Neonatal Developmental Neurotoxicity of Brominated Flame Retardants, the Polybrominated Diphenyl Ethers (PBDEs)

BY

HENRIK VIBERG
Dissertation presented at Uppsala University to be publicly examined in Lindahlsalen, EBC, Uppsala, Friday, October 29, 2004 at 09:00 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract

This thesis examines developmental neurotoxic effects of polybrominated diphenyl ethers (PBDEs), PBDE 99, PBDE 153, and the fully brominated PBDE 209, after exposure during the newborn period in rodents.

Our environment contains vast numbers of contaminants, including the flame retardants, PBDEs. The PBDEs are widely found in the environment and are increasing in human milk. Individuals can be exposed to PBDEs during their whole lifetime, and especially during the lactation period. The neonatal period, coinciding with the lactation period, is characterized in many mammalian species by rapid growth and development of the immature brain. It has been shown that numerous toxicants can induce permanent disorders in brain function when administered to the neonatal mouse during the brain growth spurt (BGS). In mice and rats this period is postnatal, spanning over the first 3-4 weeks of life, while in humans, BGS begins during the third trimester of pregnancy and continues throughout the first two years of life.

The present studies identified a defined critical period during BGS in mice when the brain is vulnerable to insults of low doses of PBDEs and that it is the presence of PBDEs or their metabolites in the brain during this critical period that is crucial to evoking neurotoxic effects. The effects observed are permanent altered spontaneous behavior, reduced habituation, deficits in learning and memory, and disturbances in the cholinergic system. These effects worsen with age.

The ability of PBDEs to induce neurotoxic effects does not appear to be gender-, strain- or species-specific, because the neurotoxic effects are induced in rats and male and female mice of different strains.

The developmental neurotoxic effects of PBDEs are similar to those observed for polychlorinated biphenyls (PCBs) and possible interactive effects of PBDEs and other environmental contaminants are therefore of concern.

Keywords: brominated flame retardants, polybrominated diphenyl ethers, PBDE, neonatal, development, neurotoxicity, behaviour, cholinergic system

Henrik Viberg, Department of Evolutionary Biology, Department of Environmental Toxicology, Norbyv. 18A, Uppsala University, SE-75236 Uppsala, Sweden

© Henrik Viberg 2004

ISSN 1104-232X
ISBN 91-554-6053-4
urn:nbn:se:uu:diva-4576 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-4576)
List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

**Paper I**

**Paper II**

**Paper III**

**Paper IV**

**Paper V**

**Paper VI**
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>OBJECTIVES</td>
<td>9</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>10</td>
</tr>
<tr>
<td>Exposure to environmental toxic agents</td>
<td>10</td>
</tr>
<tr>
<td>Polybrominated diphenyl ethers (PBDEs)</td>
<td>10</td>
</tr>
<tr>
<td>Polybrominated diphenyl ethers – environment</td>
<td>12</td>
</tr>
<tr>
<td>Polybrominated diphenyl ethers – humans</td>
<td>13</td>
</tr>
<tr>
<td>Polybrominated diphenyl ethers – general toxicology</td>
<td>15</td>
</tr>
<tr>
<td>Brain development and vulnerable periods</td>
<td>17</td>
</tr>
<tr>
<td>The cholinergic system, behavior, aging and neurodegenerative disorders</td>
<td>19</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>23</td>
</tr>
<tr>
<td>Chemicals</td>
<td>23</td>
</tr>
<tr>
<td>Animals</td>
<td>23</td>
</tr>
<tr>
<td>Treatment</td>
<td>24</td>
</tr>
<tr>
<td>Behavioral tests</td>
<td>24</td>
</tr>
<tr>
<td>Spontaneous behavior</td>
<td>24</td>
</tr>
<tr>
<td>Nicotine-induced behavior</td>
<td>25</td>
</tr>
<tr>
<td>Swim maze</td>
<td>25</td>
</tr>
<tr>
<td>Receptor assays</td>
<td>26</td>
</tr>
<tr>
<td>$^3$H-α-bungarotoxin binding</td>
<td>26</td>
</tr>
<tr>
<td>$^3$H-QNB-binding</td>
<td>27</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>27</td>
</tr>
<tr>
<td>Spontaneous behavior</td>
<td>27</td>
</tr>
<tr>
<td>Habituation capability</td>
<td>27</td>
</tr>
<tr>
<td>Nicotine-induced behavior</td>
<td>28</td>
</tr>
<tr>
<td>Swim maze</td>
<td>28</td>
</tr>
<tr>
<td>RESULTS AND DISCUSSION</td>
<td>29</td>
</tr>
<tr>
<td>Effects of neonatal exposure to PBDEs during a defined critical period of brain development on behavior, learning and memory, and cholinergic system</td>
<td>29</td>
</tr>
<tr>
<td>Effects of neonatal exposure to a highly brominated PBDE</td>
<td>37</td>
</tr>
</tbody>
</table>
Gender, strain, and species comparison of neurobehavioral effects of neonatal exposure to PBDEs ..........................................................41
General discussion.............................................................................44

CONCLUDING REMARKS....................................................................47

ACKNOWLEDGEMENTS......................................................................49

SUMMARY IN SWEDISH......................................................................50
Utvecklingsneurotoxikologiska effekter av bromerade flamskyddsmedel, polybromerade difenyletrar (PBDEer) hos neonata möss och råttor ....50

REFERENCES ..........................................................................................52
Abbreviations

AChE  Acetylcholine esterase
BDE  Brominated diphenyl ether
BFR  Brominated flame retardants
ChAT  Choline acetyltransferase
CNS  Central nervous system
DDT  1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane
EU  European Union
FR  Flame retardants
GD  Gestational day
HBCDD  Hexabromocyclododecane
MPTP  1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NMRI  Naval Medical Research Institute
PBB  Polybrominated biphenyl
PBDE  Polybrominated diphenyl ether
PBDE 153  2,2',4,4',5,5'-hexabrominated diphenyl ether
PBDE 209  2,2',3,3',4,4',5,5',6,6'-decabrominated diphenyl ether
PBDE 47  2,2',4,4'-tetrabrominated diphenyl ether
PBDE 99  2,2',4,4',5-pentabrominated diphenyl ether
PCB  Polychlorinated biphenyl
PND  Postnatal day
QNB  Quinuclidinyl benzilate
TBBPA  Tetrabromobisphenol A
WHO  World Health Organization
OBJECTIVES

The object of this thesis was to explore developmental neurotoxic effects of neonatal exposure of different polybrominated diphenyl ethers in rodents. The more specific objectives of the thesis were:

- To demonstrate whether there is a defined critical period during neonatal brain development when polybrominated diphenyl ethers can induce persistent behavioral derangements in the adult, such as changes in spontaneous behavior and learning and memory.

- To demonstrate whether neonatal exposure to polybrominated diphenyl ethers causes dose-response related neurobehavioral effects in the adult.

- To demonstrate whether neonatal exposure to polybrominated diphenyl ethers affects the cholinergic transmitter system in the adult.

- To study whether animals of different sexes, different strains, and different species are equally susceptible to neurotoxic effects of neonatal exposure of polybrominated diphenyl ethers.
INTRODUCTION

The thesis focuses on the neurotoxic effects of exposure to the brominated flame retardants, polybrominated diphenyl ethers (PBDEs), during a period of rapid brain growth and development in neonatal rodents.

Exposure to environmental toxic agents

Persistent organic contaminants have been readily found in our environment over the past decades and some of them are known to be toxic to living organisms. Individuals can be exposed to potentially toxic persistent organic substances throughout their entire lifetime, beginning with the fertilization of the egg. Embryos/fetuses may be exposed to toxic agents during the gestational period, via intake of toxic substances by the mother. Both lactation and direct exposure become possible exposure routes for the offspring after birth. In humans, perinatal developmental neurotoxicity is evident in the adverse effects of lead poisoning, as well as in fetal alcohol syndrome, methyl mercury poisoning, and drug abuse during pregnancy (Court et al. 1996).

The toxic effects of hazardous contaminants depend on the amount of the contaminant, the route of administration, and the point of time of exposure during the organism’s life cycle. A number of agents have shown the capacity to induce permanent disorders in organisms by acting as neurotoxicants perinatally. In mouse, both persistent and non-persistent agents can induce permanent neurological derangements if administered during a critical period of rapid brain development (Eriksson 1997).

Polybrominated diphenyl ethers (PBDEs)

Over the past 50 years the polymer industry has grown dramatically, and a large number of products have been introduced to the market. The polymers have different properties and use. Most of them are petroleum-based and hence flammable. In order to meet fire safety regulations, flame retardants (FRs) are added to combustible products such as plastics, electronic circuit boards, wood, computers, TV sets, construction materials, synthetic textiles, and the like (WHO 1994a).
Brominated flame retardants (BFRs) are a diverse group of chemicals that include the polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs) (WHO 1994b), and hexabromocyclododecane (HBCDD) (WHO 1997). These three are all additive BFRs. Tetrabromobisphenol A (TBBPA) (WHO 1995) can be used as either additive or reactive BFRs. Reactive BFRs are mixed with the plastic before polymerization to form covalent bonds and become a part of the polymer matrix. Additive BFRs are incorporated into the plastic mixture without chemical binding. Thus, this makes them more prone to leak out of goods and products during their lifetime, including loss at the time of disposal (Hutzinger et al. 1976; Hutzinger and Thoma 1987).

The PBDEs are a group of chemicals with 209 possible congeners, since there are a large number of positions where the bromine atom/atoms can bind onto the two phenyl rings. Their chemical formula is $\text{C}_{12}\text{H}_{(n-1)}\text{Br}_n\text{O}$ ($n \leq 10$) (Fig. 1). The PBDEs have a low vapor pressure at room temperature and high lipophilicity ($\log K_{ow}$ ranging between 4.28 and 9.9), with higher values for the more brominated congeners (WHO 1994a).

![Figure 1. General structure of PBDEs.](image)

The PBDEs are rather stable and show similar characteristics to the PCBs (WHO 1993), and are now known to be present in the environment (de Wit 2002; Hale et al. 2003). The commercial PBDE products predominately consist of so-called penta-, octa- and decabromodiphenyl ether products. Each product consists of a rather narrow range of congeners and is named, regarding bromination pattern, after the dominating congeners. Polymer products with a fire safety requirement can contain up to 30% flame retardants. The total market demand for the three major PBDE products (viz., penta-BDE, octa-BDE and deca-BDE) was reported in 2003 to be 7,500, 3,790 and 56,100 metric tons, respectively, for the year 2001 (BSEF 2003), which makes worldwide production a significant industrial sector. From 2004, penta-BDE and octa-BDE have been banned in the EU (KemI 2003b), while deca-BDE is still in use, but regulated according to the law (KemI 2003a).
Polybrominated diphenyl ethers – environment

The first signs of PBDEs in the environment came from the United States in the 1970s (Di Carlo et al. 1978; Erickson et al. 1980), when PBDEs were detected in water samples collected near plants that manufactured polybrominated compounds. The first report regarding PBDEs in higher organisms was a Swedish study from the beginning of the 1980s, in which PBDEs were found in various fish species in a Swedish river (Andersson and Blomkvist 1981). In the early 1990s, it was discovered that the PBDE levels in Sweden were increasing over time, and analyses of differences in the geographical pollution pattern showed that the variations in levels were almost identical with those previously found for PCBs and DDTs, which was also an indication of airborne pollution (Nylund et al. 1992; Sellström et al. 1993). The measurements of PBDEs in different species also indicated biomagnification. In the early 2000s, two studies measured PBDE levels in marine ecosystems and both concluded that PBDEs bio-magnify (Boon et al. 2002; Christensen et al. 2002). The bio-magnification rates of tri-, tetra-, and pentaBDEs in aquatic ecosystems have been measured to be higher than the biomagnification rates for PCBs (Burreau et al. 1999).

PBDEs have been detected in air samples collected from many different parts of the world. Air samples taken at two different locations in Sweden in 1990-1991 contained 1-8 pg PBDE/m³, with a predominance of PBDE 47, 99, and 100 (de Wit 2002), which is in agreement with the levels measured in Sweden in a recent study of PBDEs in ambient air across Europe (Jaward et al. 2004). Jeward and coworkers also conclude that urban areas of Western Europe have the highest air levels of PBDEs, with values up to 43 pg/m³. PCBs have the same type of pollution pattern, but the levels are markedly higher. In the Great Lakes region of the United States, levels of PBDEs in outdoor air have been reported to be in the range of 4.4-77 pg/m³ (Strandberg et al. 2001), while in Canadian air, sample levels have been reported to be as high as 1250 pg/m³, although they are suggested to vary with the seasons (Gouin et al. 2002). The PCB levels are still generally 2- to 3-fold higher than the PBDE levels in the Canadian study.

The PBDEs tend to be concentrated in sewage sludge with high organic carbon content, which is often spread onto farm areas. In Swedish sewage sludge samples from 1980s and 1990s, the concentration of the three congeners PBDE 47, 99, and 100 ranged from 20 to 119 ng/g dry weight (Nylund et al. 1992; Sellstrom 1999). Later studies of PBDEs in sewage sludge from Sweden and Holland showed that the most abundant congener was PBDE 209 (de Wit 2002; Sellstrom 1999), with measured levels of up to 350 ng/g dry weight. In general, the levels of PBDEs in sewage sludge from the United States are higher than in Europe, where the PBDE levels have been found to exceed the PCB levels, with a substantial contribution from PBDE 209 (Hale et al. 2003; Hale et al. 2001a).
PBDEs are now found globally in the environment, such as in sediment from rivers, lakes, and seas. Analyzing sediments provides a valuable source of temporal trends; for detailed information, see review by de Wit (de Wit 2002). In summary, levels tend to be higher in urban and industrial areas. Levels also tend to be higher in the United States compared to Europe, although the levels in both Europe and the United States have increased since the 1980s. Also interesting to note is the fact that levels of PBDE 209 markedly exceed the levels of all other congeners in certain areas.

In aquatic environments, it is not only the sediment that contains PBDEs. There are many current studies that deal with PBDE levels in fish and marine mammals; for reviews, see de Wit (2002) and Hale (2003). In general, the levels are higher in the United States than in Europe, and the dominating congeners are PBDE 47, PBDE 99, PBDE 100, and PBDE 153. It is interesting to note, however, that the highest detected levels are as high as 47,900 ng/g lipids in the United States (Hale et al. 2001b) and 36,900 ng/g lipids in Europe (Sellström et al. 1993). These reports do not contain information about PBDE 209.

In the terrestrial environment, the reports are sparser, but rabbit, moose, reindeer, and some bird species have been analyzed for PBDEs (de Wit 2002; Jansson et al. 1993; Sellström et al. 1993). PBDE 47, 99, 100, and 153 were detected, and juvenile starlings contained up to 13 ng PBDEs/g lipids. In a recent Swedish study, eggs of peregrine falcons were analyzed and all eggs from three different locations contained all the PBDE congeners that were analyzed, including the highly brominated congeners PBDE 183 and 209 (Lindberg et al. 2004). The total level of PBDEs was reported to be 2200 ng/g lipids. The authors claim that this is the first study where highly brominated PBDEs were quantified in high trophic level wildlife.

Polybrominated diphenyl ethers – humans

There are only a few studies concerning human exposure and/or levels in humans. According to a presentation by Wijesekera and coworkers, dietary intake represents 73% of human exposure and inhalation of indoor air 27% (Wijesekera et al. 2002).

Studies from Sweden, Finland, and Canada estimate the total daily intake of the most common PBDEs to be in the range of 40-50 ng in humans (Darnerud et al. 2001; Kiviranta et al. 2004; Lind et al. 2002; Ryan and Patry 2001), and in the UK, the estimated intake is 90.5 ng (Wijesekera et al. 2002). In a recent study from Spain, the total daily intake of PBDEs was estimated to be 97.3 ng, where the biggest contribution came from fish and shellfish (30.7 ng PBDE/day) (Bocio et al. 2003).

The other major route of exposure is inhalation of PBDEs through particulate matter or dust. In a study of occupational exposure to PBDEs, workers who manually dismantled computers worked in an ambient air
containing 19 and 36 ng/m$^3$ of PBDE 183 and PBDE 209, which is about 2,000 times higher than in the common office environment. The levels of lower brominated PBDEs were significantly lower (Sjödin et al. 2001a). Significant occupational exposure to PBDEs has also been seen in hospital workers who repair and maintain computers (Jakobsson et al. 2002), which increases the number of people with potential PBDE-exposure (Sjödin et al. 2003b). PBDEs have also been detected, in comparable concentrations, in indoor air from a laboratory in Norway (Thomsen et al. 2001).

Early reports of PBDEs in human blood came from Sweden in the 1990s. Blood samples from Swedish blood donors contained approximately 2.1 ng PBDEs/g lipid weight (Klasson-Wehler et al. 1997). Quantification of PBDEs in blood from electronic dismantlers (26 ng/g lipid weight) revealed significantly higher blood concentration of PBDEs, compared to blood from workers in a reference group (3.3 ng/g lipid weight), which shows that humans are exposed to PBDEs even if they work in a potentially non-contaminated environment (Sjödin et al. 1999). The higher brominated PBDEs dominated in blood from occupationally exposed workers, while lower brominated PBDEs dominated in workers from potentially non-exposed environments. Later, blood samples taken from U.S. blood donors in 1988 were analyzed, with levels of PBDEs ending up in the same range as in the group of potentially non-exposed workers mentioned above (Sjödin et al. 2001b). A time trend study of U.S. blood serum indicates an increase in PBDE-levels from 1985 to 2002 (Sjödin et al. 2003a), which is in agreement with European studies (Schröter-Kermani et al. 2000; Thomsen et al. 2002). A Swedish study of PBDE content in blood plasma from the umbilical cord showed that the total concentration of PBDEs is 4.29 ng/g lipid weight, with PBDE 47 as the most abundant congener (Guvenius et al. 2003). In the United States, on the other hand, the levels of PBDEs in fetal sera were around 20 times higher than in the Swedish study, which was also the case when comparing the PBDE levels in maternal sera (Hites et al. 2003). The same PBDE congeners were dominating in both studies. These studies show that not only adult humans are exposed to PBDEs, but also fetuses and newborn humans.

A major route of exposure to PBDEs is during the late gestational and newborn periods. Because of the lipophilic characteristics of PBDEs, they tend to be deposited in the adipose tissue and when the body fat is used, the PBDEs can be redistributed and excreted via milk. A time-related trend study of PBDEs in Swedish human milk shows that the levels increased from 0.07 to 4.02 ng/g lipid weight between 1972 and 1997 and that the most abundant congeners were PBDE 47, 99, and 153, in named order (Meironyté et al. 1999). From these measurements it is calculated that the doubling time for PBDEs in Swedish human milk is 5 years (Norén and Meironyté 2000). Comparable levels of PBDEs in Swedish human milk have later been confirmed (Lind et al. 2003). The same kind of increase has been
seen in a time trend study in Japan (1973-2000), where the sum of PBDEs in human milk was in the same range as in the Swedish study (Akutsu et al. 2003). Another Japanese study indicates that there is a strong positive relationship between PBDE concentrations in Japanese human milk and dietary intake of fish and shellfish (Ohta et al. 2002). European studies of PBDEs in human milk report similar levels with the same dominating congeners (Baumann et al. 2003; Erdogrul et al. 2004; Kalantzi et al. 2003; Pirard et al. 2003). The highest levels of PBDEs in human milk are found in the U.S., where the sum of PBDEs was 85.7 ng/g lipids, with the same dominating congeners (PBDE 47, 99, 100, and 153) (Scheer et al. 2003). All these studies contribute to establishing the fact that humans can be exposed to PBDEs as newborns.

Polybrominated diphenyl ethers – general toxicology

There are some studies available concerning the uptake, metabolism, distribution, and elimination of PBDEs. Generally, the uptake and half-life decrease with increasing bromination. Studies by Klasson-Wehler and coworkers and Orn and Klasson-Wehler show that he absorbed amount of 14C-labelled PBDE 47 in adult rats and mice, after oral exposure, is around 90-95%, with 86% and 47% of the given dose remaining in the body 5 days later in the rats and mice, respectively (Klasson-Wehler et al. 1996; Orn and Klasson-Wehler 1998). For 14C-labelled PBDE 47, most of the remaining radioactivity was found in adipose tissue. This study also concludes that there can be species difference in metabolism and excretion between rats and mice, because the excreted amount of parent PBDE 47 was 79% in rats, while in mice only 15% of the excreted amount was the parent compound. The metabolites mainly consisted of hydroxylated PBDE 47. The slow elimination of PBDE 47 in rats has also been seen by von Meyernick and coworkers (von Meyernick et al. 1990).

Oral dosing of adult male rats with 14C-labelled PBDE 99 has also shown that lower brominated PBDEs are extensively absorbed (Hakk et al. 2002). Seventy-two hours after administration, more than 50% of the dose was retained and lipophilic tissues were the preferred sites for disposition. Tissue PBDE 99 consisted mainly of the parent compound. Over 43% of the dose was excreted in the feces and mainly as the unmetabolized parent compound. The metabolites found in feces consisted mostly of hydroxylated metabolites, but oxidative debromination was also found (Hakk et al. 2002).

The uptake of 14C-labelled PBDE 209 in the adult has been shown to be low following oral exposure (el Dareer et al. 1987; Norris et al. 1975), and a previous study reported that approximately 10% of a single oral dose is absorbed in adult male rats (Morck et al. 2003). In contrast to the lower brominated PBDEs, PBDE 209 is reported to be metabolized, excreted, and marginally distributed to adipose tissue. Instead, PBDE 209 is found in
plasma and blood-rich tissues. One interesting pathway of metabolism for PBDE 209, in rodents, might be debromination to lower brominated PBDEs (Morck et al. 2003), which is also proposed in fish (Stapleton et al. 2004). Debromination of PBDE 209 has also been seen during photolysis (Soderstrom et al. 2004).

The acute toxicity for PBDEs has been reported to be low, with an oral LD₅₀ in adult rodents ranging between 0.5-5, 2-28, and 2-5 g/kg body weight for penta-, octa-, and decaBDE, respectively (WHO 1994a). For a summary of the general toxic effects of different PBDEs, see WHO, 1994a and (Hardy 2002).

As early as the beginning of the 1980s, reports were being published about PBDEs and their ability to affect the physiology of mammals. For example, it was seen in adult rats that a cumulative dose regime of penta- and octaBDE induced xenobiotic metabolism after 90 days of oral exposure (total dose 39.6 mg pentaBDE/kg body weight and 54 mg octaBDE/kg body weight), manifested as increased cytochrome P-450 and NADPH reductase. This induction was said to be long-lasting (Carlson 1980). Induction of cytochrome P-450 and increased EROD activity have also been seen after repeated oral exposure to pentaBDE in mice and rats (Fowles et al. 1994; Hallgren et al. 2001; Zhou et al. 2001), total doses > 100 mg pentaBDE/kg body weight. In the study by Fowles and coworkers, immunotoxicity was reported for 1,000 mg pentaBDE/kg body weight, concerning the plaque-forming cell response to sheep erythrocytes but not for natural killer cell (NKC) response. In contrast, two more recent in vitro studies, with lower pentaBDE doses, show no effects on the immune system (Fernlof et al. 1997; Reistad et al. 2003).

PBDEs have also been proposed as endocrine disruptors. Interest has been focused on the thyroid hormone system and subchronic treatment with pentaBDE and tetraBDE decrease levels of total thyroxine (T₄) and serum free T₄ (Fowles et al. 1994; Hallgren et al. 2001; Stoker et al. 2004). The total doses used in these experiments exceeded 100 mg/kg body weight. PBDEs have also been shown to bind to the thyroid hormone transporter transthyretin (TTR), which is one of the proposed mechanisms behind the thyroid hormone effects of PBDEs (Hallgren and Darnerud 2002; Meerts et al. 2000). Subchronic exposure to pentaBDE (> 250 mg/kg body weight/d for 14 days) has also revealed elevated serum levels of corticosterone (Fowles et al. 1994), and subchronic treatment of rats and mice with tetraBDE (36 mg/kg body weight/d for 14 days) decreased hepatic vitamin A levels (Hallgren et al. 2001). In an in vitro study with human T47D breast cancer cells, 11 different tri- to pentaBDE congeners acted as agonists of both estrogen receptors α and β, which supports evidence that high doses of PBDEs have endocrine disrupting abilities (Meerts et al. 2001).

The nervous system was shown to be a target for low doses of PBDEs in a study from 2001 (Eriksson et al. 2001). Both PBDE 47 (10.5 mg/kg body
weight) and PBDE 99 (0.8 and 12 mg/kg body weight) were shown to induce deranged spontaneous behavior in adult mice after a single oral dose on PND 10. These aberrations in spontaneous behavior were also shown to be irreversible and in fact increase with increasing age. Also, neonatal PBDE 99 exposure affected learning and memory in adult animals. In a later study by Branchi and coworkers, PBDE 99 was administered to mouse dams from GD 6 to PND 21, and offspring were studied for a number of variables of neurobehavioral development (Branchi et al. 2002). The perinatal PBDE 99 exposure (30 mg/kg body weight/day) gave rise to delayed climbing response in mice pups, increased thigmotaxis, and altered motor activity in adult offspring.

Further evidence for PBDEs as potential neurotoxicants has been reported from in vitro studies. A recent study by Mariussen and Fonnum indicates that pentaBDE can inhibit the uptake of dopamine in vesicles from adult rats, but no effect was seen on the uptake of dopamine and other neurotransmitters in rat synaptosomes (Mariussen and Fonnum 2003). It has also been seen that pentaBDE (>10 µg/ml), but not octaBDE, stimulates the release of arachidonic acid from cultured rat cerebellar granule neurons (Kodavanti and Derr-Yellin 2002), which is proposed to interfere with synaptic plasticity.

Brain development and vulnerable periods

During mammalian development, a vast number of things can go wrong, leading to a variety of malfunctions and disabilities. This is also true for neurogenesis, the development and maturation of the central nervous system (CNS). Neurogenesis is a complex and intricate process that relies on a predetermined plan for the different brain structures as well as for the connections between the different parts of the brain.

Each brain structure has its own vulnerable period, but the development of the CNS can be roughly divided into two major parts. The first part includes early embryonic brain development, a period during which the brain acquires its general adult shape, and the precursors of neurons and glia proliferate. If the brain is exposed to toxic agents or xenobiotics during this period, malformation of the brain can result. This embryonic period takes place during the first two months of gestation in humans and makes up 20% of the gestational period. It is worth noting that in mice the embryonic development constitutes 80% of the entire gestational period.

The second part, commonly referred to as the "brain growth spurt" (BGS) (Davison and Dobbing 1968), includes a series of rapid fundamental developmental changes, for example, maturation of dendritic and axonal outgrowth, establishment of neural connections, synaptogenesis, and proliferation of glia cells with accompanying myelinization (Davison and
Dobbing 1968; Kolb and Whishaw 1989). Also during the BGS, the biosynthesis and concentration of brain lipids increase, primarily in the myelin sheaths where they are relatively stable, although there are areas in the brain which are nearly free of myelinated fibers but where stable lipid components are present. Such areas are the molecular layer of cerebellum and stratum radiatum in the hippocampus (Torvik and Sidman 1965). During the BGS, mice and rats acquire many motor and sensory faculties (Bolles and Woods 1964), and their spontaneous behavior peaks (Campbell et al. 1969). This period varies from species to species as to time of onset and duration. In the human, it begins during the third trimester of pregnancy and continues during the first 2 years after birth, whereas in the guinea pig, for example, it takes place in utero. In rodents, however, the BGS is neonatal, spanning over the first 3-4 weeks of life (Fig. 2).

Figure 2. Rate curves of brain growth in relation to birth in different species. Values are calculated at different time intervals for each species. From Davison and Dobbing, 1968, and Eriksson (unpublished), with permission. Illustration by Ylva Stenlund.

In many mammalian species, such as mice, rats, and humans, the BGS coincides with the lactation period. It is well known that mice exposed to different lipophilic hydrocarbons during the neonatal period distribute them to the brain where they are retained, and the retention is most pronounced after exposure on PND 10, during the peak of the BGS. Examples of such
are DDT, chlorinated paraffin (CP), and certain PCBs (Eriksson 1984, 1998; Eriksson and Darnerud 1985). A series of studies have also shown that PCBs in the mouse dam are transferred to the offspring mainly via the milk, and only a minor part of the dam’s body burden of PCBs is transferred via transplacental transport (Gallenberg and Vodicnik 1987, 1989; Ring et al. 1990; Vodicnik 1986; Vodicnik and Lech 1980). Considering the fact that most of the PCB body burden is transferred to the offspring during a short time period in the beginning of the lactation and that the BGS is perinatal in humans, with the peak around birth, exposure of neonates to various environmental lipophilic pollutants is highly relevant.

Exposure to low doses of both persistent and non-persistent environmental agents during neonatal brain development in mouse has been shown to induce irreversible disruption in brain function in the adult. It is interesting to note that the low doses used in these studies gave no apparent irreversible disturbances when administered to adult mice. Some of the agents that have been shown to induce such neonatal developmental neurotoxic effects are DDT (Ahlbom et al. 1994; Eriksson 1992; Eriksson et al. 1992), organophosphates (Ahlbom et al. 1995), paraquat and MPTP (Fredriksson et al. 1993), pyrethroids (Eriksson 1992; Eriksson and Fredriksson 1991), nicotine (Eriksson et al. 2000; Nordberg et al. 1991), PCBs (Eriksson 1998), and PBDEs (Eriksson et al. 2001; Viberg et al. 2004). Some of these studies also indicate that the inducible, persistent effects of such substances occur only when they are administered during a critical period in the neonatal development of the mouse brain (Ahlbom et al. 1994, 1995; Eriksson 1992; Eriksson et al. 2000; Eriksson et al. 2001).

The cholinergic system, behavior, aging and neurodegenerative disorders

One of the major transmitter systems in the brain is the cholinergic system. This system is associated with many physiological processes and consciousness, such as memory, learning, audition, and vision (Karczmar 1975; Nabeshima 1993; Perry et al. 1999). The role of acetylcholine in cognitive function is well known. Studies have shown that blockage of cholinergic transmission by nicotinic or muscarinic antagonists can produce learning and memory impairments in both humans and animals (Fibiger et al. 1991; Newhouse et al. 1992).

The cholinergic receptors can be divided into two classes: nicotinic and muscarinic (Dale 1914). They belong to different gene families but are both activated by acetylcholine. The names of the receptors indicate that the alkaloids nicotine and muscarine serve as agonists to acetylcholine on the receptors.
During the development of mice and rats, the ontogenesis of most of the cholinergic system takes place during the first 3 to 4 weeks after birth. During this period, variables such as ChAT, AChE, sodium-dependent choline uptake, and muscarinic and nicotinic receptors can be observed to increase in various brain regions (Coyle and Yamamura 1976; Falkeborn et al. 1983; Fiedler et al. 1987; Hohmann et al. 1995; Kuhar et al. 1980). This coincides with the period when the animals acquire many motor and sensory faculties (Bolles and Woods 1964).

Nicotinic receptors originate in the fetal brain during neurulation. Different nicotinic subtypes seem to have different developmental patterns. Nicotinic \(^3\)H-nicotine and \(^3\)H-acetylcholine binding sites originate in the fetal brain during neurulation and rise dramatically in late gestation and after birth in mouse and rat brain (Larsson 1985; Slotkin et al. 1987). A marked decrease in \(^3\)H-nicotine binding immediately after birth has been observed. This was followed by a gradual increase during the postnatal period until the adult levels were reached, PND 28. The number of \(^3\)H-acetylcholine binding sites, however, seemed to drop from embryonic day 18 to PND 1 and remained rather constant until adult levels were reached at PND 7 (Zhang et al. 1990). Yet other studies have seen that the nicotinic binding sites, measured with \(^3\)H-nicotine and \(^125\)I-\(\alpha\)-bungarotoxin, increases until 10 days of age and decreases thereafter to adult levels, which were reached at 25 days of age (Fiedler et al. 1987; Larsson 1985).

The nicotinic acetylcholine receptors are transmitter-gated ion channels. Two snake neurotoxins, \(Naja siamensis\) and \(\alpha\)-bungarotoxin, which specifically bind to nicotinic cholinergic receptors, were the key agents in helping to isolate the nicotinic receptor (Cooper 1996). Neuronal nicotinic receptors, which contain two kinds of subunits, \(\alpha\) and \(\beta\), with \(\alpha\) occurring in at least nine different forms (\(\alpha_2\)-\(\alpha_{10}\)) and \(\beta\) in three (\(\beta_2\)-\(\beta_4\)), show regional distributions in rodent brain (see review by Lucas-Meunier) (Lucas-Meunier et al. 2003). The subunits assemble in different combinations and form a pentameric cationic channel. Some receptors contain two distinct subunits, e.g., \(\alpha_4\beta_2\). According to Flores et al., it seems that nicotine has specific affinity for this subunit set-up (Flores et al. 1992). The \(\alpha_7\) subunit is suggested to form a homo-oligomeric nicotinic channel, and \(\alpha\)-Bungarotoxin seems to have specific affinity for this subunit set-up (Couturier et al. 1990). Neuronal nicotinic receptors are permeable especially to Na\(^+\) and Ca\(^{2+}\) ions. Presynaptic nicotinic receptors mainly modulate neurotransmitter release, and postsynaptic receptors mediate a small minority of fast excitatory transmission by inducing a fast cationic inward current (Dani 2001).

Nicotinic receptors are present in a variety of brain structures, particularly the thalamus, cortex, striatum, hippocampus, and cerebellum (Court et al. 2000; Paterson and Nordberg 2000).

The muscarinic receptors are a heterogeneous group of receptors. They are G-protein coupled and exhibit a slow response time. The G-proteins act
directly on ion channels or are linked to a variety of second messenger systems (Cooper 1996). Five cloned genes, called m1 to m5, have been characterized and give rise to five different types of receptor proteins called M1 to M5 (see Lucas-Meunier et al. 2003). The muscarinic receptor proteins have seven transmembrane helices with the amino-terminus extracellularly and the carboxy terminus intracellularly.

The muscarinic receptor subtypes can generally be divided into two classes. The M1-like receptors, defined by (Hammer et al. 1980), are the M1, M3, and M5 subtypes, which stimulate the phosphoinositol pathway. The M2-like receptors, the M2 and M4 subtypes, act via inhibiting adenylate cyclase (reviewed by Lucas-Meunier et al. 2003). The classic muscarinic antagonists atropine and QNB do not distinguish between the subtypes, but bind to all equally well (Cooper 1996).

Behavior is a major function whereby animals adapt to changes in the environment. Changes in behavior may reveal evidence of the influence of chemical pollution on our natural environment. Spontaneous behavior is especially meaningful in environmental toxicology as it reflects functions that are important for survival of the individual and of the species in the wild, e.g., the mobility needed to search for food, to mate, and to elude predators (Evans 1994).

The cholinergic involvement in behavior has been known for a long time (Russell 1982). The cholinergic system is implicated in regulating general brain excitability during arousal and sleep-wake cycles, and the basal forebrain complex plays a special role in learning and memory functions (Bear 1996).

Interest of the basal forebrain complex led to the discovery that these cells are among the first cells to die during the course of Alzheimer’s disease, which is characterized by a progressive and profound loss of cognitive functions.

It has been shown in many studies that different cholinergic agonists and antagonists affect memory and learning in different behavior test in rats (see Levin 2002). Spatial learning tasks, dependent on external cues for their solution, have been found to be highly sensitive to central cholinergic dysfunctions (Levin 2002; Riekkinen et al. 1990; Sutherland et al. 1982).

Several transmitter systems undergo reductions in receptor function and density during aging, and neurotransmitter plasticity has been shown to be impaired in the aged brain, leading to a reduced ability to adjust to changes in the environment (Pedigo 1994). Aging is also associated with progressive deterioration in learning and memory functions.

Since dysfunction in the cholinergic system has been shown to impair learning and memory (Bartus et al. 1982), it has been suggested that this system in particular is involved in aging processes.

The cholinergic system is also involved in several neurological and neurodegenerative disorders, such as Alzheimer’s disease, Parkinson’s
disease, schizophrenia, and epilepsy. Consistent losses of nicotinic receptors and cholinergic innervations have been measured in brain tissue in Alzheimer’s and Parkinson’s patients (Hellstrom-Lindahl et al. 1999; Nordberg 1993; Paterson and Nordberg 2000). Whether a neonatal exposure to environmental agents can affect aging and neurodegenerative disorders is an intriguing question.
MATERIALS AND METHODS

A more detailed description of the materials and methods is presented in the individual papers.

Chemicals

To conduct the studies in this thesis, 2,2',4,4',5-pentaBDE, 2,2',4,4',5,5'-hexaBDE, 2,2',3,3',4,4',5,5',6,6'-decaBDE, 2,2',4,4',5-penta[14C]BDE, and 2,2',3,3',4,4',5,5',6,6'-deca[14C]BDE were kindly donated by the research group led by professor Åke Bergman, Department of Environmental Chemistry, Stockholm University, Stockholm, Sweden. The different PBDEs were 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% (w:w) fat emulsion vehicle. The use of a 20% fat emulsion vehicle was used to obtain a more physiologically appropriate absorption and hence distribution (Keller and Yeary 1980; Palin et al. 1982), since fat content of mouse and rat milk is around 14%.

α-Bungarotoxin, N-[propionyl-2H]-propionylated (55.0 Ci/mmol), and I-Quinuclidinyl[phenyl-4-3H]benzilate (QNB, 43.0 Ci/mmol) were obtained from Amersham, U.K. α-Bungarotoxin, (-)-nicotine-bi-(-)-tartrate and atropine sulfate were obtained from Sigma, U.S.A.

Animals

Pregnant NMRI mice, C57/Bl mice, and Sprague-Dawley rats 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 hour 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 8-12 mice or rats 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 NMRI and Sprague-Dawley females were sacrificed 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. C57/Bl
pups were separated and housed in rooms for male or female mice only, and raised under the same conditions as detailed above.

Treatment

In the experiments carried out in this thesis, given below, animals received a single oral dose of a polybrominated diphenyl ether.

In study I, NMRI mice were exposed to 8 mg (14 µmol) 2,2',4,4',5-pentaBDE (PBDE 99)/kg body weight or 1.5 MBq 2,2',4,4',5-penta[14C]BDE/kg body weight on PND 3, 10, or 19.

In study II, NMRI mice were exposed to 8 mg (14 µmol) 2,2',4,4',5-pentaBDE (PBDE 99)/kg body weight on PND 10.

In study III, NMRI mice were exposed to 0.45, 0.9, or 9.0 mg (0.7, 1.4, or 14 µmol) 2,2',4,4',5,5'-hexaBDE (PBDE 153)/kg body weight on PND 10.

In study IV, NMRI mice were exposed to 2.22 or 20.1 mg (2.3 or 21 µmol) 2,2',3,3',4,4',5,5',6,6'-decaBDE (PBDE 209)/kg body weight on PND 3 or 19, or exposed to 1.34, 13.4, or 20.1 mg (1.4, 14, or 21 µmol) 2,2',3,3',4,4',5,5',6,6'-decaBDE/kg body weight on PND 10. Also male NMRI mice were exposed to 1.5 MBq 2,2',3,3',4,4',5,5',6,6'-deca[14C]BDE/kg body weight on PND 3, 10, or 19.

In study V, C57/Bl mice were exposed to 0.4, 0.8, 4.0, 8.0, or 16 mg (0.7, 1.4, 7.0, 14, or 28 µmol) 2,2',4,4',5-pentaBDE (PBDE 99)/kg body weight on PND 10.

In study VI, Sprague-Dawley rats were exposed to 0.8, 8.0, or 16 mg (1.4, 14, or 28 µmol) 2,2',4,4',5-pentaBDE (PBDE 99)/kg body weight on PND 10.

In studies I–VI, control animals received 10 ml 20% fat emulsion vehicle/kg body weight.

Behavioral tests

Spontaneous behavior

Animals were observed for spontaneous behavior in all studies.

In study I, male NMRI mice were tested at the age of 4 months.

In study II, male NMRI mice were tested at the age of 2 months.

In study III, male NMRI mice were tested at the ages of 2, 4, and 6 months.

In study IV, male NMRI mice were tested at the ages of 2, 4, and 6 months.

In study V, male and female C57/Bl mice were tested at the ages of 2, 5, and 8 months.
In study VI, male Sprague-Dawley rats were tested at the age of 2 months.

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 8-10 animals were randomly picked from the 3-5 different litters in each treatment group, at each testing occasion. Motor activity was measured for a 60-min. period, divided into spells of 3 × 20 min. (0-20, 20-40, and 40-60 min.), 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 animal 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 animal movements, shaking (tremors), and grooming, were registered by a pick-up (mounted on a lever with a counterweight), connected to the test cage.

Nicotine-induced behavior

Nicotine induced behavior was tested in study II only, in male NMRI mice at the age of 2 months, directly after the spontaneous behavior test as previously described (Nordberg et al. 1991).

The mice received saline- or nicotine-injections (80 μg/kg body weight) directly after the spontaneous behavior test and thereafter the animals were tested for locomotion, rearing, and total activity, as described for spontaneous behavior, for 3 consecutive 20-min. periods (60-80, 80-100, and 100-120 min.).

Swim maze

The behavior test was performed in study III in male NMRI mice at age 6 months. Randomly picked mice from 3-5 different litters in each treatment group were tested.

The swim maze, of Morris water maze type (Morris 1981), was a circular, gray tub, with a diameter of 102 cm and depth of 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 for 5 consecutive days and the animals were given five trials each day. Before
each day’s first trial, the mouse was placed on the submerged platform for 30 sec. It was then released in the south position facing the wall of the tub and was allowed 30 sec. to locate the platform. If the mouse failed to find the platform within 30 sec., it was gently placed on the platform. After each trial, the mouse was left on the platform for 30 sec. This procedure was repeated for 4 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 sec. 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).

Receptor assays
The mice were sacrificed by decapitation within one week after the behavioral tests in all experiments. Brains were dissected on an ice-cold glass plate and the hippocampus was immediately frozen at -80ºC until assayed. In study III, hippocampi were pooled from two animals and analyzed, while in study VI, an individual hippocampus was assayed. Hippocampi were placed in 24 times their own weight of ice-cold sucrose (0.32 M) and thereafter homogenized using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 1,000 g for 10 min. and the supernatant further centrifuged at 17,000 g for 30 min. The resulting pellet was suspended and homogenized in the original volume of ice-cold NaKPO₄ buffer to yield a crude synaptosomal P2 fraction (Gray and Whittaker 1962) with a protein content of about 2 mg/ml, determined by the method of Udenfriend et al. (Udenfriend et al. 1972) as described by Lorenzen and Kennedy (Lorenzen and Kennedy 1993).

Measurement of the nicotinic binding sites was performed using tritium-labelled \( \text{D-Bungarotoxin (N-[propionyl-}^3\text{H]-propionylated (55.0 Ci/mmol))}} \). Measurement of the muscarinic binding sites was performed using tritium-labelled QNB \( \text{(I-Quinuclidinyl[phenyl-4-}^3\text{H]}\text{benzilate (43.0 Ci/mmol))}} \). A liquid scintillation analyzer (Packard Tri-Carb 1900 CA) was used to count radioactivity.

\( ^3\text{H-}\alpha\text{-bungarotoxin binding} \)
The specific binding was carried out following the method of Falkeborn et al. (Falkeborn et al. 1983), with certain changes. \( \alpha\text{-Bungarotoxin was used instead of tubocurarin for determination of non-specific binding.} \)
Aliquots of the P2 fraction were incubated with [\(^3\)H]-\(\alpha\)-Bungarotoxin in NaKPO\(_4\) buffer. To measure non-specific binding, parallel samples were incubated with \(\alpha\)-Bungarotoxin. Incubation was terminated by centrifugation and the radioactivity of the remaining pellet was determined.

Specific binding was determined by calculating the difference in the amount bound in the presence vs. absence of \(\alpha\)-Bungarotoxin.

\(^3\)H-QNB-binding

The specific binding was carried out following the method of Nordberg and Winblad (Nordberg and Winblad 1981), as described by Eriksson and Nordberg (Eriksson and Nordberg 1986).

Aliquots of the P2 fraction were incubated with \([\(^3\)H]-QNB\) in NaKPO\(_4\) buffer. To measure non-specific binding, parallel samples were incubated with atropine. Incubation was terminated by centrifugation and the radioactivity of the remaining pellet was determined.

Specific binding was determined by calculating the difference in the amount bound in the presence vs. absence of atropine.

Statistical analysis

**Spontaneous behavior**

The data from the spontaneous behavior tests were subjected to a split-plot ANOVA (analysis of variance), and pairwise testing between PBDE-treated groups and control group was performed using a Tukey HSD (honestly significant difference) test (Kirk 1968) (Papers I, II, III, IV, V, and VI). From the spontaneous behavior test, normal habituation is defined as a decrease in the locomotion, rearing, and total activity variables in response to the diminishing novelty of the test chamber over the 60-min. period.

**Habituation capability**

From the spontaneous behavior tests, 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 equations were used: \(100 \times (\text{counts locomotion} \ 40-60 \ \text{min.}/\text{counts locomotion} \ 0-20 \ \text{min.})\), \(100 \times (\text{counts rearing} \ 40-60 \ \text{min.}/\text{counts rearing} \ 0-20 \ \text{min.})\), and \(100 \times (\text{counts total activity} \ 40-60 \ \text{min.}/\text{counts total activity} \ 0-20 \ \text{min.})\). This ratio was used to analyze alteration in habituation between the different spontaneous behavior testing occasions within an experiment, in order to measure whether spontaneous behavior gets worse with increasing age. These data were subjected to a 2-way ANOVA (Papers III, IV, and V).
Nicotine-induced behavior

The data from the nicotine-induced behavior test were subjected to a split-plot ANOVA, and pairwise testing between PBDE-treated groups and control group was performed using a Tukey HSD test (Kirk 1968) (Paper II).

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 performances 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 behavioral data of day 5 (difference between trial 1 and 5) was submitted to one-way ANOVA and pairwise testing using Duncan’s test (Paper III).

[^H-α-Bungarotoxin]

The data from[^H]-α-Bungarotoxin binding were subjected to one-way ANOVA and pairwise testing using Duncan’s test (Paper III).

[^H-QNB]

The data from[^H]-QNB binding were subjected to one-way ANOVA and pairwise testing using Duncan’s test (Paper VI).
RESULTS AND DISCUSSION

In this thesis, the developmental neurotoxicity of PBDEs in rodents after neonatal exposure has been investigated. These studies demonstrate that there is a defined critical period during the rapid growth and development of the brain, when PBDEs can cause alterations in spontaneous behavior, habituation, and learning and memory in the adult animal. The neonatal PBDE exposure affects the cholinergic system, manifested as altered response to nicotine and changes in nicotinic and muscarinic cholinergic receptors in the brain of the adult animal. These developmental neurotoxic effects are both dose-response related and time-response related, i.e., the effects get worse with increasing age. Also, these effects do not seem to differ between the sexes or between different mouse strains or between mouse and rat.

Effects of neonatal exposure to PBDEs during a defined critical period of brain development on behavior, learning and memory, and cholinergic system

The objective of papers I, II, and III was to ascertain whether there is a defined critical period during the BGS in the neonatal mouse when it is susceptible to the effects of PBDE 99, and to study the uptake and retention of PBDE 99 in the neonatal mouse brain. A further objective was to investigate whether the cholinergic system could be affected by neonatal PBDE 99 exposure, by studying the response in the adult animal to the cholinergic agent nicotine. Another aim was to establish whether neonatal PBDE 153 exposure shows dose-response and time-response dependency and if learning and memory and cholinergic receptors were affected in the adult mouse.

In paper I, male NMRI mice were exposed to a single oral dose of 8 mg (14 μmol) PBDE 99/kg body weight on PND 3, 10, or 19 and observed for spontaneous behavior at 4 months of age, or 1.5 MBq [14C]PBDE 99/kg body weight on PND 3, 10, or 19 and sacrificed 24 hours or 7 days after exposure for detection of radioactivity in tissue samples.
The mice exposed neonatally to PBDE 99 on day 3 or 10 showed a deviation from the normal habituation, but not mice exposed to PBDE 99 on PND 19 (Fig. 3).

Figure 3. Spontaneous behavior in 4-month-old male NMRI mice after neonatal exposure to 8 mg (14 μmol) PBDE 99/kg body weight, as a single oral dose, or 20% fat emulsion (control), on day 3, 10, or 19. The statistical difference between control mice and PBDE 99-treated mice is indicated by: A) Significantly different vs. controls, P ≤ 0.01. The height of the bars represents the mean value ± SD.
The most pronounced effect was seen in mice exposed to PBDE 99 on neonatal day 10. At the age of 4 months, these mice displayed a significantly hypoactive behavior during the first 20 min. of the 60-min. period, for all three spontaneous behavior variables, locomotion, rearing, and total activity. They also showed hyperactive behavior for all three spontaneous behavior variables during the last 20 min. of the 60-min. period. This supports the earlier findings concerning developmental neurotoxicity of the brominated flame retardants PBDE 47 and PBDE 99 (Eriksson et al. 2001). In that study, male mice (NMRI) exposed neonatally on day 10 to 0.8 or 12 mg PBDE 99/kg body weight, as a single oral dose, showed differences in spontaneous behavior at 4 months of age, manifested as a hypoactive condition during the first 20-min. period, while toward the end of the 60-min. period, the mice became hyperactive.

The amount of toxic agent present in the brain at different neonatal ages may vary. Previous studies have shown a pronounced retention of lipophilic chlorinated hydrocarbons (e.g., DDT, PCB 52, PCB 153, and chlorinated paraffins) or their metabolites in the brain when administered on neonatal day 10 (Eriksson 1984, 1998; Eriksson and Darnerud 1985). The amount of PBDE 99 or its metabolites, seen after neonatal exposure in the 3 different age categories, is in the same range as seen for certain PCBs and DDT, i.e., 3-5‰ of the administered dose (Eriksson 1984, 1998; Eriksson and Darnerud 1985). Differences in the amounts of PBDE 99 present in the neonatal brain at the different neonatal ages do not appear to explain the different behavioral effects seen in adult mice exposed on day 3, 10, or 19. The amount of radioactivity was not higher in the brains of animals exposed on day 3 or 10, yet a disturbance is seen in the spontaneous behavior of animals exposed to PBDE 99 on PND 3 or 10 but not in animals exposed on PND 19. The disturbances seen after exposure on day 3 may be attributable to the fact that the amount of PBDE 99 left in the brain 7 days later was enough to induce behavioral disturbances, which indicates that there is a defined critical period during neonatal brain development when PBDEs can induce neurotoxic effects.

From paper II, it is seen that the developing cholinergic system can be a target for PBDEs. Male NMRI mice were exposed to a single oral dose of 8 mg (14 µmol) PBDE 99/kg body weight or a 20% fat emulsion vehicle on PND 10 and tested for spontaneous behavior at 2 months of age. Immediately after the spontaneous behavior testing, the mice received saline or nicotine injections (80 µg/kg body weight) and thereafter the animals were tested another 60 min. for nicotine-induced behavior.

The neonatally PBDE 99 treated mice showed the same non-habituating behavior at the age of 2 months as mice of the age 4 months in paper I. In the nicotine-induced behavior test, the response to nicotine was changed, and the PBDE 99 treated animals showed a totally opposite response compared to the vehicle treated mice. In the control animals, hyperactivity was seen after
injection of 80 µg nicotine base/kg body weight, while the neonatally PBDE 99 treated mice showed hypoactivity. This response to nicotine after neonatal exposure to PBDE 99 is the same as previously seen for animals neonatally exposed to nicotine (Eriksson et al. 2000; Nordberg et al. 1991) and known to lack or have a reduced amount of nicotinic receptors of low-affinity type (corresponding to α-Bungarotoxin binding sites). These animals showed hypoactivity after adult exposure to nicotine, in contrast to animals neonatally exposed to 20% fat emulsion vehicle or saline, which showed hyperactivity. The neonatal exposure on PND 10 coincides with the rapid development of the cholinergic system (Falkeborn et al. 1983; Fiedler et al. 1987), and this indicates that PBDE 99 has an effect on the cholinergic system. Furthermore, it is of special interest to note that the hyperactive condition in PBDE 99 treated mice is observed again at the end of the test period (100-120 min.), indicating the persistence of the adverse spontaneous motor behavior defect. This type of reaction has also been seen in mice neonatally exposed to the ortho-substituted PCB, 2,2′,5,5′-tetrachlorobiphenyl (PCB 52) (Eriksson and Fredriksson 1996b).

In paper III, male NMRI mice were exposed to a single oral dose of 0.45, 0.9, or 9.0 mg (0.7, 1.4, or 14 µmol) PBDE 153/kg body weight on PND 10 and thereafter tested for spontaneous behavior at 2, 4, and 6 months of age, in order to assess dose-response and time-response relationships. After the spontaneous behavior testing, a learning and memory test was conducted in 6-month-old mice in the Morris water maze, after which the animals were sacrificed and their brains analyzed for nicotinic binding sites with α-Bungarotoxin, with the aim of detecting possible changes in the cholinergic system.

The spontaneous motor behavior data showed a disruption of habituation in animals exposed to PBDE 153 (Fig. 4). Normal habituation was displayed in the control animals, but the animals exposed to PBDE 153 showed a decreased activity during the beginning of the 60-min. period, while toward the end of the test period they became hyperactive. This derangement in spontaneous behavior tests also indicates that the functional disorder is dose-response related. In 2-month-old mice, the two highest doses of PBDE 153 (0.9 and 9.0 mg/kg body weight) caused a significant change in spontaneous behavior. In addition, the effect seen after neonatal exposure to 9.0 mg PBDE 153/kg body weight is more pronounced than the effect seen after neonatal exposure to 0.9 mg PBDE 153/kg body weight, indicated by deviations from the control group for all three behavioral variables during the first and last 20-min. periods of the 60-min. period.

Furthermore, the spontaneous behavior tests indicated that the change in behavioral 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
Figure 4. Spontaneous behavior (locomotion) in 6-month-old male NMRI mice exposed to a single oral dose of either 20% fat emulsion vehicle or 0.45, 0.9, or 9.0 mg (0.7, 1.4, or 14 µmol) PBDE 153/kg body weight at an age of 10 days. 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 PBDE 153 dose, \( P \leq 0.01 \); b) Significantly different vs. closest lower PBDE 153 dose, \( P \leq 0.05 \). The height of the bars represents the mean value ± SD.

clearly demonstrated in mice receiving the highest dose of PBDE 153 (9.0 mg/kg body weight), where both the habituation ratio for locomotion and total activity increased significantly (Fig. 5). This means that the ability to habituate to a novel environment became worse with age after neonatal exposure to PBDE 153. 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 behavioral defects have been reported in mice neonatally exposed to PBDE 47 and PBDE 99 (Eriksson et al. 2001). The irreversibility and time-dependency of these effects, reflected in the fact that they worsen with age, indicate an acceleration of a dysfunctional process. It is interesting to see that the observed changes in spontaneous behavior are very similar to the changes seen after neonatal exposure to certain PCBs. Neonatal exposure in mice on day 10 to some ortho-substituted PCBs, such as 2,4,4'-trichlorobiphenyl (PCB 28), 2,2',5,5'-tetrachlorobiphenyl (PCB 52), 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153), and to some co-planar PCBs, 3,3',4,4'-tetrachlorobiphenyl (PCB 77) 3,3',4,4',5-pentachlorobiphenyl (PCB 126), and 3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169), has been shown to
Figure 5. Habituation capability (locomotion) in 2-, 4-, and 6-month-old male NMRI 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 (0.7, 1.4, or 14 µmol) PBDE 153/kg body weight at an age of 10 days. The habituation ratio for the variable of locomotion 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. Statistical differences are indicated by: A (P ≤ 0.01 4 or 6 months vs. 2 months); B (P ≤0.01 6 months vs. 4 months).

cause a hypoactive condition in spontaneous behavior during the first 20-min. period, while toward the end of the 60-min. period the mice became hyperactive (Eriksson 1998). These effects were shown to be both dose-response- and time-response related. The doses used in all of these studies were approximately the same as the PBDE doses on a molar level, and the effects were induced during a defined critical period of brain development. This indicates that some PBDEs have the ability to cause developmental neurotoxic effects as has been seen previously for certain PCBs.

In the swim maze of the Morris water maze type, adult mice neonatally exposed to the two highest doses of PBDE 153 (0.9 mg and 9.0 mg/kg body weight) performed significantly worse than control animals (Fig 6).
Figure 6. Swim maze performance in 6-month-old male NMRI mice exposed to a single oral dose of either 20% fat emulsion vehicle or 0.45, 0.9, or 9.0 mg (0.7, 1.4, or 14 μmol) PBDE 153/kg body weight at an age of 10 days. Latencies in locating the platform were measured during the acquisition period (days 1–4) and the relearning period (day 5). 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 PBDE 153 dose, P \leq 0.01; b) Significantly different vs. closest lower PBDE 153 dose, P \leq 0.05; C) Significantly different vs. lowest PBDE 153 dose, P \leq 0.01; c) Significantly different vs. lowest PBDE 153 dose, P \leq 0.05.

During the 4-day acquisition period, all animals decreased the time needed to locate the submerged platform, but the animals exposed to PBDE 153 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 mg and 9.0 mg PBDE 153/kg body weight displayed longer latencies in locating the platform than animals receiving 0.45 mg PBDE 153/kg 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 of advancing age in the 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 PBDE 153 (0.9 mg 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. 2001).

In paper III, it could also be seen that the animals exposed neonatally to the highest dose of PBDE 153 (9.0 mg/kg body weight) received effects on the nicotinic cholinergic receptors in the brain, manifested as a decrease in \(\alpha\)-Bungarotoxin binding in hippocampus (Tab. 1).

Table 1. Effects on nicotinic cholinergic receptors in hippocampus in 6-month-old male NMRI mice after neonatal exposure on day 10 to PBDE 153

<table>
<thead>
<tr>
<th>Treatment (mg/kg body weight)</th>
<th>(n)</th>
<th>[^{[H]}\alpha\text{-Bungarotoxin binding (pmol/g protein)}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>9</td>
<td>35.16 ± 3.30</td>
</tr>
<tr>
<td>0.9</td>
<td>6</td>
<td>32.67 ± 4.04</td>
</tr>
<tr>
<td>9.0</td>
<td>6</td>
<td>27.92 ± 5.22**</td>
</tr>
</tbody>
</table>

\(^{a)}\) Male NMRI neonatal mice were exposed to a single oral dose of 0.9 or 9.0 mg (1.4 and 14 \(\mu\)mol) PBDE 153/kg body weight and controls to 20% fat emulsion in the same manner. The animals were killed at 6 months of age and \[^{[H]}\alpha\text{-Bungarotoxin binding (mean ± SD)}\] was assessed in the P2 fraction. The statistical difference is indicated by: **\(P \leq 0.01\).

This decrease was about 21%. It has also been shown that adult animals exposed to PBDE 99 on PND 10 have a decreased amount of nicotinic receptors (Viberg et al. 2004) and an altered response to the cholinergic agent nicotine (Paper II). Altered performance in swim mazes and a reduced amount of nicotinic receptors have also been seen in mice neonatally exposed to certain PCBs (PCB 52 and PCB 126) (Eriksson and Fredriksson 1996a, 1998). It has also been seen that neonatal exposure to PCB 52 alters the adult response to nicotine (Eriksson and Fredriksson 1999b). 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 behavioral 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). Noteworthy, 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 nicotinic cholinergic receptors in cortex and hippocampus (James and Nordberg 1995; Nordberg 1993).

Effects of neonatal exposure to a highly brominated PBDE

The objective of paper IV was to study whether exposure during the neonatal brain development to PBDE 209, i.e., the fully brominated PBDE, could induce persistent neurotoxic effects on spontaneous motor behavior in the adult mouse. To evaluate whether the tissue distribution and/or a defined critical phase of brain development is the underlying cause of developmental effects, a study of the uptake and retention of $^{14}$C-labelled PBDE 209 at different stages of neonatal mouse brain development was conducted.

In paper IV, male NMRI mice were exposed to 2.22 or 20.1 mg (2.3 or 21 µmol) PBDE 209/kg body weight on PND 3 or 19, or exposed to 1.34, 13.4, or 20.1 mg (1.4, 14, or 21 µmol) PBDE 209/kg body weight on PND 10, and tested for spontaneous behavior at 2, 4, and 6 months of age. Male NMRI mice were also exposed to 1.34, 13.4, or 20.1 mg (1.34, 13.4, or 20.1 µmol) PBDE 209/kg body weight on PND 3, 10, or 19 and sacrificed 24 hours or 7 days after exposure for detection of radioactivity in tissue samples.

The spontaneous motor behavior data showed a disruption of habituation in adult mice neonatally exposed to the highest dose of PBDE 209 (20.1 mg/kg body weight) on day 3 only (Fig. 7). At the age of 2 months, these mice displayed a significantly hypoactive behavior during the first 20 min. of the 60-min. period for all three spontaneous behavior variables, locomotion, rearing and total activity, while towards the end of the 60-min. period, they showed hyperactive behavior. Although the deranged spontaneous behavior is induced in day 3 animals, the alteration in spontaneous behavior is the same as observed for mice neonatally exposed to PBDE 47, 99, and 153 on PND 10.

The data from spontaneous behavior in 4- and 6-month-old mice show the same thing. Mice exposed to PBDE 209 on PND 3 have a disrupted spontaneous behavior, but not mice exposed to PBDE 209 on PND 10 or 19. This means that the neurotoxic effect of neonatal PBDE 209 exposure is induced during a restricted period of brain development and that the effect is persistent. When comparing the habituation capability in spontaneous behavior testing at 2, 4, and 6 months of age, it is evident that the disruption of habituation worsens with age. This form of developmental neurotoxic effect is very similar to what was seen in the PBDE 153 study (paper III).
Figure 7. Spontaneous behavior (locomotion) in 6-month-old male NMRI mice after neonatal exposure to 20.1 mg (21 µmol) PBDE 209/kg body weight, as a single oral dose, or 20% fat emulsion (control), on day 3, 10, or 19. The statistical difference between control mice and PBDE 209-treated mice is indicated by: A) Significantly different vs. controls, $P < 0.01$. The height of the bars represents the mean value ± SD.

In paper I, it was demonstrated that PBDE 99 elicited the same kind of altered spontaneous behavior when administered on PND 10 as that seen for certain PCBs (Eriksson 1998). From these studies, it was also evident that effects from these substances can be seen after exposure on PND 3, but those effects are proposed to be due to the presence of the molecules in the brain during the peak of BGS, namely, around day 10 (Eriksson 1998; Eriksson et al. 1992; Eriksson et al. 2000). The amount of toxic agent present in the brain at different neonatal ages may vary. In paper I, a pronounced retention of PBDE 99 in the brain was seen when administered on neonatal day 10 and it has also been seen for other lipophilic chlorinated hydrocarbons or their metabolites (e.g., DDT, PCB 52, PCB 153 and a chlorinated paraffin) (Eriksson 1984, 1998; Eriksson and Darnerud 1985). Mice exposed to [14C]PBDE 209 on PND 3 or 10 show approximately the same amount of radioactivity in the brain after 24 hours, 4.8 and 4.0‰, respectively, as has previously been seen for animals exposed to PBDE 99 (Paper I), DDT, and PCBs (Eriksson 1984, 1998), while the amount of radioactivity in mice exposed to [14C]PBDE 209 on PND 19 was only 0.6‰ (Fig 8). During the
course of time, the amount of radioactivity from PBDE 99, DDT, and PCBs was the same or decreased over the 7-day period. In contrast, the radioactivity from PBDE 209 significantly increased, to 7.4 or 10.5‰, in animals exposed to [14C]PBDE 209 on PND 3 or 10, but for animals exposed on PND 19, no increase or decrease was seen.

There are only a few studies known to deal with the kinetics of PBDE 209. In one study, adult rats were fed with food containing [14C]PBDE 209. The uptake was low but still detectable (el Dareer et al. 1987) and radioactivity was seen in a variety of organs and tissues, including the brain. In the present study, two reference tissues, liver and heart, were studied for radioactivity from [14C]PBDE 209 administered on PND 3, 10, or 19 (Fig 8). The mean radioactivity from the liver 24 hours after oral administration on day 3, 10, or 19 was 126‰, 94‰, or 58‰, respectively, of the total radioactivity administered. Over the 7-day period, there was a significant decrease of the radioactivity in the liver to 58, 46, or 3.1‰ for animals administered on day 3, 10, or 19, respectively. The mean radioactivity from the heart 24 hours after oral administration on day 3, 10, or 19 was 2.8‰, 3.1‰, or 2.2‰, respectively, of the total radioactivity administered. Over the 7-day period, there was no change or a significant decrease of radioactivity in the heart to 3.4, 3.2, and 0.8‰ for animals administered on day 3, 10, or 19, respectively. It is of special interest that even though an increase in the amount of radioactivity was seen in the brain during the 7-day period, the mean radioactivity from the liver and the heart tissues decreased or was unchanged, after oral administration on day 3, 10, or 19. This and the fact that effects on spontaneous behavior were only seen in mice exposed neonatally on day 3 suggest that the effect is caused by one or more metabolites of the parent compound, PBDE 209. If the parent compound caused the neurotoxic effect, mice exposed to PBDE 209 on day 10 should have shown these neurotoxic effects, because of the amount of radioactivity present 24 hours after administration on day 10. For animals exposed to 20.1 mg PBDE 209/kg body weight on PND 10, the calculated amount in the brain after 24 hours would be 419 pmol, and for animals exposed to 20.1 mg PBDE 209/kg body weight on day 3, the calculated amount in the brain after 7 days would be 388 pmol, which is approximately the same amount. This is also the case when comparing these two groups according to radioactivity. This, too, indicates a defined critical period and that it is the amount present around PND 10, during the peak of BGS, that is enough to induce persistent developmental neurotoxic effects.
Figure 8. Radioactivity levels (% of total administered radioactivity) in mouse brain, heart, and liver 24 h and 7 days after a single oral administration of 1.5 MBq [14C]PBDE 209/kg body weight (40.5 µCi/kg body weight) to neonatal male NMRI mice at the age of 3 days, 10 days, or 19 days. The statistical differences between 24 h and 7 days are indicated by asterisks: **P<0.01. The height of the bars represents the mean value ± SD from 4 to 7 animals.
Gender, strain, and species comparison of neurobehavioral effects of neonatal exposure to PBDEs

The object of papers V and VI was to investigate whether neurotoxicity of PBDEs is inducible in mouse strains other than NMRI and in females as well as in males and in a different species, namely, the rat. A further objective was to investigate if the cholinergic system is affected in the rat, as earlier seen in the mouse.

In paper V, male and female C57/Bl mice were exposed to 0.4, 0.8, 4.0, 8.0, or 16 mg (0.7, 1.4, 7.0, 14, or 28 µmol) PBDE 99/kg body weight on PND 10 and tested for spontaneous behavior at 2, 5, and 8 months of age.

In male C57/Bl mice, the four highest doses of PBDE 99 (0.8, 4.0, 8.0 and 16 mg/kg body weight) caused a significant change in spontaneous behavior on all three testing occasions (Fig. 9). The spontaneous behavior tests indicated that the change in behavioral motor activity worsens with increasing age, as the aberrations appeared to be most pronounced in the 8-month-old male C57/Bl mice, which was also true when looking at habituation capabilities. This change over time is clearly demonstrated in mice receiving the highest dose of PBDE 99 (16 mg/kg body weight). This type of both dose-response and time-response behavioral defects and reduced habituation capability with age is in agreement with what we have seen in male NMRI mice neonatally exposed to PBDE 47, PBDE 99 (Eriksson et al. 2001), PBDE 153 (paper III), and PBDE 209 (paper IV). Developmental neurotoxicity of PBDE 99 has been seen in yet another mouse strain, CD-1 Swiss male offspring (Branchi et al. 2002). These studies indicate that the developmental neurotoxic effects of PBDEs are not specific for one mouse strain.

In female C57/Bl mice, the four highest doses of PBDE 99 (0.8, 4.0, 8.0, and 16 mg/kg body weight) caused a significant change in spontaneous behavior (Fig. 9). The spontaneous behavior tests indicated that the change in behavioral motor activity worsens with increasing age, as the aberrations appeared to be most pronounced in the 8-month-old female C57/Bl mice, which was also true when looking at habituation capabilities. In female mice, this is demonstrated at the doses 4.0 and 16 mg PBDE 99/kg body weight, where the ability to habituate is significantly decreased for the locomotion variable. Also, for the rearing variable, the ability to habituate decreased over time in female C57/Bl mice receiving 0.8, 8.0, or 16 mg PBDE 99/kg body weight. This type of both dose-response and time-response behavioral defects and reduced habituation capability with age corresponds well to the effects seen for male C57/Bl mice. When comparing male and female C57/Bl mice, both dose-response and time-response effects are similar. However, the time-response effect appears, from this study, to be more pronounced in female mice, where this effect was already seen in the rearing variable at the dose of 0.8 mg PBDE 99/kg body weight.
Figure 9. Spontaneous behavior (locomotion) of 8-month-old male and female C57/Bl mice exposed to a single oral dose of either 20% fat emulsion vehicle or 0.4, 0.8, 4.0, 8.0, or 16 mg (0.7, 1.4, 7.0, 14, or 28 µmol) PBDE 99/kg body weight at 10 days of age. 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. 0.4 mg PBDE 99/kg body weight, $P \leq 0.01$; b) Significantly different vs. 0.4 mg PBDE 99/kg body weight, $P \leq 0.05$; C) Significantly different vs. 0.8 mg PBDE 99/kg body weight, $P \leq 0.01$; D) Significantly different vs. 4.0 mg PBDE 99/kg body weight, $P \leq 0.01$; d) Significantly different vs. 4.0 mg PBDE 99/kg body weight, $P \leq 0.05$; E) Significantly different vs. 8.0 mg PBDE 99/kg body weight, $P \leq 0.01$. The height of the bars represents the mean value ± SD.

Furthermore, the capacity of PBDEs to induce behavioral neurotoxic effects in different strains and sexes again show similarities with the well known environmental pollutants, PCBs. Different studies have shown that PCBs can induce neurotoxic effects in both male and female mice and rats (Eriksson

In paper VI, male Sprague-Dawley rats were exposed to 0.8, 8.0, or 16
mg (1.4, 14, or 28 µmol) PBDE 99/kg body weight on PND 10 and tested for
spontaneous behavior at 2 months of age, after which the animals were
sacrificed and their brains analyzed for muscarinic binding sites with QNB,
to detect changes in the cholinergic system.

In male Sprague-Dawley rats, the two highest doses of PBDE 99 (8.0 and
16 mg/kg body weight) caused a significant change in spontaneous behavior
at 2 months of age (Fig. 10).

In male Sprague-Dawley rats, the two highest doses of PBDE 99 (8.0 and
16 mg/kg body weight) caused a significant change in spontaneous behavior
at 2 months of age (Fig. 10).

Figure 10. Spontaneous behavior (locomotion) in 2-month-old male Sprague-
Dawley rats exposed to a single oral dose of either 20% fat emulsion vehicle or 0.8,
8.0, or 16 mg PBDE 99/kg body weight (1.4, 14, or 28 µmol PBDE 99/kg body
weight) at an age of 10 days. The statistical differences are indicated as: A)
Significantly different vs. controls, \( P \leq 0.01 \); B) Significantly different vs.
closest lower PBDE 99 dose, \( P \leq 0.01 \); C) Significantly different vs. lowest PBDE 99
dose, \( P \leq 0.01 \). The height of the bars represents the mean value ± SD.

Animals from the two highest dose categories had a significantly lower
activity level than the control animals the first 20-min. period (0-20 min.)
regarding all three behavioral variables (locomotion, rearing and total
activity), while toward the end of the testing period (40-60 min.), they
showed a significantly higher activity level compared to the controls. The
spontaneous behavior test also indicated that the changes in behavioral motor activity are dose-response related, because the observed change in spontaneous behavior was more pronounced in animals exposed to 16 mg PBDE 99/kg body weight than in animals exposed to 8 mg PBDE 99/kg body weight. These types of changes in spontaneous behavior, i.e., dose-response behavioral defects, have been seen in male NMRI mice neonatally exposed to PBDE 47, PBDE 99 (Eriksson et al. 2001), PBDE 153 (paper III), and PBDE 209 (paper IV), and in male and female C57Bl mice neonatally exposed to PBDE 99 (paper V). Taken together, these studies indicate that the developmental neurotoxic effects of PBDEs are not specific for one species but can be induced in both rats and mice.

In paper VI, it could also be seen that rats exposed neonatally to the highest dose of PBDE 99 (16 mg/kg body weight) received effects on the muscarinic cholinergic receptors in the adult brain, manifested as a decrease in QNB binding in hippocampus. This decrease was about 7%. Effects in the cholinergic system have not previously been seen in rats, but are in agreement with the findings in papers II and III, where effects in the cholinergic system are seen in mice after neonatal exposure to PBDE 99 and 153. In addition, we have seen that neonatal exposure to PBDE 99 on PND 10 gives rise to a decrease in nicotinic receptors in the hippocampus in adult mice (Viberg et al. 2004). Therefore, muscarinic or nicotinic receptor changes in the cholinergic system, or both, may be one of the mechanisms behind the behavioral changes seen after neonatal PBDE exposure.

General discussion

It is known that exposure to certain xenobiotics during the fetal period can cause functional anomalies of the CNS that result in behavioral, cognitive, and motor defects. Furthermore, it is also known from developmental neuroscience that many potentially sensitive processes occur during the early postnatal period of brain maturation. Therefore, in the evaluation of developmental effects in mammals, it is important to consider the differences between animals used in research and humans. By using the mouse as an animal model, we can study the effect of a single toxicant administered directly to animals during different stages of the BGS.

This animal model allows us, in a controlled manner, to isolate the effects of certain toxicants and also to specify certain issues that may be difficult to solve in traditional developmental toxicity tests and also in epidemiological studies.

Taken together, the results of the present thesis clearly demonstrate that neonatal PBDE exposure can cause developmental neurotoxic effects, manifested in the adult mouse and rat as deranged spontaneous behavior,
impaired learning and memory, and changes in the cholinergic system, effects that are not species-, strain-, or gender-specific.

Taking into account the chemical and physical properties of PBDEs and PCBs (WHO 1993, 1994a), the doses used, and the neurotoxic effects seen from neonatal PBDE exposure, it is hard not to compare PBDEs with PCBs. These two groups of compounds seem to be equally capable of inducing this kind of neurotoxicity. From studies done by the Eriksson research group (Eriksson 1998), it has been shown that PCBs can induce developmental neurotoxic effects during a defined critical period of the BGS in mice, which is also presented for PBDEs in this thesis. The PBDE doses used in this thesis range from 0.7 to 28 µmol/kg body weight, which on a molar basis are the same as those earlier used to evaluate the developmental neurotoxicity of certain PCBs (Eriksson 1998). Therefore, the conclusion must be that PBDEs are equally capable of inducing this kind of neurotoxic effects as PCBs.

It is not only the dose and time of administration of PBDEs and PCBs that are crucial for inducing neurotoxic effects in our test system. To induce persistent effects, the presence of the neurotoxic compound or its metabolites is necessary during the defined period of the BGS. We have seen that both PBDEs and PCBs induce their neurotoxic effects when administered on PND 10, but due to the retention of these compounds or their metabolites in the brain, effects can also be seen after exposure on PND 3, if the amount of the compound is retained in sufficient amounts. This is evident for the decabrominated diphenyl ether, PBDE 209, which does not cause any neurotoxic effect when administered on PND 10 even though the parent compound is present in the brain 24 hours after administration. Administration of PBDE 209 on PND 3, on the other hand, induces behavioral deficits in the adult animal, which is probably due to metabolism of the parent compound and the presence of metabolites in sufficient amounts to induce effects during the defined limited period of the BGS, occurring around PND 10.

According to a review by Gallenberg and Vodicnik, different species retain persistent chemicals differently. The bioconcentration factors of 11 lipophilic environmental contaminants, including PCB, were between 3- and 47-fold higher in humans than in rats, which makes the amount of chemicals available for milk transfer a good deal higher in reality than expected after extrapolation from rodent toxicokinetic studies (Gallenberg and Vodicnik 1989). Also, PCB levels in human milk from over 800 women declined significantly during the course of lactation (Rogan et al. 1986), which has also been seen in a series of animal studies. In the adult mouse, PCBs are distributed to adipose tissue and mammary glands, and for both tetra- and hexaCBs, most of the dam’s body burden is transferred to the offspring during the first 4 days of lactation. After the lactation period, hardly any PCB can be found in the dam. Up to 75% of the dam’s body burden is found
in the offspring after the first week of lactation (Gallenberg and Vodicnik 1987, 1989; Ring et al. 1990; Vodicnik 1986; Vodicnik and Lech 1980). This effect suggests that large amounts of persistent lipophilic environmental contaminants, stored in a human mother, can be transferred to the offspring fairly quickly after birth.

The current margin of safety for PBDE-induced developmental neurotoxicity appears to be low for many individuals, according to a recent oral presentation by Thomas McDonald at The Third International Workshop on Brominated Flame Retardants (McDonald 2004). This opinion is based on U.S. human milk levels, daily intake of PBDEs in the U.S., and PBDE doses known to induce developmental neurotoxic effects in laboratory animals (McDonald 2004). A calculation of the amount of PBDE that will be present in the brain after neonatal exposure can be done using the results generated in the retention studies. The hypothetical amount of PBDE in the neonatal brain will be in the same order of magnitude as PCB amounts (7 ppb in cerebrum) that earlier has been seen in brains from human infants (Gallenberg and Vodicnik 1989), which make the PBDE doses used in this thesis environmentally relevant, especially since the levels of PBDEs are still increasing in different parts of the world.

Experimental studies of exposure to PCBs in mice, rats, and monkeys during development have shown long-term changes in behavior, neurotransmitters, and neuroreceptors (Seegal and Schantz 1994; Seegal and Shain 1992; Seegal 1996; Tilson and Harry 1994; Tilson et al. 1990). There are also human epidemiological studies suggesting that perinatal exposure to PCBs can induce developmental neurotoxic effects (Fein et al. 1984; Jacobson and Jacobson 1996a; Jacobson and Jacobson 1996b; Jacobson et al. 1990; Rogan et al. 1988; Schantz et al. 2003).

In yet another epidemiological study of perinatal PCB exposure, the authors show that there are interactive effects of PCBs and other environmental contaminants, such as methylmercury (Grandjean et al. 2001), which is further supported by laboratory studies in rats (Roegge et al. 2004).

PBDEs are not only a concern for neonatal exposure. In a recent study by Ankarberg and coworkers (Ankarberg 2003), it was shown that neonatal nicotine exposure during the BGS made adult animals more susceptible to PBDE exposure. The dose used for adult exposure (8 mg/kg body weight) had no effect on spontaneous behavior when no neonatal nicotine exposure had occurred, but if neonatal nicotine exposure (33 µg/kg body weight) preceded the adult PBDE exposure, a clear difference in spontaneous behavior was seen. This indicates that susceptibility to environmental pollutants in adult life is not necessarily inherited and that PBDEs alone or in combination with other contaminants can be a threat to adult individuals as well as to neonates.
CONCLUDING REMARKS

This thesis has shown that PBDEs, or their metabolites, can cause persistent developmental neurotoxic effects when present during a defined critical period of neonatal brain development in rodents.

Neonatal administration of [14C]-labelled PBDEs shows that they are taken up and distributed in the body and reach the brain in amounts similar to the amounts that earlier have been seen for PCBs and DDT. However, in contrast to the lower brominated PBDE, PBDE 99, and to some well-known persistent environmental pollutants, such as PCBs and DDT, the fully brominated PBDE, PBDE 209, shows an increase in the neonatal brain.

The developmental neurotoxic effects observed in mice neonatally exposed to the PBDEs, PBDE 99, PBDE 153, and PBDE 209, include deranged spontaneous behavior, lack of or reduction of habituation, and decreased learning and memory function. These effects are dose-response related, persistent, and can worsen with increasing age.

The developmental neurotoxic effects do not seem to be gender or strain specific. Neonatal exposure to PBDE 99 causes the same effects in male NMRI mice and in male and female C57 BI mice. Furthermore, the developmental neurotoxic effects are not restricted to one specie, because the same effects are seen in male Sprague-Dawley rats.

Exposure to PBDEs during the defined critical period of brain development is shown to affect the cholinergic system, manifested as increased adult susceptibility to nicotine, decreased numbers of nicotinic cholinergic receptors in mouse hippocampus, and decreased numbers of muscarinic cholinergic receptors in rat hippocampus.

The induction of developmental neurotoxic effects of PBDE 209 is different compared to the other investigated PBDEs. Administration of PBDE 209 has to be done before the defined limited period of BGS, since metabolism of PBDE 209 must take place for toxic metabolites to form and be present in the brain during the critical susceptible phase of neonatal brain development. This shows that it is not the time of administration that is crucial but the presence of the compound or its metabolites during this short critical phase of neonatal brain development.

The presence and increase of PBDEs to an environment already contaminated with persistent pollutants is of special concern. The effects of PBDEs and the similarities between PBDEs and PCBs call for further studies of the neonatal toxicity of PBDEs and possible interaction with other
environmental contaminants, especially contaminants that we risk being exposed to during the perinatal/neonatal brain development.
ACKNOWLEDGEMENTS

The Department of Environmental Toxicology, Uppsala University, has been the home of this work, with guest appearances at the Department of Neuroscience, Psychiatry Ulleråker, Uppsala University. I wish to express my sincerest gratitude to those who have contributed:

**Per Eriksson**, the most fantastic supervisor you can imagine. You can’t have a better one. Always supporting and understanding with great patience. Thank you for having me in your research group and letting me participate in your work. Not only that, you are a good friend with a million of good laughs that always makes me feel better. THANKS DAD.

**Anders Fredriksson**, my superb stand-in supervisor, co-author, “behavioral guy”, and friend. Thank you for all the good work we have done together and good discussions about science and other matter. Together with Per you have contributed to this thesis and the good time I have had while completing it.

**Ingvar Brandt**, head of department, for providing the facilities.

**Åke Bergman**, for “delivering the goods” that made this work possible.

**Emma Ankarberg, Anna Pettersson, Celia Fischer** and **Niclas Johansson**, who all work or worked in my research group and contributed to a nice atmosphere.

All present and past members of **Ekotox**.

Members of the **animal facility** for creating a positive atmosphere and relieving me from a lot of work.

**My parents**, for always being there for me. You are great.

The work done in this thesis was financially supported by grants from the Foundation for Strategic Environmental Research (NewS) and the European Commission (QLK4-CT-1999-01562).
Utvecklingsneurotoxikologiska effekter av bromerade flamskyddsmedel, polybromerade difenyletrar (PBDEer) hos neonata möss och råttor

Denna avhandling undersöker neurotoxiska effekter av en typ av flamskyddsmedel, polybromerade difenyletrar (PBDEer), efter exponering under den snabba utvecklings- och tillväxtperioden av hjärnan, hos nyfödda möss och råttor. Tre olika PBDE-kongener har undersökts: PBDE 99, en pentabromerad difenyleter; PBDE 153, en hexabromerad difenyleter; och PBDE 209 den decabromerade difenyletern.

Vår miljö innehåller en mängd föroreningar, däribland de bromerade flamskyddsmedlen PBDEer. PBDEer används i plaster, elektroniska kretskort, datorer, byggnadsmaterial och syntetiska textilier. Både i Sverige och globalt är PBDEerna vitt spridda och man har sett en kontinuerlig ökning av halterna i miljön under de senaste årtiondena. Även i human modersmjölk finner man PBDEer och också här har man sett en kontinuerlig ökning. En individ kan exponeras för PBDEer under hela sin livslängd, inklusive under digivningsperioden, då ämnen förs över till avkomman via modersmjölen.

Den neonatala perioden karaktäriseras hos många däggdjursarter av snabb utveckling och tillväxt av den outvecklade hjärnan, då en rad fundamentala förändringar sker. Det har tidigare visats att olika toxiska föreningar kan inducera permanenta skador i hjärnfunktionen när de administreras till neonatala möss under denna utvecklingsfas. Hos möss och råttor sker denna utveckling och tillväxt postnatalt och sträcker sig över de första 3-4 veckorna efter födseln. Hos människa börjar denna period under den sista tredjedelen av graviditeten och fortsätter under de två första levnadsåren.

De studier som presenteras i denna avhandling har identifierat en avgränsad kritisk period under den snabba utvecklings- och tillväxtperioden av hjärnan, då hjärnan är mycket känslig för låga doser av PBDEer. Studierna visar att det är närvaron i hjärnan av PBDEer och/eller deras metaboliter, under denna kritiska fas, som leder till utvecklingsneurotoxiska effekter.

Exponering för PBDEer, under den korta kritiska perioden av hjärnutveckling, leder till permanent förändrat spontanbeteende och
försämrad habituering till nya miljöer samt hyperaktivitet, hos den vuxna individen. Dessa skador kan också förvärras med åldern. Dessutom uppkommer störningar i minnes- och inlärningsförmågan hos den vuxna individen samt förändringar i det kolinerga systemet, som är kopplat till beteende, minne och inlärning.

PBDEernas förmåga att orsaka neurotoxiska effekter verkar inte vara beroende av kön, stam eller art. Studierna i denna avhandling visar att neurotoxiska effekter kan induceras hos råttor, hos möss av båda könen och hos möss av olika stammar.

Det faktum att PBDEerna själva kan induceras utvecklingsneurotoxiska effekter och att dessa effekter liknar de effekter som tidigare setts för polyklorerade bifenyler (PCBer) gör att mer fokus bör riktas mot PBDEernas neurotoxiska effekter och också mot möjliga samverkanseffekter mellan PBDEer och andra föroreningar i vår miljö.


Eriksson, P., and Darmerud, P. O. (1985). Distribution and retention of some chlorinated hydrocarbons and a phthalate in the mouse brain during the pre-weaning period. *Toxicology* 37, 189-203.


Hallgren, S., and Darnerud, P. O. (2002). Polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and chlorinated paraffins (CPs) in rats-testing interactions and mechanisms for thyroid hormone effects. *Toxicology* **177**, 227-43.

Hallgren, S., Sinjari, T., Hakansson, H., and Darnerud, P. O. (2001). Effects of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) on thyroid hormone and vitamin A levels in rats and mice. *Arch Toxicol* **75**, 200-8.


Kodavanti, P. R., and Derr-Yellin, E. C. (2002). Differential effects of polybrominated diphenyl ethers and polychlorinated biphenyls on


A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. (Prior to October, 1993, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science”.)