyperactivity disorder (ADHD), such as hyperactivity and aggression. In contrast, PFHxS was correlated with internalizing behavior such as depression and anxiety. These observations are based on PFAS serum levels and behavioral assessments.[40] More studies are needed to fully assess the potential impact of PFAS on neurological health.

A challenge in PFAS exposure studies is mimicking the environmental PFAS exposure in a laboratory setting. Many forms of PFAS toxicity are chronic, taking place over years, for example the health effects of exposure during early development may manifest only in later years.[21] PFAS are commonly detected in human serum at low part per billion levels (ng/mL) or nanomolar concentrations,[14, 41] but do not exhibit toxicity in cultured cells up to 3~100 μM.[15, 42, 43] Another factor that should be considered is PFAS, specifically long-chain ionic PFAS, reversibly binds to proteins through complex interactions that decrease bioavailability non-linearly in protein-rich cellular media used for *in vitro* studies.[44] Based on these factors, exposure studies use much higher concentrations than environmentally relevant PFAS levels.

In this study, we investigated the neurotoxic effects of PFAS in differentiated SH-SY5Y cells by transcriptomics analysis. The SH-SY5Y cell line is an established model for human neurological diseases.[45, 46] This neuroblastoma cell line can be differentiated into neuronal cells over an 11-day period following a previously established protocol.[47] We used differentiated SH-SY5Y cells to evaluate the changes in the transcriptome after exposure to six different long-chain (8 -10 carbons) PFAS, (PFOA), perfluorooctansulfonic acid (PFOS), perfluorodecanoic acid (PFDA) perfluorodecanesulfonic acid (PFDS), 8:2 fluorotelomer sulfate (8:2 FTS) and 8:2 fluorotelomer alcohol (8:2 FTOH) at 30 μM. We used a 24-hour, short-term treatment at sub-toxic concentrations that are higher than environmentally relevant concentrations of these contaminants to achieve cellular levels like *in vivo* accumulation of these compounds and the potential to capture biological effects on long-term exposure. Transcriptomics analysis revealed different impacts of PFAS tested at the gene expression levels, with PFOA inducing the highest number of differentially expressed genes ($p_{adj} < 0.05$). Gene enrichment analyses suggested different biological processes that might be perturbed by treatment with different PFAS, including neuronal function, cellular metabolism, and generation of ROS. Overall, our results highlighted diverse effects of PFAS exposure at the transcript level and the consequent changes in cellular processes.

MATERIALS AND METHODS

Materials

PFOA, PFOS, PFDA, PFDS, 8:2 FTOH, 8:2 FTS were purchased from Millipore Sigma® (Burlington, MA). M4PFOS and M4PFOS (C13 isotopically labeled standards) were purchased from Wellington Laboratories (Guelph, ON). Ammonium acetate, LC-MS grade methanol and HPLC grade methanol were purchased from J.T. Baker® (Radnor Township, PA). Dansyl chloride was purchased from Fluka Chemical (Buchs, Switzerland). Dimethyl amino pyridine (DMAP), and *N, N*-diisopropyl ethyl amine were purchased from Sigma Aldrich (St. Louis, MO). HPLC grade acetonitrile was purchased from Supelco (Bellefonte, PA). Ethyl acetate was purchased from Avantor (Radnor, PA). Sep-Pak® Vac 6cc (1g) solid phase extraction (SPE) cartridges were purchased from Waters (Milford, MA).

SH-SY5Y neuroblastoma cells were purchased from the American Type Culture collection (Manassas, VA). Dulbecco's modified Eagle media (DMEM) / Hams-F12 50:50, fetal bovine serum (FBS), trypsin, and penicillin/streptomycin antibiotic cocktail were purchased from Corning® (Corning, NY). GlutaMAX™ and Neurobasal™ media were purchased from Gibco (Billings, MT). Thiazoly Blue Tetrazolium Bromide was purchased from VWR (Radnor, PA). B-27 100X supplement was purchased from Fisher Scientific (Waltham, MA). A lactate dehydrogenase (LDH) chemical assay kit, and all-trans retinoic acid were purchased from Cayman Chemical Company (Ann Arbor, MI). Potassium Chloride was purchased from Mallinckrodt (Staines-upon-Thames, United Kingdom). Brain derived neurotrophic factor (BDNF) was purchased from Prospec™ Bio (Rehovot, Israel).

A Raptor® C18 analytical column 2.7 µm particle size, 100 x 3 mm, was purchased from Restek (Centre County, PA). Separation for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was performed using an Agilent® (Santa Clara, CA) 1200 series LC tower composed of a G13798 degasser, G1311A quaternary pump, G1367B high performance autosampler and G1330B autosampler thermostat. Mass analysis was done using a Thermo Scientific® (Waltham, MA) TSQ Quantum Ultra triple quadrupole MS equipped with a heated electrospray ionization source, operated under negative mode ionization.

Methods

Cell culture. SH-SY5Y cells were cultured based on their guidelines. Cells were grown in 10 cm plates and allowed to reach a confluency of 85% after approximately 5-6 days. After reaching 85% confluency cells were sub-cultured to new 10 cm plates at a 1:10 ratio. PBS was used to rinse cells before sub culturing and trypsin was used to uplift cells from plate. Cellular media was replaced every 3-4 days as needed.

Cellular differentiation. Cells were differentiated over an 11-day period using retinoic acid and brain derived neurotrophic factor.[47] Over the 11 days 3 different media compositions are used. The first was the base media used for general cell culturing for SH-SY5Y cells composed of DMEM/F12 media with 5% FBS and 1% penicillin/streptomycin (p/s). The second is stage 1 media which used DMEM/F12 50:50 as the base supplemented with 2.5% FBS, 1% P/S, and 10 µM all-trans retinoic acid. Lastly stage 2 media which used neurobasal media supplemented with

1X B-27, 20 mM KCl, 1% P/S, 2mM glutaMAX, and 50 ng/mL BDNF. Differentiation over 11 days was carried out as described in the literature.[47, 48]

PFAS exposure and cell collection. After the 11-day differentiation SH-SY5Y cells in 10 cm plates were exposed to 30 µM PFAS. PFAS treatments included PFOA, PFOS, PFDA, PFDS, 8:2 FTOH, and 8:2 FTS. Final treatment solutions included 1% MeOH. Treatments lasted 24 hours and were done in triplicate.

Viability assays. After day 11, treatments of PFAS in MeOH were added for final concentrations of 3, 10, 30, and 100 µM with 1% MeOH with a vehicle control of 1% MeOH. An additional treatment 10% Triton™ was added as a positive control for membrane permeabilization (n=5). Treatments lasted 24 hours.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were also performed. After a 24-hour PFAS treatment, cell plates were centrifuged at 200xg for 2 minutes at 24 ºC. Media was removed and 200 µL of 9% MTT in base media was added back. The plates were incubated at 37ºC under 5% CO_2 for 3 hours. After the plate was centrifuged a 150 µL portion of media was removed and 90 µL of DMSO was added back. Plates were then incubated at 37 ºC for 15 minutes and then mixed gently to dissolve formazan crystals. Absorbance was measured at 550 nm in a plate reader.

PFAS extraction. Frozen cell pellets were spiked with 1 mL of cold MeOH spiked with M4PFOS (deuterated standard used as an extraction control). Cell pellets were vortexed for 30 seconds and this was repeated 3 times for a total of 90 seconds. Cell pellets were sonicated at room temperature for 30 minutes. Lastly cells were centrifuged at 14,000xg for 15 minutes. Supernatant was collected and dried down under a nitrogen stream. Extract was then resuspended in 970 µL of starting mobile phase (45% 5 mM ammonium acetate/55% methanol) spiked with M4PFOA (deuterated standard used as LC-MS/MS control). PFAS analysis was done via LC-MS/MS analysis. Prior to LC-MS/MS analysis 8:2 FTOH was derivatized with dansyl chloride (procedure described in SI) LC-MS/MS analysis of PFAS is described in detail in the SI. Samples were normalized based on their protein content prior to analysis.

RNA extraction, library preparation and sequencing. Frozen cell pellets were transported on dry ice to the University at Buffalo Genomics core and stored at -80ºC until analyzed. RNA was extracted using TriZol using Invitrogen protocols. Quality of RNA was assessed using Agilent's Fragment Analyzer and Qubit. RNA was then used to prepare an RNA library using an Illumina RiboZero Total Stranded RNA library prep kit. Libraries were pooled together at 10 nM with concentration being determined with a QuantaBio Universal qPCR reaction kit. The pooled library was sequenced using a NovaSeq6000 (PE100).

RNA-seq analysis. The obtained RNA sequences were evaluated using FastQC[49] and MultiQC[50] for quality control. Two samples were eliminated from the downstream analysis due to evidence of contamination. For five out of the six treatments, we have at least three samples, a sample was discarded each from the control and the FTOH treatment category, leaving two samples for the downstream analysis for each category. The two discarded samples were flagged as contamination due to their low number of successfully assigned alignments: for Control_3_S29 less than 14.2%

of the reads were successfully mapped to the reference, while for FTOH_3_S47 a mere 14% of the total number of reads were successfully aligned. FastQC screen reports for the two samples confirmed that there are discrepancies with these samples either due to contamination or issues with the library preparation due to the presence of reads that produce multiple hits to multiple genomes. The RNA-seq reads were mapped to the human transcriptome reference (hg38) from Ensemble using hisat2[51] and quantified using featureCounts[52]. The differential expression analysis was carried out using DESeq2.[53] DESeq2 estimates the log fold-change of transcription for each gene using the Wald test of significance and performs multiple hypotheses correction on the raw read counts. DESeq2 can estimate dispersion with one 'degree of freedom,' calculated as the difference between the number of samples and the number of coefficients. In the context of our study after the removal of the two contaminants, there are four samples and two groups (represented by two coefficients), thus, two 'degrees of freedom' are available. However, it is important to note that statistical power diminishes rapidly in this context, leading to a decreased sensitivity, thereby hindering the detection of smaller log-fold changes.[54] To determine the significantly differentially expressed genes we filtered our results for an p_{adj} of 0.05 (where the p_{adj} we refer to the multiple-hypotheses-corrected p-value).

RESULTS AND DISCUSSIONS

Effect of PFAS exposure on cell viability of differentiated SH-SY5Y cells. The SH-SY5Y cells were differentiated as described previously[48] and treated with PFOA, PFOS, PFDA, PFDS, 8:2 FTOH, and 8:2 FTS for 24 hours at concentrations of 3, 10, 30, and 100 µM. Cell viability was assessed using MTT assays. None of the treatments induced a significant reduction in cell viability (p-value > 0.05), except for 8:2 FTS, which induced 30% cell death at 100 µM (p-value < 0.05) (**Figure 1**). Based on these results, we selected 30 µM as the treatment concentration for future studies, the highest non-toxic concentration tested. While 30 µM is much higher than what is observed in human serum (typically sub µM),[14, 41] PFAS levels that will be bioavailable to cells *in* vi*tro* studies are expected to be lower due to known PFAS-serum protein interactions.[44]

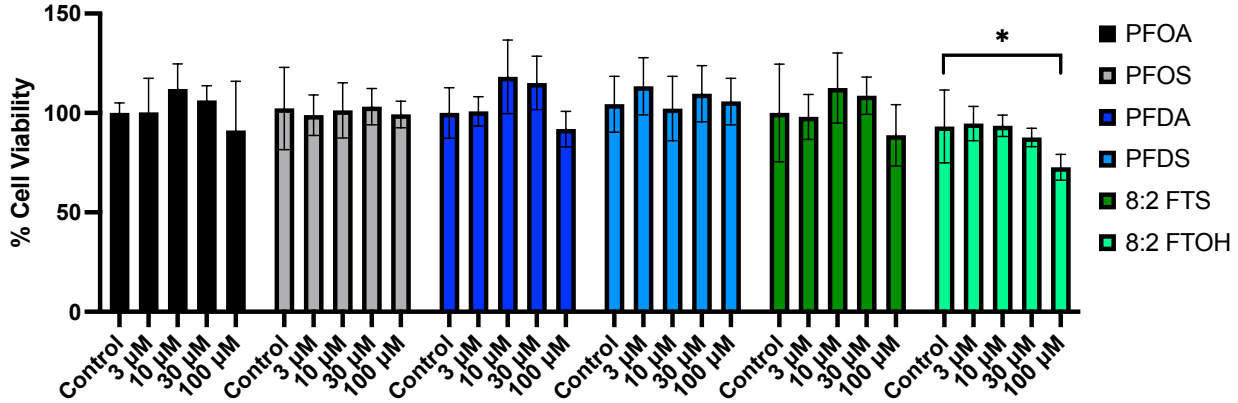

Figure 1: *MTT viability assay in SH-SY5Y cells with treatments of 3, 10, 30, and 100 µM PFOA, PFOS, PFDA, PFDS, 8:2 FTOH, and 8: FTS for 24-hours. 8:2 FTOH treatment at 100 µM showed*

a slight decrease in viability, which was statistically significant based on a paired t-test (p-value < 0.05).*

PFAS uptake in differentiated SH-SY5Y cells. Total PFAS uptake in cells was determined via LC-MS/MS with single point standard addition. Treatments were carried out as described in the previous section. Cell pellets were collected, and protein contents were measured, followed by LC-MS/MS analysis (see SI) for detailed procedures, including the derivatization of 8:2 FTOH). **Table 1** shows the average uptake for each treatment. PFOA, PFOS, PFDA, PFDS, and 8:2 FTS treatments showed comparable uptake values at ~600 ng PFAS/mg protein. 8:2 FTOH, however, was quantified at ~12 ng PFAS/mg protein, considerably lower as compared to PFOA, PFOS, PFDA, PFDS and 8:2 FTS. The larger standard deviation observed is a result of biological variability between the three replicates. The lower cellular levels of 8:2 FTOH suggests either reduced uptake or metabolism of FTOHs, which have been reported *in vitro* and *in vivo.*[55, 56] A primary metabolite of 8:2 FTOH degradation is PFOA, as well as various fluorotelomer carboxylic acids.[57] A targeted LC-MS/MS analysis of PFOA, as a degradation product, showed no appreciable levels of PFOA in 8:2 FTOH treated cells, supporting that it is the reduced uptake of 8:2 FTOH as compared to other PFAS tested that causes low cellular levels. Overall, our data suggest that cellular uptake of PFAS increases with chain length, and fully fluorinated sulfonates exhibit the highest uptake while fluorotelomer alcohol shows the least cellular uptake in neuronal cells.

Table 1: *Uptake of PFAS in SH-SY5Y cells after a 30 µM exposure for 24 hrs (N=3) with a range defined by measured standard deviation (avg +/- 1 std).*

PFAS	ng PFAS / mg protein
PFOA	526 +/- 259
PFOS	627 +/- 224
PFDA	522 +/- 190
PFDS	796 +/- 482
8:2 FTS	600 +/- 40
8:2 FTOH	9 +/- 7

Changes in gene expression in differentiated SH-SY5Y cells. To evaluate the impact of PFAS exposure in neuronal cells, we conducted a transcriptomics analysis to investigate pathways that are differently regulated after PFAS treatment. Differentiated SH-SH5Y cells were treated with PFOA, PFOS, PFDA, PFDS, 8:2 FTOH, and 8:2 FTS at 30 µM. Cells were collected as described in the experimental methods "PFAS exposure and cell collection" section. After mRNA extraction (n=3 for each condition), the mRNA content was sequenced using Illumina sequencing. Gene expression in treatments were compared to a control and was calculated as $\log_2$ fold change (Log_2 FC). Genes which showed differences in expression levels between treatment and control, and had a $p_{adj} < 0.05$, were compiled in a list for further statistical analysis and Gene Ontology (GO) gene enrichment.

Figure 2 represents data for the total pool of differently expressed genes impacted by all six treatments as compared to the control, and the distribution of the number of genes affected per treatment. Each bar represents an individual treatment, and colors represent genes from the pool affected by the specific PFAS treatment. White sections correspond to genes that are not affected by the given treatment. Gray, blue, and red represent differentially expressed genes ($p_{adj} < 0.05$) with various fold change (FC) thresholds: gray represents log_2FC <[0.5], blue represents log_2FC [0.5-.99], and red represents log_2FC >[1]). Log_2FC thresholds of [0.5] and [1] were selected for gene enrichment because they represent approximately 5% and 1% of the total gene impact in each treatment, respectively. This analysis resulted in 721 unique genes that were impacted across the six treatments combined. PFOA treatment affected the largest number of genes out of the six treatments, significantly changing the expression of 584 genes ($p_{adj} < 0.05$). In comparison, the remaining 5 PFAS chemicals each only impacted approximately 100 genes ($p_{adj} < 0.05$), (PFOS-102, PFDS-106, PFDA-147, 8:2 FTS-100, 8:2 FTOH-67). While PFOA affected the most number of genes, the extent of the effect ranged from moderate to low (log2FC < 0.5). In contrast, PFOS affected a smaller number of genes but at much higher level ($log_2FC > 1$) (**Figure 2**, see **Table S2** for a complete list of genes separated by treatment group and sorted by log_2FC).

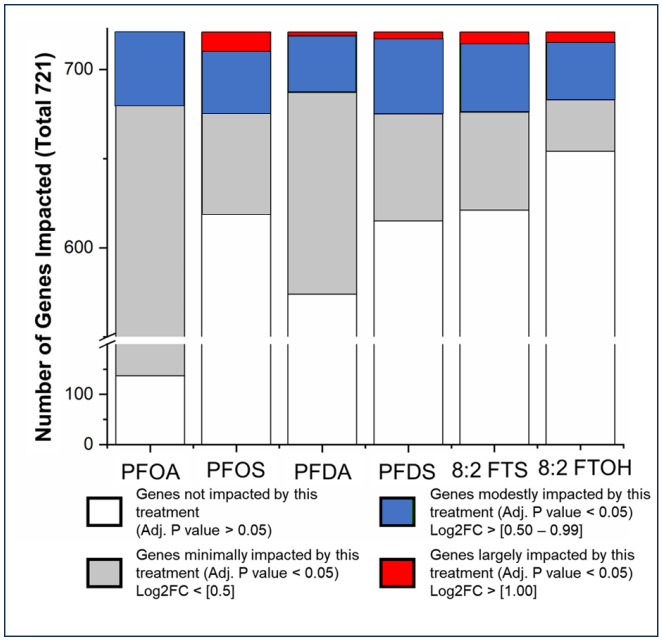

Figure 2: *Distribution of log_2FC observed for gene expression in SH-SY5Y cells after exposure to six different PFAS species. Log2FC > [0.5] were selected. Log_2FC of > [1] was chosen to represent genes that were doubled or halved in expression and comprised less than 1% of the genes impacted by the PFAS treatments.*

To visualize differentially expressed genes that are common between different treatment groups, an upset plot was generated. This plot groups impacted genes by the number of overlapping treatments (**Figure 3**). PFOA induced the highest number of changes in gene expression. Among the 721 differentially expressed genes, 425 were unique to PFOA treatment. Treatments with other PFAS uniquely affected 13 to 36 genes, while 193 genes showed differential expression under

combinations of different PFAS treatments. PFDA and PFOA treatments affected gene expression in the same 53 genes, possibly due to the carboxylate headgroup they share. Another noteworthy observation was that 11 genes (10 protein coding and one RNA coding) were differentially regulated in all six treatments (**Figure 3**). Overall, these results point towards a few important observations. First, among the PFAS tested, PFOA induced the highest number of changes in gene expression, suggesting that PFOA is more potent in perturbating cellular homeostasis than other PFAS. Second, PFDA and PFOA, which are both carboxylated, had the highest overlap among differentially expressed genes, suggesting that the type of head group significantly influences cellular effects of PFAS. Finally, our results revealed 11 genes as potential biomarkers of PFAS exposure, as their expressions were significantly affected by all six PFAS treatments.

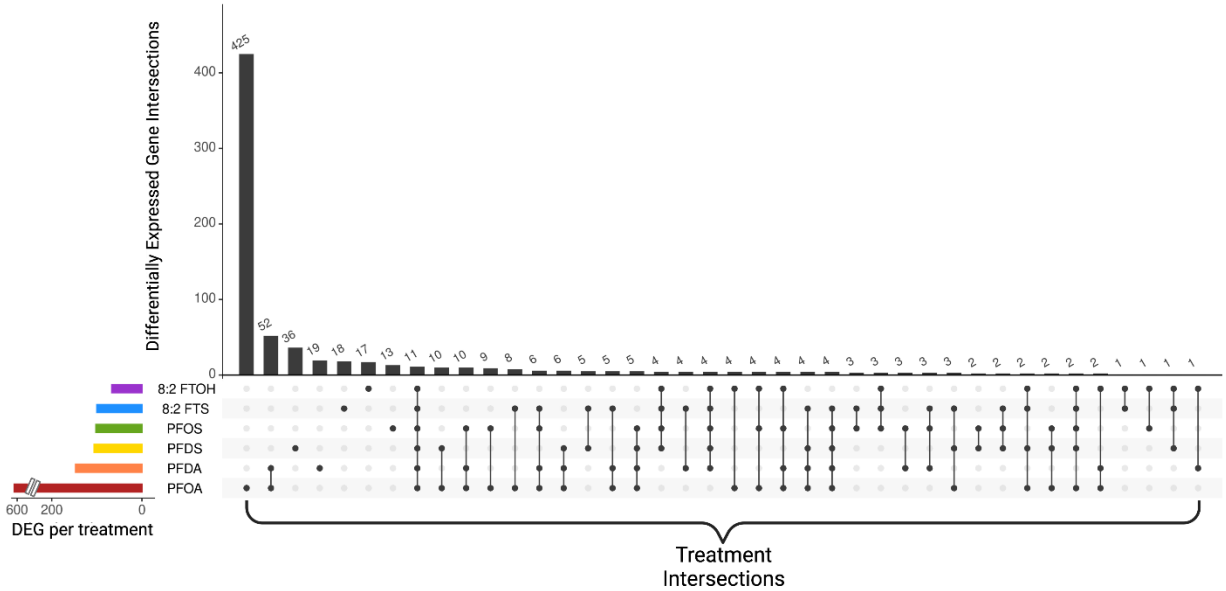

Figure 3: *An upset plot to visualize the grouping of genes that overlap in multiple treatments. Treatment intersections are defined by treatments that can be grouped by commonality and, in this context, overlapping differentially expressed genes. Differentially expressed genes (DEGs) are genes that were found to have log2FC > 0 and log2FC < 0 after a PFAS treatment compared to a control (*$p_{adj} < 0.05$*).*

Potential markers of PFAS exposure. The Organization for Economic Cooperation and Development (OECD) contains a database of 4700 unique PFAS chemicals,[58], and only about 6% (256 compounds) are compounds that are commercial products, byproducts, or known degradation material.[5] It is therefore important to develop indirect assays to assess exposure to PFAS when direct PFAS measurements from tissue are not feasible. Analysis of peripheral markers of PFAS exposure could be a powerful tool to assess exposure and risk to a potential disease progression. Previous studies in zebrafish have identified potential genetic biomarkers after exposure to the environmental pollutant phenanthrene.[59] Recent studies have shown that changes in protein expression related to neurodegenerative diseases can be measured in peripheral body fluids such

as blood and saliva.[60, 61] It has also been shown that damage to the BBB in certain neurodegenerative diseases leads to the movement of biomarkers out of the brain into the blood[62]. As such, it is possible to capture disruptions in the nervous system via monitoring changes in other tissues, most practically in blood. PFAS has previously been shown to disrupt the BBB through oxidative stress and may also lead to this leakage.[26] Therefore, it is likely that genes impacted by PFAS exposure in the brain may directly influence expression in other biofluids (e.g. saliva, blood), which could be sampled to assess exposure.

We identified 10 protein coding genes that were impacted by all six treatments using transcriptomics. These are *Transmembrane protein 106B* (TMEM106B), *transmembrane p24 trafficking protein 7* (TMED7), *T-box 3* (TBX3), *mesencephalic astrocyte derived neurotrophic factor* (MANF), *pyruvate dehydrogenase kinase 1* (PDK1), *BCL2 interacting protein 3* (BNIP3), *isoleucyl-tRNA synthetase 1* (IARS3), *mitotic spindle organizing protein 1* (MZT1), *thioredoxin interacting protein* (TXNIP) and *H4 clustered histone 13* (HIST1H4L). Each gene was differentially regulated in the same direction (i.e., upregulation or downregulation) between all six treatments, independent of the headgroup (carboxylate, sulfate, or alcohol), chain length (8 or 10 carbons) or level of fluorine saturation among the PFAS tested (**Figure 4)**. Such uniform response against multiple PFAS treatments could be used as a potential biomarker(s) to assess PFAS exposure, independently or combined. Future efforts investigating the regulation of these changes in other PFAS are warranted to explore these changes as biomarkers of PFAS exposure.

Gene regulation or dysregulation by PFAS and potential adverse health effects of exposure. We used several strategies to investigate the implications of the PFAS-induced differential expression. To focus on biological relevance, we took into account the effect size of gene expression differentiation in this analysis. Thus, in addition to the ten genes that are impacted by all treatments (Figure 4, red arrow), we focused on the genes that showed at least two-fold up or downregulation in at least three treatments (Figure 4, blue arrow), constituting less than 1% of the total genes impacted. **Figure 4** illustrates the genes selected based on these criteria.

Multiple genes that were affected in this resulting list have been implicated to various cancer pathologies. This includes BNIP3[63], HIST1H4L[64], TBX3[65] (observed in all six treatments), *early growth response factor 1* (EGR1)[66], *solute carrier family 7 member 11* (SLC7A11)[65] (observed in 5 treatments) *niban apoptosis regulator 1* (NIBAN1)[67] and *fos proto-oncogene* (FOS)[68] (observed in 3 treatments). In addition, multiple PFAS-affected genes have previously been linked to neuronal health, including MANF[69], TXNIP[70] (impacted in all six treatments), and *tolloid-like protein 2 (*TLL2)[71] (impacted in 5 treatments). MANF expression was found to be depleted in all treatments. MANF is an important binding protein for survival pathways in neuronal cells, and evidence in rats has suggested that MANF can reverse symptoms of neurodegenerative diseases.[69] TXNIP, which was upregulated in PFAS treatments, has been linked to oxidative stress resulting in neuronal death and has been implicated in neurodegenerative diseases such as Parkinson's Disease.[70] TLL2 gene regulates the extracellular membrane matrix composed of proteins such as collagen, which is important in many cells for development and equilibrium in cells, including brain cells.[71] Similarly, dysregulation of TLL2 and related tolloid like proteinase have been linked

to kidney disease and ischemic heart failure.[72] Our study is the first to reveal that PFAS exposure results in the downregulation of TLL2 expression in all treatments, except PFOA.

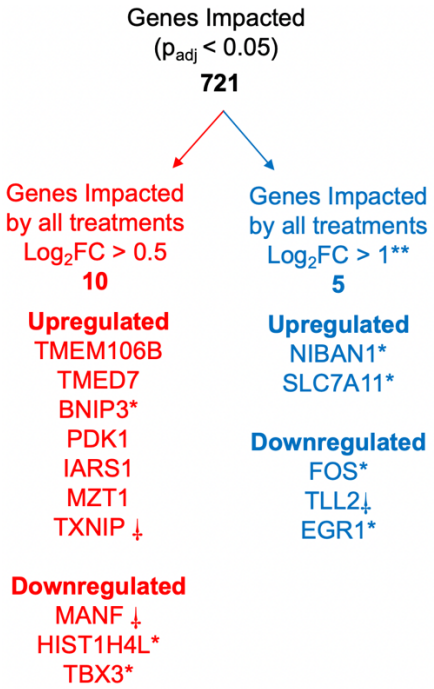

Figure 4: *Fifteen notable genes which were impacted after PFAS treatments. There were six different PFAS treatments (PFOS, PFOA, PFDS, PFDA, 8:2 FTS, 8:2 FTOH) at 30 µM for 24 hours with differentiated SH-SY5Y neuronal cells. Transcriptomics analysis revealed out of over 20,000 genes, 721 genes were differently regulated (compared to a control) and had a* $p_{adj} < 0.05$*. Of these, 10 genes were differently regulated in all six treatments. It is also important to note that there was no meaningful impact of treatment on the up or down regulation of each gene. Alternatively, there were 5 genes that were not differently regulated in all treatments but had the highest overall* log_2FC *of any impacted gene (*log_2FC *> [1]). Genes that had an impact this large account for <1% of the affected genes in this study.*
**Genes that, when dysregulated, have been linked to a cancerous pathology.*
⸸Genes that, when dysregulated, have been linked to neurodegenerative disorders.
***Genes in this list were impacted in at least three out of six treatments.*

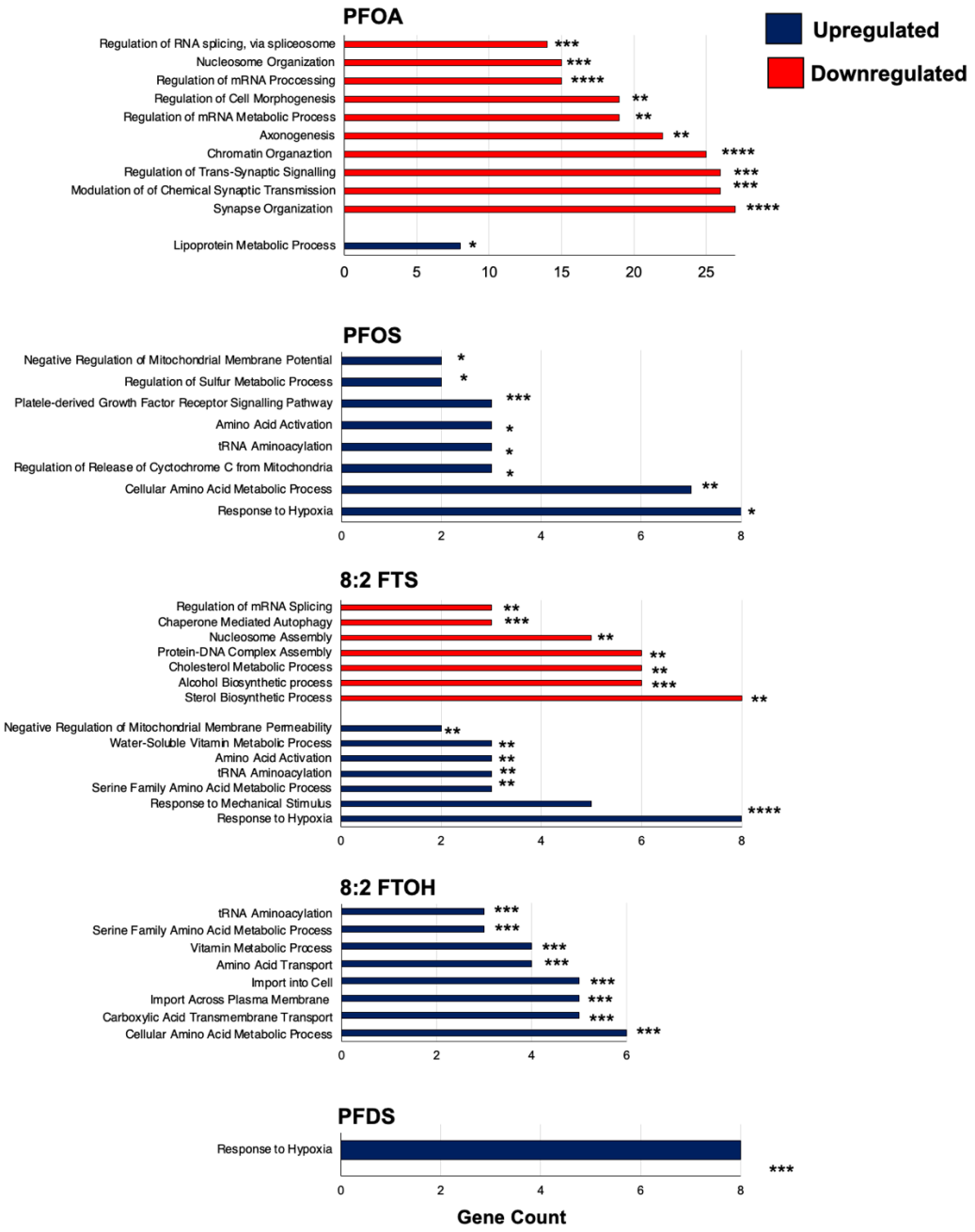

*Figure 5: GO gene enrichment analysis for discovering potential biological pathways affected by PFOA, PFOS, PFDS, 8:2 FTS, and 8:2 FTOH treatments. Genes used for this analysis were genes impacted by each treatment with log2FC>0.5 (control vs treatment) with $p_{adj} < 0.05$. The multiple hypothesis corrected p-values are shown in the figure: $p_{adj} < 0.05$). *$p_{adj} < 0.05$, **$p_{adj} < 0.01$, ***$p_{adj} < 0.001$, ****$p_{adj} < 0.0001$. Red and blue indicate enrichments among downregulated and upregulated genes, respectively.*

PFOA effect on neurodevelopment and neurotoxicity. We searched for functional enrichments among differentially expressed genes under PFAS treatment (**Figure 5** and ***Table S3*).** A total of 34 biological processes were found to be significantly enriched ($p_{adj} < 0.05$) among differentially expressed genes. Among the several enrichments we documented, we note that genes that are downregulated by PFOA treatment are enriched for neuronal processes, including axonogenesis (develop of axons from neurons to deliver action potentials), regulation of trans-synaptic signaling, modulation of chemical synaptic signaling, and synapse organization. This enrichment may suggest a PFOA-specific response to the SH-SY5Y neuronal network. Supporting this notion, previous work documented the neuronal effects of PFA treatment. For example, PFOS, PFOA, and PFDA exposure led to 20-25% reduction in neurite outgrowth.[73] Similarly, PFOA exposure in mice led to overexpression of brain-development related proteins *microtubule associated protein ta*u (MAPT), *synapsin 1* (SYN1), *calcium/calmodulin dependent protein kinase II* (CAMK2A). Even though these specific genes were not impacted in our study, potentially because of the differences in experiment models, exposure tissues, and conditions,[74] our results confirm the emerging notion that PFAS, and PFOA in particular, may affect neuronal development and function, with major implications to neurological disorders. Indeed, another study showed in differentiated SH-SY5Y cells that PFOA exposure leads to decreased neurite density and altered phenotypes for the nucleolus and mitochondria, similar to the cellular representation of neurodegenerative disorders such as Parkinson's disease.[75]

Impact of sulfate-containing PFAS on hypoxia-related genes. Hypoxic states have been previously reported to adversely affect cellular energy production and increase production of ROS in neuronal cells.[76] There is limited literature that discusses a PFAS-mediated hypoxic response. One study investigated the impact of PFOS and PFOA exposure on type 2 diabetes and related chronic kidney disease, and suggested that PFAS exposure may reduce the hypoxic state associated with these diseases due to the oxygen carrying capacity of perfluorocarbons.[77] This is inconsistent with our observation that PFAS exposure triggered a hypoxic state in our neuronal model. We found that upregulated genes under PFAS exposure are enriched are associated with cellular processes involved in response to hypoxic state. These genes include adenylate kinase family (AK4), BNIP3, and *pyruvate dehydrogenase kinase 1* (PDK1), which are linked to *hypoxia inducible factor 1 subunit alpha* (HIF-1α) functioning. Specifically, AK4 can stabilize HIF-1α and increase cellular reactive oxygen species levels.[78] BNIP3 (a HIF-1α regulated gene) is involved with triggering autophagy in a hypoxic state and has been linked to cancer formation.[63] PDK1 is activated by HIF-1α to interfere in the conversion of pyruvate to acetyl-CoA and is implicated in hypoxic states.[79] *Vascular endothelial growth factor A* (VEGFA)[80], *stanniocalcin-2* (STC2)[81], *phorbol-12-myristate-13-acetate-induced protein 1* (PMAIP1)[82], *hexokinase 2* (HK2)[83], SLC2A1[84], and *DNA damage inducible transcript 4* (DDIT4)[85] were upregulated in one or two treatments which contributed to the pathway enrichment are all known to be enriched in a hypoxic state. Lastly, while the gene encoding for HIF-1α was not upregulated, *EPAS1* (which codes for HIF-2α) that has been reported to regulate many HIF-1α targets was upregulated.[86] Overall, these upregulations resulting in the enrichment of hypoxia response processes suggest that the exposure to sulfonate-containing PFAS may perturb hypoxia signaling in differentiated neuronal cells.

PFOA, 8:2 FTS and 8:2 FTOH upregulate genes related to tRNA aminoacylation. Finally, we observed that several genes involved in tRNA aminoacylation were upregulated with PFOS, 8:2 FTOH, and 8:2 FTS treatment. Notably, *cysteinyl-tRNA synthetase 1* (CARS1), *aspartyl-tRNA synthetase 1* (DARS1), and IARS1 are upregulated in these treatments. These enzymes are responsible for the addition of cysteine, aspartic acid, and isoleucine to tRNA molecules necessary for protein synthesis, respectively. These genes are upregulated in the pro-proliferative state of cancer cells.[87] Interestingly, PFOS, 8:2 FTOH, and 8:2 FTS treatments also result in upregulating amino acid uptake/transport and metabolism-related processes, which have been linked to the increased energy requirement in cells.[88,89] While further investigations are needed to study the effects of PFAS on protein synthesis and cellular metabolism, these results suggest significant effects on critical processes involved cellular homeostasis.

CONCLUSIONS

In this study, differentiated SH-SY5Y cells were used as a model for human neurons to assess the transcriptomic impact of PFOS, PFOA, PFDS, PFDA, 8:2 FTS and 8:2 FTOH, which have all been previously detected in human and animal brain tissues. We observed similar cellular uptake levels of PFOS, PFOA, PFDS, and 8:2 FTS, while cellular 8:2 FTOH levels were much lower. The fact that 8:2 FTOH is the only neutral species tested suggest that PFAS uptake is strongly influenced by the nature of the headgroup. Using transcriptomics, we identified hundreds of genes affected by PFAS exposure. PFOA treatment impacted 3-6 times more genes (~600) than the other treatments. PFOA-affected genes are enriched for biological processes related to neurite growth, neurite organization, and action potential, indicating potential links to neurodegenerative diseases. Other PFAS affect genes enriched in biological processes related to response to hypoxic state and amino acid metabolism. Overall, results from this study provide valuable insight to the biological mechanisms through which PFAS affect neuronal biology and health. Future work will focus on understanding the mechanism of how PFAS induce these changes and potential links to different disease models.

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