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# Neonatal Exposure to PFOS and PFOA in Mice Results in Changes in Proteins which are Important for Neuronal Growth and Synaptogenesis in the Developing Brain

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**Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) belong to the family of perfluorinated compounds. They are used in industrial and consumer applications, e.g., clothing fabrics, carpets, and food packaging. PFOS and PFOA are present in the environment and are found in dust and human milk, which implies that newborns and toddlers can be directly exposed to these agents during brain development. Recently, we reported that PFOS and PFOA can cause neurobehavioral defects and changes in the cholinergic system, in the adult animal, when given directly to neonatal mice, and thereby showing similarities with other investigated persistent organic pollutants, such as dichloro-diphenyl-trichloroethan, polychlorinated biphenyls, and polybrominated diphenyl ethers (PBDEs). In recent studies, we have also seen that highly brominated PBDEs can affect the levels of proteins that are important for neuronal growth and synaptogenesis in the neonatal mouse brain. The present study shows that a single oral dose of either 21 μmol PFOS or PFOA/kg body weight (11.3 or 8.70 mg), given directly to the neonatal mice on postnatal day 10, significantly increased the levels of CaMKII, GAP-43, and synaptophysin in the hippocampus of the neonatal mouse. Both compounds significantly increased the levels of synaptophysin and tau in cerebral cortex, and PFOA also increased the levels of tau in hippocampus. These proteins are important for normal brain development, and altered levels of these proteins during a critical period of the brain growth spurts could be one of the mechanisms behind earlier reported behavioral defects.**

*Key Words:* **PFCs; neurotoxicity; CaMKII; GAP-43; synaptophysin; tau.**

Perfluorinated compounds (PFCs) are persistent in the environment. Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) belong to the family of PFCs and are globally present in the environment, wildlife, and humans (Calafat *et al*., 2006; Giesy and Kannan, 2001; Kannan *et al*., 2001). The carbon-fluorine bonds are among the strongest in organic chemistry, and this stability make these compounds persistent in the environment and practically nonbiodegradable (Key *et al*., 1997, 1998). The chemical properties of PFOS and PFOA (both of which are eight-carbon chain compounds) make them suitable as surfactants, and thus, they are used in industrial and consumer applications, including uses for clothing fabrics, carpets, paper coatings, food packaging, hydraulic fluids, and fire extinguishers (OECD, 2002; Renner, 2001). PFCs were first detected in human tissue 40 years ago (Taves, 1968). Since then, PFOS and PFOA have been detected in human blood, plasma, liver, breast milk, and umbilical blood (Apelberg *et al*., 2007; Kärrman *et al*., 2007; Olsen *et al*., 2003; So *et al*., 2006). The mean levels in umbilical blood were reported to be 4.9 and 1.6 ng/ml, for PFOS and PFOA, respectively. So *et al.* (2006) reported that breast milk concentrations of PFOS and PFOA ranged from 45 to 360 and 47 to 210 ng/l, respectively.

Recent reports indicate that young people have the same or even higher PFC levels in serum or blood, compared to older generations (Kärrman *et al*., 2006; Olsen *et al*., 2004; WWF, 2005). There are studies indicating that indoor air and dust can be a major route of exposure to PFCs. Infants, toddlers, and children, especially those in the crawling stage, tend to experience higher uptake doses than teenagers and adults. The reason for this is the higher relative uptake via food consumption, hand-to-mouth transfer of the chemicals from carpets, and ingestion of dust (Trudel *et al*., 2008). The fact that PFOS and PFOA are present in dust and human milk implies direct exposure to these agents during a period of rapid brain development in newborns and toddlers.

In a previous study, we showed that neonatal exposure to PFOS and PFOA during a period of rapid brain development can cause neurotoxic effects in adult mice, manifested as changes in spontaneous behavior, habituation, and altered susceptibility of the cholinergic system (Johansson *et al*., 2008a). In other studies, we have also shown that this period of rapid brain development is vulnerable to insult by xenobiotics (bioallethrin, dichloro-diphenyl-trichloroethan [DDT], nicotine, ketamine, polybrominated diphenyl ether [PBDE] 99, and PBDE 209) and that the presence of the compound in the brain during a defined period of this maturational process is a critical factor (Ahlbom

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*et al.*, 1994; Eriksson *et al.*, 1992, 2000, 2002; Viberg *et al.*, 2003a, 2007, 2008a,b). The doses of PFOS and PFOA were also in the same order of magnitude as those that caused behavioral changes for DDT and PBDEs. This period of rapid brain growth is known as the "brain growth spurt" (BGS) (Davison and Dobbing, 1968). This period in mammalian development is associated with numerous biochemical changes that transform the feto-neonatal brain into that of the mature adult. The BGS includes axonal and dendritical outgrowth, establishment of neuronal connections, synaptogenesis, and proliferation of glia cells with accompanying myelinization (Davison and Dobbing, 1968; Kolb and Whishaw, 1989). The BGS varies in onset and duration between species. In rats and mice, the BGS occurs in the neonate, spanning the first 3–4 weeks of life and peaking around postnatal day (PND) 10. In humans, it begins during the third trimester of pregnancy and continues throughout the first 2 years of life, coinciding with the lactation period.

During the BGS, the levels of several proteins involved in neuronal survival, growth, and synaptogenesis change. Among them are calcium/calmodulin-dependent protein kinase II (CaMKII), growth-associated protein-43 (GAP-43), synaptophysin, and tau. The function of CaMKII involves regulation of synapotogenesis and synaptic plasticity (Frankland *et al.*, 2001; Rongo and Kaplan, 1999). GAP-43 plays a key role in guiding the growth of axons and modulating the information of new connections. Due to its characteristics and pattern of expression, GAP-43 is frequently used as a marker for axonal sprouting and growth (Oestreicher *et al.*, 1997). Synaptophysin is an integral membrane glycoprotein in presynaptic vesicles and is localized in all nervous tissue (Navone *et al.*, 1986; Wiedenmann and Franke, 1985). Synaptophysin has also been identified as a useful marker for synaptic density (Hamos *et al.*, 1989; Masliah *et al.*, 1990). Tau, which is a microtubule-associated protein, has been implicated in the outgrowth of neuronal processes, the development of neuronal polarity, and the maintenance of normal morphology of the neurons, as reviewed by Wang and Liu (2008), as well as in the promotion of microtubule assembly and the maintenance of stability (Vila-Ortiz *et al.*, 2001; Weingarten *et al.*, 1975). We have recently reported that the levels of these proteins increase during the BGS in the neonatal mouse, with the most pronounced increase taking place around PND 7-14 (Viberg, submitted) (Viberg *et al.*, 2008a).

We have seen in an earlier study that neonatal exposure to PFOS and PFOA can cause developmental neurotoxic effects, manifested as defect spontaneous behavior and habituation as well as dysfunction of the cholinergic system when given during the critical period of neonatal brain development in mice. These behavioral defects are similar to those seen after neonatal exposure to the highly brominated diphenyl ethers (Johansson *et al.*, 2008b; Viberg *et al.*, 2003b, 2006). Recently, we have also shown that PBDE 209 affects the levels of CaMKII and GAP-43 (Viberg *et al.*, 2008a). Therefore, the present study was undertaken to investigate whether neonatal exposure to PFOS or PFOA can affect the protein levels of CaMKII, GAP-43, synaptophysin, and tau in the neonatal mouse brain.

## MATERIALS AND METHODS

***Chemicals and animals.*** Pregnant Naval Medical Research Institute (NMRI) mice were obtained from B&K, Sollentuna, Sweden, and were housed individually in plastic cages in a room with an ambient temperature of 22°C and a normal 12/12-h cycle of light and dark. The animals were supplied with standardized pellet food (Lactamin, Stockholm, Sweden) and tap water *ad libitum*. The pregnant NMRI mice were checked for birth twice daily (0800 and 1800 h). The day of birth was designated day 0 and pups born during the night were designated day 0 when checked at 0800 h. The size of the litters was adjusted to 10–12 mice within the first 48 h after birth by euthanizing excess pups. The litters contained pups of both sexes, in about equal numbers. Only male mice were used in order to compare with our earlier developmental neurotoxicological study on PFOS and PFOA (Johansson *et al.*, 2008a) and the highly brominated diphenyl ethers, as well as with the ontogeny studies on CAMK II, GAP-43, synaptophysin, and tau (Viberg, submitted) (Viberg *et al.*, 2008a).

The experiment was carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) after approval from the local ethical committee (Uppsala University and Agricultural Research Council) and by the Swedish Committee for Ethical Experiments on Laboratory Animals.

PFOS (potassium salt, purity $\geq$ 98%) and perfluorooctanoic acid PFOA (purity = 96%) were purchased from Sigma-Aldrich (Sweden). The substances were dissolved in a mixture of egg lecithin (Merck, Darmstadt, Germany) and peanut oil (*Oleum arachidis*) (1:10) and then sonicated together with water to yield a 20% (wt/wt) fat emulsion vehicle containing 1.13 mg PFOS/ml or 0.870 mg PFOA/ml (2.1 μmol/ml, respectively). The use of a 20% fat emulsion vehicle was to give a more physiologically appropriate absorption and hence distribution (Keller and Yeary, 1980; Palin *et al.*, 1982), since fat content of mouse milk is around 14%.

At the age of 10 days, mice were exposed to a single oral dose of either 21 μmol PFOS/kg body weight (11.3 mg/kg body weight) or 21 μmol PFOA/kg body weight (8.70 mg/kg body weight) via a metal gastric tube. In the same manner, the control animals received 10 ml/kg body weight of the 20% fat emulsion vehicle. The number of litters in each treatment group was as follows: controls three litters, PFOS four litters, and PFOA four litters. Brain tissues from seven to eight animals were used in the protein analysis. The animals were sacrificed by decapitation 24 h after exposure to the vehicle, PFOS or PFOA (i.e., on PND 11), and the brains were dissected on an ice-cold glass plate. The cortex and hippocampus were collected and frozen at −80°C until assayed.

***Slot blot analysis for CaMKII, GAP-43, synaptophysin, and tau.*** Cerebral cortex and hippocampus were homogenized in a radio-immunoprecipitation assay buffer cell lysis buffer (50mM Tris HCL, pH 7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 20mM sodium pyrophosphate, 2mM sodium orthovanadate, 1% sodium deoxycholate) with the addition of 0.5% protease inhibitor cocktail (Protease Inhibitor Cocktail set III, Calbiochem, Sweden). The homogenate was then centrifuged at 14,000 × g for 15 min at 4°C, and the protein content of the supernatant was measured using the bicinchoninic acid method (Pierce). Subsequently, the supernatant was stored at −80°C until use.

An evaluation of the antibodies used was earlier conducted by Viberg *et al.* by running the GAP-43 (Chemicon AB5220), CaMKII (Chemicon MAB8699) (Viberg *et al.*, 2008a), synaptophysin (Calbiochem 573822), and tau (Santa Cruz Biotechnology 32274) (Viberg, submitted) antibodies in a Western blot procedure, which showed that the antibodies were specific for the protein intended. It was thus concluded that the antibodies were suitable for use in the slot blotting procedure. The four antibodies recognize both the nonphosphorylated and phosphorylated form of the protein.

Four micrograms of protein for CaMKII and GAP-43, 3 μg for synaptophysin, and 3.5 μg for tau were diluted to a final volume of 200 μl

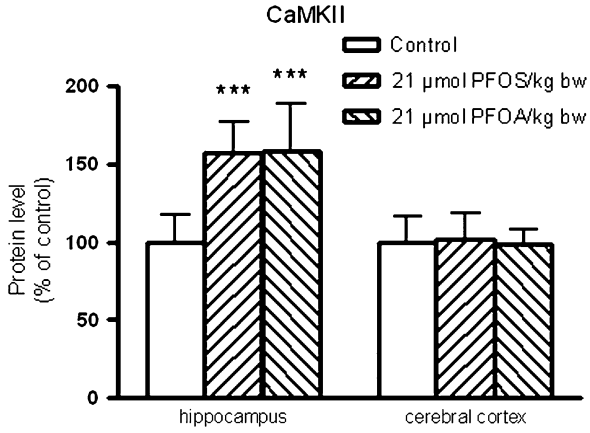


**FIG. 1.** Protein level of CaMKII in the hippocampus and cortex of animals exposed to 21 µmol PFOS/kg body weight or 21 µmol PFOA/kg body weight on PND 10 and sacrificed 24 h later. The data were subjected to one-way ANOVA and Newman-Keuls *post hoc* test. The statistical differences are indicated as (***) significantly different versus controls $p \leq 0.001$. The height of the bars represents the mean value ± SD.

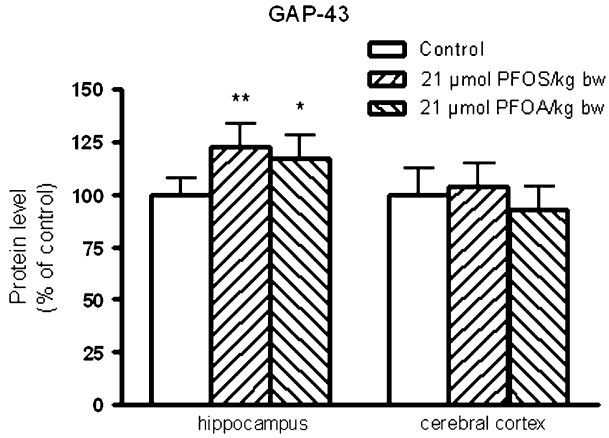


**FIG. 2.** Protein level of GAP-43 in the hippocampus and cortex of animals exposed to 21 µmol PFOS/kg body weight or 21 µmol PFOA/kg body weight on PND 10 and sacrificed 24 h later. The data were subjected to one-way ANOVA and Newman-Keuls *post hoc* test. The statistical differences are indicated as (**) significantly different versus controls $p \leq 0.01$; (*) significantly different versus controls $p \leq 0.05$. The height of the bars represents the mean value ± SD.

with sample buffer (120mM KCl, 20mM NaCl, 2mM $NaHCO_3$, 2mM$MgCl_2$, 5mM HEPES, pH 7.4, 0.05% Tween 20, 0.2% $NaN_3$) and applied in duplicates to a nitrocellulose membrane (0.45 µm, Bio-Rad, Sweden) using a Bio-Dot SF microfiltration apparatus (Bio-Rad). The membranes were fixed in 25% isopropanol and 10% acetic acid solution, washed, and blocked for 1 h at room temperature in 5% nonfat dry milk containing 0.03% Tween 20. The membranes were then incubated overnight at 4°C with a mouse monoclonal CaMKII antibody (1:5000), a rabbit polyclonal GAP-43 antibody (1:10,000), a mouse monoclonal synaptophysin antibody (1:10,000), or a mouse monoclonal tau antibody (1:1000). Immunoreactivity was detected using a horseradish peroxidase–conjugated secondary antibody against mouse (074-1806, 1:20,000) or rabbit (KPL 074-1506, 1:20,000). Immunoreactive bands were detected using an enhanced chemiluminescent substrate (Pierce, Super Signal West Dura) with imaging on a LAS-1000 (Fuji Film, Tokyo, Japan). The intensity of bands was quantified using IR-LAS 1000 Pro (Fuji Film).

***Statistical analysis.*** The body weight data from 10- and 11-day–old mice were subjected to one-way ANOVA and pairwise testing using Newman-Keuls *post hoc* test (GraphPad Prism 5.01, GraphPad Software, San Diego, CA). Differences between CaMKII, GAP-43, synaptophysin, and tau protein levels in vehicle-, PFOS-, and PFOA-treated animals were determined using one-way ANOVA. Pairwise testing between the different treatment groups was performed with Newman-Keuls *post hoc* test (GraphPad Prism 5.01).

## RESULTS

There were no overt signs of acute toxic effects in the PFOS- and PFOA-treated mice. The body weights (mean ± SD.) of the 10-day–old mice did not differ significantly ($p > 0.05$) between the vehicle-treated (6.09 ± 0.51 g), PFOS-treated (5.75 ± 0.51 g), and PFOA-treated (6.15 ± 0.31 g) mice. Nor were there any significant ($p > 0.05$) differences between the vehicle-treated (6.45 ± 0.42 g), PFOS-treated (6.20 ± 0.56 g), and PFOA-treated (6.56 ± 0.29 g) in 11-day–old mice. This shows that there were no acute effects on the body weights. The body weights for 10-day–old mice are in accordance with our earlier study on developmental toxicity study on PFOS and PFOA (Johansson *et al.*, 2008a).

### *Effects of PFOS and PFOA on CaMKII, GAP-43, Synaptophysin, and tau Protein Levels in the Hippocampus and Cerebral Cortex*

*Effects on CaMKII protein levels.* There were significant treatment effects on CaMKII levels in hippocampus [$F_{2,\ 20} = 15.64$, $p < 0.0001$], Fig. 1. CaMKII levels in the hippocampus were significantly increased ($p < 0.001$) by 57% in mice treated with PFOS and significantly increased ($p < 0.001$) by 58% in mice treated with PFOA, compared to vehicle-treated mice. No significant treatment effect [$F_{2,\ 19} = 0.0818$, $p = 0.9217$] of PFOS or PFOA treatment on CaMKII levels was seen in cerebral cortex.

*Effects on GAP-43 protein levels.* There were significant treatment effects on GAP-43 levels in the hippocampus [$F_{2,\ 14} = 7.960$, $p = 0.0049$], Fig. 2. GAP-43 levels in the hippocampus were significantly increased ($p < 0.01$) by 22% in mice treated with PFOS and significantly increased ($p < 0.05$) by 17% in mice treated with PFOA, compared to vehicle-treated mice. No significant treatment effect [$F_{2,\ 19} = 1.896$, $p = 1.776$] of PFOS or PFOA treatment on GAP-43 levels was seen in cerebral cortex.

*Effects on synaptophysin protein levels.* There were significant treatment effects on synaptophysin levels in the hippocampus [$F_{2,\ 20} = 25.30$, $p < 0.0001$] and in cerebral cortex [$F_{2,\ 20} = 12.95$, $p = 0.0002$], Fig. 3. In mice treated with PFOS, the synaptophysin levels in the hippocampus were significantly increased ($p < 0.001$) by 48% and in cerebral cortex ($p < 0.01$) by 59%, compared to vehicle-treated mice. In addition, synaptophysin levels in the hippocampus were significantly increased ($p < 0.001$) by 52% and in cerebral cortex ($p < 0.001$) by 82% in mice treated with PFOA, compared to vehicle-treated mice.

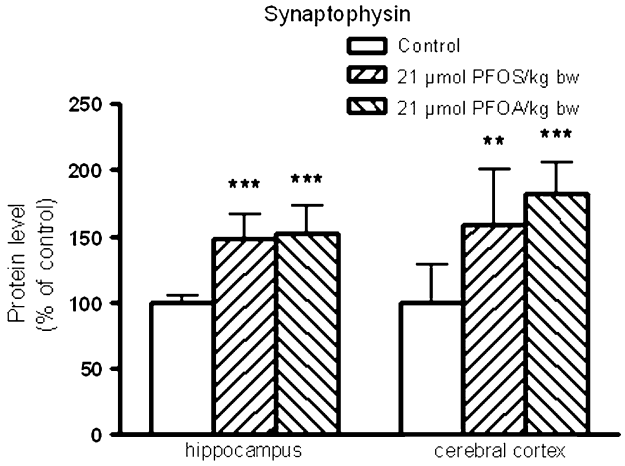


**FIG. 3.** Protein level of synaptophysin in the hippocampus and cortex of animals exposed to 21 µmol PFOS/kg body weight or 21 µmol PFOA/kg body weight on PND 10 and sacrificed 24 h later. The data were subjected to one-way ANOVA and Newman-Keuls *post hoc* test. The statistical differences are indicated as (***) significantly different versus controls $p \leq 0.001$; (**) significantly different versus controls $p \leq 0.01$. The height of the bars represents the mean value ± SD.

*Effects on tau protein levels.* There were significant treatment effects on tau levels in the hippocampus [$F_{2,\ 20} = 4.40$, $p = 0.0261$] and in cerebral cortex [$F_{2,\ 14} = 7.459$, $p = 0.0062$], Fig. 4. In mice treated with PFOS, the tau levels were not significantly different ($p > 0.05$) in the hippocampus, but significantly increased ($p < 0.05$) in cerebral cortex by 80%, compared to vehicle-treated mice. In addition, tau levels in the hippocampus were significantly increased ($p < 0.05$) by 92% and in cerebral cortex ($p < 0.01$) by 142% in mice treated with PFOA, compared to vehicle-treated mice.

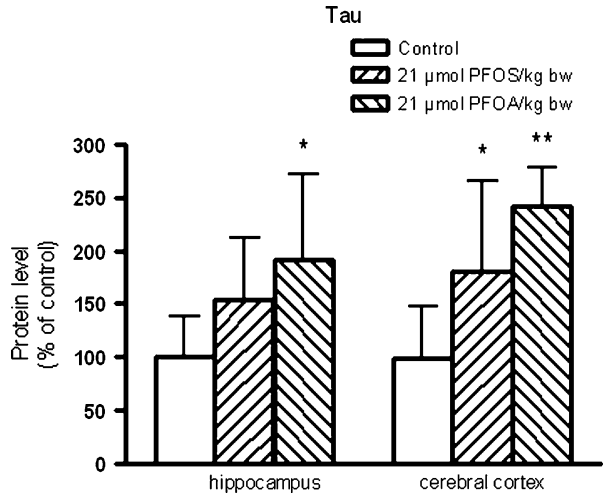


**FIG. 4.** Protein level of tau in the hippocampus and cortex of animals exposed to 21 µmol PFOS/kg body weight or 21 µmol PFOA/kg body weight on PND 10 and sacrificed 24 h later. The data were subjected to one-way ANOVA and Newman-Keuls *post hoc* test. The statistical differences are indicated as (**) significantly different versus controls $p \leq 0.01$; (*) significantly different versus controls $P \leq 0.05$. The height of the bars represents the mean value ± SD.

## DISCUSSION

We have recently reported that neonatal exposure to PFOS and PFOA during the BGS on PND 10 caused deranged spontaneous behavior, manifested as reduced and/or lack of habituation and hyperactivity, and had effects on the cholinergic system in the adult mice (Johansson *et al.*, 2008a). These effects were dose related, exacerbated with age, and occurred in the absence of any overt clinical signs of toxicity and without any change in body weight and body weight gain. The present study shows that administration of 21 µmol PFOS or 21 µmol PFOA/kg body weight to mice on PND 10 alters the amount of CaMKII, GAP-43, synaptophysin, and tau in the neonatal brain.

Neonatal exposure to PFOS and PFOA on PND 10 significantly increased the levels of CaMKII, GAP-43, and synaptophysin in the hippocampus. Increased levels of synaptophysin and tau were also seen in the cerebral cortex in mice after neonatal exposure to PFOS and PFOA. In addition, mice exposed to PFOA showed significantly increased levels of tau in the hippocampus. This indicates that the hippocampus is the most sensitive brain region investigated for neurotoxic effects of neonatal PFOS and PFOA exposure. Whether these regional differences in the effects of neonatal exposure to PFOS and PFOA could result from differences in the developmental processes in the hippocampus and cerebral cortex, or whether it is the amounts of the compounds reaching the hippocampus and cerebral cortex that are different, is not clear. During the BGS in mice, there is dramatic change in the protein levels of CaMKII, GAP-43, synaptophysin, and tau (Viberg, submitted) (Viberg *et al.*, 2008a). In the case of CaMKII, the greatest rate of increase is in the amount seen between PND 7 and PND 14 (Viberg *et al.*, 2008a). The ontogeny of CaMKII is almost the same in the two brain regions. It has also been reported, in rats, that the expression of CaMKII increased continuously during the first 4 weeks of life and that this increase was similar in the hippocampus and cerebral cortex (Kelly *et al.*, 1987; Polli *et al.*, 1990; Sugiura and Yamauchi, 1992). GAP-43, on the other hand, has a bell-shaped ontogeny, with peak levels around PND 7 in the hippocampus, whereas the peak is around PND 10 in cerebral cortex (Viberg *et al.*, 2008a). In the case of synaptophysin, the levels increase most rapidly in the hippocampus and cerebral cortex between PND 10 and PND 14 and continue to increase at least up to PND 28 (Viberg, submitted). The ontogeny of tau reaches a peak in the hippocampus and cerebral cortex at PND 3 and PND 7, respectively, and thereafter declines (Viberg, submitted). This shows that the ontogenies of the respective proteins are quite similar in the hippocampus and the cerebral cortex. Therefore, the observed increased levels of CaMKII, GAP-43, synaptophysin, and tau in the PFOS- and PFOA-treated animals do not seem to be connected to the different developmental phases of these proteins in the two different brain

regions. Regional differences have been reported after neonatal exposure to PBDE 209 and ketamine, where the protein levels of GAP-43 and CaMKII were increased in the hippocampus (Viberg *et al*., 2008a,b). Regional differences have also been seen after neonatal exposure to PBDE 209, PBDE 206, and PBDE 203, where CaMKII and synaptophysin levels were increased in the hippocampus (Viberg, submitted) (Viberg *et al*., 2008a). Furthermore, Kobayashi and coworkers found that fetal and lactational exposure to propylthiouracil in rats induced the expression of GAP-43 messenger RNA in cerebral cortex but not in the hippocampus (Kobayashi *et al*., 2005).

The increased levels of these proteins during the BGS in animals neonatally exposed to PFOS or PFOA might have consequences for normal brain development, with effects on the final architecture and function of the hippocampus and cerebral cortex. An increase in the amount of CaMKII protein, as seen in the hippocampus in mice exposed to PFOS or PFOA, can affect normal synaptogenesis as well as synaptic plasticity since CaMKII is involved in those events (Frankland *et al*., 2001; Rongo and Kaplan, 1999). Although there is limited information on the effects of developmental neurotoxicants on CaMKII, neonatal exposure to PBDE 209, PBDE 206, PBDE 203, and ketamine has been shown to increase the CaMKII protein level in the hippocampus (Viberg, submitted) (Viberg *et al*., 2008a,b). Recently, a study concluded that neonatal exposure to PBDE 47 on PND 10 affected hippocampal LTP and decreased the phosphorylated (active) form of CaMKII (Dingemans *et al*., 2007). The function of synaptophysin is not fully understood, but synaptophysin is involved in synapse formation and synaptic plasticity, and an imbalance in the synaptophysin level might affect the long-term development of the brain in ways that could result in functional impairments later in life (Janz *et al*., 1999; Tarsa and Goda, 2002). It is known that synaptophysin is activated by CaMKII phosphorylation on synaptophysin (Lynch, 2004), which may play an important role in long-term potentiation. It has been reported that depletion of GAP-43 markedly prevents neurite and growth cones formation and that overexpression on the other hand results in excessive sprouting (Aigner and Caroni 1995; Aigner *et al*., 1995). It is worth noting that these proteins are affected after neonatal exposure to PFOA, PBDE 203, 206, 209, and ketamine, at doses where we have observed functional impairments in adult mice (Fredriksson and Archer, 2004; Fredriksson *et al*., 2007; Johansson *et al*., 2008a,b; Viberg *et al*., 2003b, 2006).

In humans, the developing brain is inherently much more susceptible (Davison and Dobbing, 1968), which appears to include greater susceptibility to injury from toxic agents than the brain of an adult person (Grandjean and Landrigan, 2006). It is known that if a developmental process in the brain is halted or inhibited, there is only a slight potential for subsequent repair, and the consequences can therefore be permanent (Davison and Dobbing, 1968; Rice and Barone, 2000). In our earlier study, we observed that neonatal exposure to PFOS and PFOA led to persistent disturbances in the cholinergic system and to disturbed behavior in the adult animal (Johansson *et al*., 2008a). During the BGS, parallel with the development of CaMKII, GAP-43, synaptophysin, and tau, the ontogeny of the cholinergic system is taking place. In mice, the levels at birth of choline acetyltransferase, acetylcholinesterase, muscarinic acetylcholine receptors, and nicotinic acetylcholine receptors are about 20% of the adult amount, but adult levels are reached within 3–4 weeks (Coyle and Yamamura, 1976; Falkeborn *et al*., 1983; Fiedler *et al*., 1987; Kuhar *et al*., 1980). We have seen in earlier studies that PBDEs affect behavior in a similar way as PFOS and PFOA do. Furthermore, the cholinergic system has been affected, manifested as an altered response to the cholinergic agent (nicotine) and reduced amount of muscarinic (Viberg *et al*., 2005) and nicotinic receptors (Viberg *et al*., 2003a, 2004) in the hippocampus at adult age and that such receptors can be affected as early as during neonatal development which have been observed after exposure to certain environmental toxicants such as DDT, pyrethroids, and nicotine (Ahlbom *et al*., 1994; Eriksson, 1992; Eriksson *et al*., 2000).

Whether early changes in CaMKII, GAP-43, synaptophysin, and tau, with possible consequences on behavior (learning and memory, cognitive functions) and the cholinergic system, can affect normal development is an intriguing question. There are neurodegenerative diseases, like Alzheimer's disease (AD), that are characterized by a progressive and profound loss of cognitive functions. Cholinergic neurons are severely affected in the brains of AD patients. Consistent losses of cholinergic innervations and nicotinic receptors have been seen in brain tissue in AD and Parkinson's patients (Hellstrom-Lindahl *et al*., 1999; Nordberg, 1993; Paterson and Nordberg, 2000). In AD, the neuropathological hallmarks are senile plaques and neurofibrillary tangles. The pathogenic peptide β-amyloid$_{42}$ has been shown to induce hyperphosphorylation of tau and that this may impair the function and plasticity of the synapse. This malfunction is thought to be one of many possible abnormalities linked to AD (Muntane *et al*., 2008; Wang *et al*., 2003). Other processes that play a crucial role in AD pathology include formation of tau and neurotransmitter impairment (Mattson, 2004). With elevated levels of tau in the brain at neonatal age, as seen in the present study in mice exposed to PFOS or PFOA, and an altered response of the cholinergic system in adult mice neonatally exposed to the same compounds (Johansson *et al*., 2008a), it is possible that early exposure to certain PFCs can affect processes linked to neurodegeneration, with consequences for cognitive function.

In conclusion, the present study shows that the levels of four proteins, CaMKII, GAP-43, synaptophysin, and tau, are affected in the brain 24 h after neonatal exposure to PFOS or PFOA on PND 10 and that the hippocampus may be one of the main target regions in the brain. All four proteins are known biochemical substrates for cellular processes like neurite outgrowth and synaptogenesis, and altered levels of these proteins

may alter normal brain development. With regard to our earlier study on developmental neurotoxic effects of PFOS and PFOA (Johansson *et al.*, 2008a), the interaction between the studied proteins and the development of the cholinergic system in the hippocampus may help explain the effects on adult behavior and an altered response of the cholinergic system.

## FUNDING

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## REFERENCES

Ahlbom, J., Fredriksson, A., and Eriksson, P. (1994). Neonatal exposure to a type-I pyrethroid (bioallethrin) induces dose-response changes in brain muscarinic receptors and behaviour in neonatal and adult mice. *Brain Res.* **645,** 318–324.

Aigner, L., Arber, S., Kapfhammer, J. P., Laux, T., Schneider, C., Botteri, F., Brenner, H. R., and Caroni, P. (1995). Overexpression of the neural growth-associated protein GAP-43 induces nerve sprouting in the adult nervous system of transgenic mice. *Cell* **83,** 269–278.

Aigner, L., and Caroni, P. (1995). Absence of persistent spreading, branching, and adhesion in GAP-43-depleted growth cones. *J. Cell Biol.* **128,** 647–660.

Apelberg, B. J., Goldman, L. R., Calafat, A. M., Herbstman, J. B., Kuklenyik, Z., Heidler, J., Needham, L. L., Halden, R. U., and Witter, F. R. (2007). Determinants of fetal exposure to polyfluoroalkyl compounds in Baltimore, Maryland. *Environ. Sci. Technol.* **41,** 3891–3897.

Calafat, A. M., Kuklenyik, Z., Caudill, S. P., Reidy, J. A., and Needham, L. L. (2006). Perfluorochemicals in pooled serum samples from United States residents in 2001 and 2002. *Environ. Sci. Technol.* **40,** 2128–2134.

Coyle, J. T., and Yamamura, H. I. (1976). Neurochemical aspects of the ontogenesis of cholinergic neurons in the rat brain. *Brain Res.* **118,** 429–440.

Davison, A. N., and Dobbing, J. (1968). *Applied Neurochemistry*. Blackwell, Oxford.

Dingemans, M. M., Ramakers, G. M., Gardoni, F., van Kleef, R. G., Bergman, A., Di Luca, M., van den Berg, M., Westerink, R. H., and Vijverberg, H. P. (2007). Neonatal exposure to brominated flame retardant BDE-47 reduces long-term potentiation and postsynaptic protein levels in mouse hippocampus. *Environ. Health Perspect.* **115,** 865–870.

Eriksson, P. (1992). Neuroreceptor and behavioural effects of DDT and pyrethroids in immature and adult mammals. In *The Vulnerable Brain and Environmental Risks* (R. L. Isaacson and K. F. Jensen, Eds.), Vol. 2, pp. 235–251. Plenum Press, New York.

Eriksson, P., Ahlbom, J., and Fredriksson, A. (1992). Exposure to DDT during a defined period in neonatal life induces permanent changes in brain muscarinic receptors and behaviour in adult mice. *Brain Res.* **582,** 277–281.

Eriksson, P., Ankarberg, E., and Fredriksson, A. (2000). Exposure to nicotine during a defined period in neonatal life induces permanent changes in brain nicotinic receptors and in behaviour of adult mice. *Brain Res.* **853,** 41–48.

Eriksson, P., Viberg, H., Jakobsson, E., Orn, U., and Fredriksson, A. (2002). A brominated flame retardant, 2,2′,4,4′,5-pentabromodiphenyl ether: Uptake, retention, and induction of neurobehavioral alterations in mice during a critical phase of neonatal brain development. *Toxicol. Sci.* **67,** 98–103.

Falkeborn, Y., Larsson, C., Nordberg, A., and Slanina, P. (1983). A comparison of the regional ontogenesis of nicotine- and muscarine-like binding sites in mouse brain. *J. Dev. Neurosci.* **1,** 187–190.

Fiedler, E. P., Marks, M. J., and Collins, A. C. (1987). Postnatal development of cholinergic enzymes and receptors in mouse brain. *J. Neurochem.* **49,** 983–990.

Frankland, P. W., O'Brien, C., Ohno, M., Kirkwood, A., and Silva, A. J. (2001). Alpha-CaMKII-dependent plasticity in the cortex is required for permanent memory. *Nature* **411,** 309–313.

Fredriksson, A., and Archer, T. (2004). Neurobehavioural deficits associated with apoptotic neurodegeneration and vulnerability for ADHD. *Neurotox. Res.* **6,** 435–456.

Fredriksson, A., Ponten, E., Gordh, T., and Eriksson, P. (2007). Neonatal exposure to a combination of N-methyl-D-aspartate and gamma-aminobutyric acid type A receptor anesthetic agents potentiates apoptotic neurodegeneration and persistent behavioral deficits. *Anesthesiology* **107,** 427–436.

Giesy, J. P., and Kannan, K. (2001). Global distribution of perfluorooctane sulfonate in wildlife. *Environ. Sci. Technol.* **35,** 1339–1342.

Grandjean, P., and Landrigan, P. J. (2006). Developmental neurotoxicity of industrial chemicals. *Lancet* **368,** 2167–2178.

Hamos, J. E., DeGennaro, L. J., and Drachman, D. A. (1989). Synaptic loss in Alzheimer's disease and other dementias. *Neurology* **39,** 355–361.

Hellstrom-Lindahl, E., Mousavi, M., Zhang, X., Ravid, R., and Nordberg, A. (1999). Regional distribution of nicotinic receptor subunit mRNAs in human brain: Comparison between Alzheimer and normal brain. *Brain Res. Mol. Brain Res.* **66,** 94–103.

Janz, R., Sudhof, T. C., Hammer, R. E., Unni, V., Siegelbaum, S. A., and Bolshakov, V. Y. (1999). Essential roles in synaptic plasticity for synaptogyrin I and synaptophysin I. *Neuron* **24,** 687–700.

Johansson, N., Fredriksson, A., and Eriksson, P. (2008a). Neonatal exposure to perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) causes neurobehavioural defects in adult mice. *Neurotoxicology* **29,** 160–169.

Johansson, N., Viberg, H., Fredriksson, A., and Eriksson, P. (2008b). Neonatal exposure to deca-brominated diphenyl ether (PBDE 209) causes dose-response changes in spontaneous behaviour and cholinergic susceptibility in adult mice. *Neurotoxicology* **29,** 911–919.

Kannan, K., Koistinen, J., Beckmen, K., Evans, T., Gorzelany, J. F., Hansen, K. J., Jones, P. D., Helle, E., Nyman, M., and Giesy, J. P. (2001). Accumulation of perfluorooctane sulfonate in marine mammals. *Environ. Sci. Technol.* **35,** 1593–1598.

Kärrman, A., Ericson, I., van Bavel, B., Darnerud, P. O., Aune, M., Glynn, A., Lignell, S., and Lindstrom, G. (2007). Exposure of perfluorinated chemicals through lactation: levels of matched human milk and serum and a temporal trend, 1996–2004, in Sweden. *Environ. Health Perspect.* **115,** 226–230.

Kärrman, A., Mueller, J. F., van Bavel, B., Harden, F., Toms, L. M., and Lindstrom, G. (2006). Levels of 12 perfluorinated chemicals in pooled australian serum, collected 2002-2003, in relation to age, gender, and region. *Environ. Sci. Technol.* **40,** 3742–3748.

Keller, W. C., and Yeary, R. A. (1980). A comparison of the effects of mineral oil, vegetable oil, and sodium sulfate on the intestinal absorption of DDT in rodents. *Clin. Toxicol.* **16,** 223–231.

Kelly, P. T., Shields, S., Conway, K., Yip, R., and Burgin, K. (1987). Developmental changes in calmodulin-kinase II activity at brain synaptic junctions: Alterations in holoenzyme composition. *J. Neurochem.* **49,** 1927–1940.

Key, B. D., Howell, R. D., and Criddle, C. S. (1997). Fluorinated organics in the biosphere. *Environ. Sci. Technol.* **31,** 2445–2454.

Key, B. D., Howell, R. D., and Criddle, C. S. (1998). Defluorination of organofluorine sulfur compounds by Pseudomonas Sp. strain D2. *Environ. Sci. Technol.* **32,** 2283–2287.

Kobayashi, K., Tsuji, R., Yoshioka, T., Kushida, M., Yabushita, S., Sasaki, M., Mino, T., and Seki, T. (2005). Effects of hypothyroidism induced by perinatal exposure to PTU on rat behavior and synaptic gene expression. *Toxicology* **212,** 135–147.

Kolb, B., and Whishaw, I. Q. (1989). Plasticity in the neocortex: Mechanisms underlying recovery from early brain damage. *Prog. Neurobiol.* **32,** 235–276.

Kuhar, M. J., Birdsall, N. J., Burgen, A. S., and Hulme, E. C. (1980). Ontogeny of muscarinic receptors in rat brain. *Brain Res.* **184,** 375–383.

Lynch, M. A. (2004). Long-term potentiation and memory. *Physiol. Rev.* **84,** 87–136.

Masliah, E., Terry, R. D., Alford, M., and DeTeresa, R. (1990). Quantitative immunohistochemistry of synaptophysin in human neocortex: An alternative method to estimate density of presynaptic terminals in paraffin sections. *J. Histochem. Cytochem.* **38,** 837–844.

Mattson, M. P. (2004). Pathways towards and away from Alzheimer's disease. *Nature* **430,** 631–639.

Muntane, G., Dalfo, E., Martinez, A., and Ferrer, I. (2008). Phosphorylation of tau and alpha-synuclein in synaptic-enriched fractions of the frontal cortex in Alzheimer's disease, and in Parkinson's disease and related alpha-synucleinopathies. *Neuroscience* **152,** 913–923.

Navone, F., Jahn, R., Di Gioia, G., Stukenbrok, H., Greengard, P., and De Camilli, P. (1986). Protein p38: An integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. *J. Cell Biol.* **103,** 2511–2257.

Nordberg, A. (1993). In vivo detection of neurotransmitter changes in Alzheimer's disease. *Ann. N. Y. Acad. Sci.* **695,** 27–33.

OECD. (2002). *Co-operation on Existing Chemicals. Hazard Assessment of Perfluorooctane Sulfonate (PFOS) and its Salts.* ENV/JM/RD(2002)17/FINAL. www.oecd.org/dataoecd/23/18/2382880.pdf.

Oestreicher, A. B., De Graan, P. N. E., Gispen, W. H., Verhaagen, J., and Schrama, L. H. (1997). B-50, the growth associated protein-43: Modulation of cell morphology and communication in the nervous system. *Prog. Neurobiol.* **53,** 627–686.

Olsen, G. W., Church, T. R., Hansen, K. J., Burris, J. M., Butenhoff, J. L., Mandel, J. H., and Zobel, L. R. (2004). Quantitative evaluation of perfluorooctanesulfonate (PFOS) and other fluorochemicals in the serum of children. *J. Child Health* **2,** 53–76.

Olsen, G. W., Hansen, K. J., Stevenson, L. A., Burris, J. M., and Mandel, J. H. (2003). Human donor liver and serum concentrations of perfluorooctanesulfonate and other perfluorochemicals. *Environ. Sci. Technol.* **37,** 888–891.

Palin, K. J., Wilson, C. G., Davis, S. S., and Phillips, A. J. (1982). The effects of oil on the lymphatic absorption of DDT. *J. Pharm. Pharmacol.* **34,** 707–710.

Paterson, D., and Nordberg, A. (2000). Neuronal nicotinic receptors in the human brain. *Prog. Neurobiol.* **61,** 75–111.

Polli, J. W., Patanow, C. M., and Billingsley, M. L. (1990). Developmental expression of neuronal calmodulin-binding proteins in rat brain. *Brain Res.* **53,** 62–70.

Renner, R. (2001). Growing concern over perfluorinated chemicals. *Environ. Sci. Technol.* **35,** 154A–160A.

Rice, D., and Barone, S., Jr. (2000). Critical periods of vulnerability for the developing nervous system: Evidence from humans and animal models. *Environ. Health Perspect.* **108**(Suppl. 3), 511–533.

Rongo, C., and Kaplan, J. M. (1999). CaMKII regulates the density of central glutamatergic synapses in vivo. *Nature* **402,** 195–199.

So, M. K., Yamashita, N., Taniyasu, S., Jiang, Q., Giesy, J. P., Chen, K., and Lam, P. K. (2006). Health risks in infants associated with exposure to perfluorinated compounds in human breast milk from Zhoushan, China. *Environ. Sci. Technol.* **40,** 2924–2929.

Sugiura, H., and Yamauchi, T. (1992). Developmental changes in the levels of Ca2+/calmodulin-dependent protein kinase II alpha and beta proteins in soluble and particulate fractions of the rat brain. *Brain Res.* **593,** 97–104.

Tarsa, L., and Goda, Y. (2002). Synaptophysin regulates activity-dependent synapse formation in cultured hippocampal neurons. *Proc. Natl Acad. Sci. U.S.A.* **99,** 1012–1016.

Taves, D. R. (1968). Evidence that there are two forms of fluoride in human serum. *Nature* **217,** 1050–1051.

Trudel, D., Horowitz, L., Wormuth, M., Scheringer, M., Cousins, I. T., and Hungerbuhler, K. (2008). Estimating consumer exposure to PFOS and PFOA. *Risk Anal.* **28,** 251–269.

Viberg, H., Fredriksson, A., and Eriksson, P. (2003a). Neonatal exposure to polybrominated diphenyl ether (PBDE 153) disrupts spontaneous behaviour, impairs learning and memory, and decreases hippocampal cholinergic receptors in adult mice. *Toxicol. Appl. Pharmacol.* **192,** 95–106.

Viberg, H., Fredriksson, A., and Eriksson, P. (2004). Neonatal exposure to the brominated flame-retardant, 2,2′,4,4′,5-pentabromodiphenyl ether, decreases cholinergic nicotinic receptors in hippocampus and affects spontaneous behaviour in the adult mouse. *Environ. Toxicol. Pharmacol.* **17,** 61–65.

Viberg, H., Fredriksson, A., and Eriksson, P. (2005). Deranged spontaneous behaviour and decrease in cholinergic muscarinic receptors in hippocampus in the adult rat, after neonatal exposure to the brominated flame-retardant, 2,2′,4,4′,5-pentabromodiphenyl ether (PBDE 99). *Environ. Toxicol. Pharmacol.* **20,** 283–288.

Viberg, H., Fredriksson, A., and Eriksson, P. (2007). Changes in spontaneous behaviour and altered response to nicotine in the adult rat, after neonatal exposure to the brominated flame retardant, decabrominated diphenyl ether (PBDE 209). *Neurotoxicology* **28,** 136–142.

Viberg, H., Johansson, N., Fredriksson, A., Eriksson, J., Marsh, G., and Eriksson, P. (2006). Neonatal exposure to higher brominated diphenyl ethers, hepta-, octa-, or nonabromodiphenyl ether, impairs spontaneous behavior and learning and memory functions of adult mice. *Toxicol. Sci.* **92,** 211–218.

Viberg, H., Fredriksson, A., Jakobsson, E., Orn, U., and Eriksson, P. (2003b). Neurobehavioural derangements in adult mice receiving decabrominated diphenyl ether (PBDE 209) during a defined period of neonatal brain development. *Toxicol. Sci.* **76,** 112–120.

Viberg, H., Mundy, W., and Eriksson, P. (2008a). Neonatal exposure to decabrominated diphenyl ether (PBDE 209) results in changes in BDNF, CaMKII and GAP-43, biochemical substrates of neuronal survival, growth, and synaptogenesis. *Neurotoxicology* **29,** 152–159.

Viberg, H., Pontén, E., Eriksson, P., Gordh, T., and Fredriksson, A. (2008b). Neonatal ketamine exposure results in changes in biochemical substrates of neuronal growth and synaptogenesis, and alters adult behavior irreversibly. *Toxicology* **249,** 153–159.

Vila-Ortiz, G. J., Santa-Coloma, T. A., Carminatti, H., and Radrizzani, M. (2001). The rate of Tau synthesis is differentially regulated during postnatal development in mouse cerebellum. *Cell. Mol. Neurobiol.* **21,** 535–543.

Wang, H. Y., Li, W., Benedetti, N. J., and Lee, D. H. (2003). Alpha 7 nicotinic acetylcholine receptors mediate beta-amyloid peptide-induced tau protein phosphorylation. *J. Biol. Chem.* **278,** 31547–31553.

Wang, J. Z., and Liu, F. (2008). Microtubule-associated protein tau in development, degeneration and protection of neurons. *Prog. Neurobiol.* **85,** 148–175.

Wiedenmann, B., and Franke, W. W. (1985). Identification and localization of synaptophysin, an integral membrane glycoprotein of Mr 38,000 characteristic of presynaptic vesicles. *Cell* **41,** 1017–1028.

Weingarten, M. D., Lockwood, A. H., Hwo, S. Y., and Kirschner, M. W. (1975). A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. U.S.A.* **72,** 1858–1862.

WWF. (2005). *Generations X, Detox Campaign*, p. 59. Brussels, Belgium.