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# Exposure to Perfluorooctane Sulfonate during Pregnancy in Rat and Mouse. II: Postnatal Evaluation

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**The postnatal effects of *in utero* exposure to perfluorooctane sulfonate (PFOS, $C_8F_{17}SO_3^-$) were evaluated in the rat and mouse. Pregnant Sprague-Dawley rats were given 1, 2, 3, 5, or 10 mg/kg PFOS daily by gavage from gestation day (GD) 2 to GD 21; pregnant CD-1 mice were treated with 1, 5, 10, 15, and 20 mg/kg PFOS from GD 1 to GD 18. Controls received 0.5% Tween-20 vehicle (1 ml/kg for rats and 10 ml/kg for mice). At parturition, newborns were observed for clinical signs and survival. All animals were born alive and initially appeared to be active. In the highest dosage groups (10 mg/kg for rat and 20 mg/kg for mouse), the neonates became pale, inactive, and moribund within 30–60 min, and all died soon afterward. In the 5 mg/kg (rat) and 15 mg/kg (mouse) dosage groups, the neonates also became moribund but survived for a longer period of time (8–12 h). Over 95% of these animals died within 24 h. Approximately 50% of offspring died at 3 mg/kg for rat and 10 mg/kg for mouse. Cross-fostering the PFOS-exposed rat neonates (5 mg/kg) to control nursing dams failed to improve survival. Serum concentrations of PFOS in newborn rats mirrored the maternal administered dosage and were similar to those in the maternal circulation at GD 21; PFOS levels in the surviving neonates declined in the ensuing days. Small but significant and persistent growth lags were detected in surviving rat and mouse pups exposed to PFOS prenatally, and slight delays in eye opening were noted. Significant increases in liver weight were observed in the PFOS-exposed mouse pups. Serum thyroxine levels were suppressed in the PFOS-treated rat pups, although triiodothyronine and thyroid-stimulating hormone [TSH] levels were not altered. Choline acetyltransferase activity (an enzyme that is sensitive to thyroid status) in the prefrontal cortex of rat pups exposed to PFOS prenatally was slightly reduced, but activity in the hippocampus was not affected. Development of learning, determined by T-maze delayed alternation in weanling rats, was not affected by PFOS exposure. These results indicate that *in utero* exposure to PFOS severely compromised postnatal survival of neonatal rats and mice, and caused delays in growth and development that were accompanied by hypothyroxinemia in the surviving rat pups.**

*Key Words:* **perfluorooctane sulfonate; postnatal; toxicity; rodent.**

Perfluorooctane sulfonate (PFOS, $C_8F_{17}SO_3^-$) is a member of the stable organic fluorochemical compounds that have wide industrial and consumer applications (Renner, 2001). PFOS is well absorbed but poorly metabolized and cleared, thus producing long half-lives in rats (7.5 days), monkeys (200 days), and humans (estimated at 8.7 years) (Geary Olsen, 2002, personal communication; Johnson *et al*., 1979; Seacat *et al*., 2002). In addition, recent reports from Giesy and co-workers (Giesy and Kannan, 2001; Kannan *et al*., 2001a,b; 2002a,b,c) indicate a widespread presence of this environmental contaminant in a variety of wildlife animals. These findings prompted the chief manufacturer of PFOS in the United States, the 3M Company, to halt production at the end of 2002. However, to date, PFOS is still available in the European and Asian markets.

The potential toxicity of organic fluorinated chemicals in general, and PFOS in particular, has not been well characterized, and even less is known about mechanisms of their toxic action. In adult monkeys and rodents, hepatic toxicity and altered thyroid hormone economy are notable findings (Luebker *et al*., 2002; Seacat *et al*., 2002; 2003; Sohlenius *et al*., 1993; Thibodeaux *et al*., 2003), and interference with mitochondrial bioenergetics and cell–cell communication through gap junctions have been implicated as potential mechanisms of toxicity (Berthiaume and Wallace, 2002; Hu *et al*., 2002; Starkov and Wallace, 2002). The preceding article (Thibodeaux *et al*., 2003) describes the maternal and prenatal toxicity of PFOS in rats and mice. In this study, evaluation of

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the developmental toxicity of PFOS is extended to the postnatal period. Survival of the newborn rodents, their growth and development, as well as their thyroid status have been examined, and alterations induced by *in utero* exposure to PFOS have been correlated with maternal and neonatal body burden in rats.

## MATERIALS AND METHODS

### *Chemicals*

Perfluorooctane sulfonate (PFOS, potassium salt) was purchased from Fluka Chemical (Steinheim, Switzerland). The chemical was reported to be 91% pure by the supplier. Our analysis indicated that approximately 71% of the chemical was straight-chain, and the remaining 29% was branched. Additional analysis indicated that the chemical obtained from Fluka appeared to be identical to that produced by 3M.

### *Animal Treatment*

Pregnant Sprague-Dawley rats and CD-1 mice, bred within a 4-h period in the afternoon and overnight, respectively, were obtained from Charles River Laboratories (Raleigh, NC). Those animals with spermatozoa in a vaginal smear and/or with a copulatory plug were considered to be at gestation day (GD) 0. Animals were housed individually in polypropylene cages with heat-treated pine shavings for bedding and were provided pellet chow (LabDiet 5001, PMI Nutrition International, Brentwood, MO) and tap water *ad libitum*. Animal facilities were controlled for temperature (20–24°C) and relative humidity (40–60%), and operated under a 12-h light-dark cycle.

***Rats.*** Perfluorooctane sulfonate was freshly prepared in 0.5% Tween-20 vehicle and administered to pregnant dams daily by gavage at doses of 1, 2, 3, 5, or 10 mg/kg from GD 2 until GD 21. Controls received vehicle alone at an equivalent volume (1 ml/kg). On GD 22, rats were monitored at hourly intervals. Time of parturition for each animal, number of live offspring, and conditions of the newborns were noted. The following day was designated as postnatal day (PD) 1. The number of live pups in each litter was tabulated daily, and their body weight was determined at intervals of several days. For this study, the litter size was not adjusted, despite the loss of neonates, unless only three or fewer pups survived within a litter (typically seen in the high-dosage groups). In such cases, the surviving pups were distributed randomly to nursing dams within the same dosage group with a litter size of less than 10. The age at which the neonates opened their eyes was tracked beginning on PD 12. All pups were weaned on PD 21 and separated by gender. The age at which the rat offspring reached puberty was determined by tracking vaginal opening in females beginning on PD 30 and preputial separation in males beginning on PD 40. For the female rats, when vaginal opening was completed, estrous cycles were monitored by daily evaluation of vaginal cytology, according to the method described by Cooper and Goldman (1999).

In a follow-up study, newborns from the 5 mg/kg PFOS dosage group were cross-fostered with controls immediately after parturition. In this experiment, 10 control and 10 PFOS-exposed litters were subdivided evenly into four groups: (1) control pups remaining with their dams; (2) PFOS-exposed pups remaining with their dams; (3) PFOS-exposed pups transferred to control nursing dams; and (4) control pups transferred to PFOS-treated dams. Survival of the neonates was monitored for 3 days after birth.

In another study, pregnant rats (17–28 per dosage group) were treated with PFOS (0, 1, 2, 3, or 5 mg/kg), as previously described. Four pups from each litter were sacrificed by decapitation within 2–4 h after birth, and trunk blood and liver were collected, pooled within each litter, and stored frozen for PFOS and thyroid hormone analyses. The remaining neonates were randomized and redistributed to the nursing dams within their respective dosage groups, with litter size kept at 10–12 pups to maintain a uniform nutritive status. Redistribution of pups was repeated at intervals of several days. All pups were weaned on PD 21. On PDs 2, 5, 9, 15, 21, 28, and 35, pups of both genders were randomly chosen from several litters (one sample per litter for preweaning age point), weighed, and sacrificed by decapitation. Trunk blood was collected and serum prepared for PFOS and thyroid hormone analyses. Liver weights were recorded. Brains were removed quickly and dissected for hippocampus and prefrontal cortex on a cold plate (Thermoelectrics, Wilmington, DE); these brain tissues were then frozen immediately on dry ice and stored at −80°C for subsequent determination of choline acetyltransferase activity. At the early age points, samples were pooled from several pups within each litter: On PD 2, four pups were pooled for each sample; on PD 5, three pups were pooled; on PDs 9 and 15, two pups were pooled. In addition, at weaning, one male and one female pup were chosen randomly from eight individual litters of controls and eight litters of the 3 mg/kg dosage group for the T-maze delayed alternation test. The behavioral experiment was performed in two separate blocks, each with eight controls and eight PFOS-exposed pups.

***Mice.*** PFOS was prepared as for rats and administered by gavage at doses of 1, 5, 10, 15, and 20 mg/kg from GD 1 until GD 17. Controls received an equivalent volume of 0.5% Tween-20 vehicle (10 ml/kg). The day of birth was designated PD 0. Condition of the newborn mice was examined as described for the rat. Litter size and weight gain of the pups were monitored; all pups were weaned on PD 21. Age at eye opening was tracked beginning on PD 12. In a separate study, pregnant mice (21–22 per dosage group) were given PFOS as described above. Pups of both genders were randomly selected from several litters, weighed, and sacrificed within 2–4 h of birth and on PDs 3, 7, 14, 21, 28, and 35. Liver samples were collected and weighed. Trunk blood was collected and serum prepared for thyroid hormone analysis. At the early age points, samples were pooled from several pups within each litter: On PD 3, four neonates were pooled for each sample; on PD 7, two pups were pooled. Litters were culled to 10–12 pups at intervals of several days.

### *Radioimmunoassays*

***$T_4$ and $T_3$.*** Serum samples were thawed, and levels of total thyroxine ($T_4$), free $T_4$, and triiodothyronine ($T_3$) were measured in duplicate with the respective radioimmunoassay (RIA) kits (Diagnostics Products Corporation, Los Angeles, CA), according to the method described in detail in the preceding article (Thibodeaux *et al*., 2003). Internal standards from rat sera were used to monitor interassay differences.

***Thyroid-stimulating hormone (TSH).*** Serum samples were thawed, and the level of TSH was quantified by RIA. The assay was performed using the following materials supplied by the National Hormone and Pituitary Program (Torrance, CA): iodination preparation NIDDK-rTSH-I-9, reference preparation NIDDK-rTSH-RP-3, and antiserum NIDDK-antirat TSH-RIA-6. Iodination material was radiolabeled with $^{125}$I (Perkin Elmer/New England Nuclear, Boston, MA) by a modification of the chloramine-T method of Greenwood *et al*. (1963). Labeled TSH was separated from unreacted iodide by gel filtration chromatography, as described previously (Goldman *et al*., 1986). The assay was conducted according to procedures described in the preceding article (Thibodeaux *et al*., 2003).

### *Choline Acetyltransferase (ChAT) Assay*

Assays were conducted essentially as described by Lau *et al*. (1987). Frozen rat brain tissues were thawed and homogenized (Polytron, Brinkman Instruments, Westbury, NY) in 79 volumes of ice-cold 10-mM phosphate buffer (pH 7.4). An aliquot of 30 $\mu$l of the homogenate was mixed with 30 $\mu$l of the incubation buffer containing final concentrations of 60 mM sodium phosphate (pH 7.9), 200 mM NaCl, 20 mM choline chloride, 17 mM $MgCl_2$, 1 mM EDTA, 0.2% Triton X-100, 0.12 mM physostigmine, 0.6 mg/ml bovine serum albumin, and 0.4 mM [$^{14}$C]acetyl-coenzyme A (NEN Life Science, Boston, MA). Blanks for each treatment group were prepared from pooled homogenate and kept on ice. Samples were preincubated on ice for 15 min, then transferred to a 37$^0$C water bath for an additional 30 min of incubation. The reaction was stopped by placing the samples on ice, and labeled acetylcholine was then extracted and counted by scintillation spectrometry. Enzyme activity was expressed as nmol of acetylcholine formed per g of tissue per 30 min.

*T-Maze*

The apparatus and procedure have been described in detail by Freeman and Stanton (1991). In brief, on PD 21, rat pups were housed in individual cages and deprived of food and water overnight. Beginning in the morning of PD 22, animals were acclimated to maze running with two goal-box training sessions and a session of 12 forced runs. These sessions occurred 4 h apart (e.g., at 8 A.M., noon, and 4 P.M.). Goal-box sessions consisted of placing the animal in a goal arm (one arm for the first session and the other for the second session) containing 0.05 ml of light cream reward. This process was repeated six times for each session. The forced-run session consisted of 12 forced-run trials (six to each maze arm in random order) in which the trap door to only one arm of the T-maze was raised, and the animal was rewarded when it broke the photoelectric beam at the end of that goal arm. At the end of each training session, animals received supplementary cream in their home cages in order to maintain them at 85% of their predeprivation body weight and to equalize the amount of light cream given to each subject in a session, depending on the number of rewarded trials. In the morning of PD 23, subjects were trained on delayed alternation over two sessions that were separated by a 6-h interval. The first session consisted of two 12-trial blocks and the second of three 12-trial blocks. On each training trial, an animal was placed in the maze and given a pair of runs. On the first run, the animal was allowed to enter only one of the two goal arms of the T-maze. This forced run was followed immediately by the second run in which the animal was allowed to choose either arm of the maze but was rewarded only for choosing the arm opposite from that entered on the previous run. Performance was scored as percentage of correct determinations to enter the choice arm, with 50% reflecting random success. The studies were conducted in four separate blocks, each representing two male and two female pups (from two individual litters) for controls and two males and two females for the PFOS (3 mg/kg) group. Statistical analysis did not indicate a gender effect in the T-maze performance; therefore, results from male and female rats were pooled.

*Determination of PFOS Concentrations*

Serum samples were diluted, and liver samples were homogenized in five volumes of reagent-grade water. An aliquot of each dilution was spiked with appropriate internal standards. Acetonitrile (5 ml) was added as an extraction solvent, which also served to precipitate the proteins. The samples were shaken at 300 rpm for 20 min and centrifuged at 850 × *g* for 10 min. The supernatant was transferred to a clean tube, diluted with 40 ml of water, and passed through a preconditioned C18 SPE cartridge. PFOS was eluted from the SPE cartridge with 2 ml methanol and analyzed by high-performance liquid chromatography-electrospray tandem mass spectrometry (HPLC-ES/MS/MS), according to the method described by Hansen *et al*. (2001).

*Data Analysis*

Data are presented as means and standard errors, and evaluated by ANOVA. For the neonatal survival, cross-foster, and developmental landmark studies, the individual litter was used as the statistical unit. For other postnatal studies, because neonates were randomized at birth (within each treatment group) and litters were culled during the course of the study, the individual pup was used as the statistical unit. When a significant treatment effect or interaction was detected, Duncan's multiple-range test or Dunnett's *t*-test was performed post hoc for comparisons between treated groups and controls. Statistically significant differences were determined at $p \leq 0.05$.

The U.S. Environmental Protection Agency (EPA) now uses the benchmark dose (BMD) approach (Barnes *et al*., 1995; Crump, 1984) for noncancer risk assessment (EPA, 1995). This approach is designed to provide a more quantitative alternative to dose-response assessment than the no-observed-adverse-effect-level (NOAEL) process by constructing mathematical models to fit all data points in the dose-response study and to take data variance into consideration. In this study, $BMD_5$ and $BMDL_5$ values were calculated for maternal and developmental toxicity after PFOS exposure. $BMD_5$ refers to the central estimate of the administered dose predicted to cause a 5% increase in response above background, and $BMDL_5$ is defined as the corresponding lower limit of the 95% confidence interval on the BMD (Allen *et al*., 1994). Benchmark Dose Software (EPA, 2000) was used to calculate the $BMD_5$ values. Selection of a specific curve-fitting model for the BMD determination was based on the Akaike's Information Criterion (AIC) value. The AIC is equal to $-2L + 2p$, where $L$ is the log-likelihood at the maximum likelihood estimates for the parameters, and $p$ is the number of model parameters estimated. The model that demonstrates "goodness of fit" with the lowest AIC value is presumed to be the most appropriate.

## RESULTS

*Rat*

Prenatal PFOS exposure significantly reduced the postnatal survival of rat pups in a dose-dependent manner (Fig. 1). At parturition, all animals were born alive and pink in color, and all appeared to be active. However, in the 10 mg/kg group, the neonates became pale, inactive, and moribund within 30–60 min, and all died soon afterward. In the 5 mg/kg group, the neonates also became moribund but survived for a longer period of time (8–12 h). Over 95% of these animals did not survive the first day of postnatal life, and only five pups from one litter reached puberty. Survival improved with lower PFOS exposure and was about 50% at 3 mg/kg. Mortality was not different between control and treated groups after the first week of postnatal life. Cross-fostering the PFOS-exposed rat pups (5 mg/kg) to control nursing dams immediately after birth failed to improve survival of the neonates, but all control pups cross-fostered to PFOS-treated dams survived for the duration of the study (3 days).

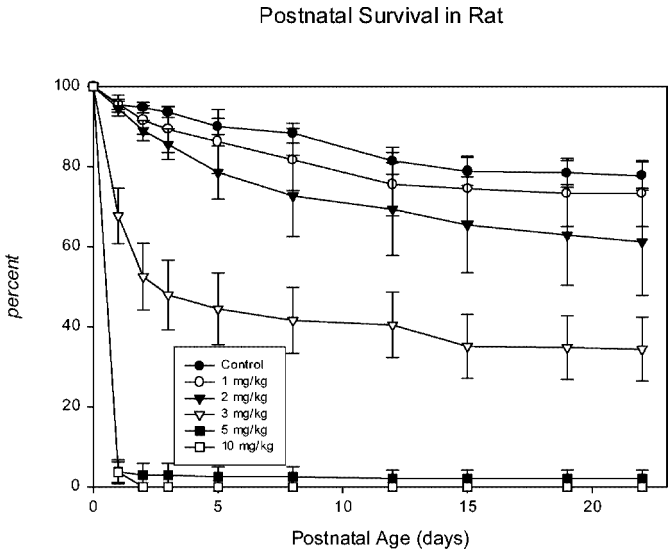


**FIG. 1.** Effects of prenatal exposure to PFOS on postnatal survival in rats. Each data point represents mean ± SE of 9–18 litters. Two-way ANOVA with repeated measure indicated significant main effects and interactions ($p < 0.0001$). Duncan's multiple-range test indicated that dose groups of 2 mg/kg and greater vary significantly from controls, whereas the 2 and 3 mg/kg groups are significantly different from the 5 and 10 mg/kg dose groups.

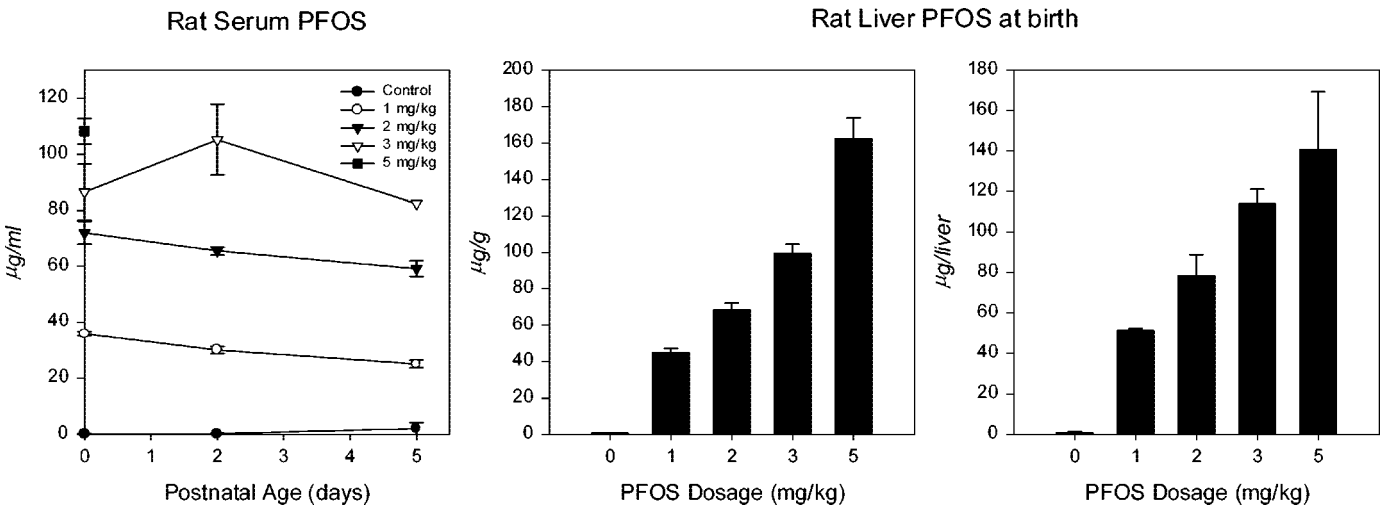


**FIG. 2.** Concentrations of PFOS in neonatal rat serum and liver. Each data point or bar represents mean ± SE of determination from 3–5 rats.

Serum PFOS levels of rat pups surviving the first 5 days of postnatal life are illustrated in Figure 2. At birth, the serum concentrations of the fluorochemical increased with exposure doses, but the relationship was not linear, especially at the higher dosage. At PD 5, the serum PFOS level of every surviving treatment group was lower than at birth. PFOS was also found in the liver of the newborn rats; however, in contrast to the findings with the dams (Thibodeaux *et al*., 2003), hepatic levels of PFOS were similar to serum levels (Fig. 2).

Postnatal growth of surviving rat pups was somewhat stunted by *in utero* exposure to PFOS. Body weights of pups in the 2 mg/kg and higher dosage groups significantly lagged behind those of the controls, and this effect persisted past weaning (Table 1). Liver weights of the PFOS-exposed pups did not differ from controls appreciably; however, when body weight deficits were taken into consideration, the relative liver weight of all PFOS dosage groups was significantly increased (Table 1). A majority of rat pups in the control group opened their eyes between PD 14 and PD 15 (Table 2); slight but significant delays (by approximately 1 day) of this developmental landmark were noted in the PFOS-exposed rats at 2 mg/kg and higher. In control rats, vaginal opening occurred by PD 33 and preputial separation by PD 42 (Table 2); prenatal exposure to PFOS did not affect these pubertal landmarks. In addition, no significant alterations were detected between the controls and PFOS-exposed rats in the onset and profiles of the estrous cycle (data not shown).

Hypothyroxinemia was detected in the PFOS-exposed neonates as early as PD 2 (the first age point where hormonal levels could be measured reliably, Fig. 3). Both total $T_4$ and free $T_4$ concentrations in the serum were depressed in all PFOS dosage groups. The levels of total $T_4$ in the fluorochemical-treated groups appeared to have recovered by weanling age, but the effects on free $T_4$ levels persisted through the duration of our study (PD 35). In contrast, no significant changes in serum $T_3$ or TSH were found in the PFOS-exposed pups, compared to the controls.

As shown in Figure 4, ChAT activity in the prefrontal cortex of neonatal rats was slightly but significantly altered by prenatal PFOS exposure (3 mg/kg), although the enzyme activity in the hippocampus was not altered. In the T-maze test, control rats acquired learning and memory behaviors after five trial blocks, achieving 90% proficiency (Fig. 4); whereas no overall significant difference was detected between the PFOS-exposed (3 mg/kg) pups and controls.

### *Mouse*

Similar to the rat, prenatal PFOS exposure reduced the postnatal survival of the mouse in a dose-dependent manner (Fig. 5). Most offspring exposed to 15 or 20 mg/kg PFOS did not survive for 24 h after birth. The $LD_{50}$ was estimated at 10 mg/kg, whereas survival of the lower dosage groups (1 and 5 mg/kg) was not different from that of controls. Among the survivors, a trend toward growth deficit was also noted in the 10 mg/kg dosage group ($p < 0.03$ vs. controls, Table 3). Compared to the rat, a more pronounced increase of liver weight was noted in PFOS-exposed neonatal mice, regardless of whether the data are expressed as absolute or relative liver weight. This pattern of hepatic enlargement persisted through the duration of the study (to PD 35). Serum thyroxine levels in the developing mice are shown in Figure 6; although the hormonal levels in the 5 and 10 mg/kg dosage groups tended to be lower, these changes were less consistent than those detected in the PFOS-exposed rat pups. A significant delay (treatment effect, $p < 0.0001$) in eye opening was detected in PFOS-exposed offspring (PD 14.8 ± 0.1 in controls vs. 15.1 ± 0.1, 15.5 ± 0.1, and 15.6 ± 0.1 at 1, 5, and 10 mg/kg/day PFOS, respectively).

**TABLE 1**
**Effects of Prenatal Exposure to PFOS on Body Weight and Absolute and Relative Liver Weights of Neonatal Rats**

| | | PFOS exposure level | | | | |
|---|---|---|---|---|---|---|
| | PD | Control | 1 mg/kg | 2 mg/kg | 3 mg/kg | 5 mg/kg |
| Body wt (g) | 0 | 5.9 ± 0.2 | 5.7 ± 0.1 | 5.4 ± 0.1* | 5.3 ± 0.1* | 5.0 ± 0.1* |
| | 1 | 6.4 ± 0.1 | 6.2 ± 0.1 | 5.5 ± 0.1* | 5.3 ± 0.1* | 4.9 ± 0* |
| | 2 | 7.2 ± 0.1 | 6.9 ± 0.2 | 6.0 ± 0.2* | 5.5 ± 0.2* | 5.1 ± 0* |
| | 3 | 8.2 ± 0.2 | 7.8 ± 0.3 | 6.8 ± 0.3* | 6.5 ± 0.2* | 5.7 ± 0* |
| | 5 | 10.6 ± 0.2 | 10.5 ± 0.2 | 8.9 ± 0.3 | 8.6 ± 0.3* | 7.5 ± 0* |
| | 8 | 15.2 ± 0.4 | 15.4 ± 0.6 | 12.1 ± 1.0 | 13.1 ± 0.5 | 9.7 ± 0* |
| | 9 | 20.2 ± 0.4 | 18.2 ± 0.9 | 15.9 ± 0.4* | — | — |
| | 12 | 23.3 ± 0.8 | 23.8 ± 0.7 | 18.8 ± 1.1 | 20.9 ± 1.6 | 16.7 ± 0* |
| | 15 | 32.2 ± 0.8 | 32.3 ± 0.7 | 29.6 ± 1.2 | 28.1 ± 0.7 | 23.5 ± 0* |
| | 19 | 39.2 ± 1.3 | 40.1 ± 0.9 | 34.6 ± 0.9 | 36.9 ± 1.6 | 28.1 ± 0* |
| | 21 | 51.0 ± 2.7 | 50.7 ± 1.0 | 46.7 ± 2.4 | 40.9 ± 2.5 | — |
| | 22 | 50.0 ± 1.4 | 50.8 ± 1.4 | 42.2 ± 1.2 | 45.6 ± 2.8 | 37.1 ± 0* |
| | 28 | 79.5 ± 2.8 | 84.4 ± 3.5 | 77.7 ± 2.3 | 68.8 ± 2.4 | — |
| | 35 | 128.7 ± 4.6 | 136.2 ± 2.6 | 119.1 ± 3.5 | 106.5 ± 5.0 | — |
| Liver wt (g) | 0 | 0.31 ± 0.01 | 0.33 ± 0.02 | 0.32 ± 0.02 | 0.30 ± 0.02 | 0.29 ± 0.01 |
| | 2 | 0.28 ± 0.01 | 0.29 ± 0.01 | 0.26 ± 0.01 | 0.25 ± 0.02 | — |
| | 5 | 0.39 ± 0.01 | 0.39 ± 0.02 | 0.36 ± 0.02 | 0.31 ± 0.02* | — |
| | 9 | 0.59 ± 0.02 | 0.59 ± 0.03 | 0.55 ± 0.01 | 0.33 ± 0.11* | — |
| | 15 | 1.10 ± 0.06 | 1.12 ± 0.03 | 1.14 ± 0.05 | 0.93 ± 0.03 | — |
| | 21 | 1.93 ± 0.13 | 2.03 ± 0.06 | 1.91 ± 0.11 | 1.61 ± 0.10 | — |
| | 28 | 3.75 ± 0.13 | 3.98 ± 0.25 | 3.96 ± 0.11 | 3.50 ± 0.14 | — |
| | 35 | 5.97 ± 0.31 | 6.50 ± 0.20 | 5.41 ± 0.10 | 5.29 ± 0.25 | — |
| Relative liver wt (%) | 0 | 5.4 ± 0.1 | 5.8 ± 0.3 | 5.9 ± 0.3 | 5.6 ± 0.3 | 5.8 ± 0.2 |
| | 2 | 3.9 ± 0.1 | 4.3 ± 0.1* | 4.3 ± 0.1 | 4.4 ± 0.1* | — |
| | 5 | 3.5 ± 0.1 | 3.7 ± 0.1 | 3.9 ± 0.1* | 3.9 ± 0.1* | — |
| | 9 | 2.9 ± 0.1 | 3.3 ± 0.1* | 3.4 ± 0.1* | 3.3 ± 0.3 | — |
| | 15 | 3.1 ± 0.1 | 3.3 ± 0.1 | 3.4 ± 0.1* | 3.5 ± 0.1 | — |
| | 21 | 3.7 ± 0.1 | 4.0 ± 0.1 | 4.1 ± 0.1* | 3.9 ± 0.1 | — |
| | 28 | 4.7 ± 0.1 | 4.7 ± 0.2 | 5.1 ± 0.1 | 5.1 ± 0.1 | — |
| | 35 | 4.7 ± 0.2 | 4.8 ± 0.1 | 4.6 ± 0.1 | 5.0 ± 0.2 | — |

*Note.* For body weight, each data point represents mean ± SE of 8–12 pups obtained from 17–28 litters, with the exception of the 5 mg/kg dose group, where $n = 5$ pups. Two-way ANOVA indicated a significant treatment effect and interaction with age ($p < 0.0001$); all PFOS dose groups except 1 mg/kg were significantly different from controls. For absolute liver weight, two-way ANOVA indicated a significant treatment effect ($p < 0.03$); however, this effect primarily reflects differences between 1 mg/kg, 2 mg/kg, and 3 mg/kg dose groups, but none of the PFOS dose groups was significantly different from controls. For relative liver weight, two-way ANOVA indicated a significant treatment effect ($p < 0.0001$), and all PFOS dose groups are significantly different from controls. PD, postnatal day.

*Significant differences ($p < 0.05$) from control values.

## DISCUSSION

Although frank teratism and weight deficits were detected in fetuses of pregnant rats exposed to 10 mg/kg PFOS during gestation (Thibodeaux *et al.*, 2003), a significant increase in neonatal mortality was observed in the offspring of animals treated with dosages as low as 2 mg/kg in this study. These findings are consistent with a preliminary report employing a different dose-regimen of PFOS (Butenhoff *et al.*, 2002). The morbidity and mortality of the newborn rats appeared to be related directly to their body burden of the fluorochemical. Indeed, although rats from all dosage groups were born alive, neonates exposed to the high dosages of PFOS (5 and 10 mg/kg, or with serum levels in excess of 110 μg/ml) survived for only a few hours. Survival rate was better in the lower dosage groups, and the first 5–7 days of postnatal life were critical to the long-term survival of the neonatal rats. Hence, $BMD_5$ and $BMDL_5$ for survival at PD 8 are estimated at 1.07 mg/kg and 0.58 mg/kg, respectively (NCTR model). Consistent with a preliminary report (Case *et al.*, 2001), results from our cross-fostering study indicated that postnatal effects of PFOS were not linked to abnormal maternal behavior, because postpartum transfer of the PFOS-exposed newborn rats to control nursing dams failed to rescue them. Although dose-dependent neonatal mortality was also noted in PFOS-ex-

**TABLE 2**
**Developmental Landmarks of Rat Pups Exposed to PFOS *in Utero***

| | PFOS exposure level | | | | |
|---|---|---|---|---|---|
| | Control | 1 mg/kg | 2 mg/kg | 3 mg/kg | 5 mg/kg |
| Age at eye opening (PD) | 14.79 ± 0.04 | 14.86 ± 0.06 | 15.23 ± 0.06* | 15.41 ± 0.06* | 15.80 ± 0.20* |
| Age at vaginal opening (PD) | 33.3 ± 0.2 | 32.8 ± 0.2 | 34.0 ± 0.3 | 34.3 ± 0.3 | 33.3 ± 1.2 |
| Body wt at vaginal opening (g) | 108 ± 2 | 109 ± 2 | 107 ± 2 | 110 ± 2 | 88.5 ± 2.5 |
| Age at preputial separation (PD) | 42.3 ± 0.2 | 43.3 ± 0.2 | 43.0 ± 0.3 | 43.4 ± 0.3 | 45.0 ± 0 |
| Body wt at preputial separation (g) | 203 ± 2 | 219 ± 3 | 194 ± 3 | 207 ± 3 | 210 ± 14 |

*Note.* Data represent means ± SE of 40 rats derived from 10 individual litters per dose, except for 5 mg/kg, where there were three females and two males from 1 litter. PD, postnatal day.

*Significant differences ($p < 0.05$) from controls.

posed mice, the estimated $BMD_5$ and $BMDL_5$ for survival at PD 6 were higher than those for the rat, 7.02 mg/kg and 3.88 mg/kg, respectively (National Center for Toxicological Research [NCTR] model).

The pathophysiological mechanisms underlying PFOS-induced neonatal mortality are largely unknown at present. However, in a preliminary study, Grasty and coworkers (2002) reported a similar pattern of neonatal death when PFOS was

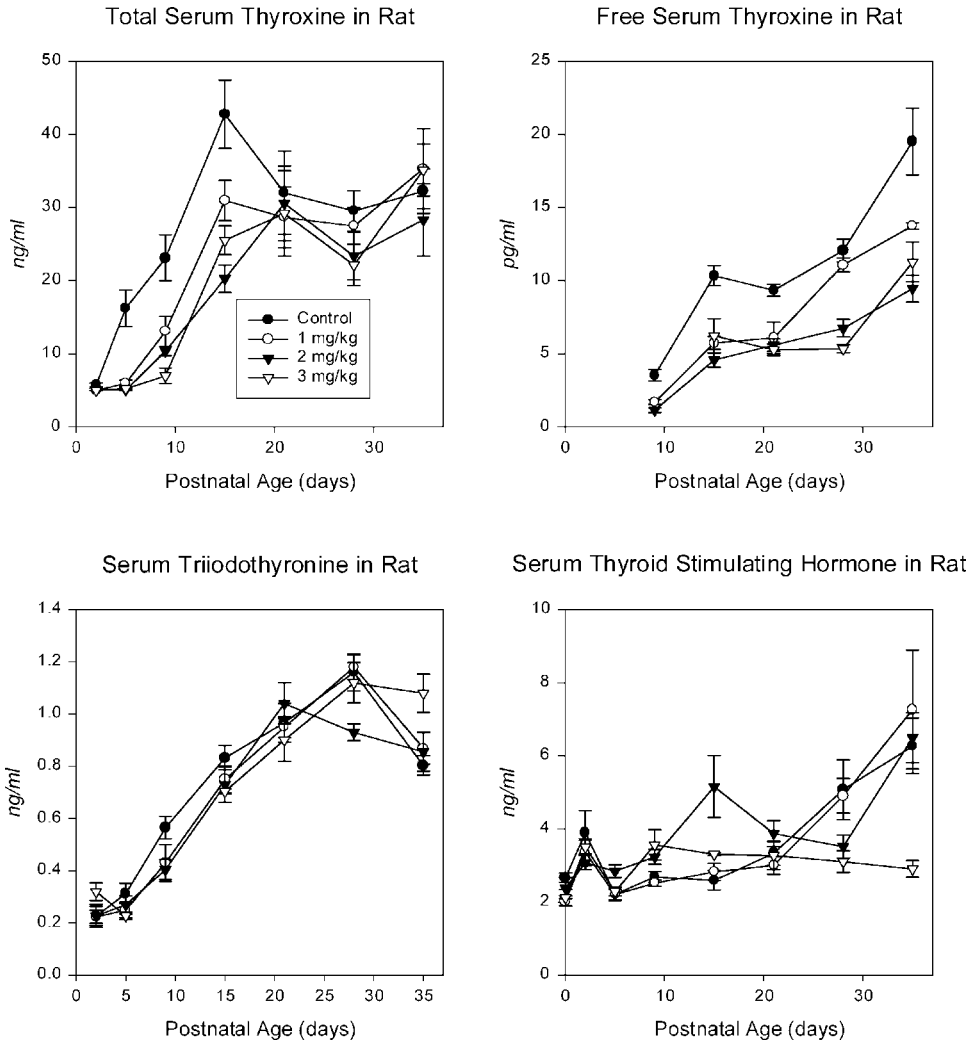


**FIG. 3.** Effects of prenatal exposure to PFOS on circulating thyroid hormones in neonatal rats. Each data point represents mean ± SE of 3–8 determinations. Two-way ANOVA indicated a significant treatment effect ($p < 0.0001$) for serum total and free $T_4$, whereas Duncan's multiple-range test indicated significant variations from controls at doses of 2 mg/kg and above, and the level of total $T_4$ of the 5 mg/kg dose group is significantly different from other groups. For serum $T_3$, two-way ANOVA indicated a significant age × treatment interaction ($p < 0.01$). For TSH, two-way ANOVA did not indicate any treatment effect or interaction.

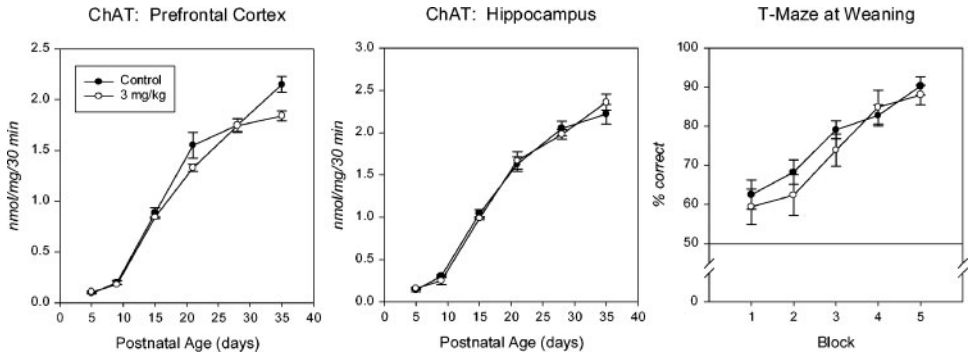


**FIG. 4.** Effects of prenatal PFOS exposure on cortical and hippocampal choline acetyltransferase activities and T-maze performance of developing rats. For ChAT activity, each data point represents mean ± SE of determination from six rats, except for PDs 9 and 15, where $n = 2$ and 3, respectively, for the 3 mg/kg dose group. For T-maze, each data point represents mean ± SE of repeated measure from eight male and eight female pups. For cortical ChAT activity, two-way ANOVA indicates a significant main effect for treatment ($p < 0.02$). For hippocampal ChAT activity and T-maze, two-way ANOVA did not indicate any significant main treatment effect or interaction.

given to the pregnant rats during only the last few days of gestation, although a higher daily dosage of the fluorochemical was used. In this regard, it will be interesting to compare the body burdens of PFOS in newborn rats after various exposure paradigms (e.g., Butenhoff *et al*., 2002; Grasty *et al*., 2002; current study) and between rat and mouse newborns to ascertain whether postnatal toxicity of the fluorochemical can be correlated to a critical body burden. These results together suggest that the organ systems developing late in gestation may be particularly vulnerable to PFOS. This hypothesis is also consistent with the relatively unremarkable teratological findings. Considering that PFOS-induced organ toxicity is incompatible with postnatal survival, maturation of the lung and pulmonary function is a plausible target of PFOS toxicity (Lau and Kavlock, 1994). Indeed, the profile of neonatal mortality induced by PFOS is reminiscent of the developmental toxicity of nitrofen, an herbicide that perturbed fetal lung maturation, ultimately leading to compromised cardiopulmonary function and death in newborn rats (Lau *et al*., 1986, 1988; Stone and Manson, 1981). The previous teratological finding of enlarged right atrium in the PFOS-exposed fetus (Thibodeaux *et al*., 2003), possibly suggesting pulmonary hypertension, is also consistent with this hypothesis.

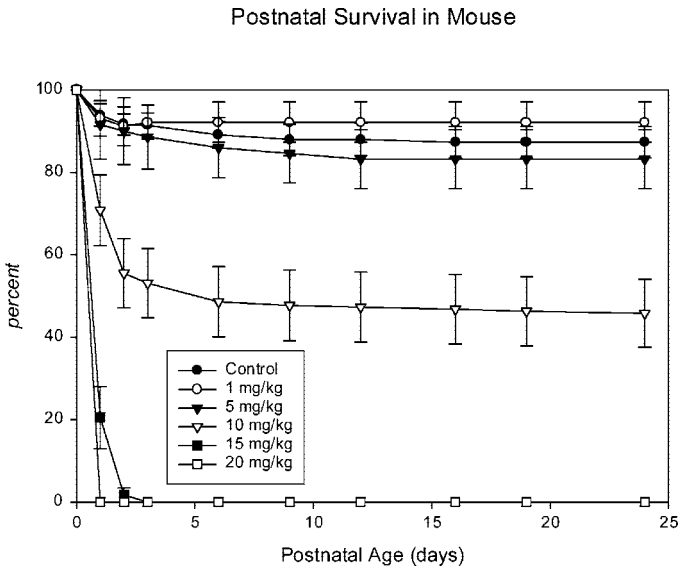


**FIG. 5.** Effects of prenatal exposure to PFOS on postnatal survival in mice. Each data point represents mean ± SE of 7–18 litters. Two-way ANOVA indicated a significant treatment effect ($p < 0.0001$) but no interaction with age. Duncan's multiple-range test indicated that doses of 10 mg/kg and higher are significantly different from controls, and the 15 and 20 mg/kg groups vary significantly from the 10 mg/kg group.

PFOS-exposed rat pups surviving beyond the first few days exhibited growth retardation. Deficits of body weight gain were seen in the 2 mg/kg and higher dosage groups, accompanied by a trend toward developmental delays, as illustrated by eye opening. This pattern of growth deficit and developmental delay also extended to neonatal mice exposed to PFOS prenatally. These results suggest that PFOS may interfere with cellular or functional maturation of target organs, possibly *via* alterations of thyroid hormones. The importance of thyroid hormones in regulating growth and development is well established (Legrand, 1986; Mussa *et al*., 2001; Porterfield, 1993; Stein *et al*., 1991). In previous studies, PFOS has been shown to depress circulating thyroid hormones in the adult monkey, rat, and mouse (Seacat *et al*., 2002, Thibodeaux *et al*., 2003). Levels of $T_4$ and $T_3$ in pregnant rodents were markedly reduced by PFOS. Prior to fetal thyroid gland development, the only source of thyroid hormones for the conceptus is the maternal circulation. Thus, the abrupt falls of $T_4$ and $T_3$ in PFOS-treated pregnant rodents would be expected to deprive the developing organism of these key endogenous signals. However, the actual availability of thyroid hormones (particularly $T_3$) in the embryonic/fetal target cells has not been determined; the impact of the maternal hypothyroid state could have been counteracted by upregulation of placental deiodinase activity (Versloot *et*

**TABLE 3**
**Effects of Prenatal Exposure to PFOS on Body Weight and Absolute and Relative Liver Weights of Neonatal Mice**

| | | PFOS exposure level | | | | | |
|---|---|---|---|---|---|---|---|
| | PD | Control | 1 mg/kg | 5 mg/kg | 10 mg/kg | 15 mg/kg | 20 mg/kg |
| Body wt (g) | 0 | 1.51 ± 0.04 | 1.51 ± 0.09 | 1.49 ± 0.04 | 1.40 ± 0.05 | 1.31 ± 0.05 | 1.28 ± 0.04 |
| | 1 | 1.65 ± 0.04 | 1.55 ± 0.03 | 1.57 ± 0.04 | 1.53 ± 0.04 | — | — |
| | 2 | 1.86 ± 0.05 | 1.79 ± 0.05 | 1.77 ± 0.06 | 1.66 ± 0.03 | — | — |
| | 3 | 2.19 ± 0.05 | 2.08 ± 0.04 | 2.11 ± 0.04 | 1.92 ± 0.05 | — | — |
| | 5 | 3.10 ± 0.14 | 3.02 ± 0.11 | 3.25 ± 0.14 | 2.61 ± 0.09 | — | — |
| | 7 | 4.01 ± 0.15 | 4.13 ± 0.15 | 3.90 ± 0.11 | 3.93 ± 0.18 | — | — |
| | 9 | 5.14 ± 0.20 | 4.57 ± 0.15 | 5.05 ± 0.17 | 4.45 ± 0.14 | — | — |
| | 12 | 6.43 ± 0.23 | 5.71 ± 0.15 | 6.50 ± 0.2 | 5.93 ± 0.16 | — | — |
| | 14 | 7.36 ± 0.50 | 7.55 ± 0.31 | 7.44 ± 0.25 | 7.07 ± 0.13 | — | — |
| | 16 | 7.80 ± 0.28 | 6.95 ± 0.21 | 7.97 ± 0.24 | 7.60 ± 0.21 | — | — |
| | 19 | 9.17 ± 0.39 | 7.85 ± 0.29 | 8.88 ± 0.28 | 8.58 ± 0.23 | — | — |
| | 21 | 12.88 ± 0.83 | 12.80 ± 0.66 | 11.55 ± 0.44 | 11.73 ± 0.58 | — | — |
| | 23 | 14.45 ± 0.82 | 12.30 ± 1.09 | 15.78 ± 0.91 | 13.65 ± 0.74 | — | — |
| | 28 | 21.71 ± 1.40 | 21.69 ± 0.82 | 21.89 ± 0.79 | 21.35 ± 0.41 | — | — |
| | 35 | 25.70 ± 1.30 | 29.47 ± 0.93 | 25.65 ± 1.34 | 27.00 ± 1.29 | — | — |
| Liver wt (mg) | 0 | 83 ± 2 | 84 ± 2 | 89 ± 2 | 93 ± 2* | 90 ± 2 | 97 ± 3* |
| | 3 | 76 ± 2 | 81 ± 2 | 92 ± 2* | 88 ± 3 | — | — |
| | 7 | 131 ± 4 | 144 ± 9 | 141 ± 4 | 165 ± 7* | — | — |
| | 14 | 265 ± 13 | 291 ± 11 | 302 ± 6* | 307 ± 8* | — | — |
| | 21 | 687 ± 47 | 755 ± 47 | 731 ± 30 | 809 ± 38 | — | — |
| | 28 | 1431 ± 94 | 1379 ± 51 | 1551 ± 66 | 1421 ± 46 | — | — |
| | 35 | 1687 ± 99 | 2054 ± 85 | 1871 ± 110 | 1921 ± 101 | — | — |
| Relative liver wt (%) | 0 | 5.5 ± 0.2 | 5.9 ± 0.1 | 6.1 ± 0.1* | 6.6 ± 0.1* | 7.0 ± 0.1* | 7.5 ± 0.2* |
| | 3 | 3.5 ± 0.1 | 3.8 ± 0.1 | 4.3 ± 0.1* | 4.3 ± 0.1* | — | — |
| | 7 | 3.3 ± 0.1 | 3.5 ± 0.2 | 3.6 ± 0.1* | 4.2 ± 0.1* | — | — |
| | 14 | 3.6 ± 0.1 | 3.8 ± 0.1* | 4.1 ± 0.1* | 4.4 ± 0.1* | — | — |
| | 21 | 5.3 ± 0.2 | 5.8 ± 0.1* | 6.2 ± 0.1* | 6.8 ± 0.1* | — | — |
| | 28 | 6.5 ± 0.2 | 6.3 ± 0.1 | 7.0 ± 0.2 | 6.8 ± 0. | — | — |
| | 35 | 6.4 ± 0.2 | 6.9 ± 0.1 | 7.1 ± 0.2 | 7.0 ± 0.2 | — | — |

*Note.* For body and liver weights, each data point represents mean ± SE of determination from 20–40 mice derived from 21–22 litters. For body weight, two-way ANOVA did not indicate a treatment effect, but a significant age × treatment interaction ($p < 0.0001$). For liver weight, two-way ANOVA indicated a significant treatment effect ($p < 0.0001$) and a significant age × treatment interaction ($p < 0.0005$); all PFOS dose groups are significantly different from controls. For relative liver weight, two-way ANOVA indicated a significant treatment effect ($p < 0.0001$); all PFOS dose groups are significantly different from controls. PD, postnatal day.

*Significant differences ($p < 0.05$) from control values.

*al*., 1998). On the other hand, prenatal exposure to PFOS was associated with hypothyroxinemia in neonatal rats, because both total and free $T_4$ levels were significantly and persistently reduced. The lack of similar effects on neonatal serum $T_3$ (unlike the responses to PFOS in the adult rats) or TSH (consistent with adult findings) is not well understood, though not necessarily unique. Previous studies with polychlorinated biphenyls and polybrominated diphenyl ethers have shown a similar profile of thyroid hormone imbalance (Goldey *et al*., 1995; Morse *et al*., 1996; Rosiak *et al*., 1997; Zahalka *et al*., 2001; Zhou *et al*., 2002). One possibility for the maintenance of serum $T_3$ in the PFOS-exposed rat pups may involve up-regulation of the liver type I deiodinase that converts $T_4$ to $T_3$.

Development of the brain in general and the cholinergic neurotransmitter system in particular are critically dependent on thyroid hormones (Gould and Butcher, 1989; Kalaria and Prince, 1986; Rami *et al*., 1989; Sawin *et al*., 1998; Virgili *et al*., 1991). The neurotransmitter biosynthetic enzyme choline acetyltransferase is exquisitely sensitive to thyroid status (Rami *et al*., 1989; Sawin *et al*., 1998). Propylthiouracil (PTU)-induced neonatal hypothyroidism has been shown to suppress ChAT activity and impair learning and memory (Christy Carter, 2003, personal communication; Sawin *et al*., 1998). Circulating $T_4$ and $T_3$ were profoundly depressed in the PTU-treated rat pups, whereas PFOS suppressed only serum $T_4$, and to a lesser extent than that produced by PTU. Yet small but significant reductions of ChAT activity were seen in the prefrontal cortex of PFOS-treated pups, whereas ChAT activity in another brain region richly endowed with cholinergic neurons, the hippocampus, was unaffected. Importantly, the

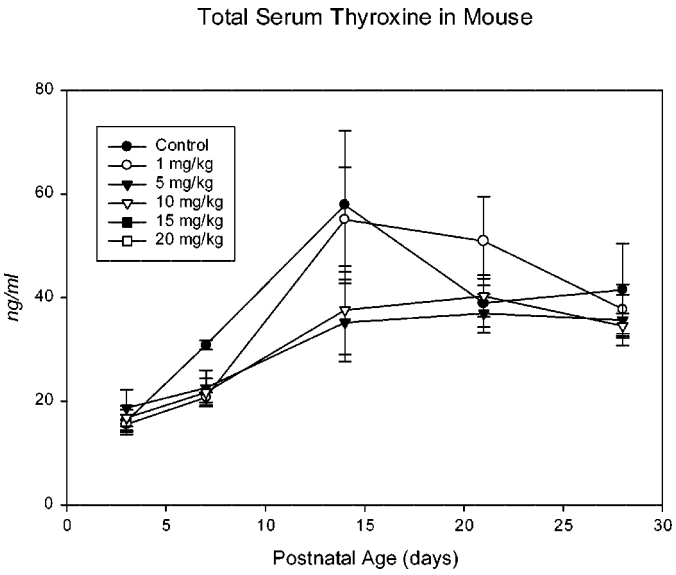


**FIG. 6.** Effects of prenatal exposure to PFOS on circulating thyroid hormones in neonatal mice. Each data point represents mean ± SE of 3–7 determinations; in the early age points, samples were pooled from 2–3 pups. Two-way ANOVA did not indicate any significant treatment effect or age × treatment interaction.

PFOS-induced hypothyroxinemia and marginal reductions of cortical ChAT activity were not accompanied by notable deficits in learning and memory acquisition, perhaps due to the mildness of the thyroid hormone imbalance. Indeed, only subtle changes in behavioral performance produced by congenital hypothyroidism have been previously reported (MacNabb *et al*., 2000).

In subchronic studies with adult rat and monkey, liver enlargement and hepatic toxicity were associated with PFOS exposure (Seacat *et al*., 2002, 2003). In our studies where rodents were exposed to PFOS only during pregnancy, maternal liver weights in rats were unaltered but those in mice were significantly elevated, compared to controls (Thibodeaux *et al*., 2003). Whereas significant increases of relative liver weight were detected in both rat and mouse offspring, these changes were more notable and consistent in the mouse. These findings suggest that the developing liver is another potential target for PFOS action.

Although the results were limited to the first few days of life, several points can be made concerning the body burden of PFOS in the neonatal rats:

(1) Serum levels of newborns were comparable to those of the dam at term, suggesting that PFOS equilibrated across the placenta.

(2) At birth, serum and liver levels of PFOS of the newborns were similar, indicating no preferential accumulation of PFOS in the liver (unlike the adult), perhaps due to minimal enterohepatic cycling *in utero* (Belknap *et al*., 1981).

(3) With the exception of one time point in the 3 mg/kg dosage group (Fig. 2), serum PFOS levels of rat pups began to decline after birth, suggesting that although PFOS might be present in the milk (John Butenhoff, 2003, personal observation), lactational transfer to the neonates was not a major source of PFOS.

In summary, *in utero* exposure of PFOS to laboratory rodents severely compromised postnatal survival. Morbidity and mortality were dose dependent, with the rat being more sensitive than the mouse. Persistent growth deficits and developmental delays were seen in the surviving animals. Hypothyroxinemia was observed in PFOS-exposed rat pups, with an attendant small reduction of ChAT activity in the prefrontal cortex, but significant deficits in learning behaviors were not detected. Thus, postnatal evaluations of rodent offspring exposed to PFOS *in utero* revealed adverse developmental outcomes at a dosage lower than that associated with teratological findings; additional investigation will be required to elucidate the pathophysiological mechanisms underlying the PFOS toxicity.

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