Neonatal Exposure to Highly Brominated Diphenyl Ethers and Perfluorinated Compounds

Developmental Dependent Toxicity and Interaction

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Abstract

This thesis investigated the developmental neurotoxic effects of neonatal exposure to highly brominated diphenyl ethers (PBDEs) and perfluorinated compounds (PFCs), alone or in combinations, during a critical period of the brains’ rapid growth and development, in mice. The compounds investigated were the decaBDE (PBDE 209), nonaBDE (PBDE 206), octaBDE (PBDE 203), heptaBDE (PBDE 183), and three PFCs, PFOS, PFOA, and PFDA.

PBDEs and PFCs have been identified as emerging classes of persistent environmental compounds, present in wildlife as well as humans, and present at higher levels in infants/children, compared to older persons. Individuals can be exposed to these compounds throughout her/his lifetime and newborn/children can be exposed to toxicants both via the mothers’ milk and directly via ingestion and inhalation.

The brain growth spurt (BGS) is defined by rapid growth and developmental of the brain. For rodents (mice and rats), the BGS is postnatal spanning the first 3-4 weeks after birth. In humans this period begins during the third trimester of pregnancy and continues throughout the first two years of life. It has been shown that several environmental toxicants can induce permanent disorders in brain function when administered to the neonatal mouse, during the BGS.

This thesis shows that highly brominated PBDEs, including PBDE 209, PBDE 206, and PBDE 203 can cause developmental neurotoxic effects, when given directly to the neonatal mouse. Of the investigated PFCs, PFOS and PFOA were shown to cause similar effects as the PBDEs. Furthermore, PBDE 209 and PFOA can at low doses interact and enhance the neurotoxic effects in mice. Effects in the adult animal included; deranged spontaneous behavior, reduced or lack of habituation, decreased learning and memory abilities, and increased susceptibility of the cholinergic system. Both classes of compounds were shown to affect proteins (CaMKII, GAP-43, synaptophysin, and tau) important for neuronal growth and synaptogenesis in the neonatal mouse brain.

Keywords: Behavior, Brain, Brominated flame retardants, Cholinergic system, Development, Neonatal, Neuroprotein, Neurotoxicity, PBDEs, PFCs

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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Abbreviations

AD Alzheimer’s disease
ADHD Attention deficit hyperactive disorder
ACh Acetylcholine
AChE Acetylcholinesterase
ANOVA Analysis of variance
BGS Brain growth spurt
BDE Brominated diphenyl ether
BFR Brominated flame retardant
CNS Central nervous system
CaMKII Calcium/calmodulin-dependent kinase II
DDT 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane
GAP-43 Growth associated protein-43
GD Gestational day
LTP Long-term potentiation
NMRI Naval Medical Research Institute
OP Organophosphorus
PBDE Polybrominated diphenyl ether
PBDE 183 2,2’3,4,4’,5,6’-heptabrominated diphenyl ether
PBDE 203 2,2’,3,4,4’,5,5’,6-octabrominated diphenyl ether
PBDE 206 2,2’,3,3’,4,4’,5,5’,6-nonabrominated diphenyl ether
PBDE 209 2,2’,3,3’,4,4’,5,5’,6,6’-decabrominated diphenyl ether
PCB Polychlorinated biphenyl
PFC Perfluorinated compound
PFCA Perfluorinated carboxylate
PFDA 2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-nonadecafluorodecanoic acid
PFSA Perfluorinated sulfonate
PFOA 2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluoroctanoic acid
PFOS Perfluorooctane sulfonate
PND Postnatal day
POP Persistent organic pollutants
WHO World Health Organization
WWF World Wildlife Fund
Introduction

Exposure to environmental toxic agents

In the environment, there are a vast number of toxicants to which wildlife and humans can become exposed. Many of these contaminants are well-known persistent organic pollutants (POPs) like PCBs (polychlorinated biphenyls) and DDT, as well as the more novel PBDEs (polybrominated diphenyl ethers) and PFCs (perfluorinated compounds). The individual can be exposed to toxic agents throughout her or his lifetime, beginning at the time of fertilization of the zygote. The embryo or fetus may be exposed to toxicants throughout gestation via the mother. After delivery the offspring can be exposed to toxicants both via the mother’s milk and via direct exposure. In humans, one child in every six has a developmental disability, and in most cases such disabilities affect the nervous system (Boyle et al. 1994). In humans, developmental neurotoxicity is evident in the adverse effects of lead exposure in children, as well as in fetal alcohol syndrome, methyl mercury poisoning, and abuse of drugs during pregnancy (Court et al. 1996). Animal studies show that xenobiotics can induce developmental neurotoxic effects at low doses, effects not seen to affect adult animals (Eriksson 1997; Tilson 2000). In developmental toxicology it is important to ascertain when a toxic agent can be harmful. Therefore, it is important to study when a toxic effect can be induced during development.

The toxic effects of a chemical depend on the route of exposure, the dose, and the point of time in the organism’s life cycle. An organism is seldom exposed to a single xenobiotic because a mix of toxic agents is present in the environment. This makes it important to study not only the individual toxicants but also how combinations or mixtures of different chemicals interact and the damage they can cause. Persistent and nonpersistent agents have been shown to be potent inducers of neurological derangements in neonatal rodents when present during the defined critical period of rapid brain development (Eriksson 1997; Eriksson et al. 1992; Eriksson et al. 2000).

Polybrominated diphenyl ethers

To meet fire safety regulations, flame retardants (FRs) are added to combustible products such as plastics, electronic circuit boards, wood, computers, TV sets, building materials, and textiles, etc. (WHO 1994a). Brominated
flame retardants (BFRs) are a diverse group of chemicals that include the PBDEs, polybrominated diphenyls (PBBs) (WHO 1994b), and hexabromocyclododecane (HBCDD) (WHO 1997), which are used as additive BFRs. Tetrabromobisphenol A (TBBPA) (WHO 1995) can be used as either an additive or as a reactive BFR. Additive BFRs are incorporated into the plastic mixture without a chemical binding, whereas reactive BFRs are covalently bonded into the polymer matrix. Additive BFRs have a tendency to leak into the environment during the lifetime of products (Hutzinger et al. 1976; Hutzinger and Thoma 1987).

The PBDEs consist of two phenyl rings where bromine atom/atoms can bind. The 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 $K_{\text{ow}}$ ranging between 4.28 and 9.9) (WHO 1994a). PBDEs have been sold in three commercial mixtures, penta-, octa-, and decaBDE, which are named after the dominating homologue group. In 2004, the penta- and octaBDE were banned from all products on the European Union market and in 10 states in the United States, followed by a cease in their production in the United States. Now only the decaBDE is still produced and applied around the world (BSEF 2007). PBDE 209 is the dominating congener in the decaBDE formula.

![Figure 1. The general structure formula of PBDEs, with 209 possible congeners.](image)

Polybrominated diphenyl ethers: levels and toxicity

PBDEs were first detected in the environment close to a plant that manufactured polybrominated compounds (Di Carlo et al. 1978; Erickson et al. 1980). This was in the USA in the 1970s. In the early 1980s, the first report regarding PBDEs in higher organisms came from Sweden, where PBDEs were discovered in various fish species collected in a river (Andersson and Blomkvist 1981). Since then, PBDEs are demonstrably present in the global environment and have been found in samples taken from both aquatic and terrestrial compartments, e.g., fish and humans (de Wit 2002; Schecter et al. 2005b; Sellström et al. 1993), and in 2000, there was an report on increasing levels of PBDEs in mothers’ milk (Noren and Meironyte 2000).
The decaBDE is found in abiotic compartments and constitutes between 95% and 99% of the total PBDE load in sediment in the Great Lakes (Song et al. 2005; Zhu and Hites 2005). PBDE 209 has also been found in biota (de Wit 2002; Hale et al. 2003; Hale et al. 2001; Johnson-Restrepo et al. 2005; Jones-Otazo et al. 2005; Lindberg et al. 2004; Sellström 1999; Wang et al. 2005; Verslycke et al. 2005), the levels tending to increase with time (Hale et al. 2006; Law et al. 2006). It appears that the PBDEs have an environmental dispersion similar to that of the well-known POPs such as PCBs and DDT (Darnerud et al. 2001; de Wit 2002; Sellström et al. 1993).

There are several studies concerning human exposure to and levels of PBDEs in humans. Given the lipophilicity of PBDEs and their presence in consumer products and house dust, it is assumed that the two routes whereby humans are exposed to these compounds are diet and domestic environment. There are several reports on PBDEs in food products (Bocio et al. 2003; Darnerud et al. 2001; Domingo 2004; Harrad et al. 2004; Lind et al. 2002; Ryan et al. 2002; Schecter et al. 2006; Wijesekera et al. 2002). In the study by Bocio et al., the total daily intake was calculated to be 97.3 ng, the bulk of it deriving from fish and shellfish.

Due to their lipophilic characteristics, PBDEs tend to accumulate in adipose tissue, and when body fat is used, PBDEs can become redistributed and excreted via milk. In newborns, a major route of exposure to POPs is via mothers’ milk. PBDEs are universally present in human mothers’ milk, the levels having increased in recent decades (Akutsu et al. 2003; Lind et al. 2003; Meironyte et al. 1999; Noren and Meironyte 2000; Ohta et al. 2002; Schecter et al. 2003).

House dust has been analyzed for PBDEs, which were found to be present (Gevao et al. 2006; Harrad et al. 2006; Jones-Otazo et al. 2005; Rudel et al. 2003; Schecter et al. 2005a; Stapleton et al. 2005; Wilford et al. 2005; Wu et al. 2007). In the report by Wu et al. (2007), a significant association was found between PBDEs in breast milk and in house dust. Inhalation of PBDEs in particulate matter or dust is suggested to be the greatest contributor to PBDE exposure in humans, from toddlers to adults (Jones-Otazo et al. 2005), and the main PBDE congener listed in house dust and indoor dust standard reference materials appears to be PBDE 209 (Stapleton et al. 2005; Stapleton et al. 2006).

The uptake of $^{14}$C-labelled PBDE 209 in adult rats has been shown to be low following oral exposure (el Dareer et al. 1987; Norris et al. 1975). Absorption of PBDE 209 has been reported to be at least 10% of a single oral dose in adult rats and reported to be metabolized, excreted, and marginally distributed to adipose tissue, but found in plasma and blood-rich tissue (Morck et al. 2003). Debromination of PBDE 209, as a pathway of metabolism, has been reported in rodents and suggested in fish (Morck et al. 2003; Stapleton et al. 2004). After an oral administration in adult rats, PBDE 209 has been shown to be metabolized and debrominated to hepta-, octa-, and nonaBDEs (Morck et al. 2003; Sandholm 2003; Sandholm et al. 2003). Rats
exposed to a commercial decaBDE via the diet showed an accumulation of PBDE 209, three nonaBDEs, and four octaBDEs (Huwe and Smith 2007). Developmental exposure of mice and rats to PBDE 209 has been reported to cause neurobehavioral defects (Rice et al. 2007; Viberg et al. 2007; Viberg et al. 2003b). The neurobehavioral defects are suggested to be due to metabolites of PBDE 209 when present during a critical period of brain development, namely around postnatal day (PND) 10 (Viberg et al. 2003b). Oral neonatal administration of $^{14}$C-PBDE 209 on PND 3, 10, or 19 showed that the compound is distributed throughout the body and increases in the brain, from 24 h after administration to 7 days after administration, in 3-day-old and 10-day-old mice. Disturbances in spontaneous behavior only occurred in mice exposed on PND 3, and the effect worsened with age. Recently, it has been shown that the protein levels of calcium/calmodulin-dependent protein kinase II (CaMKII) and growth-associated protein-43 (GAP-43) can be affected in the neonatal mouse brain after neonatal exposure to PBDE 209 (Viberg et al. 2008a). Earlier studies on the developmental neurotoxic effects of PBDEs and/or its metabolites reported that the effects are induced during a defined period of neonatal life and manifested as modified spontaneous behavior, reduced habituation, defective learning and memory, as well as changes in the cholinergic system (Eriksson et al. 2001; Eriksson et al. 2002; Viberg et al. 2003a; Viberg et al. 2003b). A recent report by Dingemans et al., also indicated that long-term potentiation in the hippocampus of neonatal mice can be affected by PBDEs, when exposed on PND 10 (Dingemans et al. 2007).

The cholinergic system has also been found to be a target for PBDEs. In both mice and rats, PBDEs have been found to modify the response of the cholinergic system and to reduce the density of cholinergic nicotinic receptors in the hippocampus. Both the cholinergic system and LTP are linked to learning and memory processes.

The toxic effects of exposure to PBDEs have also been reported in the thyroid hormone system, and subchronic treatment with tetraBDE, pentaBDE, and decaBDE can reduce the levels of total thyroxine (T4) and free T4 in serum (Fowles et al. 1994; Hallgren et al. 2001; Rice et al. 2007; Stoker et al. 2004). PBDEs have also been shown to bind to the thyroid hormone transporter, transthyretin (Hallgren and Darnerud 2002; Meerts et al. 2000).

**Perfluorinated compounds**

PFCs comprise a large group of chemicals that have been produced for the last fifty or so years. PFCs are fully fluorinated chemicals, which are man-made with unique properties and have been recognized as a class of emerging, persistent contaminants. Carbon-fluorine bonds are among the strongest in organic chemistry. This stability makes these compounds practically non-biodegradable and persistent in the environment (Key et al. 1997, 1998).
Perfluorinated sulfonates (PFSA), such as perfluorooctane sulfonate (PFOS), and perfluorinated carboxylates (PFCAs), such as 2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluorooctanoic acid (PFOA) and 2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-nonadecafluorodecanoic acid (PFDA) (Fig. 2.), are not reactive, resist hydrolysis and photolysis, and are not easily degraded in biological systems (Kissa 2001). These chemicals are produced for numerous applications in industrial processes and are used to make consumer products, such as water-, oil-, and stain-resistant coatings for clothing fabrics, leather, and carpets, and oil-resistant coatings for paper products for food contact. They are also used in surfactants, photographic emulsifier, aviation hydraulic fluids, fire-fighting foams, floor polishes, and insecticide formulations (Renner 2001; Seacat et al. 2002).

Figure 2. The structure formula of PFOS (left) and PFOA (right).

Perfluorinated compounds: levels and toxicity

In 2001, it was established that PFOS, the stable and extremely persistent end-product of degradation of various sulfonated fluorochemicals, are universally present and bioaccumulating (Giesy and Kannan 2001; Kannan et al. 2001). PFOS and PFOA are present globally in the environment, wildlife, and humans (Calafat et al. 2006; Giesy and Kannan 2001; Kannan et al. 2001). PFCs were first detected in human tissue 40 years ago (Taves 1968), and since then PFOS and PFOA have been detected in human blood, plasma, the liver, breast milk, and umbilical blood (Apelberg et al. 2007; Kärrman et al. 2007; Olsen et al. 2003; So et al. 2006). PFCs do not accumulate in the lipids as do lipophilic POPs; instead, for example, PFOS and PFOA associate with proteins (Han et al. 2003; Jones et al. 2003) and are distributed mainly to the liver and plasma (OECD 2002). The majority of studies conducted to determine the presence of PFCs based their findings on blood from the adult population, although other studies demonstrate that children have been exposed as well (Apelberg et al. 2007; Calafat et al. 2007a; Calafat et al. 2007b; Inoue et al. 2004; Olsen et al. 2004).

Recent reports indicate that young people have the same or even higher PFC levels in serum or blood than older individuals do (Kärrman et al. 2006; Olsen et al. 2004; WWF 2005). The World Wildlife Fund (WWF) reported that the median level of PFCs was 6.45 ng/g of blood in the children’s generation versus 3.11 ng/g of blood in the grandmother’s generation. Research indicates that indoor air and dust can be a major route of exposure to novel
chemicals such as PFCs. Infants, toddlers, and children, especially those who crawl, tend to experience higher uptake doses than teenagers and adults do. The reason for this is the higher relative uptake via food consumption and hand-to-mouth transfer of the chemicals from carpets and ingestion of dust (Trudel et al. 2008). PFOS and PFOA are readily absorbed but poorly excreted and are not known to be metabolized (Harada et al. 2005). In humans, the half-life of these chemicals have been reported to be 4.8 years and 3.8 years (geometric mean), respectively (Olsen et al. 2007).

Long-term effects of perfluorinated compounds are uncertain, and their toxicity is not explicitly characterized. Berthiaume and Wallace reported that perfluorinated compounds are peroxisome proliferators (Berthiaume and Wallace 2002) and tumor promoters, and that they may inhibit cell-to-cell communication by inhibiting gap junctional intercellular communication at environmentally relevant concentrations (Hu et al. 2002). PFOS have been demonstrated to alter both cellular homeostasis and cell membrane properties (Hu et al. 2003). Prenatal and postnatal toxicity of PFOS in rats and mice include increased liver weight and growth lag, as well as delayed development (Lau et al. 2003; Thibodeaux et al. 2003). Developmental toxicity of PFOS has been studied in laboratory rats and mice. Lau et al. (2003) reported that developmental neurotoxic effects in rats and mice follow PFOS exposure throughout pregnancy. The pups were born alive, active, and pink. But 30 to 60 minutes after birth, having been exposed to 10 mg/kg of PFOS, they became pale, inactive, and moribund. All neonates died soon afterward. Survival rates of the neonates improved with lower doses of PFOS exposure; when mice were exposed to 3 mg/kg, a 50% survival rate was seen. It was also reported that the development of learning was not affected by PFOS exposure (Lau et al. 2003).

In rats, effects of PFOA have been seen, namely reduced weight gain and delayed development (Butenhoff et al. 2004). PFOA and PFDA have been reported as peroxisome proliferators (Harrison et al. 1988; Ikeda et al. 1985). In rats, PFDA has been seen to cause anorexia, altered fatty acid metabolism, bradycardia, hepatotoxicity, reduction of circulating thyroid hormone, and hypothermia (George and Andersen 1986; Gutshäll et al. 1988, 1989; Langley and Pilcher 1985; Pilcher et al. 1987; Pilcher and Langley 1986; Singer et al. 1990).

The neonatal brain: a vulnerable organ

During mammalian development, several critical phases can be affected by toxicants, leading to malformations and disabilities. The development and maturation of the central nervous system (CNS), i.e. neurogenesis, relies on a predetermined plan for the different brain structures and for connections between the different parts. The development of the CNS can be roughly
divided into two major phases. The first includes the embryonic development of the brain, when the general shape of the brain takes place and precursors of glia and neurons proliferate. Embryonic development of the human brain takes place during the first two months of gestation, taking one-fifth of gestation. In mice, however, embryonic development occupies four-fifths of gestation. The second phase of brain development, known as the "brain growth spurt" (BGS), includes a series of rapid developmental changes, including maturation of dendritic and axonal outgrowth, synaptogenesis, establishment of neuronal connections, and proliferation of glia cells with accompanying myelination (Davison and Dobbing 1968; Kolb and Whishaw 1989). 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). The time frame for the BGS occurs at different times in different mammals. In several mammalian species, including mice, rats, and humans, the BGS coincides with the lactation period. In humans, it begins during the third trimester of pregnancy and continues throughout the first 2 years of life. In mice and rats, on the other hand, it is postnatal, spanning the first 3 to 4 weeks of neonatal life and peaking around postnatal day 10. In rodents, it is known that during this phase, a vast number of proteins are highly enriched in the nervous system, and many of them are signaling proteins that regulate neuronal processes such as survival, growth, and synaptogenesis. The levels of several proteins involved in neuronal survival, growth, and synaptogenesis also change. Among them are CaMKII, GAP-43, synaptophysin, and tau. The function of CaMKII involves regulation of synaptogenesis and synaptic plasticity (Frankland et al. 2001; Rongo and Kaplan 1999). GAP-43 plays a key role in guiding the growth of axons and modulating the information of new connections. Due to its characteristics and pattern of expression, GAP-43 is frequently used as a marker for axonal sprouting and growth (Oestreicher et al. 1997). Synaptophysin is an integral membrane glycoprotein in presynaptic vesicles and is localized in all nervous tissue (Navone et al. 1986; Wiedenmann and Franke 1985). Synaptophysin has also been identified as a useful marker for synaptic density (Hamos et al. 1989; Masliah et al. 1990). Tau, which is a microtubule-associated protein, has been implicated in the outgrowth of neuronal processes, the development of neuronal polarity, and the maintenance of normal morphology of the neurons, as reviewed by (Wang and Liu 2008). Tau has also been implicated in the promotion of microtubule assembly and the maintenance of stability (Weingarten et al. 1975; Vila-Ortiz et al. 2001). The levels of these proteins have been found to increase during the BGS in the neonatal mouse, with the most pronounced increase taking place around PND 7-14 (Viberg submitted) (Viberg et al. 2008a).

The developing human brain is inherently much more susceptible (Davison and Dobbing 1968) to injury caused by toxic agents than is the brain of an adult (Grandjean and Landrigan 2006). Due to the complexity of mamma-
lian brain development, windows of unique susceptibility to interference with toxicants can arise that have no counterpart in the mature brain, or in any other organ. If a developmental process in the brain is haltered or inhibited, there is only a slight potential for subsequent repair, and the consequences can therefore be permanent (Davison and Dobbing 1968; Rice and Barone 2000). Evidence has been collected for decades that industrial chemicals cause developmental damage and subclinical stages of these disorders might be common. The possibility of a link between chemicals and widespread neurobehavioral changes was first made widely known by research showing the toxicity of lead in the developing brain, across a wide range of exposures (Baghurst et al. 1987; Dietrich et al. 1987; Landrigan et al. 1975; Needleman et al. 1979). Animal studies support the notion that a wide range of industrial chemicals can induce developmental neurotoxicity at low doses that do not have the same effect in a mature animal (Eriksson 1997; Eriksson and Talts 2000; Tilson 2000).

The cholinergic system, behavior, and neurological disorders

One of the major transmitter systems in the brain is the cholinergic system, where acetylcholine (ACh) is the neurotransmitter. The cholinergic transmitter system is involved in many behavioral phenomenon (Karzmar 1975) and is closely related to cognitive functions (Drachman 1977; Herlenius and Lagercrantz 2004; Levin and Simon 1998; Paterson and Nordberg 2000; Perry et al. 1999). There are two major modulatory cholinergic systems in the brain, the pontomesencephalotegmental cholinergic complex and the basal forebrain complex. During development of mice and rats, the ontogenesis of the cholinergic system takes place during the first 3 to 4 weeks after birth. During this period, variables such as choline acetyltransferase (ChAT), acetylcholinesterase (AChE), and sodium dependent choline uptake, as well as muscarinic and nicotinic receptors, have been 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). The nicotinic subtype of the cholinergic receptor is a ligand-gated ion channel related to GABA\(_{A}\), glycine, and NMDA receptors (Karlin 2002). Activation of central nicotinic receptors presynaptically facilitates the release of several neurotransmitters, and postsynaptic receptors mediate a small minority of fast excitatory transmissions by inducing a fast cationic inward current (Dani 2001).

Behavior is a major function by which animals adapt to changes in the environment. Spontaneous behavior reflects a function dependent on the integration of a sensoric input into a motoric output, thus revealing an animal’s ability to habituate to a novel home environment and integrate new
with previously attained information, and thereby constitutes a measure of cognitive function. Spontaneous behavior is especially meaningful in environmental toxicology, as it reflects functions that are of importance for the individual/species in the wild. One such function is mobility, which is needed to search for food, to mate, and to elude predators (Evans et al. 1984). In this thesis, spontaneous behavior has been used to get information about motor activity, habituation, and cognitive function in the adult animal. It is known that lesions of cholinergic nuclei, or cholinergic neurons projecting to the hippocampus or cortex, can cause learning and memory deficits, see (Berger-Sweeney et al. 1994; Nabeshima 1993).

A wide range of genetic, neurochemical, and neuroanatomical alterations are known to increase locomotor activity in animals when exposed to a novel environment (Viggiano 2008). In the recent review by Viggiano (2008), a metaanalysis approach was used to organize a database of genetic modifications, pharmacological treatments, and brain lesions that increase locomotor activity in animal models. Analysis of the database showed that the genes, when mutated, induce hyperactive behavior do not pertain to a single neurotransmitter system. In fact, alterations in most neurotransmitter systems can give rise to a hyperactive phenotype, whereas fewer changes can decrease locomotor activity. Genetic and pharmacological alterations that enhance the dopamine, orexin, histamine, or cannabinoids systems or that antagonize the cholinergic system induce an increase in locomotor activity, according to the review. Other findings were that an imbalance in the two main neurotransmitters of the nervous system, GABA and glutamate, usually result in hyperactivity, and that few focal lesions decrease locomotor activity. Finally, the review showed that a large number of toxic events can increase locomotor activity, particularly if delivered during the prepubertal time window.

Mice knockouts for a large number of transcription factors/second messenger proteins as well as growth factors and their receptors show a hyperactive behavior. Among them are CaMKII knockouts (Chen et al. 1994) and GAP-43 knockouts (Metz and Schwab 2004).

Several studies have shown that different cholinergic agonists and antagonists affect learning and memory in different types of behavior in rats, see (Levin 2002). The Morris water-maze type of swim maze, with its submerged platform, is designed to measure spatial learning, which is suggested to be correlated with cholinergic function (Lindner and Schallert 1988; Whishaw 1985). Spatial learning tasks, which are 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). The role of CaMKII in synaptic plasticity has been examined both at the cellular and behavioral level, using pharmalogical and genetic approaches (Hinds et al. 1998; Malinow et al. 1989; Silva et al. 1992). Binding of CaMKII to the NMDA channel is crucial for LTP induction (Barria and Malinow 2005).
Aging is associated with the progressive deterioration of learning and memory functions. Several transmitter systems undergo a reduction in receptor function and density during aging, leading to a reduced ability to adjust to changes in the environment (Pedigo 1994). It has been suggested that the cholinergic system in particular is involved in the process of aging because dysfunction in this system has been shown to impair learning and memory (Bartus et al. 1982).

The cholinergic system is also involved in several neurological and neurodegenerative disorders, such as Alzheimer’s disease (AD), Parkinson’s disease, and schizophrenia. A consistent loss of cholinergic nicotinic receptors and cholinergic innervations has been seen in brain tissue in AD and Parkinson’s patients (Hellstrom-Lindahl et al. 1999; Nordberg 1993; Paterson and Nordberg 2000). The basal forebrain complex plays a crucial role in learning and memory functions, and these cells are among the first to die during the course of AD. In diseases like AD and Parkinson’s disease, it seems likely that nicotinic agonists could function as a therapy (Levin and Rezvani 2000; Rusted et al. 2000; White and Levin 1999). The use of nicotine and nicotinic agonists has also been proposed as a treatment for ADHD in adults (Levin et al. 1996; Levin 1998).
Objective

The overall objective of this thesis was to investigate the developmental neurotoxic effects of neonatal exposure to highly brominated diphenyl ethers (BDEs) and PFCs, alone or in combination, in mice. The specific aims were:

- To study whether neonatal exposure to the fully brominated BDE (decaBDE, PBDE 209) causes dose–response-related neurobehavioral effects in the adult mouse, such as changes in spontaneous behavior and response of the cholinergic transmitter system.

- To study whether neonatal exposure to highly brominated BDEs, nonaBDE (PBDE 206), octaBDE (PBDE 203), and heptaBDE (PBDE 183) can induce behavioral effects in the adult mouse, such as changes in spontaneous behavior and in learning and memory.

- To study whether neonatal exposure to perfluorinated compounds (PFOS, PFOA, and PFDA) can induce developmental neurotoxic effects, such as changes in spontaneous behavior and response of the cholinergic system.

- To study whether neonatal exposure to PFOS and PFOA can alter the levels of proteins important for normal brain development in the neonatal mouse.

- To study whether PBDE 209 and PFOA can interact during a defined critical phase of the developing neonatal brain to enhance neurobehavioral effects, such as changes in spontaneous behavior.

- To study whether PBDE 209 and PFOA can interact to alter the levels of proteins important for normal brain development in the neonatal mouse.
Materials and methods

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

Chemicals

The brominated diphenyl ethers, 2,2′,3,3′,4,4′,5,5′,6,6′-decaBDE, 2,2′,3,3′,4,4′,5,5′,6-nonaBDE, 2,2′,3,4,4′,5,5′,6-octaBDE, and 2,2′,3,4,4′,5,6-heptaBDE, were kindly donated by the research group led by Åke Bergman, Department of Environmental Chemistry, Stockholm University, Sweden. The purity of the compounds was ≥ 98%. Potassium 1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8-heptadecafluorooctane-1-sulfonate, purity ≥ 98%; 2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluorooctanoic acid, purity ≥ 96%; and, 2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-nonadecafluorodecanoic acid, purity ≥ 98% were purchased from Sigma Aldrich.

The different PBDEs and PFCs 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 with different concentrations of PBDEs and PFCs. The use of a 20% fat emulsion vehicle was used to obtained a more physiologically appropriate absorption and, hence, distribution (Keller and Yeary 1980; Palin et al. 1982).

Animals

Pregnant NMRI (Naval Medical Research Institute) mice were obtained from B&K, Sollentuna, Sweden, and were housed individually in plastic cages (40 × 25 × 15 cm) in a room with an ambient temperature of 22°C and a 12/12-h cycle of light and dark. The animals were supplied with standardized pellet food (Lactamin, Stockholm, Sweden) and tap water ad libitum. The pregnant NMRI mice were checked for birth twice daily (08.00 and 18.00 h). The day of birth was assigned day 0, and pups born during the night were assigned day 0 when checked at 08.00 h. The size of the litters was adjusted to 10 to 12 mice (studies I-IV), 8 to 12 mice (study V), and 10 to 14 mice (study VI) within the first 48 hours after birth by euthanizing excess pups. The litters contained pups of both sexes in about equal numbers.
during the neonatal period, and no separation with regard to sex was made in the preweanling mice. Animals used in studies IV and VI were euthanized at PND 11. At the age of 4 to 5 weeks, all females were sacrificed, and the males were kept in litters (in treatment groups) with their siblings and raised in groups of 4 to 7, in a room for males only and under the conditions detailed above (studies I-III and V).

Treatment
In the studies carried out in this thesis, animals received a single oral dose of 2,2′,3,3′,4,4′,5,5′,6,6′-decaBDE (PBDE 209), 2,2′,3,3′,4,4′,5,5′,6-nonaBDE (PBDE 206), 2,2′,3,4,4′,5,5′,6-octaBDE (PBDE 203), 2,2′,3,4,4′,5′,6-heptaBDE (PBDE 183), perfluorooctane sulfonate, potassium salt (PFOS), perfluorooctanoic acid (PFOA), or perfluorodecanoic acid (PFDA). The animals received a single oral dose on either PND 3 or PND 10, or on PND 3 and PND 10.

In study I, NMRI mice were exposed to 1.4 μmol (1.34 mg), 2.3 μmol (2.22 mg), 14 μmol (13.4 mg), or 21 μmol (20.1 mg) 2,2′,3,3′,4,4′,5,5′,6,6′-decaBDE/kg body wt., on PND 3.

In study II, NMRI mice were exposed to 21 μmol (18.5 mg) 2,2′,3,3′,4,4′,5,5′,6-nonaBDE/kg body wt., 21 μmol (16.8 mg) 2,2′,3,4,4′,5,5′,6-octaBDE/kg body wt., or 21 μmol (15.2 mg) 2,2′,3,4,4′,5′,6-heptaBDE/kg body wt., on PND 3 or PND 10.

In study III, NMRI mice were exposed to 1.4 μmol (0.75 mg) or 21 μmol (11.3 mg) PFOS/kg body wt., 1.4 μmol (0.58 mg) or 21 μmol (8.70 mg) PFOA/kg body wt., or 1.4 μmol (0.72 mg) or 21 μmol (10.8 mg) PFDA/kg body wt., on PND 10.

In study IV, NMRI mice were exposed to 21 μmol (11.3 mg) PFOS/kg body wt., or 21 μmol (8.70 mg) PFOA/kg body wt., on PND 10.

In study V, NMRI mice were exposed to one single oral dose on PND 3 and another single oral dose on PND 10 (PND 3-PND 10) of PBDE 209 (1.4 or 8.0 μmol/kg body wt., PFOA (1.4 or 14 μmol/kg body wt.), co-exposed to PBDE 209 and PFOA, or a vehicle (20% fat emulsion). The combinations were; 1). vehicle-vehicle, 2). vehicle-1.4 μmol PFOA/kg body wt., 3). vehicle-14 μmol PFOA/kg body wt., 4). 1.4 μmol PFOA/kg body wt-vehicle, 5). 14 μmol PFOA/kg body wt.-vehicle, 6). 1.4 μmol PBDE 209/kg body wt.-vehicle, 7). 8.0 μmol PBDE 209/kg body wt.-vehicle, 8). 1.4 μmol PBDE 209/ kg body wt. + 1.4 μmol PFOA/kg body wt.-vehicle, 9). 1.4 μmol
PBDE 209/kg body wt. + 14 μmol PFOA/kg body wt.-vehicle, 10). 8.0 μmol PBDE 209/kg body wt. + 1.4 μmol PFOA/kg body wt.-vehicle, 11). 8.0 μmol PBDE 209/kg body wt. + 14 μmol PFOA/kg body wt.-vehicle, 12). 1.4 μmol PBDE 209/kg body wt.-1.4 μmol PFOA/kg body wt., 13). 1.4 μmol PBDE 209/kg body wt.-14 μmol PFOA/kg body wt., 14). 8.0 μmol PBDE 209/kg body wt.-1.4 μmol PFOA/kg body wt., 15). 8.0 μmol PBDE 209/kg body wt.-14 μmol PFOA/kg body wt.

In study VI, NMRI mice were exposed to 1.4 μmol (1.34 mg) PBDE 209/kg body wt. on PND 3 and 14 μmol (5.80 mg) PFOA/kg body wt. on PND 10.

In studies I-VI, control animals received 10 ml of the 20% fat emulsion vehicle/kg body wt.

**Behavioral tests**

**Spontaneous behavior**

Animals were observed regarding spontaneous behavior in studies I, II, III, and V.

In study I, male NMRI mice were tested when 2 months and 4 months old.
In study II, male NMRI mice were tested when 2 months old.
In study III, male NMRI mice were tested when 2 months and 4 months old.
In study V, male NMRI mice were tested when 2 months and 4 months old.

The animals were tested between 08.00 and 13.00 h, under about the same light and temperature conditions as in their cages. Ten mice were picked randomly from three to five different litters in each treatment group, except in study I, when 16 mice were picked when 4 months old. Motor activity was measured during a 60-minute period, divided into three 20-minute intervals, in an automated device consisting of cages (40 × 25 × 15 cm, same size as the home cage) placed within two series of infrared beams (low and high level) (Rat-O-Matic, ADEA Elektronik AB, Uppsala, Sweden) (Fredriksson 1994). Twelve cages, placed in individual sound-proofed boxes with separate ventilation, were used. In each 60-min recording session, animals from each treatment group were represented.

In the spontaneous behavior test, three variables were measured. Locomotion: This was registered when the mouse moved horizontally through the low-level grid of infrared beams. Rearing: Vertical movement was registered at a rate of four counts per second, when a single high-level beam was intercepted, i.e., number of counts was proportional to time spent rearing. Total activity: This measured all types of activity within the test cage caused by the mouse,
e.g., tremor and grooming. Registration was done with a sensor composed of a needle mounted on a horizontal arm with a counterweight. All data were collected electronically through a computer interface. 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 a 60-minute period.

Nicotine-induced behavior
The nicotine-induced behavior test was used in studies I and III. In both studies, male NMRI mice were tested at 4 months of age. The test was conducted directly after the spontaneous behavior test, and the animals were tested for locomotion, rearing, and total activity, as described for the spontaneous behavior test, for another 60-min period (60-120 min). In study I, the nicotine-induced behavior was tested by giving the mice a single subcutaneous (s.c.) injection of saline or nicotine (80 μg/kg body wt.) In each treatment group eight mice received NaCl and eight mice received nicotine. In study III, the nicotine-induced behavior was tested by giving mice a single s.c. nicotine injection (80 μg/kg body wt.). In each treatment group, 10 mice were injected with nicotine. The dose of 80 μg nicotine base/kg body wt. was selected from an earlier dose–response study (Ankarberg et al. 2001) because it is known to cause increased activity in normal adult NMRI mice (Ankarberg et al. 2001; Eriksson et al. 2000; Nordberg et al. 1991; Viberg et al. 2002).

Elevated plus-maze
The elevated plus-maze (EPM) test was used in studies I and III. In both studies, male NMRI mice were tested at 4 months of age.

The test procedure, adopted from Lister (Lister 1987), measures the number of entries that the mice made into the open arms, as described below, and the time that they spent there. In the plus-maze apparatus, made of plywood, two diametrically placed open arms (white floor with no wall, 30×6 cm) face two closed arms (black floor with walls, 30×6×30 cm) mounted 50 cm above the floor. Testing took place from 09:00 to 14:00 h. The mice were transferred to the test laboratory in their “home” cages at least 60 min before they were submitted to the EPM. A mouse was placed on the central platform (white floor, 6×6 cm) of the apparatus, facing “north” toward the closed arms. A video camera was used to monitor the animal’s behavior. Ten mice were picked randomly from three to five different litters in each treatment group and were tested only once in this test. The number of entries into the open and closed arms and the time spent in each of the arms were measured for 5 min. Arm entry was defined as all four paws in the arm. The maze apparatus was cleaned after each trial with a towel soaked in hot water.
Swim maze

The Morris swim maze test was conducted in study II, in mice 3 months of age. Mice exposed to PBDE 203 on PND 3 or PND 10, or to PBDE 206 on PND 10 and their controls were observed. These treatment groups were selected with regard to significantly altered spontaneous behavior, when tested at the age of 2 months. About 15 mice, from 3 to 5 litters, from each treatment group were tested.

The Morris type (Morris 1981) swim maze was a circular grey tub, 102 cm in diameter, filled with water at 23°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’s surface. The platform consisted of a metal mesh, 12 cm in diameter. The relative positions of the pool and the observer were the same every day the tests were performed. Each mouse’s ability to locate the submerged platform was observed on five consecutive days. The mouse was given five trials each day, and its latency to locate the platform was recorded. Testing was conducted between 09.00 and 14.00 h. Before the first trial each day, 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 the task within 30 sec it was gently replaced on the platform. After each trial, the mouse remained on the platform for 30 sec. All the mice in the study were subjected to five trials each day, and the tests were performed on four consecutive days. In other words, trials 1 through 20 were performed on days 1 through 4. On day 5, the platform was moved to the center of the tub’s “northwest” quadrant, for reversal trials; otherwise the day 5 procedure was identical to that of the previous four days. Latencies to reach the platform were recorded by the observer; total search time for the five trials was set to 150 sec. The first 20 trials (days 1-4) measured each mouse’s spatial learning ability and the last five trials (day 5) its relearning ability.

Analysis of neuroproteins

Slot-blot analysis was conducted in studies IV and VI. The protein levels of CaMKII, GAP-43, synaptophysin, and tau were analyzed in the cerebral cortex and the hippocampus in animals from all treatment groups. The protein levels were expressed as a percentage of controls. The antibodies used were mouse monoclonal CaMKII (Chemicon MAB8699, 1:5,000), rabbit polyclonal GAP-43 (Chemicon AB5220, 1:10,000), mouse monoclonal synaptophysin (Calbiochem 573822, 1:10,000), and mouse monoclonal tau (Santa Cruz, 1:1,000). Immunoreactivity was detected using a horseradish peroxidase-conjugate secondary antibody against mouse (074-1806, 1:20,000) or rabbit (KPL 074-1506, 1:20,000). Immunoreactive bands were
detected using an enhanced chemiluminescent substrate with imaging on a LAS-1000 (Fuji Film, Tokyo, Japan). The intensity of the bands was quantified using IR-LAS 1000 Pro (Fuji Film). The CaMKII, GAP-43, synaptophysin, and tau antibody recognizes both the nonphosphorylated and the phosphorylated form of the protein.

Statistical analysis

**Spontaneous behavior test**
The data from the spontaneous behavior tests were subjected to a split-plot ANOVA (analysis of variance), and pairwise testing was performed using a Tukey HSD (honestly significant difference) test (Kirk 1968) for studies I, II, III, and V.

**Habituating capability**
A ratio was calculated between the performance 40-60 min and 0-20 min period for the two different variables: locomotion and rearing. The following calculations were used: 100 × (counts “locomotion” 40-60 min/counts “locomotion” 0-20 min), 100 × (counts “rearing” 40-60 min/counts “rearing” 0-20 min). This ratio was used to analyze the alteration in habituation between 2-month-old and 4-month-old mice. These data were subjected to two-way ANOVA. (In study I the ratio for locomotion and in study III the ratio for locomotion and rearing were used).

**Nicotine-induced behavior test**
The data from the nicotine-induced behavior test were subjected to a split-plot ANOVA, and pairwise testing between nicotine-injected and saline-injected animals (study I), and pairwise testing between the different nicotine-injected animals and the nicotine-injected controls (study III) were performed using the Tukey HSD test.

**Swim maze test**
The data from days 1 to 4 of the test were subjected to a general linear model (GLM) with a split-plot design and to Duncan’s multiple range test. Comparisons between the performance of the last trial on day 4 versus day 5 were submitted to a paired t-test and Kruskal-Wallis with pairwise testing, using Duncan’s test (study II).

**Elevated plus-maze test**
Time spent on the open arms and the entries into the open arms were tested using a one-way ANOVA, and pairwise testing between the different treat-
ments groups and the control group was preformed using a Tukey HSD test (study I and III).

**Analysis of neuroproteins**
Differences between CaMKII, GAP-43, synaptophysin, and tau protein levels in different treatment groups were determined using a one-way ANOVA and pairwise testing using Newman-Kuels *post hoc* test (studies IV and VI).
Results and discussion

This thesis has investigated the developmental neurotoxic effects after neonatal exposure to four highly brominated diphenyl ethers PBDEs (PBDE 209, 206, 203, and 183), and three perfluorinated compounds, PFCs (PFOS, PFOA, and PFDA). Neonatal NMRI male mice were exposed to a single compound or to two different ones administered as a single oral dose during the neonatal brain’s rapid growth and development. It was shown that PBDE 209 can cause a dose–response-related change in spontaneous behavior and altered susceptibility of the cholinergic system in adult animals. Possible debrominated products of PBDE 209, i.e., PBDE 206 and PBDE 203, were shown to cause effects in spontaneous behavior as well as alterations in memory and learning that were similar to those caused by PBDE 209. Neonatal exposure to PFOS and PFOA were shown to cause effects similar to those of the highly brominated PBDEs. Furthermore, it was shown that PBDE 209 and PFOA, at low doses, can interact during the BGS to enhance alterations in spontaneous behavior, in a time-related manner. The levels of proteins important for brain development were shown to be altered in the neonatal mouse brain after exposure to PBDE 209 and PFOA given alone or in combination. These effects are induced when the parent compound/or its metabolites are present during a defined critical period of BGS, namely on PND 10.

Developmental neurotoxic effects of deca- (PBDE 209), nona- (PBDE 206), octa- (PBDE 203), and heptaBDE (PBDE 183)

This thesis (studies I, II, V, and VI) demonstrated that exposure to highly brominated diphenyl ethers during the period of the brain’s rapid growth and development in the neonatal mouse can cause developmental neurotoxic effects. Observed effects were disturbed spontaneous behavior, habituation, and learning and memory abilities, and altered susceptibility to the cholinergic agent nicotine in adult animals. This thesis also demonstrated that the levels of important proteins for normal brain development (synaptophysin and tau) can be affected in the neonatal mouse brain. The disturbances in spontaneous behavior caused by PBDE 209 were dose–response-related and worsened with age. NonaBDE (PBDE 206) and octaBDE (PBDE 203), poss-
ible products of debrominated PBDE 209, were shown to induce neurobehav-
ioral effects that were similar to those caused by PBDE 209. These findings
show that highly brominated PBDEs can be as potent as the lower bromi-
nated PBDEs.

Neurobehavioral effects observed in the spontaneous behavior test

The first study (study I) investigated whether exposure to PBDE 209 could
alter spontaneous behavior in a dose–response manner in mice. Mice were
exposed to 1.4 μmol (1.34 mg), 2.3 μmol (2.22 mg), 14 μmol (13.4 mg), or
21 μmol (20.1 mg)/kg body wt. Spontaneous behavior was observed in mice
at 2 months and 4 months of age. The spontaneous behavior test revealed a
significant dose-related change in all three test variables. In the control mice,
there was a distinct decrease in activity in all three spontaneous behavioral
variables over the 60-min period, in both 2- and 4-month-old mice. In the
spontaneous behavior test conducted at 2 months of age, mice exposed to 2.3
to 21 μmol PBDE 209/kg body wt. were significantly less active for all three
behavioral variables during the first 20-min period (0-20 min) than the con-
trol mice and mice given 1.4 μmol PBDE 209/kg body wt. During the third
20-min period (40-60 min), mice given 14 and 21 μmol PBDE 209/kg body
wt. showed significantly increased activity in all three variables, compared
with the control mice and mice given 1.4 and 2.3 μmol/kg body wt.

Four months after neonatal exposure to PBDE 209, there was still a sig-
nificant dose-related change in all three test variables (Fig. 3). The lowest
dose of PBDE 209 to cause a decrease in total activity during the first 20-
min period was 1.4 μmol/kg body wt., and the lowest dose to cause an in-
crease in activity during the third 20-min period was 2.3 μmol/kg body wt.

From this test, it could also be concluded that the aberrations were more
pronounced in 4-month-old mice than in 2-month-old mice. This is con-
firmed by the significantly reduced habituation capability of 4-month-old
mice compared with that of 2-month-old mice exposed to 2.3, 14, and 21
μmol PBDE 209/kg body wt. These dose–response study findings were also
in agreement with the earlier observed developmental neurobehavioral de-
fects in NMRI mice exposed neonatally to 21 μmol PBDE 209/kg body wt.
(Viberg et al. 2003b). Furthermore, both the dose–response and time–
response defects in spontaneous behavior and the reduced habituation ability
with age are similar to those defects that were reported earlier to occur in
mice exposed neonatally to the lower brominated PBDEs: PBDE 47, PBDE
99, and PBDE 153 (Eriksson et al. 2001; Viberg et al. 2003a; Viberg et al.
2004b). A study by Rice and co-workers (Rice et al. 2007) has shown that
Figure 3. Spontaneous behavior of 4-month-old NMRI male mice exposed to a single oral dose of 1.4, 2.3, 14, or 21 μmol PBDE 209/kg body wt. on PND 3. In the same manner, control animals received 10 ml/kg body wt. of the 20% fat emulsion vehicle on PND 3. The statistical differences are indicated as (A) significantly different vs. controls $P \leq 0.01$; (a) sign. diff. vs. controls $P \leq 0.05$; (B) sign. diff. vs. 1.4 μmol PBDE 209/kg body wt. $P \leq 0.01$; (b) sign. diff. vs. 1.4 μmol PBDE 209/kg body wt. $P \leq 0.05$; (C) sign. diff. vs. 2.3 μmol PBDE 209/kg body wt. $P \leq 0.01$; (c) sign. diff. vs. 2.3 μmol PBDE 209/kg body wt. $P \leq 0.05$; (D) sign. diff. vs. 14 μmol PBDE 209/kg body wt. $P \leq 0.01$; (d) sign. diff. vs. 14 μmol PBDE 209/kg body wt $P \leq 0.05$. Bar heights represent mean values ± SD.

PBDE 209 can have developmental neurobehavioral effects in another strain of mice (C57BL6/J). Several studies in mice have shown that the presence of compounds, both persistent and non-persistent around neonatal day 10, can cause permanent developmental neurotoxic disturbances (Eriksson 1997; Eriksson et al. 1992; Eriksson et al. 2000). Regarding neonatal exposure to
PBDE 209 (21 μmol/kg body wt.), the developmental neurotoxic effects on spontaneous behavior and habituation capability were seen only after exposure on PND 3 but not in mice given PBDE 209 on PND 10 (Viberg et al. 2003b). It is known that the amount of a toxic agent present in the brain at different neonatal ages can vary. Earlier studies, using radio-labelled compounds, have shown a pronounced retention of lipophilic chlorinated hydrocarbons or their metabolites (e.g., PBDE 99, DDT, PCB 52, PCB 153, and chlorinated paraffins) in the brain when the compounds were administered on neonatal day 10 (Eriksson 1984; Eriksson and Darnerud 1985; Eriksson and Fredriksson 1998; Eriksson et al. 2002). The amount of radioactivity from these substances was the same or decreased gradually over the 7-day period. In contrast, the radioactivity from 14C-PBDE 209 increased significantly in animals exposed to 14C-labelled PBDE 209 on PND 3 or PND 10 (Viberg et al. 2003b). The capability of neonatal mice to metabolize persistent organic compounds, e.g., PCBs, has been reported by Vodicnik and co-workers (Vodicnik et al. 1980). Therefore, it was suggested that the developmental neurotoxic effects of PBDE 209 were caused by one or more metabolites of the parent compound.

To test the hypothesis that products of debrominated PBDE 209 can cause the effects seen in mice exposed to PBDE 209, three possible metabolites of PBDE 209, namely PBDE 206, PBDE 203, and PBDE 183, was given to neonatal NMRI male mice that were 3 days or 10 days old. The mice were exposed to 21 μmol (18.5 mg) PBDE 206/kg body wt., 21 μmol (16.8 mg) PBDE 203/kg body wt., or 21 μmol (15.2 mg) PBDE 183/kg body wt. (study II). Spontaneous behavior was observed in mice that were 2 months old. The administration of 21 μmol PBDE 206/kg body wt., or 21 μmol (16.8 mg) PBDE 203/kg body wt. to mice on PND 10 caused disturbances in spontaneous behavior, with nonhabituating behavior observed at 2 months of age. Mice exposed neonatally to PBDE 206 on PND 10 showed significantly decreased activity in all three variables during the first 20-min period as compared to that of the control animals, but an increase in all three variables during the third 20-min period. Similarly, mice exposed to PBDE 203 on PND 3 showed a significant decrease in all three variables during the first 20-min period as compared to the controls, and a significant increase in activity was seen in the three variables during the third 20-min period. Mice exposed to PBDE 203 on PND 10 showed a significant decrease in all three variables during the first 20-min period when compared to those of the controls. During the third 20-min period, a significant increase in activity was seen in the three variables. Although defects in spontaneous behavior were seen after neonatal exposure to PBDE 203 on PND 3 and PND 10, the effects were significantly more pronounced in mice exposed on PND 10, indicating that the neonatal brain is more vulnerable to PBDE 206 and PBDE 203 in 10-day-old mice than in 3-day-old mice. This is also supported by the effect seen in mice exposed to PBDE 203 on PND 10, when tested with the Morris swim maze.
Whether or not the metabolites of PBDE 209 in the neonatal brain of 10-day-old mice constitute debrominated products such as PBDE 206 and PBDE 203, after administration to neonatal mice on PND 3, the present study has shown that congeners such as PBDE 203 and PBDE 206 are capable of inducing developmental neurotoxic effects when given on PND 10.

In studies I, II, and V, a non-habituating behavior was seen in mice exposed to highly brominated PBDEs, with hypoactive behavior observed early in the 60-min test period. In the end, however, the mice became hyperactive. This nonhabituating behavior profile has also been reported to occur in adult mice neonatally exposed to PBDE 47, PBDE 99, and PBDE 153 (Eriksson et al. 2001; Viberg et al. 2003a; Viberg et al. 2004b). It is conceivable that the reduced activity at the beginning of this test might have been related to increased anxiety about a novel home environment. However, the behavior observed in the EPM failed to reveal any significant differences in the variables, entries, or time spent in the open arms (study I). It therefore appears that the reduced activity seen in mice neonatally exposed from 2.3 to 21 μmol PBDE 209/kg body wt. during the first 20-min period of spontaneous behavior was not due to anxiety-like behavior.

Effects on learning and memory abilities

Spontaneous behavior and habituation can be considered as a non-associated learning process. The animals’ ability to learn and memorize was further explored in a Morris type of water maze, which provides information about spatial learning and memory abilities. The test was performed in study II, in which mice exposed to PBDE 203 or the vehicle on PND 3 and mice exposed to PBDE 206, PBDE 203, or the vehicle on PND 10 (Fig. 4), were observed to see how they performed in the swim maze when they were 3 months old. During the acquisition period (days 1-4) of spatial learning ability, all mice, regardless of treatment, improved their ability to locate the platform. However, mice exposed to PBDE 203 on PND 10, differed significantly from control mice. This reveals a treatment effect of PBDE 203 exposure.

To study relearning, the platform was relocated on day 5. In the first trial on day 5, the control mice in each age category displayed significantly longer latencies than those in the last trial on day 4. This is a normal behavior during relearning (Morris et al. 1982). The control mice improved their ability to find the platform during the trials on day 5, indicating normal relearning in control mice. Mice exposed to PBDE 203 on PND 3 or PBDE 203 and PBDE 206 on PND 10 did not significantly differ from their control group. Lower brominated PBDEs, e.g., PBDE 99 and PBDE 153, were shown earlier to affect performance in the Morris water maze, in mice exposed on PND 10 (Eriksson et al. 2001; Viberg et al. 2003a). The reduced ability of mice to perform in the Morris swim maze have been reported in advanced age rodents (Gage et al. 1984; Gallagher and Pelleymounter 1988; Lamberty and
Gower 1989; Magnusson 1998; Pelleymounter et al. 1990). Spatial earning 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). Performance in this maze test is also connected to the function of the cholinergic system (Lindner and Schallert 1988; Whishaw 1985).

![Figure 4](image_url)

Figure 4. Swim maze performance in 3-month-old NMRI male mice exposed to a single oral dose of either 21 μmol/kg body wt. (16.8 mg) PBDE 203, (18.5 mg) PBDE 206, or 20% fat emulsion vehicle (control), at the age of 10 days. Latencies in locating the platform were measured during the acquisition period, trials 1-20 (days 1-4), and during the relearning period, trials 21-25 (day 5). A significant treatment effect was seen during the acquisition period (days 1-4) when mice exposed to PBDE 203, on PND10, significantly differed from vehicle-treated mice ($P \leq 0.01$) and from those mice administered PBDE 206 ($P \leq 0.05$). Trial 20 vs. 21 was submitted to a $t$-test, as was trial 21 vs. 25, but no significant changes were observed.

Effects on the cholinergic system

In study I, neonatal exposure to PBDE 209 was shown to alter the susceptibility of the cholinergic system in a dose–response-related manner in adult animals. In mice exposed to 14 or 21 μmol PBDE 209/kg body wt., a hypoactive response to nicotine was observed during the first 20-min period (60-80 min). An altered response was also seen in mice exposed to 1.4 or 2.3 μmol PBDE 209/kg body wt. In control animals, hyperactivity was seen after injecting 80 μg nicotine base/kg body wt. This is a normal reaction to this dose of nicotine (Eriksson et al. 2000; Eriksson and Fredriksson 1996; Viberg et al. 2002). It is known that a low dose of nicotine can induce hyperactivity in animals, whereas a high dose induces hypoactivity (Nord-
The dose–response-related reaction (susceptibility) of the cholinergic system was clearly demonstrated when evaluating the difference in reaction between saline-injected and nicotine-injected mice (Fig. 5). The altered and opposite response to nicotine was the same as that observed earlier in mice exposed neonatally to PBDE 99, PCB 52, or nicotine (Eriksson et al. 2000; Eriksson and Fredriksson 1996; Viberg et al. 2002). It was particularly interesting to see that the dose–response-related change in reaction to nicotine was similar to that found earlier in mice treated neonatally with nicotine (Ankarberg et al. 2001; Eriksson et al. 2000; Nordberg et al. 1991). In the study by Eriksson and co-workers (2000), the nicotine-induced behavioral test revealed a hypoactive response to nicotine, though only in mice exposed on PND 10-14, and not in those exposed on PND 3-7 or PND 19-23. The response to nicotine by mice in the control groups and in the other age categories was increased activity. At no time during the neonatal period could nicotinic receptors of low affinity nicotine-binding sites be found following nicotine treatment, but the persistence of this effect and the altered response to nicotine was evident only in adult mice exposed on PND 10-14 (Eriksson et al. 2000; Nordberg et al. 1991). From previous studies it is known that the neurotoxic effects of PBDE 209 are induced only when given on PND 3, but not on PND 10 or PND 19 (Viberg et al. 2007; Viberg et al. 2003b). The findings in studies I and II on the effects on spontaneous behavior, learning and memory, and susceptibility of the cholinergic system further support the finding that the effects of PBDE 209 on development are caused by metabolites (possible debrominated ones) of PBDE 209, because possible metabolites of PBDE 209, namely PBDE 206 and PBDE 203, cause effects similar to those caused by PBDE 209. The most pronounced effects were seen in mice exposed to PBDE 206 or PBDE 203 on PND 10. One of the mechanisms of the developmental neurobehavioral defects caused by PBDE 209 appears to involve changes in the cholinergic system that might have implications for induction of cognitive disorders.
Figure 5. Four-month-old NMRI male mice exposed to a single dose of either 20% fat emulsion vehicle or 1.4, 2.3, 14, or 21 μmol PBDE 209/kg body wt. on PND 3. In the same manner, control mice received 10 ml/kg body wt. of the 20% fat emulsion vehicle on PND 3. The nicotine-induced behavior was studied by using 80 μg nicotine base/kg body wt. s.c. and 10 ml 0.9% NaCl/kg body wt. s.c. The difference between locomotion mean (period 60-80 min) for nicotine- and saline-injected mice are shown for the different doses administered. Linear equation $y = -32.27x + 193.12$ with the correlation coefficient $r^2 = 0.9995$.

Effects on protein levels in the neonatal brain

In study VI, the levels of four proteins were analyzed in the cerebral cortex and the hippocampus of the neonatal brain of mice neonatally exposed to 1.4 μmol PBDE 209/kg body wt. at PND 3. The study demonstrates that exposure to 1.4 μmol PBDE 209/kg body wt. can cause significantly increased protein levels of synaptophysin and tau in the neonatal brain. In mice exposed to PBDE 209 at PND 3 and to the vehicle at PND 10, the synaptophysin level was significantly increased ($P \leq 0.001$) in the hippocampus, by 71%, compared to synaptophysin level in the control mice. The tau protein level in the cerebral cortex was significantly increased ($P \leq 0.001$), compared to that in the control mice. In mice exposed to PBDE 209, the tau protein level in the hippocampus was significantly increased ($P \leq 0.001$), compared to the tau protein level in the control mice.

The increased level of synaptophysin in the hippocampus is in agreement with unpublished data from Viberg that shows an increased synaptophysin level in the hippocampus in mice exposed to 21 μmol PBDE 209/kg body wt. (Viberg submitted). In that study, the tau levels were not significantly changed in the cerebral cortex or hippocampus.

The protein level of GAP-43 or CaMKII were not significantly changed in the cerebral cortex or hippocampus in mice exposed to the PBDE 209-vehicle. In a study conducted by Viberg and co-workers, it was reported that mice exposed on PND 3 to 21 μmol PBDE 209/kg body wt. showed in-
creased protein levels of CaMKII in the hippocampus and decreased levels of GAP-43 in the cerebral cortex (Viberg et al. 2008a). The effects on CaMKII, GAP-43, and tau need to be explored further in a dose–response study.

Developmental neurotoxic effects of PFOS, PFOA, and PFDA

This thesis demonstrates that neonatal exposure to PFCs like PFOS and PFOA during a defined critical period of the BGS can cause a disturbed spontaneous behavior, altered susceptibility of the cholinergic system in adult animals, and behavioral effects that worsen with age (studies III and V). However neonatal exposure to PFDA did not cause any significant behavioral effects. Furthermore, the levels of protein important for normal brain development were altered in the neonatal brain after neonatal exposure (studies IV and VI). It was shown that neonatal exposure to PFOS or PFOA can affect the levels of CaMKII, GAP-43, synaptophysin, and tau and increase it in the neonatal mouse brain.

Neurobehavioral effects observed in the spontaneous behavior test

Study III investigated whether neonatal exposure to a single oral dose of PFOS, PFOA, or PFDA could alter spontaneous behavior in adult mice. Neonatal mice were exposed to either 1.4 or 21 μmol/kg body wt. of PFOS (0.75 or 11.3 mg), PFOA (0.58 or 8.70 mg), or PFDA (0.72 or 10.8 mg), on PND 10. Spontaneous behavior was observed in mice at the ages of 2 months and 4 months. In the control mice, there was a distinct decrease in activity in all three spontaneous behavioral variables over the 60-min period, in 2- and 4-month-old mice (also seen in studies I, II, and V). In the spontaneous behavior test, the control mice showed a normal decrease in activity during the 60-min test period, at 2 months and 4 months of age, similar to the habituation seen in control mice in studies I and II. This behavioral pattern was not seen in mice exposed to 21 μmol PFOS or PFOA/kg body wt., in which a hypoactive behavior was seen in the beginning of the 60-min period. However, in the end they showed a hyperactive behavior when they were 2 months old. When tested at the age of 4 months, the same behavioral pattern was seen in mice exposed to 21 μmol of PFOS or PFOA (Fig. 6) (Please note that Fig. 2 in paper 3 does not show the spontaneous behavior at 4 months of age. The spontaneous behavior in 4-month-old mice is illustrated in Fig. 6, below). The lowest dose of PFOA to cause any change in any of the three variables in 4-month-old mice was 1.4 μmol/kg body wt., in the locomotion and total activity variables. In mice given 21 μmol PFOS or
PFOA/kg body wt., spontaneous behavior was seen to worsen as mice aged from 2 months old to 4 months old, where the habituation ratios for locomotion and rearing increased significantly ($P \leq 0.01$). This means that the ability to habituate to a new home environment deteriorated with age after exposure to 21 μmol PFOS or PFOA/kg body wt. These changes in behavior, both time-dependent and dose-related, indicate the advance of a brain dysfunction process induced at the time of BGS in the neonatal mouse. In study V, 1.4 or 14 μmol PFOA/kg body wt. was administered on PND 3 or PND 10. The spontaneous behavior in 2- and 4-month-old mice revealed that a neurobehavioral effect could only be induced when the compound was administered on PND 10, when no significant changes in the three variables were seen in mice exposed to 1.4 or 14 μmol PFOA/kg body wt. on PND 3, compared to the control mice.

Activity in the spontaneous behavior test was reduced in mice exposed neonatally to PFOS and PFOA during the first 20-min period, compared with the controls. In studies I, II, and V, similar non-habituating behavior was seen in mice exposed to highly brominated PBDEs. The mice demonstrated hypoactive behavior early in the 60-min test period, whereas at the end of the period, they became hyperactive. Similar behavior has been seen in adult mice neonatally exposed to PBDE 47, PBDE 99, and PBDE 153 (Eriksson et al. 2001; Viberg et al. 2003a; Viberg et al. 2004b). The EPM test failed to reveal any significant differences in the variables, entries, or time spent in the open arms in the PBDE 209 exposed mice (study I). The EPM test was also conducted in study III on 4-month-old mice neonatally exposed to PFOS or PFOA. The behavior observed in the EPM failed to reveal any significant differences in the variables, entries, or time spent in the open arms. Therefore it appears that the reduced activity of mice exposed neonatally to 21 μmol PFOS/kg body wt., or 1.4 or 21 μmol PFOA/kg body wt. observed during the first 20-min period of spontaneous behavior was not due to anxiety-like behavior.
Figure 6. Spontaneous behavior of 4-month-old NMRI male mice exposed to a single oral dose of either 1.4 or 21 μmol/kg body wt. of PFOS (0.75 or 11.3 mg/kg body wt.), PFOA (0.58 or 8.70 mg/kg body wt.), or PFDA (0.72 or 10.8 mg/kg body wt.), via a metal gastrictube at the age of 10 days. In the same manner, the control animals received 10 ml/kg body wt. of the 20% fat emulsion vehicle. 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. 1.4 μmol PFOS/kg body wt. $P \leq 0.01$; (b) significantly different vs. 1.4 μmol PFOS/kg body wt. $P \leq 0.05$; (C) significantly different vs. 1.4 μmol PFOA/kg body wt. $P \leq 0.01$; (D) significantly different vs. 21 μmol PFOA/kg body wt. $P \leq 0.01$. Bar height represents mean value ± SD.
Effects on the cholinergic system

While the disturbances seen in the spontaneous behavior of mice exposed to PFOS or PFOA are quite similar to those of mice exposed to the highly brominated PBDEs, it would be of interest to see if the susceptibility of the cholinergic system shows similarities as well. Therefore, the susceptibility of the cholinergic system was explored in a nicotine-induced behavior test in 4-month-old mice neonatally exposed to 1.4 or 21 μmol PFOS, PFOA, or PFDA/kg body wt. (study III).

In the nicotine-induced behavior test, the control animals responded with increased activity during the first 20-min period, after being injected with 80 μg nicotine base/kg body wt. During the last 20-min period of this test, the control mice showed base-line activity again. This response was also seen for control mice in study I. Mice neonatally exposed to 21 μmol PFOS or PFOA/kg body wt., responded to nicotine with decreased activity during the first 20-min period. A dose–response change in the response to nicotine was seen in the PFOA-treated mice. When mice were exposed to 1.4 μmol PFOA/kg body wt., they were significantly less active than the control mice during the first 20-min period.

In study I, mice exposed to 14 or 21 μmol PBDE 209/kg body wt. showed a hypoactive response to nicotine during the first 20-min period (60-80 min). An altered response was also seen in mice exposed to 1.4 or 2.3 μmol PBDE 209/kg body wt., see Fig. 5. This altered and opposite response to nicotine is the same as the response observed in a previous study of mice exposed neonatally to PBDE 99, PCB 52, and nicotine (Eriksson et al. 2000; Eriksson and Fredriksson 1996; Viberg et al. 2002).

This shows that PFOS and PFOA can affect the cholinergic system during its development, causing increased susceptibility at adult age, as was observed in previous studies of PBDEs and PCBs.

Effects on protein levels in the neonatal brain

Study IV investigated whether neonatal exposure to a single oral dose of PFOS or PFOA could change the protein levels of CaMKII, GAP-43, synaptophysin, and tau. Recently it has been shown that neonatal exposure to PBDE 209 can affect the levels of CaMKII and GAP-43 in the neonatal brain (Viberg et al. 2008a). Study IV was conducted to investigate whether neonatal exposure to PFOS and PFOA can affect the levels of these proteins in a similar way as PBDE 209 exposure does. Mice were exposed to either 21 μmol PFOS or PFOA/kg body wt. on PND 10. It was demonstrated that mice exposed to 21 μmol PFOS or PFOA/kg body wt. showed significantly increased protein levels of GAP-43 and CaMKII in the hippocampus, compared to levels of these proteins in vehicle-treated mice (Fig. 7A). In mice exposed to PFOA, the synaptophysin and tau protein levels were significant-
ly increased both in the hippocampus and in the cerebral cortex (Fig. 7A and 7B). In mice exposed to PFOS, the synaptophysin level was significantly increased in the hippocampus and cerebral cortex. The tau protein level, however, was only significantly increased in the cerebral cortex. In study VI, it was shown that 14 μmol PFOA/kg body wt. could affect the level of tau in the neonatal mouse brain. The level of the tau protein was significantly increased in the cerebral cortex and hippocampus, compared to the tau protein level in the control animals. The results from study IV indicate that the hippocampus is the most sensitive brain region investigated after neonatal exposure to PFOS and PFOA. Regional differences have been reported after neonatal exposure to PBDE 209 and ketamine, where the protein levels of GAP-43 and CaMKII were increased in the hippocampus (Viberg et al. 2008a; Viberg et al. 2008b). Regional differences have also been seen after neonatal exposure to PBDE 209, PBDE 206, and PBDE 203, where CaMKII and synaptophysin levels were increased in the hippocampus (Viberg submitted) (Viberg et al. 2008a).

These proteins are affected after neonatal exposure to PBDE 203, 206, 209, and ketamine at doses where functional impairments in adult mice have been observed (Fredriksson and Archer 2004; Fredriksson et al. 2007; Viberg et al. 2003b; Viberg et al. 2006). With regard to studies III, IV, and VI, PFOS and PFOA can now be included among those chemicals. The interaction between the studied proteins and the development of the cholinergic system in the hippocampus may help explain the effects of these chemicals on adult behavior and an altered response of the cholinergic system.

Interaction between PBDE 209 and PFOA during neonatal brain development

In studies I, II, and III, it was shown that neonatal exposure to PBDE 209, 206, and 203, as well as to PFOS and PFOA, can cause similar developmental neurotoxic defects in spontaneous behavior in adult animals. In study V, it was shown that PBDE 209 and PFOA can interact during the critical period of brain development and enhance the developmental neurobehavioral effect on spontaneous behavior. Exposure to PFOA was shown to affect the protein levels of CaMKII, GAP-43, synaptophysin, and tau in the neonatal brain (study IV). Similar changes in protein levels have reportedly been seen after neonatal exposure to PBDE 209 (Viberg et al. 2008a). Therefore, it was investigated whether PBDE 209 and PFOA could interact during this period and enhance the effects on the protein levels of those proteins. It was shown that the protein level of synaptophysin was significantly increased in the brains of mice exposed to PBDE 209 on PND 3 and later PFOA on PND 10, compared to mice exposed to the sole compounds (study VI).
Figure 7. Protein level of CaMKII, GAP-43, synaptophysin, and tau in (A) the hippocampus and (B) cerebral cortex of animals exposed to 21 μmol PFOS or PFOA/kg body wt. on PND 10 and sacrificed 24 h later. The statistical differences are indicated as (**) significantly different vs. controls $P \leq 0.01$; (*) significantly different vs. controls $P \leq 0.05$. The height of the bars represents the mean value ± S.D.

Neurobehavioral effects observed in the spontaneous behavior test

In study V, it was investigated whether PBDE 209 and PFOA could interact during the BGS to enhance developmental neurotoxic effects on spontaneous behavioral variables and whether the effects could be time-dependent. Mice were orally exposed on PND 3 and PND 10 to PBDE 209 (1.4 or 8.0 μmol/kg body wt.), PFOA (1.4 or 14 μmol/kg body wt.), both PBDE 209 and PFOA, or the 20% fat emulsion vehicle, as given in the table in paper V. Spontane-
ous behavior was observed in mice at ages 2 months and 4 months. In study V, it was shown that a highly brominated diphenyl ether, PBDE 209, and a PFC, PFOA, can interact during neonatal brain development to enhance developmental neurobehavioral defects in mice. This interaction is obviously dependent on both the metabolism of PBDE 209 and the presence of its metabolites (possibly debrominated ones) together with PFOA during a defined critical period of neonatal brain development, namely around PND 10. Neonatal exposure to a single oral dose of PBDE 209 on PND 3 and, later, a single oral exposure to PFOA on PND 10 caused impaired spontaneous behavior in 2- and 4-month-old mice, an effect significantly changed from single exposure to PBDE 209 or PFOA. Furthermore, these behavioral defects were also sustained over time, and at low doses, were also time dependent.

Mice exposed on PND 3 to PBDE 209 (1.4 μmol) and on PND 10 to PFOA (14 μmol) displayed significantly disrupted spontaneous behavior and defective habituation at the ages of 2 months and 4 months. Control mice displayed a normal habituation profile, as seen in studies I, II, and III, whereas mice exposed to PBDE 209 (1.4 μmol) on PND 3 + PFOA (14 μmol) on PND 10, were obviously hypoactive early in the 60-min test period, becoming hyperactive toward the end. A change in spontaneous behavior and habituation was also evident in mice neonatally exposed to high doses of PBDE 209 (8 μmol) and PFOA (14 μmol). Study V also reveals that the developmental neurotoxic effects were as pronounced in mice receiving the combined dose of PBDE 209 + PFOS (PND 3; 1.4 μmol + PND 10; 14 μmol), as in mice given the six-fold higher dose of PBDE 209 (8 μmol), and the behavior deviation was about two times higher, compared to the mice just receiving PFOA (14 μmol). Both the combination PBDE 209 + PFOA (PND 3; 1.4 μmol + PND 10; 14 μmol) and the six-fold higher dose of PBDE 209 caused a similar hypoactive condition during the first 20 min and a hyperactive condition during the last 20-min period. Of special significance was the observed hyperactive condition in 4-month-old mice neonatally exposed to the low doses of PBDE 209 and later PFOA (PND 3; 1.4 μmol + PND 10; 1.4 μmol) (Fig. 8), at which the dose of PFOA had no significant effects and PBDE 209 could be a borderline dose. This indicates that these two compounds also can interact at low doses to enhance developmental neurotoxic effects and that the interaction can be time-dependent.

Earlier studies have shown that a lower brominated PBDE can interact with other persistent environmental contaminants like PCB and MeHg to enhance developmental neurotoxic effects when present during the critical period of brain development (Eriksson et al. 2006a; Fischer et al. 2008a, b). The effects were manifested as deranged spontaneous behavior, lack and/or reduced habituation, as well as learning and memory defects. Study V shows that PBDE 209 and PFOA interact at doses that are comparable on molar levels to those other agents and that they thereby can be as potent as they are in inducing developmental neurotoxic effects.
Figure 8. Spontaneous behavior in 4-month-old NMRI male mice orally exposed to PBDE 209 on PND 3 and PFOA on PND 10, or to a 20% fat emulsion vehicle. Statistical differences: A = P ≤ 0.01, a = P ≤ 0.05, vs control. B = P ≤ 0.01, b = P ≤ 0.05, vs PBDE 209 (1.4 μmol, PND 3) and PFOA (1.4 μmol, PND 10). C = P ≤ 0.01, c = P ≤ 0.05, vs PBDE 209 (1.4 μmol, PND 3) and PFOA (14 μmol, PND 10). D = P ≤ 0.01, d = P ≤ 0.05, vs PBDE 209 (8 μmol, PND 3) and PFOA (1.4 μmol, PND 10). E = P ≤ 0.01, e = P ≤ 0.05, vs PBDE 209 (8 μmol, PND 3) and PFOA (14 μmol, PND 10). The height of each bar represents the mean ± SD of 8 animals.
Effects on protein levels in the neonatal brain

In study VI, it was investigated whether PBDE 209 and PFOA can interact and enhance the effects of the protein levels of CaMKII, GAP-43, synaptophysin, and tau in the neonatal brain. It was demonstrated that mice exposed to 1.4 μmol PBDE 209/kg body wt. on PND 3 and later to 14 μmol PFOA/kg body wt. on PND 10 showed a significantly increased level of synaptophysin in the cerebral cortex as compared both to mice exposed the sole compounds and to the control mice. The level was significantly increased by 174%, compared to the level in the control mice. The level of synaptophysin was increased in the hippocampus, compared to the level in mice exposed to PFOA and in the control mice. The level was increased by 93% compared to the level in the control mice.

In mice exposed to PBDE 209 on PND 3 and later PFOA on PND 10, the tau levels in the hippocampus were significantly increased by 209% compared to tau levels in the control mice, and they were significantly increased compared to mice exposed to PFOA. In the cerebral cortex the tau levels were significantly increased by 664% compared to levels in the control mice, and these levels were significantly increased compared to levels in mice exposed to PFOA. No significant changes in the CaMKII and GAP-43 protein levels were seen in the cerebral cortex or hippocampus in mice exposed to PBDE 209 and later to PFOA during the BGS. A possible explanation for this might be that the doses of PBDE 209 and PFOA used were too low to affect the level of these proteins in the cerebral cortex and hippocampus.

As in study V, it was shown that the two compounds can interact at low doses during the critical period of brain development to enhance the effects. Whether a change in the levels in some of these proteins during brain development and the development of the cholinergic system is the explanation for the effects seen after exposure to PBDE 209 on PND 3 and later PFOA on PND 10 (study V) is an intriguing question, and further studies are needed to clarify the relationship.
General discussion

It is known that exposure to xenobiotics (e.g. lead, alcohol, and nicotine) during the fetal period can cause functional anomalies of the CNS that results in behavioral, cognitive, and motor defects, for references see (Schettler 2001). 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 these differences between animals used in research and humans. To cover critical developmental phases occurring in the fetal and newborn periods, the mouse animal model can be used to follow the BGS. By using the mouse as an animal model one can study the effects of a single toxicant administered directly to animals during different stages of the BGS. Interacting effects between different toxicants and the interaction between neonatal and adult exposure can also be studied in a controlled manner. In this animal model, it is possible to isolate the effect of toxicants and to study certain issues that can be difficult to solve in traditional developmental toxicity tests and in epidemiological studies.

By using this animal model, previous studies have shown that the period of rapid brain development is vulnerable to insult by chemicals such as the well-known neurotoxic agents DDT (Eriksson 1992; Eriksson et al. 1992), nicotine (Eriksson et al. 2000), organophosphate (DFP) (Ahlbom et al. 1995), and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Fredriksson et al. 1993), and the anesthetic agent ketamine (Viberg et al. 2008b). Similar effects have been shown for chemicals not intended to be neurotoxic. Such chemicals include PCBs (Eriksson 1998), the BFR HBCDD (Eriksson et al. 2006b), lower and highly brominated PBDEs (PBDE 47, 99, 153, and 209) (Eriksson et al. 2001; Eriksson et al. 2002; Viberg et al. 2003a, 2004a; Viberg et al. 2003b). The presence of any of these compounds in the brain during a defined period of this maturational process is a critical factor. In this animal model, it has also been reported that agents like the BFR TBBPA and two OPs used as FR and plasticizers, namely triphenyl phosphate and tris(2-chloro-ethyl)phosphate, do not induce any developmental neurotoxic effects at corresponding doses as used above (Eriksson et al. 2001; Eriksson et al. 2004).

This thesis clearly shows that neonatal exposure to PBDE 209 can cause developmental neurotoxic effects observed as dose–response changes in spontaneous behavior and can alter the susceptibility of the cholinergic system. The lowest dose to cause a decrease in the total activity during the first
20-min period was 1.4 μmol/kg body wt. Developmental exposure of mice and rats to PBDE 209 has been reported to cause neurobehavioral defects (Rice et al. 2007; Viberg et al. 2007; Viberg et al. 2003b). In the study by Rice and co-workers (2007), C57BL6/J mice were exposed to a daily dose of 0, 6, or 20 mg PBDE 209/kg body wt. from PND 2 to 15. It was reported that male mice exposed to 20 mg/kg/day showed increased locomotor activity during the first 1.5 h of a 2 h test session at adult age. It is worth noting that the behavior test was conducted at least two hours after the onset of the dark cycle in the vivarium. This indicates that PBDE 209 can be toxic in different mouse strains and also that behavioral deviations are seen both during the active dark period and the less active light period.

The study by Viberg et al. (2003) suggested that the effects were caused by metabolites of PBDE 209 that were present during the critical period of brain development, namely around PND 10 (Viberg et al. 2003b). In this thesis, it has also been demonstrated that possible debrominated products of PBDE 209, i.e., nonaBDE (PBDE 206) and octaBDE (PBDE 203), can cause changes in spontaneous behavior that are similar to those caused by PBDE 209, when administered during the critical period of brain development in mice. When mice exposed to PBDE 206 on PND 10 were tested in the Morris swim maze at adult age, it was shown that the learning and memory functions were affected. Taken together these developmental toxicity data on PBDE 209, 206, and 203 strengthens the hypothesis that it is a metabolite of PBDE 209 that induces the effect when present around PND 10. Earlier studies have shown that lower brominated PBDEs, PBDE 47, PBDE 99, and PBDE 153 can induce developmental neurotoxic effects, at doses of 1.4-21 μmol/kg body wt. (Eriksson et al. 2001; Viberg et al. 2003a; Viberg et al. 2004b). The doses used and effects seen of the highly brominated PBDEs in this thesis shows that highly brominated PBDEs can be as potent as the lower brominated PBDEs, tested earlier.

This thesis shows that similar neurotoxic effects can be seen in adult mice neonatally exposed on PND 10 to PFOS and PFOA. These effects are dose-related and exacerbated with age. As seen for other agents, like PCB, DDT, and lower brominated PBDEs, the effect of neonatal exposure to PFOS was shown to be induced when given on PND 10. In another developmental toxicity study, it was reported that male offspring from maternal treatment (gestation day 0 through PND 20) to 1.0 mg PFOS/kg body wt./day displayed increased motor activity and reduced habituation on PND 17. This was not seen on PND 13, 21, and 61 (Butenhoff et al. 2008). The difference between the effects seen in mice neonatally exposed on PND 10 and the effects seen from maternal exposure from GD 0-PND 20 to PFOS that Butenhoff et al. (2008) reported remains to be explored. Toxicokinetic studies are required to determine how large dose the fetus/pup is actually exposed to during the gestational and fetal period, as well as how large the lactational exposure really is. A study conducted on rats, in which the pregnant rats were orally exposed to
PFOS from GD 0-PND 20, the highest level of PFOS was detected in the neonatal brain on GD 20. It was speculated that the volume expansion (i.e., brain growth) could contribute to the lowering of brain concentrations seen postnatally, even with lactational exposure. The brain [PFOS]-to-serum [PFOS] ratio in the fetuses and pup were about 0.30 on GD 0 and PND 10 in the 0.1, 0.3, and 1.0 mg/kg-day treated rats. At PND 21, the ratio was about 0.13 irrespective of the exposure dose (Chang et al.). Human and animal data from a variety of species suggest that accumulation of highly persistent chemicals via milk far exceeds the contribution made by maternal-fetal transfer, see (Gallenberg and Vodicnik 1989). In animal studies using PCB 153, it was found that about 60% of the total body burden was eliminated via milk during the first 5 days of lactation and that virtually all was eliminated by day 20 (Gallenberg and Vodicnik 1989; Vodicnik and Lech 1980).

In this thesis, it has been shown that neonatal exposure to PBDE 209, PFOS, and PFOA led to disturbances in the cholinergic system, manifested as an altered response to the cholinergic agent nicotine in adult animals. Earlier studies have shown that neonatal exposure to POPs, like PCB, DDT, and PBDEs, can lead to a reduced amount of muscarinic and nicotinic receptors in the hippocampus at adult age (Eriksson 2008; Eriksson et al. 1992; Viberg et al. 2003a; Viberg et al. 2004b, 2005). These receptors can be affected as early as during neonatal development, which has been observed after exposure to certain environmental toxicants such as DDT, pyrethroids and nicotine (Ahlbom et al. 1994; Eriksson 1992; Eriksson et al. 2000). During the BGS, parallel to the ontogeny of the cholinergic system, the development of CaMKII, GAP-43, synaptophysin, and tau is taking place. It is known that these proteins increase during the BGS in the mouse, with the most pronounced increase taking place around PND 7-14 (Viberg submitted)(Viberg et al. 2008a). These proteins are known biochemical substrates for cellular processes like neurite outgrowth and synaptogenesis, and altered levels of these proteins may inflect changes on normal brain development. In this thesis, it was also demonstrated that mice exposed to PFOS or PFOA could increase the levels of all four proteins in the neonatal brain, and the study results indicate that the hippocampus is the most sensitive brain region investigated for the neurotoxic effects of neonatal exposure to PFOS and PFOA. A similar effect was seen in mice exposed to PBDE 209, where the levels of synaptophysin and tau where increased in the neonatal brain.

There are also epidemiological studies that indicate that exposure to environmental pollutants during early human development can have deleterious effects on cognitive development in children (Saint-Amour et al. 2006; Schantz et al. 2003).

In newborns a major route of exposure to POPs is via mothers’ milk. PBDEs are universally present in human mothers’ milk, the levels having increased in recent decades (Akutsu et al. 2003; Lind et al. 2003; Meironyte et al. 1999; Noren and Meironyte 2000; Ohta et al. 2002; Schecter et al. 2003).
In a recent report by Wu et al. (2007), a significant association was found between PBDE in breast milk and in house dust (Wu et al. 2007). Inhalation of PBDEs in particulate matter or dust is suggested to be the greatest contributor to PBDE exposure of humans, from toddlers up to adult age (Jones-Otazo et al. 2005), and the main PBDE congener in house dust and indoor dust standard reference materials appears to be PBDE 209 (Stapleton et al. 2005; Stapleton et al. 2006).

There are studies showing that indoor air and dust can be a major route of exposure to PFCs. Infants, toddlers, and children, especially those in the crawling stage, tend to experience higher uptake doses than teenagers and adults do. The reason for this is the higher relative uptake via food consumption, hand-to-mouth transfer of the chemicals from carpets, and ingestion of dust (Trudel et al. 2008). Regarding PFOS, lactational exposure to PFOS has been reported in humans. Human milk samples collected from primiparous volunteers in China showed that the concentration of PFOS and PFOA ranged from 0.05-0.36 and 0.05-0.21 μg/l, respectively (So et al. 2006). The daily intake for an infant was calculated to be 0.030 μg/kg/day of PFOS and 0.017 μg/kg/day of PFOA, with the mothers having the highest concentration of the compounds in their milk. In a Swedish study, by Kärrman and co-workers, it was found that PFOS milk concentration in 12 mothers was approximately 1% of the corresponding serum concentration of PFOS. The total PFC concentration in the maternal serum was 32 ng/ml. The estimated total intake of PFCs by the infant was 200 ng/day from the mother’s milk (Kärrman et al. 2007). These study findings taken together make lactation and dust considerable sources of infants’ exposure to PFCs.

Reviews by Grandjean and co-workers (Grandjean and Landrigan 2006; Grandjean et al. 2001), including epidemiological studies, indicate that environmental toxicants can interact to induce defective cognitive development. That environmental toxicants like PCB and MeHg can interact and enhance developmental neurotoxic effects is supported in certain epidemiological studies, which have shown neuropsychological deficits during early development in children from the Faroe Islands but not in children from the Seychelles (Davidson et al. 2006; Grandjean et al. 2001; Myers and Davidson 1998). Both populations have a high consumption of MeHg-contaminated fish, but children in the Faroe Islands were additionally exposed to PCBs via the mothers’ dietary consumption of whale meat and blubber. Animal studies shows that a disrupted spontaneous behavior, lack of habituation, and reduced cognitive functions are found in adult mice neonatally co-exposed to PCB and MeHg at doses that could be related to the ones reported for exposed children (Fischer et al. 2008b).

In this thesis it has been shown that PBDE 209 and PFOA, at low doses, can interact during the neonatal brain development to enhance neurobehavioral defects in the adult animal, which are manifested as disturbed spontaneous behavior with hyperactive behavior in the end of the test period. These
effects were sustained over time and were time-dependent. The doses of PBDE 209 and PFOA, used in the interaction-study, are comparable at molar level to the doses used in those reported co-exposure studies conducted in experimental animals (Eriksson et al. 2006a; Fischer et al. 2008a, b). An interaction of the effects was also seen when the levels of proteins important for normal brain development were examined in the neonatal brain. An increasing number of reports suggest that highly brominated PBDEs and PFCs are present in dust, indicating that infants and young children may be at risk for direct exposure during brain development, which calls for further studies.
Concluding remarks

This thesis has shown that highly brominated PBDEs including PBDE 209, PBDE 206, and PBDE 203, as well as perfluorinated compounds, like PFOS and PFOA, can cause developmental neurotoxic effects when given to the neonatal mouse. The developmental neurotoxic effects include deranged spontaneous behavior, reduced or lack of habituation, decreased learning and memory abilities, and increased susceptibility of the cholinergic system, effects that are dose–response related. PBDE 209, PFOS, and PFOA were also shown to affect proteins important for neuronal growth and synaptogenesis in the developing brain.

This thesis has shown that PBDE 209 and PFOA, at low doses, can interact during neonatal brain development to enhance neurobehavioral defects in mice and that the compounds could interact and enhance the effects of the protein levels in the neonatal mouse brain.

This thesis also showed that there is a critical window in the development of the brain during which the effects can be induced and that it is not only the dose and time of administration that are crucial for inducing neurotoxic effects, but also the presence of the neurotoxic compounds or their metabolites during the defined period of the BGS.

The BFRs and PFCs are two groups that have been identified as emerging classes of persistent environmental contaminants present in mothers’ milk and in house dust. PBDEs and PFCs are of special concern because these compounds have been shown to be present at equal or higher levels in children than in older people. Exposure to these chemicals via mothers’ milk or direct exposure via inhalation and dust ingestion can be important routes of exposure for infants and toddlers.

The BGS period is characterized by rapid changes in the size and development of the brain. Direct exposure during the neonatal brain development has been shown to be a sensitive model to detect changes in the neonate and adult animal induced by environmental agents. By using the mouse animal model, it is possible to study the effects of a single toxicant, or combinations of them, administered directly to animals, during different stages of the BGS. In developmental neurotoxicology it is important to consider the interaction between toxicants when they are present during a critical period of brain development.
There are epidemiological studies indicating that environmental toxicants can interact to induce defects in cognitive development. An intriguing question is whether exposure to these environmental contaminants can affect aging and neurological disorders in humans. The cholinergic system is involved in several neurodegenerative disorders, e.g., AD. This disease is characterized by cognitive impairment and progressive memory loss. The loss of cholinergic innervations and involvement of the tau protein has been shown in previous studies. With elevated levels of tau in the brain at neonatal age, as seen in mice exposed to PFOS, PFOA, and PBDE 209, and an altered response of the cholinergic system at adult age, it is possible that early exposure to certain PFCs and PBDEs can affect processes linked to neurodegeneration, with consequences for cognitive function.

Learning disabilities and attention deficit hyperactivity disorder (ADHD) have been estimated to affect 5% to 10% and 3% to 17% of children. This thesis showed that highly brominated PBDEs and PFCs can give rise to learning and memory disabilities, lack of habituation, and hyperactivity in adult animals. These types of behaviors are included in the adult diagnosis for ADHD.

Further research is needed to investigate the link between exposure to PBDEs and PFCs and influences on an individual’s susceptibility to develop neurological diseases.
Neonatal exponering för högbromerade difenyletrar och perfluorerade kemikalier-utvecklingsberoende toxicitet och interaktion.

I denna avhandling har jag undersökt utvecklingsneurotoxiska effekter av en typ av flamskyddsmedel, polybromerade difenyletrar (PBDE), samt perfluorerade kemikalier (PFC), efter exponering under den snabba utvecklings- och tillväxtparamoden av hjärnan hos nyfödda möss. Fyra olika PBDE-kongener har undersökt: decaBDE (PBDE 209), nonaBDE (PBDE 206), octaBDE (PBDE 203), och heptaBDE (PBDE 183), samt tre stycken PFCer: PFOS, PFOA, och PFDA.

I vår miljö utsätts människan ständigt för nya kemikalier som kan bli framtida miljögifter och miljöproblem. På senare tid har intresset ökat för hur kemikalier kan påverka utvecklingen av nervsystemet under fosterstadiet och under de första levnadsåren. Detta kommer sig av att det har skett en ökning i utvecklingsstörningar, ADHD, samt autism. Vi har också en ökning av sjukdomar som påverkar inlärnings- och minnefunktioner, som Alzheimers sjukdom.

Utvecklingstoxikologi är läran om hur giftiga ämnen påverkar en individs utveckling. Graviditetsperioden delas in i två perioder: den embryonala perioden och fosterperioden. Hos människa utgör den embryonala perioden de första 20 procenten av hela graviditeten och fosterperioden 80 procent. Hos de två vanliga laboratoriedjuren, mus och råtta är förhållandet det omvända, då den embryonala perioden utgör 80 procent av dräktigheten och fosterperioden 20 procent. Exponering för kemikalier under den embryonala perioden kan orsaka missbildningar var som helst i kroppen och exponering för toxiska ämnen under fosterstadiet och nyfödhusperioden kan ge upphov till störningar i det centrala nervsystemet som kan yttra sig som beteendeförändringar.

Nyfödhusperioden karaktäriseras hos många däggdjursarter av snabb tillväxt och utveckling av den relativt utvecklade hjärnan. Det är en period när det sker en rad fundamental förändringar av hjärnan. Under den här perioden utvecklas flera av hjärnans signalsubstanssystem och däggdjurshjärnan kan nu börja bearbeta ny information från de olika sinnena och omsätta detta till ett adekvat beteende. Hos människa börjar denna tillväxt av hjärnan under den sista tredjedelen av graviditeten och fortsätter under de första två levnadsåren, medan den hos möss och råttor sker efter födseln och sträcker sig över de första 3-4 veckorna. Det mänskliga fostret kan bli indirekt expo-
nerat under graviditeten via moderns intag av toxiska ämnen samt under
nyföddhetsperioden då barnet kan exponeras genom modersmjölken och
genom direktxponering.

Tidigare forskning har visat att det hos möss finns en avgränsad kritisk
period under den snabba utvecklings- och tillväxtperioden, då hjärnan är
känslig för låga doser av toxiska ämnen. Toxiska ämnen kan framkalla per-
manenta skador i hjärnfunktionen när de finns närvarande under en begrän-
sad fas under denna utvecklingsperiod.

Tidigare forskning har visat att PBDE 209 kan orsaka utvecklingsneuro-
toxiska effekter hos möss, men att det troligen inte är PBDE 209 i sig själv
som ger effekten, utan att det är nedbrytningsprodukter av den, som närva-
rande under den kritiska nyföddhetsperioden leder till beteendestörningar,
uttbyggt som hyperaktivitet i spontanbeteende vid vuxen ålder. Spontanbete-
ende mäter djurens förmåga att bearbeta intryck från en ny hemmiljö som de
placeras i.

I denna avhandling visas att det finns ett dos-respons samband i beteende-
störningarna PBDE 209 orsakar, vilket visar att effekten hänger ihop med
dosens storlek. Vidare visas det att dess förändringar i spontanbeteende för-
värras med ökad ålder samt att det kolinerga systemet är påverkat. Det koli-
nerga systemet är ett signalsubstanssystem som är kopplat till beteende samt
minne och inlämnring.

I denna avhandling visas också att de två högbromerade PBDEerna,
PBDE 206 och PBDE 203, kan inducera effekter i de neonatala djuren som
yttrade sig som förändrat spontanbeteende vid vuxen ålder. Störst effekt
hade PBDE 206 och PBDE 203 då mössen exponerades på postnatal dag 10.
Möss exponerade för PBDE 206, uppvisade också förändringar i minne och
inlämnring i vuxen ålder.

For att undersöka om PFCer kan orsaka liknande effekter som de högb-
romrade PBDEerna, direktexponerade möss för PFOS, PFOA och PFDA
under den kritiska hjärnutvecklingsfasen. Denna avhandling visar att PFOS
och PFOA kan ge upphov till liknande neurotoxiska effekter som PBDEerna,
samt att förändringarna i spontanbeteende förvärras med ålder och att det
drönaha systemet är påverkat. Denna avhandling visar också att flera protei-
ner som är viktiga för den normala hjärnutvecklingen kan påverkas efter
exponering för PFOS och PFOA under denna hjärnutvecklingsperiod. Möss
som exponerats för PFOS och PFOA neonatalt, hade förändrade nivåer av
flera proteiner i hjärnan, som kan vara viktiga för minnesfunktionen. Ett av
proteinerna, tau, har tidigare visats vara involverat i Alzheimer’s sjukdom
som är en neurodegenerativ sjukdom.

PBDEer används i plaster, elektroniska kretskort, datorer, byggnadsmate-
rial, samt syntetiska textilier. Studier visar att det denna grupp av flams-
kyddmedel finns i högre eller lika höga nivåer i barn som vuxna. En annan
grupp av ämnen som finns i höga nivåer hos barn är PFCer, som är sam-
lingsnamn för en grupp kemikalier som har ytaktiva egenskaper och används
i bl a textilier, för att bilda vatten-, fett- och smutsavvisande ytor. Då det visat sig att nyfödda och unga människor har lika eller högre nivåer av dessa ämnen i sin kropp jämfört med äldre, tyder det på att det förekommer en direktexponering av dessa ämnen hos barn. Både PBDEer och PFCer återfinns i modersmjölk samt i damm i inomhusmiljön. Eftersom nyfödda rör sig mycket på golvet och att hand- till munkontakten är frekvent, kan det vara en trolig orsak till att de uppvisar höga nivåer i blodet.

Då denna avhandling visar att PBDEer och PFCer kan framkalla likartade utvecklingsstörningar på nervsystemet är de inte bara intressanta som enskilda kemikalier utan också för sin möjlighet att samverka.

I denna avhandling visas att PBDE 209 och PFOA kan samverka vid låga doser och ge förstärkta beteendeförändringar och att effekterna är tidsberoende, vid vuxen ålder. Vidare visas att PBDE 209 och PFOA kan samverka och förstärka effekterna i proteinnivåerna i den neonatala mushjärnan.

Denna avhandling visar att det finns ett kritiskt fönster under utvecklingen av hjärna då dessa effekter kan induceras samt att det inte bara är dosen eller tidpunkten då exponeringen sker, som är avgörande för att en neurotoxisk effekt ska uppstå, utan också närvaron av den neurotoxiska substansen eller dess metaboliter under den kritiska perioden av utvecklings- och tillväxtperioden av hjärnan.
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