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Quantification of neuronal caspase-3  
activation in the neonatal mouse brain after  
exposure to 2,2',4,4',5-pentabromodiphenyl  
ether (PBDE 99), Tetrabromobisphenol A.  
(TBBPA) or Ketamine

Mathias Södervall

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## **Summary**

Brominated flame retardants such as polybrominated diphenyl ether (PBDE) and tetrabromobisphenol A (TBBPA) are widely used in the polymer industry and are commonly used in synthetic textiles and electrical components such as in computers and TV sets. Their high availability has made them frequently detected in the environment, wildlife species, humans and human mother's milk. Exposure to PBDEs during the neonatal development of the central nervous system (CNS) may alter its critical developmental maturation and may lead to permanent neuronal damage which has shown lead to behavioural and learning defects in adult mice. In contrast TBBPA may not affect behaviour, learning and memory in adult mice. In this study PBDE 99 is investigated with TBBPA as a negative control. Ketamine, a common anaesthetic used in both human and veterinary medicine was used as a positive control because of its known ability to induce neurodegeneration in the CNS.

Neurodegeneration can be caused by apoptosis. Caspase-3 is a cystein aspartate specific protease important for the termination of apoptosis causing DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross linking of proteins and finally expression of ligands for phagocytosis.

In this study the caspase-3 activity in neonatal mouse brain was investigated at 24 h or 48 h after single oral exposure to 12 mg (21  $\mu$ mol) PBDE 99/kg body weight or 11.5 mg (21  $\mu$ mol) TBBPA/kg or subcutaneous injection in the neck of 50 mg ketamine/kg body weight. The hypothesis was that the neurotoxic effects of these compounds could be connected to neuronal apoptosis and thereby caspase activation.

This study shows that the number of caspase-3 activated cells is significantly increased 24 h after PBDE 99 exposure (100%) and 24 h after ketamine exposure (600%) in two different

structures in the neonatal mouse brain. TBBPA did not increase the number of caspase-3 activated cells. These results confirm that PBDE 99 and ketamine can act as neurotoxicants which may be related to the behavioural, learning and memory alterations earlier investigated.

## **Introduction**

### Brain development and exposure to neurotoxicants

There are two critical developmental periods of the mammalian brain. The first takes place in the early embryonic development during the organogenesis and the second, named the brain growth spurt (BGS), takes place during the prenatal and/or postnatal period, depending on species. In humans the BGS starts from the third trimester of pregnancy and continues to the age of two years and for murine rodents the BGS takes place the first 24 postnatal days and peaks on postnatal day (PND) 10 (Dobbing and Sands 1979). During this period the central nervous system (CNS) undergoes many critical developmental changes such as establishment of neuronal connections, axonal outgrowth, synaptogenesis and myelinisation together with many sensor-motorical abilities and behavioural characters (Bolles and Woods 1964, Campbell *et al.* 1969). If a harmful xenobiotic alters this critical developmental maturation of the CNS it may lead to permanent neuronal damage and malformations, (Eriksson *et al.* 2001, Eriksson *et al.* 2002, Jevtovic-Todorovic *et al.* 2003), which may lead to behavioural and cognitive effects sometimes similar to different kinds of concentration disorders such as ADHD, (Fredriksson and Archer 2004).

### Cell signalling and apoptosis

#### *Apoptosis and necrosis*

Apoptosis is a controlled cellular “shut down”, which occurs when a cell, or neighbouring cells, triggers a cascade of events for programmed cell death. This process is strictly regulated with numerous control mechanisms and checkpoints (Igney and Krammer 2002). An error in the apoptotic regulation or pathways may lead to different types of cancer or autoimmune

disorders (Elmore 2007). In eukaryotes apoptosis is a useful and necessary physiological function in growth, development and the cellular lifecycle especially when a cell is damaged or dysfunctional (Guo *et al.* 2002), for example by a xenobiotic. Many chemicals may trigger apoptosis leading to neurodegeneration in the mammalian brain when exposed during the BGS, which may lead to irreversible neuronal damage (Jevtovic-Todorovic *et al.* 2003, Rizzi *et al.* 2008). In the cell many types of stimuli can trigger apoptosis, such as cytokines, growth factors, altered calcium levels, toxins and oxidative stress (OS) (Mattson and Chan 2003). Pathological treatment like radiation and chemotherapy can also trigger apoptosis via DNA damage, through the tumour suppressor protein, p53, dependent pathway (Elmore 2007).

Apoptosis differs from necrosis which is an accidental cell death and does not require energy (Elmore 2007, Levin *et al.* 1999). The process of necrosis includes cell swelling, disrupted organelle membranes and eventually disruption of the cell membrane (Trump *et al.* 1997). The disruption of the mitochondrial inner membrane affects the proton-pump leading to energy shortage and the damage to the cell membrane will cause release of the cytoplasmic content to the surroundings of the cell leading to an inflammatory response which does not occur in apoptosis (Kurosaka *et al.* 2003).

Whether a cell dies from apoptosis or necrosis depends on a number of features (Zeiss 2003). The nature of the death stimuli, tissue type, physiological environment and the developmental stage of tissue will affect the outcome of the dying cell.

#### Caspase mediated apoptotic pathways

There are two major pathways for apoptosis in the eukaryotic cell. These pathways are referred to as intrinsic, also called mitochondrial pathway, and extrinsic, also called death receptor pathway (Elmore 2007). These pathways converge on to the same terminal execution pathway, which results in activation of caspase-3 and thereafter DNA fragmentation,

degradation of cytoskeletal and nuclear proteins, cross linking of proteins and finally expression of ligands for phagocytosis (Martinvalet *et al.* 2005). Caspases are in most cells widely expressed as inactive proenzyme forms. When activated they may trigger other procaspases allowing a protease cascade amplifying the apoptotic signalling leading to rapid cell death. To date, 10 major caspases have been identified, which are divided into three groups: initiators (caspase-2,-8,-9,-10), effectors/executors (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4, and -5). Caspase-3 is considered to be the most important execution caspase and is activated by any of the other initiator caspases (Elmore 2007), therefore it will be used as a biomarker for apoptosis in this study.

The intrinsic pathway is activated by a non-receptor mediated stimuli that produces intracellular signals. The initiation signals originate from the mitochondria and can be caused by either loss of apoptotic suppressors or toxins, radicals, hypoxia, radiation and viral infections (Elmore 2007). All these stimuli may cause changes in the mitochondrial membrane resulting in an opening of the mitochondrial permeability transition pore and eventually release of the pro-apoptotic proteins like cytochrome c and smac/direct IAP binding protein with low pI (DIABLO) (Saelens *et al.* 2004). Smac/DIABLO inhibit apoptotic inhibitors and cytochrome c activate procaspase-9. Thereby intrinsic mitochondrial pathway is activated, forming an apoptosome, and eventually carrying on to the execution pathway (Hill *et al.* 2004).

The extrinsic pathway initiates apoptosis through transmembrane receptor mediated interactions. When a ligand is bound to the receptor cytoplasmic adapter, proteins recruit to their intracellular domains (Wajant 2002). The adaptor proteins then dimerize with procaspase-8 forming a death inducing signalling complex (DISC), resulting in activation of procaspase-8 and eventually the execution pathway is activated (Kischkel *et al.* 1995).

## Brominated flame retardants

To meet fire safety regulations, the production and use of brominated flame retardants (BFR) have played a central role in the polymer industry. The property to lower a materials probability to catch fire is used in many combustible products, such as electrical components in computers and TV sets, synthetic textiles and construction materials (WHO 1994).

A list presenting the production of polymers year 2000 and its BFR content are presented in table 1 (Alaee *et al.* 2003).

Table 1. Production of polymers year 2000 and its BFR content (Alaee *et al.* 2003).

<b>Polymer</b>	<b>BFR content (%)</b>	<b>Type of BFR</b>	<b>Annual production (1000 tonnes per year, metric)</b>
Polystyrene foam	0.8-4	HBCD	600
High-impact polystyrene	11-15	d-PBDE, Br PS	350
Epoxy resin	19-33	TBBPA	300
Polyamides	13-16	d-PBDE, Br PS	200
Polyolefins	5-8	d-PBDE, D BS	200
Polyurethanes	10-18	p-PBDE Br Polyols	150
Polyterephthalate	8-11	Br Ps, der-TBBPA	150
Unsaturated polyesters	13-28	TBBPA	150
Polycarbonate	4-6	Br PS der-TBBPA	100
Styrene copolymers	12-15	o-PBDE, Br PS	50

HBCD, hexabromocyclododecane; PBDE, polybrominated diphenylethers (d: deca, o: octa, and p: penta formulations); TBBPA, tetrabromobisphenol A, (der: derivative); Br PS, brominated polystyrene; DBS, propylene dibromo styrene; Br Polyols, esters of TBBPA.

### *Polybrominated diphenyl ethers*

The PBDEs are a diverse group easily synthesized chemicals, composed of two phenyl rings with 209 possible combinations of bromine and hydrogen atoms bound to them, see figure 1.

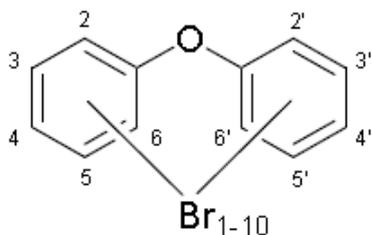


Figure 1. *The backbone of PBDEs.* The general structure allows up to 209 different congeners.

The PBDEs are additive flame-retardants, which means that they are not chemically bound to their substrate (Hutzinger *et al.* 1976). This will increase their ability to escape into the environment, which may explain their high availability and subsequently to the presence in human breast milk (Meironyte *et al.* 1999). The PBDEs are lipophilic and have therefore a tendency to accumulate in lipid rich tissue (Darnerud 2003; Darnerud *et al.* 2001). Postnatal exposure originating from various sources such as breast milk, dust, particles and indoor air (Schechter *et al.* 2003), which will make children exposed during the BGS.

In a study from 2001 (Eriksson *et al.* 2001) neonatal PBDE 99 exposure induced irreversible altered spontaneous behaviour in adult mice. This occurred after a single oral dose, 0.8 or 12 mg/kg body weight, on PND 10 and indicated that the nervous system was seriously affected. PBDE 99 has also shown to decrease in cholinergic nicotinic receptors in rat hippocampus with increasing effects with age (Viberg *et al.* 2004). Similar results have been shown after neonatal exposure to PBDE 153 in NMRI mice, 0.9mg/kg body weight, which also showed a decrease in cholinergic nicotinic receptors in hippocampus with increasing effects with age (Viberg *et al.* 2003a). Another study have shown that even higher brominated PBDEs, such as PBDE 209, can induce altered spontaneous behaviour in adult NMRI mice due to their metabolites formed after neonatal exposure, an effect increasing with age (Viberg *et al.* 2003b).

Recent research indicates that apoptotic neurodegradation may be the mechanism behind the observed neurotoxic effects of PBDE 99 (Madia *et al.* 2004). After exposure to 100 $\mu$ M PBDE 99 in human astrocytoma cell cultures an increase in apoptosis together with elevated p53 expression was observed. They suggest that the over expression of p53 leads to the apoptotic activation of the cells. Another study suggests that PBDE 47 may induce oxidative stress, DNA damage and apoptosis in cultured rat hippocampal neurons (He *et al.* 2008). They also observed alterations in the ultra-structure of neurons in hippocampal region CA1 after PBDE 47 exposure, a result which correlated with the effects in learning and memory test, swim maze, as well as with the activation of caspase-3,-8 and -9 (He *et al.* 2009).

A recent study on adult rats exposed to PBDE 99, single oral dose 1.2 mg/kg body weight, showed no behavioural effects, but a significant decrease in activity for different markers of oxidative stress (OS) (Bellés *et al.*). These decreases were region specific for different OS markers. In the cortex choline acetyl transferase (CAT) activity decreased, in the cerebellum the activity of CAT, superoxide dismutase (SOD) and glutathione reductase (GR) decreased and in the hippocampus CAT and SOD activity decreased, which together show that the antioxidant defence systems were affected by the PBDE exposure.

Due to current research PBDE 99 have been banned within the EU and phased out in the US.

#### *Tetrabromobisphenol A.*

Tetrabromobisphenol A (TBBPA) have during the last decade been one of the most widely used BRF in the world (de Wit 2002), for general structure see figure 2. It is a highly lipophilic halogenated aromatic molecule, primarily used as a chemically bound flame retardant in all sorts of electrical devices, such as computer boards, cell phones and washing machines.

Although 90% of the TBBPA used is chemical bound to its substrate, scientists have found that this chemical can leak into the environment, even though this was not expected (Sellström and Jansson 1995).

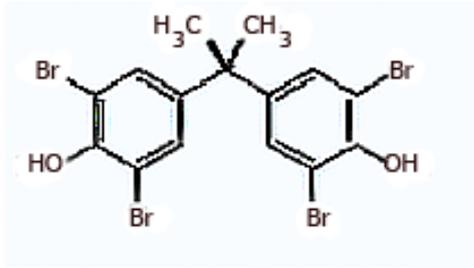


Figure 2. The backbone of TBBPA. The current most widely used BRF in the world.

In a study investigating serum concentrations of TBBPA in humans a slight increase of the compound was found between 1985 and 1999 (Thomsen *et al.* 2002). This study showed the highest serum concentrations, average 0.71 ng/g lipids, in 4 year old children. Since the half-life of TBBPA is suggested to be about 2 days in human this may indicate that we are under continuous exposure to the compound (Sjodin *et al.* 2003).

A recent study indicates that TBBPA interferes with mitogen activated protein kinase (MAPK) pathways (Strack *et al.* 2007). This exposure lead to dose dependent effects on the cell line Cal 62 (human thyroid cells), causing disturbances in cell proliferation and the cell cycle.

TBBPA does not show neurotoxic effects on spontaneous behaviour, memory or learning, after neonatal exposure in NMRI mice (Eriksson *et al.* 2001), although a recent study indicates that TBBPA may induce behavioural effects in mice 3 hours after oral exposure on PND 21 (Nakajima *et al.* 2009). This is the time point when the TBBPA plasma concentration is predicted to be highest (Schauer *et al.* 2006). Nakajima and co-workers also present results indicating an accumulation of TBBPA in the striatal region of the mouse brain.

### Ketamine

Ketamine is an N-methyl d-aspartate (NMDA) glutamate receptor blocking drug used as an anaesthetic in both traditional medicine and veterinary medicine. Many studies in rodents

show that blocking of these NMDA receptors during fetal or neonatal development leads to widespread apoptotic neurodegradation suggesting that the NMDA receptors are essential for neuronal survival (Ikonomidou *et al.* 1999; Jevtovic-Todorovic *et al.* 2003; Olney *et al.* 2002; Slikker *et al.* 2007; Turner *et al.* 2009; Wang *et al.* 2005). This neurodegradation may cause irreversible behavioural, memory and learning disorders which seems to increase with age (Fredriksson and Archer 2004; Fredriksson *et al.* 2007; Viberg *et al.* 2008). The mechanism of action for neuronal damage of ketamine is still under investigation. Researchers have found that a calcium induced loss of membrane potential in the mitochondria may be the cause of apoptosis due to up-regulation of the NMDA receptor NR1 (Slikker *et al.* 2007). A recent study presents that the calcium/calmodulin dependent kinase, CaMKII levels increased significant in mouse hippocampus along with another important protein, growth-associated protein-43 (GAP 43), which together are essential for axon growth and neuronal development during the BGS (Viberg *et al.* 2008) .

Both intrinsic and extrinsic apoptotic pathways seems to be activated after ketamine exposure together with increased Caspase-8 and -9 activity at 4 and 6 hours after exposure respectively (Jevtovic-Todorovic and Olney 2008).

### **Objectives**

The objectives of this study were: 1) create a method for immunohistochemistry (IHC) for fluorescent caspase-3 antibodies in mice brain samples; 2) evaluate and quantify neuronal apoptosis due to exposure to PBBDE 99, TBBPA and ketamine; 3) find specific brain regions or structures, which will be affected by these exposures and evaluate the affected regions probability to induce behavioural and cognitive effects.

## Method

### Chemicals

2,2',4,4',5-pentabromo diphenyl ether (PBDE 99) and tetrabromobisphenol A (TBBPA) were provided by Johan Eriksson at Wallenberg Laboratory (Stockholm University, Sweden). Ketamine (Ketalar® 50 mg/ml Pfizer Inc. New York, USA) was bought from Apoteket Uppsala, Sweden.

To mimic mouse milk (fat content around 14%) as a carrier the of the lipophilic chemicals, PBDE 99 and TBBPA were dissolved in a mixture of egg lecithin (Merck, Darmstadt, Germany) and peanut oil (*Oleum arachidis*) yielding a 20% fat emulsion. In more detail, the exposure substrates were sonicated with water to yield 20% weight water: fat emulsion vehicle containing 1.2 mg PBDE 99/ml or 1.15 mg TBBPA/ml. As a control, a 20% fat emulsion vehicle was used. The water soluble ketamine was mixed with saline (0.9% NaCl) to yield a solution of 10 mg ketamine/ml.

### Animals and exposure

Pregnant NMRI mice were purchased from B&K, Sollentuna, Sweden. The mice were kept individually in plastic cages in a room with temperature of 22°C, a 12/12-h cycle of light and dark, free access to standard pellet food (Lactamin, Stockholm, Sweden) and tap water *ad libitum*. The day of birth was assigned post natal day (PND) 0 and the cages contained both male and female pups. The size of the litters was kept intact during the whole experiment until the day of dissection. Animal experiments were conducted in accordance with and after approval from the local ethical committee (Uppsala University and Agricultural Research Council) and by the Swedish Animal Welfare Agency (license C185/9).

On PND 10 male mice were given 12 mg (21  $\mu$ mol) PBDE 99/kg body weight or 11.5 mg (21  $\mu$ mol) TBBPA/kg body weight via a metal gastric tube as one single oral dose. The volume was 10 ml/kg body weight and control mice received 10 ml/kg body weight of the 20% fat emulsion vehicle. Only male mice were used in the experiment in order to get comparable results with earlier developmental neurotoxicological studies with PBDEs (Eriksson *et al.* 2001; Eriksson *et al.* 2002; Viberg *et al.* 2002; Viberg *et al.* 2004; Viberg *et al.* 2005). 24 or 48 hours after exposure mice from all exposure groups were killed and the brain were dissected out and incubated in formaldehyde for 48 hours and thereafter stored in 70% ethanol (EtOH).

The ketamine study was conducted in the same way as the PBDE/TBBPA study, but on PND 10 the male mice were given a subcutaneous injection (5 ml/kg bw) in the neck of 50 mg ketamine/kg body weight. This dose has in earlier studies shown effects on neurodevelopment and neurodegeneration (Fredriksson *et al.* 2004). The control animals received the same volume of 0.9% saline. 24 or 48 hours after exposure, mice from both groups were killed and the brain were dissected out and incubated in formaldehyde for 48 hours and thereafter stored in 70% EtOH.

A total of 40 brains were used, 20 that were dissected 24h after exposure and 20 after 48h, which provided eight brains per exposure, four brains for each of the five exposures and exposure times.

### Histopathology

#### *Sample preparations and screening*

The two groups of mouse brain, 24 and 48 hours for all the treatments, in total 8 for each treatment: PBDE 99, TBBPA, ketamine, fat emulsion and saline were prepared and handled in exactly the same manner. To prepare the brains for slicing they were dehydrated (From

70% EtOH to 95% EtOH to 99.5% EtOH to xylene) and embedded in low temperature paraffin, 54°C. The sectioning was carried out using a Microme HM 355 microtome making 4µm thin sections that were fixated with water on super frost glass slides.

At first a total of 10 brains, one for each exposure and exposure time, were screened by collecting one section every 40µm. Of these sections, approximately 200 for each brain, 15-20 sections from different levels of the brain were investigated using IHC for localization of caspase-3 in different brain regions, covering the major areas of interest such as the striatum, corpus callosum, frontal cortex, cortex, hippocampus, sub-ventricular zone and cerebellum. After analyzing the result of the screening three different levels of the remaining 6 brains per treatment were selected for further investigation. These three levels were named P0 9 (P0 for postnatal day 0), P0 29 and P0 34 which refers to different brain levels at postnatal day 0 in rats (Paxinos 1990). Level P0 9 is positioned in the front, level P0 29 in the middle and level P0 34 in the rear of the three levels investigated. Three adjacent sections on each level, P0 9, P0 29 and P0 34 were used for quantification, resulting in 270 sections total, 9 for each treatment and exposure time.

### *Immunohistochemistry*

The IHC were carried out using a cleaved caspase-3 (Asp 175) antibody (Cell Signalling, bought February 2010) and Alexa flour 488 (Invitrogen, goat anti-rabbit IgG, bought February 2010) was used as the secondary antibody giving a flourescent marker for apoptosis. The brains were sectioned and fixated on super frost glass slides.

Day 1, the sections were deparaffinized and rinsed in phosphate buffered saline (PBS) pH 7.4. The antibody epitopes were then unmasked in sodium citrate buffer (2.94 sodium citrate/litre water), 10mM, pH 6, while gently boiled in a steam boiler for 30 minutes and cooled in the same solution for 20 minutes. After wash, first in PBS and then distilled water the sections were circumscribed with an ImmEdge pen vector to create a hydrophobic barrier. The

sections were then washed in PBS-Triton X 100 (PBS-T), and then treated with a blocking solution containing 5% normal goat serum for 1 hour in a humid chamber (dark) at room temperature (RT). Then the slides were washed in deionised water and gently dried around the hydrophobic barrier and thereafter treated with 50 µl cleaved caspase-3 antibody (diluted 1:100 in PBS) and incubated in a humid chamber at 4°C for approximately 18 hours.

Day 2, the primary antibody was washed off from the sections in PBS and then the secondary antibody (Alexa fluor 488, diluted 1:100 in PBS-T, kept dark) was applied and the sections were incubated in the humid chamber for 2 hours in the dark at RT. Then the slides were washed in PBS, dark, and mounted with invitrogen- DAPI prolonged, 4',6-diamidino-2-phenylindole (DAPI) and stored in the dark in refrigerator, 4°C.

#### Quantification of Caspase activity

The brains were screened to find areas and specific brain regions that showed caspase-3 activation. The three different levels, P0 9, P0 29 and P0 34 were investigated separately, and the investigator was blinded to the treatments. Each region with caspase-3 activation was closely investigated using a light microscope with a 500 nm filter and the number of caspase-3 activated cells was counted. Each side of the brain were counted separately. To distinguish the positive cells from red-blood cells and other objects that might give fluorescent response to Alexa-fluor 488, criteria was set: 1) the fluorescent areas had to be in direct position or overlapping with the nucleus, visible with DAPI (UV-filter) 2) the fluorescent areas could not be seen as a light-brown dot when using the UV-filter 3) the fluorescent areas had to have a shape with nonspecific structure with no sharp edges or a size that not fitted the nucleus of the cells.

## Statistical analysis

The number of caspase-3 activated cells after PBDE 99, TBBPA, fat emulsion, saline or ketamine exposure were statistically analyzed for two different areas/regional structures; retrosplenial granular cortex (RSG), level P0 29 and P034 together, and a large area on level P0 9 containing numerous structures including the diagonal band of broca, nucleus of vertical limb of diagonal band, nucleus of horizontal limb of diagonal band and accumbens nucleus, here named: structures of the diagonal band (SODB), figure 3. Difference in caspase-3 activity were analyzed for all exposures in the two areas examined using one-way ANOVA with Newman-Keuls post-hoc test (GraphPad Prism 3.03).

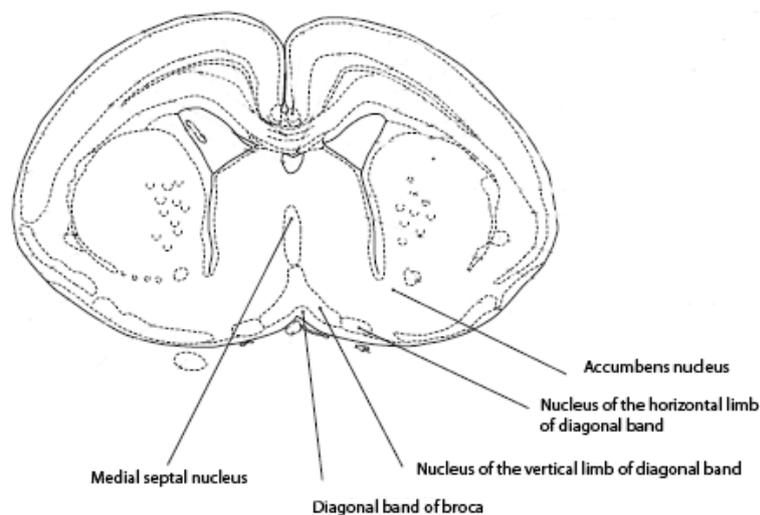


Figure 3. An overview of the structures of the diagonal band (SODB) on level P0 9 (postnatal day 0, level 9) , modified figure from (Schambra 2008).

## Results

The sub ventricular zone, striatum, major parts of the cortex and the hippocampus were screened and pre-counted without any increase in caspase-3 activation.

Two different areas were quantified, the RSG on level P0 29 and P0 34 and SODB on level P0 9. Both 24 h and 48 h exposure times were investigated for each area. RSG is positioned in the upper cortex where the two lobes of cortex face each other, (figure 4A and 4B), and the SODB is positioned in the lower part of the brain between the lateral migratory streams on level P0 9, (figure 5A and 5B).

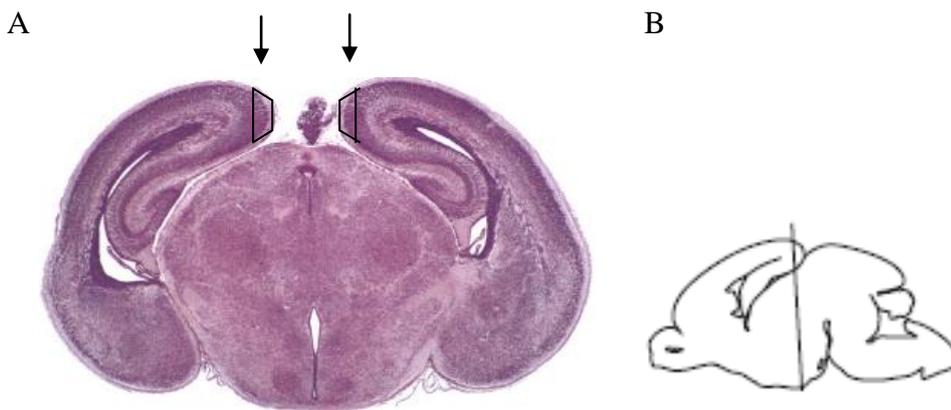
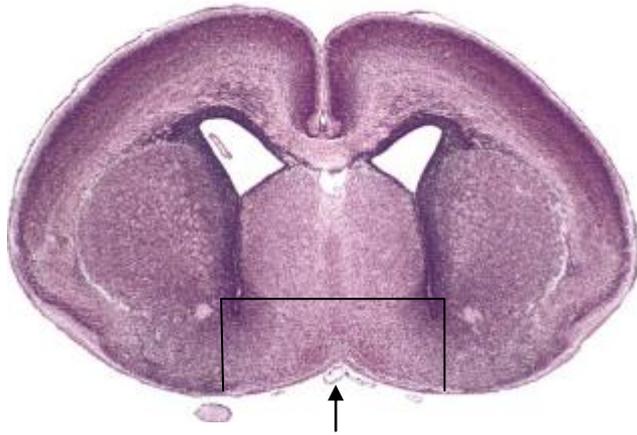


Figure 4. A) Coronal section of mouse brain (gestational day 18) representative for level P0 29 (postnatal day 0, level 29). The retrosplenial granular cortex (RSG) is marked by the boxes. B) An overview of the mouse brain seen from the left side, the line mark the physical orientation of level P0 29 (Schambra 2008).

A



B



Figure 5. A) Coronal section of mouse brain (gestational day 18) representative for level P0 9 (postnatal day 0, level 9). The structures of the diagonal band (SODB) is marked by the lines. B) An overview of the mouse brain seen from the left side, the line mark the physical orientation of level P0 9 (Schambra 2008).

There was a significant increase with almost 600% in caspase-3 activity 24 h after subcutaneous injection of ketamine (50 mg/kg bw) on PND 10, compared to injection of saline (5 ml/kg bw), in the RSG structure on both level P0 29 and P0 34 together ( $p \leq 0.001$ ), (figure 6 and 7). The increase in caspase-3 activated cells was significantly higher in the ketamine-treated group as compared to the other exposure groups, i.e. ketamine-treated animals versus fat emulsion ( $p \leq 0.01$ ), PBDE 99 ( $p \leq 0.001$ ) and TBBPA ( $p \leq 0.001$ ) respectively. There were no significant increase in caspase-3 activation 24 h after a single oral dose of either PBDE 99 (12 mg/kg bw) or TBBPA (11.5 mg/kg bw) compared to 20% fat emulsion vehicle (10 ml/kg bw) on PND 10.

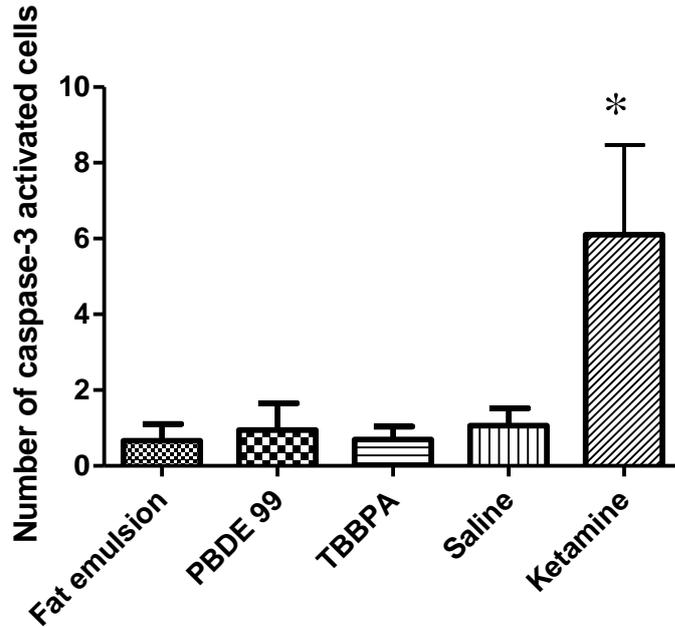


Figure 6. The mean number of caspase-3 activated cells in retrosplenial granular cortex (RSG). Mean caspase-3 activity  $\pm$  S.D. (n=6) 24 h after a single oral dose of either 20% fat emulsion vehicle (10 ml/kg bw), PBDE 99 (12 mg/kg bw), TBBPA (11.5 mg/kg bw), single subcutaneous injection of 0.9% saline (5 ml/kg bw) or ketamine (50 mg/kg bw) on PND 10. The mean values are the number of cells with caspase-3 activity in the RSG on level P0 29 (postnatal day 0, level 29) and P0 34. Each RSG region of the two brain halves were counted separately. \* Significantly different from saline, PBDE 99 and TBBPA,  $p \leq 0.001$  and significantly different from emulsion,  $p \leq 0.01$ .

A

B

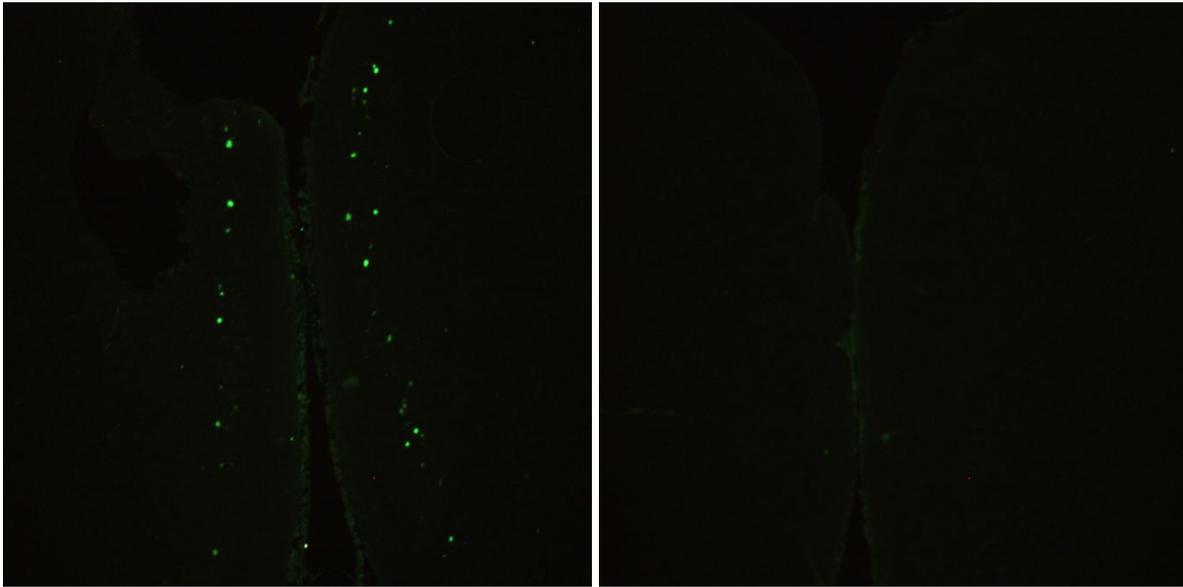


Figure 7. Staining of cells with activated caspase-3 in the retrosplenial granular cortex (RSG). A) Representative image of coronal section showing caspase-3 activation in the RSG 24 h after ketamine exposure (x10 magnification using a 500 nm filter). The caspase-3 activated cells in the RSG 24 h after subcutaneous injection of ketamine (50 mg/kg bw) on PND 10 are ordered as bands where the two lobes of cortex face each other. B) Representative image of coronal section showing caspase-3 activation in the RSG 24 h after saline exposure (x10 magnification using a 500 nm filter), no caspases-3 activated cells are present.

There were no significant difference in the caspase-3 activation 48 h after a single oral dose of either 20% fat emulsion vehicle (10 ml/kg bw), PBDE 99 (12 mg/kg bw), TBBPA (11.5 mg/kg bw), single subcutaneous injection of 0.9% saline (5 ml/kg bw) or ketamine (50 mg/kg bw) on PND 10 in the RSG structure in level P0 29 or P0 34 (figure 8).

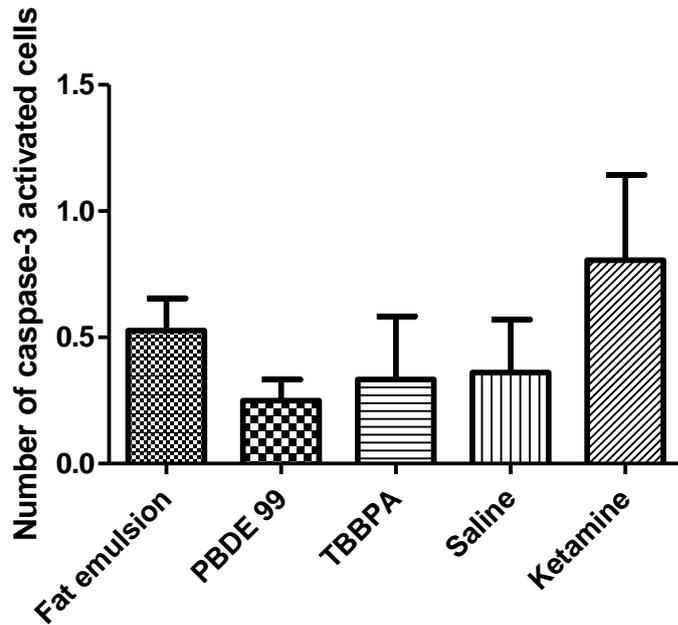


Figure 8. The mean number of caspase-3 activated cells retrosplenial granular cortex (RSG). Mean caspase-3 activity  $\pm$  S.D. (n=6) 48 h after a single oral dose of either 20% fat emulsion vehicle (10 ml/kg bw), PBDE 99 (12 mg/kg bw), TBBPA (11.5 mg/kg bw), single subcutaneous injection of 0.9% saline (5 ml/kg bw) or ketamine (50 mg/kg bw) on PND 10. The mean values are the number of cells with caspase-3 activity in the RSG on level P0 29 (postnatal day 0, level 29) and P0 34. Each RSG region of the two brain halves were counted separately.

There was a significant increase of about 100% in caspase-3 activity in the SODB structure on level P0 9 (p-value  $\leq 0.01$ ) after a single oral dose of PBDE 99 (12 mg/kg bw) compared to the control, 20% fat emulsion vehicle (10 ml/kg bw) after 24 h exposure (figure 9). The increase in caspase-3 activated cells was significantly higher in the PBDE 99-treated group as compared to all other exposure groups, i.e. PBDE 99 treated animals versus TBBPA (p  $\leq 0.01$ ), PBDE 99 versus saline (p  $\leq 0.01$ ), and PBDE 99 versus ketamine (p  $\leq 0.05$ ). There was no significant increase of caspase-3 activation 24 h after subcutaneous injection of ketamine (50 mg/kg bw) compared to injection of saline (5 ml/kg bw) on PND 10 in the SODB area on level P0 9.

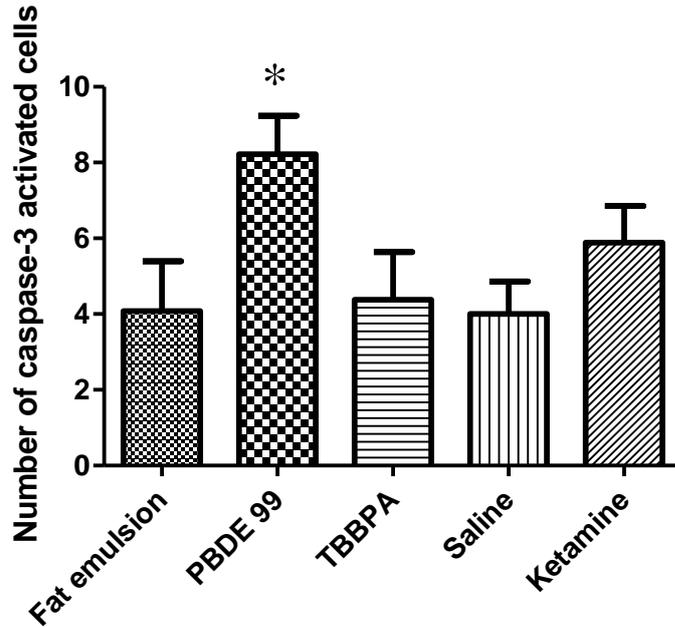


Figure 9. The mean number of caspase-3 activated cells in the structures of the diagonal band (SODB). Mean caspase-3 activity  $\pm$  S.D. (n=6) 24 h after a single oral dose of either 20% fat emulsion vehicle (10 ml/kg bw), PBDE 99 (12 mg/kg bw), TBBPA (11.5 mg/kg bw), single subcutaneous injection of 0.9% saline (5 ml/kg bw) or ketamine (50 mg/kg bw) on PND 10. The mean values are the number of cells with caspase-3 activity in the SODB on level P0 9 (postnatal day 0, level 9). Each SODB region on the two brain halves were counted separately. \* Significantly different from fat emulsion, saline and TBBPA,  $p \leq 0.01$  and significantly different from ketamine,  $p \leq 0.05$ .

The caspase-3 activated cells in the SODB 24 h after a single oral dose of PBDE 99 (12 mg/kg bw) have low structural specificity (figure 10A and 10B).

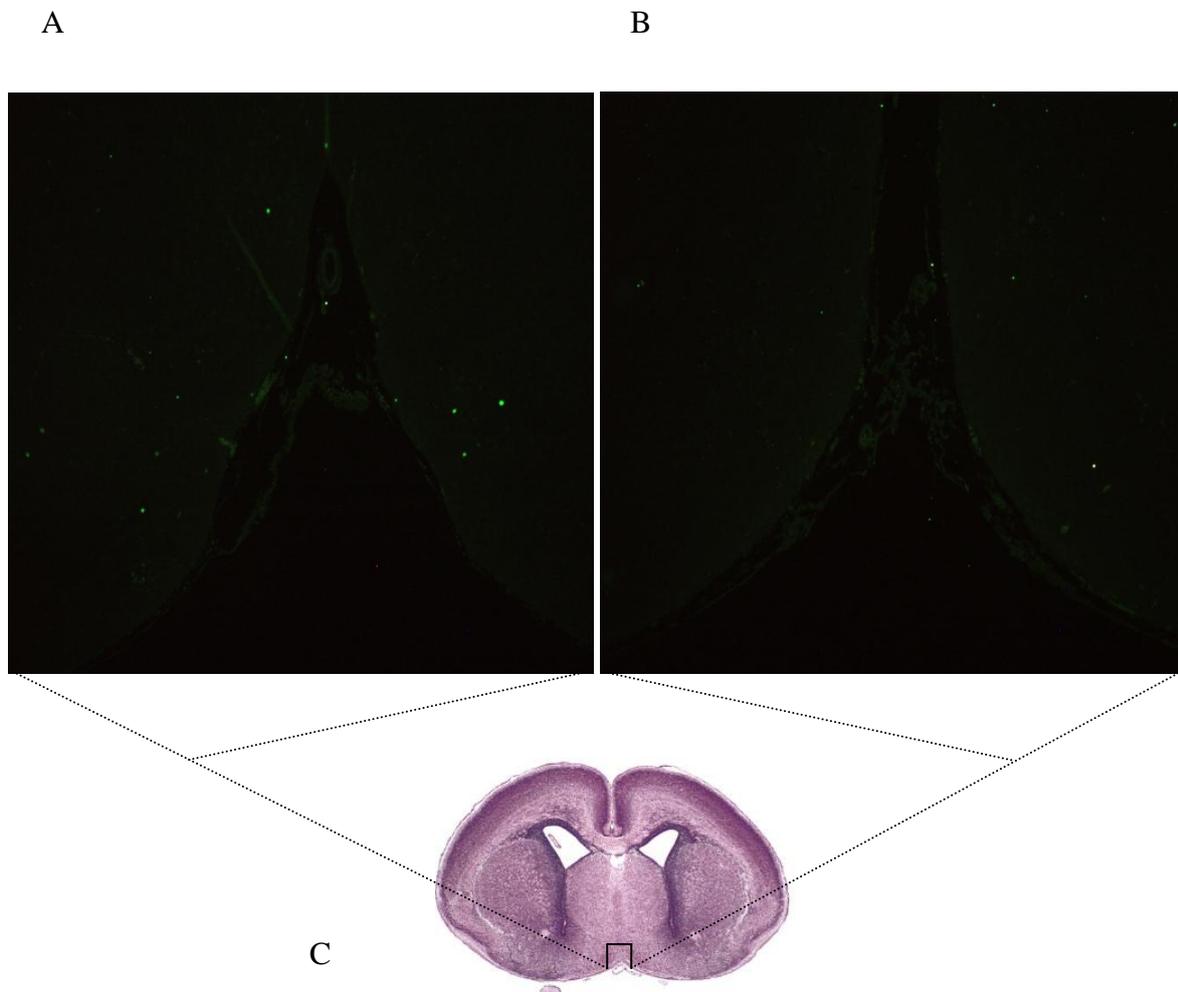


Figure 10. Staining of activated caspase-3 in the middle part of the structures of the diagonal band (SODB). A) Representative image of coronal section (x10 magnification and 500 nm filter), showing caspase-3 activation as a green signal in the SODB 24 h after PBDE 99 exposure (12 mg/kg bw). B) Representative image of coronal section showing caspase-3 activation in the SODB after fat emulsion exposure (10 ml/kg bw). C) The orientation of the photo compared to coronal section of mouse brain (gestational day 18), level P0 9 (postnatal day 0, level 9) (Schambra 2008). The photos cover the structure: nucleus of vertical limb of diagonal band and diagonal band of broca.

There were no significant differences in the caspase-3 activation in the SODB area in level P0 9 at 48 h after a single oral dose of either 20% fat emulsion vehicle (10 ml/kg bw), PBDE 99 (12 mg/kg bw), TBBPA (11.5 mg/kg bw), single subcutaneous injection of 0.9% saline (5 ml/kg bw) or ketamine (50 mg/kg bw) on PND 10 (figure 11).

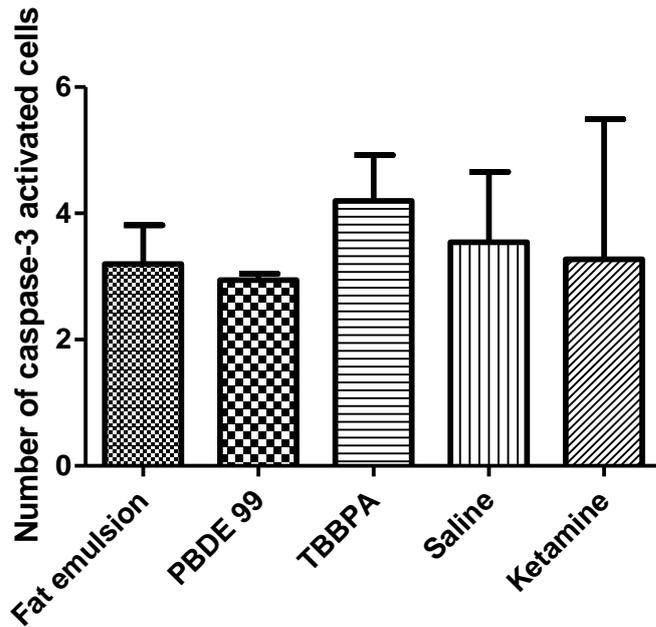


Figure 11. The mean number of caspase-3 activated cells in the structures of the diagonal band (SODB). Mean caspase-3 activity  $\pm$  S.D. (n=6) 48 h after a single oral dose of either 20% fat emulsion vehicle (10 ml/kg bw), PBDE 99 (12 mg/kg bw), TBBPA (11.5 mg/kg bw), single subcutaneous injection of 0.9% saline (5 ml/kg bw) or ketamine (50 mg/kg bw) on PND 10. The mean values are the number of cells with caspase-3 activity in the SODB on level P0 9 (postnatal day 0, level 9). Each SODB region on the two brain halves were counted separately.

## Discussion

Brominated flame retardants, such as PBDEs, have in earlier studies been shown to cause persistent developmental neurotoxic effects, when administered during the brain growth spurt (BGS). These neurotoxic effects may result in alterations in adult spontaneous behaviour and cognitive function, irreversible impacts, which worsen with age (Eriksson *et al.* 2001, Viberg *et al.* 2003b). The mechanisms behind these effects are poorly understood and the brain structures involved are yet not well defined, but neurodegeneration may play an important role in the understanding of the mechanisms involved (Dingemans *et al.* 2007, Madia *et al.* 2004).

Recent studies indicate that PBDE 47 exposure leads to neurotoxic effects in the hippocampus (He *et al.* 2008). One of the suggested mechanisms behind these effects is apoptosis along with caspase-3, -8 and -9 activation (He *et al.* 2009).

The present study showed no caspase-3 activation in the hippocampus, striatum or frontal cortex in mice neonatally exposed to PBDE 99 (12 mg/kg bw), TBBPA (11.5 mg/kg bw), or Ketamine (50 mg/kg bw). The only region which seemed to be affected by the neonatal exposure to PDBE 99 was the SODB region, where there was a significant increase of caspase-3 activation 24 h after the exposure (figure 9). The mean value of caspase-3 activated cells in the SODB region after PBDE 99 exposure was 8.2 for each brain half and 4.1 in the control group, an increase of 100%. The SODB region is a very large area and contains the structures of the diagonal bands, including the diagonal band of Broca and accumbens nucleus (figure 3 and 10). The increase in caspase-3 activity was not structure specific in the SODB region and the number of affected cells low. Therefore the actual impact of this increase in caspase-3 activation in the SODB region is complex to evaluate, whether or not an increase of

4 cells, mean, per brain half and section in an area containing thousands of cells can give rise to behavioral effects such as those shown by Eriksson and Viberg is uncertain (Eriksson *et al.* 2001, Viberg *et al.* 2003b).

The diagonal band of Broca consists of cholinergic, GABAergic and glutamatergic neurons (Colom *et al.* 2005) and provides the hippocampus with important input concerning cognitive functions such as memory (Winson 1974). Significant increase in caspase-3 activation in this area, if lead to apoptosis and thereby neurodegradation, may explain the behavioral changes earlier found by Eriksson and Viberg (Eriksson *et al.* 2001, Viberg *et al.* 2003b).

Mice exposed to the known neurotoxicant ketamine did not show any increase in caspase-3 activity in the SODB region, level P0 9. The only region that was affected by this compound was the RSG in the P0 29 and P0 34 level, which showed a significant increase in caspase-3 activation 24 h after a single subcutaneous injection of 50 mg ketamine/kg bw on PND 10 in mice (figure 5).

Ketamine is known to alter the CaMKII levels in mouse hippocampus along with another important protein, GAP-43, which together are essential for axon growth and neuronal development during the BGS (Viberg *et al.* 2008).

The RSG is important for spatial memory and learning in most mammals and is tightly connected to hippocampus and thalamus. Early Alzheimer's disease patients' shows changes in the RSG which may be linked to dementia (van Groen 2004). In humans the RSG may also play an important role in the interface between the emotions and the episodic memory (Maddock 1999).

The present study shows that the RSG is seriously affected by the ketamine exposure with a large increase in caspase-3 activated cells of almost 600% (figure 5 and 6). The RSG is a rather small structure with many connections and an increase in caspase-3 activation here is

most likely to give rise to behavioural, memory and learning disorders earlier seen after ketamine exposure (Fredriksson and Archer 2004, Fredriksson *et al.* 2007, Viberg *et al.* 2008).

As seen in figure 7 A, the caspase-3 activated cells are arranged in a thin band in each hemisphere indicating that only 1 or 2 cell layers were affected in the RSG. In the present study it is not possible to identify which these cell layers are, but it is interesting for future investigations.

In an unpublished study (LeBesconte and Viberg 2008), an increase of CaMKII, synaptophysin and GAP-43 protein levels in mouse cortex was shown 24 h after a single oral dose of PBDE 99 (12 mg/kg bw) on PND 10. CaMKII also increased in hippocampus after the PBDE 99 exposure, but no effects on these protein levels were seen after TBBPA exposure (11.5 mg/kg bw). CaMKII, synaptophysin and GAP 43 are important proteins for neuronal survival, growth and synaptogenesis (Viberg *et al.* 2008). Even though these protein levels were altered, the current study does not indicate neuronal alterations due to caspase-3 activation, in the cortex or hippocampus after PBDE 99 exposure.

TBBPA in this study, used as a negative control, did not induce any caspase-3 activity in level P0 9, 29 or 34. This may support a previous study by Eriksson and co-workers where no behavioural effects were seen in mice after neonatal exposure to 11.5 mg TBBPA/kg bw (Eriksson *et al.* 2001). On the other hand a study by Nakajima and co-workers (Nakajima *et al.* 2009) showed that a single oral dose of 0.1 and 5 mg/kg bw TBBPA to adult mice, gave rise to behavioural effects 3 h after the exposure and the present study shows the caspase-3 activity 24 h after exposure. They also saw an accumulation of TBBPA in the striatum region, localized in level P0 9, a region which in the present study showed no increase in caspase-3 activity. Nakajima and co-workers found no behavioural effects in their group of animals

exposed to 250 mg/kg bw, which brings up an uncertainty whether these results are trustworthy.

The present study shows no increase in caspase-3 activation 48 h after neonatal single subcutaneous exposure of ketamine (figure 8), or oral exposure to PBDE 99 (figure 11). The decline in caspase-3 activation from 24 h after exposure to 48 h may for the ketamine-treated animals be explained by the short half-life of the substance and may in the PBDE 99-treated animals be explained by the apoptosis either being completed or had declined because of apoptosis reversible nature.

In this study we have investigated a time window 24 h or 48 h after the exposure. It is possible that the caspase-3 activation peaks much earlier, possible 6 h after the exposure, or later after the exposure than 48 h. This may vary due to the distribution and chemistry of the compound, and therefore the total number of caspase-3 activate cells may be much higher. To get a broader view of the whole event further studies are needed to investigate the activity of other caspases and proteins connected to apoptosis.

The present study investigates the caspase-3 activity, the final caspase in the execution pathway leading to apoptosis. Caspase-3 trigger mechanisms for DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross linking of proteins and finally expression of ligands for phagocytosis (Martinvalet *et al.* 2005). Apoptosis is a very complex mechanism and the activation of caspase-3 is a late step in the process and is preceded by the activation of caspase-2,-8,-9 and 10 and cytochrome c release from the mitochondria (Elmore 2007).

One problem with the identification of caspase-3 activated cells is that they gave a weak signal in the present study. Therefore criteria were set up to be able to count the activated cells properly. It would have been convenient to get a stronger signal so that the quantification

could be done by measuring the fluorescence in a software for image analysis. To reach a stronger signal another caspase-3 antibody could be used, or a different caspase could be investigated, or multiple antibodies for more than one caspase at the time could be used in the IHC.

The main sources of PBDE 99 exposure in young children in daily life are dust particles, indoor air and mother's milk (Schechter *et al.* 2003). Therefore acute human exposures may be low, but the accumulated continuous exposure during the whole BGS can be higher, compared to the exposure situation in the present study. This study shows that PBDE 99 induce caspase-3 activation after a single oral dose which is an indication that the actual neuronal alterations connected to PBDE 99 exposure, in daily life, may be much worse.

In conclusion this study have shown that a single oral dose of PBDE 99, 12 mg/kg bw, in neonatal mice, increases the caspase-3 activity in the SODB region. This may play a central role in the explanation of behavioural changes earlier shown by Eriksson and Viberg (Eriksson *et al.* 2001, Viberg *et al.* 2003b). Ketamine, a common anaesthetic, increased the caspase-3 activation in the RSG after a single subcutaneous injection of 50 mg /kg bw, on PND 10, which may be closely connected to behavioural, memory and learning disorders seen after neonatal ketamine exposure (Fredriksson and Archer 2004, Fredriksson *et al.* 2007, Viberg *et al.* 2008).

Further studies are necessary to get a complete picture and to understand the mechanisms involved in causing the neurotoxic effects that could be connected to adult behavioural alterations.

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