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Neonatal ontogeny and effect of decabrominated diphenyl ether (PBDE 209) on synaptophysin and tau

David Friberg

CONTENTS

PREFACE

This master's thesis was carried out as part of the graduate studies program in Ecotoxicology at the Department of Environmental Toxicology, Uppsala University, Sweden. The work has been financially supported by grants from FORMAS to Dr. Henrik Viberg.

First of all I wish to express my gratitude to my excellent advisor, Dr. Henrik Viberg, for his generosity with research material, encouragement and never-failing enthusiasm. Moreover I wish to thank Niclas Johansson for good advice and practical assistance.

ABSTRACT

Flame retardants are used to suppress or inhibit combustion in an effort to reduce the risk of fire. One class of flame retardants, polybrominated diphenyl ethers (PBDEs), has been found to increase in the environment and in human milk. This is also true for the only congener still in use, decabrominated diphenyl ether (PBDE 209). During the neonatal period the mammalian brain undergoes a marked period of rapid brain growth and development, in which both synaptophysin and tau, two neuroproteins, play important roles. In the present study, brains from 1, 3, 7, 10, 14 and 28 days old mice were analyzed for the two proteins synaptophysin and tau. The level of synaptophysin increased continuously during the neonatal period, while tau has a bell-shaped ontogeny curve, with a peak around postnatal day 10. In addition to the ontogeny study, neonatal NMRI male mice were orally exposed on postnatal day 3 to 20.1 mg PBDE 209/kg body weight. The animals were euthanized 7 days after exposure to PBDE 209 and levels of synaptophysin and tau were measured in different brain regions. The protein analysis showed that synaptophysin levels increased significantly in brain tissue in exposed mice compared control animals while no significant change was seen in the levels of tau. This shows that PBDE 209 can affect an important protein involved in normal maturation of the brain and this further strengthen earlier findings concerning PBDE 209 as a developmental neurotoxicological agent.

1. INTRODUCTION

1.1 Toxic agents in the environment and exposure

Throughout the history of the human kind there have always been a will to make progress regarding the development of new methods and materials in order to make life more convenient. The usage of new materials in the manufacturing industry has shown to benefit our technical development and daily life and the demand of more effective technology has increased. Consequently, this development reduces the life span of electronic devices and more electronic-waste needs to be handled (UNEP, 2005; Wong *et al.*, 2007). Within the process of combustion or degradation of the e-waste, persistent organic pollutants (POPs) can be released into the environment. Another problem is the possibility of transformation of the compounds during degradation into products of which some are more toxic than the original products (He *et al.*, 2006; Klaasen, 2001). Many of these compounds are highly hydrophobic and are considered a potential threat to organisms due to their affinity to lipids, combined with their persistent nature and studies have confirmed this theory.

Detectable levels of POPs have been found both in wildlife and in humans. Fish for instance are exposed both via the direct contact with the water through gill respiration and via food uptake (Burreau *et al.*, 2004). The primary exposure route for POPs for humans is through food intake, mainly from fatty fishes living in contaminated areas (Sjödin *et al.*, 2000). The nursing child can be exposed to POPs through the mother's milk (Meironyté *et al.*, 1999) or via inhalation or ingestion of particulate matter or dust, and it has been seen that newborns and toddlers are exposed to higher levels of some POPs than the average adult individual (Jones-Otazo *et al.*, 2005; WWF, 2005). The toxic effects from POPs are dependent on the route of administration, the amount as well as at what time period in the life cycle the organism is exposed. In experiments some of these compounds have shown to cause permanent disorders in organisms acting as neurotoxicants. In mouse POPs can cause permanent neurological derangements if administered during a period of rapid brain development (Eriksson, 1998; Eriksson and Talts, 2000; Viberg *et al.*, 2003b).

1.2. Flame retardants

During the last 50 years advances in the polymer industry have led to development of a large number of polymers with different properties. These new properties of the

polymers have shown to be useful in a wide range of products, from clothing and furniture to vehicles and electronics (e.g. computers and TV sets) (WHO, 1994). Most of these polymers are based on petroleum products, which make them highly flammable. In order to meet the standards of fire safety regulations, manufacturing industries apply flame retardants to increase the resistance to fire (WHO, 1997). During the combustion process free radicals (highly oxidizing agents) are released and these elements are essential for the flame to propagate. When halogenated compounds are added to the polymer they provide halogen atoms to capture free radicals and hence decrease the capability for the flame to propagate. The trapping efficiency of the halogens increases with size (I <Br <Cl <F), so the most suitable organohalogen flame retardant should be the fluorinated compounds. The fluorinated compound has one disadvantage; they are too stable and release their halogen atoms at higher temperatures than most organic matter burns. Brominated and chlorinated compounds are the two most widely used compounds in flame retardants, they release their atoms at the right temperature in order to reduce the capability of the flame to propagate (Alaee *et al.*, 2003). The major producers of brominated flame retardants are Albermale Corporation (US), Great Lakes Chemical Corporation (US and UK) and Eurobrom (Netherlands). Their products contain different types of brominated organic compounds; polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA) and polybrominated biphenyls (PBBs) (OECD, 1993).

1.2.1 Polybrominated diphenyl ethers (PBDEs)

Like other members of the halogens, bromine is a reactive element and therefore it appears in different forms of salts in nature. Most of the bromine that we use today originates from brine wells that contain 0.4 – 0.5% bromine. The reactive bromine monomer can be added to the polymer in different ways, and regarding PBDEs the bromine containing monomer is simply blended to the mixture after the initial polymerization and no chemical binding occurs, which make them an additive flame retardant. With this procedure it is more likely for the flame retardant to leach out of the product into the environment (Hutzinger *et al.*, 1976; Hutzinger and Thoma, 1987). PBDEs are a group of chemical substances that consist of two phenyl rings with a various number of hydrogen and bromine atoms resulting in 209 possible congeners with the chemical formula $C_{12}H_{(9-0)}Br_{(1-10)}O$ (Fig. 1). The bromination of

the diphenyl is typically manufactured in three different variants, Penta-BDE, Octa-BDE and DecaBDE. PBDEs are relatively stable compounds with boiling temperatures ranging between 310 and 425 °C and with low vapour pressures between 3.85 to 13.3 * 10^{-3} mmHg in room temperature. PBDEs are very lipophilic substances with log K_{ow} ranging from 4.28 to 9.9 (WHO, 1994). The global demand for PBDEs has been estimated to be close to 70 000 metric tones in 1999 (BSEF, 2003). PBDEs have been identified in almost every part of the environment. POPs like PBDEs are found in locations distant from sources such as the arctic region (de Wit *et al.*, 2006). In 2004 and The European union banned the use of two formulas of PBDE, PentaBDE and OctaBDE, the same congeners were withdrawn from the North American market the same year (Betts, 2008). However, DecaPBDE is still on the market in Europe, North America and Asia.

Zhou and co-workers found that DE-71 (a commercial polybrominated diphenyl ether mixture containing mostly tetra- and penta-bromodiphenyl ethers) affected thyroid hormones and hepatic enzyme activity in rat offspring, following perinatal maternal exposure (Zhou *et al.*, 2001). Furthermore a study by Branchi *et al*. showed that perinatal exposure to PBDE 99 induced hyperactivity in mice (Branchi *et al.*, 2002). Previous work by Viberg *et al*. and Eriksson *et al.* have shown that neonatal exposure to PBDE congeners, including PBDE 209, can induce persistent neurotoxic effects in adult mice and rats, manifested as changes in spontaneous behavior, habituation, learning an memory and cholinergic neurotransmission (Eriksson *et al.*, 2001; Eriksson *et al.*, 2002; Viberg *et al.*, 2003a; Viberg *et al.*, 2007; Viberg *et al.*, 2003b; Viberg *et al.*, 2006). PBDEs need to be present in the brain around postnatal day (PND) 10 in order to induce their neurotoxic effects. Studies have shown that disturbances induced by PBDE 209 occur after exposure on PND 3 but not after exposure after PND 10. This suggests metabolism of PBDE 209, probably debromination and the presence of the metabolites in the brain to induce developmental neurotoxic effects on PND 10 (Viberg *et al.*, 2003b). In order to understand more of the neurotoxic effects caused by PBDEs, Viberg and co-workers studied how neonatal exposure to PBDE 209 affected important neuroproteins involved in normal maturation of the brain. They showed that neonatal exposure to PBDE 209 altered the levels of three proteins, important for the developing brain; CamKII, GAP-43 and BDNF (Viberg *et al.*, 2008a). Studies by Rice and co-workers

on mice showed that postnatal exposure to PBDE 209 increased the locomotor activity in young adults and reduced the levels of thyroid hormone (T4) (Rice *et al.*, 2007).

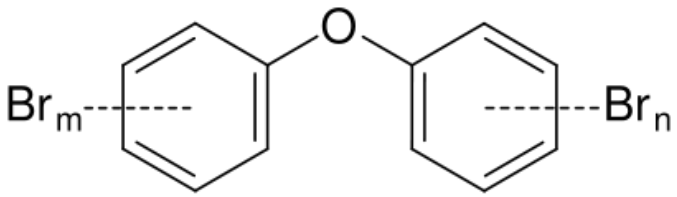


Fig 1. Structural formula for PBDEs where m + n = 1 to 10

1.3 Vulnerable periods and brain development

During the pre- and postnatal development of the mammalian brain a period of rapid brain growth takes place. This phase is known as the brain growth spurt (BGS) (Davison and Dobbing, 1968) and is characterized by axonal and denditric outgrowth, formation of neuronal connections, synaptogenesis and myelinization. During the BGS the mammal also attain new motor and sensory abilities and different neurotransmitter systems undergo rapid development, such as the cholinergic system (Campbell *et al.*, 1969; Coyle and Yamamura, 1976). The BGS varies in onset and duration between species and in mice and rats the period is spanning the first 3–4 weeks of life and the peak is reached around postnatal day (PND) 10 (Fig. 2). For humans this period of rapid brain growth begins during the third trimester of pregnancy and continues throughout the first 2 years of life, coinciding with the lactation period (Fig. 2). In recent studies it has been shown that this period of rapid brain development is vulnerable to insults from xenobiotics and that the presence of compounds like PBDEs or its metabolites in the brain during a defined period of this maturation process is a critical factor (Ahlbom *et al.*, 1994; Ahlbom *et al.*, 1995; Eriksson, 1997; Eriksson, 1998; Eriksson *et al.*, 1992; Eriksson *et al.*, 2000; Eriksson *et al.*, 2002; Viberg *et al.*, 2003b).

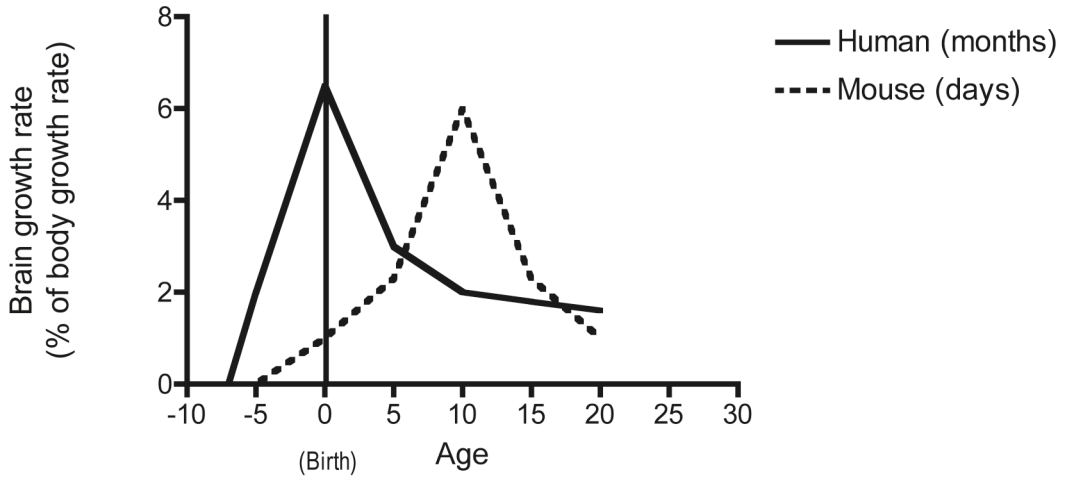


Fig. 2. Rate curves of brain growth in relation to body growth for human and mouse. Values are at different time intervals for the two species. Data from Davison and Dobbing, 1968.

1.3.1 Neuroproteins, synaptophysin and tau

Synaptophysin is a 38 kDa glycoprotein associated with the membrane of presynaptic vesicles, as such, it is highly concentrated in the axonal terminals in the neuron (Sarnat and Born, 1999). The protein is involved in processes regarding the formation and cycling of the synaptic vesicle, from which neurotransmitters are released in order to exchange information between neurons. The function of the synaptic vesicle is critical for the neurons ability to communicate and hence the function of the brain. Synaptophysin is widely used as a immunohistological marker for determination of the density of synapses in brain disorders (Valtorta *et al.*, 2004). In order to activate synaptophysin it needs to be phosphorylated. CamKII is the most abundant protein kinase in neuronal tissue and serve as a regulator of synaptophysin by phosphorylating the protein (Lynch, 2004). If two neurons are active at the same time the efficiency of the synapse will be strengthened. This mechanism is called long-termed potentation (LTP) and is believed to be fundamental for information storage in the brain. In order to reach LTP, synaptophysin, along with other proteins involved in neurotransmitter release, needs to be activated. This communication between neurons is believed to be impaired with age and causing diseases like Alzheimer's disease (Lynch, 2004).

Tau is a protein belonging to the family of microtubule-associated proteins. The protein exists in six different isoforms controlled by a single gene by alternative splicing and posttranslational modification. The tau proteins have been implicated in

outgrowth of neural processes, the development of neuronal polarity and to maintain normal morphology of neurons (Wang and Liu, 2008). Based on *in vitro* experiments tau has also been suggested to regulate microtubule assembly and stability (Vila-Ortiz *et al.*, 2001; Weingarten *et al.*, 1975). The expression of the isoforms is developmentally regulated, where the adult-specific tau isoforms may have a stronger binding affinity for microtubule and consequently results in a more stable and less dynamic microtubule in adult neurons (Brandt, 1996). The microtubule is one of the most important components in the cytoskeleton in order to control a variety of cellular processes like mitosis, cytokines and vesicular transport. Microtubule is also involved in cellular motion, maintenance and determination of the cell shape (Weingarten *et al.*, 1975). The pathogenic peptide β-amyloid$_{42}$ has shown to induce hyperphosphorylation of tau that may impair the function and the plasticity of the synapse. This malfunction is thought to be one of many possible abnormalities linked to Alzheimer's disease (Muntane *et al.*, 2008; Wang *et al.*, 2003).

2. AIMS

The objectives of this study were to; 1) verify the specificity of the antibodies against synaptophysin and tau in a Western blot analysis, 2) construct a curve using increasing concentrations of protein versus the resulting intensity from the slot-blot in order to locate where the linearity of the response for the antibodies occurs, 3) analyze changes in synaptophysin and tau levels during the neonatal brain development, 4) study effects of neonatal exposure to PBDE 209 on the neonatal levels of synaptophysin and tau.

3. MATERIALS AND METHODS

3.1 Animals

Pregnant NMRI mice were purchased from B&K, Sollentuna, Sweden and were housed individually in plastic cages in a room with a temperature of 22°C. In order to simulate natural light conditions the mice were held in 12/12-hour cycle of light and dark. The animals were supplied with standardized pellet food (Lactamin, Stockholm, Sweden) and tap water *ad libitum*. The size of the litters was adjusted to 10-14 pups, within the first 48 h after birth. The litters contained pups of both sexes. Only male mice were used in the present study in order to compare the results with results from

earlier developmental neurotoxicological studies of PBDE 209, other PBDEs, PCBs and other known neurotoxic substances (Eriksson, 1997; Eriksson, 1998; Eriksson *et al.*, 2001; Viberg *et al.*, 2003a; Viberg *et al.*, 2003b; Viberg *et al.*, 2008a).

3.2 Analysis of the normal ontogeny of synaptophysin and tau

In order to perform the ontogeny study five untreated mice per age category were sacrificed at different ages. They came from two to three different litters and were at the age of 1, 3, 7, 10, 14 and 28 days. The brains were dissected on an ice-cold glass plate and the cortex, hippocampus and whole brain were collected (Glowinski and Iversen, 1966), flash frozen in liquid nitrogen and stored at -80°C until assayed.

3.3 Exposure to PBDE 209

2,2´,3,3´,4,4´,5,5´,6,6´-decaBDE (PBDE 209) were kindly donated by Johan Eriksson at the Department of Environmental Chemistry, Stockholm University, Sweden. The purity of PBDE 209 exceeded 98%. PBDE 209 was dissolved in a mixture of lecithin from egg (Merck, Darmstadt, Germany) and peanut oil (Oleum arachidis) (1:10) and then sonicated with water to yield a 20% (w/w) fat emulsion vehicle containing 2.01 mg PBDE 209/ml (2.1 μmol/ml). At the age of 3 days mice were given 20.1 mg PBDE 209/kg body weight (21 μmol PBDE 209/kg body weight) via a metal gastric-tube, as a single oral dose. This dose is the same as has been used in earlier studies by Viberg and co-workers inducing adult behavioral disturbances (Viberg *et al.*, 2007; Viberg *et al.*, 2003b). Control mice received 10 ml of the 20% fat emulsion vehicle per kg body weight. The control group and the PBDE 209 treated group each consisted of pups from three to four litters. Animals were sacrificed by decapitation 7 days after exposure to vehicle or PBDE 209 (i.e. on PND 10) and brains were dissected on an ice-cold glass plate and the cortex and hippocampus were collected, flash frozen in liquid nitrogen and stored at -80°C until assayed.

3.4 Slot-blot analysis for synaptophysin and tau

The analysis was performed according to Viberg *et al.* (2008a & b). Cortex, hippocampus and whole brain tissue from 10 to 12 animals were homogenized in a RIPA cell lysis buffer (Assay Design) (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1 mM EGTA, 1% Triton X-100, 20 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1% sodium deoxycholate) with addition of 0.5% proteas inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem). The homogenates

were then centrifuged at 14,000 × g for 15 min at 4°C, and the protein content of the supernatant was measured using bicinchoninic acid protein assay reagent (Pierce), a method for colorimetric detection and quantitation of total protein. Subsequently, the supernatant was stored at -80°C until use.
The specificity of the antibodies for synaptophysin (Calbiochem 573822) and tau (Santa Cruz 32274) were evaluated in a Western blot analysis and both antibodies used in this study were monoclonal and derived from mouse. Both antibodies were specific for synaptophysin and tau, respectively.

Optimizations of the slot-blot analysis were performed for detection of synaptophysin and tau by plotting increasing concentrations of protein from PND 10 mouse cortex versus the intensity of the bands. The plot showed in which interval of protein the linearity appeared and consequently the right protein amount to use for the slot-blot analysis. 3.0 and 3.5 micrograms of protein for synaptophysin and tau, respectively, were diluted to a final volume of 200 μl with sample buffer (120 mM KCl, 20 mM NaCl, 2 mM $NaHCO_3$, 2 mM $MgCl_2$, 5 mM HEPES, pH 7.4, 0.05% Tween-20, 0.2% NaN_3) and applied in duplicate to a nitrocellulose membrane (0.45 μm, Bio-Rad) using a Bio-Dot SF microfiltration apparatus (Bio-Rad). The membrane was then fixed in solution containing 25% isopropanol and 10% acetic acid, washed, and then blocked in a water solution containing 5% non-fat dry milk and 0.03% Tween-20 for 1 h at room temperature in order to prevent nonspecific binding. The membranes were then incubated overnight at 4°C with the synaptophysin antibody (Calbiochem 573822, 1:10,000) or tau antibody (Santa Cruz 32274, 1:1000). Immunoreactivity was detected using a horseradish peroxidase-conjugated secondary antibody against mouse (KPL 074-1806, 1:20,000). Immunoreactive bands were detected using an enhanced chemiluminescent substrate (Pierce, Super Signal West Dura) with imaging on a LAS-1000 (Fuji Film, Tokyo, Japan). The intensity of bands was quantified using IR-LAS 1000 Pro (Fuji Film).

3.5 Statistical analysis

The levels of synaptophysin and tau were analyzed in hippocampus and cortex in PBDE 209 treated animals and control. Differences in protein levels between control and PBDE 209 treated animals were analyzed using a paired two-tailed Student's *t*-test.

4. RESULTS

4.1 Optimization of the slot-blot procedure for detection of synaptophysin and tau

In order to verify the specificity of the primary antibodies against synaptophysin and tau, Western blot technique was used to separate proteins according to their electrophoretic mobility and to detect a specific protein. Both antibodies indicated single bands in homogenates from PND 10 mouse brain at the appropriate molecular weight, independent of the amount of protein load (Fig. 3a, b). To demonstrate the linear response of the slot-blot procedure, increasing concentrations of protein were used from PND 10 mouse cortex tissue. Fig 4a and 5a show the band intensity versus the amount of protein loaded in the slot-blot analysis for synaptophysin and tau, respectively. Fig 4b and 5b show typical slot-blots for synaptophysin and tau, respectively. Regarding synaptophysin we found the linear response between 2.0-4.0 μg total brain protein (Fig. 4a) and tau showed a linear response in the range 3.0-5.0 μg total brain protein (Fig. 5a). In the slot-blot analysis a total amount of 3.0 μg and 3.5 μg total brain protein was used for synaptophysin and tau, respectively.

(a) (b)

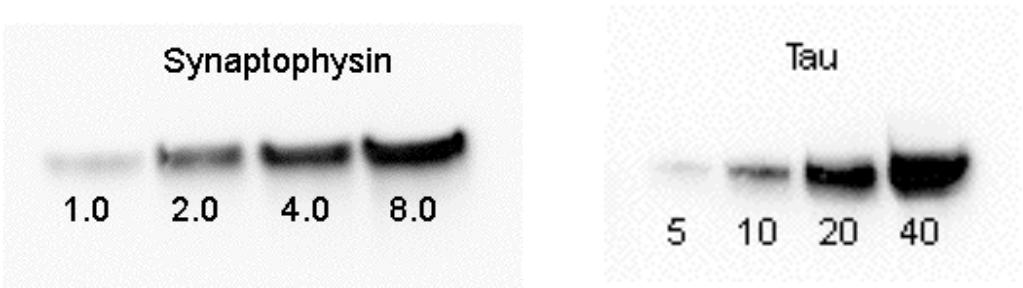


Fig. 3. Results from Western blot analysis for synaptophysin and tau. The numbers represent the total amount of protein loaded on the blot in μg. Mouse monoclonal synaptophysin antibody (Calbiochem 573822, 1:10,000) (a) and mouse monoclonal tau antibody (Santa Cruz 32274, 1:1000) (b).

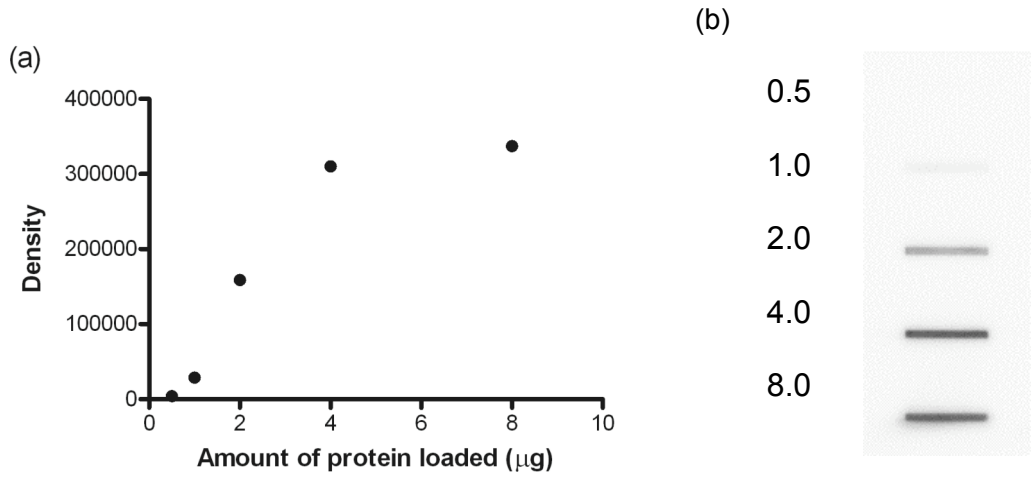


Figure 4. A plot of band intensity vs. the amount of protein loaded in the slot-blot for synaptophysin, the data were not subjected to any statistical analyzes (a). A typical slot-blot for synaptophysin, the numbers represent the total amount of protein loaded in the slot-blot in μg (b).

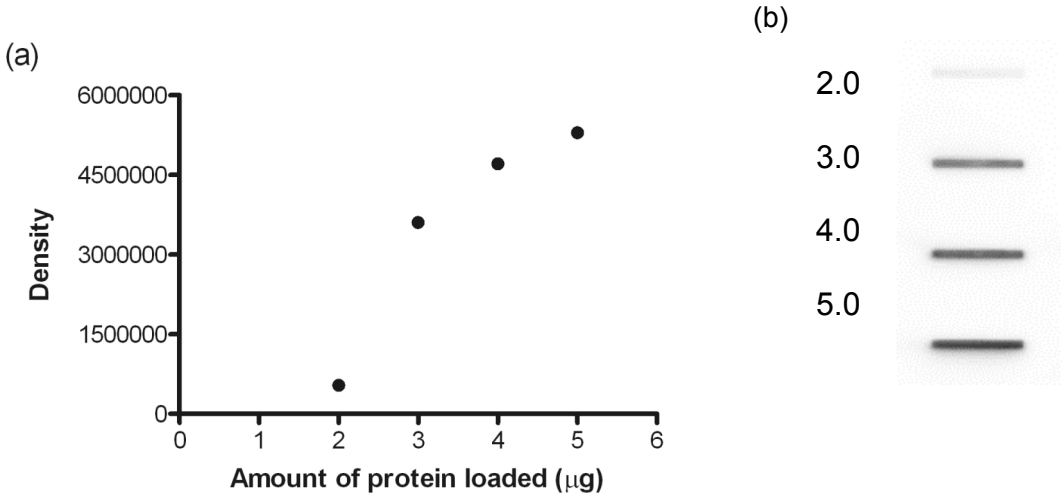


Figure 5. A plot of band intensity vs. the amount of protein loaded in the slot-blot for tau, the data were not subjected to any statistical analyzes (a). A typical slot-blot for tau, the numbers represent the total amount of protein loaded in the slot-blot in μg (b).

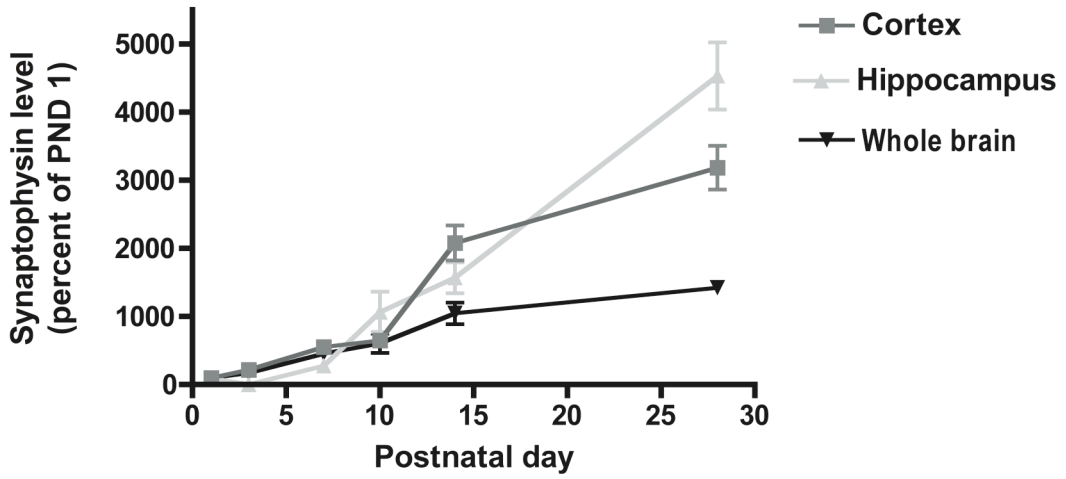
4.2 Ontogeny of synaptophysin and tau

The level of synaptophysin was quantified in brain tissues in untreated mice on PND 1, 3, 7, 10, 14 and 28 and the results are presented in Fig. 6. Levels of synaptophysin increased from PND 1 to PND 28 in cortex, hippocampus and whole brain. This increase was most rapid between PND 10 and 14. Relative to PND 1 there was a 30-, 45-, and 14-fold increase in cortex, hippocampus and whole brain, respectively, over the 4-week period.

The level of tau was quantified in brain tissues in untreated mice on PND 1, 3, 7, 10, 14 and 28 and the results are presented in Fig. 7. Levels of tau increased during the early neonatal period and subsequently decreased during the last part of the 28-day

period. In hippocampus the level of tau peaked on PND 3, while in cortex and whole brain the level of tau peaked on PND 7. Relative to PND 1 the peak level in tau was 248%, 138% and 129% higher in cortex, hippocampus and whole brain, respectively. After the peak, levels of tau decreased continuously so that by PND 28 tau was below the levels observed on PND 1.

(a)



(b)

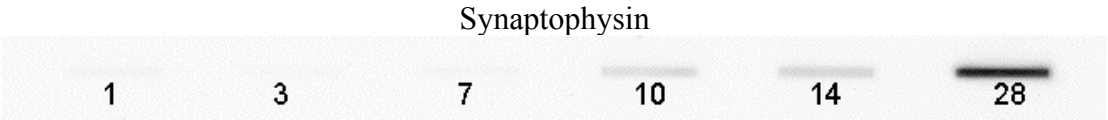
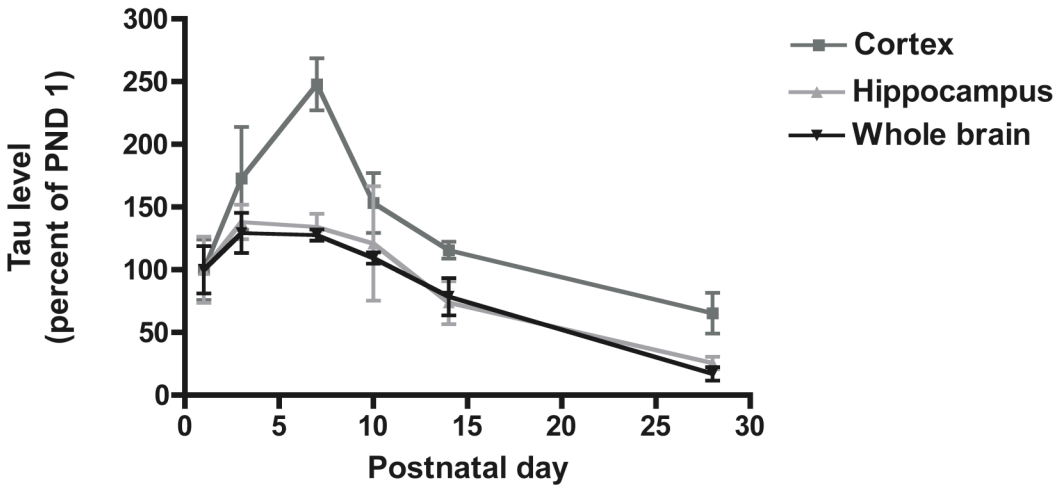


Figure 6. Postnatal ontogeny of synaptophysin shown as the level of synaptophysin ± SD in brain tissue from untreated mice on PND 1, 3, 7, 10, 14 and 28, expressed as percent of the level synaptophysin on PND 1 (a). A picture of a representative slot-blot for synaptophysin, the numbers represent the postnatal day of sacrifice (b). The number of observations (*n*) for each group is 4.

(a)



(b)

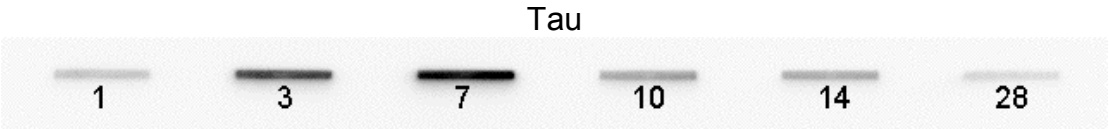


Figure 7. Postnatal ontogeny of tau shown as the level of tau ± SD in brain tissue from untreated mice on PND 1, 3, 7, 10, 14 and 28, expressed as percent of the level tau on PND 1 (a) and a picture of a representative slot-blot for tau, the figures represent the postnatal day of sacrifice (b). The number of observations (*n*) for each group is 4.

4.3 Effects of PBDE 209 on synaptophysin and tau levels in hippocampus and cortex

The levels of synaptophysin and tau were quantified in brain tissues on PND 10, 7 days after a single oral dose of 20.1 mg PBDE 209/kg body weight on PND 3. The results are presented in Fig. 8. Exposure to PBDE 209 resulted in a 41% increase in the level of synaptophysin in hippocampus. There was no significant change in the level of synaptophysin in cortex. The results presented in Fig. 8 also show that there were no significant changes in the level of tau in hippocampus or cortex on PND 10, after exposure to 20.1 mg PBDE 209/kg body weight on PND 3.

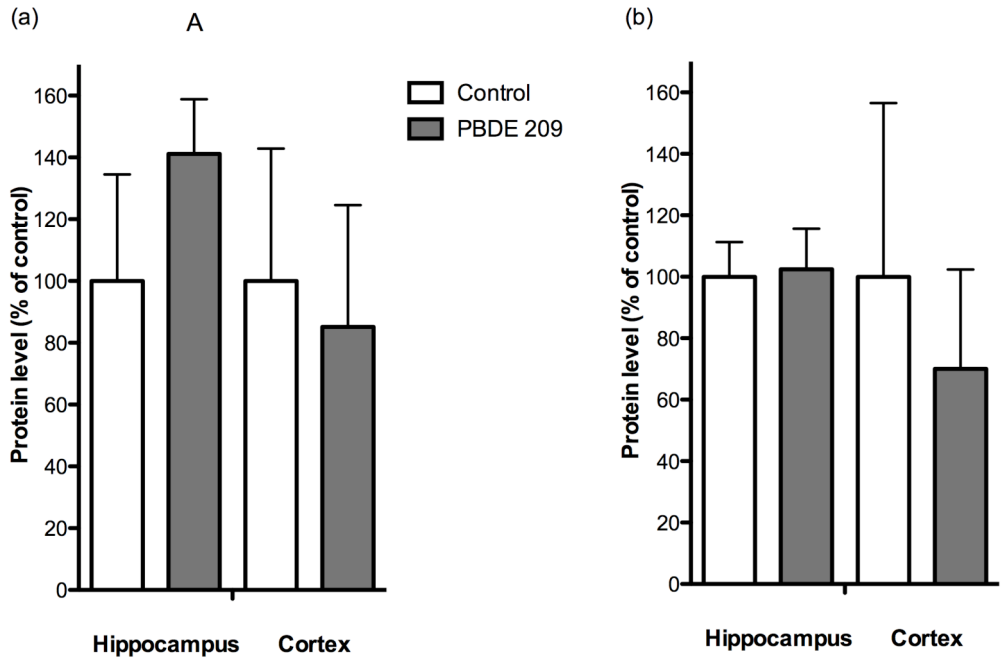


Figure 8. Levels of (a) synaptophysin and (b) tau in cortex and hippocampus of animals exposed to 20.1 mg PBDE 209/kg body weight on postnatal day 3 and sacrificed 7 days later, on PND 10. The data were subjected to Student's *t*-test and the statistical difference is indicated by A ($p < 0.05$ PBDE 209-treated vs. control). The height of the bars represents the mean value ± SD. The number of observations (*n*) in each group is 10-12.

5. DISCUSSION

Earlier studies have shown that exposure to PBDE 209 and other PBDE congeners (including PBDE 47, 99, 153, 183, 203 and 206) during the brain growth spurt results in changes in adult spontaneous behavior, nicotine induced behavior, learning and memory and density of cholinergic receptors in hippocampus in mice and rats (Eriksson *et al.*, 2001; Eriksson *et al.*, 2002; Viberg *et al.*, 2002; Viberg *et al.*, 2003a; Viberg *et al.*, 2004b; Viberg *et al.*, 2004a; Viberg *et al.*, 2005; Viberg *et al.*, 2003b; Viberg *et al.*, 2006). These effects were dose-related and worsened with age and occurred in absence of any visual clinical signs of toxicity immediately after dosing and without any changes in body weight and body weight gain. In order to understand the possible mechanisms underlying this developmental neurotoxicity of PBDEs in general and PBDE 209 in particular, the effect of PBDE 209 on two proteins involved in brain development, synaptophysin and tau, were examined. The results showed that administration of PBDE 209, to mice on PND 3, altered the level of synaptophysin in hippocampus on PND 10, around the peak of brain the growth spurt.

By analyzing the amount of certain proteins involved in the brain development it is possible to detect and understand the mechanisms by which chemicals can damage

the CNS (O'Callaghan and Miller, 1988; Viberg *et al.*, 2008a). In order to fully understand the mechanisms it is vital to know the normal pattern of protein levels during the neonatal development period of the brain. In the present study the levels of the two proteins synaptophysin and tau was studied in cortex and hippocampus during this important period of brain development.

Synaptophysin is a protein associated with the membrane of presynaptic vesicles, key organelles in regulating the release of neurotransmitters and hence the function of the neuron (Ovtscharoff *et al.*, 1993; Valtorta *et al.*, 1989). Studies have suggested that proteins like, synaptophysin, could function as good tools to study the developmental profiles of neurons. In the present study the level of synaptophysin increased continuously during the first 4 weeks of life and this increase was similar in hippocampus, whole brain and cortex. On PND 28 the level of synaptophysin in hippocampus was over 14 times higher than the level on PND 1. Regarding cortex and whole brain, the greatest increases in the level of synaptophysin occurred between PND 10 and PND 14. Furthermore, the greatest rate of increase regarding hippocampus occurred between PND 7 and PND 10. All together, the greatest rate of increase coincides with the peak in neonatal brain growth. Studies by Fujita *et al.* find the same trend, namely that the level of synaptophysin is increasing during the early development of the brain, when the synaptogenesis spurt (Fujita *et al.*, 1996).

Another protein of importance for the developing neuron is tau, a protein essential for microtubule assembly. Tau is present in association with tubulin acting as an activator for tubulin polymerization and hence a major regulator of microtubule formation in cells. Microtubule is involved in cellular motion and the maintenance and determination of the cell shape (Weingarten *et al.*, 1975). Tau showed a different developmental profile compared to synaptophysin. For hippocampus, cortex and whole brain, the level of tau increased during the early postnatal period. The peak level of tau occurred around PND 3 for hippocampus and whole brain, while cortex reached peak levels around PND 7. The increase was similar in hippocampus and whole brain, but in the cortex the increase seemed to be even more pronounced.

The peak in the level of tau, between PND 3 and PND 7, was followed by a gradual decrease during the remaining part of the 28 days period. The pattern of tau ontogeny

seems to be consistent with its function in the neuron. During the period of rapid brain growth spurt the neurons undergoes axonal and denditric outgrowth, establishment of neuronal connections (Davison and Dobbing, 1968; Kolb and Whishaw, 1989). Throughout this period tau is expressed in order to promote microtubule assembly and hence facilitate axon outgrowth and neurite stabilization (Drubin *et al.*, 1985). After the synaptic stabilization has concluded, a new situation develops in which axon elongation is no longer required, therefore tau is no longer needed and the concentration of the protein in the mouse brain is declining (Vila-Ortiz *et al.*, 2001). According to similar studies of the ontogeny of tau, in brain tissue from mice, the level of tau remained constant from PND 9 and 12, and decreased by PND 20 (Vila-Ortiz *et al.*, 2001). These results correlates with earlier studies by Viberg and co-workers where proteins like GAP-43, a phosphoprotein involved in axonal growth, have shown a similar pattern of changes in protein level (Skene, 1989; Viberg *et al.*, 2008a). GAP-43 levels peaks around PND 10 followed by a gradual decrease during the remaining part of the 28 days period. This pattern is consistent with the function of the protein in the neuron. GAP-43 and tau is only required in high levels during the phase of axonal growth and formation of synapses associated with the brain growth spurt (Casoli *et al.*, 1996; Jacobson *et al.*, 1986; Kierstein *et al.*, 1996; Oestreicher and Gispen, 1986; Vila-Ortiz *et al.*, 2001).

The present study attempts to identify how neonatal exposure to PBDE 209 could result in neurotoxicity. Two essential proteins for brain development, synaptophysin and tau, were examined and the results showed that they were affected by neonatal exposure to PBDE 209. Neonatal exposure to PBDE 209 on PND 3, at a dose which has been shown to induce persistent changes in spontaneous behavior, (Viberg *et al.*, 2007; Viberg *et al.*, 2003b) resulted in a significant increase in the level of synaptophysin in the hippocampus in mouse brain tissue examined on PND 10. The observed change in protein level could result from an up regulation of protein expression as a compensatory response to toxicant-induced changes in developmental processes or a gain of the anatomical substrate in which the protein is predominantly localized (Viberg *et al.*, 2008a). PBDE 209 exposure significantly increased synaptophysin levels in hippocampus but had no effect in cortex. The observed effect of PBDE 209 exposure occurred at a time (PND 10) when levels of synaptophysin are rapidly increasing. This change might impair the normal development of

communications between neurons in this part of the brain and hence be a possible mechanism behind the altered brain function seen in mice exposed to PBDE 209. Viberg *et al.* showed that exposure to PBDE 209 significantly increased the level of CamKII in hippocampus (Viberg *et al.*, 2008). Conclusively increased levels of synaptophysin could depend on CamKII, since CamKII is one of several proteins in charge of phosphorylation of synaptophysin to its active form (Lynch, 2004). To date there is little information of the effects of developmental neurotoxicants on synaptophysin. Earlier studies have found that offspring from rats exposed to a commercial mixture of polychlorinated biphenyl (PCB) Aroclor 1254, showed an increases in synaptophysin levels in the lateral olfactory tract in male rats (Morse *et al.*, 1996).

During the BGS the mammal attain new motor and sensory abilities and different neurotransmitter systems undergo rapid development, such as the cholinergic system (Campbell *et al.*, 1969; Coyle and Yamamura, 1976). In earlier studies Viberg and co-workers have shown that neonatal exposure to other PBDEs can cause changes in the density of hippocampal cholinergic receptors both nicotinic (Viberg *et al.*, 2003a; Viberg *et al.*, 2004b) and muscarinic (Viberg *et al.*, 2005). According to studies by Fibiger *et al.,* muscarinic and nicotinic receptor antagonists have shown to produce learning and memory impairments in both humans and rats (Fibiger *et al.*, 1991; Newhouse *et al.*, 1992). In order to understand the possible mechanisms behind the effects of PBDE 209 on behavior, habituation, learning and memory the present study suggest a possible interaction between proteins like synaptophysin and the development of the cholinergic system in the hippocampus (Viberg *et al.*, 2008a).

In conclusion the results from the ontogeny studies support the presence of a defined period of rapid brain growth spurt peaking around PND 10. Earlier studies have shown that this period of rapid development is vulnerable to insults of xenobiotics and our results confirm this hypothesis. The present results show that neonatal exposure to PBDE 209 can increase the level of synaptophysin in the developing brain and thereby change the normal pattern of the ontogeny and development. PBDE 209 exposure had no effect on the levels of tau in the developing brain. This change in synaptophysin level might then impair the normal development of communications between neurons. It is still unclear how PBDE exposure increases the level

synaptophysin in hippocampus and further studies are required in order to understand the mechanism and the exact consequences of the observed changes.

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