TOXICOLOGICAL SCIENCES **98(2)**, 589–598 (2007)
doi:10.1093/toxsci/kfm121
Advance Access publication May 15, 2007

Concurrent Exposure to Perfluorooctane Sulfonate and Restraint Stress during Pregnancy in Mice: Effects on Postnatal Development and Behavior of the Offspring

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Received March 16, 2007; accepted May 10, 2007

The combined effects of maternal restraint stress and perfluorooctane sulfonate (PFOS) on postnatal development and behavior of the offspring were assessed in mice. Thirty-four plug positive females were randomly divided into two groups. Animals were given by gavage 0 and 6 mg PFOS/kg/day on gestation days 12–18. One-half of the animals in each group was subjected to restraint stress (30 min per session, three sessions per day) during the same period. Neither restraint nor PFOS exposure significantly modified maternal food or water consumption. Pups of dams exposed to 6 mg/kg of PFOS showed a reduced body weight on postnatal days 4 and 8. Moreover, PFOS exposure induced some delay in developmental landmarks and neuromotor maturation. Maternal restraint stress reduced activity in an open-field when combined with 6 mg PFOS/kg/day. In addition, in males prenatal restraint stress impaired motor coordination in a rotarod. The current results indicate that concurrent exposure to PFOS and restraint stress during pregnancy induces opposite effects on developmental parameters in the pups. These effects consist in a general delayed maturation trend induced by PFOS exposure, and a general accelerated maturation pattern induced by prenatal stress. Interactive effects between PFOS and maternal stress were observed in young adult mice. These effects consisted mainly in a diminished activity in an open-field test.

Key Words: **perfluorooctane sulfonate (PFOS); maternal restraint; maternal behavior; interactions; developmental toxicity; postnatal behavior.**

The perfluoralkyl acids and their salts, such as perfluoralkyl carboxylates (PFCs), and telomer alcohols and their derivatives are important chemicals that have wide consumer and industrial applications including surfactants, paper, and textile coatings, lubricants, paints, polishes, food packaging, and fire-retarding foams. However, in recent years a number of studies have reported global distribution and environmental persistence of PFCs, as well as presence in humans and wildlife (Begley *et al.*, 2005; Ericson *et al.*, in press; Giesy and Kannan, 2001). It together with the adverse health effects detected in laboratory animals has generated a considerable interest and concern regarding PFCs on an international scale.

Among the PFCs, perfluorooctane sulfonate (PFOS) has been most extensively studied (Giesy and Kannan, 2001; Kannan *et al.*, 2004). Toxicological investigations in adult research animals have shown that repeated doses of PFOS can lead to reduced body weight or weight gain, enlarged, vacuolated liver cells, reductions in blood lipids, hypothryroxinemia, and reductions in body fat (Lau *et al.*, 2004, 2006). Moreover, rodents exposed to PFOS *in utero* experienced reduced birth weight, reduced postnatal growth, and increased perinatal mortality (Lau *et al.*, 2004), whereas prenatal exposure to PFOS caused malformations in rats and mice only at maternal doses in excess of those producing neonatal mortality (Fuentes *et al.*, 2006; Lau *et al.*, 2003, 2004; Luebker *et al.*, 2005a,b; Thibodeaux *et al.*, 2003). Alterations in the levels of serum thyroxine (T4), and triiodothyronine (T3) in pregnant rats and mice after exposure to PFOS have been also observed (Thibodeaux *et al.*, 2003). The importance of the thyroid hormones for the mammalian brain maturation has been known for many years. In particular, T3 controls the expression of genes involved in myelination, cell differentiation, migration, and signaling (Bernal, 2005). On the other hand, alterations in the early phase of myelination process during the weaning period, have been related to changes in emotional behavior in male rats and mice (Kikusui *et al.*, 2007).

Recent studies describe extensively the developmental toxicity of PFOS and perfluorooctanoic acid (PFOA) in rodents, most notably neonatal mortality and long-term effects. Some features were common between PFOS and PFOA, suggesting potential class-wide toxicities for PFCs (Lau *et al.*, 2006).

Concerning the developmental toxicity of PFOS, in a recent study in mice we found that exposure to this chemical led to prenatal mortality (Fuentes *et al.*, 2006). In that study, the doses were lower than those previously administered by other

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investigators. However, most effects produced by PFOS exposure have been observed when this chemical was administered at high doses (Fuentes *et al.*, 2006; Lau *et al.*, 2003, 2004; Luebker *et al.*, 2005a,b; Thibodeaux *et al.*, 2003).

On the other hand, a number of studies conducted on rodents have shown that stress during pregnancy may induce maternal toxicity and adverse effects on embryo/fetal development, including impaired learning in male offspring and behavioral alterations in response to stress situations (Domingo *et al.*, 2004). Such effects depend largely on the period of exposure and the kind of stressor (Fuentes *et al.*, 2006; Golub *et al.*, 2004). Various investigations have demonstrated that some chemicals can modulate the effects of maternal stress, and conversely, maternal stress can also modify the effects of some chemicals, especially when they are given at relatively high doses (Colomina *et al.*, 1997, 1999; Fuentes *et al.*, 2006). In our previous study, we showed that maternal restraint in pregnant mice could enhance the PFOS-induced embryo/fetal toxicity, which was evidenced by a higher prenatal mortality (Fuentes *et al.*, 2006).

Due to the wide presence and persistence of PFOS in the environment, exposure to this chemical seems to be currently probable. Pregnant women may be potentially exposed to both stress and various types of pollutants, either at home or in the workplace. Given the fact that maternal stress and PFOS can induce developmental effects on the offspring, the purpose of the present study was to evaluate in mice whether maternal stress might enhance the potential adverse effects of oral PFOS exposure during late pregnancy on the postnatal development and behavior of the offspring.

MATERIALS AND METHODS

Animals

Sexually mature male and female Charles-River CD-1 mice (28–32 g) were purchased from Criffa (Barcelona, Spain). After 7 days of quarantine, female mice were mated with males (2:1) until copulation was detected. The day on which a vaginal plug was found was designated as day 0 of gestation. Animals were housed in plastic cages in a climate-controlled facility at a temperature of 22 ± 2°C, a relative humidity of 50 ± 10%, and a constant day–night cycle (light: 08:00–20:00 h) with free access to food (Panlab rodent chow, Barcelona) and tap water. From gestation day (GD) 0 to GD 18 two or three dams were housed together. On GD 18 dams were individually housed to allow individual litter observation during delivery and weaning until postnatal day (PND) 21. On GD 18, pups were separated according to the treatment group and sex. They were maintained in the same housing conditions until the beginning of the behavioral tests (at 3 months of age). The weight of the pups was recorded before behavioral testing (Fig. 1). The use of animals and the experimental protocol were approved by the Animal Care and Use Committee of the ''Rovira i Virgili'' University.

Chemical

PFOS (potassium salt) was obtained from Fluka Chemical (Steinheim, Switzerland). It was dissolved in 0.5% Tween 20 (Bio-Rad, Hercules, CA) and administered by gavage at doses of 0 and 6 mg/kg/day from GD 12 to 18. PFOS solutions were weekly prepared and concentrations were adjusted at volumes of 0.3 ml/30 g body weight. The choice of 6 mg PFOS/kg/day was based on previous studies in which a similar dose was administered (Fuentes *et al.*, 2006; Lau *et al.*, 2003).

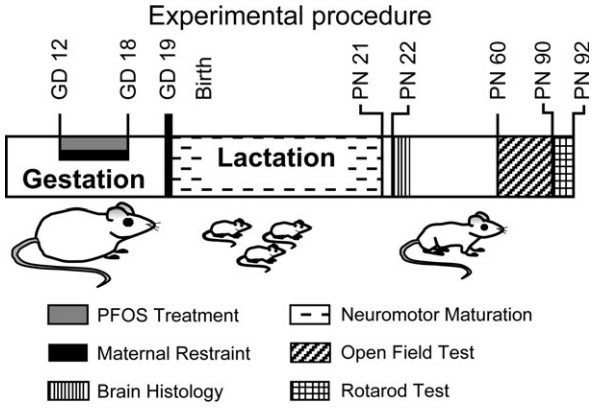

FIG. 1. Scheme of the experimental procedure. During GD 12–18 dams were treated with PFOS and daily subjected to restraint stress (30 min × three sessions). Developmental landmarks and neuromotor development were assessed during the lactation period, while motor activity and coordination were evaluated at 3 months of age.

Treatment

On GD 0, female mice were weighed and randomly assigned to one of the four treatment groups ($n = 8$–10 females per group). The experimental groups were distributed as follows: two groups received PFOS only at 0 and 6 mg/kg/day, whereas two additional groups received the same PFOS doses being also subjected to restraint. Both restraint and PFOS were given on GD 12–18 (Fig. 1). Restrained animals were immobilized three times per day (30 min each time). Mice were subjected to the first restraint session immediately after PFOS administration. A fixed time gap of 3 h was established between restraint sessions. The restraint procedure consisted in placing the mice in metacrilate cylindrical holders from Letica Scientific Instruments (Panlab, Barcelona) and maintaining them in a prone position. In previous studies, this procedure has shown to cause maternal stress in rodents (Albina *et al.*, 2005; Colomina *et al.*, 2005; Fuentes *et al.*, 2006). Maternal body weight and food and water intake of the dams were recorded on GD 0 and during the treatment period (GD 12–18) (Fig. 1).

Physical and Functional Assessment

Pregnant mice were allowed to deliver and wean their offspring. At birth, the length of gestation, maternal weight, number of live, and dead fetuses and sex and weight of every pup were recorded. Litter size was culled to 8 on PND 0. When possible, a sex ratio of 4:4 was maintained. Otherwise, pups were selected randomly.

On PND 3, a maternal care test was conducted. Pups and dam were separated and the time required by the dam to collect them recorded. On PND 4, 8, 12, and 21, body weight of pups was recorded. During these PND, maternal nesting behavior of the dams was observed and scored. Pinna detachment, incisor eruption, eye opening, vaginal opening, and testes descent were monitored as developmental landmarks in every pup of the litter. On PND 9, 11, 13, and 15, the auditory startle reflex was assessed in one pup of each sex in each litter. The test used consisted in placing a clicker behind the head of the pup, not further than 1 cm, and click it once. Response of the pup was registered as follows: 0, no response; 1, small response; 2, not coordinated whole body response, and 3, jump and return to previous position.

Neuromotor Maturation

Pups were tested with a routine testing battery in order to determine general effects. Surface righting performance was evaluated in every pup of each litter.

From PND 3 up to the achievement of the reflex within 2 s. pups were placed on their back on a horizontal board and released. Time needed to return to the dorsum-ventral position was recorded. On PND 10, 11, and 12, the development of the tail pull reflex, and cling and climb abilities were tested in one pup of each sex per litter. Pups were placed near the top on vertical screen and pulled backward by gentle pull on its tail. Resistance offered by the pup during the pull was recorded. Marks for tail pull reflex were given as follows: 0, the pup offers no resistance; 1, some resistance; 2, good resistance during part of the pull; and 3, good resistance during most of the pull. In order to test cling and climb ability, pups were placed near the bottom on vertical screen and allowed to climb. Marks for cling skill were given as follows: 0, the pup falls off immediately; 1, falls within 15 s; 2, holds on 15 s but slips back, and 3, spends 15 s at higher or same position. Marks given for climb skill were: 0, the pup falls off immediately; 1, climbs a body length using only forepaws; 2, does not reach the half of the screen, and 3, climbs to the top half of the screen.

Forelimb grip strength was measured on PND 9, 11, 13, and 15 with a grip strength meter from Ugo Basile (Panlab, Bacelona, Spain). The grip strength meter is a force transducer connected to a peak amplifier. When the mice are pulled backward across the grasping trapeze, they grasp it tightly to try to stop this movement. The peak pull force exerted on the trapeze is recorded on every trial. The maximum force from three consecutive trials was used as the measure of grasp strength.

Myelin Basic Protein Immunohistochemistry

On PND 22, two males in each group from different litters were deeply anesthetized with ketamine/xylazine (80/10 mg/kg, ip) before sacrifice, and perfused through the heart with 4% of paraformaldehyde (PFA). Brains were removed and fixed overnight in 4% PFA at 4°C. Subsequently, they were placed into a 30% sucrose/phosphate-buffered saline (PBS) for 48 h at 4°C and then snap frozen in isopentane. For myelin basic protein (MBP) immunostaining, free-floating coronal sections of brain were rinsed in 0.1% Triton X-100 (Sigma-Aldrich, Barcelona, Spain) in PBS, and H_2O_2 to inactivate endogenous phosphatase activity. Sections were blocked in 0.2% gelatin–10% fetal bovine serum (FBS)–PBS 0.1% Triton X-100 buffer for 2 h. Antibody against MBP (Chemicon, Barcelona, Spain) was diluted at 1/1000 in blocking buffer and incubated overnight at 4°C. Tissue sections were then washed with PBS and incubated with 1:200 biotinylated goat anti-rabbit antibody for 1 h at room temperature. After additional washes, the secondary antibody was detected using the avidin–biotin complex reaction (ABC Elite Kit, Vector Laboratories, Barcelona, Spain) and developed with peroxidase substrate kit DAB, SK-4100 (Vector Laboratories, Barcelona, Spain). The sections were then counterstained with (0.5%) crysil violet for 30 s, washed, dehydrated with 100% ethanol, and mounted with DPX.

Postweaning Tests

The following tests were performed at 3 months of age ($n = 10$). One male and one female from each litter were used when possible. Otherwise, a maximum of two males or females from each litter were used. The same cohort of animals was examined in both behavioral tests.

Open-field activity. General motor activity was measured in an open-field apparatus, consisting of a wood 1 × 1-m square surrounded by a 47-cm-high dark colored wall. During the test, mice were allowed to move freely around the open-field during 15 min. The path and movements of the animals were recorded by a video camera (Sony CCD-IRIS model) that was placed above the square. The video tracking program Etho-Vision (Noldus Information Technologies, Wageningen, The Netherlands) was used to measure the total distance traveled and the number of rearings (a measure of vertical activity).

Rotarod. Motor coordination and balance were tested by using an accelerating rotarod (UGO Basile Accelerating Rotarod). The rotarod test was performed by placing the animal on a rotating drum and recording every time the mouse was not able to maintain its balance and walk on top of the rod. The number of the whole flips on the rod and the latency of the first fall was also recorded. This procedure was repeated for 3 days. During the two first sessions (days 1 and 2), the speed of the rotarod was set at 16 rpm. The speed during the third session (day 3) was increased to 24 rpm. In every session, the animal was placed on the rod for 120 s. Every time the animal fell off, it was placed again on the drum.

Data Analysis

Maternal data such as body weight, body weight change, and water and food consumption were analyzed by a two-way analysis of variance (ANOVA), using PFOS treatment and restraint as factors. For the developmental study, the litter was the unit of statistical analysis. Pup body weight, developmental landmarks, and neuromotor maturation data were evaluated by a three-way ANOVA for repeated measures, using the age as the repeated measure, and PFOS exposure, restraint, and sex as factors. Sexual maturation was analyzed with a two-way ANOVA (PFOS × restraint). The postweaning tests rotarod and exploration of an open-field were analyzed with a three-way ANOVA for repeated measures, using the session or the period of time tested as the repeated measure, and PFOS exposure, restraint, and sex as factors. When no differences between sexes were noted the average of both sexes was used for each parameter. To assess differences between groups, one-way ANOVA and Tukey *post hoc* analyses for multiple comparisons were performed. Levene's test was used to determine variance homogeneity. In case of heterogeneity of variance (at $p < 0.05$), nonparametric tests (Kruskal–Wallis and Mann–Whitney *U*-test) were used. In all cases, statistical significance was set at $p < 0.05$.

RESULTS

Maternal Effects

No differences between groups were observed on food and water consumption (data not shown), or in maternal body weight change during the treatment period (Table 1). A general effect of restraint stress [$F(1,27) = 6.867$, $p = 0.015$] was noted on postnatal maternal care. Differences among groups were observed [$X^2 = 7.851$, fd 3, $p = 0.049$]. Dams from restrained only, and restrained and concurrently exposed to PFOS groups showed a significant faster response in attending the pups than dams in other groups (Table 1). No differences were seen in maternal nesting behavior (data not shown).

Fetal Outcome and Physical Maturation

Individual and/or concurrent exposure to PFOS and restraint during late gestation did not significantly affect the length of gestation or the number of live pups (Table 2). Only one PFOS-treated dam (from 10) prematurely delivered (GD 17). Dead pups at birth (PND 2) were found in one litter (from 8) in the control restrained group, and in three litters (from 9) in the group concurrently exposed to PFOS and restraint. However, the differences between groups were not statistically significant ($p = 0.068$). A three-way (PFOS × restraint × sex) ANOVA for repeated measures using the age as repeated measure, evidenced an overall effect of PFOS [$F(4,48) = 4.967$, $p = 0.030$] and restraint [$F(4,48) = 10.335$, $p = 0.002$] on pup body weight gain during lactation, indicating different growth patterns over the time. In particular, restrained groups presented a more pronounced body weight increase in the late lactation period. Because no differences between sexes were found, male and females were analyzed together to assess body weight

TABLE 1
Effects of PFOS and Maternal Restraint Stress on Body Weight Gain in Pregnant Mice

PFOS (mg/kg/day)	0	0	6	6
Restraint stress	−	+	−	+
No. of dams	8	8	10	9
Dam body weight (g) on GD 0	25.19 ± 1.20	25.27 ± 1.92	24.79 ± 1.59	24.98 ± 1.20
Dam body weight (g) on GD 12	36.99 ± 2.63	37.30 ± 3.44	38.00 ± 2.90	34.75 ± 4.24
Dam body weight (g) on GD 15	44.30 ± 4.11	43.31 ± 4.36	44.69 ± 3.44	42.49 ± 3.27
Dam body weight (g) on GD 18	53.65 ± 6.04	52.72 ± 6.36	53.63 ± 3.80	50.20 ± 4.75
Body weight change (g) treatment period (12-18)	16.66 ± 3.80	15.42 ± 3.52	15.63 ± 1.67	15.44 ± 5.75
Body weight change (g) gestation period (0-18)	28.46 ± 5.13	27.45 ± 5.97	28.84 ± 2.98	25.22 ± 4.60
Maternal care (s)	13.33 ± 6.71[a]	4.14 ± 3.85[b]	12.89 ± 13.73[a]	4.00 ± 4.94[b]

Note. Data are given as means ± SD. In each row, significant differences for comparisons between groups are indicated by the use of superscripts (a, b). Groups not showing a common superscript are significantly different at $p < 0.05$.

differences between groups, which were observed on PND 4, 8, and 21. Significant differences between groups on PND 4 and 8 consisted in a diminished body weight in the groups of unrestrained mice exposed to PFOS in comparison to restrained or unrestrained controls. These differences disappeared and returned close to control values on PND 12. As for restrained pups, both prenatally exposed and nonexposed to PFOS groups showed a significant increased body weight on PND 21 when compared to PFOS-treated or unrestrained control groups (Fig. 2).

A three-way (PFOS × restraint × sex) ANOVA test indicated no sex effect on developmental landmarks (incisor eruption, eye opening, and pinna detachment). General PFOS effects on pinna detachment [$F(1,32) = 6.909$, $p = 0.014$], on incisor eruption [$F(1,32) = 4.509$, $p = 0.042$], and on eye opening [$F(1,32) = 16.172$, $p < 0.001$] were detected. An overall effect of restraint stress was observed on incisor eruption [$F(1,32) = 5.140$, $p = 0.031$]. PFOS exposure prompted delays in pinna detachment, incisor eruption, and eye opening, while the group exposed to prenatal restraint stress presented a faster incisor eruption when compared to the PFOS-exposed unrestrained group (Table 2).

Moreover an overall effect of restraint was observed in both males and females on sexual maturation [$F(1,32) = 40.834$, $p < 0.001$; $F(1,32) = 5.423$, $p = 0.027$]. A more detailed analysis showed significant differences among groups only on male sexual maturation [$F(1,32) = 14.324$, $p < 0.001$]. Both groups of males whose dams were exposed to restraint presented a faster sexual maturation in comparison with those of the unrestrained groups (Table 2). In general terms, prenatal PFOS exposure tended to slightly delay physical maturation, while prenatal restraint tended to accelerate it.

Neuromotor and Reflexes Maturation

The effects of treatment on neuromotor development and reflexes maturation were evaluated by a three-way (PFOS × restraint × sex) ANOVA for repeated measures (at different ages). As expected, a significant improvement related to age was observed in all measures. Statistical analysis did not reveal differences between males and females. For this reason, male and females values from each litter were taken together to analyze the differences between groups. An overall effect of restraint on surface righting was observed [$F(1,27) = 4.419$,

TABLE 2
PFOS (mg/kg/day) Effects of PFOS and Prenatal Restraint Stress on Physical Maturation

PFOS (mg/kg/day)	0	0	6	6
Restraint	−	+	−	+
Length of gestation (days)	19.00 ± 0.00	19.17 ± 0.41	18.90 ± 0.32	19.00 ± 0.25
No. of litters	8	7	10	9
No. of pups per litter	12.75 ± 2.66	11.71 ± 2.06	11.20 ± 3.01	10.89 ± 2.50
No. of days at pinna detachment	4.13 ± 0.28[a]	4.11 ± 0.43[a]	4.70 ± 0.52 [b]	4.24 ± 0.32[a]
No. of days at incisor eruption	4.58 ± 0.64[ab]	3.90 ± 0.69[a]	4.87 ± 0.55 [b]	4.53 ± 0.72[ab]
No. of days at eye opening	13.73 ± 0.32[ab]	13.48 ± 0.4[a]	14.30 ± 0.66 [b]	14.27 ± 0.36[b]
No. of days at testes descent	25.12 ± 0.58[a]	23.00 ± 1.26[b]	25.30 ± 0.67 [a]	23.72 ± 0.79[b]
No. of days at vagina opening	21.94 ± 0.82	21.28 ± 0.57	21.45 ± 0.28	21.25 ± 0.27
Surface righting				
PND 4	3.56 ± 1.31[ab]	1.44 ± 0.92[a]	5.03 ± 4.24[b]	1.72 ± 0.61[a]
PND 5	1.41 ± 0.76[ab]	0.83 ± 0.79[a]	1.92 ± 0.58[b]	1.07 ± 0.93[ab]

Note. Data are given as means ± SD. In each row, significant differences for comparisons between groups are indicated by the use of superscripts (a, b). Groups not showing a common superscript are significantly different at $p < 0.05$.

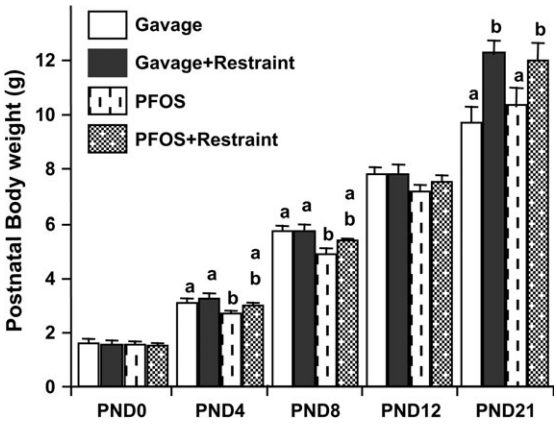

FIG. 2. Body weight of pups during the lactation period. For each PND, different letters in different groups (a, b) indicate significant differences at $p < 0.05$. Data are expressed as means ± SEM.

$p = 0.045$]. In particular, significant differences between groups were noted on PND 4 and 5, when pups exposed to restraint presented a faster ability to return to the prone position in comparison with unrestrained PFOS-exposed pups (Table 2). Concerning pull, cling, and climb tests, data revealed a significant overall effect of PFOS on tail pull resistance [$F(1,29) = 9.065$, $p = 0.005$], as well as interactions between age and PFOS [$F(2,28) = 12.793$, $p < 0.001$], and PFOS × restraint [$F(1,29) = 8.650$, $p = 0.006$]. Differences between groups were observed on PND 10 and 11 [$X^2 = 10.832$, fd 3, $p = 0.013$; $F(3,32) = 6.230$, $p = 0.002$]. Animals in the unrestrained PFOS-exposed group offered a diminished resistance during the pull in comparison with those in the remaining groups (Fig. 3). Concerning climb ability, data showed an overall effect of PFOS [$F(1,29) = 5.263$, $p = 0.029$] and an interaction between PFOS and restraint [$F(1,29) = 5.461$, $p = 0.027$]. Differences between groups were observed on PND 11 [$F(3,32) = 3.834$, $p = 0.020$], day on which climb ability in PFOS-exposed mice was reduced when compared to the respective unrestrained control group (Fig. 3). No effects on cling ability were seen (data not shown).

An overall effect of PFOS was also observed on forelimb grip strength on PND 11 [$F(1,32) = 5.844$, $p = 0.026$]. An overall effect of restraint was observed on PND 15 [$F(1,32) = 6.087$, $p = 0.023$]. However, differences between groups were only noted on PND 11, when unrestrained PFOS-exposed group showed diminished forelimb grip strength in comparison with the control unrestrained group. No significant differences between groups were observed on any other day of testing (Fig. 4). Statistical analysis of the data revealed an overall effect of restraint on auditory startle [$F(3,28) = 12.030$, $p = 0.002$]. Pups exposed to restraint tended to show a reduced reactivity in this test. Further analysis did not reveal any difference among groups (data not shown). In general terms, prenatal PFOS exposure tended to delay neuromotor maturation while the most remarkable general trend for prenatal restraint is covering up PFOS effect.

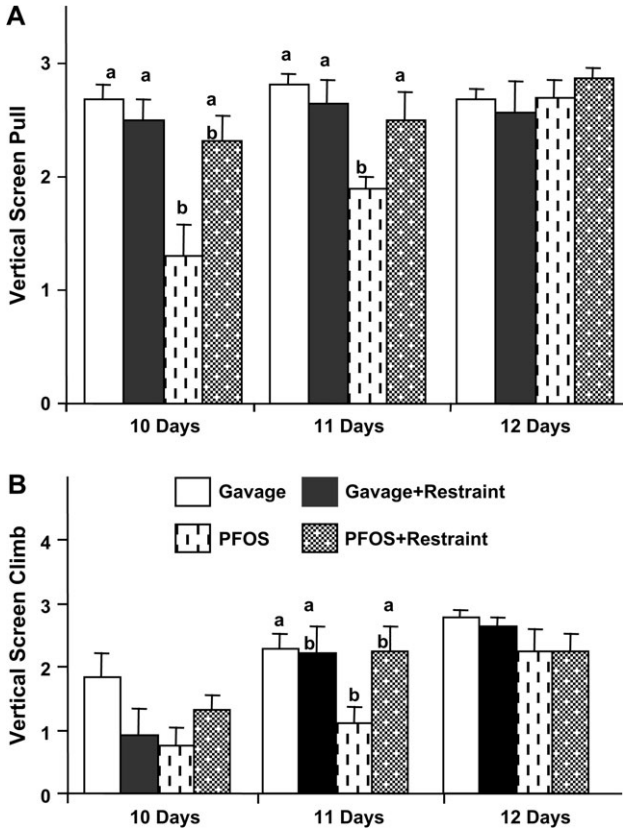

FIG. 3. (A) Pups postnatal resistance to backward pull, scored from 0 (no resistance) to 3 (good resistance). (B) Pups postnatal time to climb a vertical screen. For each PND, different letters in different groups (a, b) indicate significant differences at $p < 0.05$. Data are expressed as means ± SEM.

MBP Immunohistochemistry

No differences between mice exposed to PFOS and/or prenatal restraint stress and control groups were observed in myelination pattern at 22 days of age (Fig. 5).

Postweaning Tests

Open-field activity. Data obtained from 15-min exploratory activity measured in an open-field were analyzed by a three-way (PFOS × restraint × sex) ANOVA and repeated measures over a 15-min period. A significant decrease in the distance moved [$F(2,66) = 99.541$, $p < 0.001$] indicated habituation to the new environment. No differences in the habituation pattern were observed due to prenatal PFOS exposure or maternal restraint stress. Significant overall effects of restraint [$F(1,66) = 5.100$, $p = 0.027$], and a significant interaction between PFOS and restraint [$F(1,66) = 4.041$, $p = 0.049$] on total distance traveled were found. In relation to vertical activity, habituation over the 15-min period was also observed [$F(2,66) = 6.650$, $p = 0.002$]. However, a significant interaction between the period of time, sex, and restraint [$F(2,66) = 4.914$, $p = 0.010$] indicated a different habituation pattern depending on prenatal restraint exposure and sex.

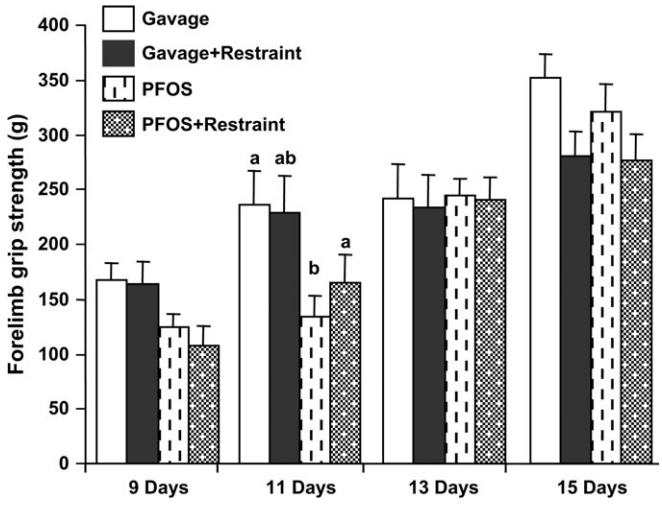

FIG. 4. Forelimb grip strength expressed in g. For each PND, different letters in different groups (a, b) indicate significant differences at $p < 0.05$. Data are expressed as means ± SEM.

Because of significant sex related effects observed in vertical activity, in order to assess differences between groups in both vertical and horizontal activity, sexes were analyzed separately. Differences in total distance traveled were seen in females, where the group exposed to both prenatal restraint stress and PFOS showed a reduced activity compared to that of the unrestrained PFOS-exposed group. In contrast, differences were not significant in males (Fig. 6). Significant differences in vertical activity were observed in both males and females exposed prenatally to maternal restraint when compared to the remaining groups in females, and when compared to PFOS-exposed groups in males (Fig. 6).

A more detailed analysis performed to assess differences between center and periphery activity showed a significant reduction in prenatally groups exposed to PFOS and maternal restraint. However, no differences in the calculated ratio distance moved in the center with respect to total distance were found, indicating that differences in center activity were only a reflex of a diminished general activity of such groups (data not shown).

In general terms, a reduction in horizontal activity in the group prenatally exposed to PFOS and maternal restraint was noted, while an increased vertical activity was observed in prenatally maternal restraint exposed males and females.

Rotarod test. Concerning motor coordination and balance measured in a rotarod, a three-way (PFOS × restraint × sex) ANOVA for repeated measures, using the session as repeated measure, was performed to analyze the learning of the animals during the two first sessions (velocity = 16 rpm). Data showed a general effect of session on the number of falls, indicating all mice improved the execution of the task in the second session

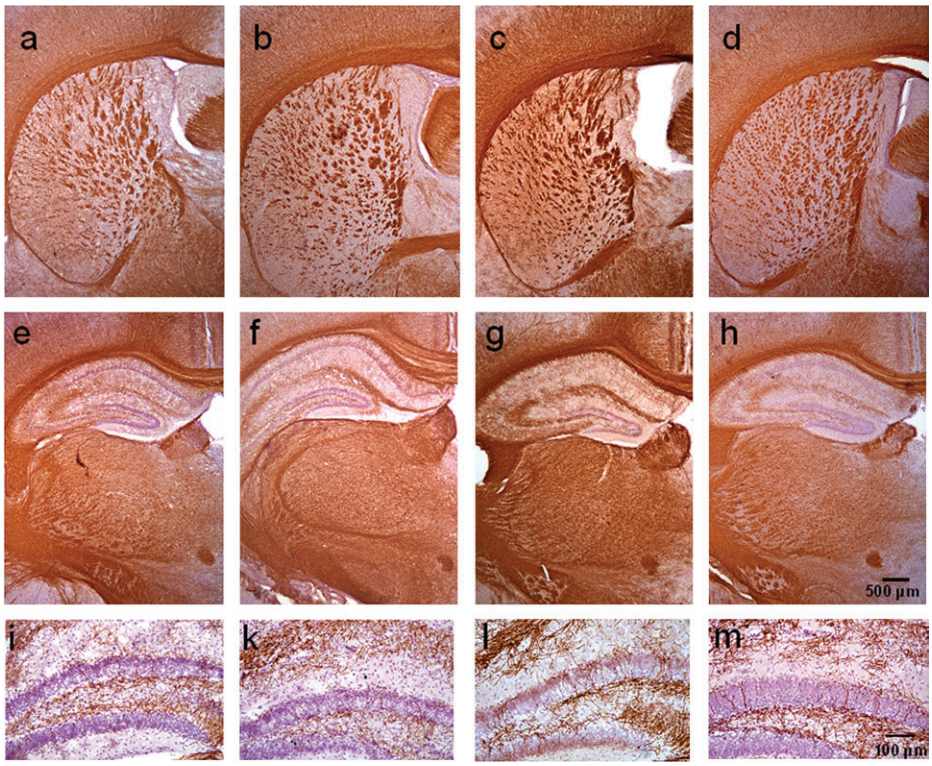

FIG. 5. MBP immunostaining counterstained with Crysil violet stain. Coronal brain slices from PND 22 pup males were stained with MBP, which binds MBP and counterstained with crysil violet to visualize cellular bodies of control group (a, e, and i), restraint group (b, f, and k), PFOS exposed (c, g, and l), and PFOS and restraint exposed group (d, h, and m). No differences between groups could be found.

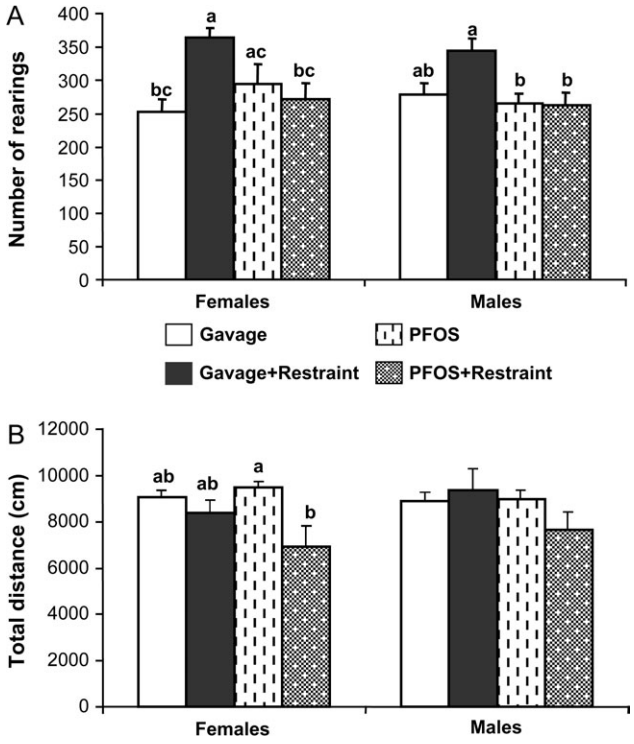

FIG. 6. (A) Vertical activity in an open-field measured as the number of total rearings during a 15-min period. (B) Total distance traveled in an open-field. Different letters (a, b, c) indicate significant differences at $p < 0.05$. Data are expressed as means ± SEM.

[$F(1,72) = 33.218$, $p < 0.001$]. Significant overall effects of sex [$F(1,72) = 11.572$, $p = 0.001$] and restraint [$F(1,72) = 9.659$, $p = 0.003$], a restraint × PFOS interaction [$F(1,72) = 4.02$, $p = 0.049$], and a restraint × sex interaction [$F(1,72) = 4.628$, $p = 0.035$] were noted. Differences between groups were observed in males. A Kruskal–Wallis test revealed a difference among male groups in the number of falls during the first session [$X^2 = 10.857$, df 3, $p = 0.013$]. This difference consisted in a greater number of falls in the groups exposed to maternal restraint either alone or combined with PFOS, when compared to the PFOS only group (Fig. 7). No other treatment effects were observed during the two first sessions, neither on the latency of the first fall, nor on the number of whole flips. No differences were observed in females for any of the assessed variables.

With respect to the third session (velocity = 24 rpm), no differences between groups, neither in males nor in females, were observed. However, significant differences between sexes emerged only in prenatally restrained groups (Fig. 7C), suggesting that prenatal stress increased differences between sexes impairing male performance in this task.

DISCUSSION

Taking into account the interest and concern raised by the perfluoroalkyl acids and their salts, in recent years several

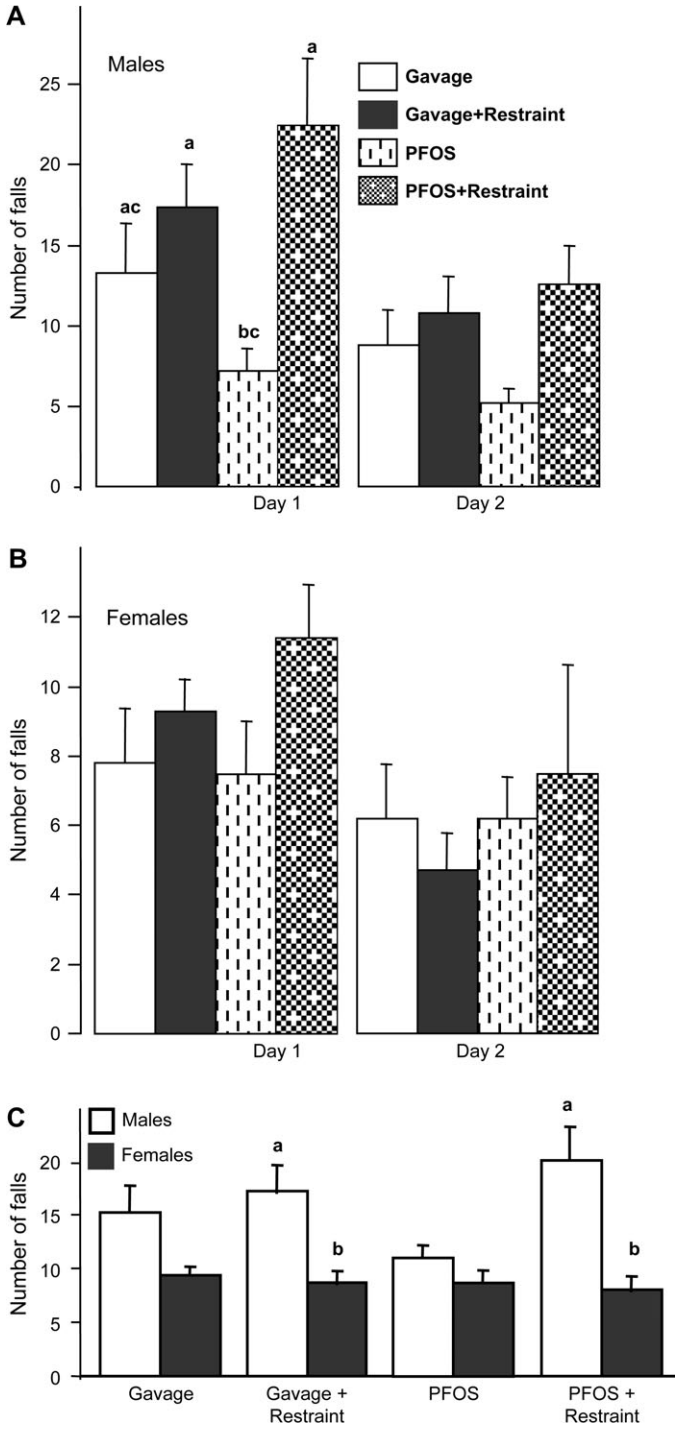

FIG. 7. (A) Males, and (B) females show the total number of falls during the first and second day sessions at a fixed velocity of 16 rpm in a rotarod test. (C) Number of total falls in males and females during the third day session at a fixed velocity of 24 rpm in a rotarod test. Different letters between days (a, b, c) indicate significant differences at $p < 0.05$. Data are expressed as means ± SEM.

workshops have been held to address trace analysis of these compounds in various media, their fate and transport, and their toxicity. Recently (14–16 February 2007), a Society of Toxicology-Contemporary Concepts in Toxicology Workshop was held in Arlington, VA. The main goal was to identify and prioritize future research directions. It was noted that toxicological studies including developmental toxicity investigations that allow understanding the mechanisms causing perinatal and developmental effects in mammals are clearly necessary.

In a recent study, Wolf *et al.* (2007) segregated the contributions of gestational and lactational exposures to PFOA and considered the impact of restricting exposure to specific gestational periods. Pregnant CD-1 mice were dosed on GD 1–17 with 0, 3, or 5 mg PFOA/kg body weight, and pups were fostered at birth to give seven treatment groups. It was concluded that the postnatal developmental effects of PFOA were due to gestational exposure. Exposure earlier in gestation produced stronger responses, but it was remarked that further study would be needed to determine if this was a function of higher total dose or if there is a developmentally sensitive period. On the other hand, when pregnant CD-1 mice received 3 or 5 mg/kg PFOA or control vehicle via oral (gavage) on GD 1–17, it was found that serum PFOA concentration was more highly associated with altered mammary gland development than exposure conditions alone (White *et al.*, 2007).

In relation to the results of the present study, maternal exposure to PFOS or restraint stress during the late gestation period (GD 12–18) did not produce observable toxic effects on the dam. Previous data on maternal restraint stress effects in both dams and pups showed important differences depending on the intensity and duration of the stress and on the gestational period in which it was administered (Fuentes *et al.*, 2006; Kofman, 2002). In a recent study, we found restraint effects on food consumption and body weight (Fuentes *et al.*, 2006), when maternal restraint and PFOS were given on GD 6–18. Decreases in maternal body weight and food intake related to maternal restraint stress have been reported (Albina *et al.*, 2005; Colomina *et al.*, 1999). In those studies, the nature, the intensity or the duration of the period of restraint stress were higher than those administered in the present investigation.

However, in spite of the absence of maternal physical effects, restraint stress altered maternal behavior, reducing the time spent by the dams to gather their pups. It has been described that stress during gestation can alter maternal behavior. In turn, it might produce changes in the offspring behavior. Notwithstanding, controversial results on stress-induced maternal behavior changes have been reported (Lehmann *et al.*, 2000; Smith *et al.*, 2004). The current results are in accordance with those of a study by Maestripieri *et al.* (1991), in which restrained dams tended to improve their maternal behavior. It seems to indicate that differences in stress procedures affect differently maternal behavior.

According to recent studies in our laboratory (Fuentes *et al.*, 2006), perinatal mortality was considerably affected in the group exposed concurrently to PFOS at 6 mg/kg/day and restraint on GD 6–18. However, in the present investigation the differences in this parameter did not reach the level of statistical significance. The current results did not show any difference neither in delivery data nor in viability of the offspring, which is in contrast with other studies using a more prolonged period of treatment. In this sense, Lau *et al.* (2003) reported high postnatal mortality in mice exposed at equal or higher 10 mg/kg/day on GD 1–17. Recently, Luebker *et al.* (2005b) reported adverse effects in rats on reproductive/developmental outcome including decreased length of gestation (at 3.2 mg/kg/day) and reduced postnatal viability and body weight gain at doses of 1.6 mg/kg/day and higher. In that experiment, males and females were treated prior and during mating, and females received also the chemical through the gestation and lactation periods. Thus, a more prolonged period of time of exposure during gestation might account for a higher accumulation of PFOS in fetuses, inducing a higher rate of mortality than that observed in the present investigation.

In the current study, body weight from PND 4 to PND 8 was lower in the group exposed to PFOS in comparison to that of the control group, while these differences disappear at PND 12. At PND 21, pups from restrained dams showed a significant increase in body weight. Because PFOS is accumulative in the organism and excretion via milk has been reported (Luebker *et al.*, 2005b; So *et al.*, 2006), a possible explanation for these changes emerging at PND 4, but not present at PND 0, could be related to PFOS exposure through maternal milk. A more limited amount of PFOS excreted via milk could be expected in the present study, in which dams were exposed to PFOS during late gestation in comparison to previous investigations using higher doses during the whole gestation period, or extending the treatment period to lactation. In the present study, this exposure would decrease over the lactation period because dams were not exposed to PFOS since GD 18. Other potential hypotheses, such as a delayed prenatal effect evidenced on PND 4, seem less plausible, but can not be discarded. Moreover, PFOS toxicity in mice requires higher doses of the chemical than those required for toxicity in rats (Lau *et al.*, 2003).

On the other hand, maternal restraint induced an increase in body weight on PND 21, which could be related to an accelerated maturation (see below) and a better ability to autonomous feeding or to alterations in energy homeostasis induced by late prenatal stress as demonstrated recently (Mueller and Bale, 2006). Once again, some data support differential maternal stress effects in the offspring depending on the intensity and the gestational time window in which stress was induced. Notwithstanding, prenatal restraint stress can mask PFOS effects on body weight observed on PND 4 and 8.

As previously reported, exposure to PFOS led to delays in eye opening and pinna detachment (effect covered up by maternal restraint), but did not affect sexual maturation (Lau *et al.*, 2003; Luebker *et al.*, 2005a; Meek *et al.*, 2000). Concerning neuromotor maturation, the group exposed to PFOS

presented a reduced ability in surface righting in comparison with the restrained control group. Luebker *et al.* (2005b) reported that rats exposed at 1.6 mg PFOS/kg/day showed a delay in the development of such skill. In the present study, PFOS also prompted difficulties in the development of skills such as pull tail reflex, climbing an inclined screen, and forelimb grip strength, supporting a delayed neuromotor maturation induced by prenatal PFOS exposure.

By contrast, maternal restraint stress seems to accelerate certain parameters at both physical and neuromotor maturation. It prompted an earlier incisor eruption and sexual maturation in males. However, Meek *et al.* (2000) described delays in incisor eruption in pups from prenatal stressed dams during the last week of gestation. In that study, stress procedure consisted in a variable stress paradigm which combined different stressors (heat, noise, and handling), and induced a higher stress response in the dam than the restraint procedure used in the present study. Our results show that maternal stress also favored the development of certain abilities like surface righting and covered up or reduced the adverse effects produced by the chemical in pull tail reflex and climb ability. These results are in agreement with those from studies using short periods of restraint stress during the late gestation (Fujioka *et al.*, 2001; Ward and Wainwright, 1989). The pattern of developmental effects due to gestational stress depends largely on the procedure and the GDs of exposure (Colomina *et al.*, 1997, 2001; Fujioka *et al.*, 2001; Golub *et al.*, 2004). Our results did not show adverse interactions between PFOS and maternal restraint on physical or neuromotor development. It has been well established that maternal stress enhances the induced developmental toxicity of some chemicals only at doses that are also clearly toxic to the dams (Domingo *et al.*, 2004). However, the period of exposure during pregnancy and the intensity and frequency of maternal stress are determinant factors in the interactive effects between exposure to chemicals and prenatal stress.

Although PFOS and maternal stress interaction did not cause adverse effects on physical maturation or in neuromotor development, it caused behavioral disturbances evidenced in adult offspring. At 3 months of age, animals exposed to both PFOS and prenatal stress presented a reduced distance traveled in the open-field test, a decrease that was mainly observed in females. In turn, prenatally stressed pups increased rearing behavior. Open-field activity is a measure of exploratory behavior in a novel environment. Studies applying prenatal stress during late gestation describe increased rearing behavior in prenatal stressed male rats (Fonseca *et al.*, 2002; Kofman, 2002). For example, Son *et al.* (2007) found a hyperactive behavior in prenatally stressed mice on GD 8–18. However, variability of the strain, method of measuring locomotion, and the type of prenatal stress may result in inconsistent results in prenatal stressed pups' motor activity (Kofman, 2002). Results in the rotarod test indicated a sex-dependent effect of prenatal restraint in this task. Both groups of males subjected to stress presented a worse performance during the first day of the test when compared to animals in the PFOS-exposed group. Moreover, an impaired coordination in prenatal stressed males when compared to females emerged in the third session. Because motor coordination is not usually an endpoint in prenatal stress studies, no data from other investigations are available. It has been recently demonstrated that prenatal maternal restraint alters the granular-to-Purkinje cell ratio in rat cerebellum (Ulupinar and Yucel, 2005; Ulupinar *et al.*, 2006). However, whether the changes observed in the present study might be related to such alteration or to modifications in other neural systems cannot be established with the current data.

In summary, it can be concluded that prenatal exposure to PFOS induced several transient delays on developmental landmarks and neuromotor maturation, while prenatal restraint stress tended to cover up such effects. Furthermore, a decreased mobility in concurrently PFOS and prenatal restraint stress exposed mice was observed, whereas prenatal stressed mice showed poor coordination in a sex-dependent way, and an increased exploratory activity. These findings indicate long lasting functional alterations, which deserve further investigations specially to assess stress challenge behavioral responses after prenatal exposure to PFOS and restraint stress.

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