A single neonatal exposure to perfluorohexane sulfonate (PFHxS) affects the levels of important neuroproteins in the developing mouse brain

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Running title: - Developmental neurotoxicity of PFHxS

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Abstract

Perfluorohexane sulfonate (PFHxS) is an industrial chemical and belongs to the group of perfluorinated compounds (PFCs). It has recently been shown to cause developmental neurobehavioral defects in mammals. These compounds are commonly used in products such as surfactant and protective coating due to their ability to repel water- and oil stains. PFCs are globally found in the environment as well as in human umbilical cord blood, serum and breast milk. In a previous study on other well-known PFCs i.e. PFOS and PFOA, it was shown that neonatal exposure caused altered neuroprotein levels in the hippocampus and cerebral cortex in neonatal male mice. The present study show that neonatal exposure to PFHxS, during the peak of the brain growth spurt, can alter neuroprotein levels e.g. CaMKII, GAP-43, synaptophysin and tau, which are essential for normal brain development in mice. This was measured for both males and females, in hippocampus and cerebral cortex. The results suggest that PFHxS may act as a developmental neurotoxicant and the effects are similar to that of PFOS and PFOA, but also to other substances such as PCBs, PBDEs and Bisphenol A.

Keywords: Perfluorohexane sulfonate (PFHxS), Neonatal, Developmental neurotoxicity, CaMKII, GAP-43, Synaptophysin, Tau

1. Introduction

Perfluorinated compounds (PFCs) are a group of synthetic chemicals that have many manufacturing and industrial applications, and comprise of two major classes; perfluoroalkyl sulfonates and perfluoroalkyl carboxylates (EPA, 2012). Because of their molecular composition PFCs are highly stable and virtually non-biodegradable and extremely persistent (Key et al., 1997). PFC's persistent nature and bioaccumulating property has led to an environmental build up and is globally found in both humans and animal wild-life (Giesy and Kannan, 2001, Calafat et al., 2007, Lau et al., 2007). The primary route of exposure in humans is believed to be via food and drinking water that have been in contact with PFC-containing products (D'Hollander et al., 2010).

Perfluorohexane sulfonate (PFHxS) is a short-chain perfluoralkyl acid and is used as a surfactant, protective coating for carpets, textiles and paper and to produce fluoropolymers (NTP, 2012). PFHxS have been detected in human umbilical blood, serum and breast milk (Kärrman et al., 2007, Monroy et al., 2008, Sundstrom et al., 2011, Glynn et al., 2012). Concentrations of PFHxS found in breast milk ranges from 0.04 ng/mL to 0.1 ng/mL (So et al., 2006, Kärrman et al., 2010). The presence of PFHxS, and other PFCs, in human breast milk is a strong indication that infants and toddlers are exposed from an early age, also there are studies that suggest that indoor air and dust are additional major exposure routes (Kubwabo et al., 2005, Strynar and Lindstrom, 2008). Toddlers and younger children tend to have a higher total intake of PFHxS due to higher relative food consumption and more frequent hand-to-mouth transfer after contact with carpets and surfaces where dust collects (Trudel et al., 2008, Beesoon et al., 2010). In humans, PFHxS has a longer half-life (~7 years) compared with other PFCs, such as perfluorooctane sulfonate (PFOS) (~5 years) and perfluorooctanoic acid (PFOA) (~4 years)

(Olsen et al., 2007). PFHxS is among the most commonly detected PFCs and trends of temporal increases have been observed (Tao et al., 2008, Glynn et al., 2012).

Still, much is unknown about the neurotoxicity of PFCs. Most studies have been conducted on PFOS and PFOA and very little or none on the remaining compounds. Others in our group have recently performed a study showing that a single neonatal exposure to PFHxS on postnatal day (PND) 10 can alter spontaneous behavior and cognitive function in male and female mice, the effects were dose-response related and long-lasting (Viberg et al., 2013). Another investigation performed on PFHxS assessed motor activity and functional observational battery (FOB) parameters in adult rats after exposure to 0.3, 1.3, 3 or 10 mg PFHxS/kg bw/day for 40-50 days (Butenhoff et al., 2009). The investigation did not reveal any neurobehavioral effects.

In previous studies, a single neonatal exposure to PFOS or PFOA induced developmental neurotoxic effects manifested as changed spontaneous behavior and habitation in adult male mice. The effects worsened with age and PFOS/PFOA also caused alterations in the developing cholinergic system (Johansson et al., 2008a). It is suggested that the altered cholinergic system could be one possible mechanism behind the disturbed adult behavior. In addition, PFOS and PFOA also affected neuroproteins important for the normal brain development after a single neonatal exposure on PND 10 (Johansson et al., 2009). Johansson et al. (2009) showed that the neonatal exposure to PFOS or PFOA on PND 10 caused an up regulation of calcium/calmodulin-dependent kinase II (CaMKII), growth-associated protein-43 (GAP-43) and synaptophysin protein levels in the hippocampus of male mice 24h after exposure. Also, increased protein levels of synaptophysin and tau were seen in the cerebral cortex. The altered levels of CaMKII, GAP-43, synaptophysin and tau could be another or contributing mechanism behind the observed adult behavioral and cognitive impairments after

neonatal exposure to PFOS and PFOA. *In utero* exposure to 3 mg PFOS/kg bw/ day from gestational day 2 to 21, in rats, did not induce effects in spatial learning and memory compared with controls (Lau et al., 2003). However, Lau et al. (2003) observed a significant decrease in the enzyme activity of choline acethyltranferase (ChAT) in the prefrontal cortex, supporting the effect on the cholinergic system. Altogether, this suggests that the timing of exposure of environmental toxicants is important in determining the manifestation of developmental neurotoxic effects.

Mammalian brain development involves several key developmental processes and stages before reaching full maturation. The brain growth spurt (BGS) is a recognized stage, where many critical developmental changes occur (Davison and Dobbing, 1968), and that the period is sensitive to insults by xenobiotics e.g. DDT, bioalltherin, nicotine, PCBs, PBDEs, PFCs, ketamine, bisphenol A (Eriksson, 1992, Ahlbom et al., 1994, Eriksson et al., 2000, Eriksson et al., 2001, Eriksson et al., 2002, Eriksson and Fredriksson, 2004, Viberg et al., 2006a, Johansson et al., 2008a, Viberg et al., 2008b, Johansson et al., 2009, Viberg, 2009a, Viberg et al., 2011, Viberg and Lee, 2012). During the BGS the establishment of neuronal connections, the maturation of axonal and dendritic outgrowth, synaptogenesis and glial multiplication and myelination take place (Dobbing and Sands, 1979, Kolb and Whishaw, 1989). CaMKII, GAP-43, synaptophysin, tau and brain-derived neurotrophic factor (BDNF) are proteins involved in the biochemical process of the BGS. The functions of these proteins involve the regulation of neuronal survival, growth and synaptogenesis (Wiedenmann and Franke, 1985, Navone et al., 1986, Benowitz and Routtenberg, 1997, Rongo and Kaplan, 1999, Cui, 2006, Wang and Liu, 2008). These proteins have all been shown to express unique ontogeny patterns, with markedly increased protein levels around the peak of the BGS in mice (Viberg et al., 2008a, Viberg, 2009b). The onset and duration of the BGS varies among mammalian species. The initiation of the BGS in humans begins around the third trimester of pregnancy and lasts throughout the first two years

of life. In mice, the BGS is entirely neonatal and continues for the first four weeks of life and peaks around PND 10 (Dobbing and Sands, 1979).

With regard to our earlier studies on PFCs, and on developmental neurobehavioral defects observed after neonatal exposure to PFHxS, the aim of this study was to investigate if neonatal exposure to a single dose of PFHxS, at the peak of the brain growth spurt, can alter the protein levels of CaMKII, GAP-43, synaptophysin, tau and BDNF in the neonatal and adult mouse brain.

2. Material and Methods

2.1. Chemicals and Animals

PFHxS (potassium salt, purity > 98%, CAS number 3871-99-6 and linear formula $C_6F_{13}KO_3S$) was purchased from Sigma-Aldrich, Stockholm, Sweden. The PFHxS was dissolved in an egg lecithin (Merck, Darmstadt, Germany) and peanut oil (*Oleum arachidis*) mixture (1:10) and sonicated with water to yield a 20% (w:w) fat emulsion vehicle containing 6.1 or 9.2 mg PFHxS/ml (14 or 21 μmol/ml) respectively. This was done to emulate the fat content of mouse milk (~14%) for a physiologically appropriate absorption and hence distribution (Keller and Yeary, 1980, Palin et al., 1982).

Pregnant NMRI mice were purchased from Scanbur, Sollentuna, Sweden and housed individually in plastic cages in a room with an ambient temperature of 22ºC and 12/12 hour cycle of light and dark. The animals had free access to standardized food pellets (Lactamin, Stockholm, Sweden) and tap water *ad libitum*. The day of birth was assigned PND 0, the litters were culled to 10-14 pups within 48h after birth. At the age of 4 weeks male and female mice were separated and were kept in their

respective treatment groups, together with their siblings. Each litter contained 4-7 animals, males and females were raised in separate rooms with the same conditions as mentioned above. Experiments were conducted 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, approval number C185/9.

On PND 10, the mice were given 6.1 or 9.2 mg PFHxS/kg body weight (14 or 21 μmol PFHxS/kg body weight) as a single oral dose via a metal gastric tube. Control mice received 10 ml of the 20% fat emulsion vehicle/kg body weight. The animals in each treatment group were randomly chosen from multiple litters, one pup per litter, meaning that the statistical unit equals the number of litters within the treatment group. The animals were euthanized 24h or 4 months after the PFHxS exposure and the cerebral cortex and hippocampus brain regions were dissected out and frozen in liquid nitrogen and stored in -80ºC until protein analysis.

2.2. Slot-blot analysis CaMKII, GAP-43, synaptophysin and tau

Cerebral cortex and hippocampus were homogenized in RIPA cell lysis buffer (50 mM Tris–HCl; pH 7.4; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 20 mM sodium pyrophosphate; 2 mM sodium orthovanadate; 1% sodium deoxycholate and 0.1% SDS) with the addition of 5 µL protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem) per mL of RIPA cell lysis buffer. The homogenate was then centrifuged at 14 000 x g for 10 min at 4ºC and the supernatant was collected and stored in -80ºC until use. The protein content of the supernatant was measured using the BCA assay method (Pierce).

The specificity of the primary antibodies; CaMKII (Upstate Millpore, 05-552), GAP-43 (Chemicon Millipore, AB5220), synaptophysin (Calbiochem, 573822) and tau (Santa Cruz, 32274) were previously evaluated by Western Blot analysis (Viberg et al., 2008a, Viberg, 2009b). The antibodies were concluded to be specific for the respective proteins, as the analysis showed only the presence of one band at the appropriate molecular weight. Therefore, the antibodies were considered suitable for use in Slot Blot analysis. In the Slot Blot analysis the chemiluminescence increased with increased protein load. The antibodies recognize both phosphorylated and nonphosphorylated forms of the proteins.

The total protein amount used for CaMKII and GAP-43 was 4 μg, for synaptophysin 3 μg and for tau 3.5 μg, which were diluted in sample buffer to a final volume of 200 μL. The diluted supernatant was then applied in duplicates to a nitrocellulose membrane (0.45 mm, Bio-Rad) soaked in TBS buffer [NaCl (0.9%), Tris–HCl (42.1 mM) and Tris–Base (7.5 mM)], using a Bio-Dot SF microfiltration apparatus (Bio-Rad). The membranes were dried at 60ºC for 5 min, fixed in a 25% isopropanol and 10% acetic acid solution, washed in 1% TBS, and blocked for 1h at room temperature in 5% non-fat dry milk containing 0.03% Tween-20. The membranes were then incubated overnight at 4°C with either a mouse monoclonal CaMKII antibody (1:5000), a rabbit monoclonal 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 (KPL 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). The protein levels were expressed as a percentage of controls.

2.3. ELISA analysis for BDNF

Cerebral cortex and hippocampus were sonicated in 10 or 20 volumes, respectively (w/v) of ice-cold lysis buffer (137 mM NaCl; 20 mM Tris–HCl, pH 8.0; 1 mM phenyl-methyl-sulfonyl fluoride; 0.5 mM sodium vanadate; 1% NP40; 10% glycerol; 10 μg/ml aprotinin; 1 μg/ml leupeptin). The homogenate was then centrifuged at 20 000 × g for 20 min at 4°C, and the supernatant was acidified (pH < 3) with HCl and neutralized back with NaOH (pH 7.6). The Promega E_{max}™ ImmunoAssay System was used to determine the amount of BDNF in the samples according to the technical bulletin supplied by the distributor. In brief, BDNF from each sample were captured with a monoclonal antibody (mAb) against BDNF; the captured BDNF were then bound to a second, specific, polyclonal antibody (pAb) against BDNF. After washing, the amounts of specifically bound pAb were detected by using a specific anti-IgY antibody conjugated to horseradish peroxidase (HRP) as a tertiary reactant. Unbound conjugates were removed by washing and following an incubation period with a chromogenic substrate, the color change was measured in a micro-plate reader at 450 nm (VICTOR^3, Perkin Elmer). The amount of BDNF was proportional to the color change generated and compared to a standard curve. The cross-reactivity to other neurotrophic factors is < 3% and the purity of the anti-BDNF antibodies was > 95%. The protein levels were expressed as a percentage of controls.

2.4. Statistical analysis

The mean values of the chemiluminescent data from the neonatal and adult animals were analyzed for all treatments by one-way ANOVA and Tukey's HSD (honest significant difference) test (GraphPad Prism 5.01). The data from the ELISA was evaluated by a one-way ANOVA. The statistical analysis compared animals from the different treatment groups, taken from multiple litters, and male and female animals were analyzed separately.

3. Results

3.1. Neuroprotein levels of CaMKII, GAP-43, synaptophysin, tau and BDNF in neonatal male and female mice in hippocampus and cerebral cortex

There were significant treatment effects on CaMKII [$F_{2, 17} = 8.26$], synaptophysin [$F_{2, 17} = 5.41$] and tau [$F_{2, 18} = 9.73$] levels in hippocampus, and GAP-43 [$F_{2, 19} = 6.54$] in cerebral cortex, 24h after exposure to PFHxS on PND 10 in the male neonatal mice (figure 1A and 1B).

The protein levels of CaMKII in hippocampus had significantly increased by 85% ($p \leq 0.05$) and 107% ($p \leq 0.01$) in the male neonates exposed to the 6.1 and 9.2 mg/kg bw dose of PFHxS, respectively, compared with the controls. The synaptophysin level in hippocampus increased significantly ($p \leq 0.05$) by 162%, in the highest exposure group, and tau protein levels in hippocampus had increased significantly ($p \leq 0.01$) by 177% and 211% in the low and the high dose exposure group, respectively. For GAP-43, a significantly decreased ($p \leq 0.05$) protein level of 13% was measured in the cortices of the male neonates compared to the control group.

There was no significant treatment effect measured on BDNF levels of the PFHxS exposed male neonatal animals compared with controls in the hippocampus or in the cerebral cortex (table 1). In the cerebral cortex there was a tendency towards decreased level of BDNF, in the animals exposed to 9.2 mg PFHxS/kg bw, as the one-way ANOVA analysis showed p-value 0.08.

There were significant treatment effects on CaMKII [$F_{2, 18} = 7.72$], synaptophysin [$F_{2, 18} = 3.97$] and tau [$F_{2, 19} = 6.66$] levels in hippocampus, and GAP-43 [$F_{2, 17} = 13.52$] in cerebral cortex, 24h after exposure to PFHxS on PND 10 in the female neonatal mice (figure 2A and 2B).

The protein levels of CaMKII in hippocampus had significantly increased ($p \leq 0.01$) by 59% in the female neonates exposed to the 6.1 or 9.2 mg/kg bw dose of PFHxS, compared with the controls. The synaptophysin level in hippocampus increased significantly ($p \leq 0.05$) by 78%, in the highest exposure group, and tau protein in hippocampus levels had increased significantly by 326% ($p \leq 0.01$) and 260% ($p \leq 0.05$) in the low and the high dose exposure group, respectively. For GAP-43, a significantly decreased ($p \leq 0.001$) protein level of 17% was measured in the cortices of the male neonates compared to the control group.

There was no significant treatment effect measured on BDNF levels of the PFHxS exposed female neonatal animals compared with controls in the hippocampus or in the cerebral cortex (table 1). In the cerebral cortex there was a tendency towards decreased level of BDNF, in the animals exposed to 9.2 mg PFHxS/kg bw, as the one-way ANOVA analysis showed p-value 0.09.

3.2. Neuroprotein levels of CaMKII, GAP-43, synaptophysin, tau in adult male mice in hippocampus and cerebral cortex

There were significant treatment effects [$F_{2, 18} = 7.72$] on tau protein level in the cerebral cortex in the adult male animals exposed PFHxS on PND 10 (figure 3A and 3B).

The protein levels on tau was significantly increased ($p \leq 0.01$) by 10% in the adult males after exposure to 6.1 mg/kg bw of PFHxS compared with the control group.

3.3. Neuroprotein levels of CaMKII, GAP-43, synaptophysin, tau in adult female mice in hippocampus and cerebral cortex

There were no significant treatment effects measured for any of the protein levels of the PFHxS exposed animals compared with controls in the hippocampus or in the cerebral cortex (figure 4A and 4B).

4. Discussion

PFCs are ubiquitously found in the environment and in humans, which are due to their chemical properties, such as extreme stability and persistency. In addition, there are indications that toddlers and young children make up a critical exposure group as they tend to have a higher body burden than adults.

The present study shows that a single neonatal exposure to PFHxS during a sensitive period of brain development can alter levels of neuroproteins in the neonatal and adult mouse brain. CaMKII, synaptophysin and tau showed significantly increased levels in the hippocampus, whereas GAP-43 showed a significantly decreased level in the cerebral cortex, 24h after the exposure in both sexes. No significant effect was measured for BDNF 24h after the exposure; however a tendency towards decreased levels in the cerebral cortex, for both sexes, was observed in the animals exposed to a high dose of PFHxS (9.2 mg/kg bw). In adult male mice, tau showed a significantly increased level in the cerebral cortex in mice exposed to a low PFHxS dose (6.1 mg/kg bw). This was not seen for the female adult mice or in the treatment group exposed to a high PFHxS dose. The altered levels of neuroproteins may cause neurotoxic effects manifested as adult aberrant behavior, as these proteins are essential for normal brain development and cognitive function. This is supported by our recent study,

showing that neonatal exposure to 9.2 mg PFHxS/kg bw cause adult effects in spontaneous behavior, cognitive function and nicotine induced behavior in mice (Viberg et al., 2013).

Viberg et al. (2013) demonstrated that male and female mice exposed to a single oral dose of 9.2 mg PFHxS/kg bw on PND 10 had a different pattern of neurotoxic effects compared to male and female animals exposed to a vehicle or lower doses (0.61 or 6.1 mg PFHxS/kg bw). The neurotoxic effects observed did not seemingly differ between the sexes. In the behavioral study by Viberg and coworkers the treatment group exposed to 9.2 mg PFHxS/kg bw exhibited significant changes in both the spontaneous behavior tests and the nicotine induced behavior test, while the effects seen in the treatment group exposed to 6.1 mg PFHxS/kg bw were much less pronounced. This corresponds to the results for the neonatal animals in present study as the animals exposed to the 9.2 mg PFHxS/kg bw dose had altered protein levels for CaMKII, GAP-43, synaptophysin and tau while the animals exposed to 6.1 mg PFHxS/kg bw only showed changes in CaMKII and tau.

CaMKII is abundantly found in the brain, enriched in the synapses, and is involved in the regulation of synaptogenesis, synaptic plasticity, behavior and memory (Silva et al., 1992, Rongo and Kaplan, 1999, Frankland et al., 2001). It plays a prominent role in the induction and maintenance of long-term potentiation (LTP), which is significant for learning and memory. LTP induction causes CaMKII to translocate to the postsynaptic density, where it further stimulates the synaptic transmission via NMDA-type glutamate receptors and enhances it through AMPA-type glutamate receptors (Lisman et al., 2012). Silva et al. (1992) established that CaMKII is essential for learning, mice deficient of CaMKII displayed specific learning impairments as a result of hippocampal LTP blockage. The study showed that CaMKII is important for spatial learning task, however not in non-spatial learning. Furthermore, cortical CaMKII has also shown to be vital in the formation of permanent memory.

Frankland et al. (2001) demonstrated that CaMKII heterozygous mouse, with normal hippocampal LTP and no cortical LTP, exhibited normal learning and memory in behavioral task but the memory was severely impaired at longer retention delays. As normal information transfer between the hippocampus and cortex is diminished, there is no consolidation of the memories. An overexpression of CaMKII may favor hippocampal long-term depression (LTD), instead of LTP, which is more associated with forgetting (Bach et al., 1995). Similar neurotoxic effects, with increased CaMKII levels in hippocampus and deranged adult behavior manifested as lack of habituation and hyperactivity, have also been seen in our previous studies where neonatal male mice were exposed to PFOS, PFOA, PBDEs or ketamine (Johansson et al., 2008a, Viberg et al., 2008a, Viberg et al., 2008b, Johansson et al., 2009, Viberg, 2009a). This supports the results from our present study and the suggestion that changes in CaMKII levels may be one of the mechanisms behind the adult behavioral and cognitive impairments seen after neonatal exposure to PFHxS.

The functions of GAP-43 mainly involve axonal growth guidance and organization of neurite endings (Benowitz and Routtenberg, 1997). GAP-43 is highly expressed during the brain development and starts to decline after synaptogenesis, except in brain regions with high plasticity e.g. hippocampus CA1 region and layer 1 of cortex (Jacobson et al., 1986, Benowitz et al., 1988). Changes in GAP-43 expression may severely affect neuronal development, synaptic plasticity of LTP and learning and memory. GAP-43 homozygous deficit mice die shortly after birth (Strittmatter et al., 1995, Maier et al., 1999), while homozygous GAP-43 overexpressing mice show enhanced learning and synaptic plasticity (Routtenberg et al., 2000). Also, transgenic GAP-43 overexpressing mice exhibit deficit spatial learning (Holahan and Routtenberg, 2008) when observed in radial arm maze, Morris water maze and contextual fear conditioning. However, Holahan and Routtenberg (2008) demonstrated that the phosphorylation state of GAP-43 plays a key role in the regulation of its activity. Significant memory impairments was observed in heterozygous GAP-43 knockout mice with levels reduced by

one-half, but with normal levels of synaptophysin (Rekart et al., 2005). Furthermore, GAP-43 phosphorylation has been shown to be correlated with the amount of LTP induction and neurotransmitter release (Benowitz and Routtenberg, 1997). Similar to our present results, down regulations of GAP-43 in the cerebral cortex and aberrant behavior have also been observed in mice neonatally exposed to PBDE 209 or ketamine in earlier studies in our group (Johansson et al., 2008b, Viberg et al., 2008a, Viberg et al., 2008b). Therefore the decrease in GAP-43 seen in cortex may contribute to the adult behavioral and cognitive impairments seen after neonatal exposure to PFHxS.

The full extent of synaptophysin function(s) is still unclear, however it is suggested to be involved in the regulation of synaptic vesicle endocytosis kinetics, especially vesicle retrieval (Kwon and Chapman, 2011). Intact information transfer is seemingly crucial for learning and memory, therefore the maintenance of neurotransmission is partially dependent on synaptic vesicle endocytosis and recycling. Synaptophysin is involved, although unclear how, in regulating the life cycle of synaptic vesicles, and that its function is controlled by phosphorylation (Evans and Cousin, 2005). Synaptophysin is mainly phosphorylated by tyrosine kinases; however synaptophysin may also be activated by CaMKII (Rubenstein et al., 1993), which indicates that synaptophysin may be regulate by changes in CaMKII. This supports our present results, and previous studies on PFOS, PFOA and PBDEs (Viberg et al., 2006b, Johansson et al., 2008a, Johansson et al., 2008b, Johansson et al., 2009, Viberg, 2009a, b), showing that disturbed synaptophysin levels may affect the brain development and give rise to behavior -and learning and memory impairments (Janz et al., 1999, Lynch, 2004, Schmitt et al., 2009).

The tau protein is a microtubule-associated protein known to modulate the assembly and stabilization of microtubules, which are essential for axonal transport of neurons, and the maintenance of normal

neuron morphology (Vila-Ortiz et al., 2001, Wang and Liu, 2008). Tau is also known for its association with neurodegenerative diseases related to tauopathies e.g. Alzheimer's disease. The precise mechanism in which it contributes to the disease progression is still unclear. However, it is recognized that hyperphosphorylation of tau causes formation of tau inclusions and aggregation, resulting into neurofibrillary tangles (NFTs) (Lee et al., 2001). The NFTs are mainly present in brain regions involved in learning and memory. The present study showed that there was an increase of tau in the hippocampus of the neonates, which is similar to the effects seen in the study by Johansson et al. (2009). This was only seen after exposure to PFOA in male mice; however Johansson et al. also measured an increase of tau in the cerebral cortex of the neonatal mice for both PFOS and PFOA 24h after exposure. In our study, we saw an increase of tau in the cerebral cortex only in the adult male mice.

In our study we have observed that there can be regional brain differences in the neuroprotein levels after neonatal exposure to PFOS, PFOA or PFHxS. Plausible explanations could be that the amount of substance reaching the hippocampus and cortex varies due to their chemical composition, the brain regions may have different degree of sensitivity and/or cellular targets of disruption. Furthermore, the neuroproteins have various functions and after the early developmental stages the functions diverge or diminish. Altered neuroprotein levels in the neonates may be a possible mechanism of action to the neurotoxic effects observed in the adult mice. Additionally, our research group showed that the abnormal behavioral in the neonatally exposed adult mice was persistent and irreversible. Also, there were indications that the cholinergic transmitter system had been affected. In a nicotine-induced behavioral test, a hypoactivity response was seen in 4 months old mice neonatally exposed to a single dose of PFHxS. This is the totally opposite response to the control animals which exhibited an instant increased level of hyperactivity. The change indicates that the cholinergic transmitter system may be affected (Viberg *et al.,* 2013).

Presently, there is very limited information on PFHxS and not much is known about the developmental neurotoxic effects of PFHxS or the mechanism of action behind it. To our knowledge there is also a lack of information about the magnitude of change in protein levels that is required to affect normal brain function. However, since the neurotoxic effects are similar to effects seen in previous studies with PFOS and PFOA by Johansson et al. (2008 and 2009), it seems reasonable to consider that PFHxS have similar neurotoxic mechanism of action.

In conclusion, the present study show that PFHxS can cause altered neuroprotein levels in neonatal and adult mice after a single neonatal exposure, at a sensitive period of the brain development. Significantly increased levels of CaMKII, synaptophysin and tau in the hippocampus and significantly decreased levels of GAP-43 in the cerebral cortex were seen in the neonatal mice, regardless of sex, already 24h after exposure. The only protein affected was tau in cerebral cortex in adult male mice after 4 months. This study, together with our recent reports on other PFCs, indicates that altered protein level may be a possible mechanism behind the disturbed adult spontaneous behavior and cognitive function recently seen in mice. Therefore, further studies are necessary to acquire a more elaborate and accurate picture of the developmental neurotoxic effect(s) of PFHxS.

5. Acknowledgement

This work was supported by grants from the Foundation for Strategic Environmental Research, Sweden and EU research project FP7:282957, DENAMIC.

6. References

Ahlbom J, Fredriksson A, 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.

Bach ME, Hawkins RD, Osman M, Kandel ER, Mayford M (1995) Impairment of spatial but not contextual memory in CaMKII mutant mice with a selective loss of hippocampal LTP in the range of the theta frequency. Cell 81:905-915.

Beesoon S, Genuis JS, Benskin JP, Martin JW (2010) Abnormally high serum levels of perfluorohexane sulfonates (PFHxS)

in a Canadian family - a case study. Organohalogen Compounds 72:1460-1463.

Benowitz LI, Apostolides PJ, Perrone-Bizzozero N, Finklestein SP, Zwiers H (1988) Anatomical distribution of the growth-associated protein GAP-43/B-50 in the adult rat brain. J Neurosci 8:339-352.

Benowitz LI, Routtenberg A (1997) GAP-43: an intrinsic determinant of neuronal development and plasticity. Trends Neurosci 20:84-91.

Butenhoff JL, Chang SC, Ehresman DJ, York RG (2009) Evaluation of potential reproductive and developmental toxicity of potassium perfluorohexanesulfonate in Sprague Dawley rats. Reprod Toxicol 27:331-341.

Calafat AM, Kuklenyik Z, Reidy JA, Caudill SP, Tully JS, Needham LL (2007) Serum concentrations of 11 polyfluoroalkyl compounds in the u.s. population: data from the national health and nutrition examination survey (NHANES). Environ Sci Technol 41:2237-2242.

Cui Q (2006) Actions of neurotrophic factors and their signaling pathways in neuronal survival and axonal regeneration. Mol Neurobiol 33:155-179.

D'Hollander W, de Voogt P, De Coen W, Bervoets L (2010) Perfluorinated substances in human food and other sources of human exposure. Rev Environ Contam Toxicol 208:179-215.

Davison AN, Dobbing J (1968) Applied Neurochemistry. pp 178-221, 253-316 Oxford: Blackwell.

Dobbing J, Sands J (1979) Comparative aspects of the brain growth spurt. Early human development 3:79-83.

EPA U (2012) Long Chain Perfluorinated Chemicals (PFCs) Action Plan Summary.

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

Eriksson P, Ankarberg E, 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, Ankarberg E, Viberg H, Fredriksson A (2001) The developing cholinergic system as target for environmental toxicants, nicotine and polychlorinated biphenyls (PCBs): implications for neurotoxicological processes in mice. Neurotox Res 3:37-51.

Eriksson P, Fredriksson A (2004) Neonatal exposure to 2,2',4,4',5,5'-hexachlorobiphenyl or 3,3',4,4',5,5'-hexachlorobiphenyl causes behavioural derangements in mouse that deteriorate with age. Submitted.

Eriksson P, Viberg H, Jakobsson E, Orn U, 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.

Evans GJ, Cousin MA (2005) Tyrosine phosphorylation of synaptophysin in synaptic vesicle recycling. Biochem Soc Trans 33:1350-1353.

Frankland PW, O'Brien C, Ohno M, Kirkwood A, Silva AJ (2001) Alpha-CaMKII-dependent plasticity in the cortex is required for permanent memory. Nature 411:309-313.

Giesy JP, Kannan K (2001) Global distribution of perfluorooctane sulfonate in wildlife. Environ Sci Technol 35:1339-1342.

Glynn A, Berger U, Bignert A, Ullah S, Aune M, Lignell S, Darnerud PO (2012) Perfluorinated Alkyl Acids in Blood Serum from Primiparous Women in Sweden: Serial Sampling during Pregnancy and Nursing, And Temporal Trends 1996-2010. Environ Sci Technol 46:9071-9079.

Holahan M, Routtenberg A (2008) The protein kinase C phosphorylation site on GAP-43 differentially regulates information storage. Hippocampus 18:1099-1102.

Jacobson RD, Virag I, Skene JH (1986) A protein associated with axon growth, GAP-43, is widely distributed and developmentally regulated in rat CNS. J Neurosci 6:1843-1855.

Janz R, Sudhof TC, Hammer RE, Unni V, Siegelbaum SA, Bolshakov VY (1999) Essential roles in synaptic plasticity for synaptogyrin I and synaptophysin I. Neuron 24:687-700.

Johansson N, Eriksson P, Viberg H (2009) 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. Toxicol Sci 108:412-418.

Johansson N, Fredriksson A, 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, 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.

Keller WC, Yeary RA (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.

Key BD, Howell RD, Criddle CS (1997) Fluorinated organics in the biosphere. Environmental Science & Technology 31:2445-2454.

Kolb B, Whishaw IQ (1989) Plasticity in the neocortex: mechanisms underlying recovery from early brain damage. Prog Neurobiol 32:235-276.

Kubwabo C, Stewart B, Zhu J, Marro L (2005) Occurrence of perfluorosulfonates and other perfluorochemicals in dust from selected homes in the city of Ottawa, Canada. J Environ Monit 7:1074-1078.

Kwon SE, Chapman ER (2011) Synaptophysin regulates the kinetics of synaptic vesicle endocytosis in central neurons. Neuron 70:847-854.

Kärrman A, Domingo JL, Llebaria X, Nadal M, Bigas E, van Bavel B, Lindstrom G (2010) Biomonitoring perfluorinated compounds in Catalonia, Spain: concentrations and trends in human liver and milk samples. Environmental science and pollution research international 17:750-758.

Kärrman A, Ericson I, van Bavel B, Darnerud PO, Aune M, Glynn A, Lignell S, 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.

Lau C, Anitole K, Hodes C, Lai D, Pfahles-Hutchens A, Seed J (2007) Perfluoroalkyl acids: a review of monitoring and toxicological findings. Toxicol Sci 99:366-394.
Lau C, Thibodeaux JR, Hanson RG, Rogers JM, Grey BE, Stanton ME, Butenhoff JL, Stevenson LA (2003) Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse. II: postnatal evaluation. Toxicol Sci 74:382-392.
Lee VM, Goedert M, Trojanowski JQ (2001) Neurodegenerative tauopathies. Annu Rev Neurosci 24:1121-1159.
Lisman J, Yasuda R, Raghavachari S (2012) Mechanisms of CaMKII action in long-term potentiation. Nat Rev Neurosci 13:169-182.
Lynch MA (2004) Long-Term Potentiation and Memory. Physiol Rev 84:87-136.
Maier DL, Mani S, Donovan SL, Soppet D, Tessarollo L, McCasland JS, Meiri KF (1999) Disrupted cortical map and absence of cortical barrels in growth-associated protein (GAP)-43 knockout mice. Proc Natl Acad Sci U S A 96:9397-9402.
Monroy R, Morrison K, Teo K, Atkinson S, Kubwabo C, Stewart B, Foster WG (2008) Serum levels of perfluoroalkyl compounds in human maternal and umbilical cord blood samples. Environ Res 108:56-62.
Navone F, Jahn R, Di Gioia G, Stukenbrok H, Greengard P, De Camilli P (1986) Protein p38: an integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. The Journal of cell biology 103:2511-2527.
NTP (2012) National Toxicology Program - Testing Status: Perfluorohexane sulfonate, PFHXS M040005
Olsen GW, Burris JM, Ehresman DJ, Froehlich JW, Seacat AM, Butenhoff JL, Zobel LR (2007) Half-life of serum elimination of perfluorooctanesulfonate,perfluorohexanesulfonate, and perfluorooctanoate in retired fluorochemical production workers. Environ Health Perspect 115:1298-1305.
Palin KJ, Wilson CG, Davis SS, Phillips AJ (1982) The effects of oil on the lymphatic absorption of DDT. J Pharm Pharmacol 34:707-710.
Rekart JL, Meiri K, Routtenberg A (2005) Hippocampal-dependent memory is impaired in heterozygous GAP-43 knockout mice. Hippocampus 15:1-7.
Rongo C, Kaplan JM (1999) CaMKII regulates the density of central glutamatergic synapses in vivo. Nature 402:195-199.
Routtenberg A, Cantallops I, Zaffuto S, Serrano P, Namgung U (2000) Enhanced learning after genetic overexpression of a brain growth protein. Proc Natl Acad Sci U S A 97:7657-7662.
Rubenstein JL, Greengard P, Czernik AJ (1993) Calcium-dependent serine phosphorylation of synaptophysin. Synapse 13:161-172.
Schmitt U, Tanimoto N, Seeliger M, Schaeffel F, Leube RE (2009) Detection of behavioral alterations and learning deficits in mice lacking synaptophysin. Neuroscience 162:234-243.
Silva AJ, Paylor R, Wehner JM, Tonegawa S (1992) Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. Science 257:206-211.
So MK, Yamashita N, Taniyasu S, Jiang Q, Giesy JP, Chen K, Lam PK (2006) Health risks in infants associated with exposure to perfluorinated compounds in human breast milk from Zhoushan, China. Environ Sci Technol 40:2924-2929.
Strittmatter SM, Fankhauser C, Huang PL, Mashimo H, Fishman MC (1995) Neuronal pathfinding is abnormal in mice lacking the neuronal growth cone protein GAP-43. Cell 80:445-452.
Strynar MJ, Lindstrom AB (2008) Perfluorinated compounds in house dust from Ohio and North Carolina, USA. Environ Sci Technol 42:3751-3756.
Sundstrom M, Ehresman DJ, Bignert A, Butenhoff JL, Olsen GW, Chang SC, Bergman A (2011) A temporal trend study (1972-2008) of perfluorooctanesulfonate, perfluorohexanesulfonate,

and perfluorooctanoate in pooled human milk samples from Stockholm, Sweden. Environ Int 37:178-183.
Tao L, Ma J, Kunisue T, Libelo EL, Tanabe S, Kannan K (2008) Perfluorinated compounds in human breast milk from several Asian countries, and in infant formula and dairy milk from the United States. Environ Sci Technol 42:8597-8602.
Trudel D, Horowitz L, Wormuth M, Scheringer M, Cousins IT, Hungerbuhler K (2008) Estimating consumer exposure to PFOS and PFOA. Risk Anal 28:251-269.
Wang JZ, Liu F (2008) Microtubule-associated protein tau in development, degeneration and protection of neurons. Prog Neurobiol 85:148-175.
Viberg H (2009a) Exposure to PBDE 203 and 206 during the neonatal brain growth spurt affects proteins important for normal neurodevelopment in mice. Toxicol Sci.
Viberg H (2009b) Neonatal ontogeny and neurotoxic effect of decabrominated diphenyl ether (PBDE 209) on levels of synaptophysin and tau. Int J Dev Neurosci 27:423-429.
Viberg H, Fredriksson A, Buratovic S, Eriksson P (2011) Dose-dependent behavioral disturbances after a single neonatal Bisphenol A dose. Toxicology.
Viberg H, Johansson N, Fredriksson A, Eriksson J, Eriksson P (2006a) Decabrominated diphenyl ether (PBDE 209) induces neurotoxic effects in the neonatal and adult mouse after neonatal exposure. The Toxicologist 90:297.
Viberg H, Johansson N, Fredriksson A, Eriksson J, Marsh G, Eriksson P (2006b) 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, Lee I (2012) A single exposure to bisphenol A alters the levels of important neuroproteins in adult male and female mice. Neurotoxicology 33:1390-1395.
Viberg H, Lee I, Eriksson P (2013) Adult dose-dependent behavioral and cognitive disturbances after a single neonatal PFHxS dose. Toxicology 304:185-191.
Viberg H, Mundy W, 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, Ponten E, Eriksson P, Gordh T, 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.
Wiedenmann B, Franke WW (1985) Identification and localization of synaptophysin, an integral membrane glycoprotein of Mr 38,000 characteristic of presynaptic vesicles. Cell 41:1017-1028.
Vila-Ortiz GJ, Santa-Coloma TA, Carminatti H, Radrizzani M (2001) The rate of Tau synthesis is differentially regulated during postnatal development in mouse cerebellum. Cellular and molecular neurobiology 21:535-543.

Figure legends

Figure 1. Neuroprotein levels in hippocampus (A) and cerebral cortex (B) in neonatal male mice exposed to 6.1 or 9.2 mg PFHxS/kg bw (14 or 21 μmol PFHxS/kg bw) on PND 10, and euthanized 24h later. The data were analyzed using one-way ANOVA and Tukey's **HSD test**. The statistical differences are indicated as: ** significantly different vs. control, $p \leq 0.01$; * significantly different vs. control, $p \leq 0.05$. The data are presented as mean ± SD, n = 6-8.

Figure 2. Neuroprotein levels in hippocampus (A) and cerebral cortex (B) in neonatal female mice exposed to 6.1 or 9.2 mg PFHxS/kg bw (14 or 21 μmol PFHxS/kg bw) on PND 10, and euthanized 24h later. The data were analyzed using one-way ANOVA and Tukey's **HSD test**. The statistical differences are indicated as: *** significantly different vs. control, $p \leq 0.001$; ** significantly different vs. control, $p \leq 0.01$; * significantly different vs. control, $p \leq 0.05$. The data are presented as mean ± SD, n = 6-8.

Figure 3. Neuroprotein levels in hippocampus (A) and cerebral cortex (B) in adult male mice exposed to 6.1 or 9.2 mg PFHxS/kg bw (14 or 21 μmol PFHxS/kg bw) on PND 10, and euthanized 4 months later. The data were analyzed using one-way ANOVA and Tukey's **HSD test**. The statistical differences are indicated as: ** significantly different vs. control, $p \leq 0.01$. The data are presented as mean ± SD, n = 4-6.

Figure 4. Neuroprotein levels in hippocampus (A) and cerebral cortex (B) in adult female mice exposed to 6.1 or 9.2 mg PFHxS/kg bw (14 or 21 μmol PFHxS/kg bw) on PND 10, and euthanized 4 months later. The data were analyzed using one-way ANOVA, no statistically significant difference was measured between the control and different exposure treatments. The data are presented as mean ± SD, n = 6.

Figure 1.

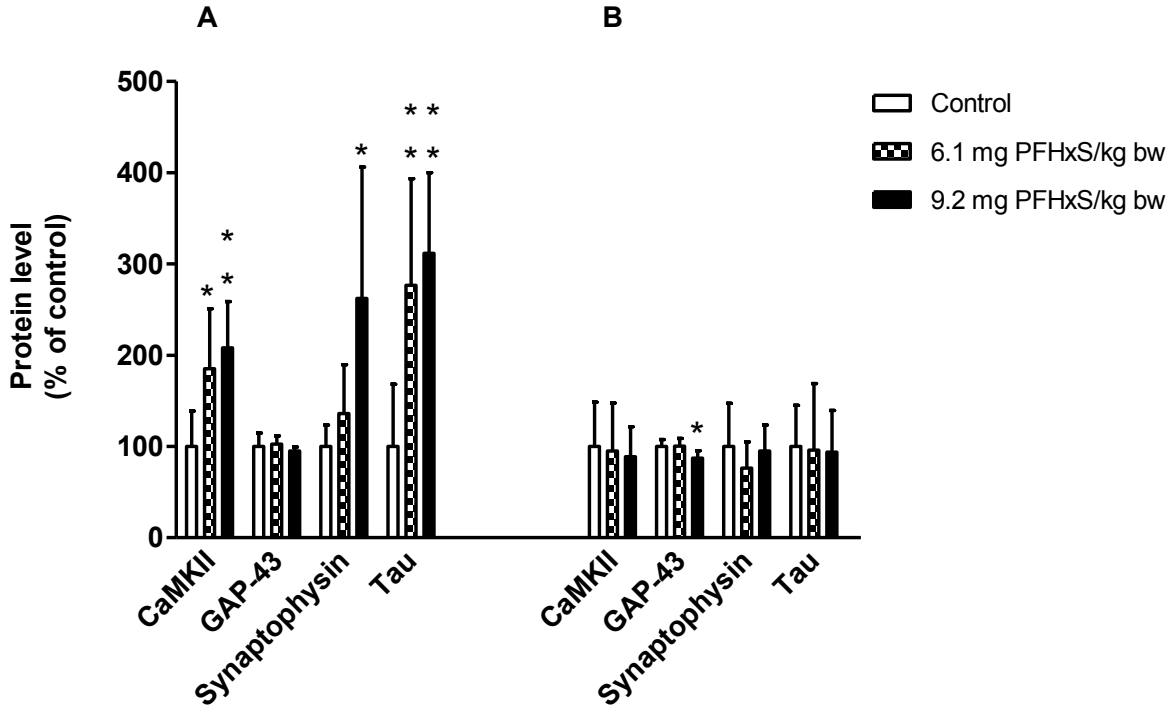
A
B
Protein level
(% of control)
500
400
300
200
100
0
Control
6.1 mg PFHxS/kg bw
9.2 mg PFHxS/kg bw
CaMKII
GAP-43
Synaptophysin
Tau
CaMKII
GAP-43
Synaptophysin
Tau

Figure 2.

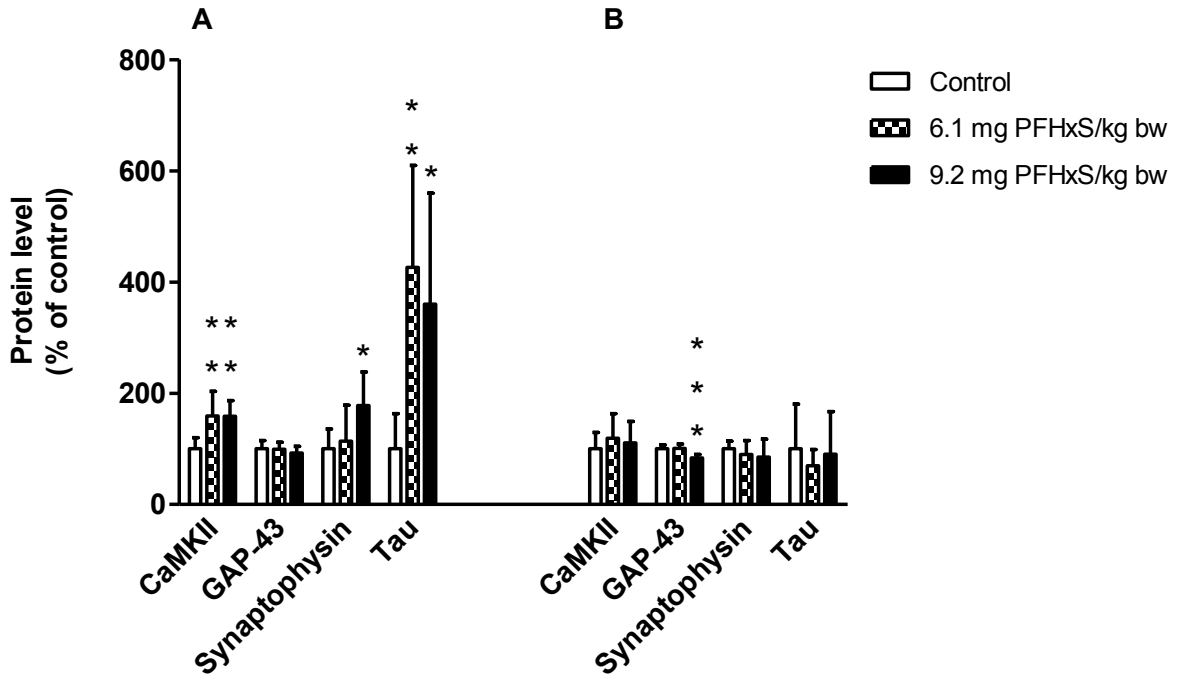
A
B
Protein level
(% of control)
800
600
400
200
0
Control
6.1 mg PFHxS/kg bw
9.2 mg PFHxS/kg bw
CaMKII
GAP-43
Synaptophysin
Tau
CaMKII
GAP-43
Synaptophysin
Tau

Figure 3.

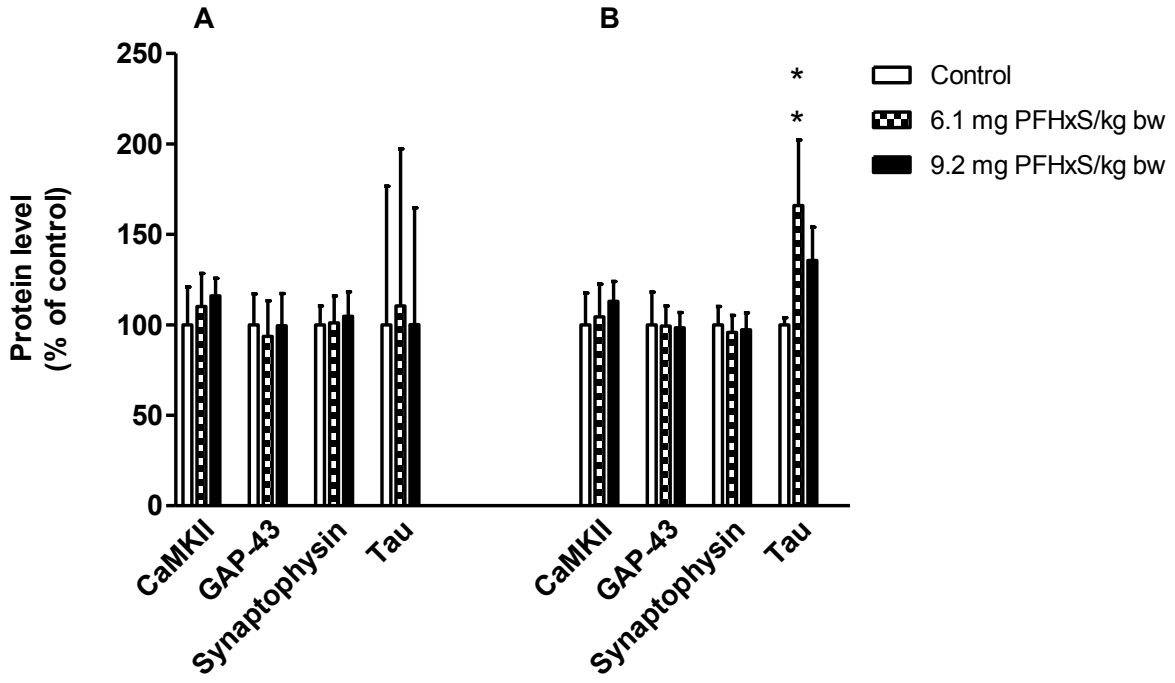
A
B
250
200
150
100
50
0
Protein level
(% of control)
*
*
Control
6.1 mg PFHxS/kg bw
9.2 mg PFHxS/kg bw
CaMKII
GAP-43
Synaptophysin
Tau
CaMKII
GAP-43
Synaptophysin
Tau

Figure 4.

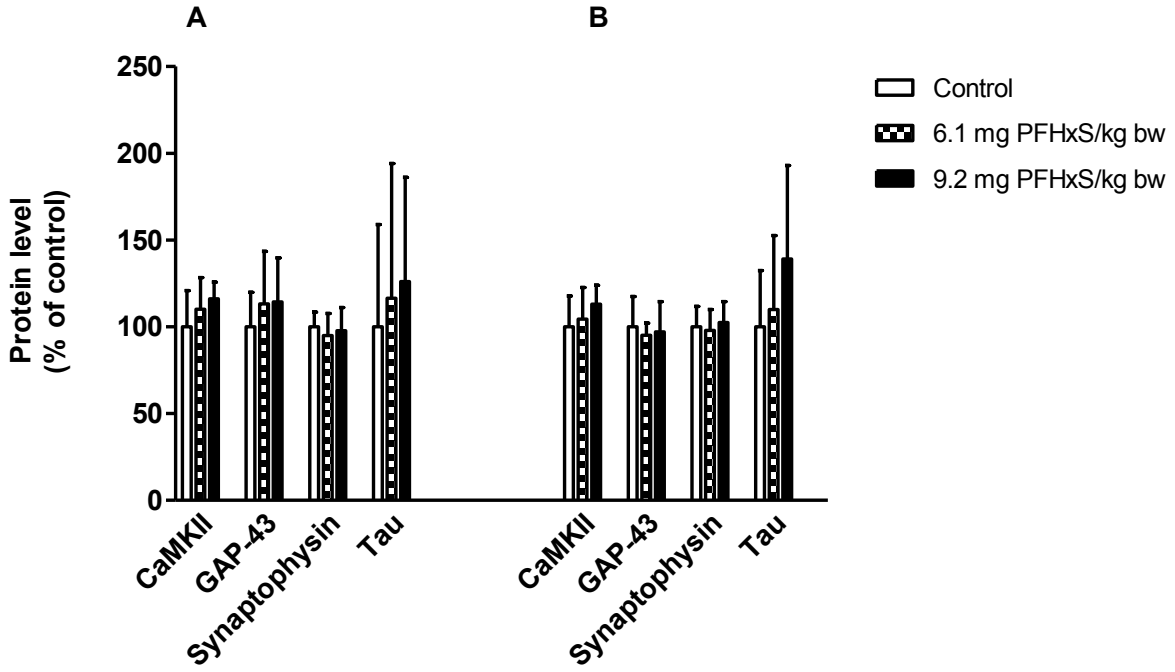
A
B
250
200
150
100
50
0
Protein level
(% of control)
CaMKII
GAP-43
Synaptophysin
Tau
CaMKII
GAP-43
Synaptophysin
Tau
Control
6.1 mg PFHxS/kg bw
9.2 mg PFHxS/kg bw

Table 1. BDNF levels in hippocampus and cerebral cortex in neonatal male and female mouse brains, 24h after exposure to 6.1 or 9.2 mg PFHxS/kg bw (14 or 21 μmol PFHxS/kg bw) at PND 10. The data were analyzed using one-way ANOVA and no statistically significant difference between the control and different exposure treatments **was** observed. The data are presented as mean ± SD, n = 5-6.

Exposure Dose	Males		Females	
	Hippocampus	**Cerebral Cortex**	**Hippocampus**	**Cerebral Cortex**
Vehicle	10.22 ± 1.31	27.18 ± 9.00	14.64 ± 1.91	12.27 ± 3.64
6.1mg PFHxS/kg bw	10.08 ± 1.29	29.63 ± 6.97	12.45 ± 4.76	12.40 ± 2.13
9.2 mg PFHxS/kg bw	8.06 ± 2.34	24.48 ± 8.35[a]	13.84 ± 1.66	11.14 ± 1.69[b]

[a] $0.05 \leq p \leq 0.1$

[b] $0.05 \leq p \leq 0.1$