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A human relevant mixture of persistent organic pollutants (POPs) and perfluorooctane sulfonic acid (PFOS) enhance nerve growth factor (NGF)-induced neurite outgrowth in PC12 cells

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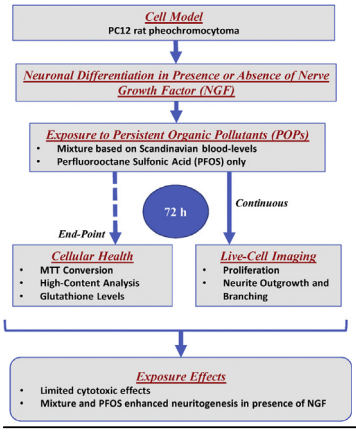
HIGHLIGHTS

- Nerve growth factor (NGF) induced neuritogenesis in PC12 rat pheochromocytoma cells.
- Persistent organic pollutants (POPs) enhanced NGF-induced neuritogenesis.
- Perfluorooctane sulfonic acid (PFOS) contributed to 50 % of the POP mixture effect.
- Nuclear and mitochondrial health was unaffected by the POP mixture or PFOS.

GRAPHICAL ABSTRACT



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ABSTRACT

Disruption of neurite outgrowth is a marker for neurotoxicity. Persistent organic pollutants (POPs) are potential developmental neurotoxicants. We investigated their effect on neurite outgrowth in PC12 rat pheochromocytoma cells, in absence or presence of nerve growth factor (NGF), an inducer of neuronal differentiation. Cells were exposed for 72 h to a defined mixture of POPs with chemical composition and concentrations based on blood levels in the Scandinavian population. We also evaluated perfluorooctane sulfonic acid (PFOS) alone, the most abundant compound in the POP mixture. Only higher concentrations of POP mixture reduced tetrazolium salt (MTT) conversion. High-content analysis showed a decrease in cell number, but no changes for nuclear and mitochondrial cellular health parameters. Robust glutathione levels were observed in NGF-differentiated cells. Live imaging, using the IncuCyte ZOOM platform indicated ongoing cell proliferation over time, but slower in presence of NGF. The pollutants did not inhibit neuritogenesis, but rather increased NGF-induced neurite length. PFOS induced neurite

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outgrowth to about 50 % of the level seen with the POP mixture. Neither the POP mixture nor PFOS affected neurite length in the absence of NGF. Our observations indicate that realistic complex mixtures of environmental pollutants can affect neuronal connectivity via NGF-induced neurite outgrowth.

1. Introduction

Normal development of the central nervous system requires the coordinated ontogeny of proliferation, migration, differentiation, synaptogenesis, gliogenesis, myelination and apoptosis (Barone et al., 2000). Perturbations of these processes through exposure to environmental toxicants during development, could affect structures and functions of the brain, leading to lifelong impairment (Grandjean and Landrigan, 2006). Pregnancy and early life stages are particularly vulnerable to exposure to toxicants including persistent organic pollutants (POPs). The growth of dendrites and synapses is fundamental for the development of neuronal circuits that underlie human cognition and behaviour. Disruption of synaptic circuits are associated with intellectual disability, epilepsy, autism spectrum disorder (ASD), schizophrenia and bipolar disorder (Forrest et al., 2018). The chemicals that disrupt the growth of these axonal and dendritic processes (collectively called “neurites”) have been linked with developmental neurotoxicity (DNT) *in vivo* (Radio et al., 2008, 2010). Many of the neurodevelopmental processes that occur *in vivo* for example cell differentiation, neurite outgrowth, and synaptogenesis, can be followed using *in vitro* neuronal systems. Among these, the measurement of neurite outgrowth has received the most attention (Radio and Mundy, 2008). Chemicals that can affect the neurite outgrowth include POPs. Various cell lines have been used to study the effects of chemicals on neurons (Radio and Mundy, 2008), including the pheochromocytoma PC12 cell line which has been widely used as a model for the study of neuronal differentiation and neurite outgrowth (Fujita et al., 1989). PC12 is a cell line established from a transplantable rat adrenal pheochromocytoma (Greene and Tischler, 1976). Serum-withdrawal together with stimulation with nerve growth factor (NGF) results in the cells undergoing one doubling and induction of neurite outgrowth (Greene, 1978).

Glutathione (GSH) acts as an important antioxidant. Because of the extensive oxidative metabolism in the brain from mitochondrial respiration, the cells of this organ continuously generate reactive oxygen species. As a result, the antioxidant function of GSH appears to be especially important for the brain. Further, a compromised GSH metabolism contributes to the progression of neurological disorders (Schmidt and Dringen, 2012).

POPs are mostly halogenated compounds that are highly resistant to environmental degradation, and that bioaccumulate and biomagnify in living organisms (UNEP, 2008). Several POPs, including polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polybrominated diphenyl ethers (PBDEs), perfluorooctane sulfonic acid (PFOS), and perfluorooctanoic acid (PFOA) are supposed to be developmental neurotoxicants (Grandjean and Landrigan, 2014; Hoyer et al., 2015; Kodavanti, 2006; Schmidt et al., 2014). Whereas many of the studies on POPs focus on single compound or a small group of contaminants, we are in real life exposed simultaneously to a complex mixture of contaminants, which could have additive, synergistic or antagonistic effects (Bopp et al., 2018; SCHER, 2012; WHO, 2017).

Previously, we designed an environmentally relevant mixture of POPs for use in animal and *in vitro* experimental studies, containing 29 different chlorinated, brominated, and perfluorinated compounds (Berntsen et al., 2017). The mixture used in the present study contained POPs concentrations based on measured human blood concentrations in Scandinavia and aimed to provide a defined and realistic mixture of environmental contaminants for toxicity studies that could reflect the relative levels of POPs to which individuals are exposed (Berntsen et al., 2017).

PFOS belongs to the chemical class of perfluorinated alkyl substances (PFAS), a family of emerging persistent pollutants of increasing concern with regards to adverse health outcomes, including cognitive effects (Spratlen et al., 2020). In our unpublished studies (manuscript under review) we found that the POP mixture at 500x fold human blood levels and PFOS at 20 μM, in a corresponding concentration, induced excitotoxicity via the involvement of N-methyl-D-aspartate receptor (NMDA-R) in cultures of chicken cerebellar granule neurons (CGNs). A similar result has been observed by our group using the same POP mixture at concentrations from 500x, and PFOS in rat as well as in chicken CGNs (Berntsen et al., 2018, 2020). PFOS-induced neurotoxicity has also previously been studied in PC12 cells (Li et al., 2017; Slotkin et al., 2008). As PFOS is the compound occurring at the highest concentration in the POP mixture, we also evaluated PFOS as an individual compound.

High Content Analysis (HCA) is a novel, high-throughput, quantitative fluorescence technique providing an important approach for the detection of compound toxicity at the level of individual cells (O'Brien et al., 2006; Wilson et al., 2016). Cellular health markers like cell number (CN), nuclear area (NA), nuclear intensity (NI), mitochondrial membrane potential (MMP), mitochondrial mass (MM) cover cellular metabolic functions (O'Brien and Haskins, 2007; Wilson et al., 2016). The detection/investigation of these multiple nuclear and mitochondrial parameters allowed us to evaluate cellular health at the end of the differentiation process. Measurements of intracellular GSH levels was used as a marker for possible effects of the POP exposures on the cellular defence mechanism.

Since neuritogenesis is a marker for neuronal differentiation, and neurite outgrowth is very sensitive to neurotoxicants we investigated the effects of POPs on neurite dynamics in the PC12 cell model. We used the IncuCyte ZOOM platform which takes non-perturbing phase-contrast images of neuronal cultures under physiological conditions over an extended period of time (Garay et al., 2016), and has been used to study neurite dynamics in different neuronal cells (Mengel et al., 2019; Yagi et al., 2015). This platform also allowed us to follow cell proliferation over time, which complemented the end point parameters described above.

2. Material and methods

2.1. Chemicals, PFOS, and the POP mixture

Dulbecco's modified Eagle's medium (DMEM) and L-glutamine were obtained from Lonza BioWhittaker (Verviers, Belgium). Penicillin-streptomycin, sodium pyruvate and horse serum were purchased from Gibco, Life Technologies (Paisley, UK). Foetal bovine serum (South America) was from BioWest (Nuaille, France). Dimethyl sulfoxide (DMSO), monochlorobimane (mBCl), thiazolyl blue tetrazolium bromide (MTT), formalin and mouse neural growth factor (NGF) were all supplied by Sigma-Aldrich (Poole, Dorset, UK). Hoechst 33342 nuclear stain and mitochondrial membrane potential dye (MitoTracker™ Red CMXRos) were provided by Thermo Scientific (UK). Valinomycin, perfluorooctanesulfonic acid potassium salt (PFOS ≥ 98

%) as well as all other reagents were from Sigma-Aldrich (St Louis, MO, USA).

In the cell toxicity test PFOS concentrations were chosen in the range of 10–100 μM, based on previous studies in PC12 cells (Berntsen et al., 2018). In the neurite outgrowth study the range was between 10–50 μM, because in the preliminary studies the concentration above 50 μM showed no effect on neurite outgrowth and was associated with fewer cells.

The defined 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 Table 1), including six perfluorinated (PFAA) (i.e., PFHxS, PFOS, PFOA, PFNA, PFDA, and PFUnDA), seven brominated (Br) (i.e., PBDE 47, PBDE 99, PBDE-100, PBDE 153, PBDE 154, PBDE 209, and HBCD), and sixteen chlorinated compounds (Cl) compounds (i.e., PCB 28, PCB 52, PCB 101, PCB 118, PCB 138, PCB 153, PCB 180, p,p'-DDE, HCB, α-chlordane, oxychlordane, trans-Nonachlor, α-HCH, β-HCH, γ-HCH and Dieldrin). The relative concentrations of the chemicals in the mixture were based on Scandinavian human blood levels and the choice of compounds were based on compounds found at high levels in blood, breastmilk and/or food. The stocks used in the present study had a concentration of 10^6 times measured human blood levels and stocks were created using DMSO as a solvent. We used the POP mixture concentration from 10 to 500x (fold human blood levels), as previously exposure with this mixture showed biological activity in the range of 1/10−500x (fold human blood levels) in a wide variety of cell systems (Doan et al., 2019; McComb et al., 2019; Shannon et al., 2019).

2.2. Cell culture and treatment

The rat pheochromocytoma cell line PC12 was cultivated in growth medium consisting of DMEM with penicillin-streptomycin, sodium pyruvate, and supplemented with 5% horse serum and 10 % foetal bovine serum as described previously (Rakkestad et al., 2014). Cells were seeded out in 96 wells plates (100 μL/well) and 35 mm diameter dishes (1 mL/dish) in a serum free medium and then incubated for 4−6 h for attachment and serum starvation. Without removing the medium, an equal volume of fresh medium containing horse serum (final 2%), NGF (final 50 ng/mL) and test compounds/controls, was thereafter added to each well. Plates were incubated further for 72 h prior to the assay. The exposure scenario is overviewed in Fig. 1.

2.3. MTT conversion assay

Cell viability was measured by MTT colorimetric assay, as described previously (McComb et al., 2019). This assay is based on the conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, a pale yellow substrate to a dark blue formazan product by the mitochondrial enzyme succinate dehydrogenase in living cells (Mosmann, 1983). Cells were seeded in clear flat-bottomed 96-well plates (Nunc, Roskilde, Denmark) at a density of 1×10^4 cells/cm^2 and exposed to test compounds either in the presence or absence of NGF. Following 72 h exposure with test compounds, the supernatant was discarded, and cells were incubated further with 2 mg/mL MTT (in PBS) solution diluted 1:6 in assay media. Again, the supernatant was removed, and 200 μL/well of DMSO was added to dissolve the formazan crystals. Subsequently, the plate was incubated at 37 °C with agitation for 10 min. Optical density was measured at 570 nm with a reference filter at 630 nm using a Sunrise spectrophotometer (TECAN, Switzerland). Cell viability was measured as the percentage absorbance of the sample compared with the absorbance of the solvent control.

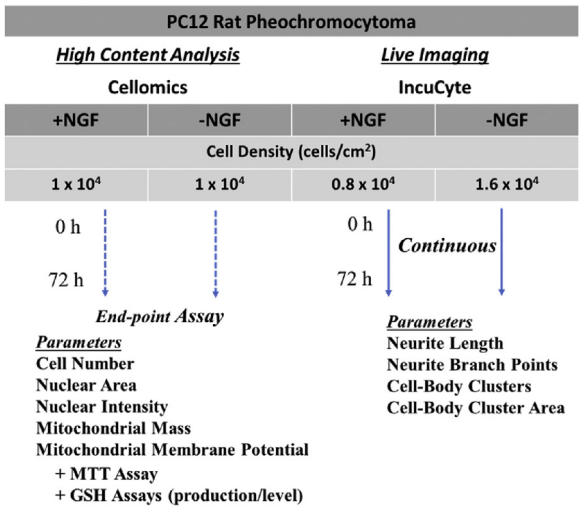

Fig. 1. Exposure overview of experiments.

Valinomycin (Vln) was used as a positive control in the MTT conversion assay and HCA assay. Exposure to Vln induces a dissipation of the mitochondrial transmembrane potential, leading to mitochondrial dysfunction (Abraham et al., 2008).

2.4. Cellular and mitochondrial health assessed by High Content Analysis (HCA)

The experimental procedure was followed as detailed previously (Shannon et al., 2017; Wilson et al., 2016). The Cellomics® HCS reagent series multiparameter cytotoxicity assay was performed following the manufacturer's instructions. Cells were seeded into collagen bio-coat BD Falcon 96 well flat bottomed microtitre plates (BD Biosciences, Bedford, MA, US) at a density of 1×10^4 cells/cm^2 and exposed to test compounds either in the presence or absence of NGF. Briefly, mitochondrial membrane potential dye was prepared by adding 117 μL of anhydrous DMSO to make a 1 mM stock. Following 72 h incubation, 50 μL of live cell stain (final 0.1 μM) was added to each well for 30 min at 37 °C and protected from light. The live stain was removed, and cells were then fixed with a 10 % formalin solution for 20 min at room temperature (RT) and washed with PBS. Hoechst 33342 dye (final 1.6 μM) was added to each well and incubated for 10 min at RT, after which cells were washed with PBS. The wells were then filled with 200 μL PBS, sealed with plate sealer and evaluated on a CellInsight™ NXT High Content Screening Platform (Thermo Fisher Scientific, UK). This instrument analyses epifluorescence of individual cell events using an automated micro-plate reader analyzer interfaced with a Dell precision 136 T5600 workstation.

Hoechst dye was used to measure CN, and nuclear morphology including NI and NA. Mitochondrial membrane potential dye (MitoTracker™ Red CMXRos) was used to measure mitochondrial health, specifically MMP and MM. Data were captured for each plate using a 10x objective magnification in the selected excitation and emission wavelengths of Hoechst dye (Ex/Em 350/461 nm) and mitochondrial membrane potential dye (Ex/Em 554/576 nm). For each well, nine fields of view images were acquired to examine each parameter.

2.5. Glutathione measurements

Total reduced GSH levels in CGNs were measured with mBCl as described previously (Sørvik et al., 2018). In brief, cells were seeded in black 96 wells plates at a density of 1×10^4 cells/cm^2 and

exposed with test compounds for 72 h. Then medium was removed and replaced with new medium containing 40 μM of mBCl and incubated further in the dark at 37 °C for 30 min. Subsequently the medium was removed, and plates were washed with freshly prepared experimental buffer containing 140 mM NaCl, 3.5 mM KCl, 15 mM Tris HCl (pH 7.4), 5 mM glucose, 1.2 mM Na_2HPO_4 (pH 7.4), and 2 mM $CaCl_2$. 100 μl of the buffer was added to each well, and mBCl fluorescence was measured at the excitation wavelength of 380 nm (15 nm bandwidth) and emission wavelength of 478 nm (21 nm bandwidth) using a CLARIOstar® plate reader. Further, in the same exposure, we also quantified cell number using nuclei staining with Hoechst 33342. Buffers were replaced with Hoechst 33342 (0.4 μg/mL) and incubated in dark for 1 min. Subsequently, the plate was read at excitation wavelengths of 350 nm (22 nm bandwidth) and emission wavelengths of 461 nm (36 nm bandwidths). This measurement was used to correct for cell number in the calculations for GSH levels after subtraction of blank values.

2.6. Transfection and luciferase assay for GCLC promoter activity assessment

The procedure has been detailed previously (Sørvik et al., 2018). Cells were seeded in Ø35 mm dishes at a density of 1.25×10^4 cells/cm^2 and allowed to attach for 24 h. On culture day 1, cells were transfected with GCLC (catalytic subunit) promoter coupled to luciferase (GCLC-luc; 0.8 μg; a kind gift from R. Blomhoff, Department of Nutrition, University of Oslo) and internal control vector pRL-TK (0.2 μg; purchased from Promega, WI, US) to a total of 1 μg DNA/mL culture medium. Transfection medium was replaced with fresh medium after 4 h. On culture day 2, cells were exposed with test compounds dissolved in serum free or serum free plus NGF medium. After 48 h luciferase was measured with a luminometer (Lumat LB9507; Berthold Technologies GmbH, Bad Wildbad, Germany) as described previously (Strom et al., 2010). *Renilla* luciferase was measured using the kit Dual-Luciferase® Reporter Assay System in accordance with the manufacturer's instructions (Promega, WI).

2.7. Live cell imaging using the IncuCyte neurite analysis

The IncuCyte ZOOM live-cell imaging system (Essen BioScience, UK) was used to analyse neurite dynamics. Different parameters were quantified over time using an automated IncuCyte® Neurotrack software module, as detailed previously (Garay et al., 2016). PC12 cells were seeded in 96-well TPP (Techno Plastic Products AG, Switzerland) plates and exposed to test compounds either in the presence or absence of NGF, as described above. Cells were seeded at a density of 0.8×10^4 cells/cm^2 in the presence of NGF and 1.6×10^4 cells/cm^2 in the absence of NGF. Plates were scanned every 60 min over a 72 h period using a 10x objective. Four images per well were captured and images were analysed for neurite length, branch points, cell-body clusters number and area. The masks/filters adjustment for Neurotrack phase contrast image analysis were as follows: Segmentation mode: Texture; Hole fill: 0; Adjust size: −5 μm; Min cell width: 8 μm; Neurite filtering: Best;

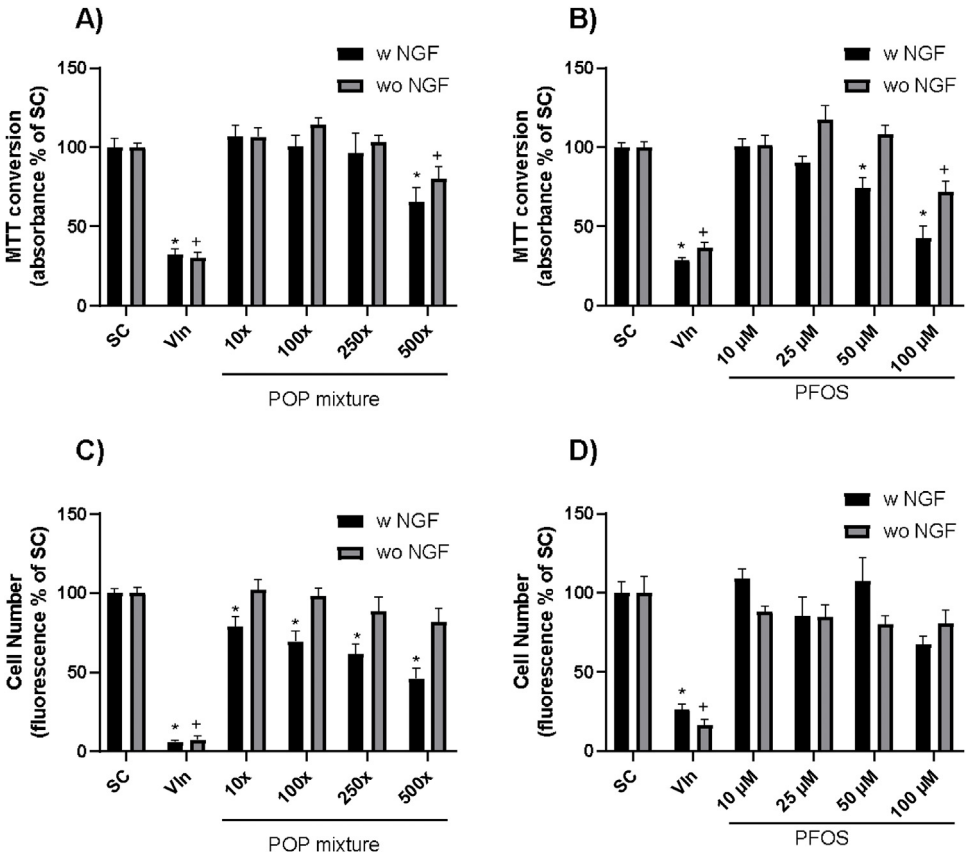

Fig. 2. Assessment of cytotoxic effects in PC12 using the tetrazolium MTT conversion assay or high content analysis (HCA). Cells were exposed in the presence or absence of NGF (50 ng/mL) with solvent control (SC, DMSO 0.1 %), POP mixture (x human blood levels) or PFOS (μM). Valinomycin (Vln, 15 μM) was included as a positive control. Following 72 h exposure to the compounds, cell viability was measured by conventional MTT conversion assay for A) POP mixture, and B) PFOS. Cell number measured by multi-parameter HCA is shown for C) POP mixture, and D) PFOS. Data are mean, n = 3-4 independent experiments, with replicate > 3. All values were presented relative to the corresponding solvent control (SC, 0.1 % DMSO) defined as 100 %, $^*p < 0.05$, compared to SC with NGF and $^*p < 0.05$ compared to SC without NGF, w = with NGF and wo = without NGF.

Neurite sensitivity: 0.35 μm; and Neurite width: 1 μm, detailed in (NeuroTrack™, 2020). The following parameters were quantified:

1 Cell-Body Clusters (CBC) = total number of cell body clusters/ area of image field;
2 Cell-Body Cluster Area (CBCA) = sum of areas of all cell-body clusters pooled/area of image;
3 Neurite Length (NL) = sum of lengths of all neurites pooled/ area of image field;
4 Neurite Branch Points (NBP) = total number of branch points/ area of image field.

2.8. Statistical analysis

Results are presented as mean ± standard error of the mean (SEM), n = 3–4 independent experiments, with replicates > 4 per group. IncuCyte data were analysed using the mixed model personality in JMP Pro 14® (SAS, Cary, USA). Dependent variables were CBC, CBCA, NL and NBP. For the statistical analysis log transformed dependent variables gave a satisfactory fit to the normal distribution. Independent fixed effect variables were: exposure group, time in culture and their interaction. Experiment and the nested effect of time within experiment were entered as random effects allowing for repeated measurements along the time scale. Dunnett's test was used to compare differences between groups exposed to chemicals and control (solvent control, 0.1 % DMSO). The effect of NGF on log transformed dependent variables (CBC, CBCA, NL and NBP) was explored in a separate mixed model using a subset of data including only controls. Independent variables included as fixed effects were time in culture, NGF and their interaction. All other data were analysed using Graph pad 8.2 (San Diego, CA, US). Outlier values were tested for by the built-in feature in GraphPad (Robust regression and Outlier removal, Q = 1%). A one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used to determine significant differences between treatments and solvent control. An unpaired *t*-test was used for comparison when appropriate between two groups. A *p*-value of < 0.05 was considered significant.

3. Results

3.1. Effects of POP mixture or PFOS on cytotoxicity as measured by MTT conversion assay

In a first approach, cytotoxic effects of pollutants on PC12 cells viability were evaluated using the MTT conversion assay. Both in the presence and absence of NGF, exposure to the POP mixture reduced MTT activity only at the highest concentration tested (Fig. 2A). PFOS alone reduced MTT activity significantly at 50 and 100 μM,which were 2.5 and 5 times higher than the corresponding concentration in the POP mixture at 500x (Fig. 2B). In the absence of NGF, reduced MTT activity was only observed at 100 μM (Fig. 2B).

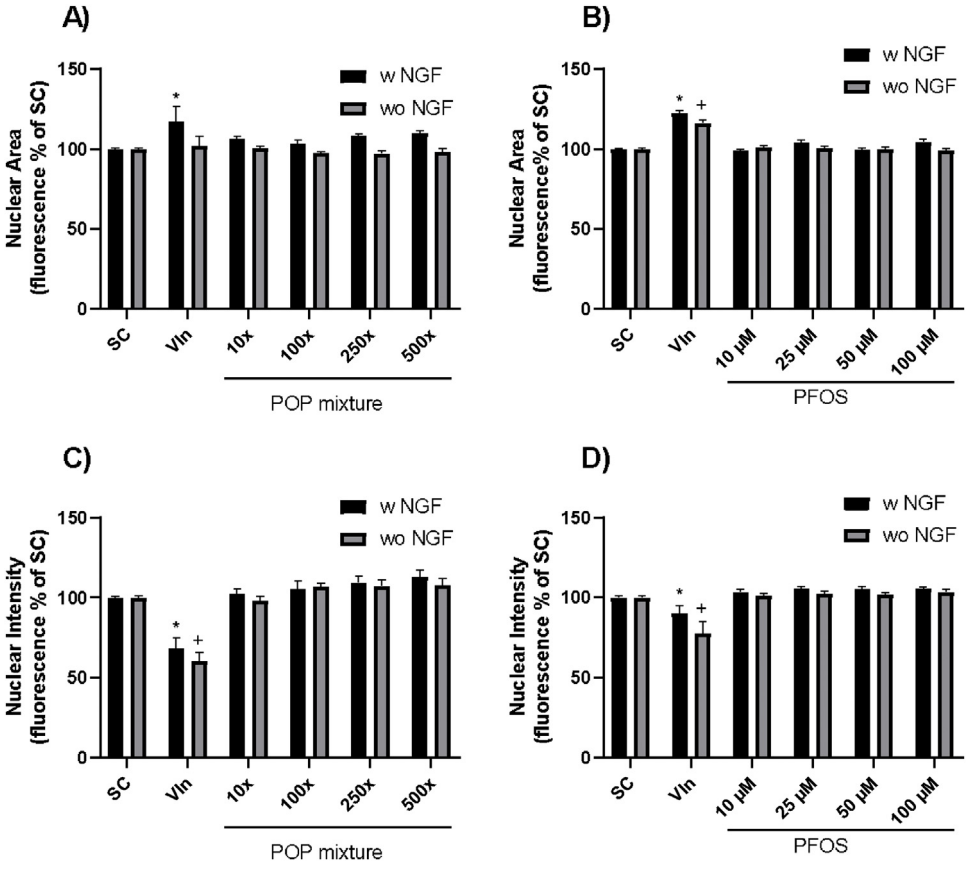

Fig. 3. Assessment of changes in nuclear health in PC12 using high content analysis (HCA). Cells were exposed in the presence or absence of NGF (50 ng/mL) with solvent control (SC, DMSO 0.1 %), POP mixture (x human blood levels) or PFOS (μM). Valinomycin (Vln, 15 μM) was included as a positive control. Multiparameter HCA endpoints were measured following 72 h exposure to the test compounds. Results for nuclear area is shown for A) POP mixture, and B) PFOS. Nuclear intensity is shown for C) POP mixture, and D) PFOS. Data are mean, n = 3-4 independent experiments, with replicate > 3. All values were presented relative to the corresponding solvent control (SC, 0.1 % DMSO) defined as 100 %. $^{*}p < 0.05$, compared to SC with NGF and $^{+}p < 0.05$ compared to SC without NGF, w = with NGF and wo = without NGF.

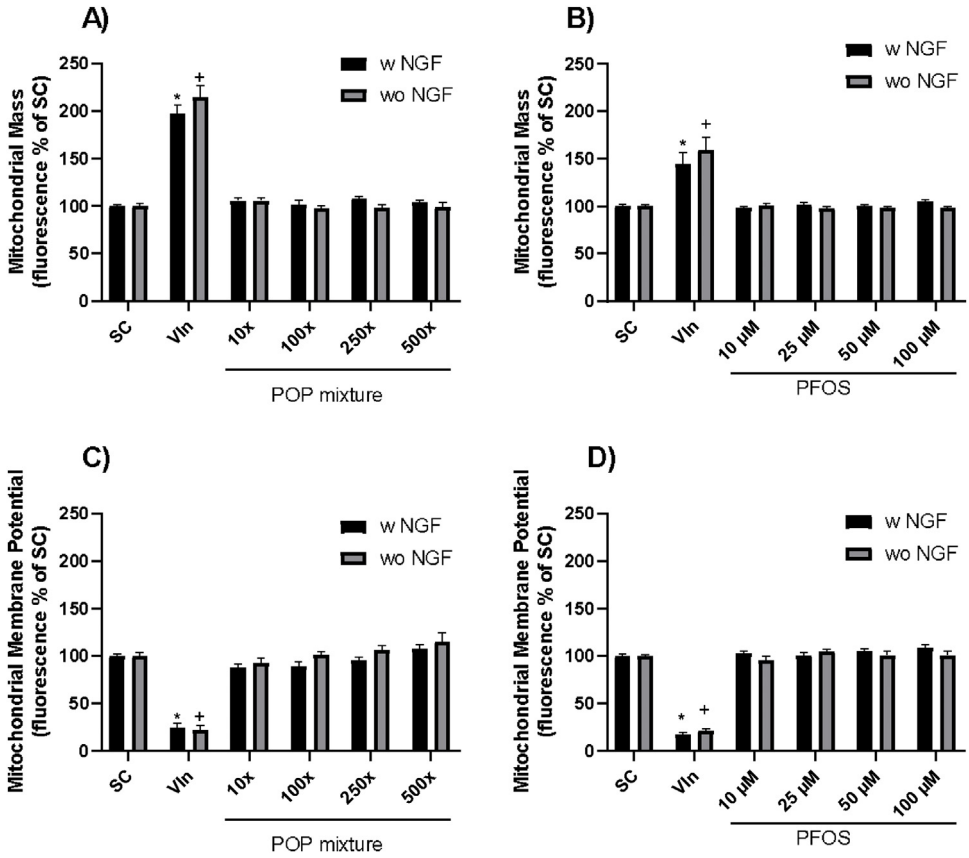

Fig. 4. Assessment of changes in mitochondrial health in PC12 using high content assay (HCA). Cells were exposed in the presence or absence of NGF (50 ng/mL) with solvent control (SC, DMSO 0.1 %), POP mixture (x human blood levels) or PFOS (µM). Valinomycin (Vln, 15 µM) was included as a positive control. Multiparameter HCA endpoints were measured following 72 h exposure to the test compounds. Results for mitochondrial mass is shown for A) POP mixture, and B) PFOS. Mitochondrial membrane potential is shown for C) POP mixture, and D) PFOS. Data are mean, n = 3-4 independent experiments, with replicate >3. All values were presented relative to the corresponding solvent control (SC, 0.1 % DMSO) defined as 100 %. $^{*}p < 0.05$, compared to SC with NGF and $^{+}p < 0.05$ compared to SC without NGF, w = with NGF and wo = without NGF.

3.2. Effect of POP mixture or PFOS on cell number measured by HCA

To investigate whether decreases in MTT activity was due to a reduction in cell number we evaluated cell number independently by HCA. For the POP mixture in the presence of NGF, reductions in cell number as measured by HCA were already significant at 10x (fold human blood levels, Fig. 2C). However, in the absence of NGF the cell number remained unaffected at any of the concentrations tested (Fig. 2C). For PFOS alone no significant concentration-response was observed for cell number in the presence or absence of NGF (Fig. 2D).

3.3. Effects of POP mixture or PFOS on cytotoxicity as measured by nuclear and mitochondrial health using HCA

Alternatively, MTT activity can be affected by diminished mitochondrial health resulting in cell loss via cell death. Thus, we investigated additional HCA toxicity parameters for nuclear and mitochondrial health. Both in the presence or absence of NGF, exposure to either the POP mixture or PFOS did not affect the nuclear (Fig. 3A–D) or mitochondrial parameters (Fig. 4A–D).

HCA images using Hoechst dye (nuclear staining) and mitochondrial potential dye (mitochondrial staining) are illustrated in Supplementary Fig. 1.

3.4. Effects of POP mixture or PFOS on glutathione levels and GCLC promoter activity

Since intracellular GSH levels are important for cell survival, we measured the effects of pollutants on total levels of GSH by mBCl probe, as well as the production of GSH by GCLC promoter activity. In the presence of NGF the POP mixture significantly increased glutathione levels at 10x blood levels, whereas at higher concentrations the glutathione levels were comparable with control levels. In the absence of NGF, glutathione levels were reduced in a concentration-dependent manner to half the level seen in the solvent control (SC, 0.1 % DMSO; Fig. 5A). Both in the presence or absence of NGF the GCLC promoter activity following 48 h exposure to POP mixture 500x remained unaffected. This suggests that the synthesis of GSH did not change following the exposure to POP mixture (Fig. 5B).

3.5. Effects of POP mixture or PFOS on cell body clusters and area in PC12 cells with and without NGF

We quantified the number of cell body clusters (CBC) and area (CBCA) using IncuCyte live cell imaging which can be interpreted as a measure of proliferation and cell body size, respectively. CBC number and CBCA increased with time in culture for all conditions

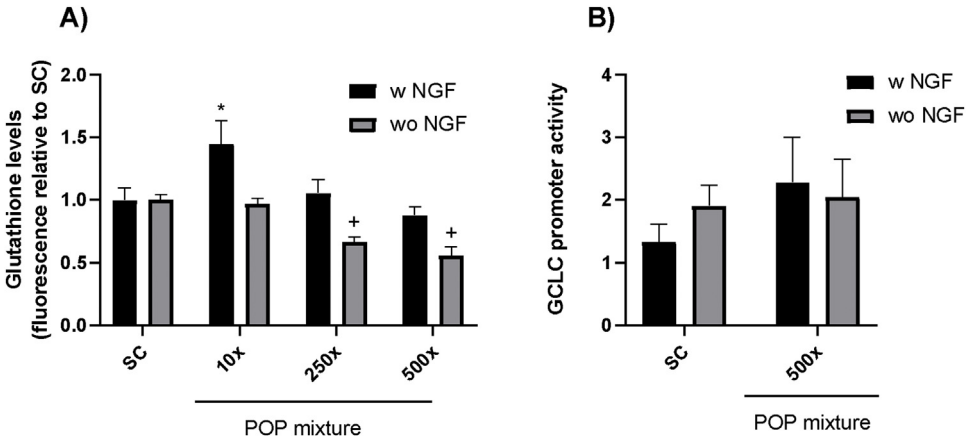

Fig. 5. Assessment of glutathione (GSH) levels and GSH production levels. Cells were exposed in the presence or absence of NGF (50 ng/mL) with solvent control (SC, DMSO 0.1 %) or POP mixture (x human blood levels) or PFOS (μM). A) GSH level using mBCl was measured following 72 h exposure, and B) GCLC promoter activity following 48 exposure. Data are mean, n = 3-4 independent experiments, with replicate > 3. All values were presented relative to the corresponding solvent control (SC, 0.1 % DMSO) defined as 1 for GSH, $^{*}p < 0.05$, compared to SC with NGF and $^{+}p < 0.05$ compared to SC without NGF, w = with NGF and wo = without NGF.

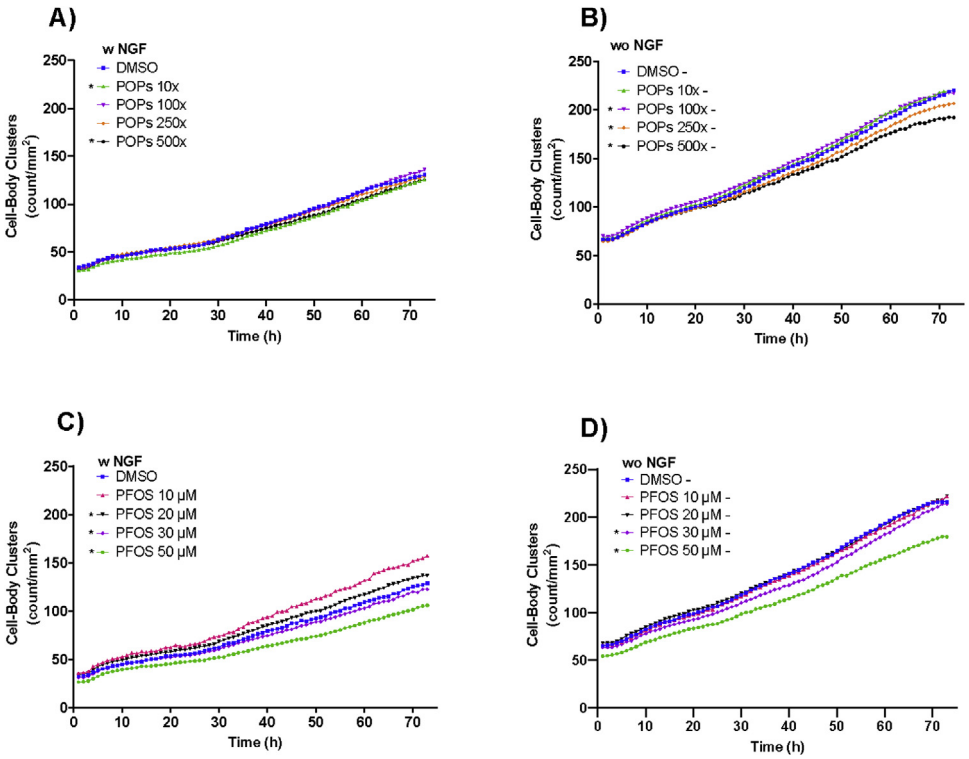

Fig. 6. Assessment of changes in cell body clusters (CBC) in PC12 cells after exposure to POP mixture or PFOS using live-cell imaging. Effect of the compounds in presence of NGF (50 ng/mL) (A and C) or its absence (B and D) were evaluated. Cell were exposed to the POP mixture (A and B), or to PFOS (C and D). Solvent control was DMSO 0.1 %. Concentration of the POP mixture is indicated as POPs x human blood levels. PFOS concentrations are in μM. Average CBC (total number of cell body clusters/area of image field) were automatically measured by Neuro Track software, every 60 min, followed for 72 h. For clarity, the graph is presented only with mean value at different time points. Data are mean, n = 3-4 independent experiments, with replicate > 3 per group. Statistical difference between the overall average of the exposed groups and the solvent control (0.1 % DMSO) is indicated on the left-hand side of the figure legends ($^{*}p < 0.05$), w = with NGF and wo = without NGF.

tested (Figs. 6 and 7). CBC number for DMSO control tripled for PC12 cells stimulated with NGF alone (Fig. 6A, C) and nearly quadrupled without NGF (Fig. 6B, D). NGF exposure was associated with a significant decrease in the overall least squares (LS) mean log CBC and log CBCA (Figs. 6 and 7; Supplementary Table 3). Exposure to high concentrations of POP mixture and PFOS was associated with a significant decrease in log CBC and log CBCA whereas low and intermediate concentrations had variable effects (Supplementary Table 2; Figs. 6 and 7).

Phase contrast IncuCyte images and images after applying Neuro Track masks for CBCs and neurites are illustrated in Supplementary Figs. 2 and 3.

3.6. Effects of POP mixture or PFOS on neurite length and branch points in PC12 cells with and without NGF

We quantified neurite length and neurite branch points as measures of neurite outgrowth using IncuCyte live cell imaging. NL

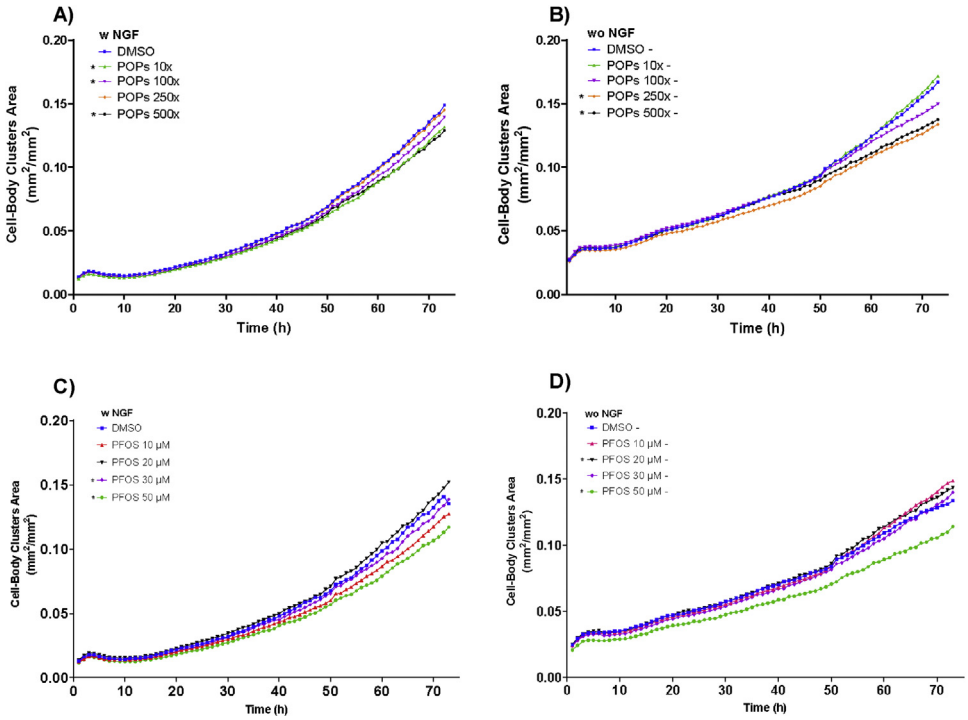

Fig. 7. Assessment of changes in Cell-Body Cluster Area (CBCA) in PC12 cells after exposure to POP mixture or PFOS using live-cell imaging. Effect of the compounds in presence of NGF (50 ng/mL) (A and C) or its absence (B and D) were evaluated. Cell were exposed to the POP mixture (A and B), or to PFOS (C and D). Solvent control was DMSO 0.1 %. Concentration of the POP mixture is indicated as POPs x human blood levels. PFOS concentrations are in μM. Average CBCA (sum of areas of all cell-body clusters pooled/area of image) were automatically measured by Neuro Track software, every 60 min, followed for 72 h. For clarity, the graph is presented only with mean value at different time points. Data are mean, n = 3-4 independent experiments, with replicate > 3 per group. Statistical difference between the overall average of the exposed groups and the solvent control (0.1 % DMSO) is indicated on the left-hand side of the figure legends ($^*p < 0.05$), w = with NGF and wo = without NGF.

and NBP increased with time in culture for all conditions in the presence of NGF (Figs. 8A, C, and 9 A, C, respectively). Only a marginal increase was observed with time in culture when NGF was not present (Figs. 8B, D and 9 B, D, respectively).

In PC12 cells stimulated with NGF, the POP mixture significantly increased the neurite length in a concentration-dependent manner compared to the effect of NGF alone (Fig. 8A; Supplementary Table 2). PFOS had a similar, but less pronounced effect. PFOS at concentrations of 20 μM and above significantly increased the neurite length compared to NGF alone over the 72 h period (Fig. 8C; Supplementary Table 2). In the absence of NGF, the effects of POPs and PFOS on NL were marginal (Fig. 8B, D; Supplementary Table 2).

Similar patterns were observed for the number of branch points (Fig. 9A–D; Supplementary Table 2).

4. Discussion

We investigated if a POP mixture based on human blood levels from the Scandinavian population can affect cellular health and neurite dynamics, using PC12 cells as a model. Outcomes were compared to PFOS alone, the most abundant compound in the POP mixture.

Cytotoxicity of the POP mixture exposure after 72 h evaluated by MTT conversion was only seen at higher concentrations. Reduced conversion of MTT to formazan by PC12 cells could indicate a reduction of mitochondrial activity, rather than reduced proliferation or loss of cells. However, a decrease in cell number was measured by HCA. Yet, the HCA nuclear and mitochondrial parameters remained unchanged and were indicative of healthy cells. This suggests that the reduction seen in MTT was mainly due to a reduced proliferation. The IncuCyte experiments allowed us to follow cell proliferation in a dynamic way over the full 72 h exposure. Indeed, the number of cell body clusters (CBC) can be interpreted as a measurement of cell proliferation, although it gives no information about the number of cells in each individual cluster. This can be gathered from the cell cluster area (CBCA), a measurement of the total area of cell clusters per image field. In all exposures, both CBC number and CBCA increased linearly over time, indicative of continued cell proliferation. However, the rise was slower in the presence of NGF, indicating a negative effect of NGF on cell proliferation. This is in accordance with the observations that NGF-induced differentiation in PC12 cells is linked with reduced cell proliferation (Greene and Tischler, 1976). During the 72 h period the most potent POP mixture concentration (500x) slowed down the rate of CBC number increase compared to solvent control. However, in the absence of NGF, the same concentration had more outspoken effect on the rate of reduction in CBC number increase. This could be related to the relative lower level of GSH as discussed below.

For PFOS in the presence of NGF, no reduction in MTT was seen at lower concentrations (10 and 25 μM), although higher concentrations (50 and 100 μM) showed a reduction compared to control. No significant changes in cell number at any concentration could be observed. As with the POP mixture, no effects were seen on nuclear and mitochondrial parameters. PFOS at lower concentration (10 and 20 μM) seemed to have a stimulating effect on the rate of CBC number increase compared to control, whereas higher concentrations (30 and 50 μM) led to lower rate. Thus, we can conclude PFOS concentrations (20 and 25 μM) relevant to those present in the total POP mixture did not adversely affect neither cell proliferation nor cellular toxicity. In the absence of NGF, only at 100 μM a reduction in MTT activity was observed, but this PFOS concentration is about 5 times higher than relevant to the POP mixture. Again, no significant effects on cell

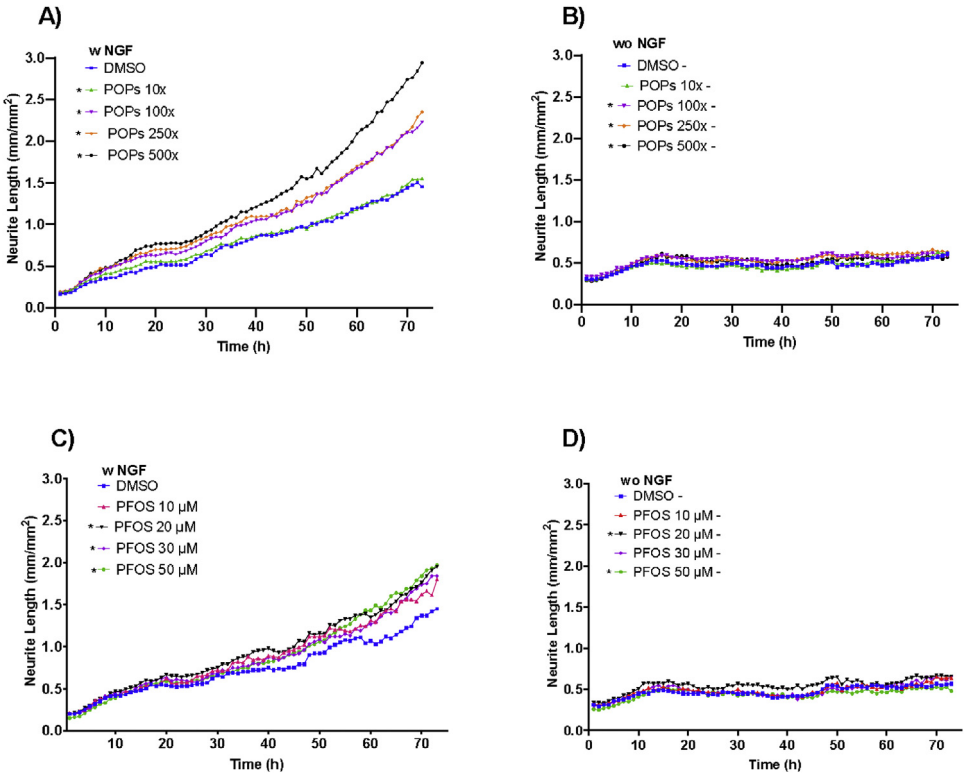

Fig. 8. Assessment of changes in neurite length over time in PC12 cells after exposure to POP mixture or PFOS using live-cell imaging. Effect of the compounds in presence of NGF (50 ng/mL) (A and C) or its absence (B and D) were evaluated. Cell were exposed to the POP mixture (A and B), or to PFOS (C and D). Solvent control was DMSO 0.1 %. Concentration of the POP mixture is indicated as POPs x human blood levels. PFOS concentrations are in µM. Average neurite lengths (sum of lengths of all neurites pooled/area of image field in mm/mm^2) were automatically measured by Neuro Track software, every 60 min, followed for 72 h. For clarity, the graph is presented only with mean value at different time points. Data are mean, n = 3-4 independent experiments, with replicate > 3 per group. Statistical difference between the overall average of the exposed groups and the solvent control (0.1 % DMSO) is indicated on the left-hand side of figure legends (*$p < 0.05$), w = with NGF and wo = without NGF.

number and cellular health parameters in HCA were observed. However, concentrations of 30 and 50 µM lead to decrease in CBC number.

Intracellular GSH levels are important for cell survival and protect the cells against injury caused by ROS. We have previously observed that the POP mixture and PFOS negatively affected GSH levels in chicken cerebellar granule cells (manuscript under review). Thus, we tested if PC12 cells were equally affected. In the presence of NGF, low concentrations of POP mixture raised GSH level, which was absent for the higher concentrations. In the absence of NGF, POP mixture exposure led to a decrease in GSH, suggesting that NGF-differentiated cells have a more robust GSH defence mechanism. Indeed, treatment with NGF has been shown to increase the level of GSH in PC12 cells (Kamata et al., 1996). We found no evidence that either in the presence or absence of NGF, the POP mixture at 500x affected GCLC promotor activity, suggesting it did not affect production rates of GSH.

NGF can stimulate neurite outgrowth in PC12 cells, and neurite outgrowth can be used as a marker for neuronal differentiation (Radio and Mundy, 2008). Indeed, in our system NGF alone was a sufficient driver for altering the cellular and neurite dynamics compared to DMSO control. Chemicals, including POPs, could potentially affect neuronal outgrowth (Addae et al., 2013; Angus and Contreras, 1995; Radio et al., 2008). However, it seems exposure to the POP mixture cannot mimic the NGF-receptor signal on its own. We found that exposure to the POP mixture did enhance NGF-induced neurite length and branched networks of neurites in a concentration- and time-dependent manner. This suggests that the POP mixture might have potentially influenced the NGF neurotrophic system. Similar to our results, Angus and Contreras (1995) found that Aroclor 1254 (a technical mixture of PCBs) enhanced NGF-stimulated neurite outgrowth in PC12 cells and caused an increase in the affinity of binding of NGF to the high-affinity NGF receptors. However, Aroclor 1254 is an industrial mixture with a lot-to-lot variation of *ortho*- and non-*ortho*-PCB composition (Kodavanti et al., 2001). In contrast, our mixture is chemically defined and contains mainly non-dioxin like PCBs. Thus, our observations indicate that non-dioxin-like PCBs might contribute to neuritogenesis. The effects of PFOS on both neurite length and branch point parameters in absolute numbers were about 50 % of the full mixture. The effect of PFOS seemed to plateau from 20 µM onwards. This also represents the concentration of PFOS present in the POP mixture at 500 ×. Thus, PFOS alone can explain part of the effect seen with the POP mixture, but additional compounds contribute to the overall effect. At present, mechanisms are not known.

Studies *in vitro* have shown the differential effects of individual POPs on neurite outgrowth using different neuronal cells. PCB 153 or PCB 180 at 100 nM facilitated the neurite outgrowth associated with a decrease in cell proliferation, indicating an increase in neuronal differentiation in primary rat embryonic neural stem cells (Tofighi et al., 2011). In the POP mixture at 500x, PCB 153 is present at 349 nM and PCB 180 at 170 nM. Generally, OCPs have been shown to reduce neurite outgrowth. p,p`-DDT and o,p`-DDT decreased the neurite outgrowth dose dependently (1–50 µM) and induced apoptosis in NGF-stimulated PC12 cells. p,p`-DDE also revealed a similar effect but to a lesser extent (Shinomiya and Shinomiya, 2003). In the POP mixture at 500x, p,p`-DDE is present at 0.5 µM. Christen et al. (2017) reported that the different organochlorine pesticides including dieldrin at 10 or 100 µM suppressed the neurite outgrowth in PC12 cells co-treated with NGF, but this compound is only present at 28 nM in the POP

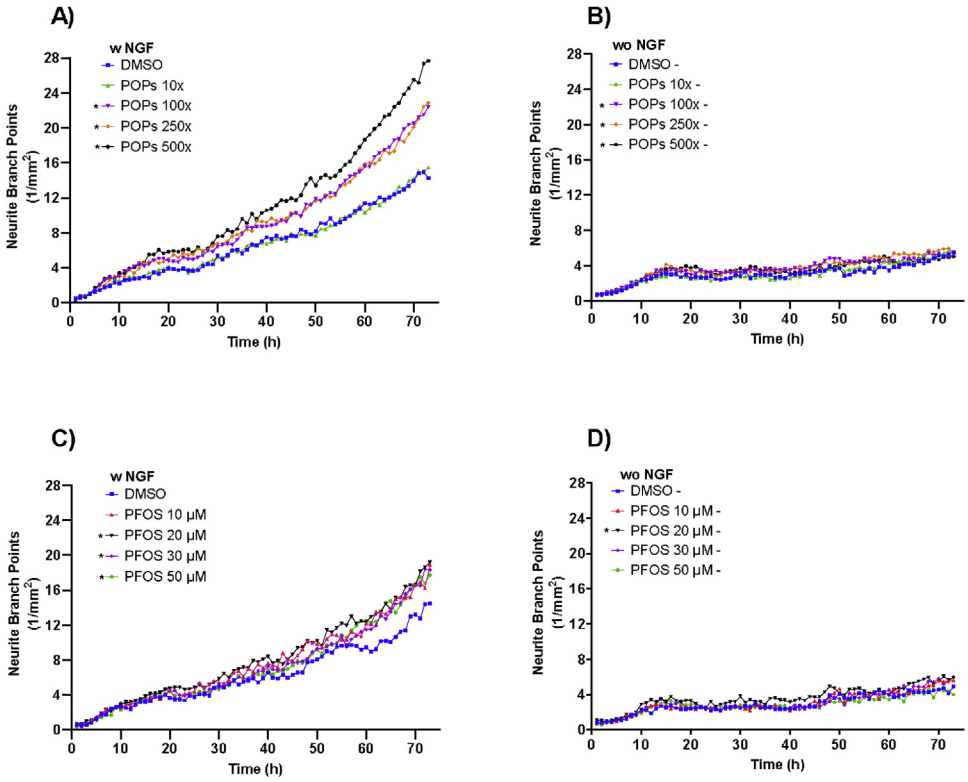

Fig. 9. Assessment of changes in neurite branch points in PC12 cells after exposure to POP mixture or PFOS using live-cell imaging. Effect of the compounds in presence of NGF (50 ng/mL) (A and C) or its absence (B and D) were evaluated. Cell were exposed to the POP mixture (A and B), or to PFOS (C and D). Solvent control was DMSO 0.1 %. Concentration of the POP mixture is indicated as POPs x human blood levels. PFOS concentrations are in µM. Average neurite branch points (total number of branch points/area of image field) were automatically measured by Neuro Track software, every 60 min, followed for 72 h. For clarity, the graph is presented only with mean value at different time points. Data are mean n = 3-4 independent experiments, with replicate > 3 per group. Statistical difference between the overall average of the exposed groups and the solvent control (0.1 % DMSO) is indicated on the left-hand side of figure legends (*$p < 0.05$), w = with NGF and wo = without NGF.

mixture at 500 ×. Treatment with HCB at 0.5 nM repressed neurite outgrowth but did not affect cell viability in mouse embryonic stem (ES) cells (Addae et al., 2013). This compound is present in the POP mixture (500x) at 114 nM. Commercial PBDEs decabromodiphenyl ether (deca-BDE) at 15 µM, inhibited the neurite growth in cultured rat hippocampal neuron (Liao et al., 2008a). In our mixture, individual BFRs are present at nanomolar range. Nanomolar concentrations of PFOS, have been shown to facilitate neurite outgrowth associated with decreased cell proliferation in rat primary embryonic neuronal cells (Wan Ibrahim et al., 2013). However, micromolar concentrations of PFAAs, comparable to ours, inhibited the neurite outgrowth in cultured rat hippocampal neurons cells (Liao et al., 2009, 2008b). These discrepancies in outcomes might be explained by many factors, such as concentration ranges, differences in different types of neurons, or the experimental conditions used. The effects of POPs on neurite outgrowth in PC12 could also be species specific or cell type specific effect. In contrast to other neurons from the central nervous system, PC12 is lacking functional expression of NMDA receptors. This receptor mediates glutamate induced excitotoxicity and can result in excessive calcium influx leading to cell death in many neurological disorders (Edwards et al., 2007). Another possible reason could be an ontogenetic effect, linked to the developmental stage of the cells used. In the present, we examined the effects of POPs in PC12 cells that were not exposed previously to NGF (i.e. not differentiated, unprimed). Although not examined in the present study, the other possible way would be to determine the effects of POPs in PC12 that have been exposed previously to NGF (primed). Parran et al. (2003) observed that in unprimed PC12 cells, mercuric chloride exposure increased NGF-stimulated neurite outgrowth. However, in primed PC12 cells, this compound inhibited NGF-stimulated neurite outgrowth. It should be noted that our POP mixture contains 29 different compounds which could have additive, synergistic or antagonistic effects.

It should be stressed that neurite length number returned by the IncuCyte image analysis software relates to the summation of the individual lengths of outgrowths per image fields, rather than a mean length of individual neurites. Similarly, the number of branch points returns a count per image field. Also, it does not differentiate between true branch points (one extension splitting in 2 or more) or crossings formed by overlapping outgrowths. Nevertheless, we found both parameters are highly correlated, and their increases suggest that cells are investing in the formation of more neurite material, and that the complexity of the outgrowths increases.

Our POP mixture did not induce immediate cell death. Rather, the inhibition of neurite outgrowth has been considered an important mode of action for specific developmental neurotoxicants (Radio et al., 2008). However, in our study POP mixture and PFOS lead to enhanced neurite outgrowth indicating lack of cytotoxicity. Some effects on the rate of cell proliferation were observed, but nuclear and mitochondrial health parameters remained unaffected.

The neurite outgrowth occurs in a strictly controlled way. During development of the nervous system, many more neurons are generated than ultimately integrate into neuronal circuits and survive (Mattson, 2008). Although pollutants showed no cytotoxic effects in these circumstances, overactive neuritogenesis at the wrong timing might also be detrimental. For example, studies have found that spine density is increased in ASD, which could suggest a developmental alteration in spine morphogenesis (Forrest et al., 2018).

Future studies could take into account effects of sub-mixtures and individual mixture constituents, and also further validate our observation of neurite outgrowth with alternative methods, such as measuring the length of individual neurites (Christen et al., 2017) or quantification by labeling with intracellular neurite specific markers, such as anti-βIII tubulin (Radio et al., 2010).

5. Conclusion

Exposure of differentiating neurons to complex mixtures of POPs might not result in neurotoxicity by cell loss, yet we cannot exclude possible adverse effects on developmental outcomes with regards to improper neuronal connectivity. PFOS contributes to a considerable part of the neuritogenesis seen with the total POP mixture, but other compounds present amplify the effect.

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 data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxlet.2020.12.007.

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