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Impairment of human dopaminergic neurons at different developmental stages by perfluoro-octanoic acid (PFOA) and differential human brain areas accumulation of perfluoroalkyl chemicals

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ABSTRACT

Perfluoroalkyl substances (PFASs) are synthetic chemicals widely used in industrial and consumer products. The environmental spreading of PFASs raises concerns for their impact on human health. In particular, the bio-accumulation in humans due to environmental exposure has been reported also in total brain samples and PFAS exposure has been associated with neurodevelopmental disorders. In this study we aimed to investigate the specific PFAS bioaccumulation in different brain areas. Our data reported major accumulation in the brainstem region, which is richly populated by dopaminergic neurons (DNs), in brain autopsy samples from people resident in a PFAS-polluted area of Italy.

Since DNs are the main source of dopamine (DA) in the mammalian central nervous system (CNS), we evaluated the possible functional consequences of perfluoro-octanoic acid (PFOA) exposure in a human model of DNs obtained by differentiation of human induced pluripotent stem cells (hiPSCs). Particularly, we analyzed the specific effect of the exposure to PFOA for 24 h, at the concentration of 10 ng/ml, at 3 different steps of dopaminergic differentiation: the neuronal commitment phase (DP1), the neuronal precursor phase (DP2) and the mature dopaminergic differentiation phase (DP3). Interestingly, compared to untreated cells, exposure to PFOA was associated with a reduced expression of Tyrosine Hydroxylase (TH) and Neurofilament Heavy (NFH), both markers of dopaminergic maturation at DP2 phase. In addition, cells at DP3 phase exposed to PFOA showed a severe reduction in the expression of the Dopamine Transporter (DAT), functionally involved in pre-synaptic dopamine reuptake.

In this proof-of-concept study we show a significant impact of PFOA exposure, mainly on the most sensitive stage of neural dopaminergic differentiation, prompting the way for further investigations more directly relevant to risk assessment of these chemicals.

1. Introduction

Perfluoroalkyl substances (PFAS) are also known as "forever chemicals" due to their strong resistance to chemical reactions and biodegradation, attributed to their hydrophobic aliphatic chain linked to fluorine atoms. (Brendel et al., 2018; Kucharzyk et al., 2017; Rahman et al., 2014). The synthesis of PFAS dates back to the 1930 s, and their amphiphilic properties (head with a functional hydrophilic group and hydrophobic per-fluorinated tail) made them widely used in various industrial applications, which resulted in a significant presence in the environment. Perfluorooctanoic acid (PFOA) and perfluoro-octane sulfonic acid (PFOS), the best known representatives of PFAS, have been used for years for the protection of fabrics, waterproof and stain-resistant coatings, metal plating, photographic films, foams aqueous

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film-forming (Brendel et al., 2018; Fujii, Polprasert, Tanaka, Lien, & Qiu, 2007; Kucharzyk et al., 2017), textiles, food packaging, water-repellent and stain-resistant fabrics, medical devices, laboratory supplies and personal care (Cousins et al., 2019; Di Nisio and Foresta, 2019). Chain length and branching can influence the physical and chemical properties of PFAS such as environmental diffusivity, degradation, partitioning, bioaccumulation, toxicity and toxicokinetics. The study by Conder et al. firstly reported that the bioaccumulation of PFAS are directly related to the length of the fluorinated carbon chain of each compound (Conder et al., 2008).

Several studies on PFAS have focused on their persistence and the long-range diffusion capacity in the environment, on the potential for bioaccumulation and on the negative effects on living organisms (Giesy and Kannan, 2001; Shoeib et al., 2006). In particular, PFAS are non-biodegradable and bioaccumulate in the environment as well as in animal and human tissues (Conder et al., 2008). In fact, PFAS can be absorbed by the intestine or inhaled and, once in the circulation, they may act as endocrine disruptors and lead to severe health consequences such as neonatal mortality, neurotoxicity, immunotoxicity, and reproductive disorders as reported in human studies (Di Nisio et al., 2020, 2019; Steenland et al., 2010). Importantly, among the others, PFAS have been identified in human brain specimens (Maestri et al., 2006; Pérez et al., 2013), suggesting the blood–brain barrier (BBB) permeability to these molecules. Similarly, animal studies have shown PFOS accumulation in specific brain area, such as cortex, hypothalamus, hippocampus and cerebellum (Austin et al., 2003). Epidemiological studies have evaluated the possible impact of PFAS on neuro-behaviour, with different results depending on age, sex and outcome. One of the most consistent effect is the increased risk of attention-deficit/hyperactivity disorder (ADHD). PFOS exposure during lactation has indeed been associated with a most frequent diagnosis of ADHD during infancy or adolescence (Lenters et al., 2019). A possible mechanism to explain this association has been identified in the alteration of the central cholinergic neurotransmission, as reported in animal models (Johansson et al., 2008), typically involved in the ADHD etiology. Other studies have shown memory impairment (Gallo et al., 2013), reduced intelligence quotient in children (Yan Wang et al., 2015; Yu Wang et al., 2015), autism (Oh et al., 2021; Shin et al., 2020) and impaired cognitive development (Oh et al., 2021). Altogether, available evidence is indicative of PFAS interference on CNS, whose effects and mechanisms are still unexplored and varying with age, the structure of the molecule and the outcome considered. The available experimental evidence supporting the biological effects of PFAS on neuronal function has been focused on reactive oxygen species (ROS) induction and apoptosis (Lee et al., 2012, 2014; Lee and Viberg, 2013; Li et al., 2017; Reistad et al., 2013). Dopaminergic neurons are typically very sensitive to ROS and, given the emerging role of PFAS in the alteration of cell membranes in other cell models (De Toni et al., 2020; Šabović et al., 2019), the understanding of cellular mechanisms involved in neural PFAS toxicity is of primary importance. In a study on amphibian animal model, both PFOA and PFOS decreased dopamine content but did not affect serotonin, norepinephrine, GABA, glutamate, or acetylcholine (Foguth et al., 2019). Accordingly, mice exposed to PFOS showed reduced levels of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the hippocampus (Long et al., 2013). These data are suggestive of PFAS interference with the dopaminergic network, thus representing a putative target of behavioral and cognitive alterations in humans. In 2013, large-scale contamination of PFAs was discovered in the Veneto region of the northern Italy, following emissions from a fluorochemical plant in the province of Vicenza (WHO, 2016). According to Pitter et al. (Pitter et al., 2020) the residents of a large territory of the Veneto region were inadvertently exposed to drinking water containing high concentrations of PFAS. Through public aqueducts, contaminated groundwater was supplied to around 140,000 people. This contamination is one of the largest cases of high residential exposure to PFAS ever reported and resembles in origin, extent and characteristics to that which occurred in the Mid-Ohio Valley, United States (Frisbee et al., 2009). In fact, in line with previous Mid-Ohio Valley reports, PFOA reached the highest concentrations in both drinking water and serum (Frisbee et al., 2009).

In this proof-of-concept study we evaluated for the first time the accumulation of a panel of PFAS in different brain areas recovered from post mortem samples of Italian subjects resident in the Veneto region, which represents a recognized hot spot of environmental pollution by PFAS, and in particular by perfluoro-octanoic acid (PFOA). Considering the high representation of dopaminergic neurons in specific areas of the CNS and the literature data pointing towards dopamine alterations in animal models, we evaluated the possible functional consequences of PFOA exposure in a human in vitro model of hiPSCs-derived. In particular, we focused on the specific effect associated with a 24 h exposure to PFOA, at the concentration of 10 ng/ml, at three different phases of dopaminergic differentiation: the neuronal commitment phase (DP1), the neuronal precursor phase (DP2) and the mature dopaminergic differentiation phase (DP3).

2. Materials and methods

2.1. Subjects

The study was performed on 5 brains taken from five adult Caucasian males who donated their bodies to the Body Donation Program of the University of Padua (Porzionato et al., 2012). Five postmortem brain samples were sampled, and when different brain regions were isolated, a total of 76 samples were analyzed, from non-occupationally exposed, resident in the red area, a hotspot of PFAS environmental pollution of the Veneto Region, Italy (Pitter et al., 2020). Mean age of subjects was 46.8 ± 6.9 years. Causes of death varied among multiple trauma, ischemic heart disease, accident or self-injury. Samples were stored at −80 °C until analysis. The study was approved by the local Ethical Committee and was performed according to the Italian laws on autopsied human tissues. Autopsies were performed within 36 h of death. In all cases, macroscopic and microscopic examination revealed the absence of acute, chronic, localized, or diffuse brain pathology. The specific brain areas collected for subsequent LC/MS analysis were as follows: thalamus, midbrain, hypothalamus, cerebellum, lenticular nucleus, caudate nucleus, frontal lobe. For each case, venous blood was sampled at autopsy from the subclavian vein. The whole blood sample, without further fractionation, was transferred into 15 mL test tube (Falcon™, Becton Dickinson, Milano, Italy) and frozen at −80 °C until use.

2.2. Extraction of PFAS from blood and brain tissues

Brain tissues were transferred in a petri dish and cut by a scalpel. Tissues was transferred in tube in aliquots of 100 mg depending on the initial amount of tissues. Specimens were then weighted and 1 mL of ^{13}C-PFOA 10 ng/ml was added as internal standard. Samples were homogenized with IKA T10 basic homogenizer, sonicated with Sonicator UP 200 s with pulse at 70 % of amplitude, centrifuged for 15 min at 13000 rpm. Supernatants were subsequently transferred in new tubes and then dried in Concentrator plus system (Eppendorf) at 60 °C and then stored at −20 °C until use. Five-hundred μl of each thawed blood sample was quantitatively transferred in a new test tube (Eppendorf, Milano, Italy) and 500 μl of ^{13}C-PFOA 10 ng/ml were added as internal standard. Samples were sonicated, centrifuged, dried as above mentioned and stored at −20 °C until use. All laboratory plasticware were tested not to release PFAS, as per previous studies (De Toni et al., 2020; Minuz et al., 2021).

Before the analysis, brain and blood samples were defrosted at room temperature and extracted in 150 μl of methanol. Suspensions were finally sonicated for 10 min, centrifuged at 13000 rpm for 10 min and supernatants were used for chromatographic analysis.

2.3. LC-MS/MS analysis

PFAS levels were measured through reversed-phase (RP) liquid chromatography coupled with triple quadrupole mass spectrometry (LC-MS/MS). A system composed by a Varian binary pump, autosampler and diode array system equipped with a triple quadrupole model MS320 (Varian/Agilent Technologies, Santa Clara, CA, USA) was used. Internal standard method was used for the quantification of PFAS in brain samples using ^{13}C-PFOA as internal standard (IS). Stock solutions were prepared by diluting in methanol ^{13}C-PFOA, PFOA, PFOS, PFHxA, PFHxS and a new generation PFAS, 2,2-difluoro-2-((2,2,4,5-tetrafluoro-5(trifluoromethoxy)-1,3-dioxolan-4-yl)oxy)-, ammonium salt (1:1), known with the commercial name of C6O4, to obtain final concentration of 500 ng/ml. The calibration curve was obtained mixing 50 µl of 500 ng/ml ISTD with different volumes (50, 25, 10, 5, 1 µl) of each PFAS standard solution in order to obtain different ^{13}C-PFOA /PFAS quantity ratios for the calibration curve. PFAS were analyzed by LC-MS/MS, using electrospray ionization (ESI) source operating in negative ion mode. Needle voltage was 5000 V, drying gas temperature was 300 °C, drying gas pressure was 22 psi, nebulizer pressure was 55 psi, capillary voltage was set at 40, and CID gas was 1.5 mbar. Mass spectrum operating in negative ion mode and specific transition for ^{13}C-PFOA, PFOA, PFOS, PFHxS, PFHxA, C6O4 were obtained using MS software. Transition were as reported: ^{13}C-PFOA 417 > 372, PFOA 413 > 369, PFOS 499 > 80, PFHxA 338 > 112, C6O4, 339 > 113. Other transitions were used to detect the presence of further PFAS based on literature data (Gremmel et al., 2016; Nakayama et al., 2020). For the chromatographic separation, an Agilent XDB C-18 column (3 × 150 mm, 3,5 µm) was used as stationary phase whilst eluents were 0.1% formic acid in water (A) and Acetonitrile (B) as previously described (Minuz et al., 2021). Elution gradient started with 90% A, achieved 50% A/50% B in 0.5 min and then 100% B in 10 min remaining isocratic up to 20 min with a constant flow rate of 0.3 mL/min. The quantification of the cell content of PFAS was performed through LC-MS/MS as described above with minor modifications. Briefly, cells were detached by scraping and extracted with acetonitrile. Fixed amounts of the stable isotope labeled internal standard were added ^{13}C-PFOA, Wellington Laboratories, Ontario, Canada). To test the analytical response and to optimize the calibration curve, standard mixture was used at increasing concentrations of PFOA together with ^{13}C-PFOA at fixed concentrations. C6O4 standard compound was purchased from Wellington Laboratories, provided as 50 µg/mL methanol solution (CAS: 1190931–41–9; Southgate, Ontario, Canada).

2.4. Cell culture

hiPScells were a kind gift from Prof. Marta Trevisan (Department of Molecular Medicine DMM, University of Padua) and were obtained as previously described (Trevisan et al., 2017). Cells at all culture stages were kept in a humidified incubator with 5% CO_2 at 37 °C. The iPS cell differentiation into DNs protocol is a modified version from Borgs et al. (Borgs et al., 2016): hiPS cells, seeded at the optimal starting density of 6.5x10^4 cells/cm^2, were induced to differentiate into neural stem cells (NSC) onto covers slips coated with Geltrex™ LDEV-Free-Reduced Growth Factor Basement Membrane Matrix and cultured for 7 days in a medium composed of 50% of DMEM F12 and 50% PSC Neural Induction medium (all items purchased from Gibco, Waltham, MA, USA). Medium was changed every 48 h and, upon the achievement of 80% confluence before day 7, cells were propagated using StemPro™ Accutase™ Cell Dissociation Reagent (Gibco, Waltham, MA, USA) with 5 µM Rho-associated kinase (ROCK Y27632; Miltenyi Biotec, Bergisch Gladbach, Germany). After 7 days of culture, the DP1 phase, aimed to obtain progenitors of neural-epithelium cells, was started by seeding cells on cover slips coated with 0.1 mg/ml of Poly-D-Lysine (Millipore, Burlington, MA, USA) and Matrigel (Corning, Glendale, AZ, USA), in Neurobasal medium with 1% penicillin/streptomycin (5,000 U/mL), 1% GlutaMAX, 1 % N-2 Supplement (100X)(all items purchased from Gibco, Waltham, MA, USA), 200 ng/ml Recombinant Human Sonic Hedgehog (Shh) and 100 ng/ml of Recombinant Human/Murine FGF-8b (all items purchased from Peprotech, Rocky Hill, NJ, USA). DP1 cells were cultured for 7 days and propagated up to 80% confluence as described above, changing the medium every other day. After 7 days of culture, the DP2 stage, aimed to obtain progenitors of dopaminergic neurons, was started by seeding cells onto cover slip coated Poly-D-Lysine and Matrigel in supplemented Neurobasal medium as for DP1 phase plus 20 ng/ml Recombinant Human BDNF, 20 ng/ml Recombinant Human GDNF, 1 ng/ml Recombinant Human TGF-β3 (all items purchased from Peprotech, Rocky Hill, NJ, USA), 0.2 mM L-Ascorbic acid, 0.5 mM dbcAMP (all items purchased from Sigma-Aldrich, Burlington, MA, USA) for 7 days, changing medium every 48 h and propagating cells up to 80% confluence. The DP3 stage, aimed to obtain mature dopaminergic neurons, was started by seeding on cover slip coated with Poly-D-Lysine and Matrigel in serum free-supplemented Neurobasal medium as for DP2 phase plus 2% B-27™ Supplement (50X, from Gibco, Waltham, MA, USA) for 7 days, changing medium every 48 h and propagating cells up to 80% confluence. At the day 6 of each differentiation stage, cells were exposed to PFOA for 24 h at the concentration of 1, 10 and 100 ng/mL and PFOA cell content was assessed by LC/MS. In the final experimental setting, cells were exposed to PFOA for 24 h at the final concentration of 10 ng/mL. Cell accumulation of PFOA was evaluated as previously described (Minuz et al., 2021). Briefly, after 24 h of exposure to PFOA, cells were detached by scraping and pellets were collected. 200 µl of ^{13}C-PFOA 10 ng/ml were then added as internal standard to each cell pellet. Samples were sonicated, centrifuged, dried as above mentioned and stored at −20 °C until use. Upon analysis, samples were defrosted at room temperature and extracted with 150 µl of methanol. Suspensions were finally sonicated for 10 min at room temperature, centrifuged at 13000 rpm for 10 min and the resulting supernatants were used for chromatographic analysis.

2.5. MTT assays

Cell proliferation was measured using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays. The cells were seeded in a 96-well culture plate at a density of 2 × 10^3 cells/well and incubated at 37 °C for 24 h. The cells were then incubated with PFOA as above for 24 h. A total of 20 mL of MTT (5 mg/mL in phosphate-buffered saline, PBS) was added to each well and the plates incubated in the dark at 37 °C for 4 h. Culture media was discarded and 200 mL of dimethyl sulfoxide (DMSO) added to dissolve the formazan crystals at room temperature for 10 min. The absorbance was measured at 490 nm using an iMark micro plate reader (BIO-RAD).

2.6. Merocyanine staining

Evaluation of plasma membrane fluidity was evaluated by Merocyanine 540 (MC540) probe as described elsewhere (Šabović et al., 2019). Briefly, a stock solution of MC540 in DMSO was diluted in the cell suspension in serum free-medium, at the final concentration of 4 µM and incubated for 15 min at 37 °C in the dark. Cells were finally analyzed by BD FACScan System (Becton–Dickinson, Milano, Italy) and at least 10.000 events per session were recorded.

2.7. Indirect immunofluorescence

Indirect immunofluorescence was used to identify the expression of Tyrosine Hydroxylase (TH), the enzyme that converts amino acid L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), of Neurofilament-Heavy (NF-H), marker of neuro-differentiation, and of Dopamine Transporter (DAT), functionally involved in pre-synaptic dopamine re-uptake. Cultured cells were fixed with 4% paraformaldehyde in PBS, blocked with 5% normal donkey serum/5% bovine serum albumin in

PBS and incubated with the following primary antibodies: rabbit anti-TH (GeneTex, Irvine, CA, USA; 1:100); chicken anti- NF-H (Life Technologies, Carlsbad, CA, USA; 1:50); mouse anti-DAT (Atlas Antibodies, Stockholm, Sweden; 1: 100). Primary immunoreaction was detected with the proper secondary antibody: goat anti-rabbit IgG (H + L)-Alexa Fluor Plus 647 conjugated (Thermo Fisher, Waltham, MA, USA), goat anti-mouse IgG (H + L)-Alexa Fluor Plus 488 conjugated (Thermo Fisher, Waltham, MA, USA), goat anti-chicken IgG (H + L) Alexa Fluor 633-conjugated (Thermo Fisher, Waltham, MA, USA). Control staining was carried out by using the secondary antibody alone. All stainings were performed in the same experimental session. After immunofluorescence staining, cells were counterstained with the nuclear dye DAPI (1 μg/ml in PBS, 0.3% Triton-X 100) for 20 min. at RT. Cells were finally washed in PBS and mounted in Mowiol (Sigma-Aldrich, Burlington, MA, USA). A Leica TCS SP5 confocal microscope was used to evaluate the expression of TH, NF-H, DAT and DAPI in the different phases of neurodifferentiation. To quantify the emitted fluorescence in gray-scale values, the Fiji ImageJ software was used. The integrated fluorescence density (I.F.D.) of each color channel was normalized to that of DAPI in order to harmonize expression data with cell density. The quantification analysis was carried out on 10 images acquired in triplicate at a magnification of 40X in mineral oil.

2.8. Statistical analysis

Results are reported as the mean value ± standard deviation derived from three independent experiments. Statistical analyses were performed with SPSS23 (SPSS, Chicago, IL, USA). Two-sided Student's *t*-test was used to compare two groups. When necessary, multiple comparisons were performed by the analysis of variance (ANOVA) with post-hoc Bonferroni's correction. Linear correlation analysis was performed through Pearson's coefficient. Results were considered significant for P values < 0.05.

3. Results

3.1. PFAS analysis in human brain autopsy samples

A total of 76 brain samples, with corresponding serum samples, were obtained from 5 male deceased subjects residing in exposed area and were analyzed by LC-MS/MS. The singular tissue/brain area concentration of detectable PFAS is reported on Table 1. The most represented perfluoroalkyl substance was PFOA, followed by perfluorohexanesulfonic acid (PFHxS) and perfluorohexanoic acid (PFHxA), respectively. Low levels of PFOS were detectable only in blood samples at very low levels (Table 1), whereas C6O4 was below LOD in all brain and blood samples. Peripheral blood serum represented the major site of accumulation of PFAS, however brain areas of basal ganglia, such as the hypothalamus, lenticular nucleus and caudate nucleus showed high levels of accumulation for all the retrieved compounds. Importantly, the mean brain concentration of each PFAS compound appeared to be linearly correlated with the corresponding serum concentration (Fig. 1), suggesting the gradient-driven *trans*-mural diffusion across the blood–brain barrier as a major mechanism of accumulation of PFAS within the brain areas.

3.2. Accumulation PFOA in differentiating hiPSCs and effects on cell membrane fluidity

The possible accumulation of PFOA in differentiating hiPSCs was evaluated by the exposure of cells to PFOA at the concentration of 1, 10 and 100 ng/mL for 24 h at the end of each differentiation phases, and quantifying the cell content of PFOA by LC-MS/MS (Scheme in Fig. 2A). In control conditions, in which exposure to PFOA was omitted, levels of PFOA were below the limit of detection (<0.2 ng/10^6 cells, data not shown). On the other hand, with the same exposure to PFOA, the cell accumulation of the perfluoro-alkyl compound showed to be dependent on the degree of differentiation towards the dopaminergic phenotype (Fig. 2B). In particular, at the earliest phase of differentiation DP1, cells displayed the highest level of PFOA accumulation, which was very low at 1 ng/mL of PFOA (0.56 ± 0.11 ng/mL) and did not differ between 10 and 100 ng/mL treatments (5.31 ± 0.36 and 5.67 ± 0.16, respectively). In the later stages of differentiation, DP2 and DP3, PFOA accumulation showed to be significantly progressively lower compared to DP1 and did not increase upon treatment with PFOA 100 ng/mL compared with 10 ng/mL (Fig. 2B). Cell viability assessed by MTT analysis showed no statistically significant effect of the exposure to PFOA, at any of the tested concentration, compared to the unexposed control condition (Supplemental Figure S2). In particular, no significant effect of either developmental stage ($p = 0.059$) or PFOA ($p = 0.145$) was observed, and neither of the interaction factor between stage and PFOA ($p = 0.053$) at multiple comparison by ANOVA. Given the non-linear dose-dependent accumulation of PFOA in vitro, in the next set of experiments we only focused on the concentration of 10 ng/mL. The membrane fluidity in developing dopaminergic neurons was assessed at each phase of differentiation by the use of Merocyanine 540 (MC540), a fluorescent probe sensitive to lipid packing (Fig. 3A). Of note, the mean fluorescence intensity of MC540, a proxy of membrane fluidity, showed a progressive and significant reduction in hiPS cells at DP2 and DP3 phases of differentiation compared to DP1 phase (respectively: 100.0 ± 1.1% DP1; 38,7 ± 1,3% DP2; 14,6 ± 1,2% DP3, both $p < 0.001$ vs DP1), suggesting an increasing lipid packing and membrane stiffness during the differentiation. In this context, the higher membrane lipid packing at DP2 and DP3 likely explains the observed trend towards a lower PFOA accumulation in later maturation steps (Fig. 2B). In previous reports we showed that PFOA accumulation in cell membrane associates with bilayer instability and increased fluidity of this organelle (De Toni et al., 2020). Thus, a reduced perturbation of membrane's biophysical properties upon PFOA exposure is expected at DP2 and DP3 compared to DP1. Accordingly, the exposure to PFOA 10 ng/mL for 24 h was associated

Table 1
Serum and brain specimens concentrations of perfluoroalkyl substances detected in 5 male deceased subjects residing in exposed area.

	PFOA, ng/g			PFOS, ng/g			PFHXs, ng/g			PFHXa[a], ng/g			∑ PFAS, ng/g		
Blood[b]	217.41	±	176.17	0.83	±	0.41	70.98	±	63.36	2.63	±	2.68	291.85	±	109.86
Thalamus	14.35	±	5.49			<LOD	2.70	±	2.59	2.69	±	0.67	19.75	±	6.73
Midbrain	14.34	±	18.75			<LOD	8.60	±	10.87	1.07	±	0.09	24.01	±	6.65
Hypothalamus[c]	158.88	±	162.00			<LOD	31.62	±	23.48	16.42	±	4.20	206.93	±	78.23
Cerebellum	32.28	±	54.55			<LOD	8.70	±	6.12	13.42	±	5.84	54.40	±	12.48
Lenticular Nucleus	31.05	±	55.35			<LOD	12.13	±	4.32	2.09	±	0.34	45.28	±	14.70
Caudate Nucleus	93.29	±	147.38			<LOD	8.43	±	8.03	7.44	±	2.50	109.15	±	49.28
Frontal lobe	22.20	±	19.69			<LOD	4.56	±	6.90	2.81	±	2.98	29.58	±	10.73
Mean brain conc.	72.98	±	79.92			<LOD	18.47	±	15.71	6.07	±	2.41	97.51	±	36.07

[a] concentration in brain tissues was > LOD only in 3 out of 5 subjects.
[b] expressed as ng/mL.
[c] includes mammillary body.

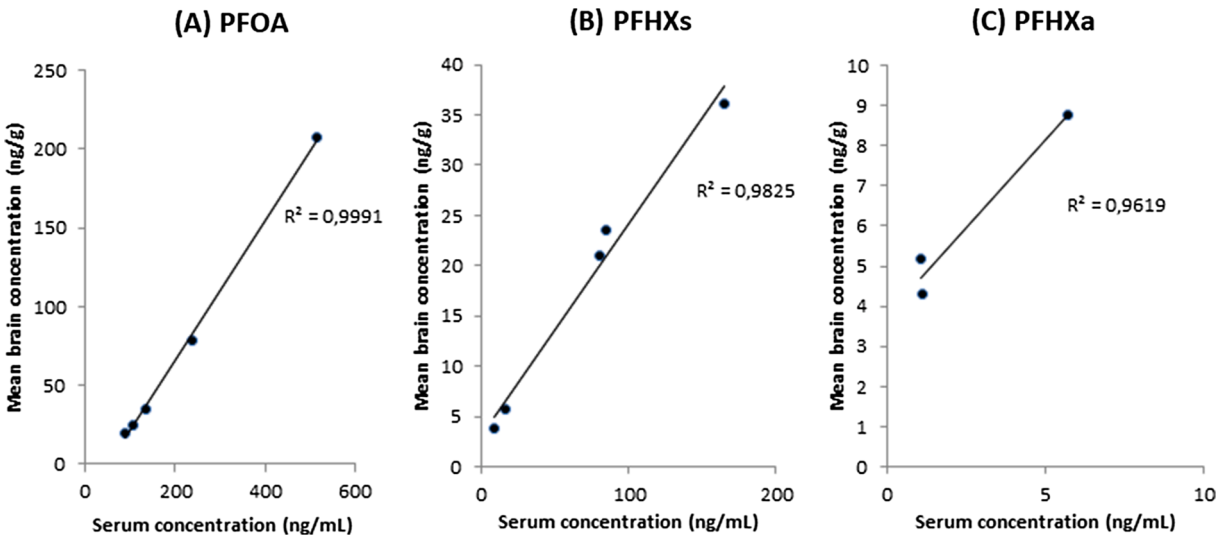

Fig. 1. Correlation analysis between serum and brain concentrations of PFOA (A), perfluoro-hexanesulfonic acid (PFHxS, B) and perfluorohexanoic acid (PFHxA, C) in post mortem specimens. Correlation's coefficient was calculated with Person's test.

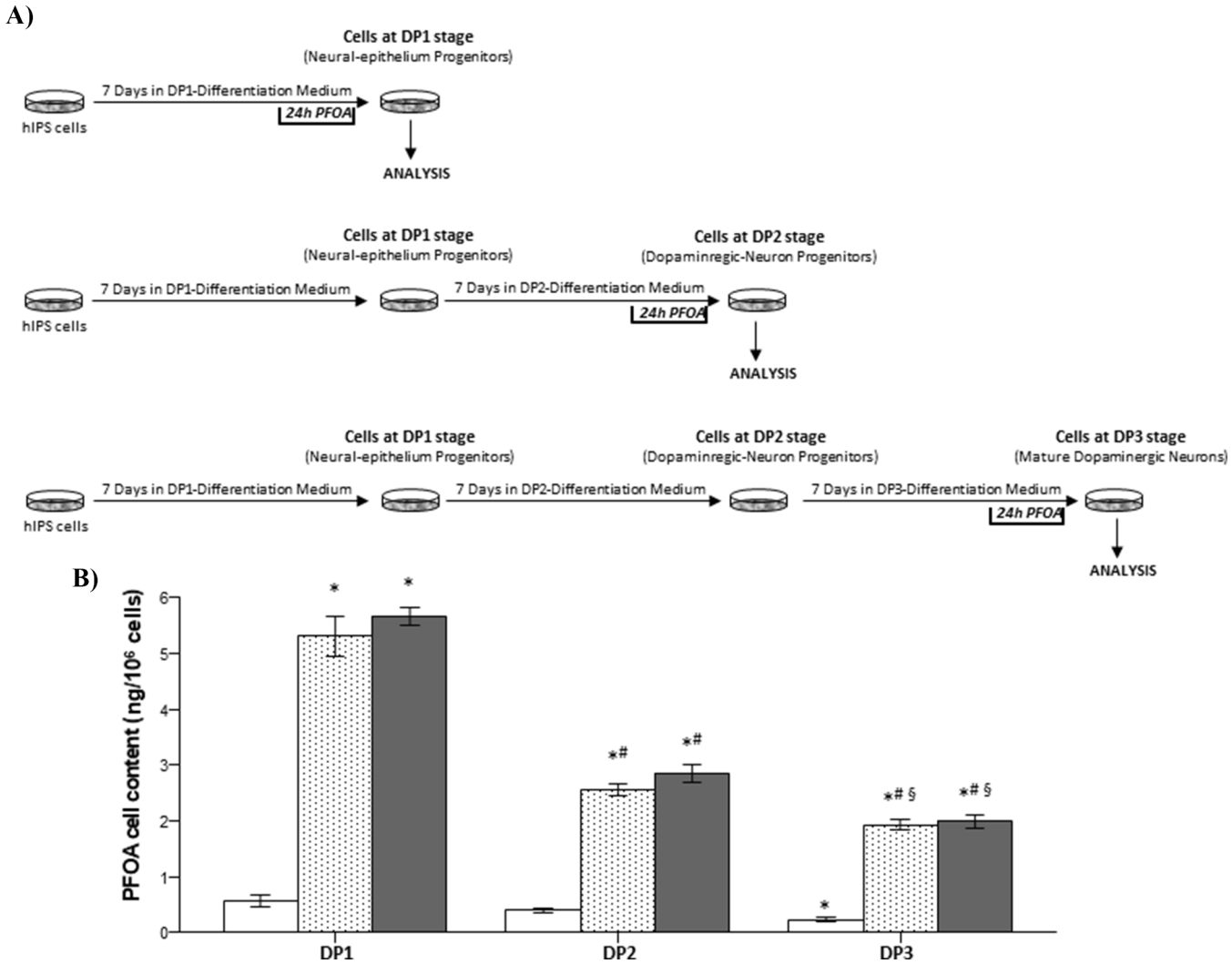

Fig. 2. A) Schematic representation of experimental design. B) PFOA accumulation during dopaminergic differentiation of hiPCs. Means ± SD of PFOA (ng/10^6 cells) in hiPCs after 24 h incubation with 1 (white bars), 10 (dotted bars) and 100 (grey bars) ng/ml of PFOA during the three stages of dopaminergic differentiation: commitment phase (DP1), the neuronal precursor phase (DP2) and the mature dopaminergic differentiation phase (DP3). *$p < 0.001$ vs 1 ng/mL treatment; $^{\#}p < 0.001$ vs DP1; $^{\S}p < 0.01$ vs DP2.

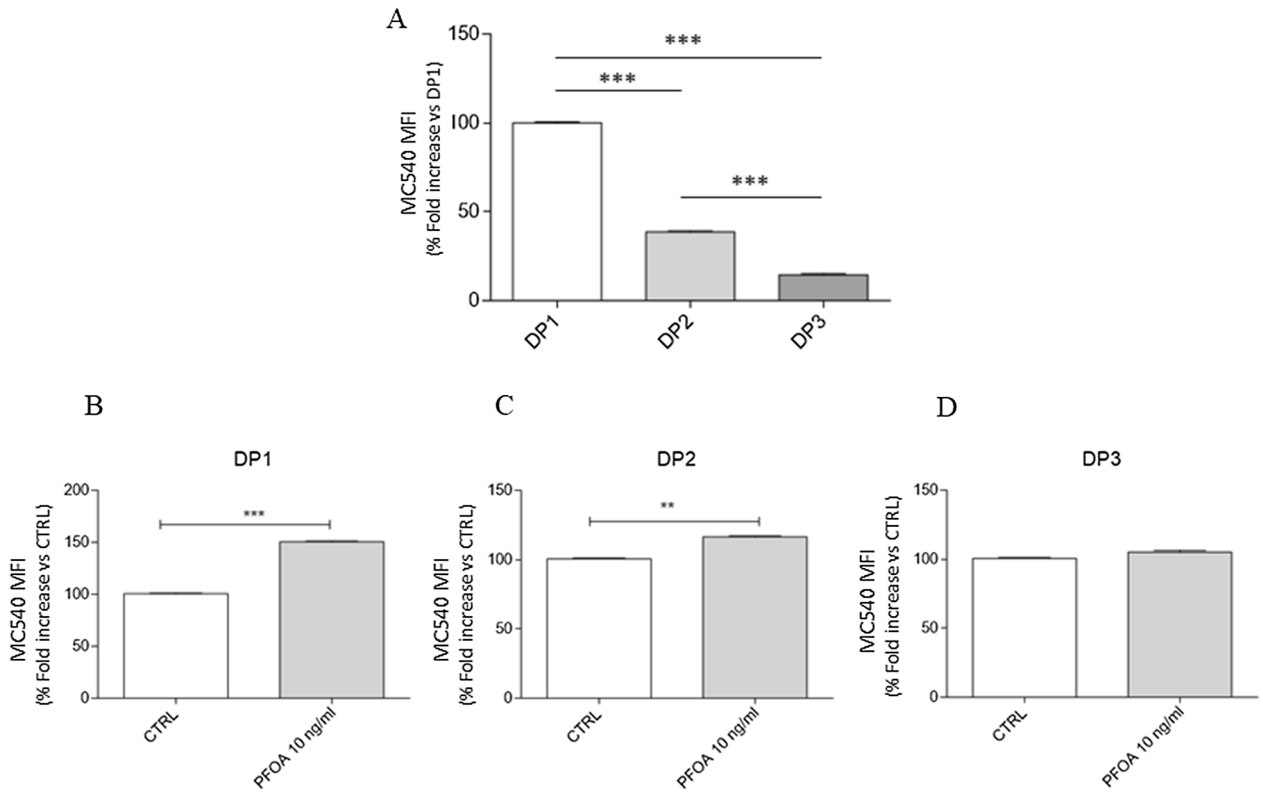

Fig. 3. Evaluation of membrane fluidity in the three phases of neurodifferentiation. Merocyanine 540 (MC540) Mean Fluorescence intensity (MFI, % of fold increase vs CTRL) at basal conditions (A) and after 24 h incubation with perfluoro-octanoic acid (PFOA 10 ng/ml) (B-D) between different stages of dopaminergic differentiation: commitment phase (DP1), the neuronal precursor phase (DP2) and the mature dopaminergic differentiation phase (DP3). ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

with a significant increase of the mean fluorescence intensity of MC540, compared to untreated control, only at DP1 (CTRL 100.0 ± 0.2%, PFOA 151.3 ± 0.3%; $p = 0.002$) and DP2 (CTRL 100.1 ± 0.5%, PFOA 116.5 ± 0.1%; $p = 0.019$); this effect was not observed at DP3 (CTRL 100.8 ± 0.3%, PFOA 104.9 ± 1.2%; $p = 0.057$; Fig. 3B-D). Taken together, these data suggest that short term-exposure of dopaminergic neurons cell model to PFOA associates with cell accumulation of the perfluoroalkyl substance according to the differentiation-dependent membrane composition, resulting in more profound perturbation of membrane properties at early stages of neuronal development.

3.3. Effect of PFOA on dopaminergic differentiation

In order to evaluate the possible effect of PFOA exposure on dopaminergic differentiation, alterations in cell morphology and the expression of different dopaminergic stages phenotype markers were evaluated in differentiating cells exposed to PFOA, in comparison with untreated cells. The comparison of cell morphology between untreated controls and cells exposed to PFOA showed minimal, or even none, morphological difference at any differentiation phase, suggesting low or no influence of PFOA on the cell cytoskeletal development (Supplemental Figure S1). The effect on the molecular phenotypes was evaluated through the expression of markers of dopaminergic differentiation, such as tyrosine hydroxylase (TH), dopamine transporter (DAT) and structural neural marker neurofilament heavy (NF-H), assessed by indirect immunofluorescence. At the early phase of dopaminergic differentiation (DP1), untreated cells showed a weak expression of TH and NFH, but no staining for DAT was detected (Fig. 4A-H). The exposure to PFOA was associated with non-major effect on the staining pattern of the whole panel of markers however, from a quantitative point of view, the normalized staining signal for NF-H showed a significant reduction compared to untreated control ($p = 0.0031$; Fig. 4I-M).

At the precursors of dopaminergic neurons phase (DP2), the normalized staining signal for NFH in untreated controls was significantly increased compared with DP1 phase (both p values < 0.001) whilst DAT staining was still almost undetectable at this stage (Fig. 5A-H). The exposure to PFOA was associated with a significant reduction of TH and NF-H normalized staining signal compared to untreated controls ($p = 0.0157$ and $p = 0.0155$, respectively; Fig. 5I-M). In mature dopaminergic neurons (DP3), untreated control cell showed a significant increase of the normalized staining signal for DAT (Fig. 6A-H; $p < 0.001$ vs DP2). The exposure to PFOA was associated with a significant reduction of the signal for the normalized staining signal of all the markers (respectively: $p = 0.0025$ for TH, $p = 0.0007$ for NFH and $p = 0.0026$ for DAT; Fig. 6I-M).

Taken together, these data show that the short-term exposure of differentiating dopaminergic neurons to PFOA was associated with a significant down-regulation of early markers on dopaminergic differentiation, such as TH and NF-H, and of late markers of dopaminergic function such as DAT, suggesting the possible impact of the perfluoroalkyl compound on the dopaminergic function, particularly in developing neuronal cells.

4. Discussion

PFAS are ubiquitous, long-lasting environmental contaminants that accumulate in human bodies, including brains. As such, understanding if and how they impact normal biological function is critical. Here, we have reported for the first time the specific accumulation of these chemicals in different brain areas, and we have investigated the impact of PFOA on dopaminergic neurons during different stages of differentiation. Our results are highly suggestive of a possible significant impact of PFAS exposure on the human brain, particularly during the most sensitive developmental phases of gestational life.

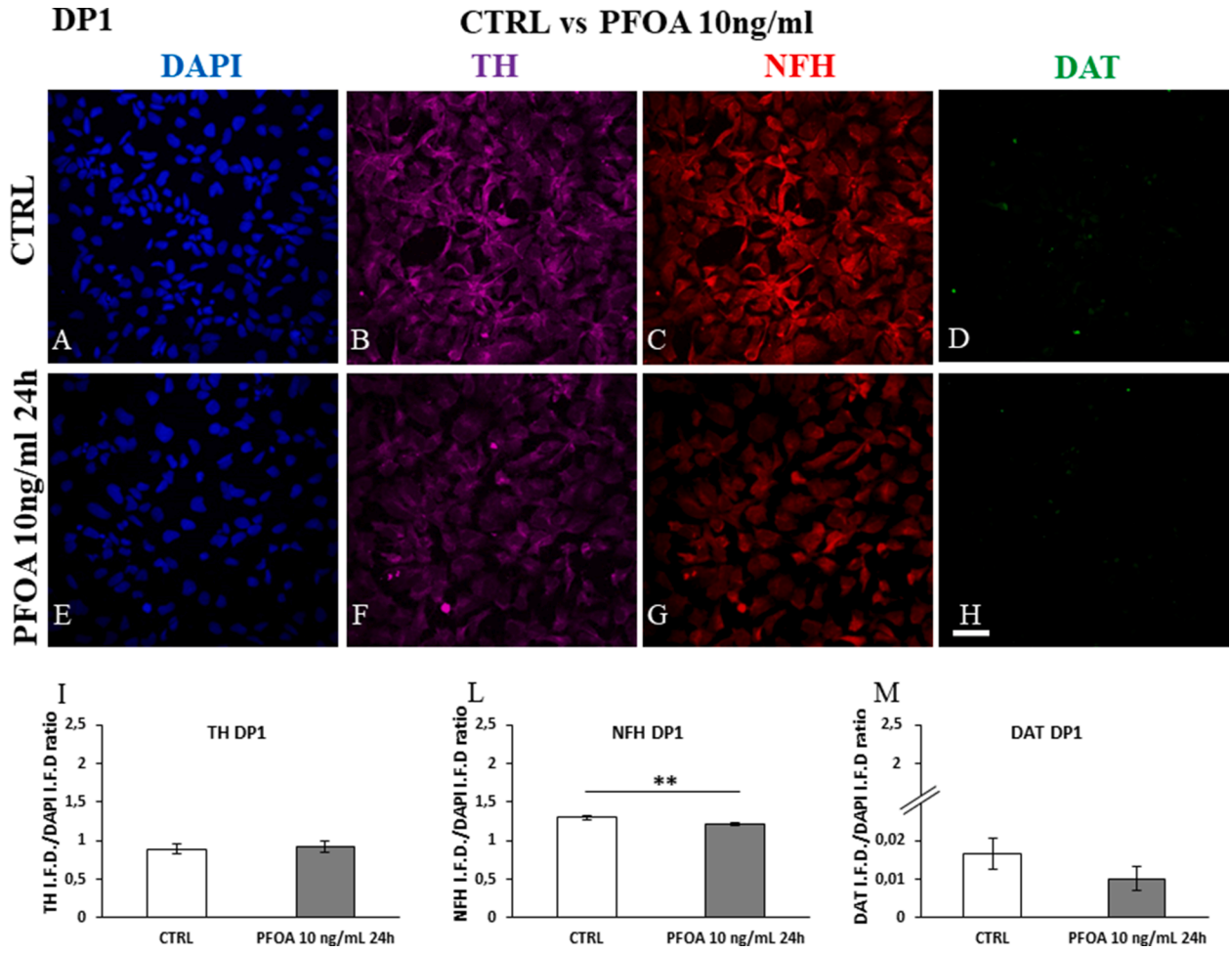

Fig. 4. Expression and quantification of tyrosine hydroxylase (TH), neurofilament heavy (NFH) and dopamine transporter (DAT) in the DP1 neurodifferentiation phase, by immunofluorescence. Cell nuclei are counterstained with 4′,6-diamidin-2-phenyindole (DAPI). Expression and fluorescence intensity quantification, reported as the ratio between the integrated fluorescence density (I.F.D.) of each color channel to the I.F.D. of DAPI, of the dopaminergic marker TH (B-F), of structural NHF (C-G) and DAT marker (D-H) in the CTRL and PFOA 10 ng/ml samples respectively. Scale bar 77.3 μm, Magnification 40X. **$p < 0.01$.

PFAS have been identified in brain specimens from human subjects (Harada et al., 2007; Maestri et al., 2006; Pérez et al., 2013), but also different animals with environmental or experimental exposure (Austin et al., 2003; Greaves et al., 2013; Pedersen et al., 2016). The hypothesized mechanism of blood–brain barrier crossing by PFAS has been ascribed to increased membrane permeability due to discontinuity of tight junctions induced on human microvascular epithelial brain cells that was likely caused by discontinuity in the tight-junctions (F. Wang et al., 2011; X. Wang et al., 2011). This discontinuity can be induced by PFOS stimulation of ROS, but only at very high levels (Lee et al., 2012; Reistad et al., 2013). Active PFAS uptake may also occur. The organic anion transporters (OATs), and organic anion transporter polypeptides (OATPs) form a class of proteins that actively shuttle molecules with specific biochemical properties across the brain barrier and have been reviewed elsewhere (Piekarski et al., 2020). More likely is the possible interference of PFAS in the cell membranes, as reported for other cellular models (De Toni et al., 2020; Šabović et al., 2019), where they induce and increase in membrane fluidity and increased structural instability. Another possible mechanism can be the simple passive diffusion, typical for molecules with a molecular weight below 400 Da approximately and involving<8 hydrogen bonds (Pardridge, 2012), therefore short-chain PFAS are more likely to cross blood–brain barrier. However, short-chain PFAS were not detected in Spanish human brain tissue samples (Pérez et al., 2013). PFOA on the other hand has a molecular weight of 414,17 Da, and its higher occurrence compared with sort-chain PFAS could be explained by the increased environmental, and serum, exposure to this molecule in this specific area. Recently, PFAS penetration was positively correlated with the barrier permeability index, indicating that barrier integrity was the main determinant of PFAS penetration across the barrier, and human data showed that PFOA, similar to serum, was still the dominant PFAS in cerebrospinal fluid (Wang et al., 2018). Moreover, we observed higher PFOA levels in the hypothalamus. This could be due to its physiological BBB higher permeability compared with other brain regions: the arcuate nucleus, located at the base of the mediobasal hypothalamus, contains neurons that sense circulating hormones and nutrients. It is morphologically located in close proximity to the median eminence, which lacks a typical BBB (Morita-Takemura and Wanaka, 2019), and accumulating evidence indicates that the arcuate nucleus has access to circulating molecules through the median eminence (Faouzi et al., 2007). Structural characteristics confer high permeability on this specific vasculature and make the median eminence a primary site of exchange between the systemic circulation and the brain. The high vascular permeability permits neuroendocrine neurons to secrete large amounts of hypothalamic releasing hormones into the bloodstream, but could also allow exogenous chemicals to passively cross BBB and reach the hypothalamus. Interestingly, we found the highest PFAS concentration specifically in arcuate nucleus and mammillothalamic tract of the hypothalamus, which are highly innervated by dopaminergic neurons (Jaber et al., 1996). Indeed, mice exposed to PFOS showed reduced levels of dopamine and DOPAC released in the

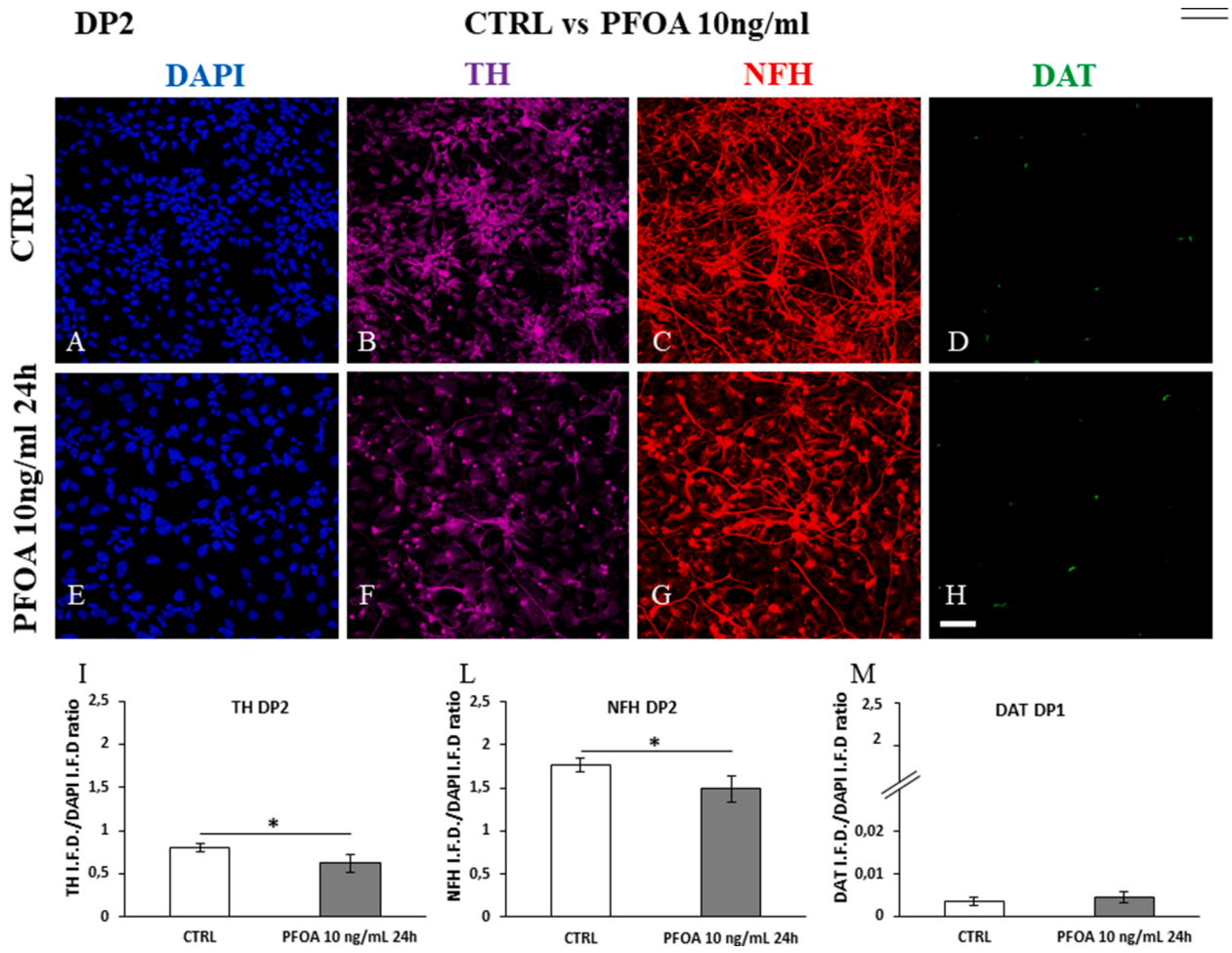

Fig. 5. Expression and quantification of tyrosine hydroxylase (TH), neurofilament heavy (NFH) and dopamine transporter (DAT) in the DP2 neurodifferentiation phase, by immunofluorescence. Cell nuclei are counterstained with 4′,6-diamidin-2-phenyindole (DAPI). Expression and fluorescence intensity quantification, reported as the ratio between the integrated fluorescence density (I.F.D.) of each color channel to the I.F.D. of DAPI, of the dopaminergic marker TH (B-F), of structural NHF (C-G) and DAT marker (D-H) in the CTRL and PFOA 10 ng/ml samples respectively. Scale bar 77.3 μm, Magnification 40X. *p < 0.05.

hippocampus (Long et al., 2013), suggesting a possible alteration of dopamine synthesis or transport. For these reasons, we focused on different stages (DP1, DP2, and DP3) of dopaminergic neural differentiation after induction of h-iPS cells. In particular, we focused on the specific effect of the exposure to PFOA for 24 h, at the concentration of 10 ng/ml, which is comparable with average PFAS levels in brain reported in non-exposed general populations (Harada et al., 2007; Maestri et al., 2006; Pérez et al., 2013) and even lower than those reported in this study on exposed population. Particularly, we chose PFOA rather than short-chain PFAS because of its higher environmental distribution in the Veneto Region specific population sampled for this study (Pitter et al., 2020). It should be noted that the blood and brain levels of some PFAS, particularly PFOA, observed in our study, are higher compared with those previously reported (La Rocca et al., 2015; Rocca et al., 2014). This difference could be likely due to the specific hotspot with severe environmental pollution from the residential area of subjects in this study, for which particularly elevated serum levels of PFOA, and comparable with our results, have already been reported (Pitter et al., 2020). Nonetheless, our in vitro results underline the possible risk also for the general population, in which the substantial PFAS exposure should not be underestimated.

On the base of the strong surfactant activity of PFAS, and particularly PFOA, major interactions of these compounds with the cell membrane have been hypothesized (Sanchez Garcia et al., 2018). In particular, PFOA has been suggested to fit within the phospholipid bilayer, destabilizing chemical-physical interactions among fatty acids and resulting in major alteration of membrane's biophysical properties, leading to higher membrane fluidity (De Toni et al., 2020). Importantly, plasma membrane of neuronal cells is acknowledged to undergo to a progressive enrichment in raft-forming lipids along with the maturation process, which is necessary to achieve peculiar bulk membrane properties sustaining the unique demands of neuronal processes and extremely fast signal transduction (Salem et al., 2001). The resulting increased domain stability has been recently evidenced by higher domain melting temperature during development (Tulodziecka et al., 2016). On these bases, a significant variation of plasma membrane biophysical properties is expected along with the maturation process of neurons. We found that PFOA accumulation in dopaminergic neurons was significantly dependent on the membrane fluidity of the respective stage, which is typically higher in the early phases of dopaminergic differentiation. Indeed, in the DP1 stage we observed the higher accumulation of PFOA, together with a greater membrane fluidity. As the maturation proceeds, cell membrane becomes more rigid and less prone to PFOA penetration, and indeed we observed lower PFOA accumulation in mature cells. Also, membrane fluidity was not altered in DP2 and DP3 stages, confirming that early DP1 differentiation stage is the most sensitive to PFOA perturbation. This resulted in a significant decrease of NFH compared with untreated controls. Nonetheless, in the later DP2 and DP3 phase we still observed a significant alteration of the molecular phenotype, as reported by a reduction in TH, NFH and DAT. These

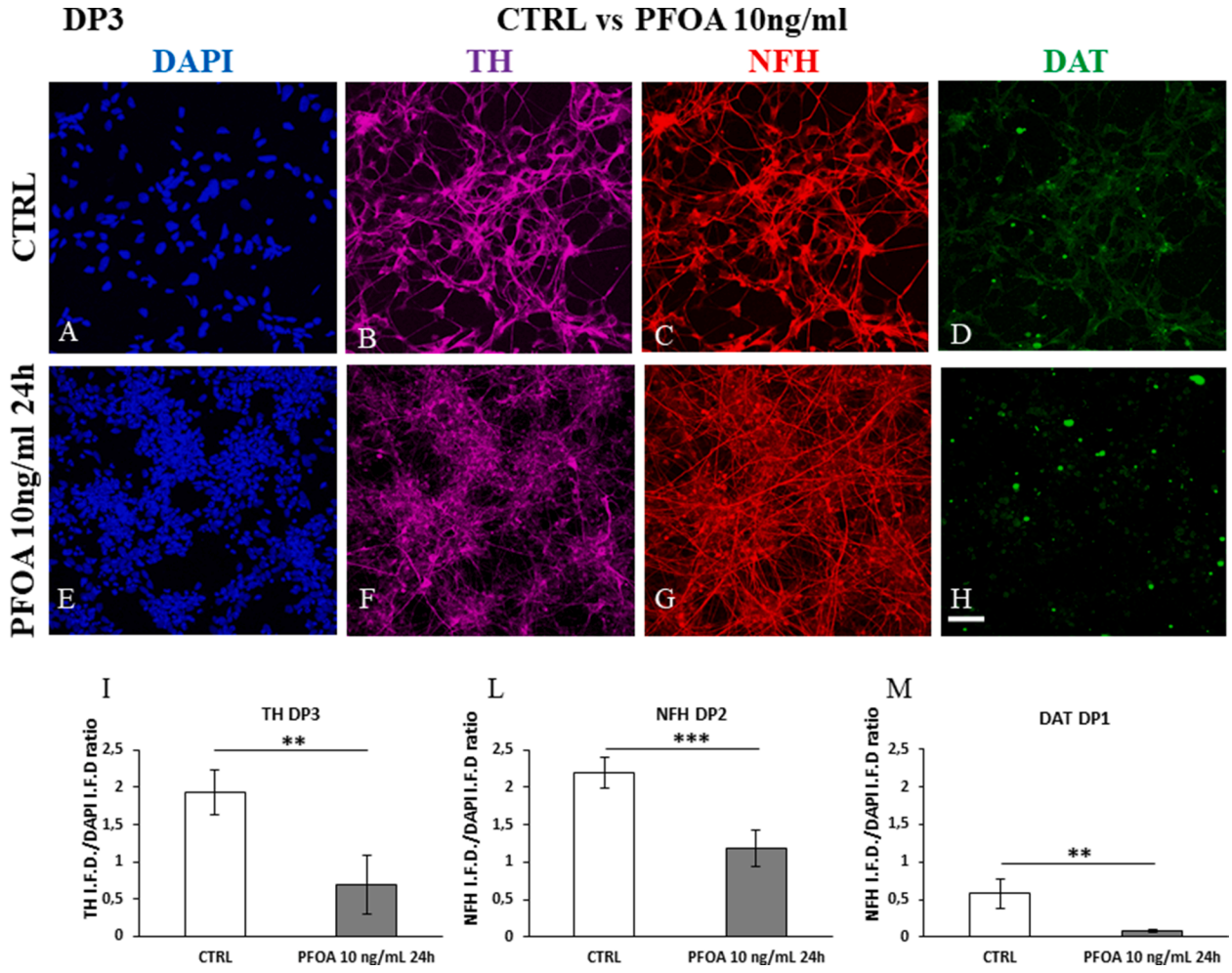

Fig. 6. Expression and quantification of tyrosine hydroxylase (TH), neurofilament heavy (NFH) and dopamine transporter (DAT) in the DP3 neurodifferentiation phase, by immunofluorescence. Cell nuclei are counterstained with 4′,6-diamidin-2-phenyindole (DAPI). Expression and fluorescence intensity quantification, reported as the ratio between the integrated fluorescence density (I.F.D.) of each color channel to the I.F.D. of DAPI, of the dopaminergic marker TH (B-F), of structural NHF (C-G) and DAT marker (D-H) in the CTRL and PFOA 10 ng/ml samples respectively. Scale bar 77.3 μm, Magnification 40X. **$p < 0.01$.

effects are probably independent from the membrane permeability, and even if at lower concentrations (5.5 ± 0.3 ng/10^6 DP1 cells; 3.5 ± 0.2 ng/10^6 DP2 cells, 1.85 ± 0.2 ng/10^6 DP3 cells), PFOA is capable of altering the expression of dopaminergic markers. The impact of PFAS on the dopaminergic system has been recently reviewed elsewhere (Piekarski et al., 2020). To this regard, although results were strongly dependent on the species considered, the exposure to PFOS for 2 months was associated with the decrease of both tyrosine hydroxylase and D2 dopamine receptor expression in NMRI mice (Hallgren and Viberg, 2016). In addition, a recent paper found that in frogs, both PFOA and PFOS decrease dopamine content but do not affect serotonin, norepinephrine, GABA, glutamate, or acetylcholine (Foguth et al., 2019). In agreement with our results, a very recent study has reported a significant reduction in dopamine and TH in PFAS-exposed male mice (Grønnestad et al., 2021). In general, much of the in vitro research into neurobiological impacts has focused on ROS induction and apoptosis-mediated cell death by PFAS. For example, cultured neurons exposed to PFOS and PFHxS for<24 h showed signs of cell death, while PFOA appeared to induce only a minimal amount of toxicity, in agreement with our data (Lee et al., 2014; Li et al., 2017). Altogether these data support that PFAS may act in a cumulative way as suggested by EFSA (D et al., 2020). Further studies are needed, possibly with dose–response curves to confirm these ancillary results.

One of the more consistent links observed is between developmental PFAS exposure and increased risk for ADHD or its symptomology (Lenters et al., 2019). Studies suggest that ADHD could be linked to the dysfunction of dopamine: a reduction in dopamine synaptic markers associated with symptoms of inattention was shown in the dopamine reward pathway of participants with ADHD (Volkow et al., 2009). Interestingly, DAT polymorphisms have been shown to influence ADHD-like traits (Jeong et al., 2015). More recently, a Norwegian cohort study has reported that Prenatal exposure to PFOA was associated with increased risk of autism spectrum disorders and ADHD in children (Skogheim et al., 2021). Therefore, PFAS interference on dopaminergic neurons could impact on the risk of developing ADHD or on its clinical manifestations, and particularly if the exposure to these contaminants happens during the most sensitive windows of CNS development in gestational life. As mentioned previously, PFAS may penetrate the BBB and, although this is particularly relevant during prenatal development when the BBB is more permeable and during neuro-differentiation, there are many circumstances in which adults may have a weakened BBB favoring PFAS brain penetration (Piekarski et al., 2020). For example, the BBB weakens with age and neurodegenerative disease (Sweeney et al., 2018). An epidemiological study indeed found an increased risk ratio for Alzheimer's disease in the same highly polluted area of the Veneto Region in Italy (Mastrantonio et al., 2018). Overall, the literature linking PFAS exposures with neurodevelopmental outcomes is still inconsistent, suggesting the need for more research to elucidate the neurotoxicological potential of PFAS, both epidemiological and experimental.

In conclusion, our results are highly suggestive of a significant accumulation of PFAS in specific areas of human brain, with an impact on dopaminergic neurons expression of specific markers of endocrine function of these cells. Altogether these data provide a possible new model of central nervous system disruption associated with the exposure to this class of chemical pollutants.

CRediT authorship contribution statement

Andrea Di Nisio: Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Project administration. **Micaela Pannella:** Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Stefania Vogiatzis:** Methodology, Data curation. **Stefania Sut:** Software, Validation, Formal analysis, Data curation. **Stefano Dall'Acqua:** Software, Validation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Maria Santa Rocca:** Validation, Formal analysis, Visualization. **Angelo Antonini:** Conceptualization, Writing – review & editing, Supervision. **Andrea Porzionato:** . **Raffaele De Caro:** Writing – review & editing, Supervision. **Mario Bortolozzi:** Methodology, Writing – original draft, Writing – review & editing. **Luca De Toni:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Investigation. **Carlo Foresta:** Conceptualization, Supervision, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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References

Austin, M.E., Kasturi, B.S., Barber, M., Kannan, K., MohanKumar, P.S., MohanKumar, S. M.J., 2003. Neuroendocrine effects of perfluorooctane sulfonate in rats. Environ. Health Perspect. 111 (12), 1485–1489. https://doi.org/10.1289/ehp.6128.

Borgs, L., Peyre, E., Alix, P., Hanon, K., Grobarczyk, B., Godin, J.D., Purnelle, A., Krusy, N., Maquet, P., Lefebvre, P., Seutin, V., Malgrange, B., Nguyen, L., 2016. Dopaminergic neurons differentiating from LRRK2 G2019S induced pluripotent stem cells show early neuritic branching defects. Sci. Reports 2016 61 6, 1–11. https://doi.org/10.1038/srep33377.

Brendel, S., Fetter, É., Staude, C., Vierke, L., Biegel-Engler, A., 2018. Short-chain perfluoroalkyl acids: environmental concerns and a regulatory strategy under REACH. Environ. Sci. Eur. 30 (1) https://doi.org/10.1186/s12302-018-0134-4.

Conder, J.M., Hoke, R.A., Wolf, W.d., Russell, M.H., Buck, R.C., 2008. Are PFCAs bioaccumulative? A critical review and comparison with regulatory criteria and persistent lipophilic compounds. Environ. Sci. Technol. 42 (4), 995–1003.

Cousins, I.T., Goldenman, G., Herzke, D., Lohmann, R., Miller, M., Ng, C.A., Patton, S., Scheringer, M., Trier, X., Vierke, L., Wang, Z., DeWitt, J.C., 2019. The concept of essential use for determining when uses of PFASs can be phased out. Environ. Sci. Process. Impacts 21 (11), 1803–1815. https://doi.org/10.1039/C9EM00163H.

D, S., M, B., L, B., JK, C., J, D.M., B, G.-K., C, H., LR, H., JC, L., CS, N., E, N., E, N., A, P., S, S., C, V., H, W., L, B., S, C., JP, C., TI, H., LS, H., N, J., HK, K., M, R., AC, R., H, V.L., G, V., K, M., F, R., T, S., 2020. Risk to human health related to the presence of perfluoroalkyl substances in food. EFSA journal. Eur. Food Saf. Auth. 18. https://doi.org/10.2903/J.EFSA.2020.6223.

De Toni, L., Radu, C.M., Sabovic, I., Di Nisio, A., Dall'Acqua, S., Guidolin, D., Spampinato, S., Campello, E., Simioni, P., Foresta, C., 2020. Increased cardiovascular risk associated with chemical sensitivity to perfluoro–octanoic acid: Role of impaired platelet aggregation. Int. J. Mol. Sci. 21 (2), 399. https://doi.org/10.3390/ijms21020399.

Di Nisio, A., Foresta, C., 2019. Water and soil pollution as determinant of water and food quality/contamination and its impact on male fertility. Reprod. Biol. Endocrinol. 17, 4. https://doi.org/10.1186/s12958-018-0449-4.

Di Nisio, A., Rocca, M.S., Sabovic, I., De Rocco Ponce, M., Corsini, C., Guidolin, D., Zanon, C., Acquasaliente, L., Carosso, A.R., De Toni, L., Foresta, C., 2020. Perfluorooctanoic acid alters progesterone activity in human endometrial cells and induces reproductive alterations in young women. Chemosphere 242, 125208. https://doi.org/10.1016/j.chemosphere.2019.125208.

Di Nisio, A., Sabovic, I., Valente, U., Tescari, S., Rocca, M.S., Guidolin, D., Dall'Acqua, S., Acquasaliente, L., Pozzi, N., Plebani, M., Garolla, A., Foresta, C., 2019. Endocrine Disruption of Androgenic Activity by Perfluoroalkyl Substances: Clinical and Experimental Evidence. J. Clin. Endocrinol. Metab. 104, 1259–1271. https://doi.org/10.1210/jc.2018-01855.

Faouzi, M., Leshan, R., Björnholm, M., Hennessey, T., Jones, J., Münzberg, H., 2007. Differential Accessibility of Circulating Leptin to Individual Hypothalamic Sites. Endocrinology 148, 5414–5423. https://doi.org/10.1210/EN.2007-0655.

Foguth, R.M., Flynn, R.W., de Perre, C., Iacchetta, M., Lee, L.S., Sepúlveda, M.S., Cannon, J.R., 2019. Developmental exposure to perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) selectively decreases brain dopamine levels in Northern leopard frogs. Toxicol. Appl. Pharmacol. 377, 114623. https://doi.org/10.1016/j.taap.2019.114623.

Frisbee, S.J., Brooks, A.P., Maher, A., Flensborg, P., Arnold, S., Fletcher, T., Steenland, K., Shankar, A., Knox, S.S., Pollard, C., Halverson, J.A., Vieira, V.M., Jin, C., Leyden, K. M., Ducatman, A.M., 2009. The C8 health project: Design, methods, and participants. Environ. Health Perspect. 117 (12), 1873–1882. https://doi.org/10.1289/ehp.080037910.1289/ehp.0800379.s1.

Fujii, S., Polprasert, C., Tanaka, S., Lien, N.P.H., Qiu, Y., 2007. New POPs in the water environment: Distribution, bioaccumulation and treatment of perfluorinated compounds - A review paper. J. Water Supply Res. Technol. - AQUA 56, 313–326. https://doi.org/10.2166/aqua.2007.005.

Gallo, V., Leonardi, G., Brayne, C., Armstrong, B., Fletcher, T., 2013. Serum perfluoroalkyl acids concentrations and memory impairment in a large cross-sectional study. BMJ Open 3 (6), e002414. https://doi.org/10.1136/bmjopen-2012-002414.

Giesy, J.P., Kannan, K., 2001. Global distribution of perfluorooctane sulfonate in wildlife. Environ. Sci. Technol. 35 (7), 1339–1342. https://doi.org/10.1021/es001834k.

Greaves, A.K., Letcher, R.J., Sonne, C., Dietz, R., 2013. Brain region distribution and patterns of bioaccumulative perfluoroalkyl carboxylates and sulfonates in East Greenland polar bears (Ursus maritimus). Environ. Toxicol. Chem. 32 (3), 713–722. https://doi.org/10.1002/etc.2107.

Gremmel, C., Frömel, T., Knepper, T.P., 2016. HPLC–MS/MS methods for the determination of 52 perfluoroalkyl and polyfluoroalkyl substances in aqueous samples. Anal. Bioanal. Chem. 2016 4096 409, 1643–1655. https://doi.org/10.1007/S00216-016-0110-Z.

Grønnestad, R., Johanson, S.M., Müller, M.H.B., Schlenk, D., Tanabe, P., Krøkje, Å., Jaspers, V.L.B., Jenssen, B.M., Ræder, E.M., Lyche, J.L., Shi, Q., Arukwe, A., 2021. Effects of an environmentally relevant PFAS mixture on dopamine and steroid hormone levels in exposed mice. Toxicol. Appl. Pharmacol. 428, 115670. https://doi.org/10.1016/j.taap.2021.115670.

Hallgren, S., Viberg, H., 2016. Postnatal exposure to PFOS, but not PBDE 99, disturb dopaminergic gene transcription in the mouse CNS. Environ. Toxicol. Pharmacol. 41, 121–126. https://doi.org/10.1016/j.etap.2015.11.016.

Harada, K.H., Hashida, S., Kaneko, T., Takenaka, K., Minata, M., Inoue, K., Saito, N., Koizumi, A., 2007. Biliary excretion and cerebrospinal fluid partition of perfluorooctanoate and perfluorooctane sulfonate in humans. Environ. Toxicol. Pharmacol. 24 (2), 134–139. https://doi.org/10.1016/j.etap.2007.04.003.

Jaber, M., Robinson, S.W., Missale, C., Caron, M.G., 1996. Dopamine receptors and brain function. Neuropharmacology 35 (11), 1503–1519. https://doi.org/10.1016/S0028-3908(96)00100-1.

Jeong, S.H., Choi, K.S., Lee, K.Y., Kim, E.J., Kim, Y.S., Joo, E.J., 2015. Association between the dopamine transporter gene (DAT1) and attention deficit hyperactivity disorder-related traits in healthy adults. Psychiatr. Genet. 25, 119–126. https://doi.org/10.1097/YPG.0000000000000086.

Johansson, S., Halleland, H., Halmøy, A., Jacobsen, K.K., Landaas, E.T., Dramsdahl, M., Fasmer, O.B., Bergsholm, P., Lundervold, A.J., Gillberg, C., Hugdahl, K., Knappskog, P.M., Haavik, J., 2008. Genetic analyses of dopamine related genes in adult ADHD patients suggest an association with the DRD5-microsatellite repeat, but not with DRD4 or SLC6A3 VNTRs. Am. J. Med. Genet. Part B Neuropsychiatr. Genet. 147B (8), 1470–1475. https://doi.org/10.1002/ajmg.b.v147b:810.1002/ajmg.b.30662.

Kucharzyk, K.H., Darlington, R., Benotti, M., Deeb, R., Hawley, E., 2017. Novel treatment technologies for PFAS compounds: A critical review. J. Environ. Manage. 204, 757–764. https://doi.org/10.1016/j.jenvman.2017.08.016.

La Rocca, C., Tait, S., Guerranti, C., Busani, L., Ciardo, F., Bergamasco, B., Perra, G., Mancini, F.R., Marci, R., Bordi, G., Caserta, D., Focardi, S., Moscarini, M., Mantovani, A., 2015. Exposure to Endocrine Disruptors and Nuclear Receptors Gene Expression in Infertile and Fertile Men from Italian Areas with Different Environmental Features. Int. J. Environ. Res. Public Health 12, 12426–12445. https://doi.org/10.3390/ijerph121012426.

Lee, H.-G., Lee, Y.J., Yang, J.-H., 2012. Perfluorooctane sulfonate induces apoptosis of cerebellar granule cells via a ROS-dependent protein kinase C signaling pathway. Neurotoxicology 33 (3), 314–320. https://doi.org/10.1016/j.neuro.2012.01.017.
Lee, I., Viberg, H., 2013. A single neonatal exposure to perfluorohexane sulfonate (PFHxS) affects the levels of important neuroproteins in the developing mouse brain. Neurotoxicology 37, 190–196. https://doi.org/10.1016/j.neuro.2013.05.007.
Lee, Y.J., Choi, S.Y., Yang, J.H., 2014. PFHxS induces apoptosis of neuronal cells via ERK1/2-mediated pathway. Chemosphere 94, 121–127. https://doi.org/10.1016/J.CHEMOSPHERE.2013.09.059.
Lenters, V., Iszatt, N., Forns, J., Čechová, E., Kočan, A., Legler, J., Leonards, P., Stigum, H., Eggesbø, M., 2019. Early-life exposure to persistent organic pollutants (OCPs, PBDEs, PCBs, PFASs) and attention-deficit/hyperactivity disorder: A multi-pollutant analysis of a Norwegian birth cohort. Environ. Int. 125, 33–42. https://doi.org/10.1016/j.envint.2019.01.020.
Li, Z., Liu, Q., Liu, C., Li, C., Li, Y., Li, S., Liu, X., Shao, J., 2017. Evaluation of PFOS-mediated neurotoxicity in rat primary neurons and astrocytes cultured separately or in co-culture. Toxicol. Vitr. 38, 77–90. https://doi.org/10.1016/j.tiv.2016.11.002.
Long, Y., Wang, Y., Ji, G., Yan, L., Hu, F., Gu, A., 2013. Neurotoxicity of Perfluorooctane Sulfonate to Hippocampal Cells in Adult Mice. PLoS One 8. https://doi.org/10.1371/journal.pone.0054176.
Maestri, L., Negri, S., Ferrari, M., Ghittori, S., Fabris, F., Danesino, P., Imbriani, M., 2006. Determination of perfluorooctanoic acid and perfluorooctanesulfonate in human tissues by liquid chromatography/single quadrupole mass spectrometry. Rapid Commun. Mass Spectrom. 20 (18), 2728–2734. https://doi.org/10.1002/(ISSN)1097-023110.1002/rcm.v20:1810.1002/rcm.2661.
Mastrantonio, M., Bai, E., Uccelli, R., Cordiano, V., Screpanti, A., Crosignani, P., 2018. Drinking water contamination from perfluoroalkyl substances (PFAS): An ecological mortality study in the Veneto Region. Italy. Eur. J. Public Health 28, 180–185. https://doi.org/10.1093/eurpub/ckx066.
Minuz, P., De Toni, L., Dall'Acqua, S., Di Nisio, A., Sabovic, I., Castelli, M., Meneguzzi, A., Foresta, C., 2021. Interference of C6O4 on platelet aggregation pathways: Cues on the new-generation of perfluoro-alkyl substance. Environ. Int. 154, 106584. https://doi.org/10.1016/j.envint.2021.106584.
Morita-Takemura, S., Wanaka, A., 2019. Blood-to-brain communication in the hypothalamus for energy intake regulation. Neurochem. Int. 128, 135–142. https://doi.org/10.1016/J.NEUINT.2019.04.007.
Nakayama, S.F., Isobe, T., Iwai-Shimada, M., Kobayashi, Y., Nishihama, Y., Taniguchi, Y. u., Sekiyama, M., Michikawa, T., Yamazaki, S., Nitta, H., Oda, M., Mitsubuchi, H., Sanefuji, M., Ohga, S., Mise, N., Ikegami, A., Suga, R., Shimono, M., 2020. Poly- and perfluoroalkyl substances in maternal serum: Method development and application in Pilot Study of the Japan Environment and Children's Study. J. Chromatogr. A 1618, 460933. https://doi.org/10.1016/j.chroma.2020.460933.
Oh, J., Schmidt, R.J., Tancredi, D., Calafat, A.M., Roa, D.L., Hertz-Picciotto, I., Shin, H.-M., 2021. Prenatal exposure to per- and polyfluoroalkyl substances and cognitive development in infancy and toddlerhood. Environ. Res. 196, 110939. https://doi.org/10.1016/j.envres.2021.110939.
Pardridge, W.M., 2012. Drug Transport across the Blood–Brain Barrier: https://doi.org/10.1038/jcbfm.2012.126 32, 1959–1972. https://doi.org/10.1038/JCBFM.2012.126.
Pedersen, K.E., Letcher, R.J., Sonne, C., Dietz, R., Styrishave, B., 2016. Per- and polyfluoroalkyl substances (PFASs) – New endocrine disruptors in polar bears (Ursus maritimus)? Environ. Int. 96, 180–189. https://doi.org/10.1016/J.ENVINT.2016.07.015.
Pérez, F., Nadal, M., Navarro-Ortega, A., Fàbrega, F., Domingo, J.L., Barceló, D., Farré, M., 2013. Accumulation of perfluoroalkyl substances in human tissues. Environ. Int. 59, 354–362. https://doi.org/10.1016/j.envint.2013.06.004.
Piekarski, D.J., Diaz, K.R., McNerney, M.W., 2020. Perfluoroalkyl chemicals in neurological health and disease: Human concerns and animal models. Neurotoxicology 77, 155–168. https://doi.org/10.1016/J.NEURO.2020.01.001.
Pitter, G., Da Re, F., Canova, C., Barbieri, G., Jeddi, M.Z., Daprà, F., Manea, F., Zolin, R., Bettega, A.M., Stopazzolo, G., Vittorii, S., Zambelli, L., Martuzzi, M., Mantoan, D., Russo, F., 2020. Serum levels of perfluoroalkyl substances (PFAS) in adolescents and young adults exposed to contaminated drinking water in the Veneto region, Italy: A cross-sectional study based on a health surveillance program. Environ. Health Perspect. 128, 1–12. https://doi.org/10.1289/EHP5337.
Porzionato, A., Macchi, V., Stecco, C., Mazzi, A., Rambaldo, A., Sarasin, G., Parenti, A., Scipioni, A., De Caro, R., 2012. Quality management of Body Donation Program at the University of Padova. Anat Sci Educ. 5 (5), 264–272. https://doi.org/10.1002/ase.v5.510.1002/ase.1285.
Rahman, M.F., Peldszus, S., Anderson, W.B., 2014. Behaviour and fate of perfluoroalkyl and polyfluoroalkyl substances (PFASs) in drinking water treatment: A review. Water Res. 50, 318–340. https://doi.org/10.1016/j.watres.2013.10.045.
Reistad, T., Fonnum, F., Mariussen, E., 2013. Perfluoroalkylated compounds induce cell death and formation of reactive oxygen species in cultured cerebellar granule cells. Toxicol. Lett. 218 (1), 56–60. https://doi.org/10.1016/j.toxlet.2013.01.006.
Rocca, C. La, Tait, S., Guerranti, C., Busani, L., Ciardo, F., Bergamasco, B., Stecca, L., Perra, G., Mancini, F.R., Marci, R., Bordi, G., Caserta, D., Focardi, S., Moscarini, M., Mantovani, A., 2014. Exposure to Endocrine Disrupters and Nuclear Receptor Gene Expression in Infertile and Fertile Women from Different Italian Areas. Int. J. Environ. Res. Public Heal. 2014, Vol. 11, Pages 10146-10164 11, 10146–10164. https://doi.org/10.3390/IJERPH111010146.
Šabović, I., Cosci, I., De Toni, L., Ferramosca, A., Stornaiuolo, M., Di Nisio, A., Dall'Acqua, S., Garolla, A., Foresta, C., 2019. Perfluoro-octanoic acid impairs sperm motility through the alteration of plasma membrane. J. Endocrinol. Investig. 2019 435 43, 641–652. https://doi.org/10.1007/S40618-019-01152-0.
Salem, N., Litman, B., Kim, H.-Y., Gawrisch, K., 2001. Mechanisms of action of docosahexaenoic acid in the nervous system. Lipids 36 (9), 945–959. https://doi.org/10.1007/s11745-001-0805-6.
Sanchez Garcia, D., Sjödin, M., Hellstrandh, M., Norinder, U., Nikiforova, V., Lindberg, J., Wincent, E., Bergman, Å., Cotgreave, I., Munic Kos, V., 2018. Cellular accumulation and lipid binding of perfluorinated alkylated substances (PFASs) – A comparison with lysosomotropic drugs. Chem. Biol. Interact. 281, 1–10. https://doi.org/10.1016/J.CBI.2017.12.021.
Shin, H.-M., Bennett, D.H., Calafat, A.M., Tancredi, D., Hertz-Picciotto, I., 2020. Modeled prenatal exposure to per- and polyfluoroalkyl substances in association with child autism spectrum disorder: A case-control study. Environ. Res. 186, 109514. https://doi.org/10.1016/j.envres.2020.109514.
Shoeib, M., Harner, T., Vlahos, P., 2006. Perfluorinated chemicals in the arctic atmosphere. Environ. Sci. Technol. 40 (24), 7577–7583. https://doi.org/10.1021/es0618999.
Skogheim, T.S., Weyde, K.V.F., Aase, H., Engel, S.M., Surén, P., Øie, M.G., Biele, G., Reichborn-Kjennerud, T., Brantsæter, A.L., Haug, L.S., Sabaredzovic, A., Auyeung, B., Villanger, G.D., 2021. Prenatal exposure to per- and polyfluoroalkyl substances (PFAS) and associations with attention-deficit/hyperactivity disorder and autism spectrum disorder in children. Environ. Res. 202, 111692. https://doi.org/10.1016/j.envres.2021.111692.
Steenland, K., Fletcher, T., Savitz, D.A., 2010. Epidemiologic evidence on the health effects of perfluorooctanoic acid (PFOA). Environ. Health Perspect. 118 (8), 1100–1108. https://doi.org/10.1289/ehp.0901827.
Sweeney, M.D., Sagare, A.P., Zlokovic, B. V., 2018. Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders. Nat. Rev. Neurol. https://doi.org/10.1038/nrneurol.2017.188.
Trevisan, M., Desole, G., Costanzi, G., Lavezzo, E., Palù, G., Barzon, L., 2017. Reprogramming methods do not affect gene expression profile of human induced pluripotent stem cells. Int. J. Mol. Sci. 18 (1), 206. https://doi.org/10.3390/ijms18010206.
Tulodziecka, K., Diaz-Rohrer, B.B., Farley, M.M., Chan, R.B., Di Paolo, G., Levental, K.R., Waxham, M.N., Levental, I., Riezman, H., 2016. Remodeling of the postsynaptic plasma membrane during neural development. Mol. Biol. Cell 27 (22), 3480–3489. https://doi.org/10.1091/mbc.e16-06-0420.
Volkow, N.D., Wang, G.-J., Kollins, S.H., Wigal, T.L., Newcorn, J.H., Telang, F., Fowler, J.S., Zhu, W., Logan, J., Ma, Y., Pradhan, K., Wong, C., Swanson, J.M., 2009. Evaluating Dopamine Reward Pathway in ADHD: Clinical Implications. JAMA 302, 1084–1091. https://doi.org/10.1001/JAMA.2009.1308.
Wang, F., Liu, W., Jin, Y., Dai, J., Zhao, H., Xie, Q., Liu, X., Yu, W., Ma, J., 2011a. Interaction of PFOS and BDE-47 Co-exposure on Thyroid Hormone Levels and TH-Related Gene and Protein Expression in Developing Rat Brains. Toxicol. Sci. 121, 279–291. https://doi.org/10.1093/TOXSCI/KFR068.
Wang, J., Pan, Y., Cui, Q., Yao, B., Wang, J., Dai, J., 2018. Penetration of PFASs Across the Blood Cerebrospinal Fluid Barrier and Its Determinants in Humans. Environ. Sci. Technol. 52 (22), 13553–13561. https://doi.org/10.1021/acs.est.8b0455010.1021/acs.est.8b04550.s001.
Wang, X., Li, B.o., Zhao, W.-D., Liu, Y.-J., Shang, D.-S., Fang, W.-G., Chen, Y.-H., 2011b. Perfluorooctane sulfonate triggers tight junction "opening" in brain endothelial cells via phosphatidylinositol 3-kinase. Biochem. Biophys. Res. Commun. 410 (2), 258–263. https://doi.org/10.1016/j.bbrc.2011.05.128.
Wang, Y., Liu, W., Zhang, Q., Zhao, H., Quan, X., 2015a. Effects of developmental perfluorooctane sulfonate exposure on spatial learning and memory ability of rats and mechanism associated with synaptic plasticity. Food Chem. Toxicol. 76, 70–76. https://doi.org/10.1016/j.fct.2014.12.008.
Wang, Y., Rogan, W.J., Chen, H.-Y., Chen, P.-C., Su, P.-H., Chen, H.-Y., Wang, S.-L., 2015b. Prenatal exposure to perfluoroalkyl substances and children's IQ: The Taiwan maternal and infant cohort study. Int. J. Hyg. Environ. Health 218 (7), 639–644. https://doi.org/10.1016/j.ijheh.2015.07.002.
WHO, 2016. Keeping our water clean: the case of water contamination in the Veneto Region, Italy. Https://Www.Euro.Who.Int/En/Publications/Abstracts/Keeping-Our-Water-Clean-the-Case-of-Water-Contamination-in-the-Veneto-Region,-Italy-2017 72.