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Perfluoroalkyl acids potentiate glutamate excitotoxicity in rat cerebellar granule neurons

Hanne Friis Berntsen [a,b,*], Angel Moldes-Anaya [c,d], Cesilie Granum Bjørklund [a], Lorenzo Ragazzi [e], Trude Marie Haug [f], Rønnaug A.U. Strandabø [g], Steven Verhaegen [a], Ragnhild Elisabeth Paulsen [h], Erik Ropstad [a], R. Andrew Tasker [i,j]

[a] Department of Production Animal Clinical Sciences NMBU-School of Veterinary Science, P.O. Box 369 sentrum, N-0102, Oslo, Norway
[b] National Institute of Occupational Health, P.O. Box 8149 Dep N-0033, Oslo, Norway
[c] Research and Development (R&D) Section, PET Imaging Center, University Hospital of North Norway (UNN), Tromsø, Norway
[d] Nuclear Medicine and Radiation Biology Research Group, Department of Clinical Medicine, UiT The Arctic University of Norway, Tromsø, Norway
[e] Neurobiology Research Group, Department of Clinical Medicine, UiT The Arctic University of Norway, Tromsø, Norway
[f] Department of Oral Biology, University of Oslo, Oslo, Norway
[g] Department of Biosciences, University of Oslo, Oslo, Norway
[h] Department of Pharmacy, Section for Pharmacology and Pharmaceutical Biosciences, University of Oslo, Oslo, Norway
[i] Department of Biomedical Sciences, University of Prince Edward Island, Charlottetown, PEI, Canada
[j] Translational Neuropsychiatry Unit, Department of Clinical Medicine, Aarhus University, Aarhus, Denmark

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ABSTRACT

Perfluoroalkyl acids (PFAAs) are persistent man-made chemicals, ubiquitous in nature and present in human samples. Although restrictions are being introduced, they are still used in industrial processes as well as in consumer products. PFAAs cross the blood-brain-barrier and have been observed to induce adverse neurobehavioural effects in humans and animals as well as adverse effects in neuronal in vitro studies. The sulfonated PFAA perfluorooctane sulfonic acid (PFOS), has been shown to induce excitotoxicity via the N-methyl-D-aspartate receptor (NMDA-R) in cultures of rat cerebellar granule neurons (CGNs). In the present study the aim was to further characterise PFOS-induced toxicity (1–60 μM) in rat CGNs, by examining interactions between PFOS and elements of glutamatergic signalling and excitotoxicity. Effects of the carboxylated PFAA, perfluorooctanoic acid (PFOA, 300–500 μM) on the same endpoints were also examined. During experiments in immature cultures at days in vitro (DIV) 8, PFOS increased both the potency and efficacy of glutamate, whereas in mature cultures at DIV 14 only increased potency was observed. PFOA also increased potency at DIV 14. PFOS-enhanced glutamate toxicity was further antagonised by the competitive NMDA-R antagonist 3-((*R*)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) at DIV 8. At DIV 8, PFOS also induced glutamate release (9–13 fold increase vs DMSO control) after 1−3 and 24 h exposure, whereas for PFOA a large (80 fold) increase was observed, but only after 24 h. PFOS and PFOA both also increased alanine and decreased serine levels after 24 h exposure. In conclusion, our results indicate that PFOS at concentrations relevant in an occupational setting, may be inducing excitotoxicity, and potentiation of glutamate signalling, via an allosteric action on the NMDA-R or by actions on other elements regulating glutamate release or NMDA-R function. Our results further support our previous findings that PFOS and PFOA at equipotent concentrations induce toxicity via different mechanisms of action.

1. Introduction

Perfluorinated compounds such as the perfluoroalkyl acids (PFAAs) are molecules composed of a fully fluorinated carbon chain attached to a functional group. The C–F bonds of the PFAAs are extremely stable, thus making the molecule resistant to degradation, and results in the accumulation of these compounds in the environment as well as in living organisms (Buck et al., 2011). Two such compounds, that have been a

* Corresponding author at: Department of Production Animal Clinical Sciences NMBU-School of Veterinary Science, P.O. Box 8146 Dep N-0033, Oslo, Norway.
E-mail addresses: hanne.friis.berntsen@nmbu.no, Hanne.Berntsen@stami.no (H.F. Berntsen).

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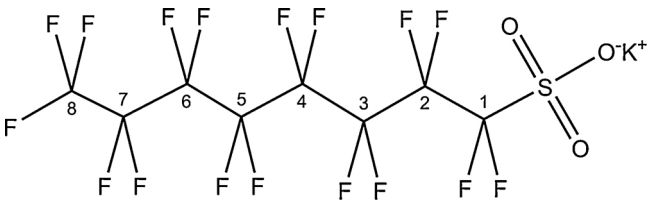
focus of attention over the two last decades, are the sulfonated perfluorooctane sulfonic acid (PFOS) and the carboxylated perfluorooctanoic acid (PFOA). Although the use of these compounds has diminished in many parts of the world over the last years due to increasing evidence of their toxic properties, and ban through the Stockholm Convention, they are still produced in several countries (UNEP, 2020). PFOS is currently still used in firefighting foam, hydraulic fluids, textiles, electric and electronic parts, and in applications like photo imaging. PFOA is used for the production of non-stick cookware and food processing equipment, and PFOA-related compounds that degrade to PFOA are used as surfactants and as surface treatment of paper and textiles as well as in paints (UNEP, 2020). Due to past and current uses, PFOS and PFOA are found at quite high levels in human blood where they bind to plasma proteins. They also cross the blood-brain barrier, and have been detected in the brain of the developing foetus (Mamsen et al., 2019).

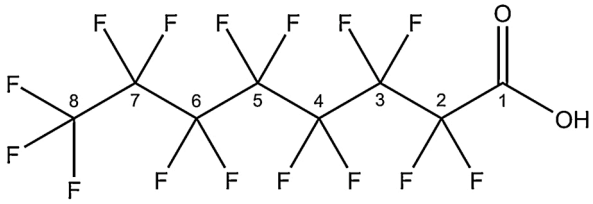
Many studies in vivo and in vitro show that PFOS and PFOA may have adverse effects on various organ systems, including the nervous system. Exposure studies of neonatal mice to PFOS and PFOA showed effects on proteins important for neuronal growth and synaptogenesis in the developing brain, and neurobehavioural effects in adults (Johansson et al., 2008, 2009). Further, daily exposure of adult mice to PFOS in another study, resulted in neurotoxic effects on the hippocampus, followed by impairment of spatial learning and memory (Long et al., 2013). Epidemiological studies have shown that prenatal exposure to PFOS and PFOA may be associated with an effect on neurodevelopment and increase hyperactivity in children (Hoyer et al., 2015), and in a recent study an association was found between early life exposure to PFOS and ADHD (Lenters et al., 2019). Prenatal exposure to PFOS was also adversely associated with gross motor development in 2-year-old children (Chen et al., 2013), as well as executive function deficits in school-age children (Vuong et al., 2016).

In previous studies of PFAAs in rat cerebellar granule neurons (CGNs) we observed that when perfluorinated molecules with the same total number of carbon atoms in the chain are attached to a sulfonate group as compared to a carboxyl group, the toxicity of the molecule was markedly increased (Berntsen et al., 2017). PFOS and PFOA both contain 8 carbon atoms in their molecular formula, but whereas PFOS is attached to a sulfonate group, PFOA contains a head carboxyl group (Fig. 1). When equipotent concentrations of PFOS and PFOA were used, PFOS induced maximum toxicity after 1 h of exposure, whereas the toxicity induced by PFOA was much slower, and maximum toxicity was reached after 24 h, the latest time-point studied (Berntsen et al., 2017). We further found that PFOS, but not PFOA caused an increase in intracellular calcium, which was completely blocked by pre-treatment with the N-methyl-D-aspartate receptor (NMDA-R) antagonist MK-801. Blocking of the NMDA-R using either competitive (CPP) or non-competitive (MK-801) NMDA-R antagonists, as well as chelation of extracellular calcium (EGTA), protected against PFOS-, but not PFOA-induced toxicity in immature cultures at days in vitro (DIV) 8. Protection against PFOS, and to some extent PFOA toxicity, was also afforded in mature cultures at DIV 14 by NMDA-R antagonists (Berntsen et al., 2018).

Effects of toxicants on the NMDA-R may be induced by direct action on the receptor itself, or by secondary processes such as release of glutamate from the cells or inhibition of glutamate re-uptake by glutamate transporters (Reistad et al., 2007). The aim of the current study was, therefore, to identify whether PFOS-induced cytotoxicity is due to direct actions on the glutamate receptor, or whether other possible mechanisms such as PFOS-induced glutamate (or other amino acids involved in neuronal function) release are involved. For consistency with our previous work PFOA was also included in these studies.



Perfluorooctanesulfonic acid potassium salt (PFOS)
$C_8F_{17}KO_3S$



Perfluorooctanoic acid (PFOA)
$C_8HF_{15}O_2$

Fig. 1. Chemical structures with molecular formula for PFOS (Mw =538.2 Da) and PFOA (Mw =414.1 Da).

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (HPLC-grade), albumin from bovine serum (BSA, ≥ 96 %), amino acid standards and internal standard norvaline for UHPLC-PDA, cytosine β-D-arabinofuranoside (ARA-C), deoxyribonuclease I from bovine pancreas (DNAse), dimethyl sulfoxide (DMSO, ≥ 99.9 %), L-glutamic acid monosodium salt hydrate (glutamate ≥ 99 %), glycine, perfluorooctanesulfonic acid potassium salt (PFOS ≥ 98 %, CAS Number: 2795−39-3), perfluorooctanoic acid (PFOA, 96 %, CAS Number: 335−67-1), poly-L-lysine hydrobromide (M_w > 70,000 g/mol), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), trypsin (type I) from bovine pancreas and trypsin inhibitor from glycine max (soybean type I–S) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Basal medium Eagle (BME), heat inactivated foetal bovine serum (FBS), Glutamax-I supplement – 200 mM, Hanks' Balanced Salt Solution (HBSS) – 10X, HEPES buffer – 1 M and penicillin (100 IU/mL)-streptomycin (100 μg/mL) were obtained from GIBCO/Invitrogen, Norway. 3-((*R*)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP, ≥ 98 %) was purchased from Tocris Biosciences (Bristol, UK). The AccQ-Tag Ultra eluent A and AccQ-Tag Ultra derivatisation kit were acquired from Waters Corporation (Milford, MA, USA). Ultrapure water was produced from a RiOs 100 Milli-Q purification system from Merck Millipore (Billerica, MA, USA).

All other chemicals and reagents used were obtained from standard commercial suppliers. Stock solutions of PFOS and PFOA were prepared by dissolution in DMSO, whereas glutamate and glycine were dissolved in Milli-Q-water. The stocks were frozen and thawed between experiments to ensure the use of the same stocks for all experiments.

2.2. Laboratory animals

For each isolation of cells, mixed-sex litters of 10 Wistar rat pups were obtained at 8 days of age from Taconic, Denmark. The pups were euthanised, by a veterinarian with FELASA C certification, without prior

use of anaesthesia, by decapitation on the day of arrival, at the Animal Laboratory Unit at the Section of Experimental Biomedicine, Norwegian University of life Sciences. The animal facility is licensed by the Norwegian Food Safety Authority (https://www.mattilsynet.no/language/english/) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (https://www.aaalac.org/). All procedures were in accordance with the Norwegian Animal Welfare Act and the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. Efforts were made to minimise animal suffering and to reduce the number of animals used.

2.3. Isolation of rat cerebellar granule neurons

Primary cultures of post-mitotic CGNs from rat pups at postnatal day (PND) 8, were prepared as described in Berntsen et al. (2013), with modifications from Mariussen et al. (2002), based on the procedures originally described in Schousboe et al. (1989) and Gallo et al. (1982). Cells from each isolation were diluted to an approximate concentration of 1–1.2 × 10^6 cells/mL in BME, supplemented with 10 % heat-inactivated FBS, 2.5 mM Glutamax, 100 IU/mL penicillin + 100 μg/ml streptomycin, 25 mM KCl and 1 % glucose, and transferred to 12 well poly-L-lysine treated (0.01 mg/mL for 2 h, 1 mL per well) cell culture plates (Nunc, 1 mL cells per well). The cytostatic drug ARA-C was added to all plates after 24 h - at DIV 1, giving a concentration of 10.3 μM in the cell medium, to prevent the replication of non-neuronal cells. That cultures consisted mainly of granule cells was confirmed during each experiment by phase contrast microscopy, but the percentage of remaining glial cells was not specifically assessed by staining in the present study. This has, however, been extensively reported by others, and primary cultures of CGNs at DIV 8 are typically found to contain around 97 % neuronal cells when the cytostatic drug ARA-C is added to the culture medium at DIV 1, as in the present study. Approximately 5 % of the cells will be GABAergic basket or Golgi neurons, whereas the number of astrocytes would be < 3% (Nicoletti et al., 1986; Perez-Gomez et al., 2004). After the addition of ARA-C, cultures to be treated on DIV 8–9 were left undisturbed in a CO_2 incubator without medium change, at 36 °C and 5 % CO_2. For CGN-cultures left until DIV 14 additional glucose (1 mg/mL) was also added to the medium on DIV 7, 9 and 12 as previously described by de Luca et al. (1996). For all chemical treatments, CGNs were exposed to toxicants in BME supplemented with 2.5 mM Glutamax, 100 IU/mL penicillin + 100 μg/ml streptomycin, 25 mM KCl and 1 % glucose, without FBS, with the exception of the measurements of extracellular concentrations of amino acids, where cells were exposed in supplemented HBSS buffer (pH 7.4) (containing 20 mM HEPES, 4.17 mM $NaHCO_3$, 5 mM glucose and 25 mM KCl). All exposures were performed in triplicate, and a mean value calculated for each concentration. Each experiment was repeated several times in separately-prepared cultures with cells from each isolation counted as one independent replicate.

2.4. Exposure of CGNs and assessment of cell viability

2.4.1. Glutamate concentration-response curves and effects of PFOS and PFOA

On DIV 8 CGNs were exposed to one of 6 concentrations of glutamate (1, 10, 20, 100, 250 or 500 μM) in conjunction with 5 μM glycine to establish a glutamate concentration-response curve. Additionally, cells were exposed to glutamate in conjunction with various concentrations of PFOS or PFOA (or to the toxicants alone) to examine their effect on glutamate-induced cytotoxicity (n = 3–6). The concentrations of PFOS (20 and 40 μM) and PFOA (300 and 450 μM) used in the initial studies were based on previous studies (Berntsen et al., 2017, 2018) and were aimed at inducing little or no toxicity on their own. Additional experiments using lower non-toxic concentrations (1 and 10 μM PFOS, and 10 μM PFOA) were thereafter conducted to establish whether observed effects were concentration dependent. To examine effects of glutamate in combination with PFOS and PFOA in cells expressing mature NMDA receptors, experiments were repeated on CGNs at DIV 14 using the same 6 concentrations of glutamate + 5 μM glycine or 1, 10, 20 and 100 μM glutamate + 5 μM glycine in conjunction with 20 μM PFOS or 300 μM PFOA. PFOS and PFOA was also tested alone (n = 4 for all). The ability of the competitive NMDA-R antagonist CPP (10 μM) to protect against glutamate + glycine toxicity alone and in conjunction with 20 μM PFOS or 300 μM PFOA was also tested in cells at DIV 8 (n = 4 for all). In our experiments the applied concentrations of PFOS were lower, whereas the concentrations of PFOA were higher, than levels reported in occupationally exposed workers by Fu et al. (2016), where serum levels up to 236 and 77 μM of the two compounds respectively, were detected.

2.4.2. The MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is based on the cleavage of the yellow tetrazolium salt MTT into the blue product formazan by the mitochondrial enzyme succinate dehydrogenase in living cells (Mosmann, 1983). In the assay, a decrease in the number of living cells results in a reduction of the amount of formazan produced compared to the control, and indicates the degree of cytotoxicity. The assay was executed as described in Berntsen et al. (2013). Dual wavelength absorbance measurements were performed at 570 and 690 nm in a VICTOR3 multilabel reader (PerkinElmer, Inc. Waltham, MA, USA).

2.5. Measurement of extracellular glutamate and other amino acids at various time-points

To measure the release of glutamate or other amino acids from the cells to the extracellular environment, CGNs were exposed in supplemented HBSS buffer to PFOS (60 μM), PFOA (500 μM) or DMSO (0.1 %) for 10 or 30 min, as well as 1, 2, 3 and 24 h. The supernatant was then removed and stored at - 20 °C until analysis with UHPLC-PDA (n = 3). The concentrations of the PFAAs were chosen based on a previous study, where time curves for toxicity were established for the same time-points using concentrations inducing equipotent effects after 24 h exposure (Berntsen et al., 2017).

2.5.1. Sample preparation and AccQ-Tag derivatisation method for amino acid analysis

Amino acid derivatisation with AccQ-tag reagents was conducted according to the manufacturer's protocol. Briefly, 10 μL of the amino acid containing solution was mixed with 70 μL of AccQ-Tag borate buffer and 20 μL of AccQ-Tag reagent solution. The reaction was allowed to proceed for 10 min at 55 °C. The amino acids included in the analysis were: serine, arginine, glycine, aspartic acid, glutamate, threonine, alanine, proline, cysteine, lysine, tyroxine, methionine, valine, isoleucine, leucine and phenylalanine. As an internal standard (IS) for quantitative determination of the analytes, a structural isomer of valine, norvaline, was chosen. Norvaline is an excellent IS for the analysis of amino acids as it has similar properties but does not interfere with the analysis. Norvaline was added at a concentration of 100 μM.

2.5.2. Amino acid analysis by UHPLC-PDA

A water Acquity UHPLC system equipped with a binary solvent manager, an autosampler and a column heater fitted with a PDA detector was used for amino acid analysis. Chromatographic separation was achieved using an Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm) heated at 43 °C. Mobile phase A and B were 10 % AccQ-Tag Ultra concentrate eluent A and 100 % of pure acetonitrile, respectively. The mobile phase flow was maintained at 0.4 mL/min during the analysis. Gradient elution was achieved as follows: 0−0.54 min 99.9 % solvent A, 5.74 min 90.0 % solvent A, 7.74 min 78.8 % solvent A, 8.04–8.64 min 40.4 % solvent A, 8.73−10 min 99.9 % solvent A. The PDA detector was set at 260 nm, with a sampling rate of 20 points/min.

The performance of the UHPLC-PDA method for the analysis was evaluated by determining important method characteristics such as reproducibility, linearity and selectivity in the analysis. Method repeatability (intra-day variability) in terms of RSD (%) was found to be < 2.6 % for all the analytes, while method reproducibility, measured over a three week period, was found to be < 3.6 % also in terms of RSD (%), for all analytes included in the analysis. Method linearity was confirmed in the range of concentrations from 25 to 200 μM, with correlation coefficients (R^2) better than 0.989 and homoscedasticity in residual analysis. The method selectivity was appropriate as no interferences were observed in the chromatograms of standard solutions made in the matrix, matrix blanks or the real samples (Supplementary Figure S1-S4). The limit of quantification (LOQ) was 25 μM for all analytes. No limit of detection (LOD) was determined as it was not necessary for the purpose of the study. In the case of the amino acids glutamate and glycine, where the chromatographic relative signal response area were under LOQ levels, relative signal response area was used as a qualitative measurement of changes in amino acid concentrations (Fig. 5 A and B).

2.6. Statistical analysis

For the studies of effects of PFOS/PFOA and CPP treatment on the glutamate concentration-response curve, as well as amino acid measurements at different time-points, significant differences were tested using a two-way ANOVA, with a Tukey-Kramer post hoc test. In all the experiments conducted, a p-value of < 0.05 was regarded as statistically significant. Best fit concentration-response curves for estimation and comparison of EC_{50} values and curves after exposure to glutamate + glycine alone or in conjunction with PFOA/PFOS were obtained by non-linear regression in PRISM 7, using a 3-parameter model with constant Hill-slope of -1. Concentrations were log transformed prior to analysis. To obtain valid confidence intervals for the EC_{50} values at DIV 14 bottom values of the model were constrained to a common value.

3. Results

3.1. Effects of PFAA exposure on the glutamate concentration-response curve

3.1.1. Glutamate concentration-response curves at DIV 8

Exposure of CGNs at DIV 8 to 1, 10, 20, 100, 250 and 500 μM glutamate in conjunction with 5 μM glycine resulted in a concentration-response curve for glutamate running from 100 % viability to between 40–60 % viability, dependent on the different experimental runs. Maximum toxicity was observed at a concentration of 100 μM glutamate, and no further increases in toxicity were observed at doses of 250 and 500 μM (Fig. 2).

Effects of co-incubation with PFOS and PFOA on the glutamate concentration-response curve were tested at DIV 8. The application of a non-toxic (20 μM) and marginally toxic (40 μM) concentration of PFOS caused a significant potentiation of glutamate-induced toxicity and shifted the glutamate concentration-response curve both down and to the left. EC_{50} values were significantly different when the values for the curves of glutamate + glycine + 20 μM PFOS (EC_{50} 2.4 μM) and glutamate + glycine + 40 μM PFOS (EC_{50} 5.2 μM) were compared to the values for the curves of glutamate + glycine controls (EC_{50} 9.3 μM) (3-parameter model, $p = 0.04$). Mean viability of cells after treatment with 20 and 40 μM PFOS only was 108 and 82 %, respectively. In a second experimental run, 1 and 10 μM PFOS were tested alone and together with glutamate + glycine, however application of these concentrations induced no significant changes to the EC_{50} values. Viability after exposure to 1 and 10 μM PFOS was 99 and 110 % of the DMSO control, respectively.

For co-exposure of CGNs to PFOA at DIV 8, concentrations of 300 and 450 μM PFOA were used. However, 450 μM PFOA reduced CGN viability to 40 %, so only the combined results after co-incubation with 300 μM PFOA were included in further analysis. PFOA (300 μM) on its own reduced viability to 78 %, but caused no significant changes to the glutamate + glycine EC_{50} values when co-applied with glutamate + glycine. When a lower concentration of PFOA was used (10 μM), this did neither have a significant effect on viability (98 % of the DMSO control), nor did it cause a significant alteration of the glutamate + glycine EC_{50} value, when co-applied.

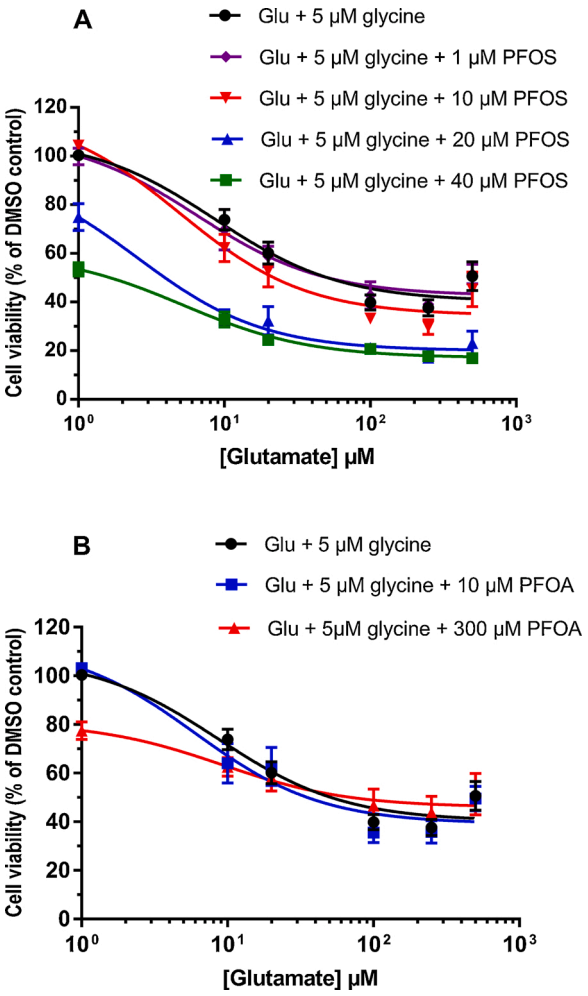


Fig. 2. Effects of PFOS and PFOA on the glutamate concentration-response curve in rat cerebellar granule neurons (CGNs) at days in vitro (DIV) 8. CGNs were exposed for 24 h to increasing concentrations of glutamate (1, 10, 20, 100, 250 or 500 μM) with 5 μM glycine alone or in conjunction with A) PFOS (1, 10, 20 or 40 μM) or B) PFOA (10 or 300 μM), before assessment of viability with the MTT assay (n = 3-10). The curve for glutamate + glycine is presented twice in A and B for illustrative purposes. Abbreviations: Glu = Glutamate.

When the curve for all experiments of glutamate + glycine was compared to the curves of glutamate + glycine in conjunction with 1, 10, 20 or 40 μM PFOS or 10 or 300 μM PFOA, the curves were found to be statistically significantly different ($p < 0.0001$). The curves for PFOS and PFOA are plotted separately in Fig. 2 A and B for illustrative purposes. EC_{50} values with 95 % CI for all the curves are presented in Supplementary Table S1.

3.1.2. Glutamate concentration-response curves at DIV 14

In cells expressing mature NMDA-receptors exposed at DIV 14, maximum toxicity after glutamate exposure was observed after treatment with 100 μM glutamate + 5 μM glycine (Fig. 3). No further increases were observed in toxicity at concentrations of 250 and 500 μM glutamate. Because of this, co-exposures of cells to glutamate in

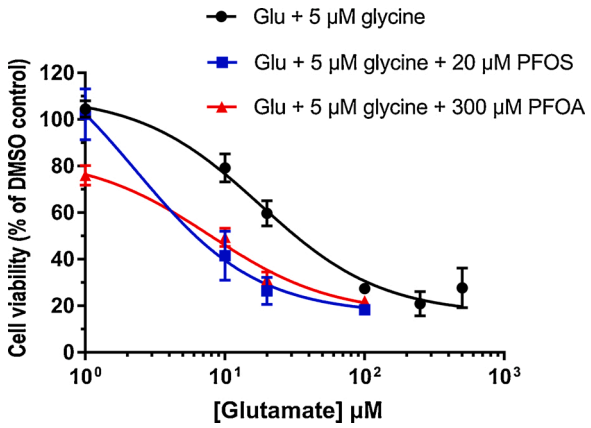


Fig. 3. Effects of PFOS and PFOA on the glutamate concentration-response curve in cerebellar granule neurons (CGNs) at days in vitro (DIV) 14. CGNs were exposed for 24 h to increasing concentrations of glutamate (1, 10, 20 or 100 μM) in conjunction with 5 μM glycine and PFOS (20 μM) or PFOA (300 μM), before assessment of viability with the MTT assay (n = 4). Glutamate and glycine (1, 10, 20, 100, 250 and 500 μM) were also tested alone in all experiments. Abbreviations: Glu = Glutamate.

conjunction with PFOS and PFOA, were only conducted at concentrations of 1, 10, 20 and 100 μM glutamate.

At DIV 14 exposure to 20 μM PFOS or 300 μM PFOA did not result in any significant toxicity (viability 116 and 94 % of the 0.1 % DMSO control, respectively). Co-incubation of CGNs to glutamate + glycine in conjunction with PFOS or PFOA, mainly resulted in a parallel left shift of the concentration-response curve (Fig. 3). In addition, PFOA also potentiated toxicity at the lowest glutamate concentration tested (1 μM) (Fig. 3). When the curves for glutamate + glycine, glutamate + glycine + 20 μM PFOS and glutamate + glycine + 300 μM PFOA were compared these were found to be significantly different ($p < 0.0001$) as were the EC_{50} values ($p < 0.01$) (Fig. 3). The EC_{50} values with 95 % CI for the curves are presented in Table 1.

3.2. Effects of CPP on the glutamate + glycine concentration-response curve

The ability of 10 μM CPP to protect against glutamate + glycine toxicity alone and in conjunction with 20 μM PFOS or 300 μM PFOA was also tested in cells at DIV 8 (Fig. 4A–F). A 2-way ANOVA with glutamate concentration and additional treatment (PFOS, PFOA, PFOS + CPP, PFOA + CPP, no additional treatment, and CPP only) as independent variables showed significant interaction between the effects of glutamate concentration and additional treatments on cell viability ($F_{25,108} = 5.104$, $p < 0.0001$). Simple main effect analysis showed that both glutamate concentration and additional treatment had significant effects on cell viability ($F_{5,108} = 16.65$, $p < 0.0001$) and ($F_{5,108} = 96.25$, $p < 0.0001$), respectively. CPP significantly antagonised glutamate/glycine at the 3 highest concentrations ($p < 0.001$ for the lower and $p < 0.01$ for the highest) and significantly reduced the toxicity of combined glutamate/glycine and PFOS from a glutamate concentration of 10 μM and above ($p < 0.001$ for all) (Fig. 4). At 10 μM and above there were also significant differences between the toxicity induced by glutamate + glycine only, and glutamate + glycine in combination with PFOS ($p < 0.05$ at 100 μM, < 0.01 at 250 μM and < 0.001 at 10, 20 and 500 μM), indicating that PFOS added to the toxicity already induced by glutamate + glycine, as previously observed. Significant antagonism by CPP was also observed at concentrations of glutamate from 100 μM and above in combination with PFOA ($p < 0.05$ at 250 and 500 μM and < 0.01 at 100 μM). There was however no difference between the toxicity induced by glutamate + glycine and glutamate + glycine + PFOA at these concentrations, indicating that PFOA did not add to the glutamate toxicity under these conditions, and that the effect of CPP was likely due to antagonism of the glutamate-induced toxicity only. Mean cell viability for PFOS and PFOA only in the same experiments was 103 and 88 % of the DMSO control, respectively.

Table 1
EC_{50} values with 95 % CI from rat CGNs for glutamate/glutamate + PFAA concentration-response curves at DIV14.

Exposure	EC_{50} (μM)	EC_{50} 95 % CI
Glutamate* + 5 μM Glycine	17.9	[10.8,30.8]
Glutamate* + 5 μM Glycine + 20 μM PFOS	2.3	[0.3,5.1]
Glutamate* + 5 μM Glycine + 300 μM PFOA	7.8	[3.1,17.1]

Abbreviations: CGN = Cerebellar granule neuron, PFAA = Perfluoroalkyl acid, DIV = Days in vitro.

* Applied concentrations of glutamate were 1, 10, 20 and 100 μM in the presence of PFAAs, and 1, 10, 20, 100, 250 and 500 in their absence.

3.3. Measurement of time-dependent release of amino acids to the extracellular space

The release of amino acids from CGNs to the extracellular environment, after exposure to PFOS or PFOA (or control solutions) was measured by UHPLC-PDA in samples of supernatant removed after 10 and 30 min, as well as 1, 2, 3 and 24 h of exposure. Of the 16 amino acids included in the analysis only alanine and serine were detected above the limit of quantification (LOQ). For glutamate (Fig. 5A) and glycine (Fig. 5B) the levels measured were below the validated LOQ and results are therefore presented as signal response area (au) relative to the area of the IS norvaline. The concentrations of alanine and serine are shown in Fig. 5C and D. Due to poor quality of one sample series, only the results from 2 experiments were used. When results were analysed statistically using 2-way ANOVA there was a significant interaction of time and exposure on glutamate concentration ($F_{15,23} = 7.003$, $p < 0.001$), and both time ($F_{5,23} = 6.64$, $p < 0.001$) and exposure ($F_{3,23} = 8.21$, $p < 0.001$) also had significant influence on glutamate release. After multiple comparisons, however, only after 24 h was a significant difference detected between PFOA and the 0.1 % DMSO control, where an 80 fold increase was observed ($p < 0.001$) (Fig. 5A). There were small increases in glutamate concentrations after PFOS exposure compared to the 0.1 % DMSO control at the earlier exposure times as well as after 24 h (2 fold increase after 10 min, 4 fold increase after 30 min, 9 fold increase after 1 and 24 h, and 13 fold increase after 2 and 3 h), however these differences were not statistically significant. For glycine there was no significant interaction detected between the effects of time and treatment on concentration (2-way ANOVA). Likewise, there was no effect of treatment. Time however, did have a significant effect on concentration. When glycine levels at 24 h were compared to all earlier time-points, there was a significant increase observed for all the four treatment groups (Fig. 5B). When serine levels were examined, a significant interaction was detected between the effect of time and treatment ($F_{15,23} = 2.76$, $p < 0.05$). Both time ($F_{5,23} = 10.89$, $p < 0.001$) and treatment ($F_{3,23} = 5.35$, $p < 0.01$) also had a significant influence on concentration. No significant differences were detected between the various exposures at the earlier time-points, however, after 24 h, both PFOS ($p < 0.05$) and PFOA ($p < 0.001$) significantly decreased serine concentrations relative to the 0.1 % DMSO control (36 μM and 23 μM versus 57 μM for PFOS and PFOA respectively). Alanine concentrations were also measured in samples taken at the various time-points. No interaction was found between the effects of time and treatment on concentration (2-way ANOVA), but both time ($F_{5,23} = 8.98$, $p < 0.001$) and treatment ($F_{3,23} = 7.28$, $p < 0.01$) did independently affect the concentration. No significant differences were found between concentrations at the earlier time-points, whereas after 24 h exposure in both the PFOS and PFOA groups, concentrations were significantly increased to 61 and 62 μM, respectively ($p < 0.001$, for both) compared to 39 μM after exposure to the 0.1 % DMSO control.

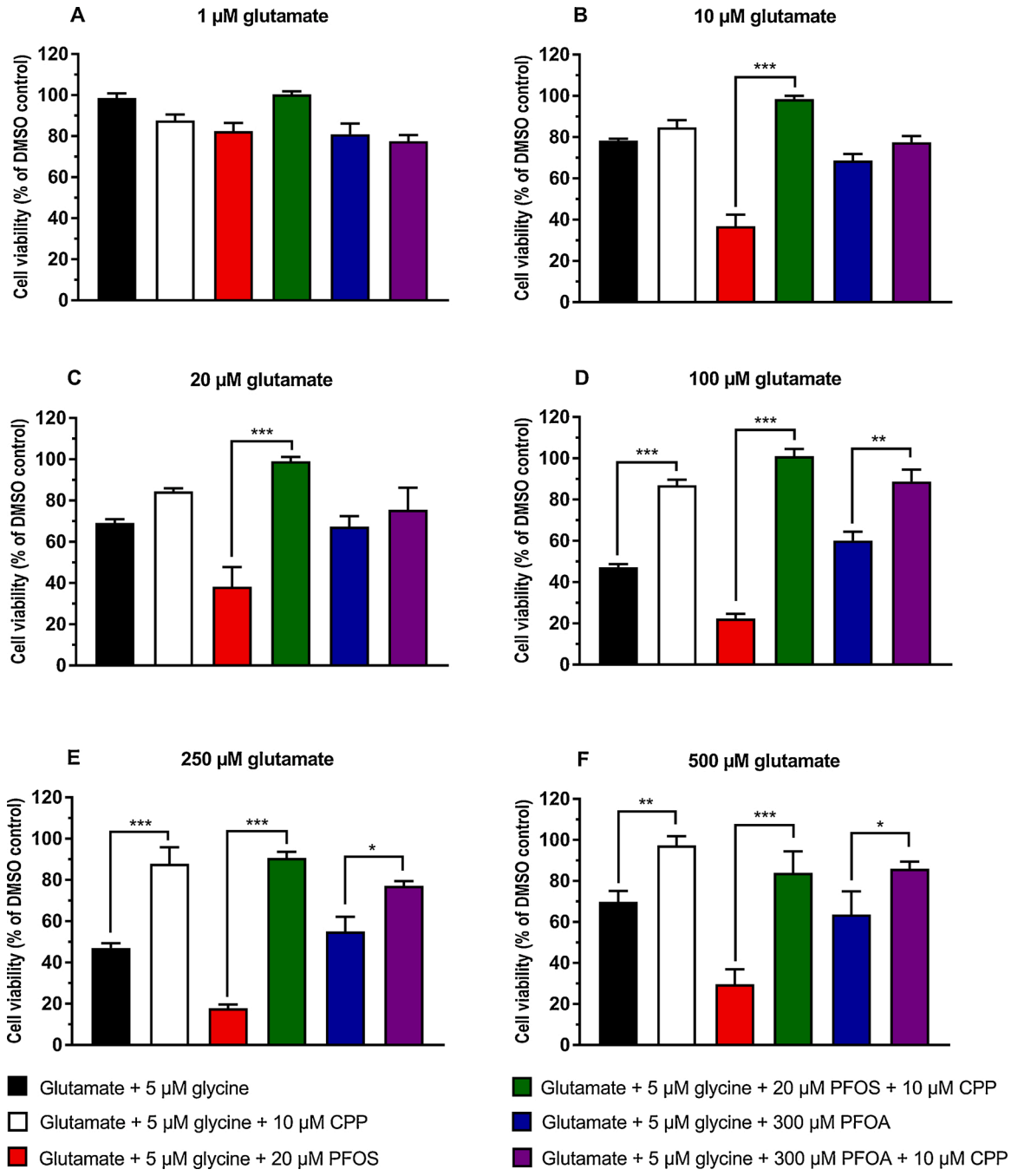


Fig. 4. Effects of CPP and PFOS/PFOA on glutamate + glycine-induced toxicity in rat cerebellar granule neurons (CGNs). CGNs were exposed at DIV 8 for 24 h to 5 µM glycine together with increasing concentrations of glutamate (1, 10, 20, 100, 250 and 500 µM) and viability assessed with the MTT assay (Figure A-F). Additionally cells were treated with glutamate and glycine in conjunction with 10 µM CPP, 20 µM PFOS, 20 µM PFOS + 10 µM CPP, 300 µM PFOA or 300 µM PFOA + 10 µM CPP (n = 4 for all). Treatment of cells with 20 µM PFOS or 300 µM PFOA only resulted in viability values at 103 and 88 % of the DMSO control (results not displayed). Significant differences between treatment combinations with and without CPP are indicated by*, ** and *** representing $p < 0.05$, $p < 0.01$ and $p < 0.001$.

4. Discussion

In the present study we observed that 24 h exposure of rat CGNs to PFOS at DIV 8 caused a concentration-dependent left and downward shift of the glutamate concentration-response curve. This enhanced toxicity of glutamate was significantly reduced by co-exposure to the competitive NMDA-R antagonist CPP. PFOA, on the other hand, had no significant effect on glutamate-induced toxicity in cells at this stage of maturation. When extracellular glutamate levels were measured after PFOS exposure, small non-significant increases were observed at all tested time-points, whereas PFOA induced a relatively large increase in extracellular glutamate, but only after 24 h. Additionally, at DIV 8, 24 h exposure of CGNs to PFOS and PFOA significantly decreased serine levels and increased alanine levels in the extracellular environment. In CGN exposed to PFOS and PFOA at DIV 14 a parallel left shift of the glutamate concentration-response curve was observed for both compounds. In our experiments the applied concentrations of PFOS were lower, whereas the concentrations of PFOA were higher, than levels reported in occupationally exposed workers.

When CGNs at DIV 8 were exposed to increasing concentrations of

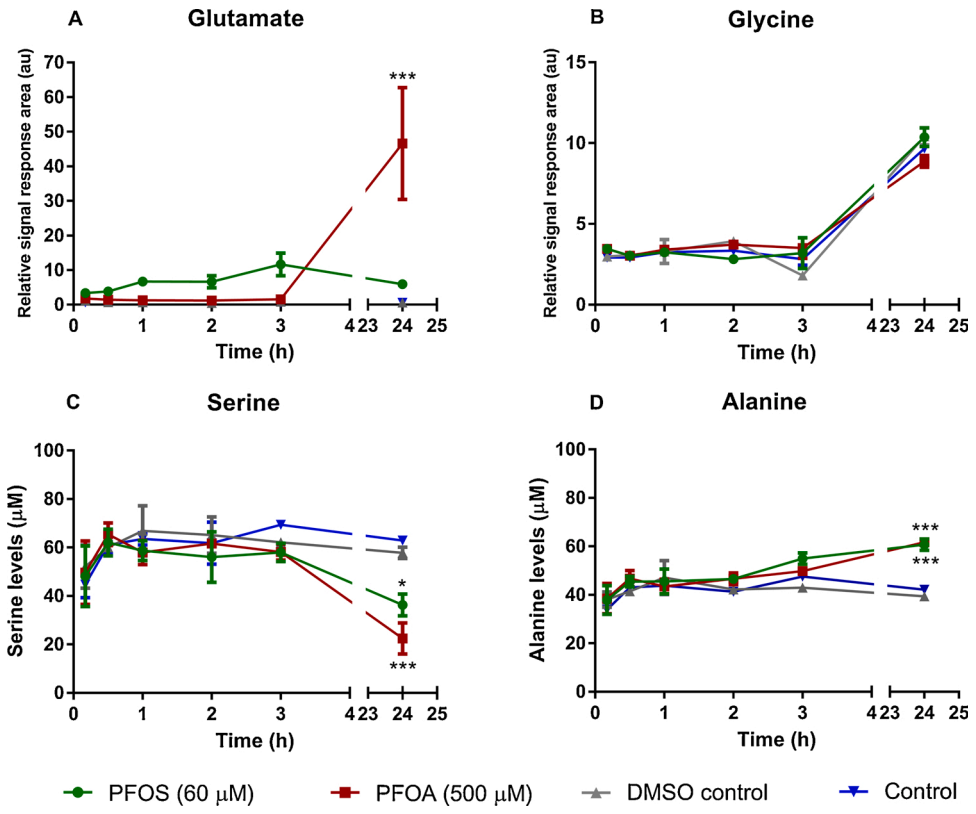


Fig. 5. Time-dependent changes in the levels of selected amino acids, released to the extracellular space after exposure to PFOS and PFOA. Levels of A) glutamate and B) glycine expressed as signal response area relative to the area of the internal standard Norvaline, as well as quantitative values of C) serine and D) alanine measured by HPLC-PDA after 30 and 60 min as well as 2, 3 and 24 h exposure to 60 μM PFOS, 500 μM PFOA, 0.1 % DMSO or HBSS buffer only (n = 2). Significant differences between the 0.1 % DMSO control and other treatment groups at a certain time-point are indicated with * and *** representing $p < 0.05$ and $p < 0.001$, respectively.

glutamate for 24 h, a concentration response curve that plateaued at 100 μM glutamate demonstrated a maximum reduction of cell viability to around 40 % of the DMSO control (Fig. 2). This is in line with previous publications (Marini and Paul, 1992; Antkiewicz-Michaluk et al., 2006). The lack of complete toxicity after glutamate exposure may be caused by the presence of some Mg^{2+} (0.81 mM $MgSO_4$) in the exposure medium, which is known to inhibit NMDA-stimulated calcium signals in a concentration-dependent manner (Marini and Novelli, 1991; Chen et al., 2005). It may also be due to subpopulations of cells in the culture that are unresponsive to glutamate. Heterogeneity amongst CGNs in culture has been reported (Milani et al., 1993). We have previously shown that PFOS induces excitotoxicity in CGNs, which likely involves influx of Ca^{2+} via the NMDA-R, and which may be blocked by specific NMDA-R antagonists (Berntsen et al., 2018). In the present study, exposure of cells to non- and marginally toxic concentrations of PFOS in addition to glutamate caused a downward and left shift of the glutamate concentration-response curve indicating enhanced efficacy and potency. This effect seemed to be concentration dependent (Fig. 2A). These results indicate that PFOS is not likely to be acting only directly on the orthosteric glutamate binding site of the NMDA-R, but that it may have other effects on glutamatergic neurons and/or other cells. One possibility could be an allosteric action on the NMDA-R at a separate site from glutamate, potentiating its effects. PFOS is known to have high affinity for proteins in general, and previous studies have shown that it tends to cluster at specific areas of the cell membrane (Gutleb et al., 2012; Berntsen et al., 2017), which could be consistent with areas dense in receptors. Binding of PFAAs to neurotransmitter receptors has been indicated by others. In a recent study Tukker et al. (2020) showed that PFOS and PFOA inhibit human $GABA_A$ receptors, with a slow restoration of the GABA-evoked current for PFOS as opposed to rapid for PFOA, indicating a higher receptor affinity for PFOS compared to PFOA. The higher PFOS affinity they assumed could be associated with the functional group. A hypothesis for the mechanism of action was that a voltage dependent pore block by PFOS and/or PFOA could be occurring through insertion of part of the molecule into the pore of the receptor. Tukker et al. (2020) also indicated that PFAAs could potentially be acting simultaneously at various kinds of neurotransmitter receptors including both glutamate and $GABA_A$ receptors. While this was not explored in the current study it is well established that glutamate and GABA contribute to the excitatory-inhibitory balance in many neural systems, so inhibition of GABA would enhance the actions of glutamate.

Another potential explanation for the observed effects of PFOS on the glutamate concentration-response curve, could be effects of PFOS on the cell membrane. PFOS has been shown to affect membrane fluidity and permeability of cells (Hu et al., 2003) and to be quickly taken up in JEG-3 cells, whereas PFOA was absorbed more slowly (Gorrocahtegui (2014); a difference that corresponds to our previous observation of a difference in the time course of toxicity between the two agents (Berntsen et al., 2017). Thus PFOS may have an effect on the cell's membrane potential. Previous studies have shown that a depolarisation of CGNs causes toxicity presumptively through the release of glutamate from endogenous stores (Schramm et al., 1990). As shown by Díaz-Trelles et al. (2000) depolarisation of CGNs in culture and glutamate release may result from pre-synaptic activation of voltage sensitive sodium channels, with the released glutamate subsequently activating NMDA-Rs and promoting the synthesis of cyclic(c)GMP (Díaz-Trelles et al., 2002) Additionally, activation of L-type voltage dependent calcium channels (L-VDCCs) has been shown necessary for the release of sufficient amounts of glutamate to promote NMDA-R-dependent cGMP formation and excitotoxicity (Díaz-Trelles et al., 2002). L-VDCCs are important for the survival of CGNs in culture, also under basal conditions, due to provision of a trophic influx of calcium and cGMP formation (Díaz-Trelles et al., 2002). PFOS-induced Ca^{2+} influx through L-VDCCs was observed by Liao et al. (2008) in primary rat hippocampal neurons, whereas the involvement of both L-VDCCs and NMDA-Rs were involved in the increase of $[Ca^{2+}]_i$ in CGNs after exposure to perfluorohexane sulfonic acid (PFHxS), a shorter chained variant of PFOS (Lee et al., 2016). In Berntsen et al. (2017) and Berntsen et al. (2018) we observed that the toxicity induced by PFOS was rapid, and mirrored the toxicity induced by glutamate in another study, where most of the

toxicity occurred within 3 h (Ankarcrona et al., 1995). In addition to resulting in release of endogenous glutamate, a potential depolarisation could have increased the cell's responsiveness to glutamate due to removal of more of the voltage dependent Mg^{2+} block. However, whether this is the case would have to be confirmed through electrophysiological studies. An increase in levels of cGMP after glutamate stimulation has previously been described in CGNs cultured in the absence of Mg^{2+} or under depolarising conditions (Novelli et al., 1987).

Another factor that should be taken into account is that PFOS and PFOA were dissolved in DMSO. Although the concentration of DMSO was not higher than 0.1 %, and by itself was not observed to induce any effects on cell viability, DMSO is known for entering cell membranes (Jacob et al., 1964a, b; de Ménorval et al., 2012), and may have facilitated access of PFOS to the intracellular space, although Makarewicz et al. (2003) reported that DMSO in concentrations 0.06–1.11 %, did not affect NMDA-induced calcium uptake in CGNs and de Ménorval et al. (2012) reported no permeabilisation of the cell membrane up to 15 Vol % DMSO.

When amino acid levels in the extracellular environment were investigated, a 9–13 fold increase in glutamate levels as compared to the 0.1 % DMSO control was observed after 1−3 h, as well as after 24 h exposure to PFOS, although the results are not statistically significant due to the small number of samples analysed (Fig. 5A). However, as the LOQ for glutamate for the applied detection method was relatively high (25 μM) it was not possible to accurately quantify these increases. It is unlikely, however, that the amount of glutamate released would exceed 25 μM. In comparison, glutamate levels in buffer from CGNs exposed for 3 h have, in a previous study, been reported to be non-detectable for DMSO, and up to 10 μM for cells exposed to another halogenated compound, tetrabromobisphenol A (TBBPA) (Reistad et al., 2007). In support of our findings, PFOS has previously been observed to increase endogenous glutamate in the hippocampus of mice orally exposed to PFOS (Long et al., 2013) and to increase extracellular glutamate in cultures of rat primary cortical astrocytes (Li et al., 2017). Although some glutamate release was detected in the present experiment, this is not likely to be the sole explanation for the enhancement of glutamate toxicity by PFOS. Even at the highest tested concentration of glutamate (500 μM) a significant toxicity potentiation was observed (Fig. 2A), and the amount of glutamate released as a result of PFOS exposure is highly unlikely to contribute to the increase in toxicity induced by 500 μM glutamate. Another interesting observation is that a maximum increase in glutamate after PFOS exposure, is not immediately detectable (Fig. 5A), and may thus also be a result of a toxic or sub-toxic excitotoxicity independent process, at least at the concentrations of PFOS applied. As synaptic glutamate release from cerebellar granule cells is known to occur in a Ca^{2+} dependent manner (Gallo et al., 1982), possible interesting future studies would be to assess whether PFOS-induced glutamate release is enhanced in the presence of a calcium ionophore, such as described by Thomas et al. (1989). In contrast to PFOS, exposure to PFOA on DIV 8 did not significantly affect the glutamate concentration-response curve (Fig. 2B), neither did it induce glutamate release, with the exception of the 24 h exposure, where a relatively large and significant 80-fold increase was observed (Fig. 5A). This is consistent with previous studies in which PFOA did not induce rapid excitotoxicity, nor was it able to acutely increase levels of Ca^{2+}, even at very high concentrations. The induced toxicity was slower than the PFOS-induced toxicity, and indicated a possible different mechanism of action from that of PFOS (Berntsen et al., 2017, 2018).

In the present study we did not measure $[Ca^{2+}]_i$. However, in Berntsen et al. (2018) an increase in $[Ca^{2+}]_i$, was observed after 30 and 60 min exposure to 60, 100 and 300 μM PFOS (only statistically significant from the control at 300 μM), which at 300 μM was completely blocked by the non-competitive NMDA-R open channel blocker MK-801. Although the lower concentrations of PFOS presently applied were not toxic on their own, there is a possibility that excitotoxicity produced by the association of a subtoxic concentration of PFOS (or PFOA) to the glutamate-induced toxicity may be due to a toxicologically significant increase in $[Ca^{2+}]_i$. As is well established, $Ca^{2+}{}_i$ may be involved both in physiological second messenger systems and in toxic processes depending on concentration. In CGNs it has been shown that after approximately 3 days in culture depolarisation-induced Ca^{2+} influx leading to the activation of Ca^{2+}/calmodulin-dependent protein kinases, is necessary for the maintenance of healthy cells, mimicking the physiological stimulation of excitatory amino acid receptors occurring in vivo by mossy fibers (Gallo et al., 1987). As primary cultures of CGNs differentiate in culture they undergo morphological, physiological and biochemical differentiation (Gallo et al., 1982; Novelli and Henneberry, 1987; Thomas et al., 1989) and acquire the capability to increase the synthesis of cGMP in response to stimulation by excitatory amino acids, which is dependent on calcium influx (Novelli and Henneberry, 1987). CGNs in culture are able to synthesis glutamate from glutamine by the aid of the enzyme glutaminase (Thomas et al., 1989). With time in culture the CGNs develop the capability of releasing glutamate in a Ca^{2+}-dependent manner after a depolarising stimulus (Gallo et al., 1982). This release of glutamate is paralleled by the induction of glutaminase as well as the formation of neurites, synaptic vesicles and synapses (Thomas et al., 1989). Further, the electrophysiological properties of CGNs have also been found to change with increasing age of the culture (Galdzicki et al., 1991). In a previous study, the toxicity of PFOS in CGNs also seemed to be dependent on the developmental stage of the culture, as exposure of cells at DIV 0 for 8 days, in contrast to exposure at DIV 8 or 14 has no effects on viability (Berntsen et al., 2018).

When CGNs were exposed to either glutamate/glycine alone or to glutamate/glycine + PFOS in conjunction with the competitive NMDA-R antagonist CPP, cell viability was significantly improved. Concentrations at or above 100 μM glutamate alone were significantly antagonised, but in combination with PFOS viability was improved at concentrations of 10 μM and above (Fig. 4). Interestingly, as may also be observed from Fig. 4, at concentrations up to 100 μM the protective effect of CPP treatment seemed to be higher in the presence of PFOS, than for cells treated with glutamate alone. This may reflect the fact that glutamate induces additional toxicity through other receptors. Another possibility is that PFOS induces a conformational change in the receptor that increases the affinity for CPP but that is speculative at this time.

As opposed to exposure at DIV 8, exposure of CGNs at DIV 14 to PFOS in conjunction with glutamate caused a leftward (i.e. increased potency), but not a downward, shift of the glutamate concentration-response curve (Fig. 3). In cells at this stage of maturation, the maximum toxicity reached by glutamate alone was also somewhat higher, which could have contributed to these differences. Cells at DIV 14 are known to express more mature NMDA-Rs with an increased number of GluN2C subunits as compared to cells at DIV 8. As receptors containing these subunits are assumed to have higher affinity for glutamate and have a reduced sensitivity to voltage dependent Mg^{2+} block (Vallano et al., 1996), this may explain the somewhat higher toxicity for glutamate observed at DIV 14. This is however only speculative at present, and would have to be confirmed by supplementary studies in Mg^{2+} free-medium. CGNs in the present study were grown under partially depolarising conditions (25 mM) necessary for the maintenance of some Ca^{2+} influx (Gallo et al., 1987), and required for the survival of cells in culture. It has been shown, that at DIV 8 depolarisation of CGNs or the absence of Mg^{2+} from the extracellular environment is necessary for the coupling of activation of NMDA-Rs by glutamate with guanylate cyclase and an increase in cGMP (Novelli et al., 1987). Further, Novelli et al. (1988) suggested that maintenance of the resting membrane potential and voltage-dependent Mg^{2+} block of the NMDA-R channel is dependent on glucose metabolism, ATP production and a functioning Na^+,K^+-ATPase. Under conditions of energy depletion, and relief of the NMDA-R Mg^{2+} block, glutamate starts acting as a neurotoxin, even at low concentrations, causing persistent activation of NMDA-Rs, opening of ion channels, and neuronal damage (Novelli et al., 1988). To compensate for energy loss CGNs are usually

cultured in the presence of glucose, and for long term cultures glucose was in the present study added repeatedly as previously described by others (Novelli et al., 1988; Schramm et al., 1990; Fernández et al., 1991; Galdzicki et al., 1991; de Luca et al., 1996). The lack of effects of PFOA exposure on the glutamate concentration-response curve at DIV 8 (Fig. 2B) were in contrast to DIV 14 where PFOA, in a similar manner to PFOS, caused a left shift of the glutamate curve (Fig. 3), which may also reflect changes in receptor subunits. Interestingly, in our previous study we found that the competitive NMDA-R antagonist CPP did provide some protection against PFOA-induced toxicity in mature cells exposed at DIV 14, as compared to cells exposed at DIV 8, where no such effect could be observed. This effect on PFOA-induced toxicity was not, however, replicated by the use of the open channel blockers MK-801or memantine (Berntsen et al., 2018).

Another interesting finding from the amino acid measurements was that serine and alanine levels were significantly different in cells exposed to PFOS and PFOA when compared to control experiments at later time points (Fig. 5C and D). During the last years it has been demonstrated that *D*-serine modulates NMDA-R function and regulates synaptic plasticity, neurodevelopment, as well as learning and memory processes (Wolosker et al., 2016) and can act as a co-agonist of NMDA-R in a similar manner to glycine (Wolosker et al., 2016). *D*-serine is synthesised in neurons by the enzyme serine racemase from *L*-serine and catabolized by *D*-amino acid oxidase (DAAO) in astrocytes. Rosenberg et al. (2013) demonstrated that *D*-serine release in the synaptic cleft occurs by the neuronal alanine-serine-cysteine transporter 1 (ASC-1), a plasma membrane antiporter that mediates tonic *D*-serine release. In line with this new paradigm, our results are showing a significant decrease of extracellular *D*-serine values in exposure bufferfrom cultures treated with PFOS and PFOA and a corresponding increase of alanine in these same cultures. This could indicate an activation of the ASC-1 transporter in CGNs and the elimination of the serine pool from the synaptic cleft. It is then expected that these neurochemical effects on serine levels, observed 24 h after exposure and coinciding with the increase in extracellular glutamate levels, may have an effect on the modulation of NMDA-R function. Confirming our findings, a newly published study has shown that PFOS-induced disruption of *D*-serine homeostasis in astrocytes affects the expression of NMDA-Rs in rat primary hippocampal neurons and leads to neurotoxicity (Wang et al., 2019).

4.1. Limitations

Although we believe our data clearly demonstrate potentiation of glutamate excitotoxicity by PFAAs the mechanism(s) by which this occurs are still unclear. Future experiments that could better identify such mechanisms include studies in Mg^{2+}-free media and the use of calcium entry blockers. Further, measures of glutaminase activity, as well as other key enzymes involved in glutamatergic signaling in CGNs exposed to PFAAs could help to understand if the metabolic state of the cultures is altered. Lastly although our quantitation of glutamate release suggested substantial increases, the result is inconclusive because the method used lacked sufficient sensitivity. Repeating these experiments using a more sensitive detection method is warranted.

5. Conclusion

In the present study we have found that PFOS induced a concentration-dependent left and downward shift of the glutamate concentration-response curve, even at a concentration of PFOS that was not by itself cytotoxic. This indicates that PFOS excitotoxicity may involve potentiation of glutamate signaling via an allosteric action on the NMDA-R or an action on other elements regulating glutamate release or otherwise affecting NMDA-R signalling. Small, non-significant increases of glutamate after 1, 2, 3 and 24 h exposure to PFOS, as opposed to a large increase in glutamate release only after 24 h exposure to PFOA, as well as differences in the response to the antagonist CPP, support the possibility that equipotent concentrations of PFOS and PFOA may be inducing cellular toxicity via different mechanisms. In mature cells at DIV 14 both PFOS and PFOA caused a left shift of the glutamate concentration-response curve without a change in efficacy. We conclude that interactions between glutamate and the perfluoroalkyl toxicants PFOS and PFOA in CGNs are complex and may vary depending on the developmental state of the cultures.

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.tox.2020.152610.

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