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A human relevant mixture of persistent organic pollutants (POPs) and perfluorooctane sulfonic acid (PFOS) differentially affect glutamate induced excitotoxic responses in chicken cerebellum granule neurons (CGNs) in vitro

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ABSTRACT

Primary cultures of cerebellar granule neurons (CGNs) derived from chicken embryos were used to explore the effects on developmental neurotoxicity by a complex defined mixture of persistent organic pollutants (POPs). Its chemical composition and concentrations were based on blood levels in the Norwegian/Scandinavian population. Perfluorooctane sulfonic acid (PFOS) alone, its most abundant compound was also evaluated. Different stages of CGNs maturation, between day *in vitro* (DIV) 1, 3, and 5 were exposed to the POP mixture, or PFOS alone. Their combination with glutamate, an excitatory endogenous neurotransmitter important in neurodevelopment, also known to cause excitotoxicity was evaluated. Outcomes with the mixture at 500x blood levels were compared to PFOS at its corresponding concentration of 20 μM. The POP mixture reduced tetrazolium salt (MTT) conversion at earlier stages of maturation, compared to PFOS alone. Glutamate-induced excitotoxicity was enhanced above the level of that induced by glutamate alone, especially in mature CGNs at DIV5. Glutathione (GSH) concentrations seemed to set the level of sensitivity for the toxic insults from exposures to the pollutants. The role of N-methyl-D-aspartate receptor (NMDA-R) mediated calcium influx in pollutant exposures was investigated using the non-competitive and competitive receptor antagonists MK-801 and CGP 39551. Observations indicate a calcium-independent, but still NMDA-R dependent mechanism in the absence of glutamate, and a calcium- and NMDA-R dependent one in the presence of glutamate. The outcomes for the POP mixture cannot be explained by PFOS alone, indicating that other chemicals in the mixture contribute its overall effect.

1. Introduction

Exposures in humans to complex mixtures of contaminants are suspected to cause adverse health outcomes. Increased prevalence of neurodevelopmental disorders, such as autism, attention-deficit hyperactivity disorder (ADHD), dyslexia, and other cognitive impairments have been linked with exposure to environmental pollutants [1]. Pregnancy and early life stages are particularly vulnerable for exposure to toxicants, including persistent organic pollutants (POPs) [2]. POPs are mostly halogenated compounds that are highly resistant to environmental degradation, and that bioaccumulate and biomagnify in living organisms [3]. Several POPs, including polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polybrominated diphenyl ethers (PBDEs), perfluorooctane sulfonic acid (PFOS), and

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perfluorooctanoic acid (PFOA) are supposed to be developmental neurotoxicants [1,4–6].

Human populations are exposed to a complex mixture of contaminants, which could have additive, synergistic or antagonistic effects. Experimental studies carried out *in vitro* and *in vivo* with mixtures showed that low-level exposure to chemicals that are not toxic on an individual basis can together lead to a biological or toxic effect if present in a mixture [7]. This kind of low-dose cocktail or mixture effects are of real concern to humans. Chemicals affecting similar adverse outcomes often act additively when present together in mixtures [8]. Chemical mixtures have often been defined by the modes of action of individual components and three basic types of action have been identified, namely: (I) *similar action* (dose or concentration addition): applied to mixtures in which the chemicals present have a similar mechanism/mode of action and may differ in their potencies (*i.e.* the impact of similar concentrations/doses will differ). In this case the effects of the mixture can be estimated directly from sum of the doses/concentrations, scaled for relative toxicity/potency of the individual substances. (II) *Dissimilar action* (independent action): applied to chemicals with dissimilar modes of action. In this case the action of mixture components can be calculated from the response of individual components (response addition) or the sum of biological responses (effects addition). (III) *Interactions* (synergism and antagonism): applied if the combined effect of two or more chemicals is either stronger (synergistic, potentiating, supra-additive) or weaker (antagonistic, inhibitive, sub-additive, infra-additive) than would be expected on the basis of dose/concentration addition or response addition. In this case the extent of interactions may vary according to the relative dose levels (*e.g.* too low to have such an effect); route of exposure (*e.g.* absorption too low or different metabolism); duration of exposure (including bio-persistence of components); and biological target [9–11].

Studies have shown that there is exposure to POPs in humans as wells in animals. In a Norwegian birth cohort study, concentrations of 27 POPs were reported in breast milk, reflecting the child's early-life exposures, and certain POPs were associated with a neurodevelopmental disorder [12]. Flores-Ramírez et al. [13] showed the levels of multiple POPs in serum samples from children living in high-risk areas in Mexico, indicating that the evaluation of the health risks posed to children living in contaminated areas is a high priority health issue. In another study, González-Mille et al. [14] reported the genotoxic effects in resident wild species exposed to complex mixture of POPs in the delta of the Coatzacoalcos river, Mexico. In the present study, we used a defined POP mixture designed by our group at the Norwegian University of Life Sciences (NMBU), Oslo. The concentration of the individual compounds was based on their respective levels in human blood in the Norwegian/Scandinavian population [15]. PFOS is the compound with the highest concentration present in the total POP mixture and a potent developmental neurotoxicant. In a multi-pollutant analysis of 27 POPs in a Norwegian birth cohort study, early-life exposure to β-hexachlorocyclohexane (HCH) and PFOS was associated with increased risk of ADHD [12]. Recently, European population-based studies including Human Milk Study (HUMIS; Norway) suggested that there may be an increased prevalence of ADHD in girls, in children from nulliparous women, and in children from low-educated mother in association with PFOS and PFOA exposure [16]. Also, our PFOS concentration is comparable to reported concentrations of 20 μM (lowest observed effect concentration) and 38 μM (lethal concentration killing 50 % of the cells) using tetrazolium salt (MTT) assay in rat CGNs [17], and all other component in the mixture had a concentration lower than its effective dose range. We have previously examined the toxicity of each individual PFAAs compounds in the POP mixture in cultured rat CGNs [18]. We observed that the two sulfonated-induced, but not perfluoroalkyl carboxylic acid-induced toxicity was associated with N-methyl-D-aspartate receptor (NMDA-R) and PFOS was the most effective on NMDA-R [18]. This receptor plays an important role in cerebellar development [19]. Here it should be pointed out that in the present study we used PFOS as a single compound with a concentration, which overlaps the concentration of the POP mixture

In addition to its role in motor control and coordination, the cerebellum is involved in learning and memory, language, executive functioning, as well as diseases such as ADHD, autism spectrum disorders, and schizophrenia [20]. The embryonic chicken cerebellum can be used as a model, since it shows developmental stages similar to those in humans [19]. Cerebellar granule neurons (CGNs) form the largest homogeneous neuronal population in the mammalian brain, and are the main excitatory neurons of the cerebellar cortical circuitry [21]. The comparative anatomy studies have shown that the cerebellum is a well-conserved structure between species [22]. The migration of granule neurons through layers of the cerebellar cortex from the external granule layer to the internal granule layer is a key feature for the development of cerebellum. This occurs prenatally before hatching in chicken and to a large extent postnatally in some mammals, including humans [19]. The migration of neurons in a developing brain is guided through N-methyl-D- aspartate receptors (NMDA-R) mediated Ca^{2+} influx, among other signals [23]. Alterations in migration could be indicative of impaired neurodevelopment [19]. CGNs isolated at embryonic day (ED) 17–18 from chicken is comparable to postnatal day 7–8 in the rat [24]. In addition, cultured chicken CGNs develops NMDA-mediated toxicity more rapidly than cultured rat CGNs and respond similarly to glutamate excitotoxicity with ROS production and activation of caspase-3 [24]. CGNs derived from chicken cerebellum serve to study brain development after exposure to different pharmaceuticals, drugs [25–28] and environmental toxins [29]. This is an advantage when considering chicken embryo CGNs as a model for NMDA receptor development and thus makes the cerebellum a potential target organ for developmental toxicity (DNT) induced by POPs.

Glutamate is a major excitatory neurotransmitter in the vertebrate central nervous system (CNS) and under normal circumstances contributes to neural transmission, development, differentiation and plasticity. However, under pathological stimuli excessive glutamate release leads to overactivation of glutamate receptors, resulting in an augmented intracellular Ca^{2+}-influx [30]. This can lead to excitotoxicity, resulting in neuronal death [31,32]. Excitotoxicity is involved in many neurodegenerative diseases such as Huntington's disease, Alzheimer's disease, lateral amyotrophic sclerosis, Parkinson's disease, stroke, or traumatic brain injury [30]. Among different types of glutamate receptors, the N-methyl-D-aspartate receptor (NMDA-R) plays an important role in mediating neuronal death observed in most primary cultured neurons [33]. In CGNs intracellular glutathione (GSH) plays a major role in protecting cells against injury caused by reactive oxygen species (ROS) following excitotoxic insults [34]. A reduction in GSH level can lead to glutamate-induced cell death [35]. Calcium influx plays a critical role in glutamate-induced neurotoxicity [32]. NMDA-R channels are highly Ca^{2+}-permeable and function as heterotetrameric assemblies that typically consist of GluN1 and GluN2 subunits, or GluN2 and GluN3 subunits. The subunit composition of NMDA-Rs changes during development in response to neuronal activity or sensory experiences [36]. Also, *in vitro* the expression of the NMDA-R subunits changes with time and maturation of CGNs [37]. Because of this previously reported time-dependent changes in NMDA-R subunits expression, we conducted the present study at different stages of maturation at day *in vitro* (DIV) 1, 3 and 5. We assume that neuronal cells exposed at DIV5 to express more mature and more sensitive neurons than cells exposed at DIV1 or 3.

A role of NMDA-R in the excitotoxity after exposure to same POP mixture, and PFOS have been shown in rat CGNs as well in chicken CGNs [18,38].The possible involvement of NMDA-Rs in excitotoxicity may be explored by using NMDA-R inhibitors. MK-801 is a non-competitive NMDA-R open channel blocker and binding sites are situated within the ion channel [39,40]. CGP-39551 is a competitive antagonist that binds to the glutamate binding site of the receptor [41].

The hypothesis of the present work was that a complex human

Table 1
The total *in vitro* mixture containing 29 different POPs based on human blood levels, detailed in [15].

Compound	Average human blood levels [a] ng/g lipid	Average human blood levels [b] ng/mL	Total mixture stock nominal concentration [c] mg/mL	Total mixture stock Measured concentration [d] μM
PCBs				
PCB 28	2.13	0.013	0.013	31.1
PCB 52	1.6	0.01	0.01	20.5
PCB 101	1.3	0.008	0.008	24.5
PCB 118	10.67	0.064	0.064	137.9
PCB 138	37	0.222	0.222	429.5
PCB 153	60.33	0.362	0.362	698.3
PCB 180	32.33	0.194	0.194	339
∑ PCBs	145.36	0.873	0.873	1680.8
OCPs				
p,p'-DDE	83.67	0.502	0.502	1065.9
HCB	19.5	0.117	0.117	228.2
α-Chlordane	1.8	0.011	0.011	23.7
Oxychlordane	3.7	0.022	0.022	33
trans-Nonachlor	6.8	0.041	0.041	99.1
α-HCH	1	0.006	0.006	16.8
β-HCH	8.77	0.053	0.053	75.6
γ-HCH	1	0.006	0.006	16.8
Dieldrin	4	0.024	0.024	56.2
∑ OCPs	130.24	0.782	0.782	1615.3
∑ PCBs + OCPs	275.6	1.655	1.655	3296.1
BFRs				
PBDE 47	1.43	0.009	0.009	17.8
PBDE 99	0.59	0.004	0.004	7.5
PBDE 100	0.36	0.002	0.002	3.8
PBDE 153	1.64	0.01	0.001*	2.1
PBDE 154	0.29	0.002	0.002	3
PBDE 209	1.81	0.011	0.011	9.4
HBCD	4.1	0.025	0.025	54.5
∑ BFRs	10.22	0.063	0.053	98.1
PFAAs				
PFHxS	N/A	3.45	3.45	7809.2
PFOS	N/A	29.425	29.425	41522.1
PFOA	N/A	4.523	4.523	4209.4
PFNA	N/A	0.8	0.8	1092.5
PFDA	N/A	0.495	0.495	375.4
PFUnDA	N/A	0.56	0.56	336.8
∑ PFAAs	N/A	39.253	39.253	55345.4

N/A - Not applicable.
Abbreviations: PCBs (polychlorinated biphenyls); OCPs (organochlorine pesticides); BFRs (brominated flame retardants); PFAAs (perfluoroalkyl acids).
[a] Average human blood levels of POPs based on a literature review of Scandinavian values, providing the basis for the *in vitro* mixture.
[b] Average human blood levels of POPs converted to ng/mL. A fat percentage of 0.6 % was used. 1 mL blood was considered to have a weight of 1 g.
[c] Nominal concentration of the various compounds in the total mixture stock – 1000000x the average concentration in blood.
[d] Measured concentrations of the various compounds in the total mixture stock converted to μM.
[*] The nominal concentration of PBDE 153 included in the total mixture stock was ten times lower than originally intended.

relevant POP mixture with defined chemical composition and concentrations could affect NMDA-R signaling and downstream calcium influx, glutathione level, and excitotoxicity, and that the mixture would share some mechanisms with PFOS, a main component of the mixture.

2. Materials and methods

2.1. Chemicals and PFOS, and POP mixture

Basal Eagle's medium (BME) and L-glutamine were purchased from Lonza BioWhittaker (Verviers, Belgium). Penicillin-streptomycin and chicken serum were acquired from Gibco, Life Technologies (Paisley, UK). Dimethyl sulfoxide (DMSO), monochlorobimane (mBCl), cytosine β-D-arabinofuranoside (Ara-C), MK-801, Poly-L-lysine and thiazolyl blue tetrazolium bromide (MTT) powder were all obtained from Sigma-Aldrich (St. Louis, US). Fura-2 AM and Hoechst 33342 were purchased from Invitrogen™ Molecular Probes (Eugene, US). CGP 39551 was from Novartis (Basel, Switzerland). Perfluorooctanesulfonic acid potassium salt (PFOS ≥ 98 %) was purchased from Sigma-Aldrich (St. Louis, MO USA). All other reagents were standard laboratory grade.

The defined POP mixture was designed and prepared by our group at the Norwegian University of Life Sciences (NMBU), Oslo (Norway), detailed in [15]. It contains 29 different compounds: six perfluorinated compounds, seven brominated compounds, and 16 chlorinated compounds (Table 1). The concentrations of the chemicals in the mixture were based on Scandinavian human blood levels. The stocks used in the present study had a concentration of 10^6 times human blood levels and stocks were created using DMSO as a solvent. Working stocks were prepared by creating suitable dilutions in DMSO in glass vials at concentrations 1000 times higher than the final exposure concentrations in the medium of 500x, where x indicates 'times human blood concentrations'. Vials were stored in −80 °C. For MTT conversion and glutathione measurements cells in the culture were exposed to test compounds for 24 h and for intracellular calcium measurements for 90 min.

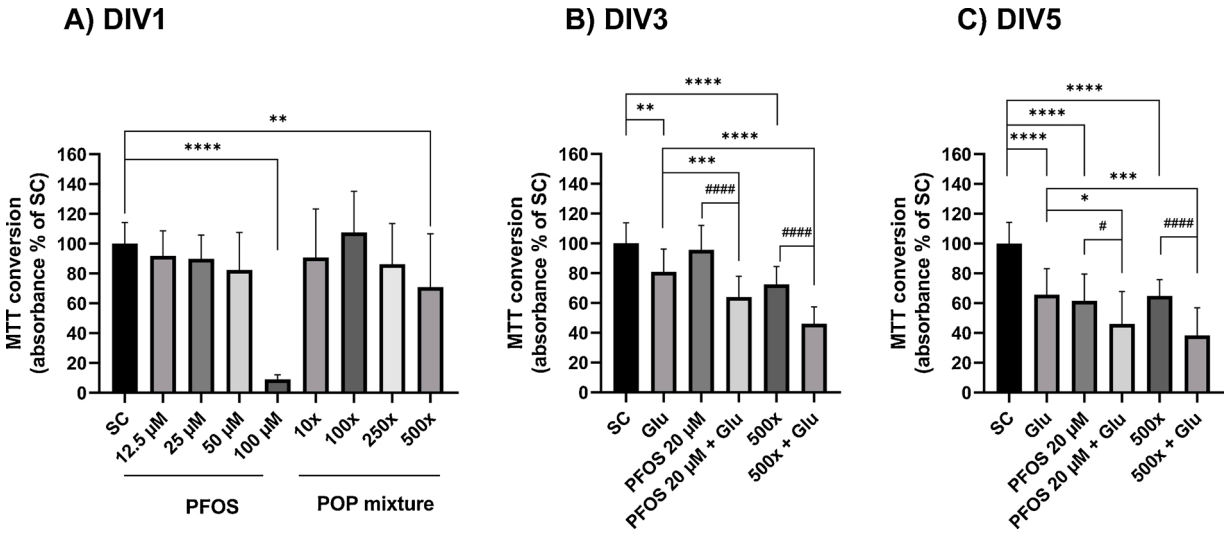

Fig. 1. Assessment of cytotoxicity in CGNs at DIV1 (A) or DIV3 (B) or DIV5 (C) using the tetrazolium MTT conversion assay. CGNs were exposed for 24 h to solvent control (SC, 0.1 % DMSO), glutamate (Glu), 100 µM (together with 10 µM glycine as co-agonist), PFOS (in µM) or POP mixture (x fold human blood levels), alone or in the presence of glutamate. All values were presented relative to the SC (defined as 100 %). Data are mean ± SD, n = 3 to 4 independent experiments (from separate cell isolates), each with replicates >4 per group/exposure, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 one-way ANOVA followed by Dunnett's multiple comparisons test, #p < 0.05, ####p < 0.0001 unpaired *t*-test.

2.2. Chicken egg incubation and isolation of cerebellar granule neurons (CGNs)

Eggs (*Gallus gallus*, weight 50−60 g) 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). In accordance with the 3R principles, the mother is excluded from the experiments and the number of fertilized eggs is easy to control. The viability of the embryos was checked with trans-illumination using a LED lamp (Brinsea) by observing spontaneous movement. Cultures of cerebellar granule neurons (CGNs) were prepared on Embryonic Day (ED) 17. The embryos *in ovo* were anaesthetized for 7 min by hypothermia in crushed ice, prior to decapitation. Animals were handled in accordance with the Norwegian Animal Welfare Act and the EU directive 2010/63/EU. The granule neurons were isolated as previously described [42]. Cells for each isolation were obtained from the pooled cerebella of 15–18 chicken embryos and diluted to a centration of 1.8×10^6 cells/mL. From each isolation, 4–6 replicates (sister wells) were prepared per exposure. Each isolation was considered as one independent experiment. In total of 3−4 independent experiments from separate cell isolates were performed. After isolation, cells were seeded onto poly-L-lysine-coated (0.01 mg/mL) dishes (diameter 35 mm) or 96 well plates in BME supplemented with chicken serum (7.5 %), KCl (25 mM), L-glutamine (2 mM), insulin (100 nM) and penicillin-streptomycin (1%) at 37 °C and 5% CO_2 for 24 h. Then the medium was replaced with serum-free BME as described previously [29] supplemented with Ara-C (10 µM) to prevent growth of non-neuronal cells. Cultures contained about 70–80 % neurons. The neurons were exposed with the test compounds on day *in vitro* (DIV) 1, 3 and 5, and then left undisturbed in an incubator until further analysis. For all the assays cells were seeded on the same day DIV0 (day of platting). MTT DIV1 experiment was performed only to find the cytotoxic concentration-range for the POP mixture and PFOS. Based on this a concentration of 500x human blood levels for POP mixture and the corresponding concentration of 20 µM PFOS was used for further mechanistic studies on DIV3 and DIV5.

2.3. Quantification of cell viability by tetrazolium MTT conversion assay

The MTT assay procedure has been described previously [18]. It is based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), a pale yellow substrate to a dark blue formazan product by the mitochondrial enzyme succinate dehydrogenase in living cells [43]. MTT was prepared freshly as a stock solution of 5 mg/mL in PBS. DIV1, 3 or 5 cultures were exposed for a further 24 h with the test compounds after which 10 µl MTT stock solution was added to each well of the 96 well plate and subsequently incubated at 37 °C for 3−4 h. Afterwards, the MTT-solution was replaced with 100 µl DMSO/well for dissolution of the formazan salt and after 30 min absorbance was measured at 570 nm in a CLARIOstar® microplate reader (BMG Labtech, Ortenberg, BW, Germany). The average of background value from wells containing cells without MTT, was subtracted from the final optical density. Cell viability was measured as a percentage absorbance of the sample compared with the absorbance of the solvent control.

2.4. Glutathione measurements using monochlorobimane (mBCl) fluorescent probe

Total reduced glutathione (GSH) levels in CGNs were measured with monochlorobimane (mBCl) as described previously [44]. mBCl is a non-fluorescent probe and forms a stable fluorescent conjugate with GSH in a reaction catalyzed by the GSH S-transferases [45]. In brief, cells were seeded in black 96 wells plates and were exposed at DIV3 or DIV5 with the test compounds for 24 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. 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 $CaCl_2$. Hundred µl of the buffer was added to each well, and mBCl fluorescence was measured at 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.

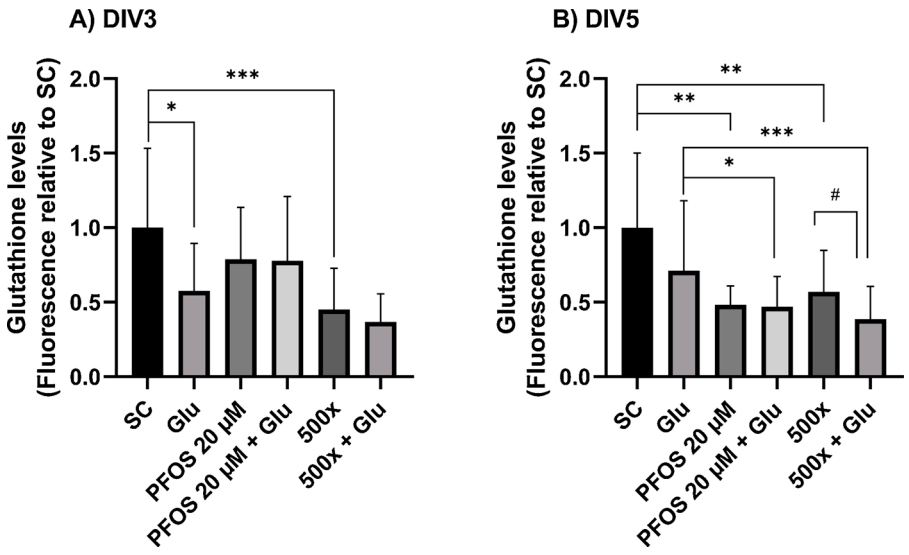

Fig. 2. Assessment of glutathione levels in CGNs at DIV3 (A) or DIV5 (B) using mBCl assay. CGNs were exposed for 24 h to solvent control (SC, 0.1 % DMSO), glutamate (Glu) 100 µM (together with 10 µM glycine as co-agonist), PFOS 20 µM, or POP mixture (500x fold human blood levels), alone or in the presence of glutamate. All values were presented relative to the SC (defined as 1). Data are mean ± SD, n = 3 to 4 experiments (from separate cell isolates), each with replicates >4 per group/exposure, *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA following Dunnett's multiple comparisons test, #p < 0.05 unpaired *t*-test.

2.5. Measurement of intracellular calcium concentrations using Fura-2 AM

The procedure has been described previously [44], with minor modifications. In brief, CGNs were seeded in CELLBIND® 96-Well Microplates, Corning® (Corning Incorporated, Kennebunk, ME, US). On DIV3 or DIV5 cells were loaded with the membrane permeable Ca^{2+}-binding fluorescent probe Fura-2 AM (5.2 µM) in medium and incubated at 37 °C for 40 min. Then the medium was replaced with experimental buffer supplemented with 1 mM $MgSO_4$ (wash buffer) for de-esterification. After 15 min of incubation baseline $[Ca^{2+}]_i$ value was measured at excitation wavelengths 340 nm and 380 nm (12 nm bandwidths) and emission at 510 nm (30 nm bandwidths) using orbital averaging. Subsequently, wash buffer was replaced with test compounds in experimental buffer and $[Ca^{2+}]_i$ value was measured 90 min post treatment. For calculation, the average $[Ca^{2+}]_i$ value obtained at 90 min after treatment was expressed relative to the average $[Ca^{2+}]_i$ value obtained prior to treatment (baseline value) at time point 0 (set to 1). Average of background autofluorescence from wells containing cells without Fura-2 AM were subtracted from excitations at 340 nm and 380 nm before the 340/380 ratio was calculated.

2.6. Statistical analysis

Results are presented as mean ± standard deviation (SD) from n = 3 to 4 independent experiments (from separate cell isolates), each with replicates >4 per group/exposure. So, the total sample size for the calculation of SD was 12–16 replicates. Data was analyzed using Microsoft Excel in addition to Graph pad 8.2 (San Diego, CA). 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 comparison test was used to determine significant differences between treatments and the corresponding 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 viability in CGNs

To explore the influence of CGNs maturation on exposure outcomes, CGNs at DIV1, DIV3, and DIV5 were used. Cell viability was evaluated by mitochondrial activity as measured by MTT (Fig. 1A-C). On DIV1, in the absence of glutamate a significant reduction in MTT was seen only at 500x by 29 % (P < 0.01) as compared to solvent control. At DIV1, PFOS only reduced MTT activity by 91 % (P < 0.0001) at 100 µM. Concentrations of PFOS in the 20 µM range did not show significant loss of MTT activity before DIV5. Therefore, a concentration of 500x was used for the POP mixture and the corresponding concentration of 20 µM PFOS was used in further studies. In the presence of glutamate, the effect of PFOS already became significant with a reduction of 33 % (P < 0.0001) at DIV3 or by 25 % (P < 0.05) at DIV5, when compared to PFOS alone; and by 21 % (P < 0.001) at DIV3 or by 30 % (P < 0.05) at DIV5 as compared to glutamate (Fig. 1B, C). For the POP mixture MTT activity was significantly reduced by 28 % (P < 0.0001) at DIV3, and by 35 % (P < 0.0001) at DIV5, when compared to solvent control (Fig. 1B, C). Again, in the presence of glutamate this reduction was more pronounced (Fig. 1B, C). In the presence of glutamate, the POP mixture led to a further significant reduction by 36 % (P < 0.0001) at DIV3 or by 41 % (P < 0.0001) at DIV5, when compared to POP mixture alone; and by 43 % (P < 0.0001) at DIV3 or by 42 % (P < 0.001) at DIV5, when compared to glutamate.

3.2. Effects of POP mixture or PFOS on the depletion of intracellular glutathione levels in CGNs

Intracellular levels of glutathione determine the sensitivity of CGNs against chemical induced cytotoxicity. Thus, we tested if the exposures resulted in depletion of the glutathione levels (Fig. 2A, B). Glutamate led to a decrease in glutathione level on both DIV3 and DIV5 by 42 % (P < 0.05 significantly) and by 29 % (P < 0.09 non-significantly), respectively as compared to solvent control. PFOS led to decrease in glutathione levels which were more pronounced on DIV5 and caused a significant reduction by 52 % (P < 0.01), when compared to solvent control. Similarly, the POP mixture resulted in decreased levels of glutathione on both days by 55 % (P < 0.001) at DIV3 and by 43 % (P < 0.01) at DIV5 as compared to solvent control. The combination with glutamate did not alter the effect compared to PFOS alone. However, when combined with the POP mixture a further reduction in intracellular glutathione levels by 18 % (non-significantly) at DIV3 or by 32 % (P < 0.05) at DIV5 as compared to POP mixture was seen; and by 36 % (non-significantly) at DIV3 or by 45 % (P < 0.001) at DV5, when compared to glutamate alone.

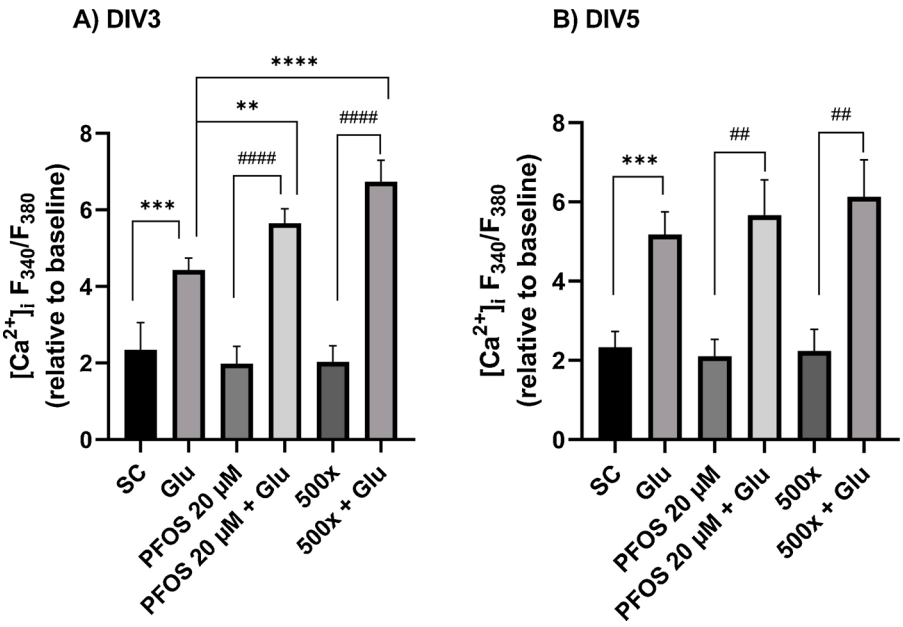

Fig. 3. Intracellular Ca^{2+} measurements in CGNs at DIV3 (A) or DIV5 (B) using Fura-2 AM assay. CGNs were exposed for 90 min to solvent control (SC, 0.1 % DMSO), glutamate (Glu) 100 µM (together with 10 µM glycine as co-agonist), PFOS 20 µM, or POP mixture (500x fold human blood levels), alone or in the presence of glutamate. Before exposure, baseline $[Ca^{2+}]_i$ was measured at time 0. The baseline was set to 1. Cells were exposed to test compounds for 90 min. Measured $[Ca^{2+}]_i$ were expressed relative to the baseline. Data are mean ± SD, n = 3 to 4 experiments (from separate cell isolates), each with replicates >4 per group/exposure, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ one-way ANOVA followed by Dunnett's multiple comparisons test, $\#\#p < 0.01$, $\#\#\#\#p < 0.0001$ unpaired *t*-test.

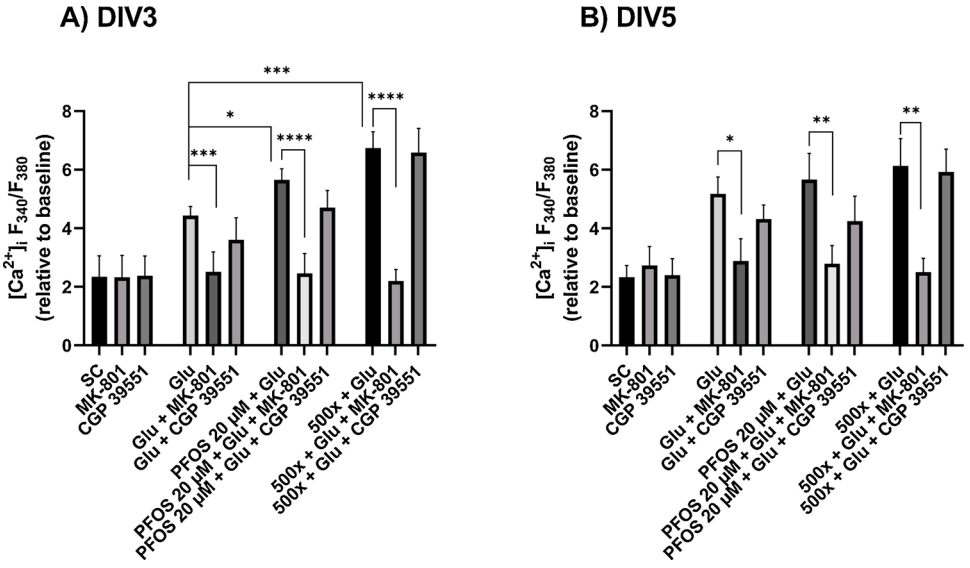

Fig. 4. Intracellular Ca^{2+} measurements in CGNs at DIV3 (A) or DIV5 (B) using Fura-2 AM assay. CGNs were exposed for 90 min to solvent control (SC, 0.1 % DMSO), glutamate (Glu) 100 µM (together with 10 µM glycine as co-agonist), PFOS 20 µM, or POP mixture (500x fold human blood levels) alone or in the presence of glutamate. Cells were treated in the presence and absence of the open NMDA-R channel blocker MK-801 (5 µM) or the competitive NMDA-R antagonist CGP 39551 (100 µM). Before exposure, baseline $[Ca^{2+}]_i$ were measured at time 0. The baseline was set to 1. Cells were exposed to test compounds for 90 min. Measured $[Ca^{2+}]_i$ were expressed relative to the baseline. Datapoints for SC, Glu, PFOS 20 µM + Glu, and 500x + Glu, are identical to Fig. 3. Data are mean ± SD, n = 3 to 4 experiments (from separate cell isolates), each with replicates >4 per group/exposure, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ one-way ANOVA followed by Dunnett's multiple comparisons test.

3.3. Effects of POP mixture or PFOS on intracellular Ca^{2+} concentration in CGNs

Since glutamate excitotoxicity raises intracellular calcium levels, we measured $[Ca^{2+}]_i$ using the Fura-2 AM fluorescent probe following exposures at DIV3 and 5 (Fig. 3A, B). As expected, glutamate alone induced a significant increase in $[Ca^{2+}]_i$ by 89 % ($P < 0.001$) at DIV3 or by 122 % ($P < 0.001$) at DIV5, when compared to solvent control. In contrast, neither PFOS, nor the POP mixture alone were able to increase $[Ca^{2+}]_i$. However, in combination with glutamate they caused a further, significant increase in $[Ca^{2+}]_i$. PFOS in combination with glutamate caused a significant increase in $[Ca^{2+}]_i$ by 185 % ($P < 0.0001$) at DIV3 or by 168 % ($P < 0.01$) at DIV5, when compared to PFOS alone; and POP mixture by 232 % ($P < 0.0001$) at DIV3 or by 174 % ($P < 0.01$) at DIV5, when compared to POP mixture alone.

3.4. Effects of POP mixture or PFOS on intracellular Ca^{2+} concentration in CGNs in combination with NMDA-R antagonists

To examine whether the increases in $[Ca^{2+}]_i$ seen in Fig. 3 could be the results of influx *via* the NMDA-R, we tested the effect of two NMDA-R antagonists, the non-competitive, open channel blocker MK-801 or the competitive antagonist CGP 39551 (Fig. 4A, B).

Neither antagonists alone affected $[Ca^{2+}]_i$ as compared to solvent control on any of the days. In the presence of MK-801, glutamate induced $[Ca^{2+}]_i$ was significantly reversed to control levels on both days ($P < 0.001$ at DIV3 and $P < 0.05$ at DIV5). The reduction by CGP 39551 was less efficient (non-significant). Interestingly, on both DIV3 and 5, MK-801 was capable of reversing the $[Ca^{2+}]_i$ to control levels for both PFOS or the POP mixture combined effect with glutamate. At either of the days, CGP 39551 also reduced $[Ca^{2+}]_i$, induced by the combined effect of PFOS and glutamate, but not statistically significant. CGP 39551 showed no inhibition effect on $[Ca^{2+}]_i$ induced by the combined effect of POP mixture and glutamate.

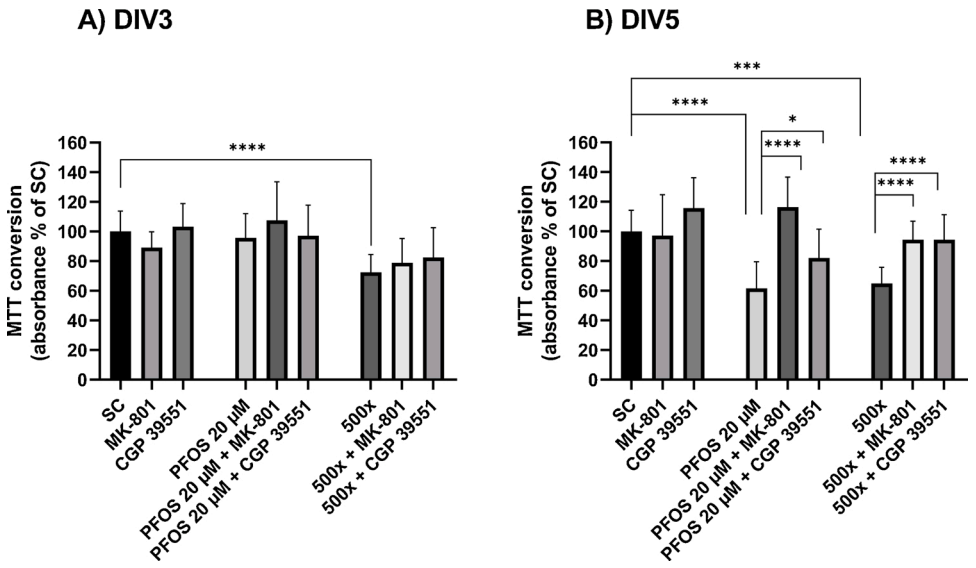

Fig. 5. Assessment of neuroprotective effects of NMDA-R antagonists in PFOS or POP mixture-induced cytotoxicity in the absence of glutamate in CGNs at DIV3 (A) or DIV5 (B) using tetrazolium MTT conversion assay. CGNs were exposed for 24 h to solvent control (SC, 0.1 % DMSO), PFOS 20 μM or POP mixture (500x fold human blood levels). Cells were treated in the presence and absence of the open NMDA-R channel blocker MK-801 (5 μM) or the competitive NMDA-R antagonist CGP 39551 (100 μM). Datapoints for SC, PFOS 20 μM, and 500x, are identical to Fig. 1B and C. All values were presented relative to the SC (defined as 100 %). Data are mean ± SD, n = 3 to 4 experiments (from separate cell isolates), each with replicates >4 per group/exposure, *p < 0.05, ***p < 0.001, ****p < 0.0001 one-way ANOVA followed by Dunnett's multiple comparisons test.

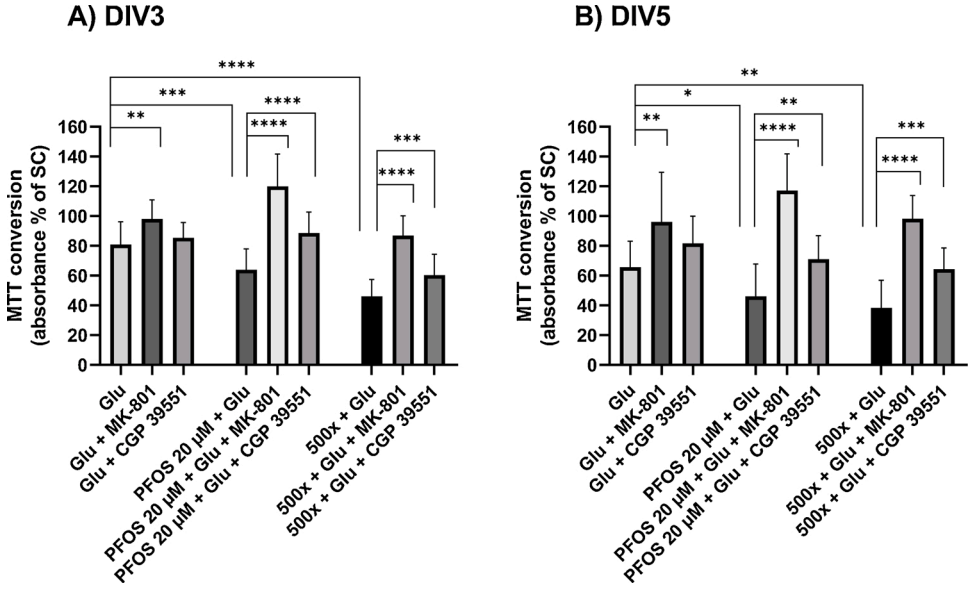

Fig. 6. Assessment of neuroprotective effects of NMDA-R antagonists in PFOS or POP mixture-induced cytotoxicity in the presence of glutamate in CGNs at DIV3 (A) or DIV5 (B) using tetrazolium MTT conversion assay. CGNs were exposed for 24 h to solvent control (SC, 0.1 % DMSO), glutamate (Glu) 100 μM (together with 10 μM glycine as co-agonist), PFOS 20 μM or POP mixture (500x fold human blood levels), alone or in the presence of glutamate. Cells were treated in the presence and absence of the open NMDA-R channel blocker MK-801 (5 μM) or the competitive NMDA-R antagonist CGP 39551 (100 μM). CGNs were exposed to glutamate. All values were presented relative to the SC (defined as 100 %). Datapoints for Glu, PFOS 20 μM + Glu, and 500x + Glu are identical to as in Fig. 1B and C. Data are mean ± SD, n = 3 to 4 experiments (from separate cell isolates), each with replicates >4 per group/exposure, *p < 0.05, **p < 0.01, ***p < 0.00, ****p < 0.0001 one-way ANOVA following Dunnett's multiple comparisons test.

3.5. Neuroprotective effects of NMDA-R antagonists on reduced MTT activity in POP mixture or PFOS-induced toxicity

We explored if the NMDA-R antagonists were also capable of rescuing cells from reduced MTT activity, alone or combined with glutamate. We observed the NMDA-R antagonists were still capable of rescuing the CGNs in situations where PFOS and the POP mixture reduced MTT activity (Fig. 1C), albeit in absence of Ca^{2+} influxes (Fig. 3). At DIV 5, MK-801 ($P < 0.0001$) proved more effective than CGP 39551 ($P < 0.05$) in rescuing PFOS-induced toxicity. But both

Table 2
The comparison between PFOS and the POP mixture exposure.

	Without Glutamate			*With Glutamate*		
A) DIV3	*PFOS 20 μM*	*POP mixture 500x*	*Difference between means ± SEM*	*PFOS 20 μM*	*POP mixture 500x*	*Difference between means ± SEM*
MTT conversion	95.61 %	72.50 %	23.11 ± 5.095 ****	63.90 %	46.07 %	17.84 ± 3.183 ****
Glutathione levels	0.7873	0.4504	−0.3369 ± 0.1151 **	0.7775	0.3676	−0.4099 ± 0.09090 ****
$[Ca^{2+}]_i$	1.984	2.030	−0.04679 ± 0.3086	5.648	6.735	−1.087 ± 0.3393 *
B) DIV5						
MTT conversion	61.48 %	64.87 %	−3.395 ± 6.093	46.11%	38.35 %	7.760 ± 5.817
Glutathione levels	0.4828	0.5692	0.08635 ± 0.08092	0.4690	0.3858	−0.08320 ± 0.05387
$[Ca^{2+}]_i$	2.105	2.236	−0.1301 ± 0.4005	5.665	6.131	−0.4660 ± 0.7437

An unpaired *t*-test was performed for the comparison between PFOS and POP mixture, in the absence of glutamate or in the presence of glutamate. *P < 0.05, **P < 0.01, and ****P < 0.0001 considered significant. MTT conversion values were presented relative to the solvent control (defined as 100 %); glutathione levels relative to the solvent control (defined as 1); and $[Ca^{2+}]_i$ relative to the baseline (before exposure, set to 1). A represents DIV3 and B DIV5.

antagonists ($P < 0.0001$) were equally effective at rescuing the POP mixture induced toxicity (Fig. 5B).

In the presence of glutamate, Ca^{2+} influxes were reduced by NMDA-R blockers (Fig. 4). In this situation we found MK-801 was very effective in rescuing cells from reduction of MTT induced by either PFOS or POP mixture (Fig. 6A, B). CGP 39551 also restored MTT activity at either day, albeit in a less efficient manner (Fig. 6A, B).

3.6. Comparison between POP mixture and PFOS at DIV 3 and 5, in absence or presence of glutamate

Table 2 summarizes the direct comparison between the effect for the POP mixture 500x with the corresponding concentration of 20 μM PFOS, at two different stages of maturation (DIV 3 and 5). We observed significant differences in MTT conversion ($P < 0.0001$ for both without and with glutamate) and glutathione levels ($P < 0.01$ without glutamate and $P < 0.0001$ with glutamate) between the exposure of POP mixture and PFOS at DIV3 either alone or in combination of glutamate. However, these differences disappeared at DIV5. Ca^{2+} influxes were at the same order of magnitude for each exposure at each day.

4. Discussion

Our work was aimed at investigating cellular mechanisms of POPs exposures, and the results showed that a POP mixture based on human blood levels from the Norwegian/Scandinavian population affects NMDA-R signaling in CGNs from chicken embryos at critical stages of cerebellar development. NMDA-R signaling is an important event in early development of cerebellum [19] and changes in the expression of this receptors have been linked to neurological disorders [46]. It is difficult to translate pure *in vitro* work into human hazard and risk assessment. Although we noticed effects at 500x blood levels, which might be considered a high concentration, it should be noted that the exposure to POPs is individualistic in nature and certain groups of people may have these levels of exposures for certain POPs similar to these concentrations (summarized in Supplementary Table 2). Our PCBs, chlordane, and PBDEs at 500x are comparable to reported levels in Inuit population [47]. DDE concentration in the 500x mixture is comparable to children in African regions with indoor residual spraying (IRS) of insecticides including DDT, which has reported levels of p, p′-DDE up to 19,772 ng/g lipid (118 ng/mL blood) and 33,605 ng/g lipid (201 ng/g blood), at 12 and 24 months of age, respectively [48]. Extremely high concentration of PFOS has been reported in Chinese factory workers [49], at levels about ten times higher than in our POP mixture at 500×. Most studies examining effects of POPs use single compounds, and often at high concentrations. However, in real life we are exposed simultaneously to a large number of chemicals, thus mixture studies at environmentally relevant concentrations are of great importance. When chemicals co-occur, they may act additively, displaying non-interaction, which is currently believed to be the most common scenario, especially at low concentrations [50,51]. Although less commonly observed, they may also display interactive synergistic (more than additive) or antagonistic (less than additive) effects. Furthermore, it should not be presumed that concentration lower than 500x can be considered safe, because of the fundamental role of the molecular target, namely NMDA-R signaling, in cerebellar development at critical stages of development. It should also be stressed that our *in vitro* exposures are of an acute short-term character, whereas real-life exposures start from conception onwards and are of a chronic nature. Measured concentrations in adult human brain tissues, thereby reflecting long-term exposure levels of PCBs or PBDEs have been reported as summarized in Supplementary Table 3. These levels were associated to neurological disorders [52,53].Together this raises concern about safe lower limits of exposure. Safe limits might potentially be lower for women during pregnancy and lactation in the work environment than for other groups. In addition, the information gained on molecular target and pathways is useful in the Adverse Outcome Pathway (AOP) framework, where it can add knowledge into existing AOPs for DNT [54] and/or form the basis of new AOPs.

PFOS belongs to the chemical class of per- and polyfluoroalkyl substances (PFAS), a family of emerging persistent pollutants of increasing concern with regards to adverse health outcomes, including cognitive outcomes [55]. The POP mixture outcomes were compared with PFOS exposure. The concentration used for PFOS exposure was set to 20 μM, equivalent to its concentration in the POP mixture 500x exposure.

We used MTT assay to quantify direct effect of the exposures on cell viability. Our observations indicated that CGNs sensitivity increases with maturation in culture. Exposure of the CGNs to the excitotoxin glutamate reduced the MTT activity significantly at DIV3, with increased sensitivity at DIV5. The increase in sensitivity at different stages of maturation could also be due to the decrease of cells. In the present study cell viability was evaluated by mitochondrial activity as measured by MTT assay. This mitochondrial integrity/activity may be interpreted as a measure of cell number, proliferation, viability, survival, or toxicity [56]. Expression of different NMDA receptors subunits at DIV3 compared to DIV5 might explain the different sensitivity of glutamate toxicity. In rat CGNs, Ceccon et al. [34] observed a similar lack of response to glutamate before DIV8, followed by increased sensitivity between DIV8 and DIV20. The POP mixture was toxic at either day, whilst PFOS only showed effect at DIV5. Recently Berntsen et al. reported that the same total POP mixture caused toxicity in rat CGNs with a significant impact on viability from 500x, and indicted an involvement of NMDA-R [38]. Similarly, Lee, Lee and Yang [57,58] showed that PFOS concentrations up to 30 μM did not cause any cytotoxicity in rat CGNs. Along the same line, Berntsen et al. [17] showed a lowest observed effect for PFOS at 20 μM in rat CGNs. It should be stressed that our PFOS concentration is comparable to reported concentrations of 26 μM in human serum levels in production workers [59]. Although the POP mixture and PFOS were capable of reducing MTT activity at DIV5, the reduction was more pronounced in presence of glutamate, suggesting an additive effect with the physiological stimulant.

It is interesting to note that the complete POP mixture had negative effects on cellular health already in early stages of CGNs maturation (starting at DIV1), whereas PFOS was most potent in later stages (DIV5). Since the concentration used for PFOS was equivalent to the one present in the total POP mixture, it is obvious PFOS alone cannot mimic the effect of the full mixture. At present, we can only hypothesize about possible mechanisms. Other PFAS compounds present in the total mixture might contribute to a more potent cytotoxic effect. Indeed, all the six PFAS compounds have been found to induce cytotoxicity after 24 h incubation in rat CGNs [17]. Moreover, the other classes of compounds, PCBs, OCPs, and BFRs might contribute to the increased potency of the total mixture. Indeed, both *in vivo* (for example in rat, mice) and *in vitro* (for example in primary CGNs and hippocampal neurons from rat and mice; PC12 and neuroblastoma cells) have shown that PCBs and BFRs could lead to disrupted calcium homeostasis, reduced glutathione levels, formation of ROS, leading to neurotoxicity [60]. Alternatively, since maturation of the CGNs is paralleled with a shift in subunit composition of the NMDA-R [36], it might be that certain compounds in the total mixture might already have an affinity for more immature stages of the receptor, compared to PFOS.

In CGNs the level of intracellular glutathione plays a major role in protecting cells against injury caused by ROS following excitotoxic insults [34]. As such, a reduction in GSH level is involved in glutamate-induced cell death [35]. Direct depletion of mitochondrial and cytoplasmic GSH could result in increased level of ROS, decrease of the mitochondrial transmembrane potential and rapid loss of mitochondrial function [61]. Our observations indicate that glutamate exposure reduced the GSH level at either day, which is in agreement with observations by Sørvik and Paulsen [44]. When exposed to POP mixture or PFOS alone, GSH levels were reduced at either day. However,

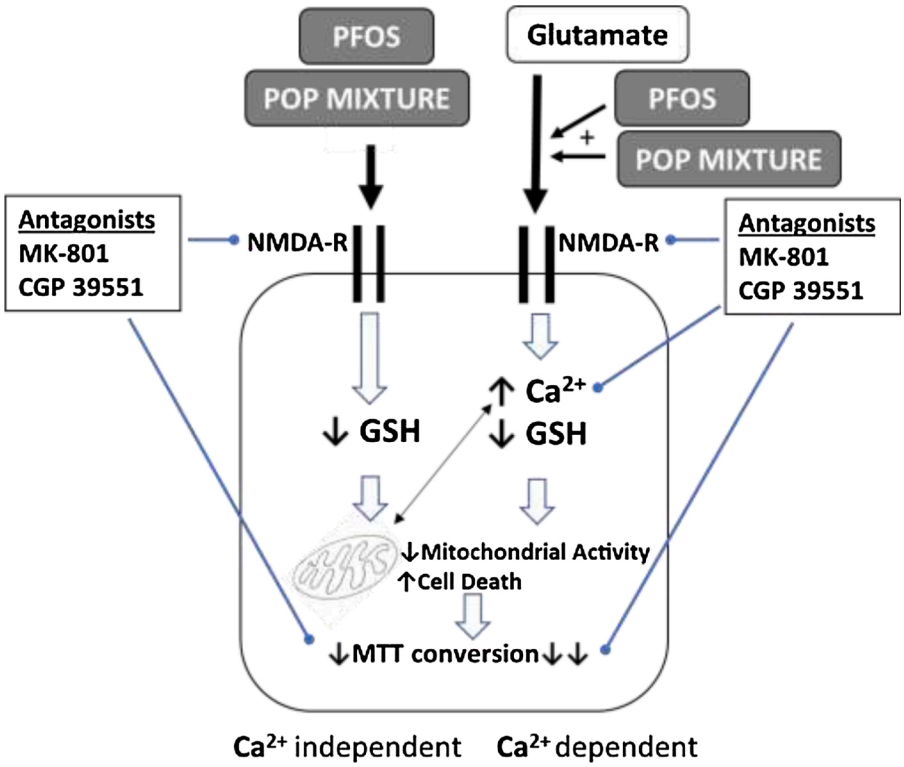

Fig. 7. Overview of the cellular mechanisms involved in CGNs toxicity induced by POP mixture or PFOS alone, in absence or presence of glutamate. Reductions (bold down-arrows) or increases (bold up-arrows) of biological effects are shown. In the absence of glutamate (left side of the figure), exposures with either POP mixture of PFOS alone, reduce intracellular glutathione (GSH) levels and induce CGNs toxicity by a Ca^{2+}-independent mechanism. In the presence of glutamate (right side of the figure), exposures with either POP mixture or PFOS alone, aggravate glutamate-induced excitotoxicity by a Ca^{2+}-dependent mechanism. Competitive (CGP 39551) and non-competitive (MK-801) NMDA-R antagonists can block the underlying mechanisms (indicated by blue lines with blunt end) and rescue CGNs cells from cytotoxicity (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

the POP mixture aggravated the effect of glutamate-induced GSH reduction, suggesting an additive effect. No such additive effect was seen for PFOS in the presence of glutamate. For the POP mixture alone, or in combination with glutamate, the reductions in GSH paralleled the reductions seen for MTT activity. This could indicate that the levels of GSH are setting the sensitivity of the CGNs to toxic insult. However, this was not the case for PFOS, suggesting it acts through a different mechanism for induction of toxicity.

NMDA-R channels are highly Ca^{2+}-permeable [36]. Their activation leads to a fast mitochondrial Ca^{2+} uptake, suggesting that Ca^{2+} influx through NMDA receptors has a privileged access to mitochondria, which could result in mitochondrial dysfunction [62,63]. In our hands the chicken CGNs responded to glutamate exposure with an increased Ca^{2+} influx, as previously described by Sørvik and Paulsen [44]. In contrast, neither the POP mixture, nor PFOS induced a Ca^{2+} influx on their own, indicating they do not reproduce the Ca^{2+} signaling induced by glutamate. The absence of a PFOS-induced Ca^{2+} influx could be due to the low concentration. Indeed, Berntsen et al. [18] did not observe Ca^{2+} influx up to the concentration of 100 μM PFOS in rat CGNs. Interestingly, our observations showed that combinations of the POP mixture or the PFOS with glutamate significantly increased the effect of glutamate on Ca^{2+} influx. This indicates that exposure to environmental pollutants can combine with existing excitotoxic processes and aggravate adverse cellular outcomes.

In a second step, we wanted to test whether known inhibitors of NMDA-R mediated Ca^{2+} influx could reverse the effects seen with combined glutamate and POP mixture or PFOS exposure. Glutamate-induced Ca^{2+} influx was reversed to control levels in presence of MK-801. The same trend was seen with CGP 39551, but non-significantly. This is in accordance with previous studies by Jacobs et al. [24,64] using MK-801. MK-801 is also very effective in reversing the Ca^{2+} influx induced by the combination of glutamate and the POP mixture or PFOS. In contrast, CGP 39551 proved far less effective in reversing this influx. It should be noted that MK-801 appeared to have very low voltage-dependency and slow unblocking kinetics to the ion channel [65], which may explain the higher protective effect of MK-801. CGP 39551 on the other hand is a competitive antagonist and acts by binding to the glutamate binding site of the receptor [41].

MK-801 also restored the reduction in MTT activity for the POP exposures in presence of glutamate. Thus, a calcium-dependent NMDA-R mediated toxicity seems at play here. Although CGP 39551 was less effective than MK-801 in blocking the Ca^{2+} influx, it also proved capable of reversing the reduction of MTT. Intriguingly, in absence of glutamate, the antagonists were also able to reverse cytotoxicity induced by either the POP mixture or PFOS. As no Ca^{2+} influx was observed under these conditions, this suggests a role for NMDA-R induced toxicity in a calcium-independent manner. Ca^{2+} flux-independent NMDA-R signaling has been reported previously. This could be due to conformational changes of NMDA-R subunits that subsequently activates intracellular signaling cascades even in the absence of Ca^{2+} influx, and p38 MAP was found to mediate this effect [66].

In general, in our hands the effects of exposures for either POP mixture or PFOS became stronger with increased age of the CGNs cultures *in vitro*. Berntsen, et al. [18] did also observe increased sensitivity of rat CGNs exposed to PFOS from DIV0 to DIV14. Also, neuroprotective effect of NMDA-R antagonists, including MK-801 was more pronounced at DIV14 as compared to DIV8. Recently, in another study Berntsen, et al. observed no toxicity in newly isolated immature rat CGNs when exposed to the same POP mixture from DIV0 to DIV8 as compared to cells exposed at DIV8 [38].This suggest that sensitivity of neurons changes with the maturation in culture. However, in our exposures there was a significant difference on DIV3 between effects of POP mixture and PFOS, whereas on DIV5 the effect of PFOS approached that of POP mixture in magnitude.

We compared the endpoints/mechanism of toxicity of most of the compounds in the mixture from other *in vitro* studies with our POP mixture at 500x (fold human blood levels). As it can be seen from Supplementary Table 1, several of the single compounds have been linked with NMDA-R, ROS and Ca^{2+} homeostasis in neuronal cell models. However, when compared to other studies *in vitro* concentration of each individual compound in the mixture is still lower than concentrations reported. Although POP mixture at 500x looks high relative to

measured blood concentrations, it should be stressed that the concentrations of the individual compounds are lower than those reported in *in vitro* studies.

Our observations indicate PFOS is the major contributor from the PFAAs group, although future experiments are needed to exclude possible additive effects amongst the members of this group. Similarly, we did not investigate the other subgroups separately or combined with PFOS. However, a recent study from our group examined the effects of same total POP mixture as well as Cl, Br, and PFAAs group alone, and their combinations on *in vitro* exposed rat CGNs. They observed that the PFAAs mixture in combination with the Br and/or Cl mixtures exerted a stronger toxic effect than the PFAA mixture alone, indicating a degree of additivity between the sub-classes [38]. Further studies with the sub-mixtures of the different classes, combinations thereof, and the single compounds would be needed to explore the underlying mechanisms.

Our study shows that exposure to POPs could interfere with NMDA glutamate receptors. Exposure to POPs have been associated with an increased prevalence of neurodevelopmental disorders including autism [1]. Changes in the expression of ionotropic glutamate receptors have been observed in autism for example, an upregulation of NMDA-R subunit protein levels has also been reported in the cerebellum of human postmortem samples [46]. In cerebellum the migration of granule neurons through layers of the cerebellar cortex is a key feature for the development of cerebellum and this neuronal migration is modulated by NMDA-R [23]. Perturbations of this process during development for example through exposure of pollutants could lead to developmental neurotoxicity and impaired brain development possibly *via* the involvement of NMDA receptors.

5. Conclusion

Primary chicken embryonal CGNs can be used as an *in vitro* model for evaluation of effects of persistent organic pollutants on developmental neurotoxicity. Our observations, schematized in Fig. 7, indicate that POPs affect different cellular and molecular endpoints in a complex fashion. The outcome depends on maturity of the CGNs. The exposures not only induce toxicity on their own, but also aggravate glutamate-induced excitotoxicity. The effect of the POP mixture cannot be explained by the action of PFOS only. With PFOS our observations in chicken embryo CGNs are similar to those previously reported in rat CGNs. Intracellular glutathione levels seem to set the levels of sensitivity to toxic insult. There seems to be two mechanisms for induction of toxicity, involving either a calcium-dependent or independent NMDA-R pathway. Importantly, antagonists of NMDA-R can inhibit the toxic effect of the exposures.

Declaration of Competing Interest

The authors declare no conflict of interest.

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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.reprotox.2021.01.008.

References

[1] P. Grandjean, P.J. Landrigan, Neurobehavioural effects of developmental toxicity, Lancet Neurol. 13 (3) (2014) 330–338.
[2] P. Grandjean, P.J. Landrigan, Developmental neurotoxicity of industrial chemicals, Lancet (London, England) 368 (9553) (2006) 2167–2178.
[3] What are POPs? UNEP, 2008 (Accessed 10 April 2020), http://chm.pops.int/TheConvention/ThePOPs/tabid/673/Default.aspx.
[4] R.J. Schmidt, K. Lyall, I. Hertz-Picciotto, Environment and Autism: Current State of the Science, Cut. Edge Psychiatry Pract. 1 (4) (2014) 21–38.
[5] P.R. Kodavanti, Neurotoxicity of persistent organic pollutants: possible mode(s) of action and further considerations, Dose. 3 (3) (2006) 273–305.
[6] B.B. Hoyer, C.H. Ramlau-Hansen, C. Obel, H.S. Pedersen, A. Hernik, V. Ogniev, B. A. Jonsson, C.H. Lindh, L. Rylander, A. Rignell-Hydbom, J.P. Bonde, G. Toft, Pregnancy serum concentrations of perfluorinated alkyl substances and offspring behaviour and motor development at age 5-9 years–a prospective study, Environ. Health 14 (2015) 2.
[7] DENAMIC Report Summary, Project ID: 282957, DENAMIC, 2012. https://cordis.europa.eu/project/id/282957/reporting.
[8] T. Svingen, A.M. Vinggaard, The risk of chemical cocktail effects and how to deal with the issue, J. Epidemiol. Community Health 70 (4) (2016) 322–323.
[9] Chemical Mixtures in Source Water and Drinking-Water, WHO, 2017. https://apps.who.int/iris/bitstream/handle/10665/255543/9789241512374-eng.pdf;jsessionid=301C7A42E1F33E57F83798D68C377636?sequence=1.
[10] Toxicity and Assessment of Chemical Mixtures, Scientific Committee on Health and Environmental Risks (SCHER), SCHER, 2012. https://ec.europa.eu/health/scientific_committees/environmental_risks/docs/scher_o_155.pdf.
[11] S.K. Bopp, R. Barouki, W. Brack, S. Dalla Costa, J.C.M. Dorne, P.E. Drakvik, M. Faust, T.K. Karjalainen, S. Kephalopoulos, J. van Klaveren, M. Kolossa-Gehring, A. Kortenkamp, E. Lebret, T. Lettieri, S. Nørager, J. Rüegg, J.V. Tarazona, X. Trier, B. van de Water, J. van Gils, Å. Bergman, Current EU research activities on combined exposure to multiple chemicals, Environ. Int. 120 (2018) 544–562.
[12] V. Lenters, N. Iszatt, J. Forns, E. Čechová, A. Kočan, J. Legler, P. Leonards, H. Stigum, M. Eggesbø, Early-life exposure to persistent organic pollutants (OCPs, PBDEs, PCBs, PFASs) and attention-deficit/hyperactivity disorder: a multi-pollutant analysis of a Norwegian birth cohort, Environ. Int. 125 (2019) 33–42.
[13] R. Flores-Ramírez, F.J. Pérez-Vázquez, M. Rodríguez-Aguilar, S.E. Medellín-Garibay, E. Van Brussel, A.C. Cubillas-Tejeda, L. Carrizales-Yáñez, F. Díaz-Barriga, Biomonitoring of persistent organic pollutants (POPs) in child populations living near contaminated sites in Mexico, Sci. Total Environ. 579 (2017) 1120–1126.
[14] D.J. González-Mille, C.A. Ilizaliturri-Hernández, G. Espinosa-Reyes, O. Cruz-Santiago, M.D.C. Cuevas-Díaz, C.C. Martín Del Campo, R. Flores-Ramírez, DNA damage in different wildlife species exposed to persistent organic pollutants (POPs) from the delta of the Coatzacoalcos river, Mexico, Ecotoxicol. Environ. Saf. 180 (2019) 403–411.
[15] H.F. Berntsen, V. Berg, C. Thomsen, E. Ropstad, K.E. Zimmer, The design of an environmentally relevant mixture of persistent organic pollutants for use in in vivo and in vitro studies, J. Toxicol. Environ. Health A (2017) 1–15.
[16] J. Forns, M.A. Verner, N. Iszatt, N. Nowack, C.C. Bach, M. Vrijheid, O. Costa, A. Andiarena, E. Sovcikova, B.B. Høyer, J. Wittsiepe, M.J. Lopez-Espinosa, J. Ibarluzea, I. Hertz-Picciotto, G. Toft, H. Stigum, M. Guxens, Z. Liew, M. Eggesbø, Early life exposure to perfluoroalkyl substances (PFAS) and ADHD: a meta-analysis of nine European population-based studies, Environ. Health Perspect. 128 (5) (2020) 57002.
[17] H.F. Berntsen, C.G. Bjorklund, J.N. Audinot, T. Hofer, S. Verhaegen, E. Lentzen, A. C. Gutleb, E. Ropstad, Time-dependent effects of perfluorinated compounds on viability in cerebellar granule neurons: dependence on carbon chain length and functional group attached, Neurotoxicology 63 (2017) 70–83.
[18] H.F. Berntsen, C.G. Bjorklund, R. Strandabo, T.M. Haug, A. Moldes-Anaya, J. Fuentes-Lazaro, S. Verhaegen, R.E. Paulsen, R.A. Tasker, E. Ropstad, PFOS-induced excitotoxicity is dependent on Ca(2+) influx via NMDA receptors in rat cerebellar granule neurons, Toxicol. Appl. Pharmacol. 357 (2018) 19–32.
[19] S. Bjornstad, L.P. Austdal, B. Roald, J.C. Glover, R.E. Paulsen, Cracking the egg: potential of the developing chicken as a model system for nonclinical safety studies of pharmaceuticals, J. Pharmacol. Exp. Ther. 355 (3) (2015) 386–396.
[20] C.J. O'Halloran, G.J. Kinsella, E. Storey, The cerebellum and neuropsychological functioning: a critical review, J. Clin. Exp. Neuropsychol. 34 (1) (2012) 35–56.
[21] M. Manto, C.I. De Zeeuw, Diversity and complexity of roles of granule cells in the cerebellar cortex. Editorial, Cerebellum (London, England) 11 (1) (2012) 1–4.
[22] F. Sultan, M. Glickstein, The cerebellum: comparative and animal studies, Cerebellum (London, England) 6 (3) (2007) 168–176.
[23] H. Komuro, P. Rakic, Modulation of neuronal migration by NMDA receptors, Science 260 (5104) (1993) 95–97.
[24] C.M. Jacobs, P. Aden, G.H. Mathisen, E. Khuong, M. Gaarder, E.M. Løberg, J. Lømo, J. Mæhlen, R.E. Paulsen, Chicken cerebellar granule neurons rapidly develop excitotoxicity in culture, J. Neurosci. Methods 156 (1) (2006) 129–135.
[25] P. Aden, I. Goverud, K. Liestol, E.M. Loberg, R.E. Paulsen, J. Maehlen, J. Lomo, Low-potency glucocorticoid hydrocortisone has similar neurotoxic effects as high-potency glucocorticoid dexamethasone on neurons in the immature chicken cerebellum, Brain Res. 1236 (2008) 39–48.
[26] P. Aden, R.E. Paulsen, J. Maehlen, E.M. Loberg, I.L. Goverud, K. Liestol, J. Lomo, Glucocorticoids dexamethasone and hydrocortisone inhibit proliferation and accelerate maturation of chicken cerebellar granule neurons, Brain Res. 1418 (2011) 32–41.
[27] L.P. Austdal, S. Bjornstad, G.H. Mathisen, P.K. Aden, I. Mikkola, R.E. Paulsen, K. E. Rakkestad, Glucocorticoid effects on cerebellar development in a chicken

embryo model: exploring changes in PAX6 and Metalloproteinase-9 after exposure to dexamethasone, J. Neuroendocrinol. 28 (12) (2016).
[28] M.F. Fjelldal, M.G. Hadera, M. Kongstorp, L.P.E. Austdal, A. Sulovic, J. M. Andersen, R.E. Paulsen, Opioid receptor-mediated changes in the NMDA receptor in developing rat and chicken, Int. J. Dev. Neurosci. 78 (2019) 19–27.
[29] G.H. Mathisen, M. Yazdani, K.E. Rakkestad, P.K. Aden, J. Bodin, M. Samuelsen, U. C. Nygaard, I.L. Goverud, M. Gaarder, E.M. Loberg, A.K. Bolling, R. Becher, R. E. Paulsen, Prenatal exposure to bisphenol A interferes with the development of cerebellar granule neurons in mice and chicken, Int. J. Dev. Neurosci. 31 (8) (2013) 762–769.
[30] A.A. Kritis, E.G. Stamoula, K.A. Paniskaki, T.D. Vavilis, Researching glutamate - induced cytotoxicity in different cell lines: a comparative/collective analysis/ study, Front. Cell. Neurosci. 9 (2015) 91.
[31] D.W. Choi, Glutamate neurotoxicity in cortical cell culture is calcium dependent, Neurosci. Lett. 58 (3) (1985) 293–297.
[32] D.W. Choi, Glutamate neurotoxicity and diseases of the nervous system, Neuron 1 (8) (1988) 623–634.
[33] A.M. Marini, S.M. Paul, N-methyl-D-aspartate receptor-mediated neuroprotection in cerebellar granule cells requires new RNA and protein synthesis, Proc. Natl. Acad. Sci. U.S.A. 89 (14) (1992) 6555–6559.
[34] M. Ceccon, P. Giusti, L. Facci, G. Borin, M. Imbesi, M. Floreani, S.D. Skaper, Intracellular glutathione levels determine cerebellar granule neuron sensitivity to excitotoxic injury by kainic acid, Brain Res. 862 (1-2) (2000) 83–89.
[35] A.B. Fallgren, G.H. Mathisen, J. Maehlen, R. Blomhoff, R.E. Paulsen, Preconditioning with estradiol abolishes its neuroprotection in cerebellar neurons, Biochem. Biophys. Res. Commun. 352 (4) (2007) 966–972.
[36] P. Paoletti, C. Bellone, Q. Zhou, NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease, Nature reviews, Neuroscience 14 (6) (2013) 383–400.
[37] M.L. Vallano, B. Lambolez, E. Audinat, J. Rossier, Neuronal activity differentially regulates NMDA receptor subunit expression in cerebellar granule cells, J. Neurosci. 16 (2) (1996) 631–639.
[38] H.F. Berntsen, N. Duale, C.G. Bjørklund, O.D. Rangel-Huerta, K. Dyrberg, T. Hofer, K.E. Rakkestad, G. Østby, R. Halsne, G. Boge, R.E. Paulsen, O. Myhre, E. Ropstad, Effects of a human-based mixture of persistent organic pollutants on the in vivo exposed cerebellum and cerebellar neuronal cultures exposed in vitro, Environ. Int. 146 (2020), 106240.
[39] K. Wedzony, K. Golembiowska, M. Zazula, Differential effects of CGP 37849 and MK-801, competitive and noncompetitive NMDA antagonists, with respect to the modulation of sensorimotor gating and dopamine outflow in the prefrontal cortex of rats, Naunyn Schmiedebergs Arch. Pharmacol. 350 (5) (1994) 555–562.
[40] E.H. Wong, J.A. Kemp, T. Priestley, A.R. Knight, G.N. Woodruff, L.L. Iversen, The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist, Proc. Natl. Acad. Sci. U.S.A. 83 (18) (1986) 7104–7108.
[41] G.E. Fagg, H.R. Olpe, M.F. Pozza, J. Baud, M. Steinmann, M. Schmutz, C. Portet, P. Baumann, K. Thedinga, H. Bittiger, et al., CGP 37849 and CGP 39551: novel and potent competitive N-methyl-D-aspartate receptor antagonists with oral activity, Br. J. Pharmacol. 99 (4) (1990) 791–797.
[42] C.M. Jacobs, P. Aden, G.H. Mathisen, E. Khuong, M. Gaarder, E.M. Loberg, J. Lomo, J. Maehlen, R.E. Paulsen, Chicken cerebellar granule neurons rapidly develop excitotoxicity in culture, J. Neurosci. Methods 156 (1-2) (2006) 129–135.
[43] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1-2) (1983) 55–63.
[44] I.B. Sørvik, R.E. Paulsen, High and low concentration of 17alpha-estradiol protect cerebellar granule neurons in different time windows, Biochem. Biophys. Res. Commun. 490 (3) (2017) 676–681.
[45] J.C. Fernandez-Checa, N. Kaplowitz, The use of monochlorobimane to determine hepatic GSH levels and synthesis, Anal. Biochem. 190 (2) (1990) 212–219.
[46] D.C. Rojas, The role of glutamate and its receptors in autism and the use of glutamate receptor antagonists in treatment, J. Neural Transm. (Vienna) 121 (8) (2014) 891–905.
[47] B.D. Laird, A.B. Goncharov, H.M. Chan, Body burden of metals and persistent organic pollutants among Inuit in the Canadian Arctic, Environ. Int. 59 (2013) 33–40.
[48] M.-A. Verner, J. Chevrier, G. Ngueta, S. Rauch, R. Bornman, B. Eskenazi, Early-life exposure to p,p'-DDT and p,p'-DDE in South African children participating in the VHEMBE study: An assessment using repeated serum measurements and pharmacokinetic modeling, Environ. Int. 119 (2018) 478–484.
[49] J. Fu, Y. Gao, L. Cui, T. Wang, Y. Liang, G. Qu, B. Yuan, Y. Wang, A. Zhang, G. Jiang, Occurrence, temporal trends, and half-lives of perfluoroalkyl acids (PFAAs) in occupational workers in China, Sci. Rep. 6 (2016) 38039.
[50] A. Kortenkamp, T. Backhaus, M. Faust, State of the Art Report on Mixture Toxicity, Final Report. Executive Summary., The School of Pharmacy University of London (ULSOP), 2009.
[51] O. Martin, M. Scholze, S. Ermler, J. McPhie, S.K. Bopp, A. Kienzler, N. Parissis, A. Kortenkamp, Ten years of research on synergisms and antagonisms in chemical mixtures: a systematic review and quantitative reappraisal of mixture studies, Environ. Int. 146 (2020), 106206.
[52] J.M. Hatcher-Martin, M. Gearing, K. Steenland, A.I. Levey, G.W. Miller, K. D. Pennell, Association between polychlorinated biphenyls and Parkinson's disease neuropathology, Neurotoxicology 33 (5) (2012) 1298–1304.
[53] M.M. Mitchell, R. Woods, L.H. Chi, R.J. Schmidt, I.N. Pessah, P.J. Kostyniak, J. M. LaSalle, Levels of select PCB and PBDE congeners in human postmortem brain reveal possible environmental involvement in 15q11-q13 duplication autism spectrum disorder, Environ. Mol. Mutagen. 53 (8) (2012) 589–598.
[54] M. Sachana, A. Rolaki, A. Bal-Price, Development of the Adverse Outcome Pathway (AOP): chronic binding of antagonist to N-methyl-d-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities of children, Toxicol. Appl. Pharmacol. 354 (2018) 153–175.
[55] M.J. Spratlen, F.P. Perera, S.A. Lederman, V.A. Rauh, M. Robinson, K. Kannan, L. Trasande, J. Herbstman, The association between prenatal exposure to perfluoroalkyl substances and childhood neurodevelopment, Environ. Pollut. 263 (Pt B)) (2020) 114444.
[56] E. Maioli, C. Torricelli, V. Fortino, F. Carlucci, V. Tommassini, A. Pacini, Critical appraisal of the MTT assay in the presence of rottlerin and uncouplers, Biol. Proced. Online 11 (2009) 227–240.
[57] H.G. Lee, Y.J. Lee, J.H. Yang, Perfluorooctane sulfonate induces apoptosis of cerebellar granule cells via a ROS-dependent protein kinase C signaling pathway, Neurotoxicology 33 (3) (2012) 314–320.
[58] Y.J. Lee, H.-G. Lee, J.-H. Yang, Perfluorooctane sulfonate-induced apoptosis of cerebellar granule cells is mediated by ERK 1/2 pathway, Chemosphere 90 (5) (2013) 1597–1602.
[59] T.A. Slotkin, E.A. MacKillop, R.L. Melnick, K.A. Thayer, F.J. Seidler, Developmental neurotoxicity of perfluorinated chemicals modeled in vitro, Environ. Health Perspect. 116 (6) (2008) 716–722.
[60] F. Fonnum, E. Mariussen, Mechanisms involved in the neurotoxic effects of environmental toxicants such as polychlorinated biphenyls and brominated flame retardants, J. Neurochem. 111 (6) (2009) 1327–1347.
[61] U. Wüllner, J. Seyfried, P. Groscurth, S. Beinroth, S. Winter, M. Gleichmann, M. Heneka, P.A. Löschmann, J.B. Schulz, M. Weller, T. Klockgether, Glutathione depletion and neuronal cell death: the role of reactive oxygen intermediates and mitochondrial function, Brain Res. 826 (1) (1999) 53–62.
[62] G. Olmos, N. DeGregorio-Rocasolano, M. Paz Regalado, T. Gasull, M. Assumpcio Boronat, R. Trullas, A. Villarroel, J. Lerma, J.A. Garcia-Sevilla, Protection by imidazol(ine) drugs and agmatine of glutamate-induced neurotoxicity in cultured cerebellar granule cells through blockade of NMDA receptor, Br. J. Pharmacol. 127 (6) (1999) 1317–1326.
[63] T.I. Peng, J.T. Greenamyre, Privileged access to mitochondria of calcium influx through N-methyl-D-aspartate receptors, Mol. Pharmacol. 53 (6) (1998) 974–980.
[64] C.M. Jacobs, M.D. Trinh, T. Rootwelt, J. Lomo, R.E. Paulsen, Dexamethasone induces cell death which may be blocked by NMDA receptor antagonists but is insensitive to Mg2+ in cerebellar granule neurons, Brain Res. 1070 (1) (2006) 116–123.
[65] T. Frankiewicz, B. Potier, Z.I. Bashir, G.L. Collingridge, C.G. Parsons, Effects of memantine and MK-801 on NMDA-induced currents in cultured neurones and on synaptic transmission and LTP in area CA1 of rat hippocampal slices, Br. J. Pharmacol. 117 (4) (1996) 689–697.
[66] P. Montes de Oca Balderas, Flux-independent NMDAR signaling: molecular mediators, cellular functions, and complexities, Int. J. Mol. Sci. 19 (12) (2018).