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# Analysis of neuroproteins GluR1 and PSD-95 in neonatal mouse brain after exposure to PFHxS

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## *Abstract*

Perfluorinated compounds are commonly used as surfactants or water and stain repellents in many different materials in products like carpets, papers and textiles. Some types of these perfluorinated compounds, such as the perfluoroalkyl acids are very persistent in the environment and easily accumulate in all kinds of environmental compartments, including humans. Recent research has shown that the perfluoroalkyl acid PFHxS can alter adult spontaneous behavior and cognitive function in mice after a single oral exposure on postnatal day 10. In this study we wanted to investigate the possible effect on neuroproteins glutamate receptor 1 (GluR1) and post-synaptic density protein 95 (PSD-95) after a single oral dose to PFHxS on postnatal 10 day. This study showed no significant changes in levels of the two neuroproteins GluR1 or PSD-95 in cortex brain tissue.

## *Contents*

## *Introduction*

### Brian development

The human brain develops in a number of different stages spanning from around week 6 of gestation until around 20 years of postnatal life (Andersen, 2003). It begins with differentiation of progenitor cells to the specific cell type. Organization and migration to proper locations is coordinated by radial glia cells and different neurotrophic factors. Once a neuron has reached its final location they begin axonal and dendritic branching. This process continues until just before, birth where a rapid elimination of neurons takes place. A process believed to occur for optimizing synaptic transmission (Andersen, 2003). After birth synaptic formation continues until about 2 years of age and myelination up to 20 years of life (fig 1).

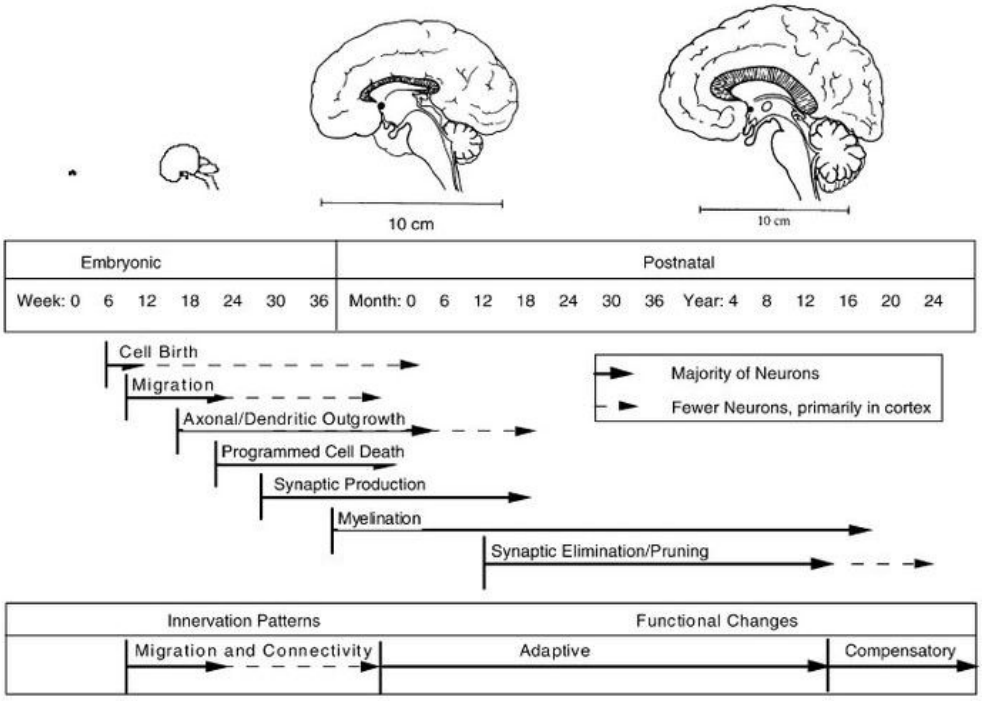


**Figure 1.** Chronologic figure over different events during brain development (Andersen S.L. 2003. Trajectories of Brain Development; Point of Vulnerability or Window of Opportunity? Neuroscience and Biobehavioral Reviews 27:3–18)

The brain growth spurt is a critical period in the development of the mammalian brain. The period is distinguished by a number of key features such as proliferation of glia cells and elongation and branching of dendrites and axons, synaptic formation and acquisition of new sensory and motor units as well as myelinization (Davidson and Dobbing, 1968; Bolles and Woods 1964).
The start and length of the brain growth spurt differ among species and in humans the period begins around the third trimester of pregnancy and proceeds until 2 years of age. In rodents on the other hand this period is postnatal and extends for the first 3-4 weeks of life (Dobbing and Sands, 1979).

Recent research has shown that this period of rapid brain development can be very vulnerable to exposure of different xenobiotics, such as perfluoroinated compounds and other industrial chemicals (Ahlbom *et al.*, 1995; Eriksson, 1997; Eriksson *et al.*, 1992; Eriksson *et al.*, 2000; Johansson *et al.,* 2008a).

## Perfluorohexane sulfonate

Perfluoroalkyl acids are a family of man-made, fluorinated organic compounds used as surfactants and water and stain repellents in carpets, paper, and textiles (NTP 2011). Perfluoroalkyl acids are resistant to biodegradation and persistent in the environment (Lau *et al.,* 2004), giving them the characteristics to bioaccumulate and biomagnify in the food chain, ultimately ending up in humans worldwide (Giesy and Kannan 2001; Yeung *et al.,* 2009).

The two most commonly detected and most studied perfluoroalkyl acids are perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA); however, PFOS, PFOA, and their precursors have been voluntarily phased out of production by major manufacturers (Martin *et al.,* 2010; Viberg and Eriksson 2011), but can of course still be found in the environment and humans. Short-chain perfluoroalkyl acids are currently being manufactured and used as substitutes for PFOS and PFOA due to their similar water-, oil-, and stain-resistant properties (Buck *et al.,* 2011). Perfluorohexane sulfonate (PFHxS) is a short-chain perfluoroalkyl acid, which also have been detected in the outdoor and indoor environments and in humans, including umbilical cord blood, human milk and child serum from all over the world (Arbuckle *et al.,* 2012; Glynn *et al.,* 2012; Kärrman *et al.,* 2007; Schecter *et al.,* 2012; So *et al.,* 2006; Stein *et al.,* 2012).
Exposure to PFHxS on postnatal day (PND) 10 has shown to result in changed spontaneous behavior and nicotine-induced behavior in adult mice (Viberg *et al.,* 2012). It is unclear whether this is caused by altered levels of neuroproteins or not, but other perfluoroinated compounds as PFOS and PFOA have been shown to cause significantly increased levels of the neuroproteins CaMKII, GAP-43, and synaptophysin in hippocampus of neonatal mice (Johansson *et al.,* 2009).

## Neuroproteins; Glutamate receptor 1 and Post-synaptic density protein 95

Glutamate receptors are the predominant excitatory receptors in the mammalian brain. The receptors are heteromeric proteins consisting of multiple subunits, which form transmembrane ligand-gated ion channels (Dingledine *et al*., 1999). The synaptic receptors are vital for the communication between neurons at the synaptic cleft. The ionotropic glutamate receptors are categorized into three different groups based on their pharmacological agonists; *N*-Methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate. The AMPA receptor is encoded by four different genes, one for each of its subunits (Hollmann and Heinemann, 1994; Wisden and Seeburg, 1993).

During brain development the glutamate receptor undergoes a number of biomolecular changes. The AMPA receptors subunit glutamate receptor 1 (GluR1) differs in abundance in different brain regions during development. In rat brain tissue there was a low expression of the subunit perinatally in neocortex and hippocampus, which then increased with age. The opposite was the case in other brain regions such as striatum, where a high expression first was observed, followed by a decrease along with maturation (Martin *et al.,* 1998). There is not only occurring changes in expression of one subunit, but also shifts in abundance of two subunits. The NMDA receptor consists of two subunits, one of them, the NR2 existing in different isoforms. During brain development of mice the NR2B subunit is solely the most abundant form of the subunit, but shortly after birth a switch occurs. The number of NR2A subunits greatly increases and later also becomes the most abundant one of the two isoforms (Liu *et al.,* 2004).

Studies have shown that alterations of the AMPA receptor subunit GluR1 have been strongly associated with behavioural disorders such as Schizophrenia and bipolar disorder (Kerner *et al.,* 2009;Magri *et al.,* 2006; O'Connor and Hemby, 2007).

Post-synaptic density protein 95 (PSD-95), is almost solely located in the post-synaptic density (Hunt *et al*., 1992), and has a molecular weight of 95 kDa, hence its name. The protein is member of the membrane-associated guanylate kinase (MAGUK) family, and like all members of the MAGUK family consists of one SH3 (SRC Homology 3) domain (Woods and Bryant, 1991; Cho *et al*., 1992; Willott *et al*., 1993) and three PDZ (acronym for the first letters of three proteins which possess domain) domains (Cho *et al*., 1992).

PSD-95 is associated with several functions in the post-synaptic density. In general it serves as a scaffolding protein, which links other proteins and signalling molecules in the post-synaptic density (Wenthold *et al*., 2003; Kim and Sheng, 2004). PSD-95 is believed to control AMPA receptor incorporation during long-term potentiation (LTP) (Ehrlich and Malinow, 2004), a process thought to be the main path of learning.
The protein is also required for localization of NMDA receptors in the synapse (Zerangu *et al*., 1999; Quinlan *et al*., 1999) and thus increases the number of ion channels and the channels opening rate in NMDA receptors (Ying Lin *et al.,* 2004) and also anchor and cluster them.
Alterations in levels of PSD-95 have been associated with different disturbance disorders and are also likely to affect the function of AMPA receptors due to its crucial role at the post-synaptic density (Jean-Claude *et al.,* 2006; Feyder *et al.,* 2010).

## *Aims*

The aims of this study were to 1) verify the antibody specificity against GluR1 and PSD-95, 2) investigate possible effects of a single dose exposure to PFHxS on PND 10 mice, on neuroproteins GluR1 and PSD-95.

## *Materials and Methods*

### Animal treatment

Pregnant NMRI mice were purchased from B&K, Sollentuna, Sweden and were housed individually in plastic cages in a room with a temperature of 22°C. In order to simulate natural circadian rythm the mice were held in 12/12-hour cycle of light and dark. The animals were supplied with standardized pellet food (Lactamin, Stockholm, Sweden) and tap water *ad libitum*. The size of the litters was adjusted to 10-14 pups, within the first 48 h after birth. The litters contained pups of both sexes. The males and females were kept in their litters (in treatment groups) with their siblings. Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), after approval from the local ethical committees (Uppsala University and Agricultural Research Council) and by the Swedish Committee for Ethical Experiments on Laboratory Animals, approval number C185/9.

## Exposure

Perfluorohexane sulfonate (PFHxS potassium salt, purity > 98%, linear formula $C_6F_{13}KO_3S$, CAS number 3871-99-6) was purchased from Sigma-Aldrich, Stockholm Sweden. PFHxS was dissolved in a mixture of egg lecithin (Merck, Darmstadt, Germany) and peanut oil (Oleum arachidis) (1:10) and then sonicated with water to yield a 20% (w:w) fat emulsion vehicle containing 0.092 or 0.92 mg PFHxS/ml (1.4 or 2.1µmol/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 the fat content of mouse milk is around 14%.

At the age of 10 days, both male and female 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. These doses are the same on a molar basis as in earlier studies of environmental pollutants, including the PFCs PFOA and PFOS, inducing adult behavioral disturbances (Eriksson 1997; Johansson *et al.* 2009; Johansson *et al.* 2008a; Viberg *et al.* 2003b). Control mice, male and female, received 10 ml of the 20% fat emulsion vehicle per kg body weight. The control group and the three different PFHxS groups each consisted of at least 15 animals from 3 different litters.

Animals were sacrificed 24 hours after exposure to PFHxS. Brains were then dissected on an ice-cold glass plate and the cortex and hippocampus were collected (Glowinski and Iversen, 1966), flash frozen in liquid nitrogen and stored at -80°C until assayed.

Cortices were homogenized, using the Potter-Elvehjem homogenizer, in cold RIPA cell lysis buffer (Assay Design) (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1 mM EGTA, 1% Triton X-100, 20 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1% sodium deoxycholate) with addition of 0.5% protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem). The homogenates were then centrifuged at 14,000 × g for 15 min at 4°C, and the protein content of the supernatant was measured using bicinchoninic acid protein assay reagent (Pierce), a method for colorimetric detection and quantification of total protein. Subsequently, the supernatant was stored at -80°C until use.

## Determination of antibody specificity

The determination of the antibody specificity was evaluated in a Western Blot analysis. The analysis was performed according to Gardoni *et al* 2006 with modifications.

The samples were denaturated in 95ºC water in 8 minutes and vortexed in order to avoid condensation. Before samples were added to SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (Pierce 10% Precise protein gels) a dyeing was made (dilution 1:1) with Sample Buffer (Laemli Buffer with 5%βME). The SDS-PAGE was conducted for15 min at 120 V and then an additional 60 minutes at 125 V. After the SDS-PAGE the gel was washed in Transfer Buffer (Bjerrum and Schafer-Nielsen, 48mM Tris-Base, 39mM Glycine, 0.00375% SDS, 20% Methanol) for 5 minutes and then an additional 15 minutes in transfer buffer. The proteins were transferred to the nitrocellulose membrane (BIORAD trans-Blot Tansfer Medium 0.2 µm) for 35 minutes at 20 V semi dry. Then washes for 2x10 minutes in Tween-Rinse (Tris-buffered saline (TBS) with 0.03% Tween-20) were conducted.
The nitrocellulose membrane was blocked in Tween-Milk Blocking Buffer (TBS with 0.03% Tween-20 and 5% BIORAD non-fat dry milk) for 60 min and then incubated with primary

antibody GluR1 (diluted in Tween-Milk: 1:1000, Millipore AB1504) or PSD-95 (0.1 µg/mL, Millipore, MABN68) over night at 4ºC.

After completed incubation the membrane was equilibrated to room temperature and washed in TBS, first two times without any attended time and then 3x5 minutes, and finally 1x10 minutes. When finished rinsing, the membrane was incubated for 60 min in horseradish peroxidase-conjugated secondary antibody, diluted in Tween-Milk blocking buffer (goat anti-rabbit, for polyclonal antibodies, diluted 1:20000 (Pierce, Rockford, IL); goat anti-mouse, for monoclonal antibodies, diluted 1:20000 (Pierce KPL 074-1806)). After incubation rigorous rinsing was performed in Tween-Wash (TBS with 0.1% Tween-20): first two times without any attended time and then 1x10 minutes, 3x5 minutes and finally 1x10 minutes. One last light-protected incubation was made in chemiluminescent substrate (Pierce, Super Signal West Dura) for 5 minutes in room temperature. The antigen-antibody complex was detected with LAS-1000 (Fuji Film, Tokyo, Japan). The intensity of bonds was quantified with the software IR-LAS 1000 Pro Image Reader (Fuji Film).

For determination of antibody specificity and linearity a titration was made. Several different protein amounts was loaded to gel with for each membrane different dilutions of primary antibody. For PFHxS exposed homogenates protein amounts of 14 µg was used for GluR1 with 1:1000 primary antibody dilution and 4 µg for PSD-95 with a concentration of 0.1 µg/mL.

## *Results*

### Determination of antibody specificity

To determine primary antibody specificity and find an appropriate protein load for future western blot analysis and slot blots, a titration was made. Ten different protein amounts were loaded for both GluR1 and PSD-95. All bonds, independent of load, was found at the correct molecular weight for both proteins, 106 kDa for GluR1 and 95 kDa for PSD-95 (figs. 1 & 3).

GluR1 showed single bonds for all protein amounts (Fig. 1). For GluR1 14 µg of protein was considered as a good amount for analysis of levels of GluR1 after PFHxS exposure. The 10 increasing protein amounts (ranging from 2 to 20 µg with an interval of 2 µg) gave an $R^2$-value of 0.9673, for the linear equation of the first order, as presented in figure 2. This means that there is a high correlation between the protein load and the chemiluminescence value, meaning that an increase in chemiluminescence will correspond to an increase in the proteinamount.

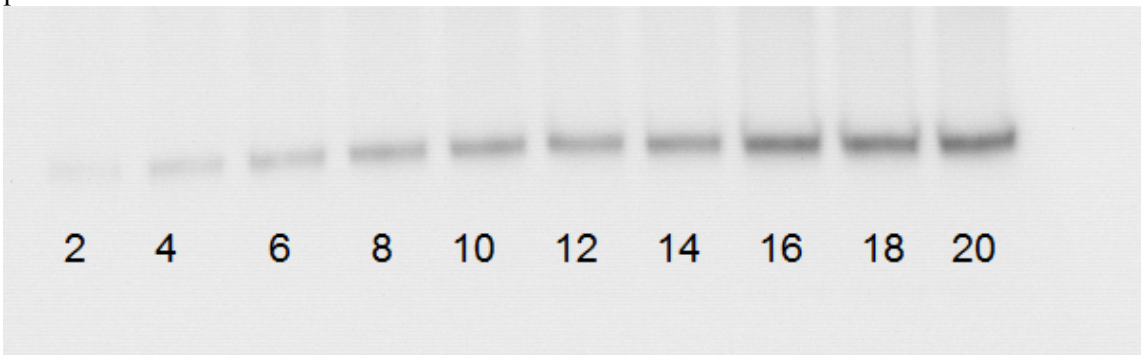


**Figure 1.** Western blot titration for GluR1. Numbers indicate protein amount (µg) loaded in each well. Rabbit polyclonal GluR1 antibody (1:1000, Millipore AB1504).

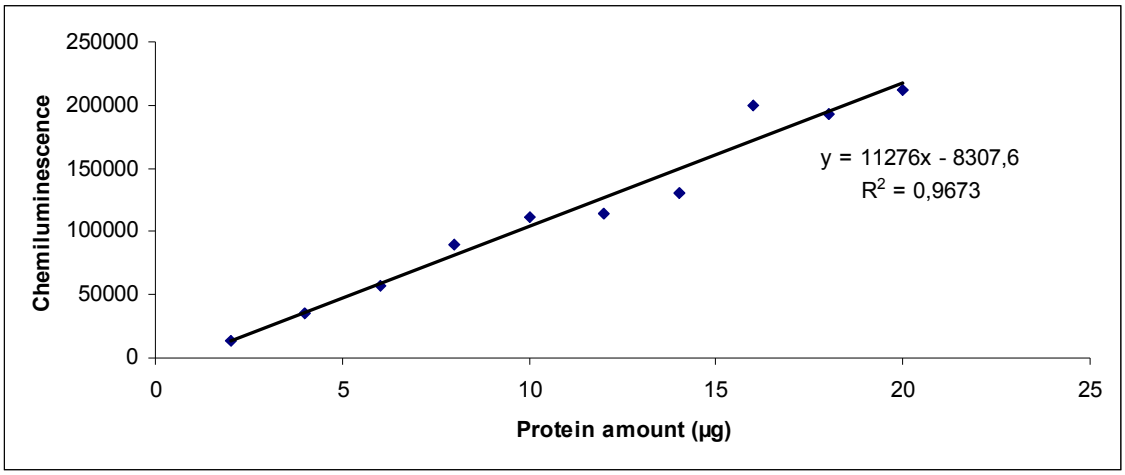


**Figure 2.** Linear response curve of the western blot titration for GluR1 with 10 different protein amounts (2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 µg).

PSD-95 showed strong visible bonds for all protein amounts between 2 and 10 µg. As seen in fig. 3 protein amounts over 5 µg was considered too strong, thus 4 µg was the protein amount used for analysis of levels of PSD-95 after PFHxS exposure.
The 10 increasing protein amounts (ranging from 1 to 10 µg with an interval of 1 µg) gave a $R^2$-value of 0.9489 for the linear equation of the first order, as presented in figure 4. This means that there is a high correlation between the protein load and the chemiluminescence value, meaning that an increase in chemiluminescence will correspond to an increase in the protein amount.

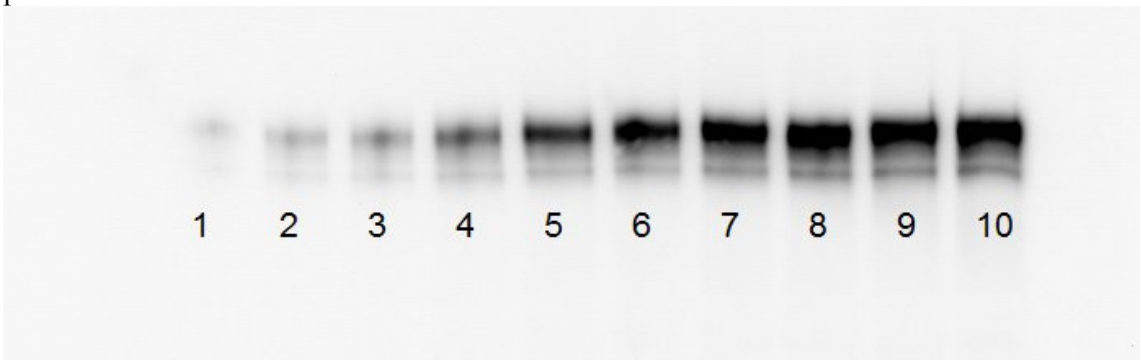


**Figure 3.** Western blot titration for PSD-95. Numbers indicate protein amount (µg) loaded in each well. Mouse monoclonal PSD-95 antibody (0.1 µg/ml, Millipore MABN68).

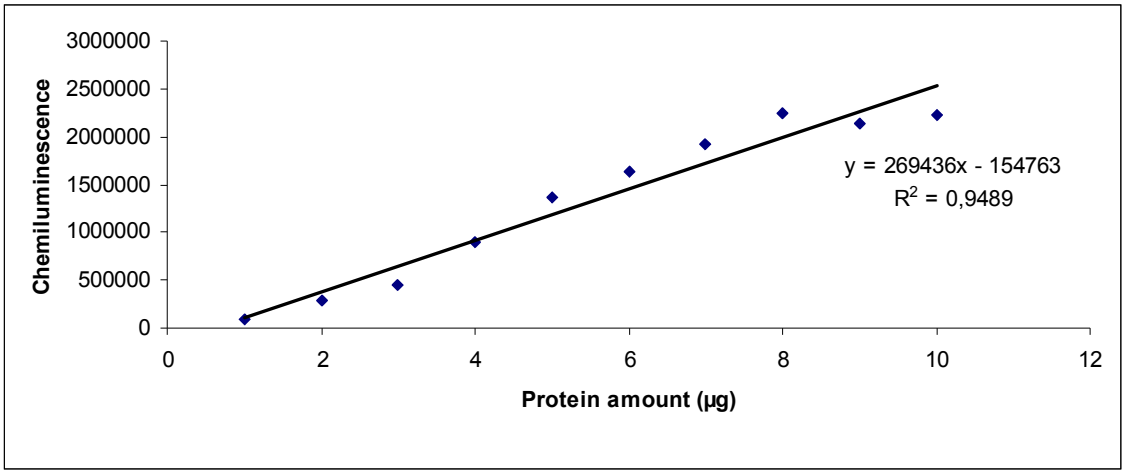


**Figure 4.** Linear response curve of the western blot titration for PSD-95 with 10 different protein amounts (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µg).

## Effects of PFHxS on GluR1 and PSD-95 in cortex

Levels of GluR1 and PSD-95 were measured in cortex of brain tissues from mice sacrificed 24h after a single oral dose of either 6.1 (low dose) or 9.2 (high dose) mg PFHxS/kg body weight on PND 10. Statistical analysis of the protein levels did not show any significant differences between the PFHxS exposed animals and the control animals.
As seen in figure 5 exposure to 6.1 mg PFHxS resulted in a 31% increase and exposure to 9.2 mg PFHxS/kg body weight resulted in a 36% increase of GluR1 levels compared to control, but the statistical analysis with one-way ANOVA gave a p-value < 0.15.

As seen in figure 6 exposure to 6.1 mg PFHxS/kg body weight resulted in a 17% increase of PSD-95 levels compared to control, but the statistical analysis with one-way ANOVA gave a p-value < 0.28.

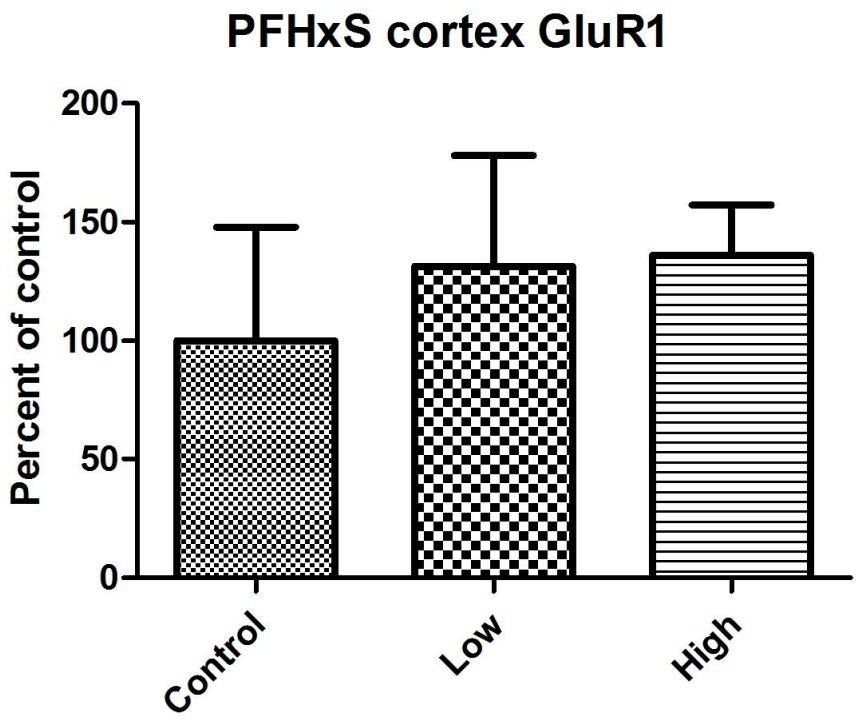


**Figure 5.** Levels of GluR1 expressed as percent of control in cortex tissue from mice exposed to a single oral dose of 6.1 (low dose) or 9.2 (high dose) mg PFHxS/kg body weight on PND 10. The data were treated with a one-way ANOVA ($p$> 0.05 PFHxS-treated vs. control). The height of the bars represents the mean value ± SD.

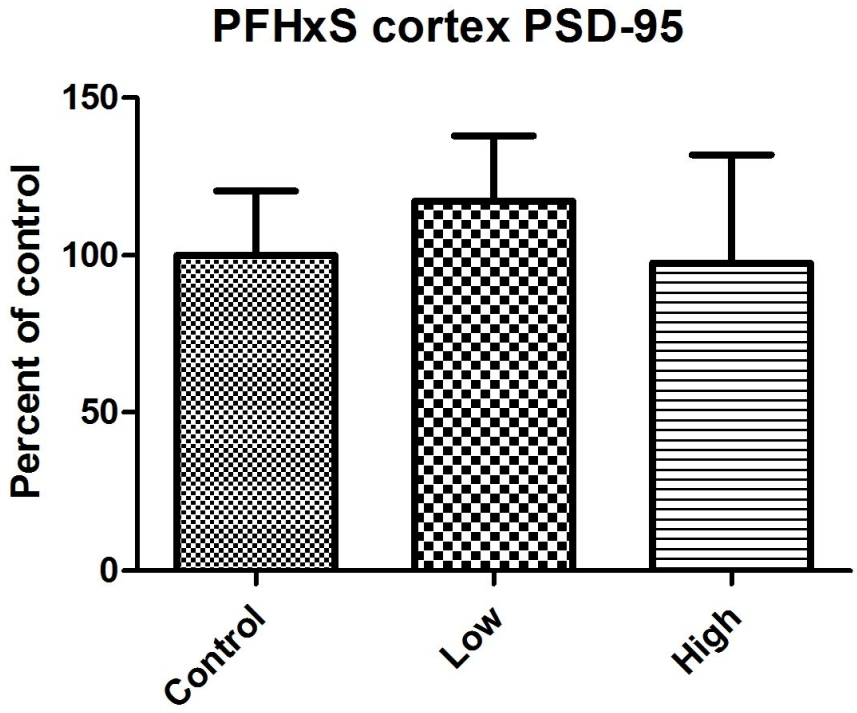


**Figure 6.** Levels of PSD-95 expressed as percent of control in cortex tissue from mice exposed to a single oral dose of 6.1 (low dose) or 9.2 (high dose) mg PFHxS/kg body weight on PND 10. The data were treated with a one-way ANOVA ($p$> 0.05 PFHxS-treated vs. control). The height of the bars represents the mean value ± SD.

## Discussion

The western blot titration demonstrated suitable protein amounts of 14 µg for GluR1 and 4 µg for PSD-95. Both antibodies indicated single bonds at correct molecular weights, showing high specificity towards proper proteins. This combined with good linear response makes them suitable for not only further western blot analysis, but also slot blot analysis, where high antibody specificity is required due to the method's lack of separation by electrophoresis properties, such as size and charge.

PFHxS has shown to induce behavioral changes in mice after a single oral exposure on postnatal day 10. Viberg *et al*. showed that after the same exposure as in this study PFHxS resulted in both impaired spontaneous behavior and changed nicotine-induced behavior in adult mice (Viberg *et al.,* 2012). Since both GluR1 and PSD-95 has been connected to disturbance disorders, such as bipolar disorder, schizophrenia and autism, it was of interest to investigate whether PFHxS could affect levels of these proteins and induce behavioural changes (Kerner *et al.,* 2009;Magri *et al.,* 2006; O'Connor and Hemby, 2007; Jean-Claude *et al.,* 2006; Feyder *et al.,* 2010). However since this study showed no significant changes in the levels of PSD-95 or GluR1 in cortex tissue, the behavioral changes seen in the above mentioned study is not likely to be caused by alterations of these neuroproteins in cortex tissue.

In this study there was no significant change in levels of GluR1 or PSD-95 in mice cerebral cortex. Previous studies has shown that neonatal exposure to other perfluoroinated compounds, such as PFOS and PFOA, during brain growth spurt results in protein changes. Johansson *et al.* showed that a single oral dose of 21 µmol PFOS or PFOA/kg body weight, given on PND 10, resulted in significant increases of the neuroproteins CaMKII, GAP-43, and synaptophysin in hippocampus, and an increase of synaptophysin and tau in cerebral cortex (Johansson *et al.,* 2009). In the present study only cortex tissue was examined, whereas in other studies which has shown changes in neuroproteins, these changes have often occurred in hippocampus tissue, but not always in cortex tissue (Johansson *et al.*, 2009; Viberg *et al.,* 2009). So, since PFHxS can induce behavioural disturbances and other perfluorinated compounds has changed neuroprotein levels and induced behavioural disturbances (Johansson *et al.,* 2008a) future studies should focus on investigating possible differences in GluR1 and PSD-95 (and other important neuroproteins) levels in hippocampus tissue.

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