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Neonatal exposure to perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) causes neurobehavioural defects in adult mice

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Abstract

Perfluorinated compounds (PFCs) are found in applications such oil/water repellents for clothing fabrics, carpets, food packaging, lubricants, surfactants and fire extinguishers. PFCs are persistent in the environment. They have been found in humans and in wildlife.

We reported earlier that persistent organic pollutants (POPs), such as DDT, PCBs and BFRs, caused developmental neurotoxic defects in mice, manifested as persistent aberrations in spontaneous behaviour, habituation capability, learning and memory, and changes in the cholinergic system in adults, when mice were exposed during a critical period of neonatal brain development.

The present study was conducted to see whether PFCs can cause similar developmental neurotoxic effects as earlier observed for POPs as PCBs and PBDEs. NMRI male mice were exposed to a single-oral dose, either 1.4 or 21 μmol/kg body weight of PFOS (0.75 or 11.3 mg), PFOA (0.58 or 8.70 mg), or PFDA (0.72 or 10.8 mg), via a metal gastric-tube at the age of 10 days. The control animals received in the same manner 10 ml/kg body weight of the 20% fat emulsion vehicle. Spontaneous behaviour (locomotion, rearing, and total activity), and habituation were observed in 2- and 4-month-old mice. The susceptibility of the cholinergic system was explored in a nicotine-induced spontaneous behaviour test in 4-month-old mice. Deranged spontaneous behaviour was observed in mice exposed to PFOS and PFOA, manifested as reduced and/or lack of habituation and hyperactivity in adult mice. These effects were also seen to worse with age. Neonatal exposure to PFOS and PFOA affected the cholinergic system, manifested as a hypoactive response to nicotine, compared to a hyperactive response to nicotine in controls. These developmental neurotoxic effects are similar to those we reported earlier for PCBs and PBDEs. This suggests that PFOS and PFOA be included in the group of POPs known to be developmental neurotoxicants.

Keywords: PFC; PFOS; PFOA; PFDA; Developmental neurotoxicity; Behaviour

1. Introduction

Perfluorinated compounds (PFCs) are found in applications such oil/water repellents for clothing fabrics and carpets, paper coatings, food packaging, lubricants, surfactants and fire extinguishers (Renner, 2001; Seacat et al., 2002). PFCs are heat stable, not subject of photolysis, and their extreme stability makes them practically non-biodegradable and very persistent in the environment. PFCs are so stable because fluorine in bound form is one of the most stable elements (Key et al., 1997, 1998). In 2001, it was published that one such PFC, perfluorooctane sulfonate (PFOS), the stable and extremely persistent end-product of degradation of various sulfonated fluorochemicals, is universally present and bioaccumulating (Giesy and Kannan, 2001; Kannan et al., 2001). PFOS is a widespread environmental pollutant. It has been detected in the Arctic, the Antarctic and the Pacific Ocean (Giesy and Kannan, 2001; Kannan et al., 2001; Martin et al., 2004). PFOS has been found in high concentrations in liver tissue in top predators (Giesy and Kannan, 2001; Smithwick et al., 2005). PFCs do not accumulate in lipids such as lipophilic POPs, whereas PFOS and PFOA are associated with proteins (Han et al., 2003; Jones et al., 2003) and are found in liver and plasma (OECD, 2002). PFOA and PFDA have been reported in various tissues of arctic biota (Ellis et al., 2004; Martin et al., 2004; Smithwick et al., 2005). These chemicals are widespread in the northern

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hemisphere and are generally more concentrated in the more populated and industrial regions. PFCs were first detected in human tissues almost 40 years ago (Taves, 1968). Since then several reports have been published of measurements of PFCs in blood, plasma and serum, showing that humans are exposed to numerous PFCs (Calafat et al., 2006; Harada et al., 2007; Kannan et al., 2004; Kärrman et al., 2006b; Olsen et al., 2004b).

The potential toxic effects of PFOS, PFOA and PFDA are summarised in (EPA, 2003; Kennedy et al., 2004; Kudo and Kawashima, 2003; Lau et al., 2004; OECD, 2002).

Lau et al. reported developmental neurotoxic effects in rats and mice following PFOS exposure throughout pregnancy. The pups were born alive, active, and pink, but 30–60 min after birth, following exposure to 10 mg PFOS/kg the neonates became pale, inactive, and moribund. All died soon afterwards. Survival rates of the neonates improved with lower doses of PFOS, with mice exposed to 3 mg/kg showed a 50% survival rate. It was also reported that the development of learning was not affected by PFOS exposure (Lau et al., 2003).

PFOA and PFDA have been reported to be peroxisome proliferators (Harrison et al., 1988; Ikeda et al., 1985). In rats PFDA has been seen to cause anorexia, alteration of fatty acid metabolism, bradycardia, hepatotoxicity, reduction of circulating thyroid hormone, and hypothermia (George and Andersen, 1986; Gutshall et al., 1988, 1989; Langley and Pilcher, 1985; Pilcher and Langley, 1986; Pilcher et al., 1987; Singer et al., 1990).

During their development, mammals can be exposed to toxicants while they are fetuses (via maternal intake of toxicants), during the neonatal period (via intake of mother's milk), by direct ingestion, or contact. Mammals can be affected by toxicants during several critical phases in their development, leading to malformations and disabilities. The development of the central nervous system (CNS) can be divided roughly into two major phases. The first includes the embryonic development of the brain, when it assumes its general shape and precursors of glia and neurons proliferate. Embryonic development of the human brain takes place during the first 2 months of gestation, occupying one-fifth of that period. In mice, however, embryonic development occupies four-fifths of gestation. The second phase of brain development, known as the ''brain growth spurt'' (BGS) (Davison and Dobbing, 1968), comprises a series of rapid developmental changes, including maturation of dendritic and axonal outgrowth, synaptogenesis, establishment of neuronal connections, proliferation of glia cells with accompanying myelinization (Davison and Dobbing, 1968; Kolb and Whishaw, 1989). The developing human brain is inherently much more susceptible (Davison and Dobbing, 1968) and appears to be more susceptible to injury caused by toxic agents than the brain of an adult (Grandjean and Landrigan, 2006). Due to the complexity of mammalian brain development, windows of unique susceptibility to interference by toxicants can arise that have no counterpart in the mature brain, or in any other organ. 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). The time frame for the BGS occurs at different times for different mammalian species. In humans it begins during the third trimester of pregnancy and continues throughout the first 2 years of life. In mice and rats on the other hand, it is postnatal, spanning the first 3–4 weeks of neonatal life and peaking around postnatal day 10. One of the major transmitter systems in the brain is the cholinergic system. The role of the cholinergic system and its transmitter, acetylcholine, in cognitive function is well known (Karczmar, 1975; Levin and Simon, 1998; Nabeshima, 1993; Paterson and Nordberg, 2000; Perry et al., 1999). In the developing brain of mice and rats, the ontogenesis of most of the cholinergic system takes place during the fist 3–4 weeks after birth. During this period, variables such as ChAT, AChE, sodium-dependent choline uptake and muscarinic and nicotinic receptors increase in various brain regions (Coyle and Yamamura, 1976; Falkeborn et al., 1983; Fiedler et al., 1987; Hohmann et al., 1995; Kuhar et al., 1980).

We have seen in previous studies that low doses of persistent environmental contaminants can disrupt adult brain function, manifested as deranged spontaneous behaviour, lack and/or reduced habituation, learning and memory defects, and changes in the cholinergic system of mice, when exposed during the BGS. Examples of environmental contaminants that induce such effects are dichlorodiphenyltrichloroethane (DDT), certain polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs) (Eriksson, 1997; Eriksson and Fredriksson, 1998; Eriksson et al., 2001a,b; Viberg et al., 2002, 2003a, 2004, 2005). PCBs and PBDE have been shown to affect the developing cholinergic system by reducing the density of cholinergic nicotinic receptors in the adult brain of mice and alter the cholinergic response to nicotine at adult age (Eriksson and Fredriksson, 1996b; Viberg et al., 2002).

Recent research indicates that indoor air and dust can be a route for exposure to PFCs. Children, especially those who crawl, ingest more dust than adults. The fact that PFCs are found in the environment and humans, and even to a higher or equal level in younger people compared to older ones, and also their presence in human milk calls for an investigation into the developmental neurotoxic effects of PFCs, *e.g.*, PFOS, PFOA, and PFDA, during the neonatal period.

The present study was undertaken to explore the developmental neurotoxic effects in mice neonatally exposed to PFOS, PFOA, or PFDA, during a critical period of brain development on (1) spontaneous behaviour and habituation (2) susceptibility of the cholinergic system, and (3) whether disrupted spontaneous behaviour can involve anxiety-like behaviour by studying the experimental animals' performance in the elevated plus-maze.

2. Materials and methods

2.1. Chemicals and animals

Pregnant NMRI (Naval Medical Research Institute) mice (from B&K, Sollentuna, Sweden) were housed individually in plastic cages in a room with an ambient temperature of 22 °C

and a 12/12-h light/dark cycle. The animals were supplied with standardized pellets (Lactamin, Stockholm, Sweden) and tap water *ad libitum*. The pregnant NMRI mice were checked for parturition twice daily (08:00 and 18:00 h). The day of birth was designated day 0; pups born at night were assigned day 0 when checked at 08:00 h. Each litter was adjusted to 10–12 pups, within the first 48 h after birth, by killing off the excess pups. Litters contained pups of both sexes during the neonatal period and no sex-based separation was made among preweanlings. At 4–5 weeks, all females were killed and the male siblings were kept in litters (in treatment groups) and raised in groups of 4–7, in a room for males only, and under the conditions detailed above. In order to compare with our earlier studies on PCBs and PBDEs, both males and females were exposed to the PFCs, but only males were used for the neurotoxicological recordings.

Perfluorooctane sulfonate (PFOS, potassium salt) purity ≥98%, perfluorooctanoic acid (PFOA) purity 96% and perfluorodecanoic acid (PFDA) purity 98%, were purchased from Sigma–Aldrich. The substances were dissolved in a mixture of egg lecithin (Merck, Darmstadt, Germany) and peanut oil (*Oleum arachidis*) (1:10) and the then sonicated together with water to yield a 20% (w:w) fat emulsion vehicle containing PFOS (perfluorooctanesulfonic acid; 0.075 or 1.13 mg/ml), PFOA (perfluorooctanoic acid; 0.058 or 0.870 mg/ml), or PFDA (perfluorodecanoic acid; 0.072 or 1.08 mg/ml).

Neonatal NMRI male mice exposed to a single-oral dose, either 1.4 or 21 μmol/kg body weight of PFOS (0.75 or 11.3 mg/kg body weight), PFOA (0.58 or 8.70 mg/kg body weight), or PFDA (0.72 or 10.8 mg/kg body weight), via a metal gastric-tube at the age of 10 days. The control animals received in the same manner 10 ml/kg body weight of the 20% fat emulsion vehicle. The two doses are within the dose range used in our earlier studies regarding developmental neurotoxicity of PCBs and PBDEs. In the same manner, mice serving as control animals were given 10 ml/kg body weight of a 20% fat emulsion vehicle.

This experimental design of neonatal exposure to xenobiotics has been used by our laboratory for several years and thereby generated historical controls as well as reproducible developmental neurotoxicological data on environmental toxicants (Eriksson, 1997, in press; Viberg et al., 2006). In this neonatal animal model each of the different treatment groups comprise mice from 3 to 4 different litters. Randomly selecting animals from at least three different litters will have the same statistical effect and power compared to the use of litter based studies to evaluate developmental neurotoxicity in neonatal mice (Eriksson and Viberg, 2005; Eriksson et al., 2005).

2.2. Behavioural tests

2.2.1. Spontaneous behaviour tests

Spontaneous behaviour was tested in males aged of 2 and 4 months. The animals were tested between 08:00 and 13:00 h, under the same light and temperature conditions as in their cages. Ten mice were picked randomly from three to five different litters in each treatment group, and were only tested once on each test occasion. Motor activity was measured during a 60-min period, divided into three 20-min spells, in a automated device consisting of cages (40 cm × 25 cm × 15 cm) placed within two series of horizontal infrared beams (low and high level) (Rat-O-Matic, ADEA Elektronik AB, Uppsala, Sweden) (Fredriksson et al., 1997).

Locomotion. Counting took place when the mouse moved horizontally through the low-level grid of infrared beams aimed 10 mm above the bedded floor. *Rearing*. Vertical plane movement was registered at a rate of 4 counts per second, when a single high-level horizontal beam was interrupted, *i.e.*, the number of counts registered was proportional to time spent rearing. These beams were aimed 80 mm above the bedded floor. *Total activity*. All types of vibration within the test cage, *i.e.*, those caused by mouse movements, shaking (tremors) and grooming were registered by a gramophone-like pick-up mounted horizontally with a counterweight, connected to the test cage.

Habituation is a relatively simple, non-associative form of learning in situations where repeated measures of behaviour are monitored. In order to assess the extent of habituation to the activity test chambers over the successive 20-min spells, a habituation quotient for each mouse was derived by dividing the count during the third 20-min spell by that obtained during the first 20-min spell, multiplied by 100. In each case the result of each division was multiplied by 100 to provide a quotient representing the reduction in activity counts from the first to the third spell, for each mouse (Eriksson et al., 2001b; Fredriksson et al., 1992). This ratio from the locomotion and rearing variables was used to analyze altered habituation between 2- and 4-month-old mice.

2.2.2. Nicotine-induced behaviour test

Directly after the spontaneous test in the 4-month-old mice, their nicotine-induced behaviour was studied. The mice were picked up from the test cage and immediately given a single s.c. injection of 80 μg nicotine base/kg body weight [nicotine-bi-(+)-tartrate (Sigma, St. Louise, MO)]. This amount of nicotine is known to elicit an increased activity in normal adult NMRI mice (Eriksson et al., 2000; Nordberg et al., 1991; Viberg et al., 2002). Furthermore, there is a dose–response increase in activity in adult mice given 1–80 μg nicotine base/kg body weight (Ankarberg et al., 2001; Eriksson et al., 2000). Directly after the nicotine injection, the mice were returned to the test chamber. Their behaviour was measured during another 60-min period (60–120 min, in the same way as during spontaneous behaviour) in which spontaneous motor activity was recorded with regard to the three variables: locomotion, rearing, and total activity. The 60-min period was divided into three 20-min spells (60–80, 80–100, 100–120 min).

2.2.3. Elevated plus-maze test

This test is based on the assumption that as normal mice prefer an enclosed environment, compared with open space, it will give information about anxiety-like behaviour in animals.

The test procedure adopting Lister (1987) and measures the number of entries made into the open arms and time spent there. The plus-maze apparatus, made of plywood has two diametrically open arms (white floor with no wall, 30 cm × 6 cm) and two opposite closed arms (black floor with walls, 30 cm × 6 cm × 30 cm) mounted 50 cm above the floor. They were tested between 09:00 and 14:00. The animals were transferred to the testing laboratory in their 'home' cages at least 60 min before being subjected to the EPM. A test mouse was placed on the central platform (white floor, 6 cm × 6 cm) of the apparatus, facing the 'north' of the closed arms. A video camera was used to monitor the animal's behaviour. The number of entries into open and closed arms and the time spent on each of the arms were measured for 5 min. Arm entry was defined as all four paws on the arm. The maze apparatus was cleaned after each trial.

2.2.4. Statistical analysis

The locomotion, rearing and total activity data over three consecutive 20-min spells (treatment, time and treatment × time; between subjects, within-subjects and interaction factors, respectively), in the spontaneous behaviour test and in the nicotine-induced behaviour test, where submitted to a split-plot ANOVA design (Kirk, 1968). The major advantages with a split-plot design compared to randomized block factorial design are that the estimates of the within-block effects are usually more accurate than estimates of the between-block estimates. Because the average experimental error over all treatments is the same for both designs, the increased precision on within-block effects is obtained by killing precision on between-block.

The habituation quotients were submitted to a two-way ANOVA and in the EPM the time spent in the open arms and the entries into the open arms, and body weight were submitted to one-way ANOVA design (Kirk, 1968). Pair-wise testing between the different treatment groups was performed with Turkey's HSD test in the behavioural studies and Duncan's test regarding body weight (Kirk, 1968).

3. Results

There were no overt signs of clinical toxicity in the PFOS-, PFOA-, or PFDA-treated mice during the experimental period. The body weights (mean ± S.D.) of the 10-day-old mice did not differ significantly ($P > 0.05$) between the vehicle treated (5.76 ± 0.72 g) and the PFOS treated (1.4 μmol, 5.92 ± 1.07 g; 21 μmol, 6.06 ± 0.89 g), PFOA treated (1.4 μmol, 5.77 ± 0.75 g; 21 μmol, 5.66 ± 0.79 g) and PFDA treated (1.4 μmol, 5.83 ± 0.77 g; 21 μmol, 5.86 ± 0.51 g) mice. Neither were there any significant ($P > 0.05$) differences between the vehicle treated (22.0 ± 2.2 g) and the PFOS treated (1.4 μmol, 22.2 ± 3.2 g; 21 μmol, 23.5 ± 2.2 g), PFOA treated (1.4 μmol, 22.6 ± 1.9 g; 21 μmol, 21.1 ± 3.1 g) and PFDA treated (1.4 μmol, 22.4 ± 2.5 g; 21 μmol, 21.0 ± 1.7 g) in 4 weeks old mice. This shows that there were no acute effects on body weight gain.

3.1. Effects on spontaneous behaviour of adult animals

The results from the spontaneous behaviour variables, locomotion, rearing, and total activity, in 2- and 4-month-old male NMRI, after exposure to a single-oral dose of either 1.4 or 21 μmol PFOS, PFOA, or PFDA/kg body weight, at the age of 10 days, are shown in Figs. 1 and 2.

Two months after the neonatal exposure to PFOS, PFOA, or PFDA there were significant group × period interactions [$F_{12,126} = 11.47$, $F_{12,126} = 43.67$, $F_{12,126} = 31.54$] for the locomotion, rearing and total activity variables, respectively. Four months after exposure there were still significant group × period interactions [$F_{12,126} = 87.85$, $F_{12,126} = 448.91$, $F_{12,126} = 139.67$] for these variables. The control mice exposed to the 20% fat

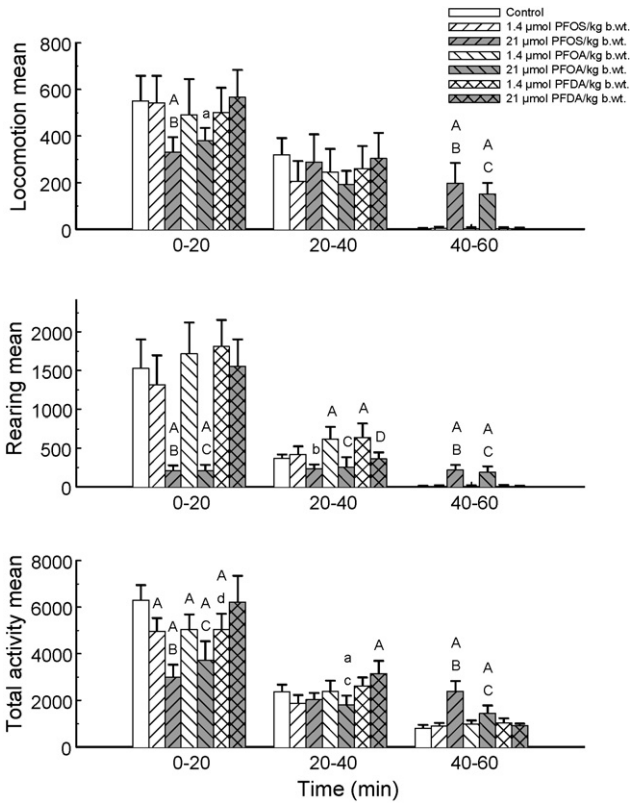

Fig. 1. Spontaneous behaviour of 2-month-old NMRI male mice exposed to a single-oral dose, either 1.4 or 21 μmol/kg body weight of PFOS (0.75 or 11.3 mg/kg body weight), PFOA (0.58 or 8.70 mg/kg body weight), or PFDA (0.72 or 10.8 mg/kg body weight), via a metal gastric-tube at the age of 10 days. The control animals received in the same manner 10 ml/kg body weight of the 20% fat emulsion vehicle. The data were subjected an ANOVA with split-plot design, and there were significant group × period interactions [$F_{12,126} = 11.47$, $F_{12,126} = 43.67$, $F_{12,126} = 31.54$] for locomotion, rearing and total activity variables, respectively. Pair-wise testing between PFOS-, PFOA-, PFDA-exposed animals and control animals was performed by using Tukey HSD tests. Statistical differences are indicated as (A) significantly different vs. controls $P \leq 0.01$; (a) significantly different vs. controls $P \leq 0.05$; (B) significantly different vs. 1.4 μmol PFOS/kg body weight $P \leq 0.01$; (b) significantly different vs. 1.4 μmol PFOS/kg body weight $P \leq 0.05$; (C) significantly different vs. 1.4 μmol PFOA/kg body weight $P \leq 0.01$; (c) significantly different vs. 1.4 μmol PFOA/kg body weight $P \leq 0.05$; (D) significantly different vs. 1.4 μmol PFDA/kg body weight $P \leq 0.01$; (d) significantly different vs. 21 μmol PFDA/kg body weight $P \leq 0.05$. Bar height represents mean value ± S.D.

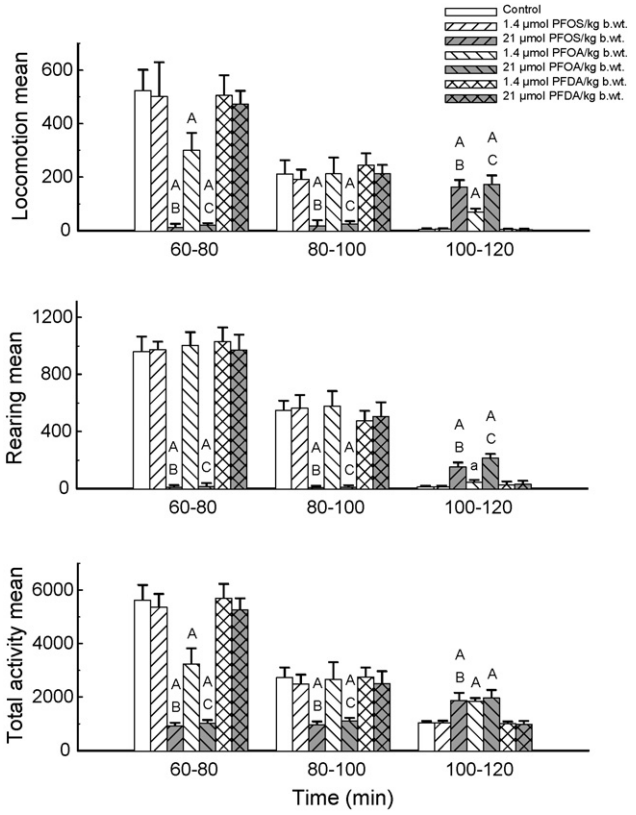

Fig. 2. Spontaneous behaviour of 4-month-old NMRI male mice exposed to a single-oral dose, either 1.4 or 21 μmol/kg body weight of PFOS (0.75 or 11.3 mg/kg body weight), PFOA (0.58 or 8.70 mg/kg body weight), or PFDA (0.72 or 10.8 mg/kg body weight), via a metal gastric-tube at the age of 10 days. The control animals received in the same manner 10 ml/kg body weight of the 20% fat emulsion vehicle. The data were subjected an ANOVA with split-plot design, and there were significant group × period interactions [$F_{12,126} = 87.85$, $F_{12,126} = 448.91$, $F_{12,126} = 139.67$] for locomotion, rearing and total activity variables, respectively. Pair-wise testing between PFOS-, PFOA-, PFDA-exposed animals and control animals was performed by using Tukey HSD tests. Statistical differences are indicated as (A) significantly different vs. controls $P \leq 0.01$; (a) significantly different vs. controls $P \leq 0.05$; (B) significantly different vs. 1.4 μmol PFOS/kg body weight $P \leq 0.01$; (b) significantly different vs. 1.4 μmol PFOS/kg body weight $P \leq 0.05$; (C) significantly different vs. 1.4 μmol PFOA/kg body weight $P \leq 0.01$; (c) significantly different vs. 1.4 μmol PFOA/kg body weight $P \leq 0.05$. Bar height represents mean value ± S.D.

emulsion vehicle on PND 10, showed a normal decrease in activity during the 60-min observation period, at 2 and 4 months.

2-Month-old mice neonatally exposed to PFOS. Mice exposed to PFOS 21 μmol/kg body weight showed a significant ($P \leq 0.01$) decrease in activity (in the locomotion, rearing, and total activity variables) during the first 20-min spell (0–20 min), vs. vehicle-treated animals and mice exposed to PFOS 1.4 μmol/kg body weight. Mice exposed to PFOS 21 μmol/kg body weight showed a significant ($P \leq 0.01$) increase in locomotion, rearing, and total activity, vs. mice exposed to PFOS 1.4 μmol/kg body weight and animals treated with the emulsion vehicle, during the third 20-min spell (40–60 min). Mice exposed to PFOS 1.4 μmol/kg body weight showed a significant ($P \leq 0.01$) decrease in total activity vs. vehicle-treated animals during the first 20-min spell.

4-Month-old mice neonatally exposed to PFOS. Mice exposed to PFOS 21 μmol/kg body weight showed a significant ($P \leq 0.01$) decrease in activity in the locomotion, rearing, and total activity variables during the first 20-min spell (0–20 min), vs. vehicle-treated animals and mice exposed to PFOS 1.4 μmol/kg body weight.

Mice exposed to PFOS 21 μmol/kg body weight showed a significant ($P \leq 0.01$) increase in the locomotion, rearing, and total activity during the third 20-min spell (40–60 min), vs. mice exposed to PFOS 1.4 μmol/kg body weight and vehicle-treated animals.

2-Month-old mice neonatally exposed to PFOA. The results from pair-wise testing of mice exposed to PFOA 21 μmol/kg body weight showed a significant ($P \leq 0.01$) decrease in activity in the locomotion, rearing, and total activity variables during the first 20-min spell, vs. vehicle-treated animals. A significant ($P \leq 0.05$) decrease in the locomotion variable was also seen, compared with vehicle-treated animals. Mice exposed to PFOA 21 μmol/kg body weight showed a significant ($P \leq 0.01$) increase in locomotion, rearing and total activity, during the third 20-min spell (40–60 min), vs. mice exposed to PFOS 1.4 μmol/kg body weight and vehicle-treated animals.

4-Month-old mice neonatally exposed to PFOA. The results from pair-wise testing of mice exposed to PFOA 21 μmol/kg body weight showed a significant ($P \leq 0.01$) decrease in activity in locomotion, rearing and total activity variables during the first 20-min spell, vs. mice exposed to PFOA 1.4 μmol/kg body weight and vehicle-treated animals. A significant decrease in the locomotion ($P \leq 0.05$) and total activity ($P \leq 0.01$) variable were also seen in mice given PFOA 1.4 μmol/kg body weight, compared with vehicle-treated animals.

During the third during 20-min spell (40–60 min), mice exposed to 21 μmol PFOA/kg body weight showed a significant ($P \leq 0.01$) increase in locomotion, rearing and total activity vs. mice exposed to PFOS 1.4 μmol/kg body weight and vehicle-treated animals.

2- and 4-month-old mice neonatally exposed to PFDA. Mice exposed to PFDA showed no significant effect on the spontaneous behaviour variables locomotion and rearing, at the age of 2 and 4 months. In the total activity variable a decrease was seen in mice given 1.4 μmol/kg body weight during the first 20-min spell vs. vehicle-treated mice.

3.2. Effects on habituation capability in adult mice

By analysing the habituation ratio between activity during the last and first (40–60 and 0–20 min) spells in the spontaneous behaviour testing, information concerning the capability to habituate to a novel environment can be obtained. This information was used to analyze changes in habituation over time. The habituation ratios, calculated from the spontaneous behaviour variables (locomotion, rearing) in 2- and 4-month-old mice, exposed on postnatal day 10 to PFOS, PFOA, and PFDA, are presented in Fig. 3. Habituation capability in the locomotion and rearing variables were shown to decrease

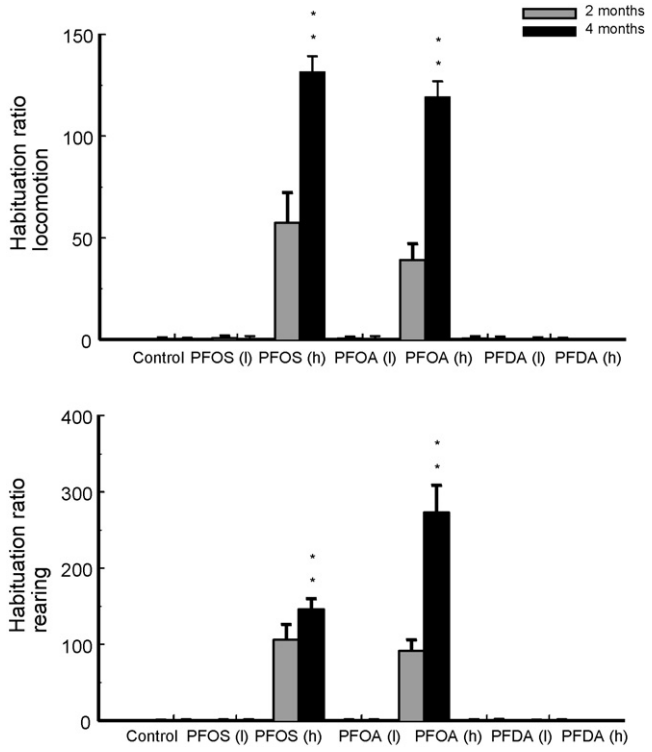

Fig. 3. Habituation capability of 2- and 4-month-old NMRI male mice exposed to a single-oral dose, either 1.4 or 21 μmol/kg body weight of PFOS (0.75 or 11.3 mg/kg body weight), or PFOA (0.58 or 8.70 mg/kg body weight), via a metal gastric-tube at the age of 10 days. The control animals received in the same manner 10 ml/kg body weight of the 20% fat emulsion vehicle. The habituation ratio for the variables locomotion and rearing was calculated by taking the value for 40–60 min, dividing it by the value for 0–20 min, and multiplying the result by 100. These data were subjected to two-way ANOVA. Pair-wise testing between 2- and 4-month-old animals was performed by using Tukey HSD tests. Statistical differences are indicated by $^{**}(P \leq 0.01$ 2 vs. 4 months).

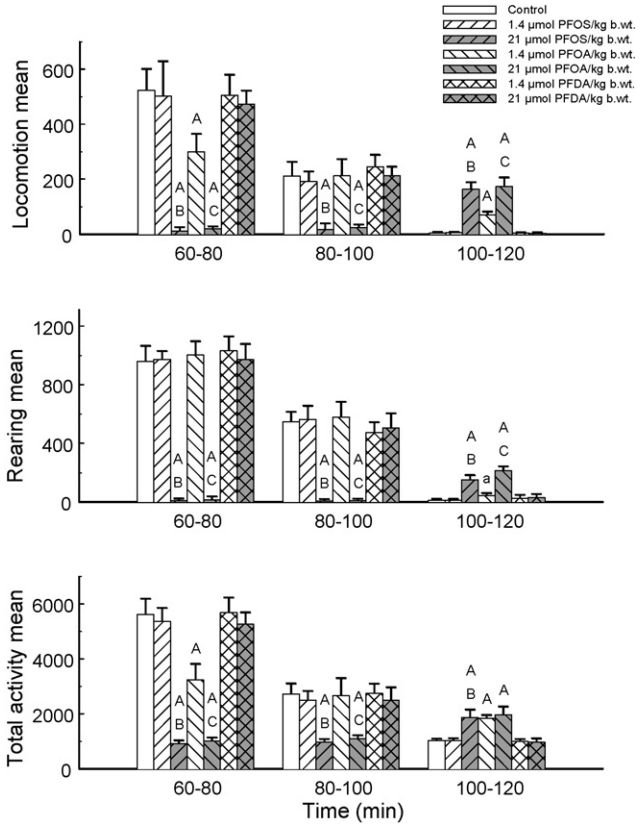

Fig. 4. Nicotine-induced behaviour of 4-month-old NMRI male mice exposed to a single-oral dose, either 1.4 or 21 μmol/kg body weight of PFOS (0.75 or 11.3 mg/kg body weight), PFOA (0.58 or 8.70 mg/kg body weight), or PFDA (0.72 or 10.8 mg/kg body weight), via a metal gastric-tube at the age of 10 days. The control animals received in the same manner 10 ml/kg body weight of the 20% fat emulsion vehicle. The nicotine-induced behaviour was studied by a s.c. injection of 80 μg nicotine base/kg body weight. The data were subjected an ANOVA with split-plot design, and there were significant group × period interactions [$F_{12,126} = 110.27$, $F_{12,126} = 191.16$, $F_{12,126} = 130.37$] for the variables locomotion, rearing and total activity, respectively. Pair-wise testing between the different nicotine-injected animals and nicotine-injected controls was performed by using Tukey HSD tests. Statistical differences are indicated as (A) significantly different vs. controls $P \leq 0.01$; (B) significantly different vs. 1.4 μmol PFOS/kg body weight $P \leq 0.01$; (C) significantly different vs. 1.4 μmol PFOA/kg body weight $P \leq 0.01$. Bar height represents mean values ± S.D.

significantly ($P \leq 0.01$) with age in mice exposed neonatally to PFOS 21 μmol/kg body weight or PFOA 21 μmol/kg body weight.

3.3. Effect on nicotine-induced behaviour in adult mice

Responses to nicotine of 4-month-old mice exposed at the age of 10 days to a single-oral dose of either 1.4 or 21 μmol PFOS/kg body weight, 1.4 or 21 μmol PFOA/kg body weight, 1.4 or 21 μmol PFDA/kg body weight, or the 20% fat emulsion vehicle are shown in Fig. 4. The mice were given a single s.c. injection of 80 μg nicotine base/kg body weight and observed for another 60 min period (60–120 min from base-line). Significant treatment × period interactions [$F_{12,126} = 110.27$, $F_{12,126} = 191.16$, $F_{12,126} = 130.37$] were observed for the variables locomotion, rearing, and total activity, respectively. Mice exposed neonatally to the vehicle responded with increased activity and displayed a high activity in all three variables during the first 20-min spell (60–80 min) compared to the last 20-min spell (40–60 min) of the spontaneous behaviour test. Mice exposed neonatally to 21 μmol PFOS/kg body responded to nicotine with a decrease in activity and were less active and significantly less so than mice exposed neonatally to the vehicle. Mice exposed neonatally to 21 μmol PFOA/kg body weight responded to nicotine with a decrease in activity, and were significantly less active in all three activity variables during the first 20-min spell (60–80 min) compared to the mice neonatally exposed to the vehicle. Mice given 1.4 μmol PFOA responded to nicotine with an increase in activity and were significantly less active than mice neonatally exposed to the vehicle. In comparison with the vehicle-treated mice, there was a dose–response change in the response to nicotine in PFOA-exposed mice. During the third 20-min spell (100–120 min), mice exposed neonatally to the vehicle, showed again base-line activity. Mice exposed neonatally to 21 μmol PFOS/kg body weight or 21 μmol PFOA/kg body weight were again hyperactive (locomotion and rearing variables). Mice exposed neonatally to 1.4 μmol

Table 1
Performance in the elevated plus-maze of 4-month-old NMRI male mice exposed to a single-oral dose[a]

Treatment	Entries made into open arms mean ± S.D. (%)	Time spent in open arms mean ± S.D. (%)
Control	29.5 ± 3.10	22.4 ± 3.81
PFOS (1.4 μmol/kg b.wt.)	29.3 ± 3.94	21.1 ± 3.21
PFOS (21 μmol/kg b.wt.)	28.9 ± 4.56	21.9 ± 3.93
PFOA (1.4 μmol/kg b.wt.)	31.5 ± 4.30	19.3 ± 3.68
PFOA (21 μmol/kg b.wt.)	31.5 ± 3.63	20.8 ± 5.01
PFDA (1.4 μmol/kg b.wt.)	28.8 ± 2.04	21.8 ± 2.78
PFDA (21 μmol/kg b.wt.)	32.9 ± 4.36	21.8 ± 3.46

[a] Either 1.4 or 21 μmol/kg body weight of PFOS (0.75 or 11.3 mg/kg body weight), PFOA (0.58 or 8.70 mg/kg body weight), or PFDA (0.72 or 10.8 mg/kg body weight), via a metal gastric-tube at the age of 10 days. The control animals received in the same manner 10 ml/kg body weight of the 20% fat emulsion vehicle. In each treatment group a total of 10 animals were tested for percent entries made and time spent in the open arms. The data were subjected to a one-way ANOVA. There were no significant treatment effects [$F_{6,63} = 1.79$, $F_{6,63} = 0.75$] for entries and time, respectively.

PFOA/kg body weight were significantly more active in all three variables than the vehicle-treated animals, an effect not seen during the last 20-min spell of the first 60-min period.

3.4. Effect in elevated plus-maze

4-Month-old male NMRI mice exposed to a single-oral dose of either 1.4 or 21 μmol PFOS/kg body weight, 1.4 or 21 μmol PFOA/kg body weight, or 1.4 or 21 μmol PFDA/kg body weight, at the age of 10 days, were tested in an elevated plus-maze regarding entry into and time spent in the open arms. There were no significant treatment effects [$F_{6,63} = 1.79$, $F_{6,63} = 0.75$] for entries and time, respectively (Table 1).

4. Discussion

The present study has shown that neonatal exposure to PFOS and PFOA (1.4 or 21 μmol/kg body weight), on postnatal day 10, can cause persistent disturbances in spontaneous behaviour of adult NMRI male mice and can modify the susceptibility of the adult cholinergic system.

The spontaneous behaviour data show a disruption of habituation in animals exposed to PFOS or PFOA. Habituation, defined here as a decrease in the variables locomotion, rearing, and total activity in response to the diminished novelty of the test chamber over 60 min was demonstrated in the control animals, whereas the PFOS- and PFOA-treated mice were obviously hypoactive during the first part of the 60-min period (0–20 min), but hyperactive during the last 20-min spell. This reduced habituation or lack of it, decreased activity in the beginning and increased activity at the end, were observed at a dose of 21 μmol PFOS/kg body weight and 21 μmol PFOA/kg body weight. The lowest dose of PFOA to cause any change in any of the three variables in 2- or 4-month-old mice was 1.4 μmol/kg body weight, in the locomotion and total activity variables. This indicates that PFOA can be as potent as certain PCBs and PBDEs in causing developmental neurotoxic defects.

Spontaneous behaviour reflects a function dependent on the integration of a sensoric input into a motoric output, and thus reveals the animals' ability to habituate to an environment and integrate new information with previously attained, and thereby be a measure of cognitive function. This indicates that PFOS and PFOA can inflict cognitive function together with modified motor behaviour.

Furthermore, the spontaneous behaviour tests indicated that the changes in spontaneous behaviour worsen with age, as the disturbances appeared to be more pronounced in the 4-month-old mice than in 2-month-old mice. This change over time is quite evident in mice given 21 μmol PFOS/kg body weight or 21 μmol PFOA/kg body weight, where the habituation ratios for locomotion and rearing increased significantly between 2- and 4-month-old mice. It is of particular interest that neonatal exposure to PFOS and PFOA can cause these types of both dose–response and time–response defects in spontaneous behaviour and reduced habituation ability with age, as seen in mice exposed neonatally to various PBDEs, such as PBDE 47, PBDE 99, PBDE 153, and PBDE 209 (Eriksson et al., 2001b; Viberg et al., 2003a,b, 2004), and certain *ortho*-substituted and co-planar PCBs (Eriksson, 1998; Eriksson and Fredriksson, 1996a, 1998). These changes in behaviour, both time-dependent and dose-related, indicate the advance of a brain dysfunction process induced at the time of BGS in the neonatal mouse.

The present study has shown that neonatal to PFOS and PFOA can affect and alter the susceptibility of the cholinergic system in adult animals. In the nicotine-induced behaviour test the response to nicotine was quite opposite in mice exposed neonatally to 21 μmol PFOS/kg body weight or to 21 μmol PFOA/kg body weight, compared with mice exposed neonatally to the vehicle. In control animals, hyperactivity was seen after injecting 80 μg nicotine base/kg body weight. It is known that low doses of nicotine can induce hyperactivity in animals whereas high doses induces hypoactivity (Nordberg and Bergh, 1985). It is seen that mice react with an increased activity when given 1–80 μg nicotine/kg body weight, whereas the opposite is seen in mice with reduced density of LA-binding sites for nicotine (Ankarberg et al., 2001; Eriksson et al., 2000). These altered and opposite responses to nicotine is the same as we observed earlier in mice exposed neonatally to PBDE 99 or PCB 52 (Eriksson and Fredriksson, 1996b; Viberg et al., 2002). In the animals exposed neonatally to PBDE 99 or PCB 52 it was also found that the cholinergic receptors were affected, showing a reduced density of cholinergic nicotinic receptors. These effects are also dose–response related in the reaction to

nicotine. Cholinergic receptors (nicotinic receptors) play an important role during later fetal development and in the growing child. It is reported that manipulation of the perinatal cholinergic system can be correlated to cognitive deficits but do not affect motor behaviour (see Herlenius and Lagercrantz, 2004).

In the spontaneous behaviour test we observed a reduced activity during the first 20-min spell in mice exposed neonatally to PFOA and PFOA, compared with controls. It is conceivable that the reduced activity in the beginning of this test might have resulted from increased anxiety engendered by a novel environment. However, the behaviour observed in the elevated plus-maze (EPM) failed to reveal any significant differences in the variables, entries made into, or time spent in the open arms. It would therefore seem that the reduced activity observed in mice exposed neonatally to PFOS and PFOA was not due to anxiety-like behaviour.

Today it is not known which pathways are the most important for human exposure for PFCs, but indoor air and dust have been found to contain PFCs (Kubwabo et al., 2005; Moriwaki et al., 2003; Shoeib et al., 2004, 2005). Exposure to chemicals via inhalation and dust ingestion may constitute important exposure way in a non-dietary manner for humans. Young children, especially those in the crawling stage, ingest more dust than adults. Recent report indicate that young people have the same or even higher PFCs levels in serum or blood, compared with older generations (Kärrman et al., 2006c; Olsen et al., 2004a; WWF, 2005). In children, 2–12 years old, living in the USA. PFOS concentrations ranged between 6.7 and 515 ng/ml. In the study by Kärrman et al. it was shown that the median concentration, on whole-blood basis, of the sum of PFC was 20–50-fold compared to the sum of PCBs and *p*,*p*′-DDE, and 300–450-fold that of hexachlorobenzene (HCB), sum of chlordanes, and sum of PBDEs. The mechanism of lactational transfer and the influence of PFC quantity of body burden or blood levels transferred to the breast milk are not clear. Human breast milk has been analysed for PFCs in the USA, Sweden, and China (Kärrman et al., 2006a, 2007; Kuklenyik et al., 2004; So et al., 2006). In women living in China, levels of PFOS ranged from 45 to 360 ng/l and PFOA from 47 to 210 ng/l. In 12 Swedish primiparous women the PFOS mean level was 201 ng/l and that of PFC in human milk was about 1% of the corresponding level in serum. Considering the present study, a dose of 1.4 μg PFOA/kg body weight is about equal to the doses of PCBs and PBDEs known to induce developmental neurotoxic effects. The doses of PCBs and PBDEs are considered to be of toxicological and physiological relevance to human exposure to these agents (Eriksson, in press; McDonald, 2005). Of concern is therefore that the body burden of PFOA and/or PFCs are in the same order of magnitude or higher than those of PCBs and PBDEs.

We have reported earlier that persistent effects of agents known to affect neuronal activity, such as DDT, nicotine, and organophosphates (OPs) only occur when administered during a defined critical period in the neonatal development of the mouse brain (Ahlbom et al., 1995; Eriksson et al., 1992, 2000, 2001b). Mice exposed neonatally to PFDA did not show any aberrations in spontaneous behaviour, or increased susceptibility to nicotine, as adults. Whether PFDA is not taken up in the neonatal brain or needs to be metabolized to induce developmental neurotoxicological effects, is not clear. Our research group have earlier reported that PBDE 209 can induce developmental neurotoxic effects when neonatal mice are exposed on PND 3, but not on PND 10. In the case of PBDE 209, it is thought that it is a metabolite of PBDE 209, possible a debrominated one, that is present during a critical window (PND 10) of brain development (Viberg et al., 2003b).

In conclusion, the present study shows that neonatal exposure to the PFCs, PFOS and PFOA can give irreversible changes in the adult mouse brain. These changes are manifested as deranged spontaneous behaviour in and lack of habituation, and increased susceptibility of the cholinergic system, manifested in adult animals as changes in the response to nicotine. This indicates that PFOS and PFOA may interact with other toxicants, known to affect the developing brain and the cholinergic system.

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