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Exposure to a human relevant mixture of persistent organic pollutants or to perfluorooctane sulfonic acid alone dysregulates the developing cerebellum of chicken embryo

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ABSTRACT

Prenatal exposure to persistent organic pollutants (POPs) is associated with neurodevelopmental disorders. In the present study, we explored whether a human-relevant POP mixture affects the development of chicken embryo cerebellum. We used a defined mixture of 29 POPs, with chemical composition and concentrations based on blood levels in the Scandinavian population. We also evaluated exposure to a prominent compound in the mixture, perfluorooctane sulfonic acid (PFOS), alone. Embryos (n = 7–9 per exposure group) were exposed by injection directly into the allantois at embryonic day 13 (E13). Cerebella were isolated at E17 and subjected to morphological, RNA-seq and shot-gun proteomics analyses. There was a reduction in thickness of the molecular layer of cerebellar cortex in both exposure scenarios. Exposure to the POP mixture significantly affected expression of 65 of 13,800 transcripts, and 43 of 2,568 proteins, when compared to solvent control. PFOS alone affected expression of 80 of 13,859 transcripts, and 69 of 2,555 proteins. Twenty-five genes and 15 proteins were common for both exposure groups. These findings point to alterations in molecular events linked to retinoid X receptor (RXR) signalling, neuronal cell proliferation and migration, cellular stress responses including unfolded protein response, lipid metabolism, and myelination. Exposure to the POP mixture increased methionine oxidation, whereas PFOS decreased oxidation. Several of the altered genes and proteins are involved in a wide variety of neurological disorders. We conclude that POP exposure can interfere with fundamental aspects of neurodevelopment, altering molecular pathways that are associated with adverse neurocognitive and behavioural outcomes.

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1. Introduction:

Exposure to man-made chemicals, together with other factors, including nutrition, stress, and gene-environment interactions, are suspected as underlying causes for the rise in neurodevelopmental impairments, such as attention deficit hyperactivity disorder (ADHD), autism spectrum disorders (ASD), and cognitive impairment (Grandjean et al., 2017). Environmental contaminants tend to biomagnify in food chains and consequently, diet is a major route of lifelong exposures. Mother-child transfer during pregnancy and lactation results in perinatal exposure of the developing infant. This may impair neurodevelopmental processes, which could affect brain structures and functions, leading to lifelong behavioural and cognitive impairments (Rice and Barone, 2000, Grandjean and Landrigan, 2014, Sunyer and Dadvand, 2019) associated with huge societal costs (Bellanger et al., 2015).

Several studies in cultured neuronal cells have reported that exposure to persistent organic pollutants (POPs) can induce oxidative stress as a mechanism of toxicity (Chen et al., 2010, Costa et al., 2007, Mariussen, 2012, Mariussen et al., 2002). Similarly, exposure to a POP mixture led to up-regulation of genes related to the antioxidant defence in cultured rat cerebellar granule neurons (CGNs), also indicating that mixture toxicity involves oxidative stress (Berntsen et al., 2020). In animal studies exposure to POPs induced oxidative stress in the brain tissues of rats (Hassoun et al., 2000, Hassoun et al., 2002) and POPs at low levels commonly present in the environment are found to be associated with increased oxidative stress in healthy humans from a general population (Kumar et al., 2014).

The migration of neurons in a developing brain is modulated by N-methyl-D-aspartate receptor (NMDA-R) mediated Ca^{2+} influx, where the NR2B subunit is especially important (Komuro and Rakic, 1993, Llansola et al., 2005). Like NR2B, Pax6 (a paired-box transcription factor) has been identified as a key regulator for differentiation of granule cells and for migration of neurons (Engelkamp et al., 1999). Pax6 has been suggested as a relevant biomarker for maturation in the cerebellum (Mathisen et al., 2013). Alterations in migration could be indicative of impaired neurodevelopment (Bjornstad et al., 2015). In our previous studies, we observed that Pax6 and NR2B are important markers for the differentiation of cerebellar granule neurons (CGNs) from mouse and chicken, and that their expression may be altered by xenobiotic exposure (Berntsen et al., 2021, Fjelldal et al., 2019, Mathisen et al., 2013).

The cerebellum is a well-conserved structure between species (Sultan and Glickstein, 2007). In addition to its role in motor control and coordination, the cerebellum is involved in learning and memory, language and executive functioning (O'Halloran et al., 2012). The chicken cerebellum enters a growth spurt around E13 that lasts until around E17 and after E17 the cerebellar growth rate continues to decelerate (Austdal et al., 2016). Chicken cerebellar development from E12 - E21 roughly corresponds to the last gestational trimester and first postnatal year in humans (Abrahám et al., 2001, Austdal et al., 2016, Bjornstad et al., 2015, Volpe, 2009). Previously, we have shown that exposure to environmental toxicants and pharmaceuticals affects genes and proteins important for cerebellar development in the chicken embryo from E13-E17 (Austdal et al., 2016, Berntsen et al., 2021, Fjelldal et al., 2019, Mathisen et al., 2013, Yadav et al., 2021b). Thus, POPs administration in the present study was scheduled so that the exposure to POPs coincide with peak cerebellar development.

We have previously designed an environmentally relevant mixture of POPs, containing 29 different chlorinated, brominated, and perfluorinated substances (Berntsen et al., 2017). The mixture contains POPs at concentrations based on those measured in human blood in Scandinavia, and provides a defined and realistic mixture of environmental contaminants for toxicity studies (Berntsen et al., 2017). Perfluorooctanesulfonic acid (PFOS) is the compound with the highest concentration in the POP mixture and is a potent developmental neurotoxicant. In our previous studies the exposure with this POP mixture, or PFOS alone, has affected NMDA-R signalling and downstream Ca^{2+}-influx, glutathione levels, and excitotoxicity in cultured chicken CGNs (Yadav et al., 2021b). Prenatal exposure to a POP mixture of similar composition in mice, and PFOS exposure *in ovo* in chicken affected NR2B protein expression in cerebellum, indicating a developmental disruption caused by POP exposure (Berntsen et al., 2020). Following maternal mouse exposure to this POP mixture, POPs were detected in the brains of the offspring, which also showed gene expression changes in hippocampus related to brain function (Myhre et al., 2021). Injection of this POP mixture into the allantoic fluid led to human-relevant exposure concentrations of POPs in the developing chicken brain (Yadav et al., 2022). In addition, the same POP mixture had adverse effects on neuronal cell function and development in differentiating human neural stem cells and PC12 cells (Davidsen et al., 2021, Yadav et al., 2021a).

Associations between POP exposure and neurodevelopmental effects have also been reported in human epidemiological studies. For example, in a multi-pollutant analysis of 27 POPs in a Norwegian birth cohort study, early-life exposure to β-hexachlorocyclohexane (β-HCH) and PFOS was associated with increased risk of ADHD (Lenters et al., 2019). Further, Sagiv et al. (2015) reported associations of prenatal exposure to polybrominated diphenyl ethers (PBDEs) with poorer attention and executive function, measured with parent report and direct neuropsychological testing of the child.

Based on the role of NMDA receptors and Pax6 in cerebellar development described above we predicted that exposure to a mixture of POPs and PFOS (as a single compound with a concentration that overlaps with the concentration of the POP mixture) at human-relevant composition and concentrations would alter the morphology of cerebellum in developing chicken embryo. A precise proliferation of granule cell precursors, the source of granular cells, is required for normal growth and development of the cerebellum (Fang et al., 2020). Thus, we investigated the levels of proliferating cell nuclear antigen (PCNA), a proliferation marker in cerebellar cortical layers. We expected changes in molecules related to the NMDA receptor pathway and down-stream effects, such as altered Ca^{2+} homeostasis, as well as glutathione and antioxidant defences. Since NMDA excitotoxicity leads to increased ROS formation, we expected higher levels of protein oxidation. Finally, findings from the RNA-seq and proteome study could give insight in novel mechanisms for disturbed neurodevelopment and provide links to adverse neurobehavioral outcomes.

2. Material and methods

2.1. POP mixture and PFOS

The POP mixture was designed and prepared at the Norwegian University of Life Sciences (NMBU), Oslo, Norway (Berntsen et al., 2017). The mixture contained 29 different compounds (Supplementary Material Table 1), including six PFAAs (perfluorohexanesulfonic acid (PFHxS), PFOS, PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), and perfluoroundecanoic acid (PFUnDA)); seven brominated (Br) compounds (PBDE 47, PBDE 99, PBDE 100, PBDE 153, PBDE 154, PBDE 209, and hexabromocyclododecane (HBCD)); and sixteen chlorinated (Cl) compounds (PCB 28, PCB 52, PCB 101, PCB 118, PCB 138, PCB 153, PCB 180, *p,p'*-dichlorodiphenyldichloroethylene (DDE), hexachlorobenzene (HCB), α-chlordane, oxychlordane, *trans*-nonachlor, α-hexachlorocyclohexane (HCH), β-HCH, γ-HCH (lindane) and dieldrin). The compounds were selected from their respective compound groups based on prevalence in blood, breastmilk and/or food, and their relative concentrations based on Scandinavian human blood levels. The stocks used in the present study had a concentration of 10^6 times blood levels in DMSO and were stored in glass vials at −80 °C (Berntsen et al., 2017).

Perfluorooctanesulfonic acid potassium salt (PFOS ≥ 98%) was obtained from Sigma-Aldrich (St Louis, MO, USA). All other reagents were standard laboratory grade.

Table 1
Common transcripts between POP mixture and PFOS exposure based on cut-off values for significance of $p < 0.05$ and with a log2 fold change $>\pm 0.7$.

RNA-seq transcripts		POP mixture		PFOS	
ID	Gene	P Value	LogFC	P Value	LogFC
Upregulated					
ENSGALG00000038364	***CCN3***	0.002	1.661	0.014	1.385
ENSGALG00000040730	***RXRG***	0.007	1.304	0.009	1.367
ENSGALG00000016791	***SLC9A2***	0.036	1.296	0.041	1.293
ENSGALG00000041491	***ACKR4***	0.000	1.097	0.000	1.506
ENSGALG00000016558	***VEGF-D***	0.007	1.051	0.033	0.794
ENSGALG00000015897	***IMPG1***	0.007	1.036	0.001	1.405
ENSGALG00000006120	***PKD2L2***	0.019	0.805	0.049	0.703
ENSGALG00000039826	***CNGA3***	0.031	0.731	0.035	0.884
ENSGALG00000009497	***AVPR2***	0.049	0.719	0.001	1.275
ENSGALG00000001141	***HES5***	0.001	0.713	0.000	0.753
ENSGALG00000002152	***GABRP***	0.010	0.709	0.005	0.824
Downregulated					
ENSGALG00000000112	***PLP1***	0.048	−0.700	0.002	−1.130
ENSGALG00000033376	***APOH***	0.003	−0.829	0.006	−1.058
ENSGALG00000053697	***TMEM125***	0.003	−0.851	0.005	−1.066
ENSGALG00000014268	***CHRNA9***	0.002	−0.858	0.011	−0.776
ENSGALG00000001391	***PLLP***	0.035	−0.872	0.009	−1.375
ENSGALG00000019716	***KRT40***	0.012	−0.886	0.000	−1.323
ENSGALG00000013775	***CDH19***	0.003	−0.896	0.003	−1.160
ENSGALG00000027514	***SCEL***	0.001	−0.898	0.001	−1.000
ENSGALG00000047990	***SCRG1***	0.001	−1.019	0.039	−0.782
ENSGALG00000019211	***MAEL***	0.000	−1.068	0.009	−0.960
ENSGALG00000004246	***SLC6A4***	0.005	−1.150	0.038	−0.847
ENSGALG00000005360	***CA4***	0.003	−1.162	0.029	−0.804
ENSGALG00000038540	***ZBTB32***	0.000	−1.167	0.012	−1.079
ENSGALG00000027874	***CHAC1***	0.000	−1.936	0.000	−1.967

In the present study we exposed chicken embryo with POP mixture at 10x human blood levels (Yadav et al., 2022) or PFOS alone at 0.4 μM, a similar concentration as present in the POP mixture.

2.2. Injection of chicken eggs and exposure of developing embryos to POPs

Injection of chicken eggs and exposure procedures were followed as described previously (Yadav et al., 2022). Eggs (*Gallus gallus*, weight 50–55 g, fertilized) were obtained from Nortura Samvirkekylling (Våler, Norway) and incubated at 37.5 °C in 45% relative humidity in an OvaEasy 380 Advance EXII Incubator (Brinsea,Weston-super-Mare, UK). Sex determination of embryos was not performed. Prior to injection, eggs were weighed, and the POP mixture was diluted in 0.9% saline. On E13, eggs were *trans*-illuminated with a LED lamp (Brinsea) to visualize spontaneous movements confirming living embryos. For injection, the POP mixture or PFOS stock was diluted 1/100 in saline. This solution was injected into each egg (1 μL saline solution/gram egg weight). Injection was performed through the chorioallantoic membrane (CAM) into the allantois with a 29-gauge needle. Injection was guided by *trans*-illumination with the LED lamp to avoid injecting into blood vessels. This resulted in a final exposure concentration of POP mixture at 10x human blood levels, and PFOS at 0.4 μM, assuming uniform distribution of the compounds throughout all compartments of the egg. Each egg received only a single administration of the POP mixture. On E17 the embryos were anesthetised by hypothermia by submerging the eggs in crushed ice for 7 min, hatched, and immediately decapitated. The whole brains or cerebella were isolated with a spatula and the meninges were removed with forceps. The brains or cerebella were snap-frozen in liquid nitrogen and stored at −80 °C until further processing. Animals were handled in accordance with the Norwegian Animal Welfare Act and the EU directive 2010/63/EU, and the study was approved by the Norwegian Food Safety Authority (application ID: FOTS 13896). The exposure scenario is shown in Fig. 1.

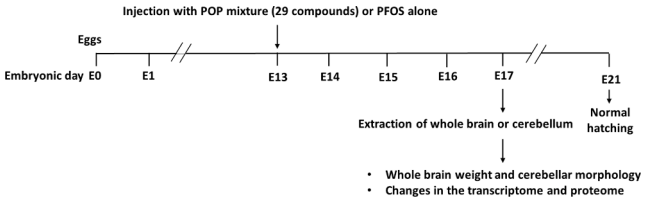

Fig. 1. POP mixture (29 compounds) or PFOS was administrated by injection through CAM into the allantois at embryonic day (E) 13. At E17 after administration, the brain or cerebellum was isolated and a study of changes in brain weight and cerebellar morphology was performed. Further, we studied changes in the transcriptome and proteome in developing cerebellum.

2.3. Brain weight, histology and immunohistochemistry

The embryos were sacrificed at E17 and the brains were removed and weighed. Brains for histology were collected as described by (Austdal et al., 2016). The skulls of chicken embryos prepared for histology were opened along the cranial sutures and heads were fixed for 24 h in 10% buffered formalin (VWR Chemicals). After fixation, the brains were divided along the coronary plane, and the dorsal part was routinely processed and embedded in paraffin. Tissue blocks were trimmed until at least three cerebellar lobuli were visible and sections of 3 μm thickness were cut, mounted, and stained with haematoxylin and eosin. Histological slides were examined blindly by a board-certified veterinary pathologist. Photomicrographs of cerebellar lobuli were taken (Supplementary Material Fig. 1) and image analysis performed using ImageJ (NIH, Bethesda, MD, USA). Quantifications of cerebellar cortical layers: external granular layer (EGL), molecular layer (ML) and internal granular layer (IGL) were performed, and only optimally oriented slides were included. Thickness of EGL, ML and IGL were measured in five locations per slide. When accounting for layer thickness, the selected slices were representative and comparable. They were from the close sections of different cerebellum and the same cerebellar lobuli.

Immunohistochemistry (IHC) staining of cerebellar tissue sections was performed using an avidin–biotin-peroxidase method (Vectastain Elite ABC-HRP Kit, Peroxidase (Rabbit IgG, PK-6101 and Mouse IgG PK-6102), Vector Laboratories USA). Anti-Pax6 (dilution 1:12 000,

AB2237, Millipore, USA) and PCNA (1:200, AM50151PU-T, OriGene, Herford, Germany) were used as primary antibodies. The immunoreaction was visualized using the chromogen 3, 3-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co.) and contrast staining with Mayer's hematoxylin (H). For IHC analysis of Pax6 and PCNA expression levels in cerebellar cortical layers (EGL, ML and IGL), we quantified DAB intensity (expressed as Intensity/Area in square pixel) by using Fiji software (Schindelin et al., 2012). A detailed procedure for IHC analysis can be found in Supplementary Material. Staining intensities were measured in 3–4 lobuli locations per section in an experimenter-blind manner and values were presented after subtraction of background intensity. Representative images of Pax6 stained cerebellar cortical layers are shown in Supplementary Material Fig. 2 and PCNA in Supplementary Material Fig. 3.

2.4. RNA extraction and transcriptomic analyses

RNA from cerebella (n = 7–8 per exposure group) was extracted following Qiagen miRNeasy mini protocol. Cerebellar tissues were homogenized by using Qiagen TissueRuptor II, and residual DNA was removed by optional on-column DNase treatment. RNA quantity and quality were assessed by Nanodrop and Bioanalyzer (Total RNA Nano kit). RNA-seq libraries were prepared using TruSeq stranded RNA-prep (Illumina, USA) following manufacturer's protocol. The libraries were pooled in equimolar amounts and 75 bp paired end sequencing were performed on a single HiSeq 3/4000 (Illumina, USA) run. Raw data from the sequencer was demultiplexed using bcl2fastq v2.20.0.422. Low

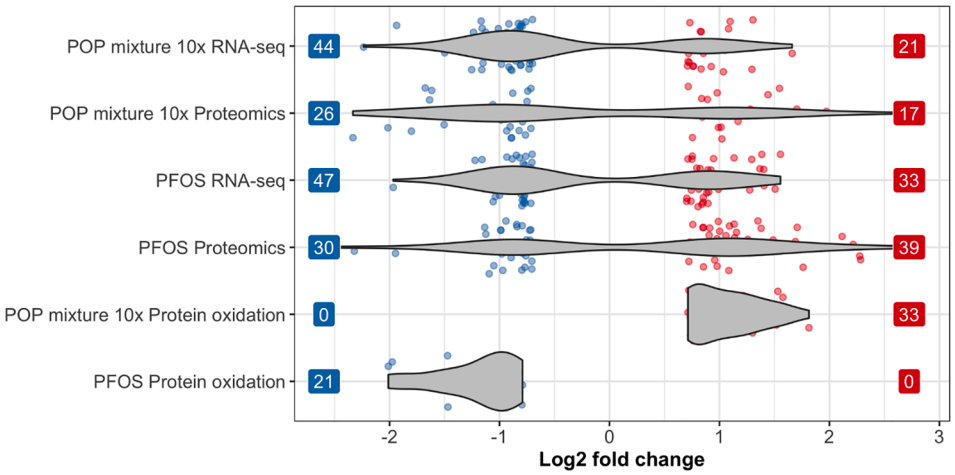

Fig. 2. Proportion of down-regulated and up-regulated genes or proteins. Significant differently expressed genes or proteins ($p < 0.05$ and with a log2 fold change $>\pm 0.7$) are represented by jittered dots: blue = down-regulated and red = up-regulated. Violin plot represents significant log2 fold change score: DMSO vs POP mixture 10x RNA-seq, DMSO vs POP mixture 10x proteomics, DMSO vs PFOS RNA-seq, DMSO vs PFOS proteomics, DMSO vs POP mixture 10x protein oxidation and DMSO vs PFOS Protein oxidation.

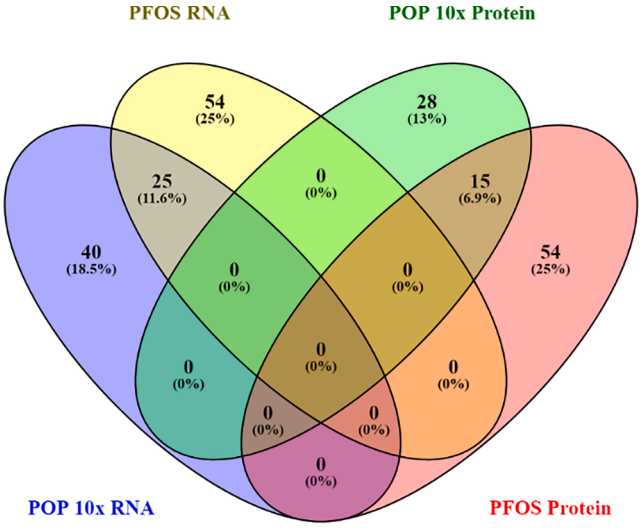

Fig. 3. Venn diagrams showing the number (percentage) of significantly differentially expressed genes or proteins in chicken cerebella following exposure to POP mixture or PFOS. Within either the POP mixture exposure or the PFOS exposure, no commonality was found between genes and proteins. However, within the RNA-seq set, twenty-five common gene transcripts were found between the POP mixture and PFOS exposure. Similarly, within the proteomics set, 15 common proteins were found between the POP mixture and PFOS exposure.

quality reads and adapter sequences were trimmed/removed along with any reads mapping to the PhiX spike-in used during sequencing using BBDuk (2021) (part of BBMap v34.56). Cleaned reads were mapped to the Chicken genome (ENSEMBL GRCg6a) using HISAT v2.1.0 (parameter:rna-strandness RF) (Kim et al., 2019). The featureCounts program was used for counting the raw reads, setting two parameters "-s 2" and "-p" to specify reverse strand specific and paired fragments (Liao et al., 2013). Resulting sam files were handled using samtools v1.2 (Li et al., 2009).

2.5. Protein extraction and proteomics analysis

Protein expression measured by label-free LC-MS-detection based proteomics was carried out at the Aberdeen Proteomics facility. Protein from cerebellar tissues was extracted using Qiagen AllPrep kits (#80004, Qiagen, Manchester, UK), following manufacturer's instructions. Briefly, whole cerebella were lysed using appropriate amounts of RLT buffer (600 µL per 30 mg of tissue). 600 µL of lysate were processed to protein pellets, dissolved in 100 µL of resuspension buffer (6.8 M Urea, 2 M thiourea, 20 mM dithiothreitol, and 0.1% w/v Rapigest detergent), and stored at − 80 °C until further analysis.

Tissue proteins were identified and quantified using a Q Exactive Plus hybrid quadrupole Orbitrap mass spectrometer fitted with an EASY-Spray nano-ESI source (Thermo Scientific) as detailed in (Filis et al., 2018, Siemienowicz et al., 2019). Briefly, 10 µg of tissue proteins was diluted to a final volume of 100 µL in 50 mM NH_4HCO_3 (BioUltra grade, Sigma Aldrich). Proteins were digested in solution according to the PRIME-XS protocol and reduced by using 2 mM dithiothreitol (Sigma Aldrich, > 99%) for 25 min at 60 °C and S-alkylated in 4 mM iodoacetamide (Sigma Aldrich, > 99%) for 30 min at 25 °C in the dark, then digested by sequencing-grade modified trypsin (Promega, Southampton, UK, cat.no. V5111) at a 1:10 ratio of trypsin:protein overnight at 37 °C. The reaction was stopped by freezing at − 80 °C. Then samples were thawed and dried by vacuum centrifugation (SpeedVac Plus SC110A, Savant), and dissolved in 10 µL 2% acetonitrile/0.1% formic acid. The equivalent of 2 µg of peptides (assuming no losses) were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Raw mass spectrophotometric output files were processed by MaxQuant (v 1.5.3.30) (Cox and Mann, 2008). MaxQuant runs were performed under the default parameters except that trypsin was set as the digestion enzyme and matching between runs was enabled. All peptide searches were performed against all the Refseq proteins retrieved from NCBI. Protein intensities across samples were normalized using the maxLFQ algorithm (Cox et al., 2014).

2.6. Statistical analysis

Each exposure group consisted of 9 samples per group for brain weight, histological and IHC analysis, and 7–8 individual eggs per group for transcriptome and proteome.

The thickness of the external granular layer, the molecular and internal granular layers as well as the DAB intensity for Pax6 and PCNA showed a satisfactory fit to the normal distribution and were analyzed by a mixed model where exposure group and the individual were included as independent variables. Exposure group was entered as a fixed effect and the individual as a random effect. Differences between exposure groups were assessed using the Tukey HSD test. Comparison of brain weights between exposure groups was analyzed by ANOVA followed by a Tukey HSD test.

The raw gene counts were used for downstream transcriptomic analyses. Genes were filtered, normalised and differential expression was performed using the default parameters of *edgeR* package in R v3.5.1. The normalised protein intensities and the site occupancies for methionine oxidation were used for the downstream proteomic analyses. Only

proteins and site occupancies with 75% of valid values across the samples compared were used for differential expression analysis. The *limma* package in R v3.5.1 was used for statistical comparisons.

3. Results

3.1. Effect of POP mixture or PFOS on brain weight, histopathological changes, and DAB intensity for Pax6 and PCNA

We observed no significant alteration in brain weight of chicken embryos exposed to POP mixture or PFOS. Results are summarized in Supplementary Material Table 2. Microscopic examination did not show strong visual histopathological changes for either the POP mixture, or the PFOS exposure. However, quantitative image analysis revealed that the ML was significantly thinner following exposure to the POP mixture, and PFOS alone compared to the control group. The thickness of the ML was reduced by 15 and 19% in response to POP mixture and PFOS exposure, respectively (Supplementary Material Fig. 1 and Supplementary Material Table 3). The thickness of the EGL and IGL were unaffected by either exposure (Supplementary Material Fig. 1 and Supplementary Material Table 3). Further, immunohistochemical analysis showed no significant differences in the DAB intensity for Pax6 or PCNA expression in any of the three cerebellar cortical layers either exposed to POP mixture or PFOS compared to the control (Supplementary Material Table 4).

3.2. Expression profile of genes and proteins after exposure to POP mixture

After quality filtering, a total of 13,800 transcripts were compared between the control and POP mixture exposure groups (Supplementary Data 1). Using $P < 0.05$ and log2 fold change $>\pm 0.7$ (equivalent to $\pm$ 1.6 fold) cut-offs, 65 transcripts (Fig. 2 and Supplementary Data 1) were shown to be affected by the POP mixture. Similarly, 2,568 proteins were compared between the control and POP mixture exposure groups (Supplementary Data 2). Using $P < 0.05$ and log2 fold change $>\pm 0.7$ (equivalent to $\pm$ 1.6 fold) cut-offs, POP mixture affected expression of 43 proteins (Fig. 2 and Supplementary Data 2). No common overlap was identified in the gene and protein sets (Fig. 3). However, several genes and proteins described a common pathway or process. Thus, transcriptional regulators were affected at the level of genes (*HES5*, *ZBTB32*), as well as proteins (PBX1, ZNF423, EIF2B2). Similarly, the insulin-signalling pathway was also affected at the gene (*IGF-I*, *CCN3*) and protein level (IGFBP7). In addition, members of the RXR signalling pathway were affected (*RXRG*, *RDH10,* RBP4). Growth factor genes including *VEGF-D*, *BMP3* were modulated. Several myelination-related genes were found to be down-regulated (*CDH19, PLP1, PLLP*). Several cell surface transporter genes were affected (*ABCB11*, *SLC1A2*, *SLC6A4*, *SLC27A6*). Protein levels for ANXA1, a membrane-localized protein that binds phospholipids and has anti-inflammatory activity and multiple roles in cell proliferation, differentiation, and migration was found downregulated. Indeed, many systems involved in cell survival and death, and cellular stress responses were affected: regulators of cell cycle and apoptosis (CCAR1, DAD1); the unfolded protein response and ubiquitination (*CHAC1*, *CA4*, *SCRG-1*, UBE2D3, NACAD); protection against DNA damage and apoptosis (FAM168A); mitochondrial health (OPA1).

Genes related to cellular trafficking were affected, including chemokine receptors (*ACKR4, S100A12*) and regulators of immune cell infiltration (*AvBD1, LECT2*). The neuronal cell adhesion gene *IMPG1* was up-regulated, whereas *DCX*, a marker of migrating and immature neurons was found to be down-regulated.

Molecules controlling cellular architecture were affected. For example, a gene coding for a member of the superfamily of intermediate filament proteins was down-regulated (*KRT40*). Proteins controlling neuronal cytoskeletal stability (TPM1, RMDN1) and axon formation (CDH13, NAA25) were affected, as well as a component of the linker of nucleoskeleton and cytoskeleton complex, SYNE1.

Table 2
Common proteins between POP mixture and PFOS exposure based on cut-off values for significance of $p < 0.05$ and with a log2 fold change $>\pm 0.7$.

Proteomics		POP mixture		PFOS	
ID	**Protein**	**P Value**	**logFC**	**P Value**	**logFC**
Upregulated					
XP_004939171	**NACAD**	0.001	2.826	0.018	2.779
XP_025004922	**LOC776992**	0.002	1.972	0.033	1.691
NP_001001760	**CDH13**	0.043	1.704	0.017	2.275
NP_001034398	**OPA1**	0.001	1.169	0.017	1.258
NP_001025835	**NAA25**	0.028	0.990	0.043	1.059
XP_015136489	**FAM168A**	0.040	0.838	0.035	0.911
Downregulated					
NP_990077	**PBX1**	0.016	−0.715	0.014	−0.721
NP_001004390	**HBBR**	0.005	−0.783	0.001	−0.990
NP_990569	**RBP4A**	0.005	−0.791	0.003	−0.783
NP_001026324	**UBE2D3**	0.011	−0.822	0.024	−0.893
NP_996789	**ANXA1**	0.018	−0.880	0.046	−0.760
XP_004944240	**ZNF423**	0.047	−0.891	0.024	−0.850
NP_001264341	**RMDN1**	0.013	−0.935	0.025	−0.979
NP_001004374	**HBZ**	0.000	−1.061	0.000	−1.094
NP_990732	**TPM1**	0.045	−1.636	0.015	−2.321

Table 3
Examples of genes and proteins affected by POP mixture or PFOS exposure in context of the biological functions and outcomes. Additional supportive published evidence is also referenced.

Biological function/ Outcome	Gene/Protein symbol	Other POPs and/or PFOS observations
RARA/RXR signalling	*RXRG*, *RDH10,* RBP4A	
Glucocorticoid signalling	ANXA1, *SCRG1*	(1) POP mixture affects stress response (Hudecova et al., 2018). (2) Glucocorticoids effects on cerebellar development in a chicken embryo development (Austdal et al., 2016)
Cell proliferation, differentiation, migration	PBX1, *CCN3*, *CHAC1*, ANXA1	
Vascularization, blood–brain barrier	*VEGFD*, *CCN3*, *SLC9A2*, *CA4*	(3) POP mixture distributes to the developing chicken brain at human relevant concentration level (Yadav et al., 2022)
Oligodendrocytes	*CDH19*	
Myelination	*HES5*, *PLP1, PLLP*	
Neuronal networking & Synaptic health	*SLC6A4*, *GABRP*	(4) POPs and PFOS increase neuritogenesis (Yadav et al., 2021a).
Neurotransmitters	RMDN1	
Ca^{2+}-homeostasis	*CHAC1*,	(5) POP mixture and PFOS aggravate glutamate-excitotoxicity; Ca^{2+} dependent and independent mechanism exist (Yadav et al., 2021b)
Redox homeostasis	HEBP2	
Mitochondrial health	OPA1, HEBP2	(6) POP mixture and single POPs affect mitochondrial mass and mitochondrial membrane potential (Shannon et al., 2019, Wilson et al., 2016a).
ER stress	*CHAC*1,	
Unfolded protein response	*CHAC1*, *CA4*	
Ubiquitination	UBE2D3, *ZBT16*, NACAD1	
Inflammation	*ACKR4*, *SELE*, *CCN3*, ANXA1	
DNA damage	UBE2D3	

Table 4
An overview of genes and proteins differentially regulated by POP mixture or PFOS exposure with a known link to neurocognitive and neurobehavioral outcomes, as well as neurodegenerative disorders.

Gene/ Protein symbol	Function	Disorders association	Reference
ANX1	Neuroinflammatory, neurovascular and metabolic disease, repairing blood–brain barrier damage	Multiple sclerosis, Alzheimer's disease, neurovascular disease, and stroke	(McArthur et al., 2016, Purvis et al., 2019)
BMP	Neural stem cell fate and maturation, neural development	Cognitive decline associated with normal aging and neurodegenerative diseases	(Bond et al., 2012)
CA4	Carbon dioxide and bicarbonate homeostasis	phobias, obsessive–compulsive disorder, generalized anxiety, and post-traumatic stress disorders	(Blandina et al., 2020)
GABRP	Associated with GABA	Autism, schizophrenia, bipolar I disorder	(Ma et al., 2005, Lang et al., 2007, Ren et al., 2018)
IGF-1	Cellular proliferation and differentiation during embryonic and postnatal development including brain growth	Autism	(Riikonen, 2016)
NACAD	Prevents mistargeting of nascent polypeptide chains to the endoplasmic reticulum membranes	Alzheimer's disease	(Kim et al., 2002)
PLP1	Major structural protein maintaining the compaction of CNS myelin sheaths	Parkinson's disease	(Hentrich et al., 2020)
PPAR-α	Lipid catabolism, brain inflammation	Alzheimer's, Parkinson's, Huntington's disease	(Zolezzi et al., 2017)
RMDN1	Calcium and lipid transfer	Alzheimer's disease	(Fecher et al., 2019)
RXR-G	Remyelination	Multiple sclerosis	(Huang et al., 2011)
SCRG1	Mouse scrapie responsive gene, cell growth suppression and differentiation, glucocorticoid signalling	Transmissible spongiform encephalopathies	(Dandoy-Dron et al., 2003, Dron et al., 2005)
SLC6A4	Reuptake of Serotonin and terminates Serotonin transmission	Mood and personality disorders, obsessive compulsive disorders, anxiety, insomnia, and eating disorders, ADHD	(Zhou et al., 1996, van der Meer et al., 2016)
UBE2D3	E2 ubiquitin-conjugating enzyme family	Gordon Holmes syndrome (characterized by reproductive and neurological problems)	From GeneCards and MalaCards database

In addition, neurotransmitter receptors were found to be modulated (*GABPR*, *CHRNA9*). *CNGA3*, a marker for synaptic plasticity was upregulated. A number of channel related genes were affected. The ion channel *PKD2L2* gene was upregulated whereas the potassium voltage-gated channel subfamily gene *KCNQ2* was down-regulated. The gene for the receptor for arginine vasotocin, *AVPR2* was up-regulated.

The plasma lipoprotein APOH and Golgi-lipoprotein transporter (MIA3) were found down-regulated. A cholesterol transfer protein that regulates Golgi structure and function (OSBLPL1) was up-regulated. A protein HCAD2 related to fatty acid metabolism was down-regulated.

Two haemoglobin-related proteins were found to be downregulated (HBZ, HBBR). SRSF7 (spliceosome) was upregulated. The Protein level of an enzyme that catalyses branched-chain amino acids (BCAT1) was down-regulated.

Some genes and proteins not previously described in brain were down-regulated (*TMEM125, SCEL, MAEL,* SYCP2).

3.3. Expression profile of genes and proteins after exposure to PFOS

After quality filtering, a total of 13,859 transcripts were compared between the control and PFOS exposure groups (Supplementary Data 3). Using $P < 0.05$ and log2 fold change $>\pm 0.7$ (equivalent to $\pm$ 1.6 fold) cut-offs, PFOS affected expression of a total 80 transcripts (Fig. 2 and Supplementary Data 3). Among others, PFOS exposure affected 25 genes that were common with POP mixture exposure (Fig. 3 and Table 1). Similarly, a total of 2,555 proteins were compared between the control and PFOS exposure groups (Supplementary Data 4). Using $P < 0.05$ and log2 fold change $>\pm 0.7$ (equivalent to $\pm$ 1.6 fold) cut-offs, PFOS affected expression of a total 69 proteins (Fig. 2 and Supplementary Data 4). Among others, PFOS exposure affected 15 proteins that were common with POP mixture (Fig. 3 and Table 2).

In addition to genes and proteins in common with POP mixture, the following were also strongly affected by PFOS exposure. The transcriptional regulators *SIX6* and *ZBTB20* were downregulated. Two genes for small nucleolar RNAs, *SNORD14* and *SNORD79,* were up-regulated. Membrane transporters (*SLC38A4, SLC35D5*) were found to be affected. Sodium-voltage regulated channel (*SCN4B*), a potassium voltage-gated channel subfamily member (*KCNQ1*) and the myelin marker (*MBP*) were down-regulated. In addition, the ten strongest up-regulated proteins included a nuclear receptor binding protein (NRBP1), the cytoskeletal component beta-actin (ACTB), a mitochondrial inner membrane transport molecule (TIMM23B), the solute carrier protein SLC27A4 involved in fatty acid transport, a neuroprotective transcription factor (ADNP), and LIN7A, a molecule involved in correct localization of NMDA-R subunits. Among the bottom ten down-regulated transcripts we report the non-receptor phosphatase PTPN12, mitochondrial 3-hydroxyisobutyrate dehydrogenase enzyme (HIBADH), co-chaperone with mitochondrial function (DNAJA1), an acyl-CoA dehydrogenase involved in fatty acid metabolism ACADSB, and the vacuolar protein sorting member (VPS11).

3.4. Protein oxidation after exposure to POP mixture or PFOS

The POP mixture exposure group showed a trend for increased oxidation ratios. Amongst 213 peptides that were oxidised on a methionine residue, 33 showed significantly increased oxidation ratios ($p < 0.05$ and log2 fold change $>\pm 0.7$) when compared with control (Fig. 2 and Supplementary Data 5). The altered oxidised peptide ratios were derived from proteins from different sites: the blood (e.g., ALB, HBA), the cytosol (e.g., ARHGDIA), the nucleus (e.g., Histone H2B), and the cytoskeleton (e.g., VIM, TUBA4A).

In the PFOS exposure group there was a trend of decreased methionine oxidation ratio. Amongst 166 peptides oxidised on a methionine residue, 21 showed significantly decreased oxidation ratios ($p < 0.05$ and log2 fold change $>\pm 0.7$), (Fig. 2 and Supplementary Data 6). The altered oxidation ratios were not derived from blood proteins and there were almost no proteins from the nucleus. Many of them were cytoskeletal e.g., tubulin (TUBB2B, TUBB3, TUBB2B) or actin (ACTC1, ACTC2).

4. Discussion

It is thought that POPs reaching the fetal brain can interfere with neurodevelopmental processes and cause developmental neurotoxicity (DNT). Previously, we covered the pharmacokinetic studies and modelled the distribution of POPs into the brain of developing chicken

embryo (Yadav et al., 2022). We exposed chicken embryos at E13 by injecting the same POP mixture, containing 29 compounds (based on blood levels measured in the Scandinavian human population) into the allantois. This resulted in a final concentration of 10x human blood levels in the egg. The concentrations of selected PCBs in chicken embryo brains were from 1 to 4x the concentrations measured in human brain samples (Dewailly et al., 1999, Mitchell et al., 2012). Similarly, OCPs DDE and HCB were 1.2x and 0.2x the concentrations measured in human brain samples, respectively (Dewailly et al., 1999). However, the levels of selected PFASs were relatively high in the chicken brain e.g., PFOS was 48 to 183x and PFOA was 12x the levels measured in human brain samples (Maestri et al., 2006, Pérez et al., 2013). As exposure to POPs is individualistic in nature, certain groups of people may have higher body burden of certain POPs. A Chinese study reported levels of PFOS up to 118000 ng/mL and PFOA up to 32000 ng/mL in serum from occupationally exposed workers (Fu et al., 2016). Although there are few studies reporting levels of PFASs in the human brain, according to Maestri et al. (2006) the concentrations in the brain of PFOS and PFOA can be expected to be the 25% and 17% of their concentrations in serum, respectively. Thus, this would correspond to brain concentrations up to 29500 ng/mL (124x the chicken brain levels) for PFOS and 5440 ng/mL (906x the chicken brain levels) for PFOA in Fu et al. (2016). Our pharmacokinetic data showed that the POP mixture at 10x human blood levels reached into the brain of chicken embryo at concentrations (most of the measured compounds), which are relevant for human exposure (Yadav et al., 2022). Similar human-based mixtures of POPs designed by

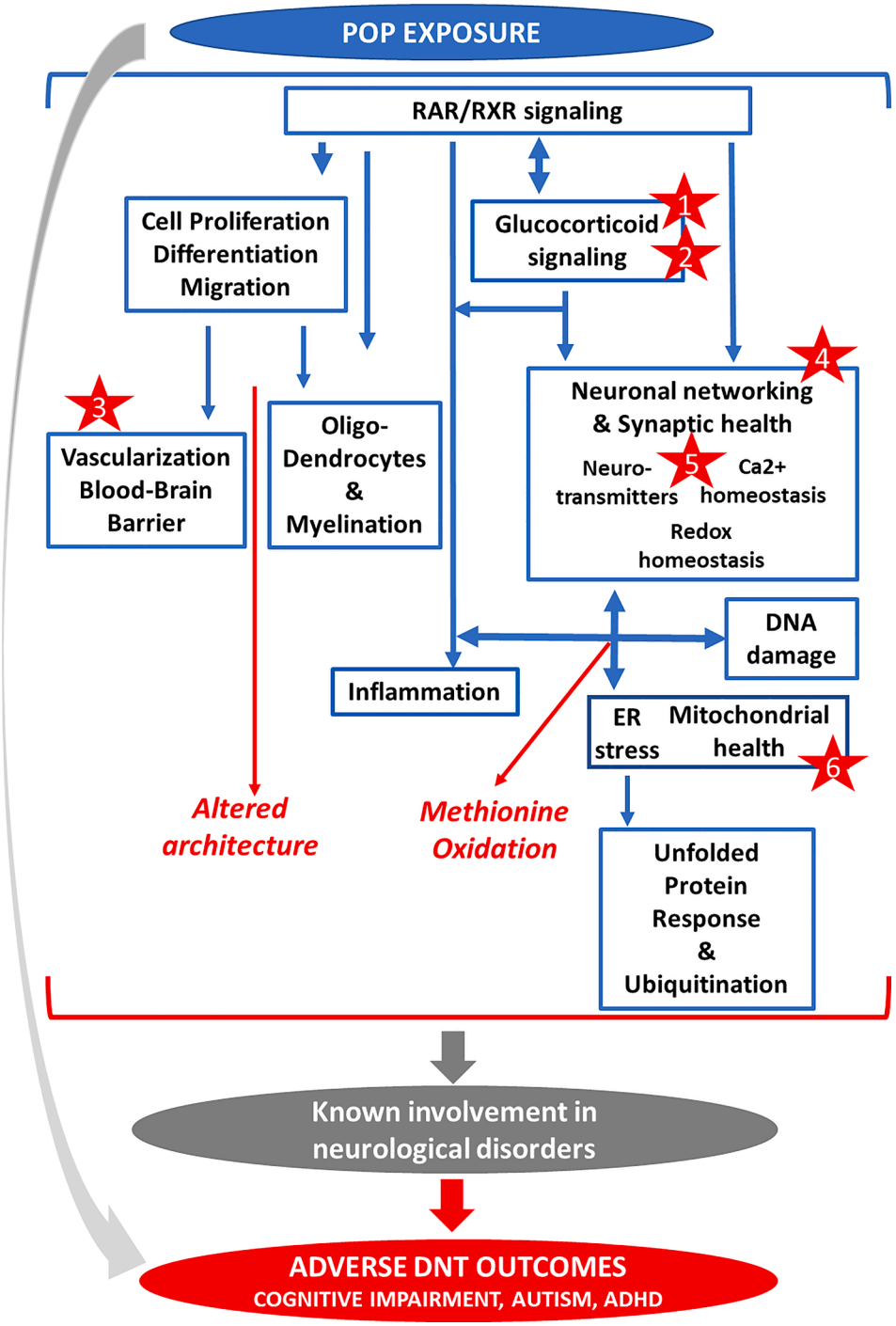

Fig. 4. Overview of biological pathways and processes affected by exposure to POP mixture or PFOS alone. Exposure to environmental toxicants has been associated with adverse neurodevelopmental outcomes (light grey curved arrow). Developing chicken cerebella exposed to POP mixture or PFOS alone showed alterations in morphology and protein oxidation. Also, expression of a set of common genes and proteins. These signalling pathways and biochemical functions are schematized above. Several of these pathways and processes are interlinked (arrows). Specific examples of these genes and proteins are listed in Table 3, as well as additional supportive observations in other studies (number in stars). A number of these genes and proteins have been shown to be involved in a wide variety of neurological disorders (references in Table 4). This provides possible causal mechanistic understanding between exposure to environmental pollutants and adverse developmental neurotoxicity (DNT) outcomes, strengthening the existing associative epidemiological links.

our group were used in previous studies to study the adverse effects of POPs in various *in vitro* and *in vivo* models (Berntsen et al., 2022, Davidsen et al., 2021, Johanson et al., 2020, Shannon et al., 2019, Yadav et al., 2021a, Yadav et al., 2021b). Thus, in the present study a POP mixture at 10x human blood levels was used to identify further mechanisms of (developmental) neurotoxicity. Our results indicate that either exposure to the POP mixture or PFOS alone elicited alterations in a common set of genes and proteins involved in pathways and processes resulting in dysregulation of normal CNS development as illustrated in Fig. 4.

We did not identify overlapping genes and/or proteins between the POP mixture exposure and the PFOS exposure, but several genes and proteins identified shared common pathways or processes that are involved in key neurodevelopmental processes. The genetic information is translated with high precision. However, mRNA molecules originating from the same gene can produce different amounts of protein and can synthesize entirely different polypeptides, suggesting that translation is highly heterogeneous (Sonneveld et al., 2020). Both transcription and translation are coordinated by many participating factors and pathways. For example, mRNA processing includes splicing, polyadenylation, modifications, transport and degradation (Buccitelli and Selbach, 2020). The potentially large number of transcript isoforms can be generated from the same gene via alternative splicing presenting an important complication for the comparison of protein and mRNA (Liu et al., 2016). Further protein translation is itself a complex multistep process that is subject to extensive regulation at the levels of initiation, elongation, localization, ribosome composition and degradation. In addition, post-translational modifications of proteins, their interaction with other proteins (and other biomolecules) and their catalytic activity give rise to phenotypes (Buccitelli and Selbach, 2020). The direct comparison between protein and mRNA abundances from the same location or from the same cell type may not be appropriate (Liu et al., 2016). The lack of overlap between genes and proteins could also be due to technological reasons, in the present study protein and RNA were retrieved from different individuals/samples.

The migration of postmitotic CGNs through cerebellar cortical layers i.e., from the EGL through the ML to the IGL (where they mature) is a key feature for cerebellar development (Bjornstad et al., 2015). While migrating from the EGL to the IGL, granule cells extend their axons, the parallel fibres, which then synapse on Purkinje cell dendrites within the ML. It has been suggested that ML thickness is a function of Purkinje cell dendritic development (Schwartz et al., 1997). The EGL, a transient layer during development, progressively reduces in thickness and disappears, while ML and IGL continue to increase after a time interval that varies according to the species (Lavezzi et al., 2006, Volpe, 2009). A reduction in the thickness of the cerebellar cortical ML was the only histopathological change following POP exposure, a neuropathological change possibly linked with neurological disorders. For example, previous neuropathological studies reported thinning of the ML in the cerebellar cortex of mentally retarded autistic cases as well as other histological changes (Lee et al., 2002). The neurophysiological significance of ML thickness is not well understood. However, ML interneurons of cerebellar cortex are important for neuronal activity (e.g., action potential production, synaptic activity) and changes of physiological function may be observed at the neuronal network level (Gill and Silliitoe, 2019, Lauritzen and Gold, 2003). This ML thinning could well be due to the observed dysregulation of genes and proteins involved in proliferative and/or migratory pathways. For example, *IGF-1* and *VEGF-D*, are modulators of migration of interneurons in different layers of cerebellum. *DCX* has also been reported to be strongly expressed by neurons in the cerebellum during periods of migration, and mutations in the human gene cause a disruption of cortical neuronal migration (Freemyer et al., 2019, Gleeson et al., 1999). Our expectation that these changes would be reflected in the expression of Pax6 and PCNA was not confirmed in the present study. However, other studies have reported that prenatal or perinatal exposure to environmental toxicants could alter the cerebellar level of these markers in chicken embryo, as well as in offspring rats and mice (Fang et al., 2020, Mathisen et al., 2013). One of the reasons for the lack of effect on Pax6 expression in the present compared to previous studies could be the time points used, i.e., the length of exposure. Although not examined in the present study, it is possible that different results may have been achieved had we used other time points. The protein level of Pax6 varied in a biphasic manner during the foetal period in the developing chicken cerebellum, with two peaks at E14–15 and E19, respectively (Austdal et al., 2016). Mathisen et al. (2013) did not find any significant differences in Pax6 level in chicken CGNs (on day *in vitro* 3) cultured from bisphenol A injected eggs on embryonic day 16 for 24 h, however on day *in vitro* 6 Pax6 level was increased. In addition, long-time exposure (11 days old offspring from prenatally exposed mice) resulted in a significant increase in the Pax6 level in the cerebellum. Further, Fang et al. (2020) found much more Pax6 positive cells in the offspring cerebellum of rats exposed prenatally to POPs compared with control pups on postnatal day 7 (PND) and PND14, and there was no significant difference in Pax6 positive cells among groups on PDN21. For immunohistological analysis in the present study we chose only Pax6 as a marker for migration, and PCNA as a marker for proliferation. However, it should be noted there are several other important markers for migration of neurons into the cerebral cortex. For example, Doublecortin (DCX) is strongly expressed by migratory granule cells (GCs), NR2B is an important marker for the differentiation of CGNs (Llansola et al., 2005), whereas NeuroD1 is an early marker of differentiated GCs (Rahimi-Balaei et al., 2018). Similarly, Ki67 and minichromosome maintenance (MCM) proteins are standard markers of proliferation that are commonly used to assess the growth fraction of a cell population (Juríková et al., 2016). Future experiments need to be performed for the conformity of these markers in different cerebellar cortical layers.

The NMDA-R is important for neural stem cell proliferation and differentiation (Chakraborty et al., 2017), establishment or elimination of synapses and migration of neurons (Komuro and Rakic, 1993). Exposure to POPs induces excitotoxicity in cultured chicken CGNs via either a Ca^{2+}-dependent or independent NMDA-R pathway (Yadav et al., 2021b). In the present data, no significant changes in glutamate ionotropic NMDA-Rs or subunit types were observed. However, *VEGF-D*, is a ligand for the VEGF receptor VEGFR-2 (Flk1) (Achen et al., 1998) and in complexing with NMDAR subunits can enhance Ca^{2+} influx in immature granular cells before synapse formation (Meissirel et al., 2011). *IGF-1* has also been reported to alleviate NMDA-induced neurotoxicity through the IGF-AKT-mTor pathway in microglia (Riikonen, 2016). We also found changes in RMDN1, a paralog of RMDN3 which is involved in calcium homeostasis (Fecher et al., 2019). This indicates that POP exposure affected regulators of the NMDA-R systems and calcium signalling, and thus could modulate excitotoxicity.

Our POP mixture reduced GSH levels in chicken CGNs in culture (Yadav et al., 2021b), but contrary to our expectations, the genes and proteins in our data set do not point strongly to changes in GSH metabolism or antioxidant defence systems. However, the proteomics analysis revealed increased methionine oxidation of peptides following exposure to the POP mixture. This oxidation is an abundant non-enzymatic post-translational modification which is indicative of oxidative stress and can act as a removal mechanism for cellular ROS, preventing the irreversible oxidation of other biomolecules (Walker et al., 2019). Cytosolic as well as nuclear proteins were affected. In addition, oxidised proteins from blood (e.g., ALB, HBA) were detected. This is suggestive of an organism-wide increase in oxidative stress. Strikingly, the PFOS only exposure led to decreased methionine oxidation. Since the concentration used for PFOS was equivalent to that in the total POP mixture, PFOS alone cannot mimic the effect of the full mixture, in accordance with previous *in vitro* studies using chicken CGNs and rat PC12 cells (Yadav et al., 2021a, Yadav et al., 2021b). We suggest that compounds other than PFOS present in the POP mixture may be responsible for stimulating methionine protein oxidation. For instance,

PBDE congeners (PBDE-47, PBDE-99, PBDE-100, PBDE-153, and PBDE-209) which are also present in our POP mixture, can induce oxidative stress (Huang et al., 2010).

Oxidation of proteins will lead to unfolded and/or misfolded proteins and this can cause ER stress, a condition where the accumulation of unfolded and misfolded proteins takes place in the ER lumen. This results in activation of the unfolded protein response (UPR) and ubiquitination process. Indeed, our data set included several important components of pathways related to ER stress, unfolded protein response or ubiquitination, such as *CHAC*1, *CA4*, UBE2D3, *ZBT32* and NACAD. The *CHAC*1 enzyme acts as an inhibitor of Notch signalling that promotes embryonic neurogenesis (Chi et al., 2012). *CHAC1* degrades glutathione and is associated with ER stress and apoptosis pathways (Perra et al., 2018). As well as being a component of the UPR pathway, *CHAC1* is also a probable substrate-recognition component of an E3 ubiquitin-protein ligase complex which mediates the ubiquitination and subsequent proteasomal degradation of target proteins (Furukawa et al., 2003). Furthermore, *ZBTB32* is involved in the control of autophagy-related proteins (Zhang et al., 2015) and autophagy is an important intracellular catabolic mechanism involved in the removal of misfolded proteins. In different wildlife species, exposure to POPs led to genotoxic effects (DNA damage) (González-Mille et al., 2019). In our dataset we found UBE2D3 and FAM168A associated with DNA damage, thus it is conceivable that exposure to the POP chemicals might lead to direct or indirect DNA damage, for example through generation of reactive oxygen or nitrogen species.

Both increased oxidative stress and altered calcium homeostasis due to ER stress can cause mitochondrial dysfunction (Chaudhari et al., 2014), leading to lowered mitochondrial transmembrane potentials and rapid loss of mitochondrial function (Wüllner et al., 1999). In *vitro* studies using the same POP mixture or different per- and polyfluoroalkyl substances (PFASs) exposures, including PFOS revealed changes in mitochondrial mass and mitochondrial membrane potentials in several non-neuronal cell systems (Shannon et al., 2019, Wilson et al., 2016a), so a similar effect in neuronal cells cannot be excluded. Indeed, in the present analysis following exposure to either POP mixture or PFOS alone, we found alterations in OPA1 and *HEBP2* which are related to mitochondrial health.

In addition to the above targets linked to known modes of actions of POPs, we also identified novel networks affected by POP exposure. These include transcription factors and their regulators (PBX1, *RXRG*, *HES5*, *CHAC1*) with known roles in the cerebellum. As a pioneer transcription factor conferring regional identity in the embryo, PBX1 is particularly well-suited to initiate cell fate changes (Grebbin and Schulte, 2017). It also increases expression of the neuroprotective gene Nfe2L1. A PBX1 transcriptional network controls dopaminergic neuron development and is impaired in Parkinson's disease (Villaescusa et al., 2016). Thus, POP exposure can affect central regulators of neuroprotective mechanisms thereby interfering with processes protecting against DNT.

RXRs are ligand-activated transcription factors that play a central role in early embryonic morphogenetic patterning, inflammation and the immune system, and the physiology of the CNS (Mey, 2017). Thus, we placed it as top-regulatory mechanism in our schematic overview in Fig. 4. The presence of RXR-G and two additional entities RBP4A and *RDH10* in our data set suggests that POP exposure might affect retinoid acid receptor FRAR/RXR signalling. *RXR-G* acts as a positive regulator of endogenous oligodendrocyte precursor cell differentiation and CNS remyelination (Huang et al., 2011). Despite its up-regulation, several genes or proteins that regulate myelin forming cells (oligodendrocyte) or myelination were down-regulated. This might be explained by the observed up-regulation of the transcription factor *HES5*, a negative regulator of myelin gene expression. Indeed, Liu et al. (2006) reported up-regulated myelin gene expression in *HES5* knock-out mice compared to wild-type siblings. *CDH19* is a marker for myelin-producing cells. Interestingly, *CDH19* is expressed both in Schwann cells (in the peripheral nervous system) and oligodendrocytes (in the brain) in the chicken embryo throughout development, suggesting that *CDH19* is selectively expressed by myelin-forming cells and might play a role in myelin formation (Lin et al., 2010). Myelin proteolipid *PLP1* is the major structural protein maintaining the compaction of CNS myelin sheaths. In *PLP1* deletion patients, myelin sheaths showed splitting and decompaction of myelin (Laukka et al., 2016). In the PLP-null (mice) CNS, axons large enough to be myelinated often lacked myelin entirely or were surrounded by abnormally thin sheaths (Rosenbluth et al., 2006). Plasmolipin (*PLLP*) is a plasma membrane proteolipid and a major myelin membrane component in the vertebrate nervous system and is detected in developing rat cerebellum (Sapirstein et al., 1992). This however requires further histological and immunohistochemical confirmation.

Excess prenatal glucocorticoid stimulation disturbs normal development of the cerebellum in the chicken embryo model (Austdal et al., 2016). The present exposures did not lead to changes in glucocorticoid receptor (GR) levels. However, *ANXA1*, which is regulated by GR, was down-regulated. ANXA1 is a potent endogenous, broad-spectrum anti-inflammatory effector (Zub et al., 2019), suggesting POP exposure might facilitate inflammatory responses. When tested in a GR translocation assay *in vitro* the POP mixture did not affect GR transactivation, although the individual POPs, *p,p'*-DDE, PFOS, PFDA and PBDE 47 modulate GR activity (Wilson et al., 2016b). However, *in vivo*, a similar POP mixture affected the stress response as measured by corticosterone level, in female mice and their offspring (Hudecova et al., 2018). Ligated RARα/RXR can interact with ligated GR, resulting in an enhanced transcriptional activity of the GR (Tóth et al., 2011). Our observation that POP exposure up-regulate *RXR-G* provides a new mechanism for the dysregulation of the glucocorticoid axis. Indeed, a dexamethasone-responsive gene, *SCRG1* (Ochi et al., 2006) was also down-regulated. *SCRG1* is involved in the maintenance of pluripotency (Ochi et al., 2006) and autophagy and has also been linked with neurodegenerative diseases (Dron et al., 2006, López-Pérez et al., 2020).

Maturation of blood–brain barrier (BBB) in chick embryos occurs from E13 onwards (Wakai and Hirokawa, 1978). Following injection of the POP mixture, 27 of 29 of the individual compounds could be detected in the developing brain between E13 and E16 (Yadav et al., 2022). ANXA1 is expressed on BBB endothelial cells during human fetal development, supporting a role for the protein in prenatal brain development (McArthur et al., 2016). *ANXA1*$^{-/-}$ mice exhibited an increase in permeability of the blood brain barrier (Purvis et al., 2019). In our experiments, ANXA1 expression decreases, suggesting that POP exposure might negatively be affecting the integrity of the BBB. However, *VEGF-D*, a mitogenic for endothelial cells (Achen et al., 1998) was up-regulated, whereas *BMP3* a regulator of pericytes (Lei et al., 2017) was down-regulated. *CA4*, which is located on the luminal surface of cerebral capillaries, and a regulator of carbon dioxide and bicarbonate homeostasis (Halmi et al., 2006) was down-regulated. This suggests POP exposure can affect the development of brain vascularization, as well as function and integrity of the BBB on several levels.

Finally, POP exposure affected genes or proteins related to cytokines and their receptors, and regulators of inflammation and immune cell infiltration such as *SELE*, *CCN3*, ANXA1, *AvBD1*, *LECT-2*, *IGF-1*, *EPX*. Currently, we cannot distinguish whether this indicates alteration of normal neuroimmunological development or if POP exposure elicits an inflammatory response.

Although the concentration of PFOS in the POP mixture is the same as PFOS alone, our results showed that more genes and proteins are altered in the group exposed to PFOS alone. In general, when chemicals co-occur, they may act additively, displaying non-interactions, which is currently considered to be the most common scenario, especially at low concentrations (Kortenkamp et al., 2009, Martin et al., 2020). Although less commonly observed, chemicals may also display interactive synergistic (more than additive) or antagonistic (less than additive) effects. As our POP mixture contains 29 different compounds, scenarios other than

simple additive outcomes may occur as a result of contradictory mechanisms of action or contrasting biological responses. It is possible that toxicant co-exposures in the mixture might have masked the effects of individual components on gene or protein expression profiles in the developing chicken cerebellum. Similarly, other components in the mixture may possibly have interfered with the developmental neurotoxicity of PFOS. For example, Padhi et al. (2008) studied the neurodevelopmental effects of a mixture (reflecting the blood contaminant profiles of Canadian arctic populations and its individual components) in perinatally exposed rats and found contaminant co-exposure significantly masked the effects of individual mixture components on cerebellum gene expression. Our previous studies have shown that among PFAAs, Br and Cl sub-groups present in the POP mixture, the PFAAs sub-mixture exerted a stronger toxic effect than the other two sub-mixtures in cultured rat CGNs (Berntsen et al., 2021). Further, among other PFAAs compounds, PFOS was the most potent in terms of NMDA-receptor mediated toxicity in cultured rat CGNs (Berntsen et al., 2018). We identified PFOS as the driving agent for the behavioural changes in zebrafish larvae exposed to the same POP mixture while there was also only a limited overlap in CNS-related genes that were significantly altered by the POP mixture and PFOS (Khezri et al., 2017).

The mixture used in the present study aimed to provide a defined and realistic mixture of environmental contaminants for toxicity studies that could reflect the relative levels of POPs to which the general human population are exposed. Some of the chlorinated compounds, such as polychlorinated dibenzodioxins/polychlorinated dibenzofurans (PCDD/PCDF), as well as most dioxin-like PCBs (with the exception of PCB 118), were excluded from the mixture because of their relatively high toxicity at a lower concentration. Toxicity at low concentration would have risked masking the effects of less potent POPs without clear mechanisms of action. Some other compounds such as toxaphenes and endosulfan were also excluded from the mixture due to few studies and limited information on relevant Scandinavian exposure levels. If the study population had been performed based on a non-Scandinavian setting, a different composition of POPs would have been needed. For example, one should consider regional differences in POP exposures such as the comparably high seafood intake in Scandinavia that could result in an increased body burden of certain POPs. One study reported that dietary exposure to brominated flame retardants correlated with blood levels in a selected group of Norwegians with a wide range of seafood consumption (Knutsen et al., 2008). Further, it has also been shown that BFR exposure through dust is/has been higher e.g. in the U.S. population as compared to other regions (Varshavsky et al., 2020). In the present study we used PFOS as a single compound. In comparison to the levels of the brominated and chlorinated compounds the concentration of PFAAs in blood in the Scandinavian population was high, likely due to their binding to serum proteins (Berntsen et al., 2017).

5. Conclusion

Our study showed that exposure to a POP mixture or to PFOS alone dysregulates genes and proteins important in cerebellar development, with plausible linkage to affected pathways. These pathways common to the two exposures are involved in several key neurodevelopmental processes including proliferation, migration, differentiation, neuronal networking and synaptic health, Ca^{2+} homeostasis, mitochondrial dysfunction, neuroinflammation, oxidative stress and unfolded protein response (Fig. 4). Disturbance of these processes are associated with adverse outcomes such as impairment of learning and memory (Bal-Price et al., 2018, Spinu et al., 2019). These pathways reflect many mechanisms/processes relevant for human (developmental) neurotoxicity. Our previous studies highlight many of the same genes, proteins and pathways involved in brain development and function (Table 3). The genes or proteins affected by POP exposures are involved in neurological disorders (summarized in Table 4). Pathway members are involved in GABAergic and serotonergic neurotransmitter systems which have a primary function in some cognitive processes (Myhrer, 2003). Structural and functional cerebellar abnormalities including thinning of the ML have been reported to be linked with neurological disorders (Lackey et al., 2018, Lee et al., 2002). Our results showed a reduction in thickness of the ML of cerebellar cortical layers in both exposure scenarios. This could be indicative of neuropathological changes. However, there were exposure differences with regards to protein oxidation: the POP mixture leading to increased protein oxidation whereas PFOS decreased protein oxidation. The present study identifies several molecules which may provide a possible mechanistic explanation for the epidemiological associations between POP exposure and adverse neurodevelopmental and cognitive outcomes. In addition, our studies add to the knowledge on existing adverse outcome pathway for developmental neurotoxicity (Spinu et al., 2019) and could identify new modes of action.

CRediT authorship contribution statement

Ajay Yadav: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Steven Verhaegen:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Panagiotis Filis:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Diana Domanska:** Writing – review & editing, Formal analysis. **Robert Lyle:** Writing – review & editing, Formal analysis. **Arvind Y.M. Sundaram:** Writing – review & editing, Formal analysis. **Magnus Leithaug:** Writing – review & editing, Formal analysis, Investigation. **Gunn Charlotte Østby:** Writing – review & editing, Formal analysis, Investigation. **Mona Aleksandersen:** Writing – review & editing, Formal analysis. **Hanne Friis Berntsen:** Writing – review & editing, Resources. **Karin Elisabeth Zimmer:** Writing – review & editing, Resources, Supervision. **Paul A. Fowler:** Conceptualization, Methodology, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Ragnhild Elisabeth Paulsen:** Conceptualization, Methodology, Resources, Formal analysis, Writing – review & editing, Visualization, Supervision. **Erik Ropstad:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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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