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Interaction of PFOS and BDE-47 Co-exposure on Thyroid Hormone Levels and TH-Related Gene and Protein Expression in Developing Rat Brains

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Perfluorooctane sulfonate (PFOS) and 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) are two persistent environmental contaminants that are toxic to developing nervous systems, particularly via their disruption of thyroid hormone (TH) function. To investigate whether an interaction existed between PFOS and BDE-47 on TH-mediated pathways, adult female Wistar rats were exposed to 3.2 and 32 mg/kg of PFOS or BDE-47 in their diet and co-exposed to a combination of each chemical (3.2 mg/kg) from gestational day 1 to postnatal day (PND) 14. Serum and brain tissues from both male and female neonates were collected on PNDs 1, 7, and 14 to examine TH-regulated gene and protein expression. The results revealed that (1) a significant accumulation difference occurred between the two chemicals; (2) On a equimolar basis, BDE-47 and PFOS affected serum total triiodothyronine and total thyroxine differently in adults and offspring; (3) there were region-specific and exposure- and time-dependent alterations in TH concentrations and tested gene and protein expression levels; and (4) interaction for the combined chemicals was only observed for brain-derived neurotrophic factor (BDNF), which exhibited a synergistic effect on PND 1 in the cortex and an antagonistic effect on PND 14 in the hippocampus. Our results suggest a complex TH-mediated gene and protein response to BDE-47 and/or PFOS exposure that seems little related to TH homeostasis and that little combined interaction of co-exposures was observed except on BDNF. The underlying mechanisms remain uncertain but seem to involve more actions than just TH-regulated pathway.

Key Words: **PFOS; BDE-47; developmental neurotoxicity; *TRβ*; *BTEB*; *BDNF*; *GAP-43*; *NCAM1*; thyroid hormone; TH-mediated transcription; combined toxicity.**

Perfluorooctane sulfonate (PFOS) is both intentionally produced and an unintended degradation product related to anthropogenic chemicals. It is widely used in commercial products and has been included in the list of nine new persistent organic pollutants (POPs) since May 2009 due to its extreme persistence, potential toxicity, and substantial bioaccumulation and biomagnification properties. Unlike more lipophilically POPs, such as polychlorinated biphenyls and dioxins, PFOS frequently binds to serum protein and accumulates in the liver and serum (Chang *et al.*, 2009). It can also penetrate the brain-blood barrier (BBB) and placental barrier and cause potential neurobehavioral defects and neurobiochemical alterations in developing fetuses (Chang *et al.*, 2009; Johansson *et al.*, 2008; Luebker *et al.*, 2005; Wang *et al.*, 2010). These neural and behavioral impacts are thought to be partly caused by the indirect action of PFOS through thyroid hormones (THs) and TH-regulated pathways.

2,2′,4,4′-Tetrabromodiphenyl ether (BDE-47) is a major congener of polybrominated diphenyl ethers (PBDEs) and a flame-retardant additive (McDonald, 2002). As it structurally resembles triiodothyronine (T3) and thyroxine (T4), it is thought to disrupt TH levels and TH-related functions in both adult and developing animals (Lema *et al.*, 2008; Skarman *et al.*, 2005; Talsness *et al.*, 2008). Unlike PFOS, however, rodent metabolism of BDE-47 results in the formation of five different hydroxylated tetra-BDE metabolites, which have high binding affinity to thyroid hormone receptors (TRs) (McDonald, 2002). Exposure to PBDEs during brain growth spurt can cause persistent dysfunction in adult animals, manifested as spontaneous deranged behavior, learning and memory defects, and dysfunctions in the cholinergic system that worsen with age (McDonald, 2002; Viberg *et al.*, 2003). These developmental neurotoxic effects are similar to those caused by PFOS exposure and are thought to be involved in the disturbance of TH-mediated pathways (Lema *et al.*, 2008).

Dietary exposure to BDE-47 resulting in disruption of TH-responsive pathways has been studied in species such as minnows (Lema *et al.*, 2008). Additionally, the T3-regulated proteins growth-associated protein 43 (GAP-43) and brain-derived neurotrophic factor (BDNF) demonstrate significant changes in the hippocampus and cortex after exposure to

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BDE-209 (Viberg *et al.*, 2008). Similar to BDE-47, PFOS induces changes in several TH-related transcripts and proteins. The GAP-43 protein level significantly increases in the hippocampus of neonatal mice given a single oral dose of PFOS (Johansson *et al.*, 2009). Two T3-mediated genes of $TR\beta$ and basic transcription element–binding protein (*BTEB*), which are influenced by BDE-47 (Lema *et al.*, 2008), show differential expression by PFOS in developing rat brains (Wang *et al.*, 2010). In addition, PFOS and BDE-47 similarly affect TH homeostasis as they both decrease circulating T4 and T3 without thyroid stimulating hormone (TSH) feedback even when the dosage is high (Skarman *et al.*, 2005; Talsness *et al.*, 2008; Yu *et al.*, 2009a). This is very different from Polychlorodiphenyls (PCBs), which are characterized by significant hypothyroidism (Gauger *et al.*, 2004). Both PFOS and BDE-47 share a similar mechanism of TH disruption, considered to result from increased biliary excretion of conjugated TH, with the production process seemingly unaffected (Skarman *et al.*, 2005; Chang *et al.*, 2008). It is, therefore, conceivable that PFOS and BDE-47 may interact on some TH-associated neurobiological pathways and show the combined effect, which may be different from the effect of single exposure. Due to the coexistence of PFOS and PBDEs in the environmental and biological samples (Roosens *et al.*, 2010) and that BDE-47 is the dominant congener detected in the blood, milk, and fat of wildlife and humans (Lind *et al.*, 2003; Mazdai *et al.*, 2003), recognition of the combined effects of the two compounds will help estimate the prevalence of POPs-induced risks and lead to evidence-based programs of prevention.

We investigated the potency and efficacy of equimolar exposure to PFOS and BDE-47 both individually and in combination on TH-mediated targets by examining serum and brain tissue from offspring. We examined the effect of single and combined exposure in relation to chemical accumulation, serum TH concentrations, and transcriptional and translational expression of several TH-mediated genes. The tested genes of focus were *BDNF*, *GAP-43*, and neural cell adhesion molecule 1 (*NCAM1*), three critical factors in neurodevelopment and synaptogenesis. To determine which effective transactivators contribute to disruption of TH-mediated pathways, we also examined messenger RNA (mRNA) levels of $TR\beta$ and *BTEB*, which are influenced by either PFOS or BDE-47 alone (Lema *et al.*, 2008; Wang *et al.*, 2010), and function as transcriptional activator and co-activator, respectively, in the T3-mediated process. All tests were conducted for both male and female animals to determine if gender differences were apparent.

MATERIALS AND METHODS

Chemicals. Potassium PFOS (C8F17KO3S; Chemical Abstract Service, no. 2795-39-3; purity > 98%) was purchased from Fluka Chemical and BDE-47 from ChemServices (West Chester, PA; > 99% purity; no. 31610). All other chemicals were commercially available and of appropriate grades.

Animals. Adult male (250–300 g) and female (180–200 g) albino Wistar rats (certification number: SCXK2008-0002) and standard laboratory rodent diet were purchased from the Experimental Animal Care Center of Dalian Medical University. Male and female animals were housed separately with four to five animals per cage in standard plastic shoebox cages and left undisturbed for a week in a temperature-controlled (22°C–24°C), moderate humidity (40 ± 5%) colony room with corncob bedding. Food and water were available *ad libitum*. All animals were treated following animal welfare guidelines, and the experimental protocol was approved by the Institute of Zoology, Chinese Academy of Sciences Institutional Animal Care Committee.

Exposure. The fertilization process began after 1 week acclimatization (Wang *et al.*, 2010). Specifically, females were housed overnight with sexually mature stimulus males (one female and three males in each cage) at the onset of the estrus phase of their reproductive cycle. The next morning females were smeared, and the presence of sperm was determined by microscope to establish gestational day (GD) 1. Pregnant females were individually housed for subsequent exposure, and the nonpregnant females were returned to their previous home cages for rebreeding. Administration of experimental chemicals started at GD 1 and continued for the entire gestation period until postnatal day (PND) 14. The dosing solution used for both K^+PFOS and BDE-47 was 2% Tween 20 in deionized water as these two chemicals completely dissolved in Tween 20 solution when heated in a water bath at 45°C. The dosing solution for each chemical was prepared as 0.16 and 1.6 mg/ml, with 10 ml of PFOS and BDE-47 solution added to 0.5 kg of diet powder for an oral dietary exposure dose of 3.2 and 32 mg/kg feed. Five dosing groups were established: specifically, PFOS (3.2 mg/kg feed), BDE-47 (3.2 mg/kg feed), BDE-47 (32 mg/kg feed), PFOS (32 mg/kg feed), and BDE-47 (3.2 mg/kg feed) + PFOS (3.2 mg/kg feed). A sixth group (the control) was given diet incorporated with 2% Tween 20 only. Each treatment group was comprised of rats from three to four different litters, and each group contained five to eight females and five to eight males. The PFOS dose (3.2 mg/kg feed) was the same as used in our previous studies, in which serum T4 was significantly reduced and some TH-related genes were affected but no obvious exosyndromes were observed (Wang *et al.*, 2010; Yu *et al.*, 2009b). A preliminary laboratory study showed no evident mortality or changes in body weight in 14-day-old male rats after BDE-47 (3.2 mg/kg feed) exposure. Therefore, to compare the potency of PFOS- and BDE-47–induced effects, BDE-47 was dosed equally with PFOS in the single and the combined groups. We ensured that the dose level for BDE-47 and PFOS did not lead to one chemical contributing disproportionately to the overall mixture effect. The highest dosed group (32 mg/kg feed) was added to clarify dose-response and help determine the interactive effect of co-exposure (Eriksson and Fischer, 2006). Prior to initial dosing, aliquots of the chemicals were collected to assess homogeneity and stability, which was determined by chemical concentration using high-performance liquid chromatography (LC)/tandem mass spectrometry. Dams and weaning litters were given free access to treated food.

Serum and tissue collection. On PNDs 1, 7, and 14 of exposure, selected animals from each group were lightly anesthetized with diethyl ether before decapitation. Blood was collected by cardiac puncture and allowed to clot for 2 h. The blood was centrifuged at 1200 × g for 20 min, and the serum was removed to determine PFOS and BDE-47 levels. The outer layer of the frontal cerebral cortex and the hippocampus were dissected from removed brains, washed to remove residual blood, and stored in liquid nitrogen until further processing.

Measurement of PFOS. The serum and brain samples were extracted following previous studies (Hansen *et al.*, 2001). The concentration of PFOS in extracted solution was quantified by LC-MS (Shimadzu, 2010 A) (Wang *et al.*, 2010).

Measurement of BDE-47. The extraction and cleanup of BDE-47 was performed as per Hovander *et al.* (2000). Briefly, surrogate standard (PCB-204) (ChemServices Int.) was spiked in the serum and brain tissue homogenate in a test tube, vortexed, and left to equilibrate overnight. Hydrochloric acid and 2-propanol were added to denaturize the proteins and release the lipids and the organohalogen compounds. The analytes were extracted by hexane and methyl

tert-butyl ether (MTBE) (vol/vol, 1:1). The organic phase was washed with a solution of potassium chloride (1%), followed by evaporation to dryness for gravimetric determination of extracted lipid content. The analytes and lipids were redissolved in hexane. An aqueous ethanolic solution of potassium hydroxide was added to remove acidic impurities. The neutral extract underwent further sulfuric acid cleanup according to standard U.S. Environmental Protection Agency (EPA) protocols (U.S. EPA Method, 1996). The samples were then dried under a gentle stream of nitrogen. The internal standard (BDE-166) was added prior to gas chromatography (GC)/MS analysis. The BDE-47 was analyzed by an Agilent HP 6890 gas chromatograph coupled with a quadrupole mass selective detector HP 5975 N, a DB-XLB column (15 m × 0.25 mm, 0.1 μm film thickness; J&W Scientific, Folsom, CA). The injector temperature was 280°C, and autoinjection of the 1-μl sample was conducted in splitless mode, with split mode turned on after 2 min. Methane was used as the chemical ionization moderating gas and helium as the carrier gas at a flow rate of 1.2 ml/min. Ion source and interface temperatures were set at 150°C and 300°C, respectively. The temperature of the GC oven was programmed as isothermal at 90°C for 2 min and 15°C/min to 320°C for 7 min. Two ions of m/z 326 and 486 were monitored in the electron ionization (EI) selected ion-monitoring mode (mass resolution = 5000; ionization voltage, 40 eV; ion source temperature = 300°C).

Measurement of THs. Total T4 (TT4) and total T3 (TT3) levels in rat serum were determined by specific and highly sensitive radioimmunoassay kits obtained from the Shanghai Institute of Radioimmunological Technology. The limits of detection for TT4 and TT3 were 5.0 and 0.12 μg/l, respectively.

Semiquantitative real-timePCR for several T3-mediated genes. Total RNA was extracted from the cerebral cortex and hippocampus of each group using the RNAiso reagent (Takara) according to the manufacturer's recommended protocols. The detection of total RNA integrity and purity, reverse transcription, and the quantitative PCR were carried out following our previous study (Wang *et al.*, 2010). Primer sequences for endogenous control (glyceraldehyde-3-phosphate dehydrogenase) and target genes including *BTEB, TRβ, BDNF, GAP-43*, and *NCAM1* are listed in Supplementary table 1. To compare gene expression levels among examined individuals, we chose the moderate amount of *BTEB* transcript in the control rat cortex on PND 1 as a suitable normalizer gene. After verifying that the amplification efficiencies of the target genes and glyceraldehyde-3-phosphate dehydrogenase were approximately equal, the subsequent gene expression level was represented by the relative value to the normalizer gene using the $2^{-\Delta\Delta CT}$ method for multiple comparisons among dose groups (Livak and Schmittgen, 2001).

***Quantification of protein levels of* BDNF, NCAM1, *and* GAP-43.** Brain tissue samples were homogenized in ice-cold lysis buffer containing 137mM NaCl, 20mM Tris-HCl (pH 8.0), 1% nonyl phenoxypolyethoxylethanol, 10% glycerol, 1mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 1 mg/ml leupeptin, and 0.5mM sodium vanadate. The tissue homogenate solutions were centrifuged at 14,000 × g for 15 min at 4°C. The concentration of total protein in the supernatant was detected using bicinchoninic acid protein assay kit (Pierce, Rockford, IL). The BDNF and NCAM1 protein levels were directly measured by rat BDNF ELISA kit (USCN; E0011r) and rat NCAM1 ELISA kit (USCN; E91225Ra), respectively, according to manufacturer's instructions. In brief, flat-bottomed 96-well microplates were coated with the BDNF and NCAM1 capture antibodies. The captured BDNF and NCAM1 were bound by their second specific antibodies, which were detected using a species-specific antibody conjugated to horseradish peroxidase as a tertiary reactant. All unbound conjugates were removed by subsequent wash steps. After incubation with chromogenic substrate, color change was measured in an ELISA plate reader at 450 nm. The method for examining GAP-43 protein concentration was adjusted from a previous study (Schreyer *et al.*, 1997). Immunoassay was carried out on a 96-well vinyl plate (Costar) in a volume of 100 milliliters per well of sample buffer (100mM Tris:HCl, pH 7, containing 2% bovine serum albumin [BSA], 1M NaCl, 4mM EDTA.Na_2, 0.2% Triton X-100, and 0.1% NaN_3) unless otherwise specified. The GAP-43 protein was partially purified from rat brains by preparative isoelectric focusing (Radola, 1984). The amount of GAP-43 protein in these preparations was measured by densitometric scanning of the GAP-43 band in Coomassie-stained gels and compared with bovine serum albumin (Sigma; A-2153) standard. Plates were coated with 2 mg/ml of the monoclonal antibody (mAb) 9-1E12 (Chemicon, MAB347) in 25mM Na-carbonate/bicarbonate buffer (pH 9.6) overnight at 4°C. Non-specific binding sites within the wells were blocked with 1% BSA in the same buffer (150 ml, 1 h, room temperature), followed by an overnight incubation at 4°C with triplicate standards (0–500 pg GAP-43) or tissue extracts. The GAP-43 protein was then detected with peroxidase-conjugated mouse mAb in sample buffer containing 0.01% thimerosal as a preservative (6 h, room temperature). Plates were washed thoroughly (5 × 3 min) with washing buffer consisting of 50mM Tris-HCl, pH 7, containing 500mM NaCl, 1mM EDTA, and 0.1% Triton X-100. Peroxidase activity was visualized by color reaction product using the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) according to the manufacturer's instructions (TMB Peroxidase EIA Substrate Kit, Bio-Rad). The plates were incubated at room temperature for 8–10 min until color was sufficiently developed. The reaction was stopped with 1M sulfuric acid, and the plates were read at 450 nm. The wells without cells or GAP-43 protein but with antibodies were used as blanks. The concentration of the three target proteins was calculated as picogram target per milligram of total protein.

Statistical analysis. Data analyses were performed using SPSS software (Ver13.0; SPSS) with statistical significance set at $p < 0.05$. Univariate ANOVA was used for chemical concentrations, TT3 and TT4 levels, and the five gene (*BDNF, GAP-43, NCAM1, TRβ*, and *BTEB*) expression levels, and two-variate ANOVA was used for the three gene and protein (BDNF, GAP-43, and NCAM1) expression levels to test the main effect of factor and differences among subjects. When results from the overall significance test lead to rejection of the null hypothesis, a *post hoc* test was performed to determine the influence source. The *post hoc* test was comprised of least significant difference (LSD) for pairwise comparisons of means and by Duncan's multiple range test for multiple comparisons among groups. All data were expressed as the mean ± SE. All individual values were analyzed by Duncan's test, with the exception of chemical concentration data which were analyzed using the Student's *t*-test for pairwise comparison of groups of interest. The relationships among examined endpoints were assessed by correlation analysis using the Pearson method.

RESULTS

Pregnancy and Litter Data

No clinical signs of general toxicity were detected during daily observations. Male and female body weight of the rat offspring was significantly decreased (5.60 ± 0.37 g compared with 6.77 ± 0.73 g in the control group at PND 1, 9.77 ± 0.68 g compared with 13.82 ± 0.99 g at PND 7, and 16.23 ± 1.62 g compared with 20.79 ± 2.23 g at PND 14) under the highest maternal exposure of PFOS (32 mg/kg feed). No other groups provoked this effect (Supplementary fig. 1). Pregnancy length, litter sizes, mortality, and sex ratios in the litters showed no obvious diversities among treatment groups (Supplementary table 2). Litters exposed to the highest dose of PFOS appeared pale and delicate.

Serum and Brain PFOS and BDE-47 Levels

Chemical concentrations are shown in Table 1. No significant sex effect was observed on chemical concentrations ($F_{1,305} = 0.70$, $p = 0.52$). After pooling data on the two sexes, exposure ($F_{5,305} = 82.3$, $p < 0.001$), tissue (serum and cortex)

TABLE 1
PFOS and BDE-47 Concentration in Serum and Cerebral Cortex of Dams and Pups (Mean ± SE)

	Serum		Cortex		Cortex/serum[a]	
Dose (mg/kg feed)	BDE-47 (μg/ml)	PFOS (μg/ml)	BDE-47 (μg/g tissue)	PFOS (μg/g tissue)	BDE-47	PFOS
Dam						
PND 1						
0	< LLOQ[d](3)	< LLOQ[e](3)	< LLOQ[d] (3)	< LLOQ[f](3)	NA[g]	NA[g]
3.2	1.14 ± 0.12 (3)	2.29 ± 0.15[b] (4)	0.22 ± 0.01 (3)	—	0.20 ± 0.05[A]	—
32	7.15 ± 0.35 (3)	16.9 ± 0.43[b] (3)	0.92 ± 0.05 (3)	0.76 ± 0.05 (3)	0.13 ± 0.003[B]	0.046 ± 0.002[B]
Combined	0.65 ± 0.03[c] (3)	1.85 ± 0.12[b],[c] (3)	—	—	—	—
PND 7						
0	< LLOQ[d] (3)	< LLOQ[e] (3)	< LLOQ[d] (3)	< LLOQ[f] (3)	NA[g]	NA[g]
3.2	1.42 ± 0.11 (3)	4.16 ± 0.04[b] (3)	0.33 ± 0.02 (3)	—	0.24 ± 0.05[A]	—
32	8.21 ± 0.47 (4)	27.3 ± 0.43[b] (4)	1.38 ± 0.27 (4)	1.33 ± 0.03 (4)	0.17 ± 0.08	0.050 ± 0.002[B]
Combined	0.99 ± 0.06[c] (4)	3.43 ± 0.22[b],[c] (4)	—	—	—	—
PND 14						
0	< LLOQ[d] (3)	< LLOQ[e] (3)	< LLOQ[d] (3)	< LLOQ[f] (3)	NA[g]	NA[g]
3.2	2.04 ± 0.06 (6)	3.15 ± 0.21[b] (6)	0.42 ± 0.03 (6)	—	0.20 ± 0.01[A]	—
32	9.58 ± 0.37 (6)	28.7 ± 1.44[b] (6)	1.13 ± 0.07 (6)	1.04 ± 0.02 (6)	0.12 ± 0.01[B]	0.035 ± 0.003[B]
Combined	1.72 ± 0.04[c] (6)	3.32 ± 0.23[b] (6)	—	—	—	—
PUP						
PND 1						
0	< LLOQ[d] (3)	< LLOQ[e] (3)	< LLOQ[d] (3)	< LLOQ[f] (3)	NA[g]	NA[g]
3.2	1.27 ± 0.08 (6)	5.85 ± 0.33[b] (7)	0.34 ± 0.02 (6)	2.05 ± 0.13[b] (7)	0.27 ± 0.01	0.36 ± 0.07
32	7.09 ± 0.44 (6)	32.9 ± 0.81[b] (6)	1.63 ± 0.07 (6)	11.5 ± 0.82 (6)	0.23 ± 0.04[A]	0.37 ± 0.05
Combined	0.88 ± 0.05[c] (6)	4.52 ± 0.28[b],[c] (6)	—	1.44 ± 0.08 (6)	—	0.32 ± 0.01
PND 7						
0	< LLOQ[d] (3)	< LLOQ[e] (3)	< LLOQ[d] (3)	< LLOQ[f] (3)	NA[g]	NA[g]
3.2	0.80 ± 0.04 (7)	3.65 ± 0.23[b] (6)	0.23 ± 0.02 (7)	1.52 ± 0.10[b] (6)	0.29 ± 0.03[A]	0.42 ± 0.01
32	5.26 ± 0.30 (6)	21.3 ± 1.06[b] (5)	1.26 ± 0.08 (6)	6.79 ± 0.48[b] (5)	0.24 ± 0.02[A]	0.32 ± 0.03
Combined	0.64 ± 0.02[c] (7)	3.26 ± 0.19[b] (7)	—	—	—	—
PND 14						
0	< LLOQ[d] (3)	< LLOQ[e] (3)	< LLOQ[d] (3)	< LLOQ[f] (3)	NA[g]	NA[g]
3.2	0.82 ± 0.06 (6)	4.89 ± 0.29[b] (5)	—	1.45 ± 0.06 (5)	—	0.30 ± 0.01
32	6.85 ± 0.37 (7)	25.2 ± 1.27[b] (6)	0.77 ± 0.05 (7)	4.92 ± 0.29[b] (6)	0.11 ± 0.01[A]	0.20 ± 0.04
Combined	0.57 ± 0.04[c] (6)	3.73 ± 0.23[b],[c] (6)	—	1.14 ± 0.08[c] (6)	—	0.31 ± 0.01

Note. ''—'' no samples available. ''Combined'' represents the combined exposure of 3.2 mg/kg feed PFOS + 3.2 mg/kg feed BDE-47.
[a]Brain level:serum level ratio. [b]$p < 0.05$ BDE-47 accumulation compared with PFOS accumulation checked by Student's *t*-test. [c]$p < 0.05$ single low-dose group of 3.2 mg/kg feed compared with combined group checked by Student's *t*-test. [d]Lower limit of quantitation (LLOQ) for serum and brain BDE-47 is 0.008–0.027 ng/g. [e]LLOQ for serum PFOS is 0.010 μg/ml. [f]LLOQ for brain PFOS is 0.025 μg/g. [g]NA, not applicable; ratio could not be calculated as chemical concentrations in serum and brain were below the LLOQ.
[A]$p < 0.05$ cortex/serum ratio for PFOS compared with the BDE-47 in dam and neonate checked by Student's *t*-test. [B]$p < 0.05$ cortex/serum ratio for PFOS or BDE-47 in neonate compared with dam checked by Student's *t*-test.

($F_{1,305} = 103$, $p < 0.001$), and chemical (PFOS and BDE-47) ($F_{1,305} = 65.4$, $p < 0.001$) showed significant effects on chemical concentrations, but no significant effects of animal (dam and neonate) ($F_{1,305} = 0.97$, $p = 0.33$) and exposure time (PNDs 1, 7, and 14) ($F_{2,305} = 0.11$, $p = 0.89$) were observed. Serum concentrations of PFOS were significantly higher than BDE-47 in both dams and neonates, and the cortex levels of PFOS were significantly higher in neonates ($p < 0.05$, LSD). In addition, a comparison of chemical effects on cortex/serum ratios revealed that ratios of PFOS were significantly lower than BDE-47 in dams ($p < 0.05$, LSD) but higher than BDE-47 in neonates ($p < 0.05$, LSD). These findings indicate an accumulation difference between the two chemicals. We also found that the ratios for both chemicals were lower in dams than in neonates ($p < 0.05$, LSD) and on PND 14 than on PNDs 1 and 7 ($p < 0.05$, Duncan), indicating that the accumulation of these chemicals in rats was dependent on development state. The serum and cortex concentrations of both chemicals seem increased with exposure time in dams but decreased in neonates. This was reflected in the significant effects of exposure time on BDE-47 and PFOS levels in the serum and cortex of both dams and neonates, with the exception of cortex BDE-47 levels in dams ($F_{42,2} = 2.84$, $p = 0.09$) (Supplementary table 8). The *post hoc* Duncan test used to determine exposure effect revealed that both 32 mg/kg dose groups were significantly higher than other groups ($p <$

0.05). Interestingly, for both PFOS and BDE-47, the 10-fold higher exposure did not translate to a 10-fold greater accumulation of these chemicals. In addition, both PFOS and BDE-47 levels in the combined groups were lower than the corresponding single exposure groups at any time point, with some showing statistical significance by *t*-test ($p < 0.05$), although no significant difference was found between single and combined exposures when ignoring effects of tissue, animal, and exposure time ($p > 0.05$, Duncan). Significant positive correlations were found in chemical concentrations between dam serum and cortex ($R = 0.634$, $p = 0.011$), between neonate serum and cortex ($R = 0.938$, $p < 0.001$), and between dam and neonate serum ($R = 0.894$, $p < 0.001$) (Supplementary table 4). Significant negative correlations between chemical concentrations and levels of TT3 ($R = -0.614$, $p = 0.007$) and TT4 ($R = -0.808$, $p < 0.001$) were found in dam serum but not in neonate serum, and no correlation was found between chemical levels and gene and protein expression levels, except weakly for the *GAP-43* transcript ($R = 0.447$, $p = 0.048$) (Supplementary table 4). This implies that serum TH homeostasis in dams may be the first direct target of the two chemicals in the current study as the chemicals were directly administrated in dams.

Serum THs

Serum TT4 and TT3 levels are shown in Table 2. Significant effects of exposure ($F_{5,566} = 7.33$, $p < 0.001$), exposure time ($F_{2,566} = 44.4$, $p < 0.001$), and TH form (TT3 and TT4) ($F_{1,566} = 309$, $p < 0.001$) were observed in neonates, but no significant sex effect was observed on TH levels ($F_{1,566} = 0.41$, $p = 0.52$). After pooling the data of the two sexes, a significant animal effect was observed ($F_{1,566} = 39.5$, $p < 0.001$). The dose-dependent reductions in TT4 or TT3 levels for both chemicals were observed in dams on PND 1, neonates on PND 7, and both dams and neonates on PND 14 ($p < 0.05$, Duncan) (Table 2). Combined exposure showed significant difference relative to controls ($p < 0.05$, Duncan) but no significant difference compared with other exposures. This implies little interaction of PFOS and BDE-47 on serum TH status in rats. Conversely, however, an antagonistic effect on TT4 levels was observed in 7-day-old neonates, as determined by significantly less reduction in TT4 levels in the combined exposure than each single exposure (3.2 mg/kg feed) ($p < 0.05$), as well as a synergistic-like effect observed in 14-day-old neonates, as determined by significantly more reduction in TT4 levels in the combined exposure than each single exposure (3.2 mg/kg feed) ($p < 0.05$) and the combined exposure showing no significant difference from high BDE-47 exposure ($p > 0.05$) (Table 2). These combined effects resulted in a different time trend in neonatal TT4 variations than in other groups, demonstrated by TT4 levels increasing then decreasing from PNDs 1 to 14 with co-exposure compared with continuous increasing with single exposure. The Duncan's test for exposure effect revealed that neonatal TT3 levels following BDE-47 single exposure were significantly lower than with PFOS single exposure for both low and high doses ($p < 0.05$), whereas neonatal TT4 levels

TABLE 2
TT3 and TT4 Concentration in Serum of Dams and Neonates (Mean ± SE)

		TT3 (ng/ml)		TT4 (ng/ml)	
Age (PNDs)	Groups (mg/kg feed)	Neonate	Dam	Neonate	Dam
1	0	0.22 ± 0.02 (12)	0.46 ± 0.03^{b} (4)	11.2 ± 0.3^{b} (12)	43.2 ± 1.6^{d} (4)
	PFOS (3.2)	0.21 ± 0.02 (12)	0.46 ± 0.01^{b} (4)	11.5 ± 0.4^{b} (12)	29.5 ± 0.8^{b} (4)
	PBDE-47 (3.2)	0.21 ± 0.02 (12)	0.46 ± 0.03^{b} (4)	11.4 ± 0.4^{b} (12)	30.3 ± 0.6^{bc} (4)
	PFOS (3.2) + PBDE-47 (3.2)	0.21 ± 0.02 (12)	0.46 ± 0.01^{b} (4)	10.4 ± 0.9^{b} (12)	33.1 ± 1.4^{c} (4)
	PFOS (32)	0.18 ± 0.03 (9)	0.39 ± 0.01^{a} (4)	6.5 ± 0.3^{a} (9)	20.3 ± 0.5^{a} (4)
	PBDE-47 (32)	0.21 ± 0.02 (12)	0.45 ± 0.01^{b} (4)	11.0 ± 0.6^{b} (12)	30.8 ± 1.5^{bc} (4)
7	0	0.43 ± 0.02 (12)	0.46 ± 0.01 (4)	40.3 ± 0.5^{f} (12)	42.9 ± 1.8^{b} (4)
	PFOS (3.2)	0.41 ± 0.02 (9)	0.46 ± 0.02 (3)	24.8 ± 1.2^{c} (9)	30.9 ± 1.0^{a} (3)
	PBDE-47 (3.2)	0.46 ± 0.03 (9)	0.46 ± 0.01 (4)	28.8 ± 1.7^{d} (9)	30.7 ± 0.7^{a} (4)
	PFOS (3.2) + PBDE-47 (3.2)	0.41 ± 0.02 (9)	—	31.5 ± 1.3^{e} (9)	—
	PFOS (32)	0.43 ± 0.02 (12)	—	18.9 ± 0.9^{a} (12)	—
	PBDE-47 (32)	0.43 ± 0.02 (12)	0.46 ± 0.01 (4)	21.6 ± 1.1^{b} (12)	29.8 ± 0.5^{a} (4)
14	0	0.72 ± 0.04^{cd} (12)	—	73.7 ± 2.9^{d} (12)	—
	PFOS (3.2)	0.78 ± 0.04^{d} (9)	0.46 ± 0.02^{b} (9)	53.5 ± 1.4^{c} (9)	17.2 ± 0.7^{b} (9)
	PBDE-47 (3.2)	0.68 ± 0.03^{bc} (12)	0.48 ± 0.03^{b} (9)	51.9 ± 2.8^{c} (12)	23.1 ± 0.9^{c} (9)
	PFOS (3.2) + PBDE-47 (3.2)	0.62 ± 0.02^{bc} (9)	0.46 ± 0.02^{b} (9)	29.7 ± 1.4^{b} (9)	21.5 ± 0.8^{c} (9)
	PFOS (32)	0.58 ± 0.03^{b} (12)	0.36 ± 0.02^{a} (9)	24.7 ± 1.5^{a} (12)	13.8 ± 0.5^{a} (9)
	PBDE-47 (32)	0.45 ± 0.02^{a} (13)	0.36 ± 0.03^{a} (9)	26.3 ± 1.4^{ab} (13)	15.6 ± 0.6^{b} (9)

Note. Means followed by the same letter within a column on each day indicate no significance ($p \geq 0.05$), whereas different letters in the same column indicate significance ($p < 0.05$) by Duncan's multiple range test (ANOVA). ''—'' no samples available.

were significantly lower with PFOS single exposure than BDE-47 single exposure at the high dose ($p < 0.05$). For dams, only TT4 levels were significantly lower than the same dose of BDE-47 exposure with high PFOS exposures ($p < 0.05$, Duncan). These results suggest a chemical difference between PFOS and BDE-47 in TH regulation, which seems related to the life stage of rat. The Pearson analysis of correlations between levels of THs and gene and protein expressions revealed significant positive correlations between dam TT3 and TT4 levels ($R = 0.69$, $p < 0.01$), between neonate TT3 and TT4 levels ($R = 0.85$, $p < 0.01$), between *BDNF* transcript and neonatal TT4 ($R = 0.38$, $p < 0.05$) and TT3 levels ($R = 0.55$, $p < 0.01$), and between proteins of BDNF and NCAM1 with neonatal TT3 and TT4 levels (Supplementary table 5). Other variables showed no significant correlation with TH levels. No significant correlation was observed in either TT4 or TT3 levels between dams and neonates, which may have partly resulted from the significant time-dependent changes in TT3 and TT4 levels from PNDs 1 to 14 ($p < 0.05$, Duncan) in neonates in contrast to lack of changes in dams.

TRβ, BTEB, BDNF, GAP-43, *and* NCAM1 *Transcripts*

The expression of the five genes (*TRβ, BTEB, BDNF, GAP-43*, and *NCAM1*) in the cortex and hippocampus of neonates were examined and are shown in Figure 1. No significant sex effects were observed in either tissues for all five genes ($F_{1,1778} = 0.54$, $p = 0.46$). No hippocampus data for the tested genes were collected on PND 1 as the tissue from 1-day-old neonate was insufficient for subsequent measurements. Significant effects of exposure ($F_{5,1778} = 18.7$, $p < 0.001$), tissue (cortex and hippocampus) ($F_{1,1778} = 24.8$, $p < 0.001$), exposure time ($F_{2,1778} = 296$, $p < 0.001$), and gene title (*TRβ, BTEB, BDNF, GAP-43*, and *NCAM1*) ($F_{4,1778} = 486$, $p < 0.001$) were observed on gene expression levels. Duncan's test revealed that exposure effect was due to significant differences between 32 mg/kg feed PFOS and other exposures ($p < 0.05$), with no significant differences observed among the other exposures ($p > 0.05$). This implies that PFOS was more effective at high dose than BDE-47 at regulating gene expression. If dose-dependent variations based on the two chemical exposures existed, this lack of significant difference in gene expression between combined and single exposures (3.2 mg/kg feed) would indicate an antagonistic effect between the two chemicals. From the overall analysis of gene expressions, however, no significant dose-dependent variation was observed for either chemical ($p > 0.05$, Duncan), indicating that little interaction of PFOS with BDE-47 existed on overall gene expression. Nevertheless, after statistical analysis of each transcript, we observed that, on PND 1, the combined exposure dramatically elevated *BDNF* mRNA levels relative to the control ($p < 0.05$) comparable to the 10 times higher exposure of PFOS, whereas BDE-47 and lower PFOS exposures showed no significant effects ($p > 0.05$). This indicates that BDE-47 had no effect in the single exposure but enhanced the effect of PFOS in combined exposure on cortex *BDNF* gene expression, implying a synergistic effect at PND 1. On PND 14, however, *BDNF* showed an antagonistic effect on mRNA levels in the hippocampus, demonstrated by significantly higher expression in the high PFOS exposure ($p < 0.05$), lower expression with combined exposure compared with control ($p < 0.05$), and no significant change in other exposures compared with control ($p > 0.05$) (Fig. 1). Similar to *BDNF* on PND 1, GAP-43 on PND 14 in the hippocampus, and *BTEB* on PND 1 in the cortex both showed synergistic effects on mRNA levels (Fig. 1). Duncan's test for exposure effect on *BDNF, GAP-43*, and *NCAM1* expression across all exposure times and both tissues reflected significant differences in the combined exposures than other exposures for *BDNF* and *GAP-43* ($p < 0.05$, Duncan) but no significant difference for *NCAM1* ($p > 0.05$, Duncan). This implies that *BDNF* and *GAP-43* but not *NCAM1* mRNA levels were influenced differently by single exposure than combined exposure. The comparison of exposure effects of the two chemicals revealed that *BDNF, GAP-43, BTEB*, and *TRβ* mRNA levels under the 32 mg/kg feed PFOS exposure were significantly higher compared with other exposures ($p < 0.05$, Duncan), whereas no significant difference was observed between low dose of PFOS and BDE-47 exposures ($p > 0.05$, Duncan). For *NCAM1*, PFOS resulted in a significantly higher expression than BDE-47 in both high and low exposures ($p < 0.05$, Duncan). The mRNA levels were significantly lower on PND 1 ($p < 0.05$, Duncan) followed by PND 7 ($p < 0.05$, Duncan), with PND 14 showing significantly higher expression levels ($p < 0.05$, Duncan). This increasing time trend in gene expression for neonatal rats was consistent with the brain growth spurt during early development. There was no significant difference between *BDNF* and *GAP-43* expression ($p > 0.05$, Duncan) and between *BTEB* and *TRβ* expression ($p > 0.05$, Duncan). The *BDNF* and *GAP-43* mRNA levels were significantly higher than *BTEB, TRβ*, and *NCAM1* ($p < 0.05$, Duncan), and *NCAM1* transcripts were significantly higher than *BTEB* and *TRβ* ($p < 0.05$, Duncan). For pairwise comparison of the two regions' effects, *BTEB* and *TRβ* showed significantly higher expression in the cortex than hippocampus ($p < 0.05$, LSD), and *BDNF, GAP-43*, and *NCAM1* showed significantly higher expression in the hippocampus than cortex ($p < 0.05$, LSD). With the exception of *BDNF*, no significant correlations between neonatal TT4 and TT3 levels and transcript levels were observed (Supplementary table 5), even though the five gene expressions were all T3 mediated. This indicates that PFOS and/or BDE-47 exposure affected the other four gene expressions not through T3 but by other pathways during early development. In addition, strong positive correlations were found in gene expression levels between *BTEB* and *BDNF* on PND 1 in the cortex ($R = 0.99$, $p < 0.01$), between *BDNF* and *NCAM1* ($R = 0.92$, $p = 0.01$) and TRβ ($R = 0.99$, $p < 0.01$) on PND 7 in the hippocampus, and between *BTEB* and *TRβ* ($R = 0.94$, $p < 0.01$) on PND 14 in the cortex, and negative correlations were found significantly

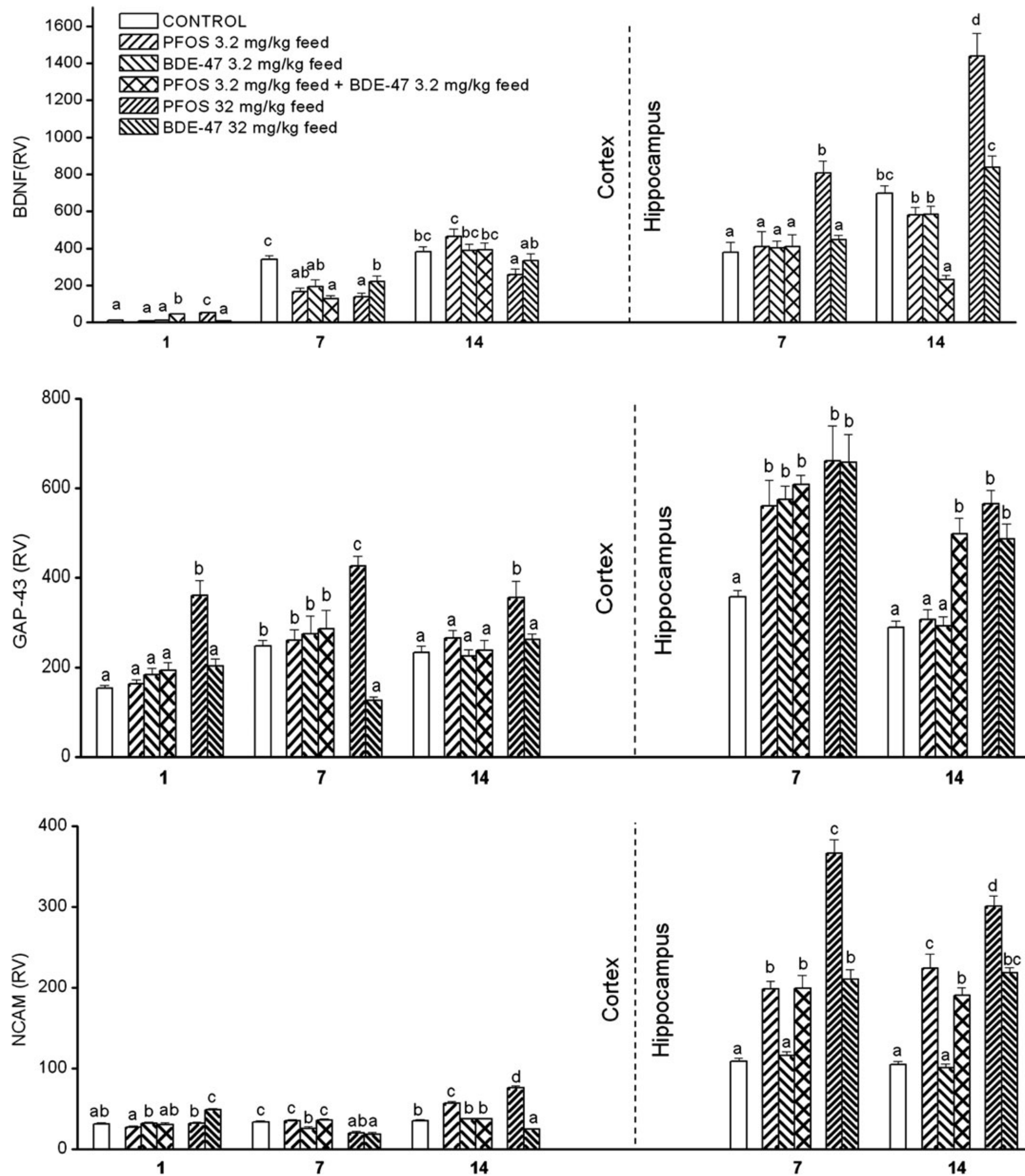

FIG. 1. Effects of PFOS and/or BDE-47 exposure on the expression of five genes (*BDNF, GAP-43, NCAM1, BTEB*, and *TRβ*) in the neonatal cortex (bars in the left area of the lash line labeled ''Cortex'') and hippocampus (bars in the right area of the lash line labeled ''Hippocampus'') during the postnatal period. For each gene, bars labeled with the same letter on the same PND in the cortex or hippocampus indicate no significance ($p \geq 0.05$) and with different letters indicate significance ($p < 0.05$) by Duncan's multiple range test (ANOVA). ''RV'' means relative value of each mRNA to the normalizer gene of BTEB in the control cortex on PND 1. Each group contained 6–12 pups.

between TH levels and levels of *BDNF* and *GAP-43* mRNA or protein on individual PNDs (Supplementary table 6). When exposure time was ignored, overall correlation analysis showed that the expression of the three genes, *BDNF, GAP-43*, and *NCAM1*, were positively correlated with each other ($R = 0.54$–0.82), and *BTEB* and *TRβ* correlated well ($R = 0.839$, $p < 0.01$) (Supplementary table 5). Significant correlations between gene and protein expressions were also observed for each transcript of *BDNF*, *GAP-43*, and *NCAM1* (Supplementary tables 5 and 6).

Protein Levels for BDNF, GAP-43, *and* NCAM1

Protein expression in neonatal cortex and hippocampus are shown in Figure 2. No significant sex ($F_{1,899} = 1.97$, $p = 0.16$) effect was observed on protein levels, but significant effects of exposure ($F_{5,899} = 4.28$, $p = 0.001$), tissue ($F_{1,899} = 290$, $p < 0.001$), exposure time ($F_{2,899} = 47.4$, $p < 0.001$), and protein title ($F_{2,899} = 591$, $p < 0.001$) (Supplementary tables 7 and 8) were observed. Comparison of exposure effects revealed that 32 mg/kg feed PFOS exposure significantly increased protein levels compared with other exposures ($p < 0.05$,

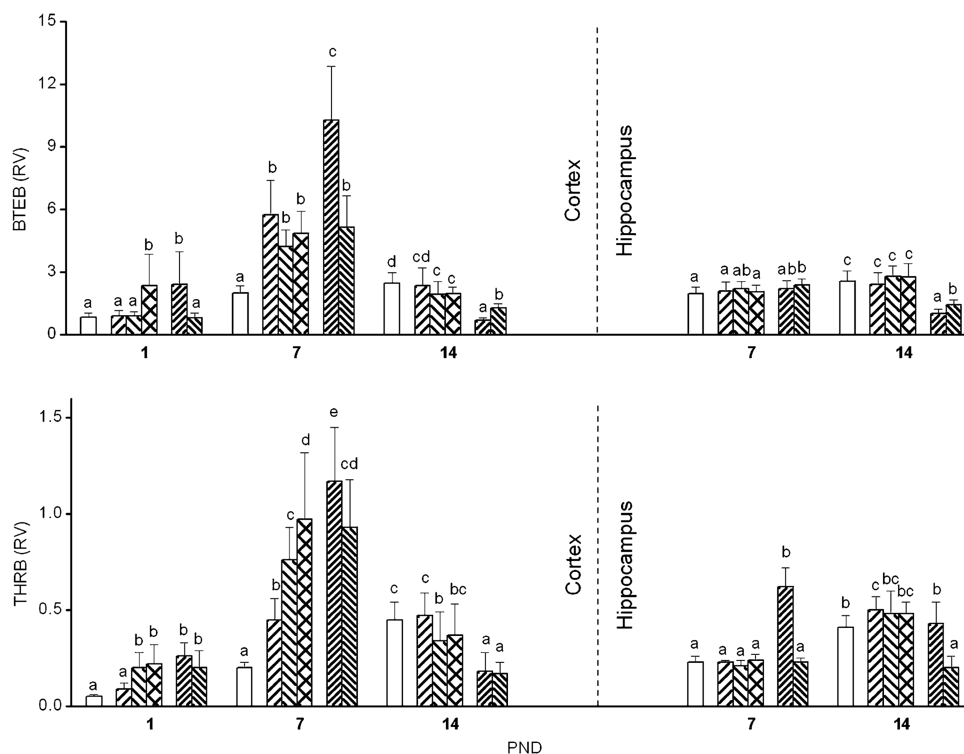

FIG. 1. Continued

Duncan), and no significant difference was observed among other exposures ($p > 0.05$, Duncan). The levels of *GAP-43* and *NCAM1* showed no significant difference from each other but were significantly lower than *BDNF* levels ($p < 0.05$, Duncan). Proteins in the hippocampus showed significantly higher levels than in the cortex ($p < 0.05$, LSD). A significantly increasing time trend across PNDs 1, 7, and 14 ($p < 0.05$, Duncan) was also observed in protein expression, showing a parallel time-dependent variation between gene and protein expressions. This was also reflected in the significant correlation between protein and gene expressions ($R = 0.609$, $p < 0.01$). However, changes in protein levels appeared less sensitive than in mRNA levels. This was reflected by protein levels showing no change, whereas significant changes in mRNA levels were observed in cortex *BDNF* on PND 7 with low PFOS, BDE-47, and combined exposure; *GAP-43* with high PFOS exposure at PND 14 in the cortex and at PND 7 in the hippocampus; hippocampus *GAP-43* in the combined exposure at PND 14; *NCAM1* with high BDE-47 exposure at PND 7 and with PFOS exposures at PND 14 in the cortex; and hippocampus *NCAM1* with low PFOS exposure and combined exposure on PNDs 7 and 14 (Figs. 1 and 2). These discrepancies between gene and protein expressions implied that a posttranscriptional or translational regulation occurred in response to the two chemical exposures. In addition, significant correlations between protein levels of *BDNF* and *GAP-43* ($R = 0.60$, $p < 0.01$), *BDNF* and *NCAM1* ($R = 0.86$, $p < 0.01$), and *GAP-43* and *NCAM1* ($R = 0.74$, $p < 0.01$) were found. For *BDNF*, the 32 mg/kg feed PFOS exposure showed significantly different effects from other exposures, whereas no significant difference was found among the other exposures. Nevertheless, we observed that *BDNF* protein in the combined exposure shared a similar pattern with its coding gene expressed synergistically on PND 1 and antagonistically on PND 14. This was also reflected in the significant correlation between *BDNF* mRNA and protein levels ($R = 0.86$, $p < 0.01$). The parallel synergistic effect on mRNA level of *BTEB* with *BDNF* observed on PND 1 was reflected in the strong positive correlation between *BTEB* and *BDNF* gene expression ($R = 0.99$, $p < 0.01$). These findings indicate that PFOS and BDE-47 may interact on *BDNF* expression partly through *BTEB*-associated pathways, and this interaction seems time dependent and tissue specific. The *GAP-43* levels in the high PFOS exposure were significantly higher than at the same dose of BDE-47 exposure ($p < 0.05$), and no significant difference between low PFOS, low BDE-47, and combined exposures was observed ($p > 0.05$, Duncan), suggesting no combined effects on overall GAP-43 protein expression. For *NCAM1*, no significant difference between PFOS and BDE-47 exposures ($p > 0.05$, Duncan) at high and low doses was observed. Additionally, no significant difference was shown between the combined exposure, low PFOS and BDE-47 exposures, and the controls ($p > 0.05$, Duncan), suggesting no combined effect on overall NCAM1 expression. Nor was any combined effect

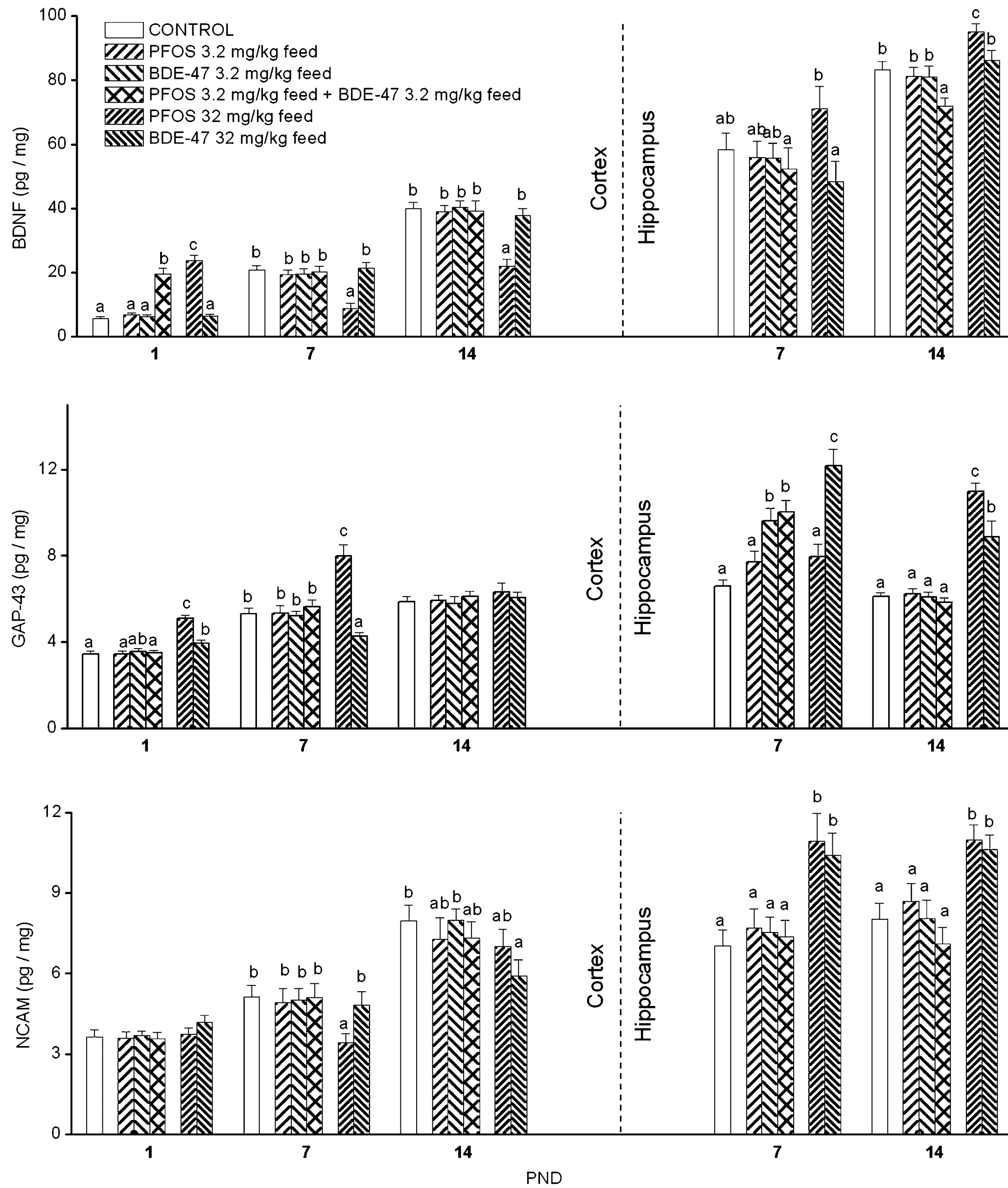

FIG. 2. Effects of PFOS and/or BDE-47 exposure on expression of three proteins (*BDNF, GAP-43*, and *NCAM1*) in neonatal cortex (bars in the left area of the lash line labeled ''Cortex'') and hippocampus (bars in the right area of the lash line labeled ''Hippocampus'') during the postnatal period. For each protein, bars labeled with the same letter on the same PND in the cortex or hippocampus indicate no significance ($p \geq 0.05$) and with different letters indicate significance ($p < 0.05$) by Duncan's multiple range test (ANOVA). Each group contained 6–12 pups.

observed on GAP-43 and NCAM1 protein levels on individual PNDs in certain brain regions, indicating little interaction of the two chemicals on GAP-43 and NCAM1 expressions. In addition, significant correlations were observed between all three protein levels (Supplementary tables 5 and 6). In the present study, we were unable to determine which chemical, PFOS or BDE-47, was more potent in gene and protein expression changes, as no significant difference was observed in either gene or protein expression between PFOS and BDE-47 exposures in the low dose and the high dose of PFOS exposure showed significantly different effects from any other exposure ($p < 0.05$, Duncan). It seems that PFOS was more effective than BDE-47, but this effectiveness appeared dose dependent as significant chemical differences were only observed in the high exposure.

DISCUSSION

No significant sex effect on chemical accumulation, TH levels, and gene and protein expressions was observed. Although experimental animals were exposed to equal amounts

of PFOS or BDE-47, we found much higher PFOS concentrations in both serum and brain tissue. This finding is similar to previous research that shows disparity between perfluorochemicals and BFC accumulation in human serum (Roosens *et al.*, 2010). The observed difference was likely dependent on chemical structure properties and patterns of entering a cell and possibly due to specific metabolism of BDE-47 into hydroxylated forms without further transformation of PFOS in the internal bio-circumstance. In the present study, we can only provide parent BDE-47 levels and not its hydroxylated or methoxylated forms, so it is possible that accumulation of internal BDE-47 was underestimated. The time-dependent reduction in both serum and brain concentrations of the two chemicals in neonates was largely due to the faster volume expansion of developing bodies and brains than the total amount of increasing chemical concentrations. The observed decrease in the brain to serum ratio observed on PND 14 compared with PND 1 and PND 7 for both chemicals might be due to the inhibition of PFOS or BDE-47 translocation into brain by a relatively well-developed BBB. This may also explain why the ratio for PFOS and BDE-47 in dams was lower than in neonates. These results agree with previous studies that demonstrate differences in PFOS or BDE-47 brain and serum accumulation between adults and offspring (Chang *et al.*, 2009; Gebbink *et al.*, 2008; Weijs *et al.*, 2010). The lower ratios for PFOS observed in dams with fully developed BBB, compared with the higher ratios observed for neonates with undeveloped BBB, indicate that the inhibition of BBB on PFOS might be more effective than on BDE-47. The characteristics observed in PFOS and BDE-47 accumulation were also partly associated with different capabilities of hepatic metabolism and exertion of chemicals between dam and neonates, between PFOS and BDE-47, and between high and low exposure dose. Both BDE-47 and PFOS induce metabolizing enzymes such as phase I, II, III, and cytochrome P450 in a positive dose-dependent manner (Szabo *et al.*, 2009; Watanabe *et al.*, 2009). The observation of higher concentrations for both chemicals in the single exposure compared with the combined exposure is likely due to enzymes being activated differently between the two exposures.

The balance of TT4 and TT3 in serum is mainly regulated by thyroid production and liver metabolism and is dependent on iodination/deiodination operated by relative enzymes (Szabo *et al.*, 2009). Our results showed greater reduction in TT4 levels by PFOS than BDE-47 but the opposite for TT3 levels in neonates. This implies that deiodination of T4 into T3 in the thyroid or the further metabolism of serum T3 may be affected differently by BDE-47 than by PFOS in offspring. Although many studies have reported on PFOS- or PBDE-47–induced responsiveness to deiodinase in thyroid and liver (Szabo *et al.*, 2009; Yu *et al.*, 2009a), comparative studies on the potencies of deiodinase with equal PFOS or BDE-47 exposure are limited. For dam, however, this chemical difference was only observed in high PFOS exposure on TT4 levels, showing less sensitiveness in TH variation in dams compared with neonates. This result agrees with previous reports that offspring are more susceptible to TH disruption than pregnant and post-wean dams (Chang *et al.*, 2008). Studies on PFOS- and BDE-47–induced disruption of circulating THs have shown a similar pattern of action and a common mechanism (Lema *et al.*, 2008; Skarman *et al.*, 2005; Yu *et al.*, 2009a). The most accepted explanation for TT4 and TT3 reduction without elevation in serum TSH is that the negative feedback expected on the pituitary was not activated due to inadequate exposure to toxicants or was impaired by excessive exposure to toxicants (Thibodeaux, 2005). In some instances, however, temporary changes in TSH have been observed (Lema *et al.*, 2008; Skarman *et al.*, 2005). Although the present study revealed a different time trend variation in neonatal TT4 levels between single and combined exposure, this was more likely attributable to the complicated feedback on TH regulation than the combined interaction between the two chemicals on neonatal TT4 regulation. It is probable that the dose magnitude of co-exposure to PFOS and BDE-47 was just sufficient to activate the transient feedback of TSH, leading to temporary higher TT4 levels in the combined group on PND 7.

Both *BTEB* and *TRβ* genes are developmentally regulated and exhibit variations in expression that parallel increases in plasma T3 levels (Iwamuro *et al.*, 2006). Previous studies have demonstrated that once one factor changes, the other responds coincidently (Bagamasbad *et al.*, 2008). This could explain the significantly correlated variations observed between *BTEB* and *TRβ* gene expression. As *TRβ* and *BTEB* proteins play significant roles in various neural processes in cooperation with T3 and other co-activators, our results imply a potential impairment in TR-mediated functions by PFOS and/or BDE-47 exposure (Denver *et al.*, 1999). Whether these protein levels change correspondingly, however, requires further examination.

The BDE-47–induced changes in *BDNF* protein levels observed in neonates in the present study were rarely consistent with that of BDE-209 (Viberg *et al.*, 2008). The *GAP-43* protein levels in neonatal rats exposed to PFOS or BDE-47 also exhibited divergence from that observed in neonatal mice exposed to PFOS or BDE-209 (Johansson *et al.*, 2009; Viberg *et al.*, 2008). These differences suggest some species-, congener-, or dosage-specific variation in BDNF and *GAP-43* protein expression. *BDNF*, *GAP-43*, and *NCAM1* are three T3-responsive genes regulated by TRs and co-activators such as *TRβ* and *BTEB*. It has been demonstrated that *BDNF* and *NCAM1* can be positively and negatively regulated by T3, respectively (Thompson and Potter, 2000), and *GAP-43* can be positively regulated at the protein level with no changes in the mRNA level (Iñiguez *et al.*, 1993). The positive relationship of *NCAM1* and negative relationships of *BDNF* and *GAP-43* with TH levels in mRNA or protein level observed on some individual PNDs were opposite to that reported with T3, suggesting these variations were not attributable to TH regulation but likely responsive to other factors. From the

present study, *BDNF* showed significant positive correlations with *BTEB* and *TRβ* on PNDs 1 and 7 in gene expression and with neonatal TT3 and TT4 levels in both gene and protein expressions across overall PNDs, indicating that changes in *BDNF* caused by PFOS and/or BDE-47 exposure were partly through TH-mediated regulations. No such effective relationships were found in *GAP-43* and *NCAM1* mRNA and protein levels, suggesting TH-mediated pathways were unlikely to participate in PFOS- and/or BDE-47–induced variations in *GAP-43* and *NCAM1* expression. Because the three factors, known as neuroplasticity effectors, usually interact to elicit and regulate neurofunctions and once one factor is triggered, the others may change in accordance, the significant relationships observed among their changes may result from some common inductions by chemicals and reflect a cooperation in associated neural functions. The developmentally induced changes in *BDNF, GAP-43*, and *NCAM1* protein levels suggest potential disturbances by PFOS and/or BDE-47 on associated neural functions such as synaptic transmission, long-term potential, neurogenesis, and neurite outgrowth (Benowitz and Routtenberg, 1997; Viberg *et al*., 2008).

The present results showed very little interaction on most tested targets except on *BDNF*, which exhibited a synergistic effect on PND 1 in the cortex and an antagonistic effect on PND 14 in the hippocampus. This combined effect was characterized by the fact that BDE-47 alone, either in high or low dose, did not induce any changes in *BDNF* expressions compared with controls but enhanced or attenuated the PFOS-induced effects in combination. This suggests a possible interaction between BDE-47 and PFOS on *BDNF*-related pathways. The precise mechanism by which the combination of PFOS and BDE-47 elicited these two effects remains undetermined. This may be partly through *BTEB* regulation on PND 1 but seems little implicated in TH regulation because of no parallel combined effects observed on TH level. In addition, it has been reported that *BDNF* can be affected by phosphorylated cAMP response element binding protein (Mantelas *et al*., 2003), estrogen (Liu *et al*., 2007), and progesterone (Morita and Her, 2008) and is related to cerebral ischemia (Onishchenko *et al*., 2008), which is responsive to PFOS and BDE-47 exposure. Our previous studies reported on the elevation of phosphorylated cAMP response element binding protein (CREB) and differential expression of cerebral ischemia-associated genes in neonatal rat brains exposed to PFOS (Liu *et al*., 2010; Wang *et al*., 2010). In addition, *in vitro* analysis has demonstrated that BDE-47 has an estrogenic effect (Kojima *et al*., 2009), whereas PFOS has both an estrogenic and anti-estrogenic effect, which is dependent on whether it competes with E2-binding sites (Liu *et al*., 2001). *In vitro* and *in vivo* studies have also demonstrated that BDE-47 enhances progesterone and testosterone secretion (Morita and Her, 2008), and PFOS lowers testosterone levels (Jensen and Leffers, 2008). It seems, therefore, that neurobiological regulations specific to *BDNF* may have also led to the observed interaction of the two chemicals.

CONCLUSIONS

We investigated the effects of PFOS and BDE-47 exposure on chemical accumulation, serum THs, and TH-mediated gene and protein expression in the developing rat brain. The results showed some region-specific and exposure- and time-dependent alterations in TH and gene and protein expression levels. Marked divergence was found between the potency of PFOS and BDE-47 in the highest exposure group. Although the two chemicals showed many toxic characteristics in common, especially related to the TH-associated regulations, our findings demonstrated little combined interactions from co-exposure except on *BDNF*. Whether these two chemicals have combined actions on other toxic targets cannot be predicted by this study. The dose used in the combined exposure for each chemical resulted in BDE-47 with 0.69–1.49 μg/g serum lipid and PFOS with 1.1–4.5 mg/l in serum, which are in the range reported for humans or occupational workers and have not led to evident external signs of toxicity in experimental animals (Olsen *et al*., 2003; Talsness *et al*., 2008; Yu *et al*., 2009b). Understanding the combined biological effects are important, therefore, in developing appropriate risk assessment paradigms that protect human health and evaluate environmental pollution and safety state without under- or overestimation.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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