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Maternal Transfer of BDE-47 to Offspring and Neurobehavioral Development in C57BL/6J Mice

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Abstract

Polybrominated diphenyl ethers (PBDEs) are flame retardants used worldwide in a variety of commercial goods, and are now widely found in both environmental and biological samples. BDE-47 is one of the most pervasive of these PBDE congeners and therefore is of particular concern. In this study C57BL/6J mice were exposed perinatally to 0.03, 0.1 or 1 mg/kg/day of BDE-47, a dose range chosen to encompass human exposure levels. Tissue levels of BDE-47 were measured in the blood, brain, fat and milk of dams and in whole fetal homogenate and blood and brain of pups on gestational day (GD) 15, and postnatal days (PND) 1, 10 and 21. From GD 15 to PND 1 levels of BDE-47 increased within dam tissue and then decreased from PND 1 to 21. Over the period of lactation levels in dam milk were comparatively high when compared to both brain and blood for all dose groups. Measurable levels of BDE-47 were found in the fetus on GD 15 confirming gestational exposure. From PND 1 to 21, levels of BDE-47 in pup tissue increased over the period of lactation due to the transfer of BDE-47 through milk. Behavioral tests of fine motor function and learning and memory were carried out between postnatal weeks 5–17 in order to evaluate the neurobehavioral toxicity of BDE-47. Behavioral deficits were only seen in the Barnes spatial maze where mice in the three exposure groups had longer latencies and traveled longer distances to find the escape hole when compared to vehicle control mice. These results support the conclusions that perinatal exposure to BDE-47 can have neurodevelopmental consequences, and that lactational exposure represents a significant exposure risk during development.

Keywords

Flame retardant; BDE-47; Polybrominated Diphenyl Ether; Neurotoxicity; Neurodevelopment; Behavior; Bioaccumulation; GC/MS

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

1. Introduction

Polybrominated Diphenyl Ethers (PBDEs) are used as flame retardants though their manufacture has recently been banned in both the US and Europe. In total there are 209 possible PBDE congeners based on the number and position of bromine atoms along the diphenyl ring structure. 2,2',4,4'-brominated diphenyl ether (BDE-47) is a tetra-PBDE congener that makes up 40 % of the available penta- commercial mixture [33]. The penta-formulation, comprised mostly of both penta- and tetra- congeners, is almost exclusively used in polyurethane (PUR) foam where it makes up approximately 40 % of the foam by weight [66]. PUR foam is frequently used for furniture and upholstery in homes and vehicles and in packaging; whereas, non-foamed PUR is used in casings and electronic equipment. When PBDEs are added to PUR products however they do not stably bind to the matrix and can leach into the environment leading to exposure [73].

Human exposure to PBDEs can occur through several routes. Work by Frederiksen et al., Schecter et al. and Huwe et al. [18,26,54,55] have found measurable levels of PBDEs in a variety of food products indicating the potential for oral exposure. Studies by Law et al. and Lober [34,66] have demonstrated the presence of PBDEs in both indoor and outdoor air samples suggesting inhalation as a possible route of exposure. Further, a study by Staskal et al. [59] identified dermal absorption as a route of exposure to PBDEs, including BDE-47. This information, paired with that fact that almost 100,000 tons of PBDEs have been used in the US alone from 1970–2001 [66], is reason for concern.

Detectable levels of PBDEs have been reported in wildlife and human tissue samples all over the world [4,6,12,23,50, 64, 65, 75, 77]. Of significant concern are reports that PBDEs have been found in umbilical cord serum, placenta, amniotic fluid, and breast milk, indicating the potential for early developmental exposure to these compounds [18,22,31,32,46,47]. In these biological samples, BDE-47 usually makes up 50 % of the congener profile [14]. Therefore, it is likely that there is considerable early life exposure to BDE-47 when compared to other congeners.

Developmental studies by Roze et al, Herbtsman et al, Shy et al. and Gascon et al. [19,24,52,56] have reported that PBDE levels in umbilical cord samples taken either during gestation or at birth are significantly correlated with altered behavior later in life. Evaluation of BDE-47 in the Herbtsman et al. study [24] specifically found that a median umbilical cord blood concentration of 11 ng/g lipid weight (lw) BDE-47, a level similar to that recorded on gestational day (GD) 15 in the 1 mg/kg/day dose group from the current study, was negatively associated with neurodevelopmental indices including psychomotor development and mental development. Further for every natural log (ln) unit increase in BDE-47 concentration there was an average 2–3 point drop in quantitative measures of these developmental indices. The study by Gascon et al. [19] found that 2 ng/g lw BDE-47 in umbilical cord serum was associated with an increased risk of symptoms on the attention deficit subscale of ADHD-DSM-IV and a significantly higher risk of poor social competence in children at 4 years of age.

There are also several studies in murine models indicating that developmental exposures to PBDEs, including BDE-47, can disrupt normal brain development [20,61,68,70]. A single acute exposure to either 8 or 12 mg/kg of 2,2',4,4'-brominated diphenyl ether (BDE-99) on postnatal day (PND) 10 alters spontaneous motor behaviors in adult mice [15], decreases locomotor behavior in response to nicotine [70], alters levels of proteins associated with neurodegeneration and neuroplasticity in the striatum, and alters energy metabolism in the hippocampus [1]. Exposure to higher brominated congeners such as 153, 183, 203, 206, and

209 at concentrations ranging from 0.4 to 20.1 mg/kg has also been associated with developmental toxicity [11,28,67,69–72]. Animal studies on BDE-47 indicate that spontaneous motor activity is a sensitive domain to exposure. Recent research has demonstrated that early developmental exposure to BDE-47 in mice can lead to hyperactivity and decreased habituation to a novel environment [15,20,61].

Considered in aggregate, the available evidence indicates that exposure to PBDEs, including BDE-47, results in abnormal neurodevelopment. The majority of studies on PBDE developmental neurotoxicity, however, rely on data from studies using acute high level dosing schemes, which do not accurately mimic relevant human exposure. The goal of our research on BDE-47 is to evaluate neurodevelopmental toxicity associated with a low level chronic perinatal exposure that is similar to that experienced by humans. Additionally, we want to gain an understanding of how BDE-47 is transferred from mother to child throughout gestation and lactation.

In a previous study conducted in our laboratory [63], we found that chronic low level perinatal oral exposure of dams to BDE-47 lead to altered spontaneous locomotor activity in the open field, and resulted in slower swim speed in the Morris Water Maze (MWM) in mouse pups. Furthermore, in our prior study mouse pups exposed to 1 mg/kg/day BDE-47 also swam a shorter distance to reach the hidden escape platform in the MWM suggesting altered learning. Motivational effects and/or motor effects related to swimming, however, could not be ruled out as explanations for the shorter swim distance in the Morris water maze because group differences were apparent on the first trial of training. Finally this study suggested a significant mobilization of BDE-47 from tissue stores (e.g. blood, brain, liver, and fat) of the dam into breast milk, which was presumably transferred to pups during lactation. Specifically, we found that there was significant accumulation of BDE-47 in the fat of dams from the high 1 mg/kg/day dose group, followed by liver, brain and blood, and that these levels decreased over the course of lactation. However, lactational transfer could not be examined in detail because levels of BDE-47 were not quantitated in breast milk or in the tissues of pups from the lower dose groups. Levels of BDE-47 in pup brain were found to be higher than those recorded for dam brain at the time of birth, but over the course of lactation levels of BDE-47 in pup brain and blood steadily decreased suggesting a decrease in exposure to BDE-47 over the postnatal period.

The present study was undertaken to further examine the motor deficits, Morris water maze performance, and to better characterize perinatal transfer of BDE-47 in mice using the same exposure procedures. Specifically, new motor tests were carried out (i.e., ladder rung test) to further examine locomotor behavior. We also tested mice for locomotor deficits using testing procedures previously reported to reveal abnormal locomotor activity in mice following a single high dose injection of BDE-47 on postnatal day 10 [15,20]. To further assess learning and memory performance mice were trained in the Barnes maze which requires the use of spatial cues to locate an escape box but does not require mice to swim [48]. Finally to gain a better understanding of how BDE-47 is transferred from dams to pups following perinatal exposure, as well as to determine how closely our exposure paradigm mimics human exposure, we used GC/MS to quantify BDE-47 in milk collected during lactation, and in additional tissues collected from dams and pups at multiple time points throughout gestation and lactation.

2. Materials & Methods

2.1 Chemical Reagents

All chemicals used in this study were A.C.S. Reagent grade and all solvents used were HPLC Grade. Certified BDE-47 (Cat. No. BDE-047N, Lot. No.100901MT) and a 50.1 µg/

ml solution of BDE-47 in isooctane (Cat. No. BDE-047S, Lot. No. 209111007) were obtained from AccuStandard (New Haven, CT, USA). A 50 µg/ml solution of [¹³C]-BDE-47 (Cat. No. EO-4982-0, Lot. No. SCHF-009) for use as an internal standard was acquired from Cambridge Isotope Laboratories, Inc., (Andover, MA, USA). Hexane, isooctane, water, Sigmacote (SL2) and DMSO were acquired from Sigma-Aldrich (St. Louis, MO, USA). Euthasol® was purchased from Virbac AH, Inc. (Fort Worth, TX, USA).

2.1.1 Dosing Solutions—A stock solution of BDE-47 was made by sonicating 5 mg of certified BDE-47 in 250 µl DMSO until dissolved. Aliquots from this stock solution were then diluted with Mazola® corn oil to create the 0.03, 0.1 and 1 mg/kg/day BDE-47 dosing solutions. These doses were selected to encompass the range of human exposure to BDE-47. Dosing solution concentration was validated before the start of dosing using the extraction and quantification methods described in sections 2.3 and 2.4.

2.1.2 Analytical Standards—A solution of 50 µg/ml [¹³C]-BDE-47 in nonane was used to make a stock internal standard (IS) solution of 500 ng/ml [¹³C]-BDE-47 in hexane. At the start of extraction 0.5ml of this stock solution, equaling 250 ng, was added to every tissue sample. Concentration of the IS stock solution was then measured during every GC/MS analysis to accurately measure percent recovery of BDE-47 from tissue samples. Concentration standards for [¹²C]-BDE-47 and [¹³C]-BDE-47 were made from stock solutions at concentrations of 0.064, 0.32, 1.6, 8.0, 40.0, 200.0 ng/ml and used to quantify tissue levels. All standards were stored in the dark at 4 °C.

2.2 Animals

Adult male and female C57Bl/6J mice used in this study were acquired from Jackson Laboratory at approximately 7 weeks of age and were not littermates (Jackson Laboratory – West, Sacramento, CA). Upon arrival, the general health of the animals was checked. Mice were maintained in an animal facility at the Center for Laboratory Animal Research (CLAS), University of California, Davis. Mice were housed on a 12 h light/12 h dark cycle, with the light cycle between 6 AM and 6 PM. Ambient temperature was maintained between 68 ±2 °F and humidity between 40 & 60 %. Mice were fed standard mouse chow (LabDiet, 5001 Rodent Diet, Purina, Farmington, MA).

2.2.1 Exposure—Following arrival at the animal facility, female mice were individually housed and allowed one-week acclimation before the start of BDE-47 exposure. During this week mice were fed 1–2 corn flakes (Kellogg's®, Battle Creek, MI) each day to familiarize them with the novel food that was used as a means for oral BDE-47 dosing. Once exposure began, female mice were weighed daily and then fed 1–2 corn flakes with BDE-47 (0.03, 0.1, or 1 mg/kg/day in corn oil) or corn oil alone (vehicle control). The daily BDE-47 dosage was determined individually for each mouse based on body weight. Mice readily ate BDE-47 dosed cornflakes and consumption was verified by direct observation. Daily dosing of dams began 4 weeks prior to breeding, and continued throughout gestation and lactation, ending on postnatal day (PND) 21. This dosing regimen resulted in approximately 70–80 days total of exposure to BDE-47, with the precise number of days dependent on how many days were required for successful mating as determined by presence of copulatory plug. This exposure procedure is identical to that used in our earlier study [63].

2.2.2 Breeding—After 4 weeks of exposure, the female mice were bred 1:1 with untreated male mice of approximately the same age. To ensure conception mating pairs were kept together for a total of 5 days even after presence of copulatory plug. Mating pairs were determined at random.

2.2.3 Litter Size and Neonatal Weight Gain—The size of litters used in both analysis of tissue levels and behavior ranged from 4 to 9 pups with a mean of 6.0 ± 0.3 pups per litter. There were no statistically significant differences in litter size between treatment groups, and the ratio of male:female pups within litters did not differ significantly between groups. The average weight for each litter was therefore used for statistical analysis. Pups weights were measured daily from PND 8 to 21 using an Ohaus balance (Ohaus Corp., Parsippany, NJ, USA), model FD6 (readability 0.001 kg).

2.2.4 Milk Collection—Milk was collected from dams on PND 10 and 21 following a protocol previously described by Depeters et al. [10]. Dams were removed from pups for approximately two hours prior to milking on the day of sacrifice. Dams were then injected intraperitoneally (i.p.) with 0.1 ml (2 IU) of oxytocin using a 27-gauge needle attached to a 1ml syringe. Milk was extracted using a vacuum system as described in Depeters et al. [10]. Approximately 100 μ l of milk was collected from each dam. After collection the milk was stored at -80°C until extraction. Mice used for collection of milk were not used in the behavioral studies.

2.2.5 Tissue collection—Four time points were chosen for tissue analysis of BDE-47: gestational day (GD) 15, PND 1, 10 and 21. For GD 15 the recorded date of the copulatory plug was used to determine the age of the embryo. The day of birth was designated PND 1. Both dams and pups were euthanized by an i.p. injection of 100 mg/kg Euthasol[®]. Samples of dam and pup blood were taken first, from the heart, and stored at -20°C . PND 1 pup blood was evaluated using whole blood from the entire litter rather than an individual pup due to the small amount of blood that could be collected at this age. Fat tissue samples from the dam abdomen and brain tissue samples from dams and pups were collected, flash frozen in liquid nitrogen and stored at -80°C until extraction. For analysis of dam brain tissue the right hemisphere (including cerebellum and brainstem) was used and for pups the whole brain was used. At PND 10 and 21, blood and brain were collected from one male and one female pup per litter for a comparison of levels between male and female pups.

2.3 Tissue Extraction

Tissue samples were weighed and transferred to test tubes containing 2 ml of HPLC grade water. The approximate wet weights of the tissue samples extracted were 0.5 g for dam blood, 0.3 g for dam brain, 0.1 g for milk, 0.1 g for fat, 0.3 g for whole fetal homogenate, 0.1 g for pup blood, and 0.3 g for pup brain. Samples were spiked with 500 μ l of the [¹³C]-BDE-47 internal standard and homogenized with a polytron (PT 1200, Kinematica AG, Switzerland) at maximal speed for 2 minutes. Samples were then left overnight at 4°C . The following day samples were frozen, lyophilized to a dry powder, and then stored at -20°C until extraction.

For sample extraction, 5 ml of hexane were added directly to the test tube containing the powdered tissue. The sample was then homogenized for 3 minutes using a polytron at maximal speed. Once the sample settled the hexane was decanted. This process was repeated two additional times. The decanted hexane was filtered through a solid phase extraction (SPE) cartridge pre-packed with 5 g of silica (Supelclean-LC-SI-SPE, Sigma-Aldrich) to remove lipid. Before the addition of sample, the SPE column was pre-conditioned with 10 ml of hexane. To elute any remaining sample off the column 20 ml of hexane was poured through after sample addition. The collected sample was then evaporated to dryness using a Sevant SpeedVac Concentrator (SVC-200H, Thermo Electron Corporation, Marietta, OH), re-suspended in 1ml of isooctane and transferred to a 2 ml crimp top amber vial using a 250 μ l deactivated insert (Agilent Technologies, Santa Clara, CA, USA). Samples were then stored at -20°C until analyzed by GC/MS.

2.4 Analytical Procedures

All the samples were analyzed using an Agilent GC/MS system with the following components: 5975B Inert XL EI/CI MSD, 7890A GC and 7683B autosampler/injector module (Agilent Technologies, Santa Clara, CA, USA). All the experiments were done in electron ionization (EI) mode, using selected ion monitoring acquisition method for quantification. For analysis 1 μ l of sample volume was injected in splitless mode with 0.5 min purge activation time. The injection port was held at 300 °C and the transfer line was held at 280 °C. For GC separation, a non-polar DB-XLB (#122-1232) column was used (J&W Scientific, Folsom, CA, USA) with the following physical characteristics: length 30 m, internal diameter 250 μ m, film thickness 0.25 μ m. Helium was used as the carrier gas (UHP 250, Airgas, Inc., Radnor, PA, USA) at a flow rate of 1ml/min with an average velocity of 37.293 cm/sec. The oven temperature program was as follows: 2 min at 100 °C; ramp-up at 20 °C/min to 325 °C; 12 min at 325 °C. Mass spectral parameters were as follows: ionization source was kept on 310 °C, quadrupole analyzer was set to 176 °C, electron energy at 70 eV, EM voltage at 1400 V and solvent delay of 5 minutes.

2.4.1 Calibration Curve—The peak area was used to determine the concentration of BDE-47 in samples. For the calibration curve, a linear first order polynomial was used, excluding the point of origin. For fit weighting, a 1/x function was used. For integration, the default parameters provided by the QuanLynx module were used, including automatic noise measurement. Peak smoothing was applied before integration using a 2 $\times$ 1 scan window and employing Savitsky-Golay smoothing method.

2.4.2 Quantification—Both [^{12}C]-BDE-47 and [^{13}C]-BDE-47 have identical retention times under the chromatographic conditions used for separation, thus the most abundant molecular ions were monitored for quantification: [^{12}C]-BDE-47, 485.85 Da ($\text{C}_{12}\text{H}_6^{79}\text{Br}_2^{81}\text{Br}_2\text{O}$); [^{13}C]-BDE-47, 497.85 Da ($^{13}\text{C}_{12}\text{H}_6^{79}\text{Br}_2^{81}\text{Br}_2\text{O}$). For data evaluation the ChemStation data files were transferred to AIA format (netCDF), then to MassLynx raw data format using “Databridge” software module (MassLynx v4.0, Waters, Manchester, UK). The quantification of all samples was done using MassLynx v4.0, standard QuanLynx module (Waters, Manchester, UK). The Limit of Detection (LOD) at which a particular peak can be reliably detected was set at a signal to noise ratio value greater than 3 ($s/n > 3$). Ratios below this level were considered to be below the limit of detection (LOD) and therefore undetected. The s/n values were determined automatically by the QuanLynx module.

2.5 Behavioral Testing

Behavioral tests are described in the order they were administered along with the approximate age of testing in parenthesis. All tests were conducted during the light phase of the light/dark cycle. One male and one female pup were randomly selected from each litter for behavioral testing, and all animals were administered all behavioral tests. Mice were taken from 9 litters for the Vehicle treated group, 11 litters for the 0.03, 12 litters for the 0.1 and 10 litters for the 1.0 mg/kg/day BDE-47 exposure groups.

2.5.1 Grip Strength (Week 5)—Neuromuscular function was assessed by measuring grip strength [45]. Briefly, mice were individually suspended by the tail and pulled along a strain gauge attached to a recording device (San Diego Instruments, San Diego, CA), and maximum resistance was recorded. Three trials each were conducted separately for fore- and hind- limb grip strength and then averaged for analysis.

2.5.2 Ladder Walk (Week 6)—Coordinated locomotor activity was examined using a ladder walk task modified from previous protocols [16,44] as previously described [25].

Mice were given a single two minute time period to traverse a series of horizontal parallel rods 1 mm in diameter and spaced 5 mm apart. The walls of the apparatus were constructed of clear Plexiglas, and were 10 cm high and 75 cm in length. Animals were videotaped from the side of the apparatus using an automated tracking system (Smart Tracking System, San Diego Instruments, San Diego, CA), which provided data on distance traveled and speed. The number of fore-limb and hind-limb foot slips through the rungs was manually scored from the video recorded sessions. The videos were viewed using a VideoLAN Client (VLC) video player (VideoLAN Organization) with the speed set to 40 %. A foot slip was defined as a slip of the paw greater than or equal to 5 mm below the parallel beams.

2.5.3 Barnes Maze (Week 8)—Spatial learning and memory were assessed using the Barnes spatial maze following a protocol adapted from Patil et al. [48]. Briefly a single bright light (Utilitech 500-Watt Halogen Portable Work Light, Lowe's Hardware) suspended directly above the surface of the maze was used as the aversive stimulus. The maze surface, 92 cm in diameter, was constructed of white Plexiglas with 20 circular holes 5 cm in diameter. An escape box (4 × 4 × 5 inches) was located directly below a designated target hole. Before training mice were adapted to the maze by placing them under a black Plexiglas start box in the center of the maze. After 10 seconds the box was lifted, the light was switched on and the mouse was guided to the escape hole, and allowed to enter the dark escape below where it remained for 2 minutes. During acquisition the mouse was placed under the black start box in the center of the maze for 10 seconds after which time it was lifted and light was switched on. The mouse was then given a total of 3 minutes to locate the escape hole and enter the dark escape box. Each trial ended when either the mouse found the escape box (nose poke over the hole opening) or after the 3 minute time period elapsed. If the mouse was unable to find the escape box during the 3 minute time period, it was guided there at the end of the trial. The mouse was given 1 minute in the escape box before being returned to its cage. Spatial cues were visible on the test walls surrounding the maze. The maze surface was thoroughly cleaned between trials using a diluted Nolvasan cleaning and disinfectant solution to remove olfactory cues.

Maze performance was monitored using an automated tracking system (Polytrack, SD Instruments, San Diego, CA) which measured locomotor speed, distance traveled in the maze, and latency to find the escape hole. Errors consisted of running to an incorrect escape hole (i.e., no escape box) and were scored manually from the video tapes. On the first day of testing there was 1 adaptation trial followed by 4 spatial acquisition trials. On days 2, 3, and 4 of training there were 4 spatial acquisition trials per day. On the last day of testing there was one probe trial where the escape box was removed and the mouse was given a 90 second time period to explore the maze. The number of head pokes over the hole that was originally above the escape box relative the number of head pokes over other holes was used as a measure of spatial memory. Changes in latency and distance to find the escape hole over training were used to measure learning. During training the inter-trial intervals were kept between 12–15 minutes.

2.5.4 Locomotor Activity (Weeks 9 & 17)—Locomotor activity was assessed at two separate time points for the same mice using the Integra apparatus (Accuscan Instruments, Columbus, OH). Mice were individually placed in the center of the automated apparatus (40 × 40 × 15 cm) and allowed to freely explore for one hour. Vertical and horizontal activities were analyzed as previously described [21,63].

2.5.5 Gait Analysis (Week 13)—Gait was assessed using TreadScan (CleverSys, Inc., Reston, VA.). Mice were individually placed into the TreadScan apparatus and allowed 1 minute to habituate. The TreadScan apparatus was then turned on and the speed slowly increased until it reached between 11.5 –12.5 rpm (with rotary dial set at 20 cm/sec). A 10

second continuous walking interval was then recorded. TreadScan version 3.0 software was used to evaluate a minimum of 3 uninterrupted strides (with stride defined as two steps by the same foot) similar to a protocol by Beare et al. [3]

2.6 Statistics

Data in figures represent mean $\pm$ standard error of the mean (SEM). Statistical analysis of behavioral data was carried out as previously described [63] using version 18 of SPSS (SPSS, Chicago, IL). Litter means were used for analysis of pup body weight from PND 8 to PND 21. The ratios of male:female pups in litters were analyzed by the Chi square test. For all other statistical analyses, data from no more than one male and one female mouse from each litter were used, with treatment and sex (and sex X treatment when appropriate) used as fixed effects variables and litter as a random effects variable. A mixed effects repeated measures ANOVA was used when appropriate. Individual *post hoc* group comparisons were made using the Tukey-Kramer test for multiple comparisons. Data were examined for individual outliers, and one outlier in the analysis of distance data in the Barnes maze data was identified ($Z=4.33$, $p<0.05$) and was not used in the data analysis. The minimum level set for statistical significant was $p = 0.05$.

3. Results

3.1 Reproductive Success

Maternal and fetal toxicity was evaluated in both the previous study by Ta et al. [87], and the current study using percent of successful pregnancies, dam weight during gestation and lactation, length of gestation, number of pups per litter, male:female pup ratio and animal lethargy. No signs of overt toxicity were observed across treatment groups in dams or pups over the perinatal period. Chi square analysis also indicated that the male:female pup ratio did not differ significantly across treatment groups.

3.2 Tissue Levels of BDE-47

3.2.1 Percent Recovery of [^{13}C]-BDE-47 from Tissue—Table 1 shows the percent recovery of [^{13}C]-BDE-47 internal standard (IS) from the different tissues evaluated in this study. The greatest recovery of [^{13}C]-BDE-47 was seen in dam fat and milk with average recoveries of 98 and 97 % respectively. Dam whole blood and pup whole blood had the lowest recoveries at 62 and 66 % respectively.

3.2.2 BDE-47 Tissue Levels in Dams—Table 2 shows levels of BDE-47 in blood, brain, and fat from the dams for the 0.03, 0.1 and 1 mg/kg/day and vehicle exposure groups. The time points evaluated in the dams were gestational day (GD) 15, PND 1, 10 and 21. BDE-47 levels in milk were also measured on PND 10 and 21. The developmental time points chosen for examination coincide approximately with high levels of neurogenesis in pup brain (i.e. GD 15) [17], birth of pups (i.e. PND 1), a period of rapid growth in pup brain (i.e. PND 10) [9] and weaning (i.e. PND 21). As shown in Table 2, levels of BDE-47 in dams generally increased for all dose groups from GD 15 to PND 1 and then declined from PND 1 to PND 21 in blood, brain and fat. Specifically, in the 1 mg/kg/day group there were significantly higher levels ($p = 0.05$) of BDE-47 in blood on GD 15 and PND 1, and in brain on GD 15, PND 1 and PND 10 when compared to tissue levels on PND 21. Fat levels of BDE-47 were also significantly higher in all three dose groups on GD 15, PND 1 and PND 10 when compared to levels on PND 21. As considered in the Discussion section, this pattern of results could be due to an initial accumulation of BDE-47 in tissue stores during gestation followed by a mobilization of BDE-47 from tissue stores to milk during lactation. Blood levels of BDE-47 for the 0.03 and 0.1 mg/kg/day groups were below the limit of detection (LOD) on GD 15 and PND 21, possibly due to efficient sequestration of BDE-47

from blood into more lipophilic tissue stores. Detectable levels of BDE-47 were found in tissues with the following relative accumulation: fat > milk > brain for all dose groups at all time points tested. Also, as shown in Table 2, there was a significantly higher concentration of BDE-47 in the 1 mg/kg/day group when compared to the two lower exposure doses ($p < 0.05$). Specifically, the 1 mg/kg/day BDE-47 group had significantly higher concentrations of BDE-47 when compared to the 0.03 and 0.1 mg/kg/day groups in blood on PND 1, and in brain on GD 15, PND 1. The level of BDE-47 in brain on PND 10 for the 1 mg/kg/day group was only significant higher than the 0.03 mg/kg/day group ($p < 0.05$). In fat tissues and milk the 1 mg/kg/day group was significantly higher than the two lower doses on all days of sample collection ($p < 0.05$).

3.2.3 BDE-47 Tissue Levels in Pups—Table 3 shows the levels of BDE-47 in pup tissues for the 0.03, 0.1 and 1 mg/kg/day and vehicle exposure groups. On GD 15 there are measurable levels of BDE-47 in whole fetal homogenate in all exposure groups, demonstrating direct exposure of the fetus to BDE-47 *in utero*. An initial analysis of levels of BDE-47 was done separately for both males and females on PND 10 and 21. However, there were no significant differences in levels of BDE-47 between sexes. Therefore average BDE-47 tissues levels across males and females are presented in Table 3. In both blood and brain of pups, levels of BDE-47 increased from PND 1 to PND 21 documenting continued exposure during lactation. As shown in Table 3 significantly higher levels of BDE-47 ($p = 0.05$) were seen on PND 10 in blood for the 0.03 and 0.1 mg/kg/day groups and in brain on PND 21 for the 0.1 and 1 mg/kg/day groups when compared to levels of BDE-47 in blood and brain on PND 1. Tissues levels in the 1 mg/kg/day group were again significantly higher ($p = 0.05$) than levels measured in tissues obtained from animals in the 0.03 and 0.1 mg/kg/day exposure groups. Specifically, blood levels in the 1 mg/kg/day group were significantly higher than the 0.03 mg/kg/day group on PND 10, and were significantly higher than both the 0.03 and 0.1 mg/kg/day groups on PND 21. In brain, levels of BDE-47 in the 1 mg/kg/day group were significantly higher than levels in brains collected from the 0.03 and 0.1 mg/kg/day groups on PND 10 and 21.

3.2.4 Litter size, sex ratio and pup weight gain—There were no significant differences in the number of pups in each litter ($F_{3,43} = 1.5$, $p = 0.23$), and no significant differences in male:female ratios in litters for the four treatment groups analyzed by Chi square analyses. There were no significant treatment effects on body weight across groups from PNDs 8 to 21 ($F_{3,38} = 0.92$, $p = 0.44$), no significant dose by day interaction ($F_{39,494} = 0.64$, $p = 0.96$), but there was a significant day effect reflecting increasing body weight for all groups from PND 8 through 21 ($F_{13,468} = 153.7$, $p < 0.0001$).

3.3 Behavioral Tests

The numbers of animals used in tests for grip strength, Barnes maze spatial learning, locomotor activity, and gait analysis were: 9 males and 7 females for vehicle, 10 males and 11 females for 0.03 mg/kg/day, 12 males and 11 females for 0.1 mg/kg/day, and 10 males and 10 females for 1 mg/kg/day. The numbers of animals used in the ladder walk test were 8 males and 8 females for the vehicle, and 9 males and 9 females for the 0.03, 0.1 and 1 mg/kg/day treatment groups.

3.3.1 Grip Strength—There were no significant differences among treatment groups in the average grip strength of the fore ($F_{3,82} = 0.52$, $p = 0.67$) and hind paws ($F_{3,82} = 0.97$, $p = 0.41$) using weight as a covariate. No statistically significant differences between sexes were found in this test.

3.3.2 Ladder Walk—There were no statistically significant differences among exposure groups in speed ($F_{3,72}=0.72$, $p=.35$), distance traveled ($F_{3,82}=0.35$, $p=0.79$) number of foot slips ($F_{3,72}=0.97$, $p=0.41$), or the ratio of the number of foot slips over distance traveled ($F_{3,72}=1.06$, $p=0.37$). No significant differences between sexes were found in this test.

3.3.3 Barnes Maze—Statistical analysis of latency to find the escape hole showed a significant effect of treatment ($F_{3,73} = 3.21$, $p<0.05$) and a significant treatment by training day interaction ($F_{9,219} = 4.01$, $p<0.001$). Further analysis on individual training days showed a significant effect of treatment on the first day of training ($F_{3,77} = 4.98$, $p<0.005$), but not on days 2–4 or in the Probe trial on day 5. Individual group comparisons on day 1 showed that the 0.03 ($p<0.01$), 0.1 ($p<0.05$), and 1 ($p<0.05$) mg/kg/day BDE-47 treated groups had longer escape latencies than the vehicle control group as shown in Figure 1A. There was also a significant treatment X day interaction for distance traveled to the escape hole in the Barnes maze ($F_{9,216} = 2.97$, $p<0.005$). Individual analysis on each day of training showed a significant treatment effect on day 1 ($F_{3,76} = 4.45$, $p<0.01$), and individual group comparisons on this day showed that the 0.03 ($p<0.01$) and 0.1 ($p<0.05$) BDE-47 treated groups traveled longer distances to the escape hole than the vehicle control group, as shown in Figure 1B. The difference between the 1 mg/kg/day and vehicle groups approached, but did not reach statistical significance ($p=0.07$). There were no significant differences among treatment groups in the other measures, including locomotor speed ($F_{3,80}=0.48$, $p=0.70$) and number of errors in finding the escape hole ($F_{3,80}=0.91$, $p=0.44$). There were no significant sex differences in Barnes maze performance.

3.3.4 Locomotor Activity—There were no statistically significant differences between exposure groups in any measure of locomotor activity when tested at 9 and 17 weeks of age. The data collected were evaluated across the entire hour of recording, as well as for each 20 minute segment making up the hour to evaluate possible changes in activity over time [15]. There were no statistically significant sex differences in this test.

3.3.5 Gait Analysis—There were no statistically significant differences between groups in any measure of gait pattern analyzed. In addition, no significant effects of treatment were seen when body weight and/or body length were used as covariates. There were no statistically significant sex differences in this test.

4. Discussion

4.1 Blood and Tissue Levels of BDE-47

Low-level perinatal exposure resulted in substantial accumulation of BDE-47 in dams and pup offspring for all tissues measured. As expected from previous work [60,63], accumulation of BDE-47 in the present study was highest in fat stores followed by milk and then brain, likely due to the relative lipid content in each respective tissue. Staskal et al. [59] reported differences in the elimination half-life of BDE-47 for different tissues after a single oral 1 mg/kg exposure in female mice. Fat was reported to have a longer elimination half-life than brain which may explain, at least in part, the very high levels of BDE-47 in fat in the present study [59]. Further, levels of BDE-47 were unexpectedly high in all tissues for the highest 1 mg/kg/day dose when compared to the 0.03 and 0.1 mg/kg/day doses. This could be due to a saturation of urinary elimination pathways by the highest dose, through binding of BDE-47 to MUP-1, the major isoform of mouse major urinary protein (MUP) that facilitates urinary excretion [60]. Staskal et al. [60] showed that 24 hours after a 1 mg/kg i.v. dose, 98.6 % of BDE-47 in urine was bound to MUPs, and by day five 40 % of the total dose had been eliminated in urine presumably through this pathway. Staskal et al. [59] also reported that higher relative doses of BDE-47 resulted in a decreasing percentage of

urinary elimination, a relationship not found in fecal elimination of BDE-47. Analysis of the urine showed that it was the parent compound being eliminated, essentially ruling out a major role for metabolism in explaining differences in elimination kinetics [58,59]. Dosing schedule may also be a factor contributing to the observed tissue accumulation of BDE-47. Specifically, a decrease in urinary elimination occurred after 10 days of repeated dosing to BDE-47 when compared to a single dose of 1 mg/kg of BDE-47 [58].

On PND 10 during lactation there was a marked decrease in levels of BDE-47 in dam tissues when compared to PND 1, and these levels continued to drop from PND 10 to 21. This pattern of loss of BDE-47 from dam tissues is similar to findings by Ta et al. [63] where a substantial decrease in levels of BDE-47 were seen in dams similarly dosed with 1 mg/kg/day group over the course for lactation. The largest decrease in BDE-47 however was from dam fat stores and likely reflects a mobilization of BDE-47 from fat stores into milk. This is supported by the observation that there were measurable levels of BDE-47 in milk that followed a dose-response pattern similar to that seen in fat. Although levels of BDE-47 in milk appeared to decrease slightly from PND 10 to 21, they still remained high compared to other tissues. As levels of BDE-47 decreased in dams from PND 1 to PND 21, there was an apparent increase of BDE-47 in pup tissues during the same period and by the end of lactation levels in pups exceeded levels reported for dams. This finding is also consistent with the previous reports that pre-weaning pups can achieve a higher accumulation of BDE-47 than adults when exposed to the same concentration of BDE-47 over the same time period [57,63].

BDE-47 was also present in whole fetal homogenate on GD 15 documenting the placental transfer of BDE-47 from dam to the fetus following chronic low-level exposure. Somewhat more limited fetal accumulation following a single dose of ^{14}C -BDE-47 to pregnant C57BL/6 dams was shown by Darnerud & Risberg using whole-body autoradiography [8].

At birth significant brain levels of BDE-47 were found in both male and female pups that averaged 7.7, 13.5, and 132.6 ng/g ww for the 0.03, 0.1 and 1 mg/kg/day groups, respectively. Again, these levels increased during lactation so that levels on PND 21 were 10.9, 33.6, and 327.1 ng/g ww for the 0.03, 0.1 and 1 mg/kg/day groups respectively. During lactation (i.e., PNDs 10 and 21) when brain levels were decreasing in dams, brain levels of BDE-47 in pups brains increased, and matched or exceeded levels in dams for the same tissue and dose. This inverse relationship is similar to the findings of the initial study by Ta et al. [87] and is likely due to mobilization of BDE-47 into milk, resulting in the transfer of high levels to pups through lactation. Mobilization of lipophilic compounds to milk stores is a well-established phenomenon [43], and has been described for several compounds including PCBs which share structural and chemical similarities to PBDEs (halogenated aromatic compounds that are lipophilic in nature) [41].

Differences in levels of BDE-47 reported in this study when compared to the previous study by Ta et al. [87] could be the result of differences in the extraction methods used. In the Ta et al. study a soxhlet extractor was used with a 3:1 hexane to acetone solvent ratio, while in the current study extraction was carried out through homogenization of tissue in hexane. Furthermore, in the study by Ta et al. [87] the data on levels of BDE-47 in dams and pup tissue was more variable than in the current study. When this variability is considered the tissue levels measured in the present study would still fall within the ranges reported by Ta et al. [87]. Additional factors that could have contributed to the differences between the two studies in BDE-47 tissue levels include differences in dam exposure duration before successful mating, litter size and tissue sampling. However, the exposure and mating procedures were identical in the two studies, litter sizes in the present study (6.0 ± 0.3) and

the previous study (6.6 ± 0.4) differed but not markedly so, and both studies analyzed whole brain homogenates and took fat from the same body depot.

Tissue levels of BDE-47 were similar in both male and female pups on PND 10 and 21. This was somewhat unexpected because the rate of elimination has been reported to be greater in male than female mice [53,58]. However, earlier studies were carried out in adult mice, and adult males have 3 times the level of MUP proteins circulating in their blood compared to females. Gonadotropic hormones (e.g., testosterone) are thought to promote higher levels of MUP proteins in adult males, so sex differences might not be expected until puberty (approximately PND 28) [7,42,74]. This hypothesis is also supported by a report that MUP proteins are equivalent between sexes at 1 month of age, but by 4 months males have significantly higher levels than females [51].

A goal of this study was to achieve BDE-47 tissue levels with a chronic low-dose perinatal dosing protocol in mice that resulted in tissue levels within the range reported in humans. Published data for levels of BDE-47 reported in human samples have ranged from 0.25 – 46 ng/g lipid weight (lw) in blood [18], 0.84 – 1100 ng/g lw in milk [29], and 1.3 – 2720 ng/g lw in fat [30]. The range of levels found in our study appears to be similar at 5.34 – 28.06 ng/g wet weight (ww) in blood, 15.75 – 745.39 ng/g ww in milk, and 146.8 – 32747.4 ng/g ww in fat. Because our concentrations were measured in wet tissue weight rather than lipid weight, the tissue levels found in the present study would tend to be more conservative (i.e., underestimate) than those reported for humans when corrected for lipid weight. Regardless, the 0.03 and 0.1 mg/kg/day doses used in the present study would still result in blood and milk levels that fall within the range reported in humans. However, the level of BDE-47 in fat from this study is substantially higher than that reported in humans, particularly for the 1 mg/kg/day dose. It should be noted that there is only one published study in the US that reported levels of BDE-47 in human fat and data from this study, therefore, may not accurately represent the degree of accumulation in human fat tissues [30]. Furthermore, metabolism of BDE-47 is higher in humans [39,76] when compared to mice [58,59] and this may explain in part why levels of the parent compound, BDE-47, are substantially higher in mouse tissue samples, and in particular fat, when compared to human data. However additional studies measuring BDE-47 levels in human fat should be carried out because while BDE-47 may be less biologically active when sequestered in fat, it appears to be readily mobilized into milk which may put the developing infant at risk [49].

Considered together, the tissue data from the present study documents substantial transfer of BDE-47 from the dam to the fetus, and that increasing levels found in brain and other tissues from nursing pups, points to a previously unappreciated risk of developmental BDE-47 exposure through lactation. The present results with chronic low-level exposure suggest that epidemiological studies evaluating a single developmental time point of exposure for the mother and developing child may not accurately reflect exposure risk and its relationship to later developmental outcomes. This is supported by work by Lunder et al. [36] which found that levels of PBDEs differed significantly between children and mothers during early development even when the same environment and diet were shared. Lunder et al. suggested that the differences in exposure levels are likely due to exposure from breast milk and hand to mouth activity. It will be important for future studies to evaluate tissue accumulation and neurobehavioral outcomes at several time points during perinatal exposure to BDE-47, and to relate the results of such studies to specific physiological systems known to be affected by BDE-47, including thyroid function and immune system development.

4.2 Behavioral Analysis

BDE-47 exposed pups showed a significant increase in latency and a significant increase in the distance traveled to find the escape hole in the Barnes spatial maze, but only on the first

day of training. These results suggest a possible learning impairment associated with perinatal BDE-47 exposure. However, the observation that performance was only affected on day one of training could also indicate impairment in other cognitive processes, including sensorimotor function, could underlie the behavioral deficits. This is supported by studies showing that NMDA receptor antagonists can cause behavioral disturbances in the Morris water maze, a spatial learning task similar to the Barnes maze [2,5], and the report that early postnatal exposure to BDE-47 can alter the subunit composition of the NMDA receptor [11]. In a previous study by our laboratory, using the same BDE-47 dosing strategy, mice exposed perinatally to 1 mg/kg/day BDE-47 swam more slowly in the Morris water maze, and also swam a shorter distance to find the hidden platform, suggesting effects on both motor activity and ability to navigate the maze [63].

The current study was designed to build on our previous data showing behavioral impairment in the Morris water maze, and included new tests of spatial learning and locomotor activity using the same perinatal BDE-47 exposure procedures. Specifically, the Barnes spatial maze was used to evaluate spatial learning, and it was used because it does not require swimming and avoids associated stress produced by water immersion. Similar to our earlier findings in the MWM [40], BDE-47 exposed mice showed altered performance in the Barnes spatial maze. However, while performance in the Barnes maze was impaired in all BDE-47 exposure groups in the present study (i.e., longer escape latency and longer distance traveled), performance of exposed mice in the MWM in our earlier study appeared to be better than controls (i.e., shorter swim distance to locate the submerged escape platform). The reason for opposite effects in the two studies is unclear, but one explanation could be related to stress associated with swimming in the MWM [13]. Suvorov et al. [62] found that exposure of rat dams to 0.2 mg/kg BDE-47 every 5th day from GD 15 to PND 20 decreased levels of circulating corticosterone (CS), caused adrenal atrophy, impaired adrenal zonation, and reduced expression of steroidogenic enzymes in offspring. Decreased levels of CS have been found to improve cognitive performance in a variety of tests (e.g., Morris water maze) as well as decrease reactivity to external stressors [37,38]. Therefore, it is possible that perinatal exposure to BDE-47 could lower CS levels, resulting in reduced stress in the MWM and better performance, while altered levels of CS in the less stressful Barnes maze might impair performance through decreased motivation. Stress (e.g., restraint stress) has been shown to have biphasic effects, either improving or impairing spatial memory depending on the duration of stress and corticosterone levels [35]. Additionally there is research suggesting that both sensorimotor as well as a lack in subordinate skills could account for the differences seen in performance learning and memory tasks [3,75,76], and thus could explain the differing results in both the MWM from the previous study and Barnes maze from the current study. To better assess the differences in effects between possible learning and memory deficits and stress, additional tests such as reversal phase MWM should be administered in future studies.

The ladder rung task was used to assess visuomotor performance and gait was analyzed using an automated Treadscan apparatus. However, no significant effects on motor performance were seen in either test. Furthermore, when we evaluated locomotor activity in the open field at the same age and following a similar protocol as Eriksson et al. [15] no effects of BDE-47 exposure were seen. Finally, neuromuscular strength did not appear to be affected in the grip strength test. These data suggest that the motor effects of BDE-47 exposure are subtle and not evident in all tests of motor function, and/or that chronic low-level perinatal dosing with BDE-47 may not affect motor performance to the same extent as a single high-dose exposure during neonatal development in mice as was reported earlier [32]. Of course, other factors that differ between studies could also explain the differing results, including mouse strain differences, BDE-47 exposure levels and timing of exposure.

In the studies by Eriksson et al. male NMRI mice were used, and previous work indicates that this strain of mice shows increased cognitive impairment and decreased in spontaneous activity and exploration with increasing age [40]. Therefore the results from the Eriksson et al. study which demonstrated a lack of habituation to a novel environment following an acute exposure to BDE-47 could be due in part to their choice of mouse strain and age of testing. Although the Gee and Moser reported increased rearing in mice exposed to 1 mg/kg BDE-47 using the same strain of mice as our study (i.e., C57BL/6), differences in behavior could be attributed to the use of a single acute dose of BDE-47 on PND 10 [20], a dosing paradigm very similar to the Eriksson et al. study which also showed similar behavioral outcomes. As discussed previously a single acute exposure to BDE-47 has different elimination kinetics than a low chronic exposure leading to possible differences in body distribution after exposure and in behavioral outcomes. Considered together, reported effects of perinatal BDE-47 exposure on locomotor activity have been inconsistent, with both hypo-, hyperactivity or no effect reported [15,20,61,63]. In view of human studies suggesting that prenatal exposure to PBDEs are associated with psychomotor deficits and poor fine manipulative abilities, possible motor effects of exposure to PBDEs may be more complex than previous appreciated [24,52].

4.3 Effects of Human Exposure to PBDEs

The results of the present study did find altered performance in a spatial learning task (i.e., Barnes maze), underscoring the fact that chronic, low-level perinatal exposure to BDE-47 can have deleterious effects on behavior. This conclusion is consistent with a growing body of evidence that perinatal exposure to PBDEs, including BDE-47, represents a significant risk for altered neurodevelopment in children [24,52]. Shy et al. [56] found that prenatal exposure to PBDEs in umbilical cord blood altered both cognition and adaptive behavior in infants 8–12 months of old. Roze et al. [52] reported that higher levels of PBDEs in serum of pregnant mothers during the 35th week of pregnancy correlated with worse fine manipulative abilities and poorer attention, but also better coordination, visual perception and general behavior in children at 5–6 years of age. In a study by Herbstman et al. [24] higher levels of PBDEs in umbilical cord serum at birth correlated with lower scores on tests of mental and physical development at 1–4 and 6 years of age. Specifically, using the Bayley Scales of Infant Development, Herbstman et al. found that higher levels of BDE-47 were significantly associated with lower scores on the Psychomotor Developmental Index (PDI) at 12 months of age and the Mental Development Index (MDI) at 24 months of age. At 48 months of age a decrease in both full and verbal IQ scores was also found to be associated with increased BDE-47 levels. Additionally Herbstman et al. determined that children who were in the highest 20% of cord blood concentrations for BDE-47, -99, or -100 had significantly lower developmental scores compared to children who were in the lower 80 % of the exposure distribution. Finally a more recent study by Gascon et al. [27] found that postnatal exposure to BDE-47, measured in the serum at age 4, was significantly related to an increase in risk of symptoms on the attention deficit subscale of ADHD as well as a significant higher risk of poor social competence. Together these studies demonstrate changes in motor function, behavior, and learning through early developmental exposure to PBDEs, including BDE-47, and highlight the concern for human development from exposure to these compounds.

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Highlights

- Chronic low-level perinatal exposure to BDE-47 in C57BL/6J mice impairs spatial learning and memory in the Barnes maze.
- Levels of BDE-47 in milk and blood of dams were found to be within the range of reported human exposure levels.
- Tissue levels dropped dramatically in lactating dams, while high levels were maintained in milk through most of the lactation period.
- Substantial transfer of BDE-47 to the offspring during lactation was found, highlighting the exposure risk to the newborn through lactation.

Figure 1. Latency (A) and Distance (B) to find the escape hole in the Barnes Maze. Mice in the 0.03, 0.1 and 1 mg/kg/day BDE-47 treatment groups had significantly longer latencies and traveled a longer distance to escape when compared to vehicle controls on the first day of training. No significant effects were seen on days 2–4 of training or the Probe Trial on day 5. Not shown in figure: No significant differences between groups in the number of visits to the escape hole were found during the Probe Trial. Group means $\pm$ SEM were 5.71 ± 0.59 , 4.50 ± 0.41 , 5.61 ± 0.51 , and 4.95 ± 0.43 for the vehicle, 0.03, 0.1 and 1 mg/kg/day groups, respectively. * $p < 0.05$ and # $p < 0.01$ versus vehicle control group.

Table 1Percent Recovery of [^{13}C]-BDE-47 from Different Tissues

Sample Type	n _a	Average	Range
Dam Blood	66	62%	25% – 83%
Dam Brain	69	87%	63% – 106%
Dam Fat	71	98%	79% – 118%
Dam Milk	30	97%	84% – 106%
Fetus	17	77%	69% – 85%
Pup Blood	69	66%	47% – 82%
Pup Brain	82	76%	37% – 98%

a. Number of animals for each group (n)

Table 2

Levels of BDE-47 in Tissues of Exposed Dams on E15, PND1, 10 & 21

Sample Type	Treatment (mg/kg/day)	ED15 (n _α)	PND1 (n)	PND10 (n)	PND21 (n)
Blood (ng/g)	0.03	<LOD _β <LOD 24.2 ± 3.0 (6)	5.3 ± 1.5 (4)	6.7 ± 0.74 (3)	<LOD
	0.1		8.5 ± 2.6 (4) 28.0 ± 2.7 (5)	6.9 ± 3.5 (3) 7.5 ± 1.8 (5)	<LOD 6.5 ± 1.2 (5)
Brain (ng/g)	0.03	12.1 ± 0.5 (4) 13.3 ± 2.3 (4) 147 ± 32.4 (5)	9.9 ± 1.5 (5)	4.1 ± 1.0 (3)	<LOD
	0.1		25.1 ± 2.9 (5) 243 ± 30.5 (5)	14.3 ± 2.8 (3) 51.7 ± 13.8 (4)	6.4 ± 0.54 (4) 24.8 ± 5.1 (5)
Fat (ng/g)	0.03	438 ± 30 (4) 1920 ± 359 (4) 18293 ± 1621 (6)	804 ± 131 (5)	462 ± 143 (6)	146 ± 20.3 (5)
	0.1		2424 ± 723 (6) 32747 ± 2442 (5)	1465 ± 391 (4) 10651 ± 120 (3)	465 ± 193 (4) 4907 ± 1353 (5)
Milk (ng/g)	0.03	μ ^a - -	-	36.4 ± 8.1 (3)	15.7 ± 4.8 (3)
	0.1		-	146 ± 51.9 (3) 745 ± 156 (4)	59.7 ± 13.7 (4) 726 ± 91.4 (5)

^α Number of animals for each group (n),^β Below Limit of Detection (<LOD),^μ Tissue not collected at this time point (-)

Table 3

Levels of BDE-47 in Tissues of Exposed Pups on E15, PND1, 10 & 21

Sample Type	Treatment (mg/kg/day)	E15 (n _α)	PND1 (n)	PND10 (n)	PND21 (n)
Fetus (ng/g)	0.03	6.2 ± 3.2 (5)	μ	-	-
	0.1	10.4 ± 1.1 (3)	-	-	-
Blood (ng/g)	0.03	50.4 ± 16.6 (5)	<LOD _β	<LOD	10.3 ± 1.5 (7)
	0.1	-	9.3 ± 1.8 (3)	<LOD	16.78 ± 1.5 (6)
Brain (ng/g)	0.03	-	45.4 ± 9.1 (3)	54.5 ± 6.0 (8)	77.1 ± 9.8 (9)
	0.1	-	7.7 ± 2.51 (3)	6.8 ± 1.4 (8)	11.7 ± 2.7 (9)
	0.1	-	13.5 ± 1.9 (5)	22.2 ± 5.4 (8)	33.1 ± 7.4 (8)
	1	-	132.6 ± 26.3 (3)	131.2 ± 17.1 (8)	330.3 ± 27.7 (9)

α. Number of animals for each group (n),

μ Tissue not collected at this time point (-),

β Below Limit of Detection (<LOD)