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Prenatal Exposure to PFOS or PFOA Alters Motor Function in Mice in a Sex-Related Manner

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Abstract Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are organic surfactants widely used in various industrial and consumer applications. Due to their chemical properties, these perfluorinated compounds (PFCs) have also become persistent contaminants. The risk of possible intrauterine and lactational exposure to these chemicals poses a significant health concern for potential developmental effects. In the present study we have found that dietary exposure of mice to 0.3 mg/kg of PFOS or PFOA throughout pregnancy results in different distribution pattern in the offspring brain and liver. In particular, exposure to PFOS led to four times higher accumulation of the chemical in the brains of newborn mice than PFOA. We have used a battery of behavioral tests to evaluate motor function, circadian activity, and emotion-related behavior in the exposed offspring. Exposure to PFOS resulted in decreased locomotion in a novel environment and reduced muscle strength only in male offspring. Prenatal exposure to PFOA was associated with changes in exploratory behavior in male and female offspring, as well as with increased global activity in males in their home cage. The neurobehavioral outcome of prenatal exposure to PFCs in mice is characterized by mild alterations in motor function and it appears to be sex-related.

Keywords PFOS · PFOA · Behavior · Mice

Introduction

Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) belong to a group of perfluorinated compounds (PFCs), which have been extensively used in various industrial and consumer products including surfactants, fire retardants, pesticides, detergents, cosmetics, oil, and water repellents. Due to their high chemical stability and lack of environmental degradation, these chemicals have become persistent pollutants and detectable levels of PFCs have been found in living organisms, including humans and wildlife (Fromme et al. 2009; Harada and Koizumi 2009). Considerable sources of non-occupational exposure in humans can be consumption of contaminated fish or other food products (through packaging material or cookware), as well as inhalation of household dust (EFSA 2008). PFOS and PFOA are able to cross the placental barrier and can also be excreted with milk, which provides a route for fetal and neonatal exposure (Tao et al. 2008; Olsen et al. 2009). Analyses of human and animal samples have shown that PFOS accumulates in the developing brain before formation of the blood–brain barrier (BBB) but can also cross mature BBB to a certain extent (Maestri et al. 2006; Harada et al. 2007; Chang et al. 2009).

The developing central nervous system (CNS) is particularly sensitive to chemical insults (Grandjean and Landrigan 2006) because of increased vulnerability to adverse effects not present in the mature brain (Barone

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et al. 2000). Thus, the potential consequences of exposure to PFCs during development are a matter of public health concern and research interest. It has been reported that developmental PFOS exposure in experimental animals induces alterations in CNS functions, including neuroendocrine disturbances and neurodevelopmental delays (Austin et al. 2003; Lau et al. 2003).

Due to considerable interspecies and sex-related variation in toxicokinetics of PFCs, the choice of animal experimental models, exposure procedure and dose should be carefully considered to enable further extrapolation of the data. Mature female rats have a short elimination half-life for PFOA (2–3 h), thus it is difficult to adjust the exposure procedure in a way that results in stable serum levels of the chemical in pregnant dams (Lau et al. 2004). Female mice seem to have a longer elimination half-life for PFOA (similar to male mice and rats) and steady-state serum levels of the chemical can be reached within 1 week of daily single exposure (Lau et al. 2006). Both chemicals can be excreted in milk, but lactational exposure does not seem to be as critical as in utero exposure for the induction of developmental toxicity (Wolf et al. 2007). Benchmark dose lower confidence limit ($BMDL_5$) affecting neonatal survival in mice has been estimated as 3.88 and 1.09 mg/kg for PFOS and PFOA, respectively (Lau et al. 2003, 2006), but even lower doses of PFOA (0.86 mg/kg as estimated $BMDL_5$) have been shown to affect postnatal body weight gain (Lau et al. 2006). To investigate whether prenatal exposure to PFOS and PFOA may have developmental neurotoxic effects at lower doses, we have exposed pregnant mice to 0.3 mg/kg of either chemical and analyzed behavior of male and female offspring in a battery of tests including assessment of motor function, circadian activity, and emotion-related behavior.

Materials and Methods

Animals and Treatment

All experiments were performed in accordance with the rules of the Swedish animal protection legislation and were approved by the local Animal Ethics Committee (Stockholms Norra Djurförsöksetiska Nämnd). Exposures to PFOS or PFOA were performed as two separate experiments with their own control group (set 1: PFOS + Control1; set 2: PFOA + Control2). C57BL/6/Bkl (Scanbur BK, Sweden) female mice were mated with males overnight and the next morning was considered gestation day (GD) 1 if a vaginal plug was observed. Pregnant dams received PFOS ($n = 6$) as heptadecafluorooctanesulfonic acid potassium salt (purity $\geq$ 98%, Sigma-Aldrich) or PFOA ($n = 6$) (purity 96%, Sigma-Aldrich) at the dose of 0.3 mg/kg/day via food from GD1 throughout pregnancy. PFCs were dissolved in 95% ethanol at the concentration of 1 μg/μl, and the solutions were applied on palatable food in a volume adjusted according to the individual body weight to reach the exposure dose of 0.3 mg/kg. The food bits were kept on the bench for 2 h to let ethanol evaporate and then placed in the cages. Control females ($n = 10$ in total) received similar bits of the palatable food with the vehicle applied and then evaporated. This route of exposure was chosen instead of gastric gavage to avoid stressful procedures that may negatively affect pregnancy.

Offspring were separated from mothers on postnatal day (PND) 21 and were injected subcutaneously with sterile microtransponders (ID-100A, Trovan, Ltd., UK) under 4% isoflurane inhalation anesthesia. Each transponder had an individual number that was used for animal identification. One or two offspring from the same litter were randomly selected for inclusion in the experimental groups. Control and PFOS-exposed groups of both sexes consisted of 8 animals. PFOA-exposed groups included 6 males and 10 females, their respective control groups consisted of 8 male and 10 female mice. The mice were housed in groups of 3–4 animals per cage and the social groups were preserved throughout the experiments. All animals were kept under standard laboratory conditions (21°C, 12 h light–dark cycle with a light phase between 6.00 and 18.00) with free access to food and water.

Measurement of PFOS and PFOA Levels in Tissues

Tissue samples (whole brain and liver) ($n = 4$, one pup per litter) were collected from pups at birth. The concentration of PFOS and PFOA in the samples was measured with HPLC–MS by adapting the method previously described by Maestri et al. (2006). A detailed description of the method is provided in the Supplementary material (S1).

Behavioral Assessments

Tests for locomotor and circadian activity were performed at the age of 5–8 weeks. Afterward, animals were tested for emotion-related behavior in elevated plus maze and forced swim tests. Tests for muscle strength and motor coordination were performed in animals 3- to 4-month old.

Locomotor Activity

The locomotor activity test was performed in cages made of transparent Plexiglas (42.5 × 26.6 × 18.5 cm) with sawdust bedding covering the floor of the cage. Mice were individually placed in the new cages; behavior was video recorded and data on the walked distance were collected by using the automated video tracking system TopScan™

(Clever Systems Inc., Reston, VA, USA). Distance traveled was registered in 5 min intervals over 30 min and used for statistical analysis.

Circadian Activity in the Home Cage

The mice were moved to the experimental room and placed in new cages preserving the social groups immediately before starting the activity recording. The cages were placed on TraffiCage™ platforms (NewBehavior, Zurich, Switzerland). The platforms consisted of a plastic base (42.5 × 27 × 1.7 cm) with 5 embedded circular antennas (9 cm in diameter), which detect the presence of the injected transponders. When a mouse crossed from one antenna to another, the movement was recorded as crossing and used as activity count.

The experiment started at 10 a.m. and the activity of the mice was monitored for 48 consecutive hours. The animals were kept under the same conditions (temperature, light-dark cycle, food and water access) as described above throughout the duration of the experiment. The recordings were divided as follows: the first 3 h were analyzed separately as adaptation to a novel environment. Unlike tests of locomotor activity, animals evaluated for circadian activity remained in their original social group, instead of monitoring activity in mice removed from their homecage for a short period of time. Typically, the novelty-induced hyperactivity drops within 3 h. The remainder was considered normal homecage locomotor activity and was further divided into light and dark phases, according to the intensity of the light provided in the experimental room.

In addition, we analyzed the resting time as the total duration of inactive periods. An "inactive period" was defined as a lag longer than 10 min between two consecutive crossings based on two observations: (1) close visual observation of the behavior in the homecage not detecting any activity (eating, drinking, grooming) in the territory of one single antenna that would last longer than 10 min; (2) the analysis of lags between crossings showed low incidence of lags longer than 7 min, but shorter than 12 min. Thus, by setting the threshold at 10 min, we were able to consistently distinguish long (inactive periods) from short lags (inherent to normal activity in the homecage), and assumed that lags longer than 10 min were indicative for resting. Because of the trailing effect of novelty-induced hyperactivity, we used only the second light and dark periods for analysis of resting time.

Elevated Plus Maze Test

We used a maze with a shape of a plus sign formed by two open arms (40 × 10 cm), two enclosed arms (40 × 10 cm) and a central platform (10 × 10 cm). The apparatus, made in gray plastic, was placed 50 cm above the floor. Animals were released on the central platform facing one of the open arms, and allowed to explore the maze for 5 min. After the end of each test, the arena was carefully cleaned. Data were collected by using a TSE video tracking system (TSE Systems, Bad Homburg, Germany). Number of entries and time spent in the open arms as well as preference for visits to open or closed arms were used as parameters of anxiety-like behavior.

Forced Swimming Test

Animals were individually placed in a glass cylinder (24 cm height, 12 cm diameter) filled with water (27°C) up to a height of 16 cm for 10 min (pretest) and for 6 min (test) 24 h after the previous session. Test sessions were video recorded and later analyzed for immobility duration. Immobility was defined as floating passively in the water at least 2 s or longer, without any movements or just small ones necessary to keep the head above the water surface. The inactive state (immobility) is considered to be a measure of depression-like behavior.

Muscle Strength in the Hanging Wire Test

Mice were individually placed on the top of a wire cage lid and when it griped the wires, the lid was turned upside down. The lid was held upside down approx. 20 cm above the cage floor covered with sawdust bedding. Latency to fall off the lid was measured with 60-s cutoff time. The test was repeated 3 times with 30-min intervals. The average value over three trials was used for statistical analysis.

Accelerating Rotarod Test

Motor coordination in the exposed and control mice was evaluated using a rotarod (LE 8200, Letica Scientific Instruments, Barcelona, Spain). The test trials on the accelerating rod were performed after three training sessions (habituation with the stationary and rotating rod, 4 rpm). The speed of the rotarod accelerated from 4 to 40 rpm over a 5-min period. Mice were placed on the rotating drum, and the time they remained on the rotarod was registered automatically. Mice were given four consecutive test trials with a maximum trial time of 300 s and 30 min intertrial rest intervals.

Statistics

Results from the behavioral tests were analyzed separately for PFOS or PFOA-exposed animals and their respective controls using two-factor ANOVA with exposure and sex as factors (predictors). For tests based on sequential

measurements, such as novelty-induced activity, adaptation to a novel environment in the TraffiCage system, and accelerating rotarod, we used repeated measures ANOVA, followed by Fisher's least significant difference (LSD) post-hoc test. $P < 0.05$ was set as threshold for statistical significance. Values are reported as mean ± S.E.M.

Results

Dams exposed to PFOS or PFOA gained weight normally during pregnancy and did not differ from control females at any gestational age. Litter size and sex ratio were similar in control and exposed groups (data not shown). There were no differences in offspring body or brain weights between groups at birth. Liver weights were normal in PFOS-exposed pups, but significantly increased in PFOA-exposed mice (77 ± 2 mg vs. 58 ± 1 mg in control, $P < 0.001$, *t*-test).

PFOS and PFOA Concentrations in Tissues

PFOS and PFOA concentrations in tissues from the exposed animals are shown in Table 1. Levels of the chemicals in control samples were below detection limit. Prenatal exposure to the same dose of the chemicals (0.3 mg/kg, GD1–20) resulted in a lower liver level and higher brain concentration of PFOS as compared to PFOA.

Behavioral Assessment

Locomotor and Exploratory Activity in the Individual Test

We found significant effects of PFOS exposure ($F_{1,27} = 5.06$, $P = 0.033$) and sex ($F_{1,27} = 10.19$, $P = 0.003$) on the results of locomotor activity test. Thus, PFOS-exposed males walked significantly less than controls when exploring a new environment (Fig. 1a), while the exposed females did not differ significantly from their respective controls. PFOA-exposure did not have a significant effect on locomotor activity in either males ($P = 0.460$) or females ($P = 0.146$).

Circadian Activity in the Social Group and Home Cage

We measured circadian activity of mice housed in social groups by using the TraffiCage system (Fig. 2). Novelty of the environment evoked increased exploratory activity in all groups lasting 2–3 h (Fig. 2a–d). Analysis of the activity during this period revealed effects that depended on sex and type of the chemical exposure. Thus, PFOS-exposed males displayed decreased activity during the first 2 h with almost twofold difference compared to control group during the second hour of the test ($P = 0.008$) (Fig. 2a). A similar trend was observed in PFOS-exposed females, but the difference during the second hour of the test did not reach significance ($P = 0.09$) (Fig. 2b). In the PFOA groups, activity levels in the exposed and control animals differed during the first hour of the test in a sex-related manner. PFOA-exposed males were more active ($P = 0.013$) (Fig. 2c), while PFOA-exposed females showed a decreased activity ($P = 0.036$) (Fig. 2d) than the controls. After habituation to the new home cage, animal activity declined to a low, diurnal level. All groups of animals had a normal circadian pattern with higher levels of activity during the dark phase and early morning hours, followed by lower activity levels during the light phase. As shown in Fig. 2e and f, activity of the control and exposed mice differed at some time points. However, there was no significant difference in total activity counts over light or dark periods between control and PFOS-exposed groups, either in males or females (Table 2). Activity of PFOA-exposed males was higher than in controls, especially during the dark phase (Table 2; Fig. 2g). Total activity counts were also higher during the light phase in PFOA-exposed male group (Table 2) due to more prominent activity peaks in the morning hours (Fig. 2g). PFOA-exposed females did not differ significantly from the respective controls (Fig. 2h).

According to our experience with TraffiCage, mouse behavior recorded during the first 24 h is influenced by adaptation to a novel environment, while the second day of monitoring (25–48 h) provides data on the mice basal activity levels (unpublished observation). The signs of altered locomotor activity in the exposed groups prompted us to extend the analysis of behavioral data by calculating resting time and its circadian distribution over the second day of the experiment. Interestingly, we found no significant differences between any groups in total resting time (data not shown), but in the number of inactive periods (Fig. 3).

We found that control females consistently had less inactive periods than control males during the dark phase. Exposure to PFOS caused an increase in the total number of inactive periods in both males and females, with differential increase for both light and dark phases only in the females. Prenatal exposure to PFOA had an opposite effect decreasing the total number of inactive periods in both males and females. The effect was significant during the light phase in both sexes; in addition, in males, the decrease was significant also in the dark phase of the circadian cycle.

Table 1 PFOS and PFOA concentrations in the tissue samples collected from the exposed offspring at birth

Exposure	Brain (μg/g)	Liver (μg/g)
PFOS	3.1 ± 0.3	11.8 ± 1.5
PFOA	0.7 ± 0.1	16.3 ± 4.1

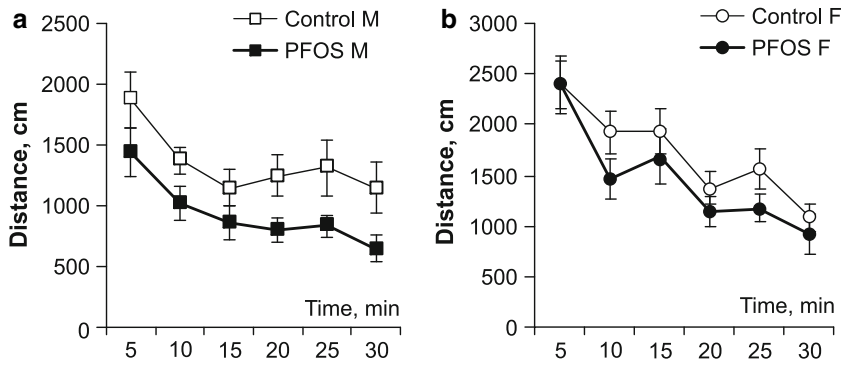

Fig. 1 Locomotor activity of control and PFOS-exposed male (**a**) and female (**b**) mice in the individual test. Total distance that animals covered over 30 min of the test was significantly lower in PFOS-exposed male group ($P < 0.05$, $n = 8$) but did not differ from control in the exposed female group ($P = 0.279$, $n = 8$)

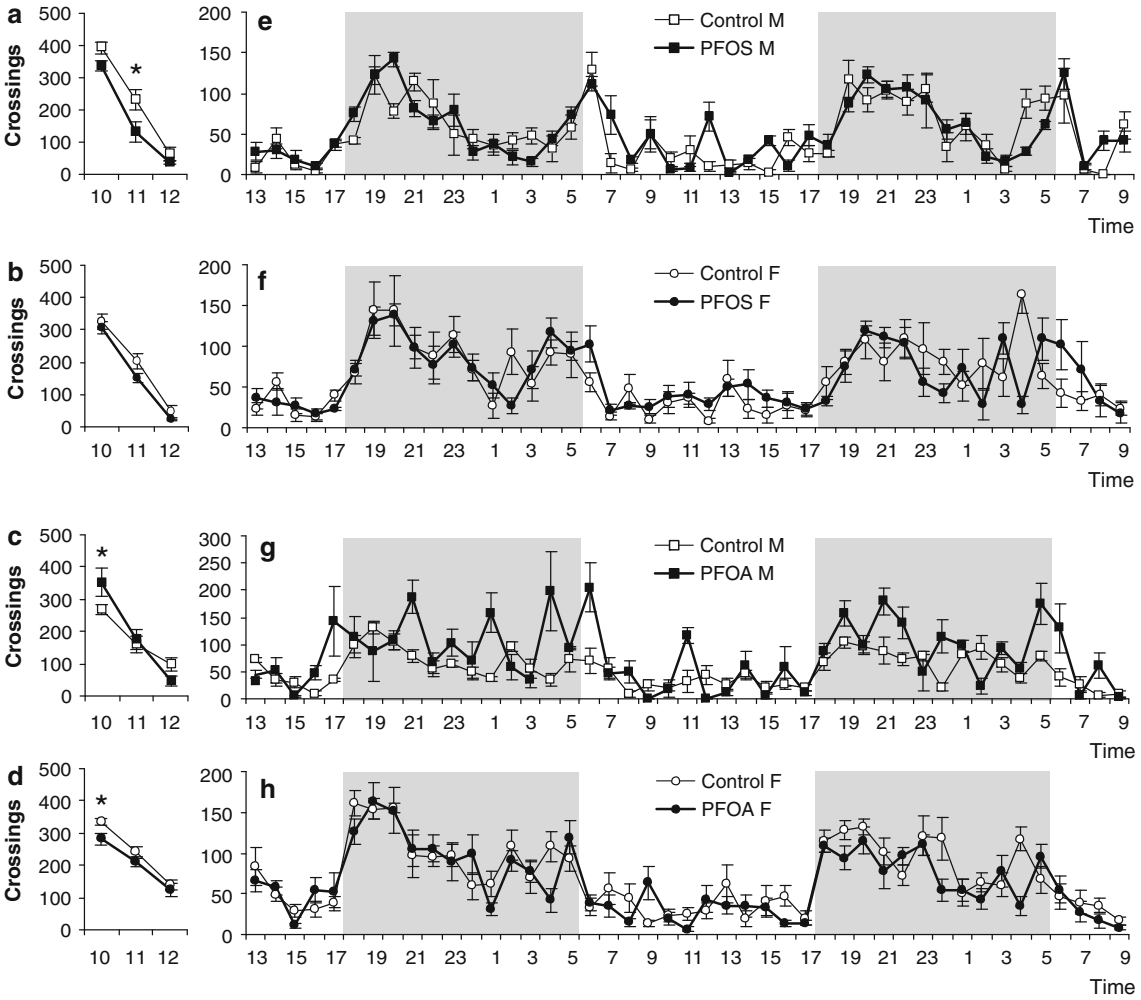

Fig. 2 Novelty-induced (**a–d**) and circadian activity (**e–h**) over 48 h in the home cage and social group in male and female mice prenatally exposed to PFOS or PFOA. Activity counts presented as number of antenna crossings in the TraffiCage (see "Materials and Methods" section for details). Gray areas correspond to a dark phase of the light–dark cycle. * $P < 0.05$, $n = 6$–10)

Anxiety-Related Behavior in the Elevated Plus Maze

Results from the elevated plus maze test are summarized in Table 3. PFOS-exposed male mice traveled equally long distance exploring the closed arms as controls, but the exposed animals spent significantly more time being inactive than controls. The total distance walked in all areas of the maze was shorter for PFOS-exposed male mice than controls, in agreement with the low level of locomotion observed in the previously described tests. PFOS-exposed male mice spent less time in the open arms ($P = 0.011$) but the number of entries was not significantly different from controls ($P = 0.075$). The ratio between open and closed arms visits was similar in both groups:

Table 2 Circadian activity of the exposed mice

Test	Phase (clock time)	Males		Females		Males		Females	
		Control	PFOS	Control	PFOS	Control	PFOA	Control	PFOA
Circadian activity in the TraffiCage	Novelty (10.00–13.00)	688.3 ± 59.7	506.3 ± 41.9*	572.9 ± 55.9	482.0 ± 12.8	523.4 ± 43.9	576.0 ± 68.4	716.7 ± 34.1	617.9 ± 45.0
	Day 1 Light (13.00–18.00)	106.3 ± 8.1	123.0 ± 19.0	147.7 ± 16	133.7 ± 15.9	183.3 ± 11.5	290.6 ± 90.1	231.8 ± 29.6	238.8 ± 34.2
	Day 1 Dark (18.00–06.00)	748.5 ± 87.7	780.5 ± 16.7	1081.7 ± 200.7	1052.7 ± 59.8	874.3 ± 64.1	1280.0 ± 140.7*	1255.2 ± 65.8	1201.2 ± 133.1
	Day 2 Light (06.00–18.00)	356.4 ± 48.5	459.4 ± 68.7	351.6 ± 39.4	475.1 ± 48.7	395.4 ± 32.1	585.6 ± 51.0*	415.7 ± 63.1	354.0 ± 43.7
	Day 2 Dark (18.00–06.00)	847.1 ± 82.9	794.0 ± 73.2	1030.1 ± 181.2	890.1 ± 70.5	884.7 ± 79.7	1273.6 ± 31.1*	1145.7 ± 71.7	964.4 ± 70.6

Data are presented as total activity counts (crossings) over light or dark phase

* $P < 0.05$ vs. control group of the same sex

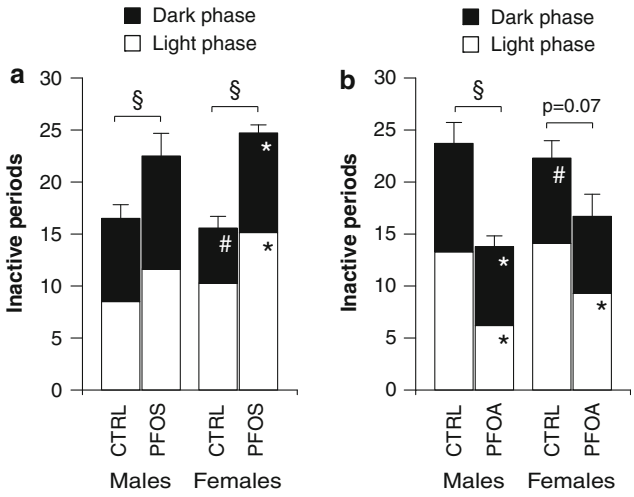

Fig. 3 Number of inactive periods in mice prenatally exposed to PFOS (**a**) or PFOA (**b**) vs. their respective controls. Note that inactive period is defined as a period at least 10 min long during which no antenna crossing was recorded for an individual animal (see also text for details). Two-way ANOVA followed by LSD test. $^{\#}$ $P < 0.05$ control males vs. control females during dark phase; §, * $P < 0.05$ exposed vs. control males or females, respectively—total number of inactive periods (§), or during light or dark phase of the cycle (*)

41:59% and 39:61% in control and PFOS-exposed mice, respectively. Therefore, the preference for exploration of open (potentially dangerous) versus closed (safe) areas did not seem to be altered in the exposed animals. PFOS-exposed females as well as all PFOA-exposed groups did not differ from their respective controls in any behavioral parameter tested in the elevated plus maze (Table 3).

Depression-Like Behavior in the Forced Swimming Test

This test evaluates the ability of animals to cope with stress caused by an inescapable aversive situation (forced swimming), and predisposition to develop depression-like "behavior of despair" (passive floating) as a response to inescapable stress. Neither exposure to the selected chemicals, nor sex had a significant effect on immobility time in the forced swimming test (Table 3).

Muscle Strength in the Hanging Wire Test

There was a significant effect of sex on the results of this test ($F_{1,27} = 12.14$, $P = 0.007$). PFOS-exposed male mice had significantly shorter fall latency than controls ($P = 0.040$) (Fig. 4a). Latency to fall did not correlate with body weight in either group ($r = -0.296$, $P = 0.48$ in controls and $r = -0.33$, $P = 0.42$ in PFOS group). Muscle strength of the PFOS-exposed female mice was not affected ($P = 0.985$ vs. female controls) (Fig. 4a). Exposure to PFOA did not cause significant effects on muscle strength in mice of either sex (Fig. 4b).

Table 3 Results of the elevated plus maze (EPM) and forced swim tests (FST)

Test	Parameter	Males		Females		Males		Females	
		Control	PFOS	Control	PFOS	Control	PFOA	Control	PFOA
EPM	Time spent in the open arms (s)	51.4 ± 13.2	19.0 ± 4.0*	20.4 ± 4.3	19.3 ± 7.4	18.7 ± 4.5	17.1 ± 4.7	36.6 ± 11.4	34.7 ± 13.4
	Entries in the open arms	15.1 ± 2.3	10.3 ± 1.8	8.1 ± 1.5	7.3 ± 1.5	6.1 ± 1.2	6.7 ± 1.5	8.0 ± 1.6	6.7 ± 1.0
	Time spent in the closed arms (s)	189.9 ± 14.6	241.0 ± 5.8*	253.9 ± 6.9	262.4 ± 10.6	255.4 ± 4.7	259.3 ± 6.1	218.0 ± 12.0	218.5 ± 15.5
	Entries in the closed arms	22.0 ± 3.2	15.9 ± 1.9	13.1 ± 2.0	12.8 ± 2.3	10.5 ± 1.1	9.0 ± 1.0	10.9 ± 0.7	9.2 ± 1.4
	Total distance (cm)	249.5 ± 7.9	202.5 ± 9.4*	199.3 ± 14.3	178.2 ± 21.0	161.0 ± 9.4	167.8 ± 11.2	188.1 ± 9.2	252.7 ± 7.5
FST	Immobility (s)	211.4 ± 37.1	231.6 ± 22.9	219.0 ± 13.9	232.2 ± 19.4	142.4 ± 20.0	120.3 ± 28.4	107.8 ± 25.4	120.9 ± 22.3

* $P < 0.05$ vs. control group of the same sex

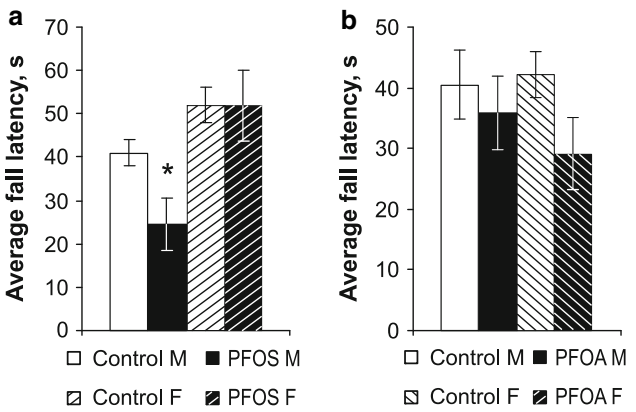

Fig. 4 Muscle strength of PFOS (**a**) or PFOA-exposed (**b**) mice in the hanging wire test. PFOS-exposed male mice had shorter latency to fall ($P = 0.040$, LSD test, $n = 8$) indicating lower muscle strength/endurance. PFOA-exposed female mice also showed a shorter latency to fall but difference with controls has not reached significant level ($P = 0.148$, $n = 10$)

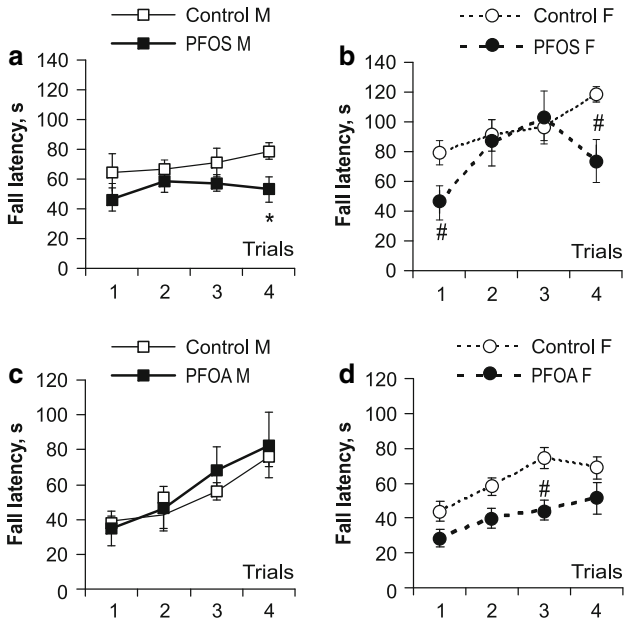

Fig. 5 Motor coordination of PFOS (**a, b**) or PFOA-exposed (**c, d**) male and female mice in the accelerating rotarod test. PFOS-exposed male mice had shorter fall latency in the last trial of the accelerating rotarod test ($n = 8$). PFOS-exposed female mice also had shorter fall latency in the first and last trials of the test ($n = 8$). However, in the other trials they performed as efficiently as their respective controls * $P < 0.05$ vs. male control; # $P < 0.05$ vs. female control (repeated measures ANOVA followed by LSD test)

Motor Coordination in the Accelerating Rotarod Test

Only minor effects of exposures were observed in the rotarod test. PFOS-exposed males were able to stay for shorter time on the accelerating rotarod during the last trial as compared to controls (Fig. 5a). PFOS-exposed female mice had shorter fall latency in the first and last trials of the test. However, results of the other trials showed that PFOS-exposed females were able to learn the skill of walking on the accelerating rotarod as efficiently as their respective controls (Fig. 5b). There was no effect of PFOA-exposure on motor coordination in males (Fig. 5c). PFOA-exposed females performed worse than the respective controls in every trial, but the statistical analysis showed that differences reached significance only in the third trial (Fig. 5d).

Discussion

In the present study we have shown that dietary exposure of mice to 0.3 mg/kg of PFOS or PFOA throughout gestation results in altered locomotor activity level and circadian distribution, muscle strength and motor coordination in the exposed offspring. Moreover, the outcome of prenatal

exposure appears to be sex-related and differs between PFOS and PFOA.

In Butenhoff et al. (2009), the no-observed-adverse-effect-level (NOAEL) for developmental neurotoxicity in rats was considered to be 0.3 mg/kg/day of PFOS received prenatally. In our experiment, exposure of pregnant mice to 0.3 mg/kg/day of PFOS resulted in PFOS tissue levels (brain and liver from pups at PND1) similar to those observed in rat fetuses at GD20 (Butenhoff et al. 2009) and induced neurobehavioral alterations in the offspring. Prenatal exposure to the same dose of PFOA led to approximately four times lower accumulation in the brain, as compared to PFOS, and it was also associated with behavioral alterations in the exposed offspring.

Effects of prenatal or early postnatal PFOS exposure on locomotor activity have been described in both rats and mice. Gestational exposure of rats to 1 mg/kg of PFOS caused increased motor activity and reduced habituation in young male offspring (Butenhoff et al. 2009). Single exposure to a high dose of PFOS in mice (11.3 mg/kg) during early life caused a decreased locomotor activity in young and adult male animals over the first 20 min in a novel environment followed by an increase in activity and impaired habituation ability (Johansson et al. 2008). In contrast, mice prenatally exposed to PFOS (6 mg/kg, GD12–18) showed no difference in habituation to a new environment, as reported in the study by Fuentes et al. (2007). In our experiment, male mice exposed prenatally to PFOS showed significantly less locomotor activity than controls in 30-min individual test. Similarly, the exposed animals were less active in a new home cage when observed for a longer period of time in their social groups. Thus, we did not detect an increase in locomotor/exploratory activity suggestive of an altered habituation ability.

The decrease in locomotor activity of exposed male mice seemed to affect the results of the elevated plus maze test. PFOS-exposed male mice spent longer time being inactive, which resulted in a shorter distance covered in the maze and longer time spent in the closed arms. However, the ratio between visits to open vs. closed arms of the maze was not affected. Therefore, the results of the elevated plus maze do not suggest altered anxiety-like behavior in the exposed animals. In addition, the forced swimming tests revealed no motivational disturbances.

Long-term monitoring of home cage activity revealed that PFOS-exposed animals had a higher number of inactive periods during the dark phase. This indicates fragmentation of the activity pattern and suggests that the exposed mice require more frequent resting periods for levels of activity similar to the control animals. We also found that environment novelty evoked activity levels 3- to 3.5-fold higher than average activity during nocturnal peaks. Thus, the novelty represented a powerful stimulus, and less locomotion might indicate a faster development of fatigue in the PFOS-exposed male mice. These results are in agreement with the poor performance in the last run on the accelerating rotarod, as well as with lower muscle strength found in exposed males evaluated by the hanging wire test. Decreased hind limb grip strength in young male rats exposed prenatally to 1 mg/kg of PFOS was earlier reported by Butenhoff et al. (2009), but this effect was transient and no other muscle strength abnormalities were observed. In contrast, we found that PFOS-exposed females had somewhat lower locomotor activity in the individual and group tests, but these alterations did not reach significance, and muscle strength was not affected.

Prenatal exposure to PFOA also had sex-related behavioral effects. Control female mice displayed higher exploratory activity than males, but the difference was reversed in mice prenatally exposed to PFOA. Interestingly, these alterations were observed when animals were tested in social groups, while individual testing did not reveal any differences.

Both wild-living and laboratory rodents display circadian fluctuations in exploratory activity and are known to be more active during the dark phase of the 24-h cycle. Therefore, continuous monitoring can provide important information about undisturbed animals' activity level and distribution during active (dark) and inactive (light) phases. By monitoring mice continuously over 48 h in the home cage, we found that PFOA-exposed males were more active than controls, as shown by higher peaks of activity and lower number of resting periods during the dark phase.

Data on the potential mechanisms behind the neurobehavioral effects of PFCs on the developing brain are, so far, very limited. It has been found that early life exposure to PFOS affects expression of the proteins involved in synaptogenesis and outgrowth of neuronal processes and calcium-mediated signaling in the hippocampus (Johansson et al. 2009; Liu et al. 2009b). The latter were prominent shortly after the exposure, but diminished with time as brain levels of PFOS were decreasing (Liu et al. 2009b). This suggests that elimination rate can be one of the factors determining magnitude and duration of the neurodevelopmental alterations caused by PFC exposure. There are data indicating that sex-related differences in toxicokinetics of PFCs appear in rats after sexual maturation with higher elimination rate of PFOS from serum in males than in females (Chang et al. 2009). Despite the variation in serum levels, brain and liver concentrations of PFOS seem to be more similar in rats and mice of different sexes (Chang et al. 2009; Liu et al. 2009a). Sex-related differences in the renal clearance of PFOA become apparent during the onset of sexual maturation in rats (Hinderliter et al. 2006) but not in mice (Kudo and Kawashima 2003; Lou et al. 2009). Therefore, we think it is unlikely that sex-related

behavioral differences observed in the present study may be due to toxicokinetic differences between males and females.

The endocrine disrupting potential of PFCs has been discussed in recent reviews (Jensen and Leffers 2008; Boas et al. 2009). In particular, exposure of adult rats to ammonium perfluorooctanoate can cause alterations in sex hormone levels (Biegel et al. 1995). Alterations in hormonal status induced by endocrine disruptors during early life may induce long-lasting gender-specific behavioral deviations (Panzica et al. 2007; Wilson and Davies 2007). It is not known whether PFCs can trigger similar hormonal changes during murine development and this issue requires further investigations.

In conclusion, we provide evidence that under similar conditions of developmental exposure, there are differences in the distribution pattern of PFOS and PFOA in newborn mice. Exposure to PFOS during pregnancy caused alterations in motor behavior in adult mice including a decrease in exploratory activity, increased number of resting periods during circadian cycle, and decreased muscle strength. These effects were more pronounced in the exposed males, while in females they were either attenuated or absent. Prenatal exposure to PFOA was associated with inversed effects on exploratory behavior in male and female offspring, as well as elevated activity of males in the home cage. Even if the behavioral changes found in the animals exposed to low doses of PFCs had rather small magnitude, our data support previous studies pointing to PFCs as potential developmental toxicants. Comprehensive analyses of behavior, including home cage monitoring in social groups, appear to provide a number of sensitive end points to detect neurodevelopmental toxicity.

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Measurement of PFOS levels in the tissues

The analytical procedure was carried out using an Alliance 2695 HPLC system (Waters, Milford, MA, USA) coupled to a single quadruple Waters ZQ 2000 mass spectrometer. For quantitative analysis the MS detector was used with an electrospray ionisation interface in negative ion mode (ESI-), with acquisition in single ion monitoring (SIM). The *m/z* ratios 499.0 for PFOS and 463.6 for PFNA were recorded simultaneously. HPLC separation was performed on a mixed bed Waters XTerra® MS C18 column, 4.6 (I.D.) x 150 mm, 3.5 µm, kept at 28°C, by gradient elution with a mixture containing variable proportions of 3 mM ammonium acetate and methanol. The detection limits for PFOS (signal-to-noise ratio = 3) was 0.1 ng/g.

About sample treatment, preliminary determinations on pool tissues were necessary to establish the range of concentration for each tissue: to avoid saturation of the chromatographic signal it was necessary to dilute samples with water before purification steps.

Each kind of tissue was first homogenized using an ultrasonic homogenizer Omni Ruptor 250 (100 mg of tissue in 800µL of water), liquid-liquid extracted with acetonitrile and then purified with solid phase extraction cartridges in two steps (Sep-Pak tC18,Waters, 1g/6 mL followed by SAX, Isolute, 25 mg/1mL) according with the method of Maestri et al.

For each kind of tissue, a pool of homogenate was constructed to obtain the calibration curve: the five calibration standards were prepared by spiking 200µL of homogenate with PFOS to final added concentrations of 0, 4.0, 16.0, 80.0, 400.0 ng/g of

tissue; twenty microliters of internal standard (PFNA, 0.5 mg/L in water) were added to all samples.

Briefly, two hundred microliters of the homogenate were liquid-liquid extracted with 1 mL of acetonitrile and, after a centrifugation step, supernatant was diluted with 3.5 mL of water and loaded onto C18 disposable SPE cartridges previously activated with 10mL of methanol and 5mL of water; the cartridges were washed with 4mL of water and 1mL of methanol. The analytes and the internal standard (PFNA) were eluted with 2mL of methanol. So, each eluate from the C18 cartridge was loaded onto a SAX disposable SPE cartridge previously activated with 0.5 mL of methanol and 0.5 mL of water. The cartridges were washed with 1mL of 1% acetic acid and then eluted with 0.2 mL of 50% acetic acid/2M ammonium acetate solution (50:50, v/v). Finally, 25 µL of each purified sample were injected onto the chromatographic system.