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Neonatal exposure to polybrominated diphenyl ether (PBDE 153) disrupts spontaneous behaviour, impairs learning and memory, and decreases hippocampal cholinergic receptors in adult mice

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Abstract

Neonatal exposure to polybrominated diphenyl ether (PBDE 153) disrupts spontaneous behaviour, impairs learning and memory, and decreases hippocampal cholinergic receptors in adult mice. Flame retardants are used to suppress or inhibit combustion processes in an effort to reduce the risk of fire. One class of flame retardants, polybrominated diphenyl ethers (PBDEs), are present and increasing in the environment and in human milk. The present study shows that neonatal exposure to 2,2′,4,4′,5,5′-hexaBDE (PBDE 153), a PBDE persistent both in environment and in human milk, can induce developmental neurotoxic effects, such as changes in spontaneous behaviour (hyperactivity), impairments in learning and memory, and reduced amounts of nicotinic receptors, effects that get worse with age. Neonatal NMRI male mice were orally exposed on day 10 to 0.45, 0.9, or 9.0 mg of PBDE 153/kg of body weight. Spontaneous behaviour (locomotion, rearing, and total activity) was observed in 2−, 4−, and 6-month-old mice, Morris water maze at an age of 6 months. The behaviour tests showed that the effects were dose-response and time-response related. Animals showing defects in learning and memory also showed significantly reduced amounts of nicotinic receptors in hippocampus, using α-bungarotoxin binding assay. The observed developmental neurotoxic effects seen for PBDE 153 are similar to those seen for PBDE 99 and for certain PCBs. Furthermore, PBDEs appear to as potent as the PCBs.

Keywords: Spontaneous behaviour; Learning and memory; Cholinergic system; Neonatal; Flame retardants; Polybrominated diphenyl ethers

Introduction

Brominated flame retardants are a diverse group of industrial compounds used to suppress or inhibit combustion processes in an effort to reduce the risk of fire, mainly in polymer products. Polybrominated diphenyl ethers (PBDEs), which constitute one group of chemical substances used as additive flame retardants, have the chemical formula $C_{12}H_{(n-1)}Br_{(n)}O$ ($n \leq 10$), the theoretical number of possible congeners 209, and characteristics very similar to those of the polychlorinated biphenyls (PCBs) (IPCS, 1993, 1994). Products that contain flame retardants are electrical appliances such as computers, television sets, textiles, and building materials (IPCS, 1994) that contribute to a significant worldwide industrial sector. PBDEs are not bound in polymer products and can thus leak into the environment (Hutzinger et al., 1976; Huttzinger and Thoma, 1987). Studies have shown that PBDEs are present in the global environment, such as sediment and fish (de Boer et al., 1998; Johnson and Olson, 2001; Manchester-Neesvig et al., 2001; Moisey et al., 2001; Strandberg et al., 2001) and that levels of PBDEs are increasing in the Swedish environment (Andersson and Blomkvist, 1981; Nylund et al., 1992; Sellstro¨m et al., 1993). A recent report has shown the presence of PBDEs in Swedish human milk and also that the PBDEs have increased exponentially since 1972, whereas PCBs are steadily decreasing (Meironyte´ and Nore´n, 1999; Nore´n and Meironyte´, 2000). The sum of PBDEs found in Swedish human milk increased from 0.07 ng/g of lipids, 1972, to 4.02 ng/g of lipids, 1997. The most abundant

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PBDE congeners in Swedish human milk, 1997, were 2,2′,4,4′-tetraBDE (PBDE 47, 2.28 ng/g of lipids), 2,2′,4,4′,5-pentaBDE (PBDE 99, 0.48 ng/g of lipids), 2,2′,4,4′,5,5′-hexaBDE (PBDE 153, 0.46 ng/g of lipids), and 2,2′,4,4′,6-pentaBDE (PBDE 100, 0.42 ng/g of lipids) (Meironyte´ and Nore´n, 1999). In human blood plasma the total PBDE concentration was 2.1 ng/g of lipids (Klasson-Wehler et al., 1997). Occupational exposure to PBDEs has been demonstrated and in blood serum the total concentration was 37 pmol/g of lipids and PBDE 153 made up 7.0 pmol/g of lipids of the total concentration (Sjo¨din et al., 1999). This indicates that humans are exposed to PBDEs both as infants and as adults.

During their development, mammals can be exposed to toxicants either as fetuses via maternal intake of toxicants, during the newborn period via intake of human milk, or by direct ingestion or contact with toxicants. We have earlier reported developmental neurotoxic effects after exposure to environmental agents, such as DDT and certain PCBs, and agents affecting the cholinergic system, nicotine, organophosphates (Eriksson, 1997, 1998; Eriksson et al., 1992, 2001a). In recent studies we have shown that neonatal exposure to PBDE congeners 2,2′,4,4′-tetraBDE (PBDE 47) and 2,2′,4,4′,5-pentaBDE (PBDE 99) (Eriksson et al., 2001b, 2002) can induce persistent dysfunction in adult mice, manifested as deranged spontaneous behaviour, e.g., hyperactivity, and altered behavioural response to the cholinergic agent nicotine (Eriksson et al., 2001b; Viberg et al., 2002). We have also shown that the effects are inducible during a restricted period of neonatal life and that the behavioural effect gets worse with age (Eriksson et al., 2001b, 2002).

These disturbances in behavior and cholinergic transmitter systems have all been shown to be induced during the rapid growth of the neonatal mouse brain. This period, the brain growth spurt (BGS) (Davison and Dobbing, 1968), is characterized by a series of rapid fundamental developmental changes, for example, maturation of dendritic and axonal outgrowth, the establishment of neural connections, and synaptogenesis and proliferation of glia cells with accompanying myelinization (Davison and Dobbing, 1968; Kolb and Whishaw, 1989). This is also the period when animals acquire many new motor and sensory abilities (Bolles and Woods, 1964) and when spontaneous motor behaviour peaks (Campbell et al., 1969). The cholinergic transmitter system undergoes rapid development during BGS (Coyle and Yamamura, 1976; Fiedler et al., 1987) and the numbers of receptors (nicotinic and muscarinic) increase in hippocampus and cerebral cortex (Fiedler et al., 1987; Falkeborn et al., 1993; Kuhar et al., 1980). Many behavioural characteristics (Karzmar, 1975) and cognitive functions (Bartus et al., 1982; Drachman, 1977) are closely linked to the cholinergic transmitter system. In mammals, the period of BGS in terms of onset and duration varies from species to species. In the human, it begins during the third trimester of pregnancy and continues throughout the first 2 years of life, whereas in the guinea pig it takes place in utero. In rodents, however, the BGS is neonatal, spanning the first 3–4 weeks of life and reaching its peak around postnatal day 10.

Can environmental agents contribute to an earlier onset of aging and/or neurodegenerative processes? PBDE 153 is a brominated flame retardant that has the same substitution of halogen as PCB 153 (2,2′,4,4′,5,5′-hexaCB), which is considered one of the most persistent and widespread PCB congeners and has been shown to induce irreversible brain dysfunctions in the adult mouse, after neonatal exposure (Eriksson, 1998). Against the background of our earlier observations of developmental neurotoxic effects and effects that get worse with age, caused by certain PCBs and PBDEs, as well as the similarities between the PBDEs and PCBs in regard to chemical and physical properties, geographical distribution pattern, and occurrence in mothers' milk, the present study was undertaken to ascertain whether 2,2′,4,4′,5,5′-hexaBDE has the potency to evoke effects on the spontaneous behaviour and affect learning and memory in the adult mouse, after neonatal exposure during the BGS. A further objective was to investigate whether neonatal exposure to 2,2′,4,4′,5,5′-hexaBDE can affect the cholinergic system, especially nicotinic receptors, in the adult mouse brain.

Materials and methods

Chemicals and animals

To conduct the study, 2,2′,4,4′,5,5′-hexaBDE (analysis showed 92.5% 2,2′,4,4′,5,5′-hexaBDE and 7.5% 2,2′,3,4,4′,5′,6-heptaBDE) was obtained from Eva Jakobsson at Wallenberg Laboratory, Stockholm. First, it was dissolved in a mixture of egg lecithin (Merck, Darmstadt, Germany) and peanut oil (Oleum arachidis) (1:10) and then sonicated with water to yield a 20% (wt:wt) fat emulsion vehicle containing 0.045, 0.09, or 0.9 mg 2,2′,4,4′,5,5′-hexaBDE/ml (0.07, 0.14, or 1.4 μmol/ml, respectively). The use of a 20% fat emulsion vehicle was to give a more physiologically appropriate absorption and hence distribution (Keller and Yeary, 1980; Palin et al., 1982), since fat content of mouse milk is around 14%.

Pregnant NMRI mice were purchased from B&K, Sollentuna, Sweden, and were housed individually in plastic cages in a room with an ambient temperature of 22°C and a 12/12-h cycle of light and dark. The animals were supplied with standardized pellet food (Lactamin, Stockholm, Sweden) and tap water ad libitum. The size of the litters was adjusted to 10–12 mice, within the first 48 h after birth, by the killing of excess pups. The litters contained pups of both sexes and at the age of 4–5 weeks, all females were euthanized and the males were placed in groups of 4–7, in a room for male mice only, and raised under the same conditions as detailed above. Only male mice were used in order to

compare with our earlier developmental neurotoxicological studies on PBDEs, PCBs, and other known neurotoxic substances (Eriksson, 1997).

Mice, at the age of 10 days, were given 0.45, 0.9, or 9.0 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight (0.7, 1.4, or 14 μmol 2,2′,4,4′,5,5′-hexaBDE/kg body weight) via a metal gastric tube, as one single oral dose. Control mice received 10 ml of the 20% fat emulsion vehicle per kilogram of body weight. Each of the different dosage categories contained three to five litters.

Behaviour tests

Spontaneous behaviour test

Spontaneous behaviour was tested in the male mice at the age of 2, 4, and 6 months, as described by Eriksson et al. (1992, 2001b). The experimenter was blinded to the different treatments of the mice. The animals were tested between 8 a.m. and 12 p.m. under the same ambient light and temperature conditions as their housing conditions. A total of 10 mice were randomly picked from the three to five different litters in each treatment group, at each testing occasion. Motor activity was measured for a 60-min period, divided into 3 × 20-min spells, in an automated device consisting of cages (40 × 25 × 15 cm) placed within two series of infrared beams (low and high level) (Rat-O-Matic, ADEA Elektronik AB, Uppsala, Sweden) (Fredriksson, 1994).

Locomotion. Counting took place when the mouse moved horizontally through the low-level grid of infrared beams.

Rearing. Movement in the vertical plane was registered at a rate of 4 counts per second, when a single high level beam was interrupted, i.e., the number of counts obtained was proportional to time spent rearing.

Total activity. All types of vibration within the cage, i.e., those caused by mouse movements, shaking (tremors), and grooming, were registered by a pick-up (mounted on a lever with a counterweight), connected to the test cage.

Swim maze

At an age of 6 months, the mice were observed for performance in a swim maze. Nineteen to 24 mice, from three to five litters and from each treatment group, were tested.

The swim maze, of the Morris water maze type (Morris, 1981), was a circular, gray tub, diameter 102 cm, depth 35 cm, filled with water at 22°C to a depth of 15 cm from the brim. In the center of the northeast quadrant of the tub, a platform was submerged 1 cm beneath the water surface. This platform, consisting of metal mesh, was 12 cm in diameter. The relative positions of pool and observer were the same every day. The mouse's ability to locate the submerged platform was observed on five consecutive days and the animals were given five trials each day. Before the first trial each day, the mouse was placed on the submerged platform for 30 s. It was then released in the south position facing the wall of the tub and was allowed 30 s to locate the platform. If the mouse failed to find the platform within 30 s, it was gently placed on the platform. After each trial, the mouse was left on the platform for 30 s. This procedure was repeated five times a day on four consecutive days. On the fifth day, the platform was moved to the northwest quadrant of the tub for reversal trials; otherwise, the procedure was identical. Latencies to reach the platform were measured by the observer and the total search time of five trials was set at 150 s. The first 20 trials (days 1–4) measured the mouse's spatial learning ability, and the last five trials (day 5) its relearning ability. Spatial learning tasks, being dependent on external cues for their solution, have been found to be sensitive to central cholinergic dysfunction (Riekkinen et al., 1990; Sutherland et al., 1982; Whishaw, 1985).

Cholinergic receptor assay

One week after completion of the behavioural tests, the mice were killed by decapitation. The brains were dissected on an ice-cold glass plate and the hippocampus was frozen at −80°C until assayed. Hippocampi were pooled from two animals and placed in ice-cold sucrose buffer (0.32 M) 24 times their own weight and thereafter homogenized with a Potter-Elvehjem homogenizer.

The homogenate was centrifuged for 10 min at 1000 *g* and the supernatant was further centrifuged for 30 min at 17,000 *g.* The remaining pellet was suspended and homogenized in the original volume of ice-cold $NaKPO_4$ buffer (0.05 M, pH 7.4) to yield a crude synaptosomal P2 fraction (Gray and Whittaker, 1962) with a protein content of about 1–2 mg, determined with fluorescamine according to Udenfriend et al. (1972).

Measurement of nicotine-binding sites was performed by using tritium-labeled α-bungarotoxin, specific activity 1.54 TBq/mmol, obtained from Amersham, Bucks, England.

[^{3}H]-α-Bungarotoxin binding

The specific binding was carried out following the method of Falkeborn et al. (1983), with certain changes. α-Bungarotoxin was used instead of tubocurarin for determination of nonspecific binding.

Aliquots of the P2 fraction (50 μl, protein content 0.1–0.2 mg) were incubated with 20 μl of [^{3}H]-α-bungarotoxin, *N*-[propionyl-^{3}H]-propionylated (41.0 Ci/mmol, 20 nM in 0.1% bovine serum albumin) for 120 min at 25°C in $NaKPO_4$ buffer (pH 7.4) in a total volume of 200 μl. To measure nonspecific binding, parallel samples were incubated with α-bungarotoxin (20 μl, 5 μM). Each binding was determined in triplicate. Incubation was terminated by centrifugation for 5 min at 20,000 *g.* The remaining pellet was washed with 500 μl of ice-cold $NaKPO_4$ buffer and the

pellet was placed in mini-scintillation vials. Five milliliters of Aquasafe 300+ scintillation liquid (Zinsser Analytic, Ltd., U.K.) was added to each vial and the radioactivity was determined in a scintillation analyzer (Packard Tri-Carb 1900 CA) after the samples had been kept in the dark for 8 h.

Specific binding was determined by calculating the difference in the amount bound in the presence vs. absence of α-bungarotoxin. The counting efficiency was about 50%, and the quench correction was made by using the external standard method.

Statistical analysis

Spontaneous behaviour. The data were subjected to a split-plot ANOVA (analysis of variance), and pairwise testing between treated groups and the control group was performed using a Tukey HSD (honestly significant difference) test (Kirk, 1968).

Habituation capability. From the spontaneous behaviour test, a ratio was calculated between the performance period 40–60 min and 0–20 min for the three different variables locomotion, rearing, and total activity. The following equation was used: 100 × (counts locomotion 40–60 min/counts locomotion 0–20 min), 100 × (counts rearing 40–60 min/counts rearing 0–20 min), and 100 × (counts total activity 40–60 min/counts total activity 0–20 min). This ratio was used to analyze alteration in habituation between 2-, 4-, and 6-month-old mice. These data were subjected to a split-plot ANOVA.

Swim maze. The data from days 1 to 4 of the test were subjected to general linear model with a split-plot design and pairwise testing using Duncan's test. Comparison between the performance of the last trial on day 4 vs. the first trial on day 5 was submitted to a paired *t* test. The statistical analysis of the behavioural data of day 5 (difference between trial 1 and 5) was submitted to one-way ANOVA and pairwise testing using Duncan's test.

[^{3}H]-α-Bungarotoxin binding. The data from [^{3}H]-α-bungarotoxin binding were subjected to one-way ANOVA and pairwise testing using Duncan's test.

Results

There were no clinical signs of toxicity in the 2,2′,4,4′,5,5′-hexaBDE-treated mice at any given time during the experimental period, nor was there any significant difference in body weight gain or adult weight between the 2,2′,4,4′,5,5′-hexaBDE-treated and the vehicle-treated mice in the three different dosage categories.

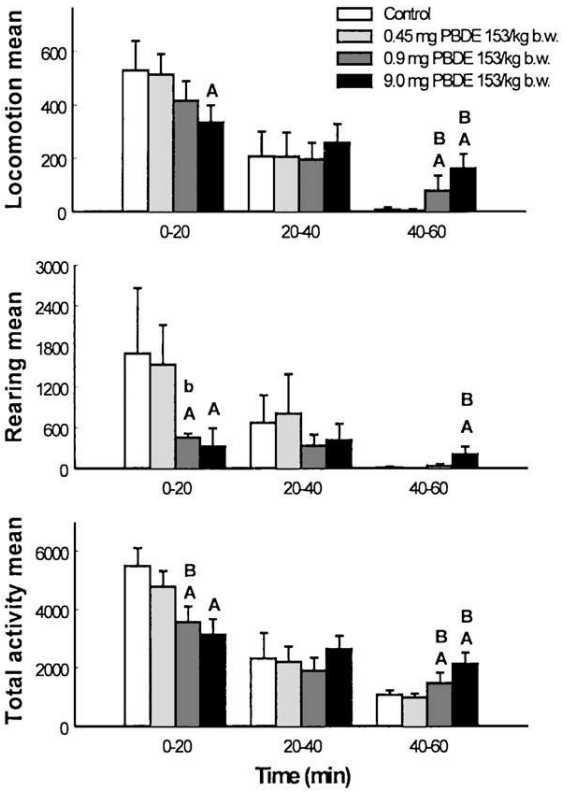

Fig. 1. Spontaneous behaviour of 2-month-old NMRI male mice exposed to a single oral dose of either 20% fat emulsion vehicle or 0.45, 0.9, or 9.0 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight (0.7, 1.4, or 14 μmol 2,2′,4,4′,5,5′-hexaBDE/kg of body weight) at an age of 10 days. The data were subjected to an ANOVA with split-plot design and there were significant group × period interactions [$F_{6,72} = 16.08$; $F_{6,72} = 11.47$; $F_{6,72} = 24.75$] for the variables locomotion, rearing, and total activity, respectively. Pairwise testing between 2,2′,4,4′,5,5′-hexaBDE-exposed and control animals was performed by using Tukey HSD tests. The statistical differences are indicated as: (A) significantly different vs. controls, $P \leq 0.01$; (a) significantly different vs. controls, $P \leq 0.05$; (B) significantly different vs. closest lower 2,2′,4,4′,5,5′-hexaBDE dose, $P \leq 0.01$; (b) significantly different vs. closest lower 2,2′,4,4′,5,5′-hexaBDE dose, $P \leq 0.05$. The height of the bars represents the mean value ± SD.

Effects on spontaneous behaviour in adult mice

The results from the spontaneous behavioural variables locomotion, rearing, and total activity in 2-, 4-, and 6-month-old male mice, after exposure to a single oral dose of 0.45-, 0.9, or 9.0 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight at an age of 10 days, are shown in Figs. 1, 2, and 3, respectively.

Two months after the neonatal exposure to 2,2′,4,4′,5,5′-hexaBDE, there were significant group × period interactions [$F_{6,72} = 16.08$; $F_{6,72} = 11.47$; $F_{6,72} = 24.75$] for the locomotion, rearing, and total activity variables, respectively (Fig. 1). Pairwise testing between 2,2′,4,4′,5,5′-hexaBDE and control groups showed a significant dose-related change in all three test variables. In control mice, there was a distinct decrease in activity in all three behavioural variables over the 60-min period. Mice exposed neonatally to

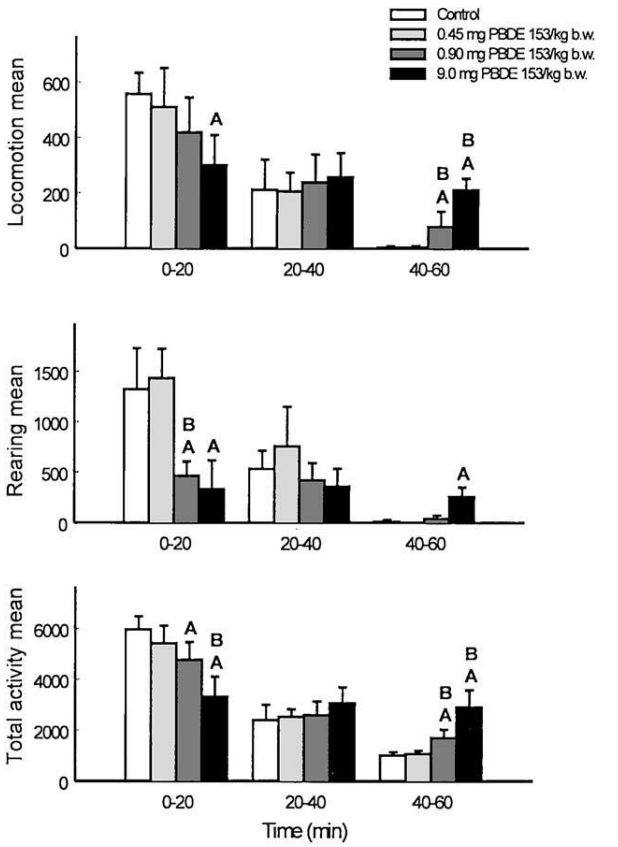

Fig. 2. Spontaneous behaviour of 4-month-old NMRI male mice exposed to a single oral dose of either 20% fat emulsion vehicle or 0.45, 0.9, or 9.0 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight (0.7, 1.4, or 14 μmol 2,2′,4,4′,5,5′-hexaBDE/kg of body weight) at an age of 10 days. The data were subjected to an ANOVA with split-plot design and there were significant group × period interactions [$F_{6,72} = 14.09$; $F_{6,72} = 20.87$; $F_{6,72} = 33.07$] for the variables locomotion, rearing, and total activity, respectively. Pairwise testing between 2,2′,4,4′,5,5′-hexaBDE-exposed and control animals was performed by using Tukey HSD tests. The statistical differences are indicated as: (A) significantly different vs. controls, $P \leq 0.01$; (a) significantly different vs. controls, $P \leq 0.05$; (B) significantly different vs. closest lower 2,2′,4,4′,5,5′-hexaBDE dose, $P \leq 0.01$; (b) significantly different vs. closest lower 2,2′,4,4′,5,5′-hexaBDE dose, $P \leq 0.05$. The height of the bars represents the mean value ± SD.

the highest dose of 2,2′,4,4′,5,5′-hexaBDE (9.0 mg/kg of body weight) displayed significantly less activity, for all three behavioural variables, during the first 20-min period (0–20 min) compared with the controls, while during the third 20-min period (40–60 min), they were significantly more active than the control animals in relation to all three behavioural variables. These mice also showed significantly higher activity, for all three variables, during the third period (40–60 min), compared with the mice receiving the middle dose of 2,2′,4,4′,5,5′-hexaBDE (0.9 mg/kg of body weight). Mice receiving the middle dose of 2,2′,4,4′,5,5′-hexaBDE (0.9 mg/kg of body weight) showed significantly lower activity during the first 20-min period (0–20 min) for the two variables rearing and total activity, compared with the controls, but during the third 20-min period (40–60 min), they showed a significantly higher activity compared with the control animals regarding the two behavioural variables locomotion and total activity. These mice also showed significantly lower activity for the rearing and total activity variables during the first 20-min period (0–20 min), compared with the mice receiving the lowest dose of 2,2′,4,4′,5,5′-hexaBDE (0.45 mg/kg of body weight). In addition, these mice showed significantly higher activity for the behavioural variables locomotion and total activity during the third 20-min period (40–60 min), compared with the mice receiving the lowest dose of 2,2′,4,4′,5,5′-hexaBDE. Mice receiving the lowest dose of 2,2′,4,4′,5,5′-hexaBDE (0.45 mg/kg of body weight) showed no significant differences in activity for any of the three behavioural variables

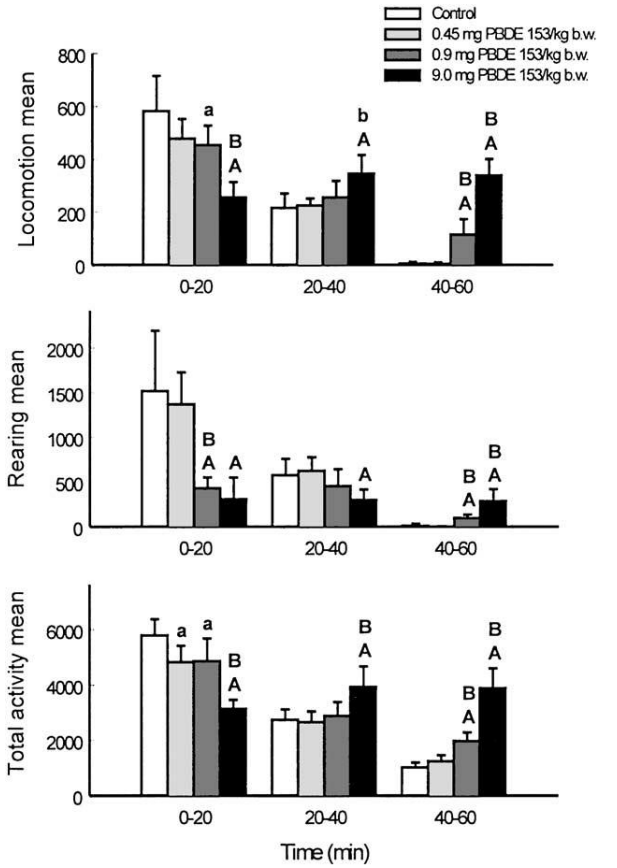

Fig. 3. Spontaneous behaviour of 6-month-old NMRI male mice exposed to a single oral dose of either 20% fat emulsion vehicle or 0.45, 0.9, or 9.0 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight (0.7, 1.4, or 14 μmol 2,2′,4,4′,5,5′-hexaBDE/kg of body weight) at an age of 10 days. The data were subjected to an ANOVA with split-plot design and there were significant group × period interactions [$F_{6,72} = 51.36$; $F_{6,72} = 24.52$; $F_{6,72} = 57.51$] for the variables locomotion, rearing, and total activity, respectively. Pairwise testing between 2,2′,4,4′,5,5′-hexaBDE-exposed and control animals was performed by using Tukey HSD tests. The statistical differences are indicated as: (A) significantly different vs. controls, $P \leq 0.01$; (a) significantly different vs. controls, $P \leq 0.05$; (B) significantly different vs. closest lower 2,2′,4,4′,5,5′-hexaBDE dose, $P \leq 0.01$; (b) significantly different vs. closest lower 2,2′,4,4′,5,5′-hexaBDE dose, $P \leq 0.05$. The height of the bars represents the mean value ± SD.

locomotion, rearing, and total activity, compared with the control animals.

Four months after the neonatal exposure to 2,2′,4,4′,5,5′-hexaBDE, there were still significant group × period interactions [$F_{6,72} = 14.09$; $F_{6,72} = 20.87$; $F_{6,72} = 33.07$] for the locomotion, rearing, and total activity variables, respectively (Fig. 2). Pairwise testing between 2,2′,4,4′,5,5′-hexaBDE and control groups showed a significant dose-related change in all three test variables. In control mice, there was a distinct decrease in activity in all three behavioural variables over the 60-min period. Mice exposed neonatally to the highest dose of 2,2′,4,4′,5,5′-hexaBDE (9.0 mg/kg of body weight) displayed significantly less activity for all three behavioural variables, during the first 20-min period (0–20 min), compared with the controls, while during the third 20-min period (40–60 min), they were significantly more active than the control animals regarding all three behavioural variables. These mice also showed significantly lower activity for the behavioural variable total activity during the first period (0–20 min), compared with the mice receiving the middle dose of 2,2′,4,4′,5,5′-hexaBDE (0.9 mg/kg of body weight), while they also displayed significantly higher activity for the two variables locomotion and total activity during the last 20-min period (40–60 min), compared with the same mice. Mice receiving the middle dose of 2,2′,4,4′,5,5′-hexaBDE (0.9 mg/kg of body weight) showed significantly lower activity during the first 20-min period (0–20 min), concerning the two variables rearing and total activity, compared with the controls, but during the third 20-min period (40–60 min), they showed a significantly higher activity compared with the control animals regarding the two behavioural variables locomotion and total activity. These mice also showed significantly lower activity for the rearing variable, during the first 20-min period (0–20 min), compared with the mice receiving the lowest dose of 2,2′,4,4′,5,5′-hexaBDE (0.45 mg/kg of body weight). Moreover, these mice showed significantly higher activity for the behavioural variables locomotion and total activity during the third 20-min period (40–60 min), compared with the mice receiving the lowest dose of 2,2′,4,4′,5,5′-hexaBDE. Mice receiving the lowest dose of 2,2′,4,4′,5,5′-hexaBDE (0.45 mg/kg of body weight) showed no significant differences in activity for any of the three behavioural variables, compared with the control animals.

Six months after the neonatal exposure to 2,2′,4,4′,5,5′-hexaBDE, there were still significant group × period interactions [$F_{6,72} = 51.36$; $F_{6,72} = 24.52$; $F_{6,72} = 57.51$] for the locomotion, rearing, and total activity variables, respectively (Fig. 3). Pairwise testing between 2,2′,4,4′,5,5′-hexaBDE and control groups showed a significant dose-related change in all three test variables. In control mice, there was a distinct decrease in activity in all three behavioural variables over the 60-min period. Mice exposed neonatally to the highest dose of 2,2′,4,4′,5,5′-hexaBDE (9.0 mg/kg of body weight) displayed significantly less activity, for all three behavioural variables, during the first 20-min period (0–20 min) compared with the controls, while during the third 20-min period (40–60 min), they were significantly more active than the control animals, regarding all three behavioural variables. During the second 20-min period, a difference could be observed for all three behavioural variables, compared with the control animals. These mice also showed significantly lower activity for the behavioural variables locomotion and total activity during the first period (0–20 min), compared with the mice receiving the middle dose of 2,2′,4,4′,5,5′-hexaBDE (0.9 mg/kg of body weight), and a difference was also evident for the two variables locomotion and total activity, during the second 20-min period (20–40 min). In addition, they displayed significantly higher activity for all three variables locomotion, rearing, and total activity, during the last 20-min period (40–60 min), compared with the same mice. Mice receiving the middle dose of 2,2′,4,4′,5,5′-hexaBDE (0.9 mg/kg of body weight) showed significantly lower activity during the first 20-min period (0–20 min) concerning the three variables locomotion, rearing, and total activity, compared with the controls, but during the third 20-min period (40–60 min), they showed a significantly higher activity compared with the control animals regarding all three behavioural variables. These mice also showed significantly lower activity for the rearing variable, during the first 20-min period (0–20 min), compared with the mice receiving the lowest dose of 2,2′,4,4′,5,5′-hexaBDE (0.45 mg/kg of body weight). In addition, these mice showed significantly higher activity for the three behavioural variables locomotion, rearing, and total activity during the third 20-min period (40–60 min), compared with the mice receiving the lowest dose of 2,2′,4,4′,5,5′-hexaBDE. Mice receiving the lowest dose of 2,2′,4,4′,5,5′-hexaBDE (0.45 mg/kg of body weight) showed significantly lower activity for the behavioural variable total activity during the first 20-min period (0–20 min), compared with the control animals.

Effects on habituation capability in adult mice

By analyzing the habituation ratio between the performance periods of 40–60 and 0–20 min in the spontaneous behaviour testing, information concerning the capability to habituate to a novel environment is obtained, and this information can be used to analyze changes in habituation over time. The results for the habituation ratio, calculated from the spontaneous behaviour variables locomotion, rearing, and total activity in 2-, 4-, and 6-month-old NMRI male mice, are presented in Fig. 4. The habituation capability concerning the locomotion and total activity variables was shown to significantly decrease ($P \leq 0.01$) with age in mice exposed neonatally to 9.0 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight. Mice exposed neonatally to 0.9 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight also showed a significant decrease ($P \leq 0.01$) in habituation capability with age, in regard to the rearing variable. In mice exposed

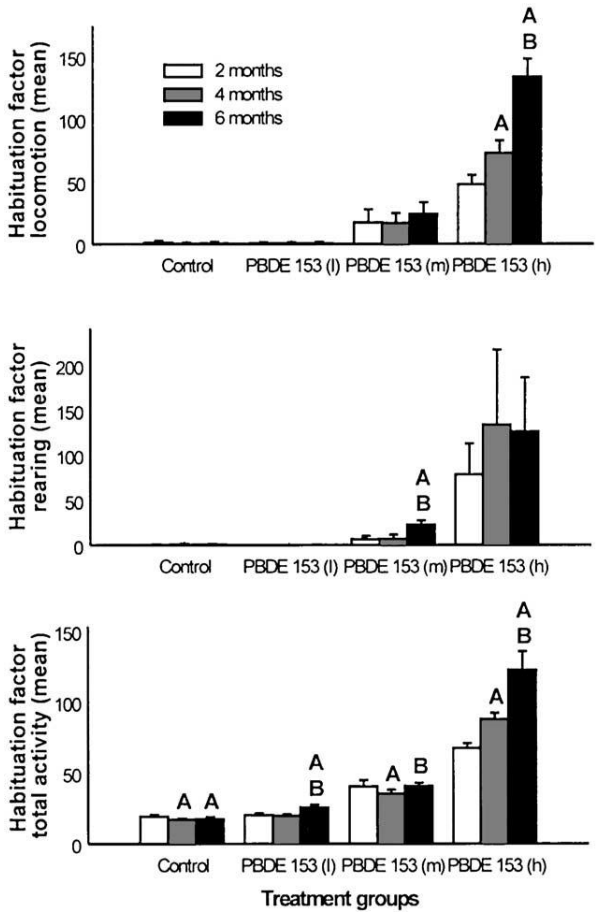

Fig. 4. Habituation capability in 2-, 4-, and 6-month-old NMRI male mice exposed to a single oral dose of either 20% fat emulsion vehicle or 0.45 (l), 0.9 (m) or 9.0 (h) mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight (0.7, 1.4, or 14 μmol 2,2′,4,4′,5,5′-hexaBDE/kg of body weight) at an age of 10 days. The habituation ratio for the variables locomotion, rearing, and total activity was calculated by taking the value for 40–60 min, dividing it with the value for 0–20 min, and multiplying the result by 100. The data were subjected to an ANOVA with split-plot design. Pairwise testing between 2-, 4-, and 6-month-old animals was performed by using Tukey HSD tests. Statistical differences are indicated by: A ($P \leq 0.01$ 4 or 6 months vs. 2 months); B ($P \leq 0.01$ 6 months vs. 4 months).

neonatally to 0.45 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight, a significantly decreased ($P \leq 0.01$) habituation capability was seen concerning the total activity variable.

Effects on learning and memory in adult mice

Swim maze performances of 6-month-old male mice, exposed to a single oral dose of 0.45, 0.9, or 9.0 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight at an age of 10 days, are presented in Fig. 5 and Table 1. During the acquisition period (days 1–4) of spatial learning ability, mice exposed to any of the three 2,2′,4,4′,5,5′-hexaBDE doses significantly improved their ability to locate the platform [$F_{3,239} = 113$], as did the control animals. Split-plot ANOVA revealed significant group × period interactions for 2,2′,4,4′,5,5′-hexaBDE- and vehicle-treated mice [$F_{9,239} = 3.55$, $P \leq 0.01$]. The mice exposed neonatally to 0.9 or 9.0 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight showed significantly longer latencies ($P \leq 0.05$) in locating the platform on the second day of the acquisition period, compared to the control group. On the third day of the acquisition period, the animals exposed to the highest dose of 2,2′,4,4′,5,5′-hexaBDE (9.0 mg/kg of body weight) displayed significantly longer latencies ($P \leq 0.01$) in locating the submerged platform. On the last day of the acquisition period (day 4), both the animals exposed to the highest dose of 2,2′,4,4′,5,5′-hexaBDE (9.0 mg/kg of body weight) and the animals exposed to 0.9 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight showed significantly longer latencies ($P \leq 0.01$) in locating the platform. On day 5, the platform was relocated in order to measure relearning ability in reversal trials. In the first trial (trial 21) on day 5, the control mice displayed significantly longer latencies ($P \leq 0.001$) than in the last trial (trial 20) of day 4, which was also true ($P \leq 0.05$) for the animals exposed neonatally to the lowest dose of 2,2′,4,4′,5,5′-hexaBDE (0.45 mg/kg of body weight). This is normal behaviour during relearning, as the mouse initially searches close to the previous location of the platform (Morris et al., 1982). Strikingly, mice exposed neonatally to the middle dose of 2,2′,4,4′,5,5′-hexaBDE (0.9 mg/kg of body weight) and the highest dose of 2,2′,4,4′,5,5′-hexaBDE (9.0 mg/kg of body weight) did not show any significant discrepancy in latency when the last trial on day 4 was compared to the first trial on day 5 ($P > 0.1$ in both cases) (Table 1). During the relearning period (day 5), mice exposed to any of the three 2,2′,4,4′,5,5′-hexaBDE doses significantly improved their ability to locate the platform, as did the control animals. However, the mice exposed neonatally to 0.9 or 9.0 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight showed significantly longer latencies ($P \leq 0.05$) in locating the platform on day 5, compared to the control group.

Effects on nicotinic receptors in hippocampus in adult mice

The densities of nicotinic receptors in the hippocampus of 6-month-old mice treated with 0.9 or 9.0 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight at the postnatal age of 10 days are presented in Table 2. There was a significant decrease ($P \leq 0.01$) in the density of specific [^{3}H]-α-bungarotoxin binding sites in the hippocampus in mice given 9.0 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight. The densities of specific [^{3}H]-α-bungarotoxin binding sites in the hippocampus in the 2,2′,4,4′,5,5′-hexaBDE-treated animals were decreased by 20.6% compared to the control animals. In mice exposed neonatally to 0.9 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight, there were no significant alterations in the densities of specific [^{3}H]-α-bungarotoxin binding sites in the hippocampus.

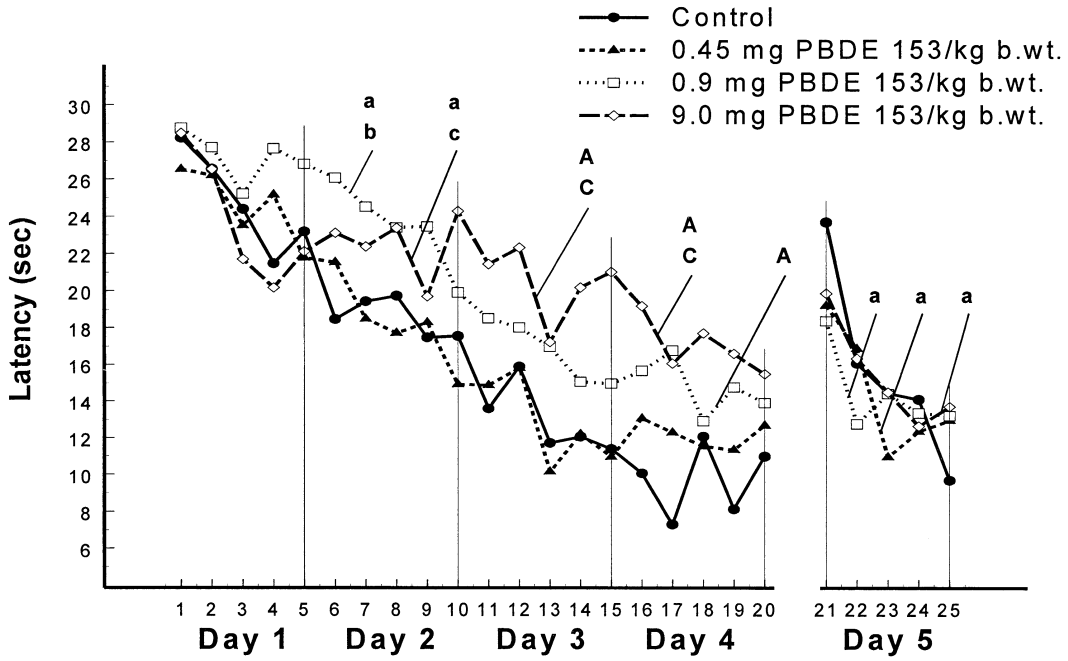

Fig. 5. Swim maze performance in 6-month-old NMRI male mice exposed to a single oral dose of either 20% fat emulsion vehicle or 0.45, 0.9, or 9.0 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight (0.7, 1.4, or 14 μmol 2,2′,4,4′,5,5′-hexaBDE/kg of body weight) at an age of 10 days. Latencies in locating the platform were measured during acquisition period (days 1–4) and during the relearning period (day 5). Statistical analysis: the behavioural data, day 1 to 4, were submitted to an ANOVA using a split-plot design with Duncan's test. Statistical analysis of the behavioural data from day 5 (difference between trial 1 and 5) was done with one-way ANOVA and pairwise testing using Duncan's test. The statistical differences are indicated as: (A) significantly different vs. controls, $P \leq 0.01$; (a) significantly different vs. controls, $P \leq 0.05$; (B) significantly different vs. closest lower 2,2′,4,4′,5,5′-hexaBDE dose, $P \leq 0.01$; (b) significantly different vs. closest lower 2,2′,4,4′,5,5′-hexaBDE dose, $P \leq 0.05$; (C) significantly different vs. lowest 2,2′,4,4′,5,5′-hexaBDE dose, $P \leq 0.01$; (c) significantly different vs. lowest 2,2′,4,4′,5,5′-hexaBDE dose, $P \leq 0.05$.

Discussion

The present investigation shows that exposure to low doses of 2,2′,4,4′,5,5′-hexaBDE, 0.45, 0.9, or 9.0 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight, on postnatal day 10, can give rise to irreversible disturbances in the spontaneous behaviour in the adult mice. These disturbances are both dose-response and time-response related. Furthermore, neonatal exposure to 2,2′,4,4′,5,5′-hexaBDE also affected learning and memory functions. This neonatal exposure to 2,2′,4,4′,5,5′-hexaBDE also caused a decrease in α-bungarotoxin binding in hippocampus, indicating that the cholinergic nicotinic receptors in the adult animals can be one of the targets for PBDEs developmental neurotoxicological effects.

The spontaneous motor behaviour data showed a disruption of habituation in animals exposed to 2,2′,4,4′,5,5′-hexaBDE. Habituation, here defined as a decrease in the locomotion, rearing, and total activity variables in response to the diminishing novelty of the test chamber over a 60-min period, was displayed in the control animals, but the animals exposed to the three different doses of 2,2′,4,4′,5,5′-hexaBDE were clearly hypoactive during the beginning of the 60-min period, while toward the end of the test period they

Table 1
Effects on learning performance between last trial of the acquisition period (trial 20) and first trial of the relearning period (trial 21) in swim maze in 6-month-old mice after neonatal exposure, on day 10, to 2,2′,4,4′,5,5′-hexaBDE[a]

Treatment group (mg/kg of body weight)	*n*	Latency trial 20 (*s*)	Latency trial 21 (s)	Trial 20 vs. 21
Control	24	10.96 ± 8.12	23.67 ± 8.26	$P \leq 0.001$
0.45	21	12.67 ± 10.62	19.19 ± 10.79	$P \leq 0.05$
0.9	20	13.90 ± 10.86	18.35 ± 8.49	$P > 0.1$
9.0	19	15.47 ± 9.95	19.82 ± 9.23	$P > 0.1$

[a] Neonatal mice were exposed to a single oral dose of 0.45, 0.9, or 9.0 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight (0.7, or 1.4 or 14 μmol 2,2′,4,4′,5,5′-hexaBDE/kg of body weight) and controls to 20% fat emulsion in the same manner. Latency of last trial (trial 20) of the acquisition period, mean ± SD, versus latency of first trial (trial 21) of the relearning period, day 5, mean ± SD, evaluated with paired Student's *t* test.

Table 2
Effects on nicotinic receptors in hippocampus in 6-month-old mice after neonatal exposure, on day 10, to 2,2′,4,4′,5,5′-hexaBDE[a]

Treatment (mg/kg of body weight)	*n*	[^{3}H]α-Bungarotoxin binding (pmol/g of protein)
Vehicle	9	35.16 ± 3.30
0.9	6	32.67 ± 4.04
9.0	6	27.92 ± 5.22[b]

[a] Male NMRI neonatal mice were exposed to a single oral dose of 0.9 or 9.0 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight (14 μmol 2,2′,4,4′,5,5′-hexaBDE/kg of body weight) and controls to 20% fat emulsion in the same manner. The animals were killed at 6 months of age and [^{3}H]-α-bungarotoxin binding (mean ± SD) was assessed in the P2 fraction. The statistical evaluation was made by using one-way ANOVA and pairwise testing using Duncan's test.
[b] $P \leq 0.01$.

were hyperactive. This derangement in spontaneous behaviour tests also indicated that the functional disorder is dose-response related. In 2-month-old mice, the two highest doses of 2,2′,4,4′,5,5′-hexaBDE (0.9 and 9.0 mg/kg of body weight) caused a significant change in spontaneous behaviour. In addition, the effect seen after neonatal exposure to 9.0 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight is more pronounced than the effect seen after neonatal exposure to 0.9 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight, indicated by deviations from the control group for all three behavioural variables during the first and the last 20-min period of the 60-min period. Furthermore, the spontaneous behaviour tests indicated that the change in behavioural motor activity worsen with increasing age, as the aberrations appeared to be most pronounced in the 6-month-old mice, which was also true when looking at the habituation capabilities. This change over time is clearly demonstrated in mice receiving the highest dose of 2,2′,4,4′,5,5′-hexaBDE (9.0 mg/kg of body weight), where both the habituation ratio for locomotion and total activity increased significantly. This means that the ability to habituate to a novel environment became worse with age after neonatal exposure to 2,2′,4,4′,5,5′-hexaBDE. This effect is seen both when comparing 2-month-old mice with 4-month-old mice and when comparing 4-month-old mice with 6-month-old mice. This type of both dose-response and time-response behavioural defects and reduced habituation capability with age have been seen in mice neonatally exposed to PBDE 47 and PBDE 99 (Eriksson et al., 2001b, 2002). Interesting to note is that, in comparison to developmental effects after neonatal exposure to PCB, it has been seen that certain ortho-substituted PCBs, such as PCB 52 and PCB 153 (Eriksson, 1998; Eriksson and Fredriksson, 1996a,b), can induce these kinds of dose-response related and time-response related changes in spontaneous behaviour, together with reduced habituation capability with increasing age. Animals neonatally exposed to PCB 153 did show differences in spontaneous behaviour 4 months after exposure, and 2 months later the deranged spontaneous behaviour was even more pronounced. The doses used in all these studies were the same, on a molar level, and the effects were induced during a defined critical period of brain development. The irreversibility and time dependency of these effects, reflected in the fact that they worsen with age, indicate an acceleration of a dysfunctional process.

In the swim maze of the Morris water maze type, adult mice neonatally exposed to the two higher doses of 2,2′,4,4′,5,5′-hexaBDE (0.9 and 9.0 mg/kg body weight) performed significantly worse than control animals. During the 4-day acquisition period, all animals decreased the time needed to locate the submerged platform, but during this acquisition period, the animals exposed to 2,2′,4,4′,5,5′-hexaBDE spent more time locating the submerged platform, an effect that was dose-response related. This deterioration during the acquisition period could already be seen on the second day, when the animals neonatally exposed to 0.9 and 9.0 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight displayed longer latencies in locating the platform than animals receiving 0.45 mg 2,2′,4,4′,5,5′-hexaBDE/kg of body weight or the 20% fat emulsion vehicle. This deviation from the control animals persisted throughout the acquisition period and became even more pronounced on days 3 and 4. This reduced ability of neonatally PBDE-exposed mice to perform in a swim maze is similar to the impairments in spatial learning tasks that have been seen in rodents with advancing age in Morris water maze (Gage et al., 1984; Gallagher and Pelleymounter, 1988; Lamberty and Gower, 1989; Magnusson, 1998; Pelleymounter et al., 1990). Spatial learning is one form of memory in which humans, too, show significant impairments as they age (Barnes, 1988; Caplan and Lipman, 1995; Evans et al., 1984). This indicates that neonatal PBDE 153 exposure can accelerate this kind of aging process. During the reversal trials on day 5, all mice improved their ability to locate the new position of the platform, but the animals exposed neonatally to the two highest doses of 2,2′,4,4′,5,5′-hexaBDE (0.9 and 9.0 mg/kg body weight) differed from the controls by longer latencies. This kind of altered reversal learning in swim maze performance has also recently been seen in adult mice neonatally exposed to PBDE 99 (Eriksson et al., 2001b). From our earlier studies regarding developmental effects from PBDE exposure, we have seen that neonatal exposure to PBDE 99 (8 mg of PBDE 99/kg of body weight) altered the adult response to nicotine, in nicotine-induced behaviour tests, which indicates an effect on the cholinergic system (Viberg et al., 2001). In the present study, the cholinergic receptors were affected, and changes in cholinergic receptors have been proposed to affect learning and memory (Bartus et al., 1982). During normal human aging, it has earlier been seen that cholinergic nicotinic receptors decrease in cortex (Court et al., 1992) and hippocampus (Court and Clementi, 1995). Also, functional alterations in motor activity and memory are seen during aging. In the present study, it could be seen that the animals exposed neonatally to the highest dose of 2,2′,4,4′,5,5′-hexaBDE (9.0 mg/kg of body weight)

received effects on the nicotinic receptors in the brain, manifested as a decrease in α-bungarotoxin binding in hippocampus. This decrease was about 21%. Altered performance in swim mazes and reduced amount of nicotinic have also been seen in mice neonatally exposed to certain PCBs (PCB 52) (Eriksson and Fredriksson, 1996a). Therefore, one of the mechanisms behind the developmental neurotoxic effects of PBDE 153, PBDE 99, and certain PCBs involves changes in the cholinergic system. The behavioural performance in tasks requiring attention and rapid processing of information in humans, and reversal learning and working memory in animals, has been suggested to involve cholinergic transmission (Hodges et al., 1991) and the cholinergic system is one of the major transmitter systems that correlate closely to cognitive function (Drachman, 1977; Fibiger, 1992). Taken together, these observations can be compared to the effects of neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, which are known to be accompanied by changes in the cholinergic nicotinic receptors in cortex and hippocampus (James and Nordberg, 1995; Nordberg, 1993).

There is of special concern that neonatal exposure to 2,2′,4,4′,5,5′-hexaBDE affected spontaneous motor behaviour, learning and memory processes, and the cholinergic transmitter system in adult mice in a way similar to those previously observed for certain PCBs. There are human epidemiological studies suggesting that perinatal exposure to PCBs can have developmental neurotoxic effects (Fein et al., 1984; Jacobson and Jacobson, 1996; Jacobson et al., 1990; Rogan et al., 1988). Experimental studies in mice, rats, and monkeys, to PCBs during development, has been shown to produce long-term changes in behaviour, neurotransmitters, and neuroreceptors (Seegal and Schantz, 1994; Seegal and Shain, 1992; Seegal, 1996; Tilson and Harry, 1994; Tilson et al., 1990, Eriksson, 1998). Exposure of rats to PCB 153 during the neonatal period (postnatal day 3 to 13) resulted in long-lasting behavioural defects (Holene et al., 1998, 1999). These results support the findings in mice, that PCB 153 and other PCB congeners can cause changes in behaviour when administered during the critical phase of neonatal brain development (Eriksson, 1998). PCBs are well known contaminants of human breast milk, which is also true for 2,2′,4,4′,5,5′-hexaBDE (Meironyte´ and Nove´n, 1999). The transfer of environmental contaminants via human milk is one of the major routes of exposure to young animals, and it has been shown that the major route for PCB 153 is via this route and not via placental transport (Vodicnic and Lech, 1980). This is interesting because our experimental setup has been shown to induce persistent neurotoxic effects, when PCBs and the structurally similar PBDEs are administered during a defined phase of the rapid development of the brain in neonatal life (Eriksson et al., 2001b, 2002; Eriksson, 1998).

In conclusion, the present investigation has shown that neonatal exposure to 2,2′,4,4′,5,5′-hexaBDE causes dose-response and time-response changes in adult spontaneous behaviour (hyperactivity), impaired learning and memory, and reduced amounts of nicotinic receptors in hippocampus. This, and the facts that PBDEs are increasing in levels in human milk and in the environment and that PCBs are still found in human milk and in the environment, calls for further investigations of PBDEs as neurotoxicants, especially since PBDEs seem to be as potent as PCBs as developmental neurotoxicants, acting via a mechanism involving the cholinergic system. Therefore, possible interactive neurotoxic effects between PBDEs and PCBs also constitute an interesting approach for the future.

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