Neurotoxicity of PBDEs and metabolites: concern for the developing brain?

Milou Maria Louisa Dingemans

ISBN 978-90-393-5290-8
Copyright © 2010 Milou M.L. Dingemans

Printed in the Netherlands by Ridderprint Offsetdrukkerij BV, Ridderkerk
Printing of this thesis was financially supported by the J.E. Jurriaanse Stichting
Layout by Harry Otter
Cover design by Marije Brouwer

The research described in this thesis was performed at the Institute of Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University.

Neurotoxicity of PBDEs and metabolites:
concern for the developing brain?

Neurotoxiciteit van PBDEs en hun metabolieten:
zorg voor het ontwikkelende brein?
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op dinsdag 2 maart 2010
des middags te 12.45 uur

door

Milou Maria Louisa Dingemans

geboren op 31 januari 1981 te Nijmegen

Promotor: Prof.dr. M. van den Berg

Co-promotoren: Dr. R.H.S. Westerink
Dr. H.P.M. Vijverberg

Contents

Chapter 1	**General introduction**	**1**
	1.1. Brominated flame retardants	3
	1.2. Environmental and human exposure to PBDEs	5
	1.3. Toxicity of PBDEs	7
	1.4. OH-PBDEs: exposure and toxicity	13
	1.5. Environmental and human exposure to HBCD	15
	1.6. Toxicity of HBCD	17
	1.7. Thesis outline	18
Chapter 2	**Toxic endpoints and applied methodologies**	**21**
	2.1. PBDEs and HBCD	23
	2.2. *Ex vivo* and *in vitro* test systems used in this research	23
	2.3. Synaptic plasticity in hippocampal CA1 region	25
	2.4. *f*-EPSP-recordings in hippocampal slices to measure LTP and PPF	25
	2.5. Cell viability tests	26
	2.6. Ca^{2+} homeostasis	27
	2.7. Intracellular Ca^{2+} imaging with Fura-2	27
	2.8. Vesicular neurotransmitter release	29
	2.9. Detection of catecholamines by amperometry	30
Chapter 3	**Neonatal exposure to brominated flame retardant BDE-47 reduces long-term potentiation and postsynaptic protein levels in mouse hippocampus**	**31**
Chapter 4	**Hydroxylation increases the neurotoxic potential of BDE-47 to affect exocytosis and calcium homeostasis in PC12 cells**	**45**
Chapter 5	**Bromination pattern of hydroxylated metabolites of BDE-47 affects their potency to release calcium from intracellular stores in PC12 cells**	**61**
Chapter 6	**Calcium-related processes involved in the inhibition of depolarization-induced calcium increase by hydroxylated PBDEs in PC12 cells**	**89**
Chapter 7	**BDE-47 and its hydroxylated metabolite 6-OH-BDE-47 modulate calcium homeostasis in primary human neural progenitor cells**	**103**

Chapter 8 **Hexabromocyclododecane inhibits depolarization-induced increase in intracellular calcium levels and neurotransmitter release in PC12 cells** **115**

Chapter 9 **Summary and human risk assessment** **129**
9.1. Introduction 131
9.2. BDE-47 decreases LTP: observed effects and human risk assessment 131
9.3. Acute effects of parent PBDEs, OH-PBDEs and HBCD on $[Ca^{2+}]_i$ and vesicular neurotransmitter release: observed effects and human risk assessment 134

Chapter 10 **Issues concerning (OH)PBDE and HBCD neurotoxicity risk assessment** **141**
10.1. The developing brain 143
10.2. Brain concentrations versus serum concentrations 145
10.3. Chemical mixtures 147

Chapter 11 **Conclusions and recommendations** **153**

References **159**

Nederlandse samenvatting **179**

Curriculum vitae **195**

Publication list **196**

Dankwoord **197**

Abbreviations

Σ	sum
AA	arachidonic acid
AB	Alamar Blue
ACh	acetylcholine
ACSF	artificial cerebrospinal fluid
Ah	aryl hydrocarbon
AM	acetoxymethyl
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANOVA	analysis of variance
ATP	adenosine triphosphate
ATCC	American Type Culture Collection
BDE	brominated diphenyl ether
BFR	brominated flame retardant
BNDF	brain-derived-neurotrophic factor
BSEF	Bromine Science and Environmental Forum
bw	body weight
$[Ca^{2+}]_i$	intracellular calcium concentration
CaMKII	Ca^{2+}/calmodulin kinase II
CSF	cerebrospinal fluid
CYP	cytochrome P450
DE-71	commercial PentaBDE product
DE-79	commercial OctaBDE product
DecaBDE	commercial PBDE mixture (containing mainly BDE-209)
DMSO	dimethyl sulfoxide
DNT	developmental neurotoxicity
EC_{50}	half maximal effective concentration
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
F_{340}	fluorescence evoked by 340 nm
F_{380}	fluorescence evoked by 380 nm
FCCP	carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone
f-EPSP	field excitatory postsynaptic potential
GAP-43	growth-associated-protein-43
GD	gestational day
HBCD	hexabromocyclododecane
hNPC	human neural progenitor cell
IC_{50}	half maximal inhibitory concentration
IP_3	inositol trisphosphate
IUF	Institut für umweltmedizinische Forschung
IUPAC	International Union of Pure and Applied Chemistry

K_{d*}	dissociation constant of Fura-2 in the experimental set-up
K_{ow}	octanol-water partition coefficient
LC_{50}	half maximal lethal concentration
LDCV	large dense-core vesicle
LDH	lactate-dehydrogenase
LOEC	lowest observed-effect concentration
LOEL	lowest observed-effect level
LTP	long-term potentiation
MeO-PBDE	methoxylated PBDE
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n.a.	not applicable
nACh receptor	nicotinic acetylcholine receptor
NADPH	nicotinamide adenine dinucleotide phosphate
NCX	sodium calcium exchanger
NMDA	*N*-methyl-D-aspartic acid
NOAEL	no-observed adverse effect level
NOEC	no observed-effect concentration
NOEL	no observed-effect level
NR	Neutral Red
NSF	N-ethylmaleimide-sensitive factor
OctaBDE	commercial PBDE mixture (containing mainly hepta-octaBDEs)
OH-PBDE	hydroxylated PBDE
OH-PCB	hydroxylated PCB
PBDE	polybrominated diphenyl ether
PCB	polychlorinated biphenyl
PentaBDE	commercial PBDE mixture (containing mainly tetra-hexaBDEs)
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMCA	plasma membrane calcium/ATPases
PND	postnatal day
PPF	paired-pulse facilitation
PPR	paired-pulse ratio
PSD	postsynaptic density
PTP	post-tetanic potentiation
Q	time integral
R	F_{340}/F_{380} ratio
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
R_{max}	maximum ratio
R_{min}	minimum ratio
RoHS	Restriction of Hazardous Substances
ROS	reactive oxygen species
SE	standard error

SEM	standard error of mean
SERCA	sarco/endoplasmic reticulum calcium ATPase
SNARE	soluble NSF attachment receptors
SOCA	store-operated calcium entry
$t_{1/2}$	half-width (time)
T_3	triiodothyronine
T_4	thyroxin
TBBPA	tetrabrominated bisphenol A
TBS	tris-buffered saline
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TG	thapsigargin
TIF	triton insoluble fraction
VGCC	voltage-gated calcium channel
VMAT	vesicular monoamine transporter
WEEE	waste electrical and electronic equipment

Full names of discussed PBDEs and PCBs and their metabolites

BDE-28	2,4,4’-tribrominated diphenyl ether
BDE-47	2,2’,4,4’-tetrabrominated diphenyl ether
BDE-49	2,2’,4,5’-tetrabrominated diphenyl ether
BDE-77	3,3’,4,4’-tetrabrominated diphenyl ether
BDE-85	2,2’,3,4,4’-pentabrominated diphenyl ether
BDE-99	2,2’,4,4’,5-pentabrominated diphenyl ether
BDE-100	2,2’,4,4’,6-pentabrominated diphenyl ether
BDE-153	2,2’,4,4’,5,5’-hexabrominated diphenyl ether
BDE-154	2,2’,4,4’,5,6’-hexabrominated diphenyl ether
BDE-183	2,2’,3,4,4’,5’,6-heptabrominated diphenyl ether
BDE-196	2,2’,3,3’,4,4’,5,6’-octabrominated diphenyl ether
BDE-197	2,2’,3,3’,4,4’,6,6’-octabrominated diphenyl ether
BDE-203	2,2’,3,4,4’,5,5’,6-octabrominated diphenyl ether
BDE-206	2,2’,3,3’,4,4’,5,5’,6-nonabrominated diphenyl ether
BDE-209	2,2’,3,3’,4,4’,5,5’,6,6’-decabrominated diphenyl ether
PCB-52	2,2’,5,5’-tetrachlorinated biphenyl
PCB-153	2,2’,4,4’,5,5’-hexachlorinated biphenyl
3-OH-BDE-47	3-hydroxy-2,2’,4,4’-tetrabrominated diphenyl ether
4-OH-BDE-42	4-hydroxy-2,2’,3,4’-tetrabrominated diphenyl ether
4’-OH-BDE-49	4’-hydroxy-2,2’,4,5’-tetrabrominated diphenyl ether
4-OH-PCB-106	4-hydroxy-2,3,3’,4,5-pentachlorinated biphenyl
4-OH-PCB-107	4-hydroxy-2,3,3’,4,5’-pentachlorinated biphenyl

4-OH-PCB-109	4-hydroxy-2,3,3’,4’,5-pentachlorinated biphenyl
5-OH-BDE-47	5-hydroxy-2,2’,4,4’-tetrabrominated diphenyl ether
6-MeO-BDE-47	6-methoxy-2,2’,4,4’-tetrabrominated diphenyl ether
6-OH-BDE-47	6-hydroxy-2,2’,4,4’-tetrabrominated diphenyl ether
6’-OH-BDE-49	6’-hydroxy-2,2’,4,5’-tetrabrominated diphenyl ether

Species scientific classification

beluga whale	*Delphinapterus leucas*
chicken	*Gallus gallus domesticus*
dog	*Canis lupus familiaris*
fathead minnow	*Pimephales promelas*
herring gull	*Larus argentatus*
human	*Homo sapiens sapiens*
kestrels (American)	*Falco sparverius*
killifish	*Fundulus heteroclitus*
lake trout	*Salvelinus namaycush*
mink	*Mustela vison*
mouse	*Mus musculus*
polar bear	*Ursus maritimus*
rainbow trout	*Oncorhynchus mykiss*
rat	*Rattus norvegicus*
river otter (North American)	*Lontra canadensis*
white-sides dolphin (Atlantic)	*Lagenorhynchus acutus*
xenopus	*Xenopus laevis*
zebrafish	*Danio rerio*

Chapter 1

General introduction

1.1 Brominated flame retardants

To reduce deaths and injuries, as well as economical impact of fires, fire safety standards have been established in modern societies. An important fire safety measure is the application of flame retardant chemicals. Flame retardants are chemicals added to a product to prevent or delay combustion, thereby increasing the chance to extinguish or escape a fire. In the last decades, increasing production of petroleum-based polymer materials has gone together with increasingly strict fire regulations. Therefore, increasing amounts of flame retardants are being produced and used during the last decades. Four main classes of flame retardants (inorganic, halogenated organic, organophosphorus and nitrogen-based flame retardants) are produced and used, comprising together more than 175 chemicals (reviewed in Alaee et al. 2003).

In Europe and North America, brominated flame retardants (BFRs) are regularly used flame retardants. Bromine, like other halogens, eliminates free radicals generated in a fire with high efficiency, thereby preventing the propagation of a flame. BFRs are a structurally diverse group of chemicals, which were introduced in the 1970s as flame retardants in industrial and consumer products after the discovery of the adverse effects of polychlorinated biphenyls (PCBs) on human health and in experimental studies (reviewed in Fonnum and Mariussen 2009; Seegal 1996).

Products in which BFRs are applied are for example electronic equipment, furniture, upholstery, construction materials and textiles. BFRs with the highest production volumes are polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD) and tetra-brominated bisphenol-A (TBBPA; Bromine Science and Environmental Forum; BSEF 2009a; table 1.1). Both PBDEs and HBCD are additive flame retardants, thus lacking chemical bonds with the polymers they are added to. This lack of chemical bonds makes these BFRs prone to leakage from the products in which they are used. Although TBBPA can also be used in an additive manner, it is mainly used as a reactive flame retardant (covalently bound to the polymers; Alaee et al. 2003), and therefore not investigated in this research.

The molecular structure of PBDEs consists of two phenyl rings connected by an ether bond, with varying bromination pattern and degree. Bromine substitution of PBDEs can be located at 10 different carbon-atoms in the two phenyl rings. Therefore, 209 different bromination patterns are possible, thus 209 PBDE congeners divided in 10 different congener groups (mono- to decabrominated diphenyl ether). PBDE congeners are numbered using the International Union of Pure and Applied Chemistry (IUPAC) system, similar to PCBs (Maervoet et al. 2004). In practice, the use of individual PBDE congeners is limited to research purposes, as PBDEs have been marketed in the form of different commercial products: PentaBDE, OctaBDE and DecaBDE (Table 1.1).

Table 1.1. Different classes of brominated flame retardants and some of their characteristics (Birnbaum and Staskal 2004; BSEF 2009c, 2009d, 2009e; Covaci et al. 2006; Hardy 2002).

	polybrominated diphenyl ether (PBDE)	hexabromocyclododecane (HBCD)	tetrabrominated bisphenol-A (TBBPA)
molecular formula molecular structure	$C_{12}H_{10-x}Br_xO$ example: 6-OH-BDE-47	$C_{12}H_{18}Br_6$	$C_{15}H_{12}Br_4O_2$
CAS number	PentaBDE: 32534-81-9 OctaBDE: 32536-52-0 DecaBDE: 1163-19-5	25637-99-4	79-94-7
molecular weight	249.1 - 959.2 (BDE-47: 485.8)	641.7	543.9
log K_{ow}	> 5 BDE-209: ~10 - 12	5.6	4.5
worldwide production volume (* 1000 metric tons, 2001)	67.4	16.7	119.6
worldwide production volume (* 1000 metric tons, 2009)	unknown	unknown	unknown
main isomers/congeners in commercial product(s)	PentaBDE: BDE-47, BDE-99, BDE-100, BDE-153 OctaBDE: BDE-183, BDE-196, BDE-197, BDE-203 DecaBDE: BDE-209	α-HBCD β-HBCD γ-HBCD	n.a.
application method	additive	additive	reactive
applications	electronics automobiles and airplanes construction and building furniture textiles	polystyrene insulation foams textile coatings electronics	printed circuit boards electronics

n.a.: not applicable

HBCD is marketed as an isomeric mixture in which the diastereoisomers α-HBCD, β-HBCD and γ-HBCD are predominant, although other diastereoisomers have also been identified (Heeb et al. 2005). The commercial HBCD mixture consists for 70 - 90% of γ-HBCD (Covaci et al. 2006).

Additive flame retardants, like PBDEs and HBCD, are generally considered persistent organic pollutants (Hale et al. 2006), although this is a matter of debate (BSEF 2009b). To prevent and reduce environmental and human exposure, PBDEs are regulated in Europe under the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) framework by the European Chemicals Agency, the Restriction of Hazardous Substances used in electrical and electronic devices (RoHS) directive, Waste Electrical and Electronic Equipment (WEEE) directive and the Water Framework Directive. Furthermore, BFR industry and down-stream users execute product stewardship initiatives to reduce environmental and human exposure (BSEF 2009a).

1.2 Environmental and human exposure to PBDEs

During the last decades, mainly PBDEs were used in consumer products (Alaee et al. 2003). Since 2004, voluntary action and legislative measures have been taken to phase out the penta- and octabrominated diphenyl ethers from the market in Europe and North America. Recently, a European Court ruling removed the exemption status of DecaBDE in the RoHS directive in the EU, meaning that DecaBDE is no longer allowed to be used as a flame retardant in electronics. It has also only just been announced (December 2009) that DecaBDE will be voluntarily phased out in North America within the following years. However, DecaBDE is still in use with few restrictions in other parts of the world. Although no longer in commercial production, the lower brominated PBDEs are still being found globally in both the environment and human tissues (Frederiksen et al. 2009; Yogui and Sericano 2009).

The use patterns of different commercial products of PBDEs vary globally (Law et al. 2006a), which is reflected in congener patterns and levels measured in the environment, wildlife and humans (Birnbaum and Cohen Hubal 2006). PBDEs are mainly released into the environment at production sites, recycling facilities and via waste water. Particularly important for the indoor environment is the release of PBDEs from flame-retarded consumer products through volatilization and abrasion (Alcock et al. 2003; Law et al. 2006a; Lorber 2008). Upon introduction into the environment, PBDEs particularly accumulate in species at a higher trophic level in mainly aquatic and arctic food chains (de Wit et al. 2006; Jenssen et al. 2007; Johnson-Restrepo et al. 2005a; Letcher et al. 2009; Losada et al. 2009; Shaw et al. 2009; Wan et al. 2008; Wolkers et al. 2004). In wildlife as well as human tissues, PBDE congeners BDE-28, BDE-47, BDE-99, BDE-100, BDE-153 and BDE-183 are particularly observed, as well as, although at lower concentrations, BDE-209 (reviewed in Hakk and Letcher 2003). Usually, BDE-47 is the predominant congener in biotic samples (reviewed in Frederiksen et al. 2009; Hites 2004).

PBDEs have been detected in air (Wilford et al. 2004) and in-door house dust (Wilford et al. 2005). Consequently, humans are exposed to PBDEs via air and ingestion of house dust, but also through intake of vegetable and animal products (Johnson-Restrepo and Kannan 2009; Frederiksen et al. 2009). Dietary exposure of especially the lower brominated PBDEs is mainly associated with fish consumption (Knutsen et al. 2008), though other food items with high lipid content, such as dairy and meat products, also contain considerable amounts of PBDEs (Schecter et al. 2008; Schecter et al. 2006). PBDEs have been detected in liver, blood, milk and adipose tissues, occasionally at high concentrations, in both wildlife (reviewed in de Wit 2002; Law et al. 2003) and human tissues, including breast milk (Bradman et al. 2007; Petreas et al. 2003; Schecter et al. 2003; Sjödin et al. 2004; reviewed in Frederiksen et al. 2009).

Exponentially increasing concentrations of PBDEs have been determined (with an approximate doubling time of 5 years determined during the last decade of the 20th century) in both wildlife (Ikonomou et al. 2002; Norstrom et al. 2002; Sellström et al. 2003) and human tissues (Norén and Meironyté 2000; reviewed in Frederiksen et al. 2009). In Europe, human levels are approaching a plateau (Guvenius et al. 2003; Lind et al. 2003; reviewed in Frederiksen et al. 2009), while in North America, human PBDE levels are not only approximately 1 to 2 orders of magnitude higher compared to Europe or Japan but may also still be increasing (Inoue et al. 2006; Johnson-Restrepo et al. 2005b; Petreas et al. 2003; Sandanger et al. 2007; Schecter et al. 2003; Sjödin et al. 2008). Despite the absence of restrictions on the production or use of PBDEs, human serum PBDE levels in Asia are comparable to those in Europe (reviewed in Martin et al. 2004; Zhu et al. 2009). However, the number of studies on human internal exposure in Asia is very low, particularly when excluding Japan.

Higher levels of PBDEs, including DecaBDE, compared to the general human population have been detected in people working at electronics waste recycling plants and incinerators (Bi et al. 2007; Sjödin et al. 1999; Stapleton et al. 2008b; Thuresson et al. 2005; reviewed in Schecter et al. 2009). Computer technicians are exposed occupationally to higher PBDE concentrations compared to the general population, particularly to higher brominated PBDEs (Jakobsson et al. 2002). Half-lives of higher brominated PBDEs for elimination after cessation of occupational exposure are estimated to be ~15 days for BDE-209 and ~30 and ~65 days for nona- and octaBDEs, respectively (Thuresson et al. 2006). In contrast, lower brominated PBDE have been demonstrated to bioaccumulate (reviewed in Frederiksen et al. 2009; Hites 2004).

Toxicokinetics studies in rodents (Chen et al. 2006; Hakk et al. 2002; Örn and Klasson-Wehler 1998; Sanders et al. 2006a; Staskal et al. 2006b; von Meyerinck et al. 1990; reviewed in Darnerud et al. 2001; Hakk and Letcher 2003) demonstrated high absorption and slow elimination as well as accumulation in adipose tissue after a single oral dose of tetra-, penta and hexaBDEs. Studies in fish showed an efficient absorbance of PBDEs, with a negative correlation with bromination degree (Burreau et al. 1997; Burreau et al. 2004). BDE-209 has also been detected in bird's eggs, birds, fish and marine mammals (reviewed in Law et al.

2006a) as well as human tissues (reviewed in Frederiksen et al. 2009) despite its poor absorption in the gastrointestinal tract, low solubility, high log octanol-water partition coefficient (K_{ow}) and molecular weight (Mörck et al. 2003). Ecotoxicological concern has arisen from the observation of very high concentrations of BDE-209 in birds of prey in North-China. This observation is a sign of significant biomagnifications of BDE-209 in terrestrial food chains (Chen et al. 2007).

Moreover, toxicokinetics studies in rodents revealed the formation of hydroxylated and methoxylated metabolites from man-made PBDEs (OH-PBDEs and MeO-PBDEs), which were previously also detected in wildlife and considered natural products (discussed in §1.4).

Several exposure studies have demonstrated that exposure to PBDEs is higher in young children compared to adults (Fischer et al. 2006; Toms et al. 2008, 2009). This is probably due to differences in external exposure, especially dust ingestion via child specific hand-to-mouth behavior (Jones-Otazo et al. 2005; Wilford et al. 2005). An additional source of exposure for young children is breast milk, in which especially lower brominated PBDEs have been detected in increasing concentrations (Fängström et al. 2005; Meironyté et al. 1999; Schecter et al. 2003; reviewed in Frederiksen et al. 2009). Moreover, prenatal exposure also occurs through placental transfer of maternal PBDEs (Bi et al. 2006; Gómara et al. 2007; Guvenius et al. 2003; Mazdai et al. 2003; Meijer et al. 2008; Schecter et al. 2007). Another contributing factor to the higher internal exposure of children to PBDEs compared to adults has been suggested to be lower renal excretion capacity in children (Staskal et al. 2006a, 2005).

1.3 Toxicity of PBDEs

Acute toxicity of PBDEs. Acute toxicity studies revealed only moderate effects at high exposure concentrations, mainly in the liver and thyroid gland (reviewed in Hardy 2002). These data resulted in no-observed adverse effect levels (NOAELs) of 1 mg/kg bw, <100 mg/kg bw and 1000 mg/kg bw (in repeated dose studies) for respectively the Penta-, Octa- and DecaBDE commercial products.

Hepatic toxicity of PBDEs. Because of their structural resemblance to aryl-hydrocarbon (Ah) receptor activating compounds (2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD and PCBs), possible effects of PBDEs on Ah receptor-mediated processes were investigated. Commercial PBDE mixtures have been shown to increase gene induction or activity of hepatic enzymes in rodents and zebrafish (Dunnick and Nyska 2009; Fowles et al. 1994; Kuiper et al. 2006; Pacyniak et al. 2007; Sanders et al. 2005; Stoker et al. 2004; Szabo et al. 2009; van der Ven et al. 2008b; Zhou et al. 2001), which has also been detected *in vitro* in hepatocytes and hepatic cell lines (Fery et al. 2009). Single PBDE congeners also induced cytochrome P450 (CYP) activity (Chen et al. 2001; Hallgren and Darnerud 2002), although others detected no effects on hepatic CYP activity or even antagonism (Chen and Bunce 2003; Kuiper et al. 2004; Peters

et al. 2004, 2006). BDE-99 and BDE-209 caused gene induction of CYP enzymes in human hepatocytes (Stapleton et al. 2009). Nonetheless, the observed Ah receptor-mediated effects of commercial PBDE mixtures is probably primarily due to trace amounts of brominated dioxins and furans, which are potent inducers of CYP enzymes (Sanders et al. 2005; Wahl et al. 2008).

Endocrine toxicity of PBDEs. Endocrine toxicity of PBDEs has been extensively investigated (reviewed in Darnerud 2008; Legler 2008). Endocrine and reproductive effects were detected in wild birds (Fernie et al. 2008, 2009; Henny et al. 2009). *In vivo* estrogenic effects of PBDEs have also been detected in experimental studies with rodents (Dang et al. 2007; Mercado-Feliciano and Bigsby 2008a), as well as decreased sperm count or function (Kuriyama et al. 2005; Tseng et al. 2006, van der Ven et al. 2008b) and histological changes in ovaries (Talsness et al. 2005). Effects on sexual development and sexually dimorphic behavior were detected in rats (Lilienthal et al. 2006). *In vitro* studies demonstrated that PBDEs bind to estrogen, proestrogen and androgen receptors, thereby exerting agonistic and antagonistic effects (Harju et al. 2007; Meerts et al. 2001; Stoker et al. 2005) and that PBDEs inhibit CYP enzymes involved in steroidogenesis (Cantón et al. 2005, 2006). Decreased activity of CYP enzymes involved in steroidogenesis was also demonstrated *in vivo* after exposure to BDE-209 (van der Ven et al. 2008a).

Additionally, evidence is accumulating for a PBDE-induced disturbance of the thyroid hormone system. Thyroid hormones are involved in the basal cellular metabolic rate, but also essential for normal brain development (Horn and Heuer 2009; Leonard 2008). Even subclinical maternal hypothyroidism has been associated with impaired psychomotor development in humans (Pop et al. 1999). Effects of PBDEs and PBDE mixtures on the thyroid hormone system, mainly decrease in thyroxin (T_4), have been observed *in vivo* in rodents exposed pre- and/or neonatally (Driscoll et al. 2009; Rice et al. 2007; Zhou et al. 2001, 2002) or in adulthood (Darnerud et al. 2007; Fowles et al. 1994; Hallgren et al. 2001; Richardson et al. 2008). At a low dose, relevant to the human exposure situation, changes in thyroid gland histology and morphology were detected in rats (Talsness et al. 2008). After developmental exposure to BDE-209 (Gestational day; GD 6 - 17), a decrease in triiodothyronine (T_3), but not T_4, was observed in mice offspring (Tseng et al. 2008).

Effects on the thyroid hormone system have also been detected in wild birds (Fernie et al. 2005), mink (Zhang et al. 2009a), fathead minnow (Lema et al. 2008), lake trout (Tomy et al. 2004b) and xenopus tadpoles (Schriks et al. 2006b). PBDEs and in particular OH-PBDEs, which have a structural resemblance to the thyroid hormone T_4, bind to thyroid hormone transport proteins (Marchesini et al. 2008; Meerts et al. 2000; Schriks et al. 2006a; Ucán-Marín et al. 2009).

Associations of PBDEs with thyroid hormone levels have also been investigated in humans. Serum concentrations of PBDEs (median: 38 ng/g lipids) in consumers of fish caught in the

Great Lakes (US) were related to thyroid hormone concentrations. The test subjects in this study had serum levels comparable to the general US population, and no thyroid-related afflictions (Turyk et al. 2008). Relationships between serum PBDE levels and thyroid hormone were also detected in men living close by the Baltic See (Hagmar et al. 2001), and in more highly exposed employees at an e-waste dismantling site (Yuan et al. 2008). However, other studies found no significant correlations (Bloom et al. 2008; Julander et al. 2005).

One epidemiological study indicates endocrine disruption by PBDEs. Exposure to PBDEs via breast milk was associated with congenital cryptorchidism in Danish children (Main et al. 2007).

Neurobehavioral effects of PBDEs. In 2001, effects of a single neonatal (postnatal day; PND 10) dose of BDE-47 or BDE-99 on spontaneous behavior and relearning in a Morris water maze were described (Eriksson et al. 2001b). Habituation capability (adjusting to a new environment) was measured as a read-out for learning by dividing activity within 40 - 60 min by activity within 0 - 20 min from placement in the test cage. The Morris water maze (Morris 1984) was used to investigate spatial learning. In a learning period, mice are placed in the water to find the hidden platform. After the learning period, the hidden platform is moved to another location, and the ability of the mice to relearn the new position is assessed. A dose-related change in locomotion, rearing and total activity was observed in mice exposed to BDE-47 or BDE-99 both 2 and 4 months after the exposure to a single oral dose. The habituation capability decreased with age in mice exposed to BDE-47 and BDE-99 compared to controls. Relearning in the Morris water maze was impaired only at the highest dose of BDE-99.

This exposure and testing regime (excluding the Morris water maze relearning in most studies) has been used to investigate the effects of different PBDE congeners. Similar long-lasting neurobehavioral changes have been detected after a single oral dose administered in neonatal mice of BDE-153, BDE-183, BDE-203, BDE-206 or BDE-209 (Johansson et al. 2008; Viberg et al. 2003a, 2003b, 2006).

A study in which BDE-99 was administered at PND 3, 10 or 19 (Eriksson et al. 2002) revealed that brain development in mice is most sensitive to exposure to PBDEs in the first 2 weeks after birth. In rodents, a rapid growth of the brain as well as synaptogenesis, myelination and other important processes in brain development occur in this period (Davison and Dobbing 1968).

Other groups also investigated effects of PBDEs in rodents after neonatal administration of a single oral dose. Several behavioral endpoints of neurodevelopment were affected in mice exposed to a single oral dose of BDE-47 (Gee and Moser 2008). A single oral dose of BDE-99 to pregnant rats at a low dose relevant to the human exposure situation resulted in hyperactivity in offspring (Kuriyama et al. 2005).

Neurobehavioral effects of PBDEs in rodents were also studied after repeated neonatal dosing. When exposed to BDE-99 from gestation through lactation, hyperactivity was

observed in adolescent rats but not in adults (Branchi et al. 2005), while effects on other behavioral parameters worsened with age (Branchi et al. 2002). By testing mice in behavioral tasks (pushing a lever for food when visually stimulated), effects on test performance were observed in aging mice after neonatal exposure to BDE-209, while no effects were observed in littermates tested at a young age. Mainly increased impulsivity was observed, and this effect worsened with age (Rice et al. 2009). In one study, rats were exposed chronically to commercial PentaBDE mixture DE-71 through lactation and food (continued through testing) to mimic low-level chronic exposure as in humans. No effects were observed on a nose poking task to receive food upon visual stimulation in adult rats, although attention was impaired (Driscoll et al. 2009). Impaired attention is consistent with previously reported hyperactivity/increased locomotor activity in animals postnatally exposed to PBDEs.

PBDE-induced effects on behavior have also been detected in fish. Embryonic exposure of killifish to DE-71 decreased motor activity, but increased predation and hyperactivity were also observed (Timme-Laragy et al. 2006).

From these combined studies it can be concluded that PBDEs can disrupt neurobehavior in rodents when exposed during brain development. Moreover, although different parameters were investigated, the presence of neurobehavioral effects is not specific for one testing regime, but occurs at different exposure and test systems.

Recently, it has been described that motor, cognitive and behavioral performance in 6-year old children is correlated with maternal serum levels of PBDEs measured in the 35th week of pregnancy (Roze et al. 2009).

Additional studies indicated effects of BDE-99 and BDE-209 on the cholinergic neurotransmitter system as exposed animals had a hypoactive response to nicotine administration (Johansson et al. 2008; Viberg et al. 2002, 2007). A decrease in numbers of nicotinic acetylcholine (nACh) receptors was observed in mice exposed to BDE-153 (Viberg et al. 2003a). When exposed to DE-71 at PND 6 - 12, performance in a food motivated visual discrimination task was impaired in rats, and sensitivity to muscarinic antagonist scopolamine suggests effects on cholinergic functioning (Dufault et al. 2005). However, no effects on muscarinic acetylcholine receptor and nACh receptor binding, cholinesterase activity or acetylcholine (ACh) concentration were detected in the cerebral cortex of mink after exposure to DE-71 from gestation through adulthood (Bull et al. 2007).

Reflexes and locating of the platform in the Morris water maze were delayed in rats exposed from gestation through lactation to BDE-99. In these animals, also increased oxidative stress was observed in the hippocampus, while this was not observed in cerebellum and cerebral cortex (Cheng et al. 2009).

In rodents in which neonatal exposure to DE-71 and BDE-209 caused effects on neurobehavior, also lower serum T_4 levels were observed (Driscoll et al. 2009; Rice et al. 2007). After dietary exposure of fathead minnows to BDE-47, decreased serum thyroid hormone levels were observed. Also, transcription of thyroid hormone receptors was altered

in the brain, while this was not changed in the liver (Lema et al. 2008). As thyroid hormones are essential for brain development (Horn and Heuer 2009; Leonard 2008), these studies indicate that the previously observed PBDE-induced hypothyroidism could play a role in the neurobehavioral effects of PBDEs. Moreover, experimentally induced hypothyroidism has been shown to result in alterations in the cholinergic neurotransmitter system (Sawin et al. 1998). Therefore, the disruption of normal brain development by PBDE-induced hypothyroidism may be an underlying mechanism for the observed PBDE-induced alterations in the cholinergic neurotransmitter system in rodents. But, when exposing mice at PND 10 to a single oral dose BDE-47, known to result in behavioral effects, no effects were detected on thyroid hormone levels (Gee et al. 2008). This suggests the involvement of additional underlying mechanisms in the observed neurobehavioral effects of PBDEs.

Effects of PBDEs on brain function and structure. In mice exposed to BDE-203, BDE-206 or BDE-209, which resulted in neurobehavioral changes, levels of brain proteins important during early postnatal brain development were investigated in hippocampus and cortex (Viberg et al. 2008; Viberg 2009a, 2009b). Levels of Ca^{2+}/calmodulin kinase II (CaMKII), synaptophysin, growth-associated-protein-43 (GAP-43), tau and brain-derived-neurotrophic factor (BNDF) were investigated. These proteins are critically involved in regulation of synaptogenesis and synaptic plasticity (Wayman et al. 2008), vesicular neurotransmitter release (Evans and Cousin 2005), axon formation and growth (Denny 2006), neuronal morphology (Wang and Liu 2008) and cell migration and synapse formation (Cui 2006), respectively. Increased levels of CaMKII and synaptophysin were observed in hippocampus after exposure to BDE-203 or BDE-206, while no changes were observed for GAP-43, tau or any of the investigated proteins in cortex. After exposure to BDE-209, CAMKII increased only in hippocampus. GAP-43 was increased in hippocampus and decreased in cortex. BDNF was decreased in cortex, but not in hippocampus. Synaptophysin was increased in hippocampus, but not in cortex. No effects were observed on tau levels.

Using a proteomics approach, changes in striatal proteins associated with neurodegeneration and neuroplasticity, hippocampal proteins associated with metabolism and energy production, and cortical proteins involved in cytoskeletal regulation, neuronal sprouting, and mitochondrial metabolism were detected after exposure to BDE-99 (Alm et al. 2006, 2008).

Developmental exposure (GD 2 - 9) to BDE-99 resulted in increased activity of the glutamate-nitric oxide-cyclic guanosine monophosphate pathway in rat cerebellum. Surprisingly, this resulted in slightly enhanced performance in a learning task. The enhancement of this pathway is probably related to the observed increase in calmodulin (Llansola et al. 2007, 2009).

These combined studies demonstrated relatively subtle structural and functional alterations in brains of animals exposed to PBDEs, mainly in the hippocampus. These alterations in brain structure could be related to the observed neurobehavioral effects.

In vitro neurotoxicity of PBDEs. *In vitro* neurotoxic effects of PBDEs involve effects on cell viability, oxidative stress in neuronal cell types, neurotransmitter homeostasis and ion channels.

At high concentrations (half maximal inhibitory concentration; IC_{50} = ~50 µM), BDE-99 causes apoptotic cell death in human astrocytoma cells (Madia et al. 2004). Apoptotic cell death was also induced by DE-71 in primary rat cerebellar granule cells with a lowest observed-effect concentration (LOEC) of 10 µM, while effects on intracellular Ca^{2+} levels or formation of reactive oxygen species (ROS) were not observed in this study (Reistad et al. 2006). The cytotoxic effects of DE-71 in primary mouse neurons and astrocytes have been shown to be modulated by intracellular levels of glutathione, which indicates involvement of cellular antioxidant defense capacity (Giordano et al. 2008). In the human neuroblastoma cell line SK-N-SH, apoptotic effects of DE-71 appeared related to intracellular Ca^{2+} levels, but oxidative stress was not observed in this study (Yu et al. 2008). Exposure of herring gull embryonic neuronal cells to DE-71 decreased cell viability. However, no effects were observed for the individual congeners BDE-47, BDE-99 or BDE-100. In this study, mRNA levels of proteins involved in the thyroid hormone system and nACh receptors were also studied, yet no effects were detected (Crump et al. 2008).

In gill and hepatic rainbow trout cell lines (RTgill-W1 and RTL-W1), primary rat hippocampal neurons as well as a human neuroblastoma cell line (SH-SY5Y), BDE-47 induced formation of ROS (He et al. 2008a, 2008b; Shao et al. 2008a). In addition, oxidative stress has also been observed in human neutrophilic granulocytes after exposure to DE-71 (Reistad and Mariussen 2005).

Inhibition of neurotransmitter re-uptake in synaptosomes and neurotransmitter vesicles has previously been described as a toxic endpoint of PCBs (reviewed in Fonnum and Mariussen 2009). This effect on neurotransmitter recycling has also been detected for DE-71, while in contrast, Octa- and DecaBDE mixtures did not have such an effect (Mariussen and Fonnum 2003).

BDE-99 (50 µM) causes translocation of protein kinase C (PKC) isozymes in human astrocytoma 132-1N1 cells (Madia et al. 2004). PKC translocation has also been observed for DE-71, BDE-47, BDE-77, BDE-99, BDE-100 and BDE-153 in primary rat cerebellar granule cells (Kodavanti and Ward 2005; Kodavanti et al. 2005). Uptake of Ca^{2+} in microsomes and mitochondria, isolated from rat cortex, cerebellum and hippocampus, was inhibited by DE-71 (Kodavanti and Ward 2005) as well as by BDE-47 and BDE-99 (Coburn et al. 2008). Also, release of the second messenger arachonic acid (which is involved in synaptic plasticity; Farooqui et al. 1997) from cerebellar granule cells, probably by activation of phospholipase A, was detected for DE-71, but not for DE-79 (Kodavanti and Derr-Yellin 2002).

Recently, it was also revealed that BDE-209 dose-dependently decreases voltage-gated sodium channel currents with a LOEC of 0.1 µM (Xing et al. 2009b).

PBDE-induced cytotoxicity and oxidative stress are not specific for neuronal cells, as they have also been observed in various other cell types, among others human hepatoma

HepG2 cells and primary human stem cells (Hu et al. 2007; Shao et al. 2008b). Nonetheless, a combination of these observed acute effects on neuronal cells could be involved in the observed neurobehavioral impairments, although the concentrations at which effects of parent PBDEs are observed are in general not relevant for the human exposure situation, also because of possible underestimation of intracellular PBDE concentrations (Mundy et al. 2004; Huang et al. 2009).

1.4 OH-PBDEs: exposure and toxicity

Besides parent PBDE congeners, hydroxylated and methoxylated PBDEs (OH- and MeO-PBDEs) have also been detected in marine and freshwater fish, sea birds as well as dolphins, seals and polar bears (Gebbink et al. 2008b; Houde et al. 2009; Kelly et al. 2008; Kierkegaard et al. 2004; Malmvärn et al. 2005; Marsh et al. 2004; McKinney et al. 2006a; Olsson et al. 2000; Routti et al. 2009; Verreault et al. 2005; Wan et al. 2009).

Toxicokinetics studies revealed the *in vivo* formation of OH-PBDEs. In *in vivo* toxicokinetics studies, OH-PBDEs were detected in liver, lung, plasma, feces and bile after oral administration of BDE-47 or BDE-99 to rats (Chen et al. 2006; Hakk et al. 2002; Marsh et al. 2006; Örn and Klasson-Wehler 1998). OH-PBDEs have also been observed in plasma after intraperitonal administration of an equimolar mixture of environmentally relevant PBDEs to rats (Malmberg et al. 2005).

Intravenous administration of BDE-47, BDE-99, BDE-100 or BDE-153 to mice revealed that hydroxylated metabolites were formed from all four PBDEs. BDE-99 was observed to be most readily metabolized by oxidation and oxidation/debromination, while debromination was not observed for the other PBDEs (Staskal et al. 2006b). In contrast, metabolism of BDE-153 after oral administration is minimal (Qiu et al. 2007; Sanders et al. 2006b), which is suggested to be due to the absence of Br-atoms with 2 adjacent unsubstituted C-atoms. In support of this explanation, the presence of several OH-PBDEs was observed in feces after oral administration of BDE-154, in which a Br-atom with two unsubstituted adjacent C-atoms is present (Hakk et al. 2009). After oral administration of BDE-209 to rats, several methoxylated and acetylated metabolites were detected in bile and feces (Mörck et al. 2003). In addition, hydroxylated octa- and nonaBDEs were also detected in plasma and the liver after oral or intravenous administration of BDE-209 to rats (Sandholm et al. 2003; Riu et al. 2008).

After subchronic low dose administration of DE-71 through the feed to rats, OH-PBDEs were identified in feces (Huwe et al. 2007). In mice, OH-PBDEs were observed in plasma after oral or subcutaneous exposure to DE-71 (Qiu et al. 2007). A study in mink demonstrated the presence of OH-PBDEs in plasma, liver and feces, after exposure to DE-71 from gestation through adolescence (Zhang et al. 2008a).

Formation of OH-PBDEs from BDE-47 was also demonstrated using phenobarbital-induced rat liver microsomes (Hamers et al. 2008). For ecotoxicological risk assessment,

interspecies differences also have to be taken into account. Using hepatic microsomes from beluga whale indicated that especially the lower-brominated PBDEs were degradated, while the formation of an OH-PBDE was only observed for one out of the five investigated PBDEs (McKinney et al. 2006b).

Recently, the formation of OH-PBDEs was also investigated in human primary hepatocytes exposed to BDE-99 or BDE-209. These cells metabolized BDE-99 into OH-PBDEs while in contrast, OH-PBDEs were not detected after exposure to BDE-209 (Stapleton et al. 2009).

Toxicokinetics studies in fish have shown that the formation of OH-PBDEs is limited and mainly debrominated metabolites are formed (Burreau et al. 2000; Nyholm et al. 2009; Stapleton et al. 2004b, 2004c). The lack of hydroxylation observed in experimental fish studies suggests that OH-PBDEs found in wild fish most likely originate from their diet.

Interestingly, OH-PBDEs and MeO-PBDEs are also natural products that have previously been detected in marine algae, cyanobacteria and sponges (Kelly et al. 2008; Malmvärn et al. 2005, 2008), sometimes in higher concentrations than PBDEs (Covaci et al. 2007a; Teuten et al. 2005). In view of the occurrence of OH-PBDEs in fish, this might be an additional source of human exposure.

In Greenland sledge dogs fed with an organohalogen-contaminated diet, a lack of association was observed between sum (Σ)PBDE and ΣOH-PBDE levels (Verreault et al. 2009). This observation suggests a small contribution of CYP mediated transformation. This would confirm a primary accumulation of OH-PBDEs via the diet, if this includes a large proportion of fish or aquatic top predators (reviewed in Letcher et al. 2009).

Recently, *in vitro* biotransformation of parent, OH- and MeO-PBDEs in rainbow trout, chicken and rat microsomes suggested an additional metabolic pathway, i.e., formation of OH-PBDEs from MeO-PBDEs (Wan et al. 2009). Because of the dietary exposure to MeO-PBDEs, particularly via intake of fish and fish oil (Covaci et al. 2007b), this could also be an additional exposure route of humans to OH-PBDEs.

Introduction in and distribution of OH-PBDEs through the environment has been shown to occur via wastewater and formation in air and sewage treatment plants (Ueno et al. 2008). In 2002, OH-PBDEs were detected in human serum (Hovander et al. 2002). Only very recently, the accumulation of hydroxylated metabolites was confirmed in humans. Several OH-PBDEs were detected in children that were highly exposed by living and working on a waste-dump site in Manague, Nicaragua (Athanasiadou et al. 2008). In Inuit adults, the presence of OH-PBDEs in plasma was also investigated and confirmed (Dallaire et al. 2009). A very recent study detected OH-PBDEs in US maternal and fetal serum samples and confirmed the bio-accumulation of these metabolites (Qiu et al. 2009).

Endocrine studies have revealed that the potency of OH-PBDEs for a number of endpoints is higher compared to their parent compounds. Aromatase and CYP17 activity in human

adrenocorticol carcinoma H295R cells were inhibited by OH-PBDEs but not by their parent PBDEs (Cantón et al. 2005, 2006). In placental microsomes, all investigated OH-PBDEs inhibited aromatase activity, while none of their methoxylated analogues showed this effect (Cantón et al. 2008b). Increased binding of OH-PBDEs to estrogen receptors α and β compared to parent PBDEs was detected in cells stably transfected with an estrogen-responsive luciferase reporter gene (Meerts et al. 2001). Agonism on the estrogen receptor has also been detected of (*in vivo* generated) hydroxylated metabolites of DE-71 in similar *in vitro* test systems (Harju et al. 2007; Kojima et al. 2009; Mercado-Feliciano and Bigsby 2008b). In addition, antagonistic effects have been observed for several parent, OH- and/or MeO-PBDEs on the estrogen, progesterone, androgen and glucocorticoid receptors (Hamers et al. 2006; Harju et al. 2007; Kojima et al. 2009). OH-PBDEs were also shown to alter the expression of genes involved in steroidogenesis much more strongly than their methoxylated analogues (Song et al. 2008).

Because of structural resemblance of OH-PBDEs to the thyroid hormone T_4, binding to thyroid receptors α and β and thyroid hormone transport protein (TTR) has been investigated and confirmed (Kojima et al. 2009; Hamers et al. 2006; Meerts et al. 2000).

More recently, an additional toxic effect of OH-PBDEs was discovered. In zebrafish exposed during development, 6-OH-BDE-47 disrupted oxidative phosphorylation, resulting in developmental delays and defects. This effect was not observed when the zebrafish were exposed to BDE-47 or its methoxylated analogue (van Boxtel et al. 2008).

1.5 Environmental and human exposure to HBCD

Much like PBDEs, HBCD is dispersed widely throughout the environment (reviewed in Covaci et al. 2006; Law et al. 2008b). Early studies in Sweden (Remberger et al. 2004) showed that HBCD was present in all investigated matrices (environmental samples, biota and food), despite the fact that in Europe HBCD is only produced in the Netherlands (BSEF 2009d). HBCD has already been detected in the arctic region, suggesting long-range oceanic and/or atmospheric transport similar to other persistent organic pollutants (de Wit et al. 2006; Gebbink et al. 2008a; Verreault et al. 2007). Toxicokinetics studies in rodents and fish showed that HBCD accumulates via the diet and is distributed throughout the body (Brandsma et al. 2009; Haukås et al. 2009b; Law et al. 2006c; Nyholm et al. 2009). It is a lipophilic compound that is slowly metabolized and therefore accumulates in lipid-rich tissues such as liver, gonads and adipose tissue (Janák et al. 2005; Peck et al. 2008; Xian et al. 2008). Like PBDEs, HBCD bioaccumulates in terrestrial, arctic and aquatic food webs (Jenssen et al. 2007; Letcher et al. 2009; Sørmo et al. 2006; Tomy et al. 2004a; reviewed in Covaci et al. 2006). Increasing levels of HBCD have been observed in the last two decades in seabird eggs (Helgason et al. 2009).

After cessation of the use of penta- and octaBDEs in consumer products, a sharp rise in levels of HBCD in marine mammals that were stranded on the UK coast was observed. This

increase is probably due to an increased use of HBCD as a replacement of PBDEs, mainly in polystyrene products (Law et al. 2006b; reviewed in Law et al. 2008b). Since 2005, these levels have stabilized, possibly due to improved reduction of emission (Law et al. 2008a). This 'leveling off' situation has also been observed in marine mammals stranded on the US East coast (Peck et al. 2008). However, regional and temporal differences have to be kept in mind as in China and Japan, increasing levels of HBCD in marine mammals have recently been observed (Lam et al. 2009; Tanabe 2008).

The routes of human exposure to HBCD also resemble those of PBDEs, although exposure appears to be mainly linked to fish consumption (Thomsen et al. 2008; van Leeuwen and de Boer 2008). HBCD as well as its metabolite pentabromocyclododecene have been detected in e.g. chicken eggs for human consumption (Hiebl and Vetter 2007) and indoor air and dust (Abdallah et al. 2008; Saito et al. 2007; reviewed in Covaci et al. 2006).

A recent exposure study suggests that, in contrast to PBDEs, HBCD levels in human adipose tissues in the US are lower compared to those in Europe (Johnson-Restrepo et al. 2008). Occupational internal exposure to HBCD, higher compared to the general population, has been detected in employees at a production site of polystyrene in Norway (Thomsen et al. 2007).

HBCD can also be transferred across the placenta to the fetus (Meijer et al. 2008) and is present in human breast milk (Eljarrat et al. 2009; Shi et al. 2009). Investigation of temporal trends revealed a stable situation regarding HBCD levels in Swedish human breast milk (Fängström et al. 2008), although in Japan, levels appear to increase (Kakimoto et al. 2008).

For the risk assessment of HBCD, it should be taken into account that although the technical mixture predominantly contains γ-HBCD, biotic samples generally contain α-HBCD as a major diastereoisomer (Haukås et al. 2009a; reviewed in Covaci et al. 2006). Evidence exists for several possible underlying mechanisms. Firstly, α-, β- and γ-HBCD have different solubilities, respectively 48.8, 14.7 and 2.1 µg/l (reviewed in Covaci et al. 2006). Also, isomer composition changes are expected during heating of the technical mixture (Heeb et al. 2008; Köppen et al. 2008). Moreover, CYP-mediated biotransformation of β- and γ-HBCD has been observed in rat and harbor seal microsomes (Zegers et al. 2005) and rainbow trout (Law et al. 2006c), but much less for α-HBCD. Finally, selective uptake of α- and β-HBCD over γ-HBCD was observed in a rainbow trout disposition study (Haukås et al. 2009b). Some or all of these aspects are involved in the selective bioaccumulation of α-HBCD in an Arctic food web, in contrast to trophic dilution of γ-HBCD (Tomy et al. 2008).

1.6 Toxicity of HBCD

As for PBDEs, acute toxic effects of HBCD are limited, if not virtually absent (reviewed in Darnerud 2003).

Effects of HBCD on hepatic gene expression and hepatic enzymes have been observed in rats (Cantón et al. 2008a; Germer et al. 2006), fish (Ronisz et al. 2004; Zhang et al. 2008c) and in rat hepatocytes and hepatoma cell lines (Fery et al. 2009). Alterations in hepatic enzyme activity and thyroid hormone levels were detected in HBCD-exposed rainbow trout, showing higher activity of γ-HBCD compared to α- and β-HBCD to affect the thyroid system (Palace et al. 2008). HBCD-induced developmental toxicity and apoptosis in zebrafish embryos was shown to be related to oxidative stress (Deng et al. 2009; Hu et al. 2009).

Endocrine effects have also been detected for HBCD in wild birds. Eggshell thinning and reduced reproduction were observed in wild kestrels (Fernie et al. 2009). In reproductive studies with rats, decreased testis weight, aromatase induction in ovaries (mainly associated with γ-HBCD) and a decreased number of primordial follicles in ovaries were observed (Ema et al. 2008; van der Ven et al. 2009). *In vitro*, agonistic and antagonistic effects by binding to the estrogen, progesterone and thyroid hormone receptors were detected (Hamers et al. 2006). A subchronic *in vivo* study in rats revealed effects of HBCD on thyroid gland and thyroid hormone levels (van der Ven et al. 2006). Because of the importance of thyroid hormones in the development of the auditory systems, effects of HBCD on auditory function were further investigated and confirmed in offspring of exposed rats (Lilienthal et al. 2009). After exposure of rats from gestation through lactation to HBCD, decreased thyroid hormone levels and changes in thyroid size and histology were observed. Possibly related to the observed hypothyroidism, a reduction in oligodendrocyte density was observed in the hippocampal CA1 region (Saegusa et al. 2009).

Similar neurobehavioral effects as observed for PBDEs were detected in mice after neonatal exposure to HBCD. Mice neonatally exposed to HBCD showed hyperactivity and reduced habituation and, at a higher dose level, impairment of performance in the Morris water maze (Eriksson et al. 2006b). In offspring of rats exposed to HBCD, the response to the dopamine receptor blocker haloperidol was also changed, suggesting effects on the dopaminergic neurotransmitter system (Lilienthal et al. 2009).

In addition, HBCD induced apoptotic cell death in primary rat cerebellar granule cells (Reistad et al. 2006), but effects on intracellular Ca^{2+} levels or ROS formation were not observed in this study. At lower concentrations than DE-71, HBCD inhibits re-uptake of neurotransmitter in synaptosomes and neurotransmitter vesicles (Mariussen and Fonnum 2003).

1.7 Thesis outline

The abovementioned research clearly indicates a neurotoxic potential of PBDEs and HBCD. However, (causal) relations between the observed *in vivo* and *in vitro* effects have not yet been resolved. Therefore, the aim of this PhD project was to gain insight in the mechanisms involved in neurobehavioral effects observed after exposure to PBDEs and HBCD during brain development (as discussed in §1.3 and §1.6). Firstly, possible effects on synaptic plasticity after exposure to BDE-47 were investigated *ex vivo*. Secondly, the main part of this thesis describes acute presynaptic *in vitro* effects of these compounds. Toxic endpoints investigated for PBDEs and HBCDs, as well as applied experimental methodologies, are discussed in **chapter 2**.

The first aim of this research was to investigate whether the observed neurobehavioral effects of BDE-47 would be reflected in changes in neural transmission in a brain region known to be involved in learning and memory, the hippocampus (see also §2.3). In **chapter 3**, the effects of BDE-47 on synaptic plasticity in hippocampal slices are described.

Mice were exposed to BDE-47 via an exposure regiment (a single oral dose of 6.8 mg/kg bw BDE-47 at PND 10) known to result in behavioral effects as described by Eriksson et al. (2001b). Effects of postnatal exposure to BDE-47 on synaptic plasticity were investigated *ex vivo* by measuring *N*-methyl-D-aspartic acid (NMDA) dependent long-term potentiation (LTP) of field-excitatory postsynaptic potentials (*f*-EPSPs) in hippocampal slices (see also §2.4). LTP is used as a neurophysiological model for learning and memory (see also §2.3). The timing of exposure and measurements was based on the timeframe of neonatal rapid brain growth (‘brain growth spurt’) that peaks (in rodents) around PND 10. During the brain growth spurt, rapid axonal and dendritic growth, glial proliferation and myelination take place (Davison and Dobbing 1968). Hippocampal slices were prepared for *f*-EPSP measurements (see §2.2 - 2.4) at PND 17 - 19, just prior to the end of the brain growth spurt.

Possible effects on a set of selected postsynaptic density (PSD) proteins involved in hippocampal LTP (see §2.3) were investigated by Western Blotting in hippocampal tissue from the animals that were also used for the LTP-recordings. The investigated proteins included PSD scaffolding proteins PSD-95 and SAP97 (a protein involved in α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate, AMPA, receptor trafficking), phosphorylated-active and unphosphorylated αCaMKII, AMPA receptor subunit GluR1 and NMDA receptor subunits NR1, NR2A and NR2B.

Possible presynaptic effects after postnatal exposure to BDE-47 were investigated by measuring paired-pulse facilitation (PPF; see also §2.3) in hippocampal slices in which LTP was also measured. Since the main presynaptic neuronal function is release of neurotransmitter (see also §2.8), basal and depolarization-evoked catecholamine release was measured from chromaffin cells (see §2.2 and §2.9) isolated from animals exposed to vehicle or 68 mg/kg bw BDE-47.

As in other studies presynaptic effects of PBDEs and HBCD were described (see §1.3 and §1.6), acute presynaptic effects of these BFRs were also investigated in this research. As proper neurotransmission critically depends on strict regulation of Ca^{2+} homeostasis and neurotransmitter release (see also §2.6 and §2.8), acute effects of PBDEs and HBCD on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and closely associated vesicular neurotransmitter release were investigated in PC12 cells and neurospheres of neural progenitor cells (**chapters 3 - 8)**. In all studies, possible confounding due to cytotoxicity was investigated by measuring cell viability (see §2.5). $[Ca^{2+}]_i$ was measured using the Ca^{2+}-sensitive fluorescent dye Fura-2 (see §2.7). Release of catecholaminergic neurotransmitter was measured using amperometry (see §2.9).

Endocrine studies investigating interactions with hormone receptor systems (mainly estrogen and thyroid hormone receptors) revealed a higher potency of OH-PBDEs compared to parent and methoxylated PBDEs (Cantón et al. 2005, 2006, 2008b; Harju et al. 2007; Kojima et al. 2009; Meerts et al. 2001; Mercado-Feliciano and Bigsby 2008b; Song et al. 2008). Another, recently discovered toxic effect is disruption of oxidative phosphorylation that is only induced by 6-OH-BDE-47 but not by parent congener BDE-47 or the methoxylated analogue (van Boxtel et al. 2008). To investigate whether hydroxylated metabolites play a role in neurotoxicity of PBDEs, the effects of parent BDE-47 and a major hydroxylated metabolite (6-OH-BDE-47) were compared **(chapter 4)**.

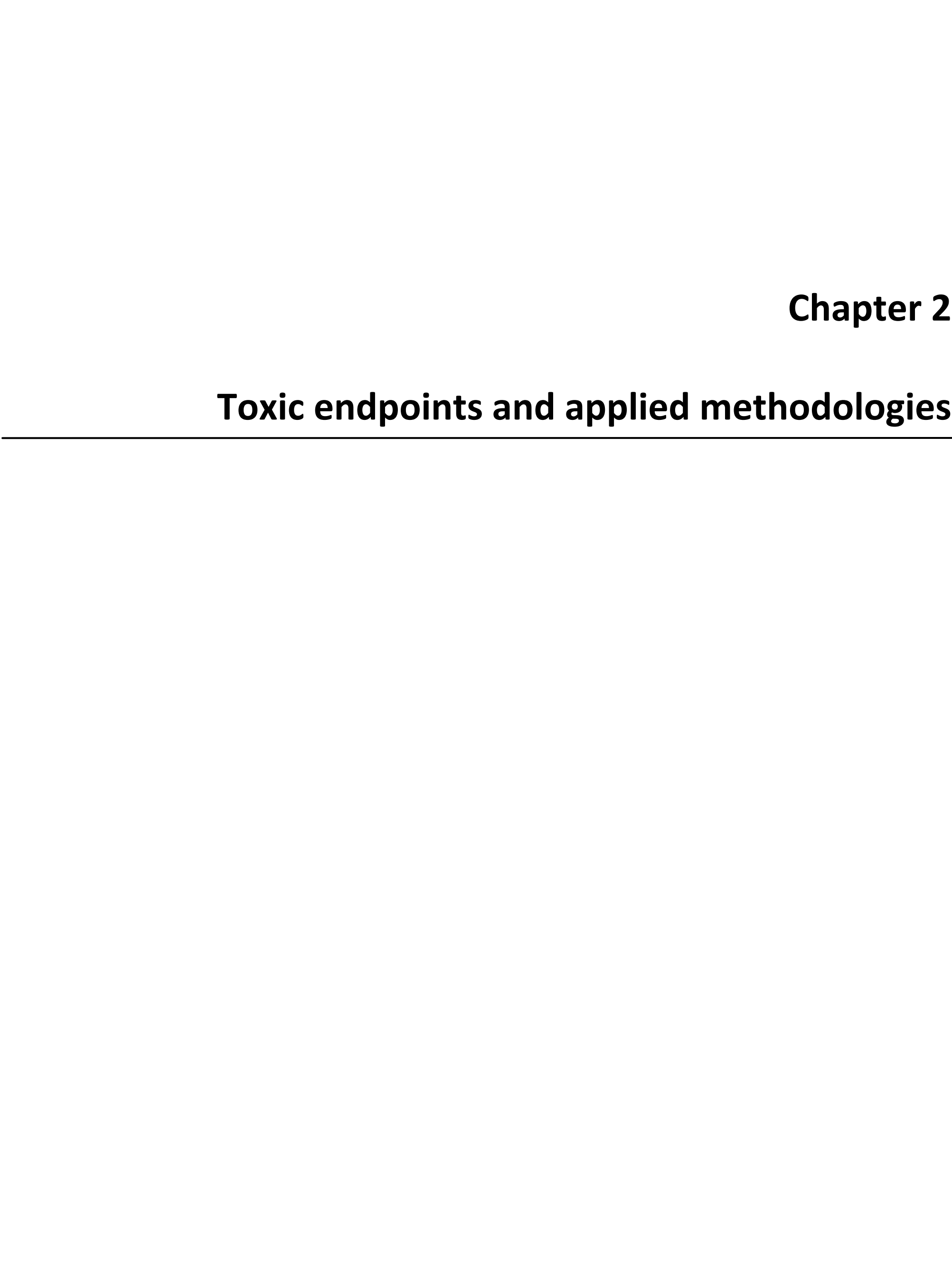
To investigate whether the observed higher activity of OH-PBDEs could be predicted by its structure, the effects on basal $[Ca^{2+}]_i$ were investigated for a set of mono-hydroxylated metabolites of BDE-47 with variations in hydroxylation and bromination pattern **(chapter 5)**.

Voltage-gated Ca^{2+} channels (VGCCs) are important for maintaining Ca^{2+} homeostasis and neuronal signaling (García et al. 2006). Therefore, the effects of the hydroxylated metabolites on depolarization-evoked $[Ca^{2+}]_i$ increase were also further investigated **(chapter 6)**.

Behavioral studies have revealed that exposure to PBDEs has to occur during brain development to induce behavioral neurotoxicity (Eriksson et al. 2002). Effects of PCBs and PBDEs on proliferation, differentiation and migration have previously been investigated in human neural progenitor cells, showing effects primarily on migration and differentiation of the progenitor cells in neuron-, astrocyte- and oligodendrocyte-like cells (Fritsche et al. 2005; Schreiber et al. 2009). To investigate whether (transient) effects on $[Ca^{2+}]_i$ could play a role in the observed effects on neurodevelopment, acute effects of BDE-47 and 6-OH-BDE-47 on $[Ca^{2+}]_i$ were investigated in these cells **(chapter 7)**.

Since the phasing out of commercial PBDE products, HBCD has been suggested as a possible replacement BFR for a number of common applications, although this has not been confirmed by involved industry. Possible effects of the commercial HBCD mixture and individual HBCD diastereoisomers on $[Ca^{2+}]_i$ were investigated **(chapter 8)**.

In the general discussion **(chapter 9)**, the observed effects described in chapters 3 - 8 are placed into context with proposed mechanisms of action, relevant human exposure and toxicity data from other researchers. A risk assessment of possible neurotoxicity in humans induced by (OH-)PBDEs and HBCD is also included. Additional issues concerning sensitive subgroups, data gaps and recommendations for future investigations are discussed in **chapter 10**. The conclusions from this PhD-research are summarized in **chapter 11**.



Chapter 2

Toxic endpoints and applied methodologies

2.1 PBDEs and HBCD

The individual polybrominated diphenyl ethers (PBDEs), their metabolites, commercial hexabromocyclododecane (HBCD) and HBCD diastereoisomers used in this research project (Table 2.1) were synthesized as described by Marsh et al. (1999) and Fång (2007) and purified (to approximately 99%) at the Department of Environmental Chemistry of Stockholm University. It has to be taken into account that (commercial mixtures of) PBDEs also contain trace amounts of polybrominated dibenzofurans and dibenzo-*p*-dioxins (Hanari et al. 2006), that cause aryl hydrocarbon (Ah) receptor-mediated toxicity (reviewed in Birnbaum et al. 2003). Therefore, potentially present polybrominated dibenzofurans or polybrominated dibenzo-*p*-dioxins were removed from the PBDEs by elution through a charcoal column (Örn et al. 1996).

Stock solutions of PBDEs and HBCDs were made in dimethylsulfoxide (DMSO) and stored cold and protected from light, because these compounds are subject to photolysis (Kajiwara et al. 2008; Söderstrom et al. 2004).

Table 2.1. Brominated flame retardants investigated in this research.

parent PBDEs	BDE-47	2,2’,4,4’-tetrabromodiphenyl ether
	BDE-49	2,2’,4,5’-tetrabromodiphenyl ether
	BDE-99	2,2’,4,4’,5-pentabromodiphenyl ether
	BDE-100	2,2’,4,4’,6-pentabromodiphenyl ether
	BDE-153	2,2’,4,4’,5,5’-hexabromodiphenyl ether
hydroxylated/ methoxylated PBDEs	6-MeO-BDE-47	6-methoxy-2,2’,4,4’-tetrabromodiphenyl ether
	6-OH-BDE-47	6-hydroxy-2,2’,4,4’-tetrabromodiphenyl ether
	6’-OH-BDE-49	6’-hydroxy-2,2’,4,5’-tetrabromodiphenyl ether
	5-OH-BDE-47	5-hydroxy-2,2’,4,4’-tetrabromodiphenyl ether
	3-OH-BDE-47	3-hydroxy-2,2’,4,4’-tetrabromodiphenyl ether
	4’-OH-BDE-49	4’-hydroxy-2,2’,4,5’-tetrabromodiphenyl ether
HBCDs	HBCD (commercial product)	1,2,5,6,9,10-hexabromocyclododecane
	α-HBCD	diastereoisomers
	β-HBCD	
	γ-HBCD	

2.2 *Ex vivo* and *in vitro* test systems used in this research

Hippocampal slices (for more details: chapter 3). The hippocampal brain region has since long (from early clinical observations) been known to be involved in learning and memory (reviewed in Blundon and Zakharenko 2008). Also, the hippocampus is involved in spatial

learning (reviewed in Lynch 2004). As behavioral effects were observed after exposure to PBDEs, particularly on spontaneous motor activity and habituation to a new environment (Eriksson et al. 2001b), hippocampus was considered to be a probable target region. Also, synaptic plasticity in the form of *N*-methyl-D-aspartic acid (NMDA)-dependent long-term potentiation (LTP) is commonly measured in hippocampal slices (although LTP occurs in most brain regions).

Mouse hippocampal slices were processed as described previously by van der Heide et al. (2005) with minor modifications. After inhalation anesthesia (isoflurane), mice were quickly decapitated and the brain rapidly dissected on ice. Transverse hippocampal slices (450 µm) were cut in ice-cold carbogenated artificial cerebrospinal fluid (ACSF) using a vibrotome. From one animal, approximately 6 slices could be processed. After cutting, the slices were incubated at room temperature in carbogenated ACSF to stabilize for at least 1.5 h.

Primary chromaffin cells (for more details: chapter 3). To investigate possible effects on vesicular neurotransmitter release after exposure to BDE-47, adrenal chromaffin cells were isolated as described previously by Westerink et al. (2006). Briefly, chromaffin cells were isolated by digesting mouse adrenal glands using digestive enzymes.

Rat pheochromocytoma (PC12) cells (for more details: chapter 4). To investigate effects on intracellular calcium concentration ($[Ca^{2+}]_i$) and neurotransmitter release on a single-vesicle level, the rat pheochromocytoma (PC12) cell line was used. This cell line was isolated from a rat adrenal pheochromocytoma (Greene and Tischler 1976). Especially after differentiation with dexamethasone (Schubert et al. 1980), PC12 cells excrete considerable amounts of catecholaminergic neurotransmitter, which can be detected by amperometry (Westerink and Vijverberg 2002). Although PC12 cells are chromaffin-derived cells, the balance of processes involved in regulation of Ca^{2+} homeostasis in undifferentiated PC12 cells resembles more that of sympathetic neurons (Duman et al. 2008). Therefore, (undifferentiated) PC12 cells can be used as an *in vitro* model to study neurotoxicity (Westerink and Ewing 2008).

Neural human progenitor cells (for more details: chapter 7). Rodent studies revealed that effects of PBDEs on behavior are induced during a defined period of brain development (Eriksson et al. 2002). This indicates that acute effects of PBDEs during brain development can cause persistent alterations in the brain. To reduce the amount of animals to be used in developmental neurotoxicity testing, an *in vitro* model for neurodevelopment has been developed in the Institut für umweltmedizinische Forschung (IUF) of the Heinrich-Heine-Universität Düsseldorf (Fritsche et al. 2005). Primary human neural progenitor cells are cultured in the form of neurospheres. Upon withdrawal of growth factors, the cells differentiate and migrate.

Effects of PBDEs on cell proliferation, differentiation and migration have been investigated in this system (Schreiber et al. 2009). In collaboration with the IUF, we have investigated whether effects on $[Ca^{2+}]_i$ could be involved in the observed effects of PBDEs.

2.3 Synaptic plasticity in hippocampal CA1 region

The stimulation protocol used for LTP-induction in this research is known to induce NMDA-dependent LTP, first recognized by Bliss and Lømo (1973). NMDA-dependent LTP, which involves both pre- and postsynaptic processes, is used as an neurophysiological model for learning and memory (reviewed in Blundon and Zakharenko 2008; Lynch 2004). This form of LTP is induced by tetanic stimulation, resulting in strong depolarization and a large increase in $[Ca^{2+}]_i$ (reviewed in Malenka and Nicoll 1999; Soderling and Derkach 2000). In the postsynaptic density (PSD) protein scaffolding complex, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and NMDA glutamate receptors are located, as well as modulators of synaptic transmission. Modulators are mainly kinases, but also scaffolding proteins such as PSD-95 have been shown to interact with NMDA receptors (Gardoni et al. 2006b). NMDA receptors are usually blocked by Mg^{2+}, which is removed by strong postsynaptic depolarization, after which these ion channels become permeable to Na^{2+} and Ca^{2+}. This activation of NMDA receptors initiates LTP, while expression and maintenance of LTP are mediated primarily by AMPA receptors and Ca^{2+}/calmodulin kinase II (CaMKII) activity (reviewed in Soderling and Derkach 2000). After activation by binding of Ca^{2+} and calmodulin, CaMKII is autophosphorylated, even after Ca^{2+} levels have returned to basal. Autophosphorylated CaMKII potentiates the postsynaptic current by phosphorylation of the AMPA GluR1 subunit (reviewed in Soderling and Derkach 2000). Activated kinase activity also results in long-lasting recruitment of additional AMPA receptor to the postsynaptic membrane (reviewed in Lynch 2004).

Paired-pulse facilitation (PPF) is a short-term form of synaptic plasticity, mainly driven by presynaptic accumulation of Ca^{2+} (reviewed in Blundon and Zakharenko 2008). PPF has been measured in this research to identify possible effects on presynaptic processes involved in LTP.

2.4 *f*-EPSP-recordings in hippocampal slices to measure LTP and PPF (for more details: chapter 3)

A field-excitatory postsynaptic potential (*f*-EPSP) is the combined electrical postsynaptic depolarization response upon stimulation measured from a population ('field') of neurons in close vicinity of the measuring electrode (Purves et al. 1997).

f-EPSPs were measured in the CA1 region in hippocampal slices as described previously (van der Heide et al. 2005) with minor modifications. Hippocampal slices were placed in a recording chamber at 30°C, where they were superfused with carbogenated ACSF. The afferent fibers of the stratum radiatum of the hippocampal CA1 region were stimulated by a bipolar stainless steel electrode (diameter: 0.1 mm). *f*-EPSPs were recorded with ACSF-filled glass microelectrodes connected to an Axoclamp-2B amplifier. Half-maximum *f*-EPSPs were evoked every 30 s for investigation of LTP, which was evoked after a baseline recording by a single tetanic stimulation. The slope of *f*-EPSPs during the post-tetanic potentiation (caused by presynaptic Ca^{2+} accumulation) and after stabilization of LTP were analyzed as a measure for synaptic plasticity. PPF in *f*-EPSPs evoked with a short interval was determined by dividing the slope of the second evoked *f*-EPSP with the slope of the first.

2.5 Cell viability tests (for more details: chapters 4, 5 and 8)

Effects on cell viability were investigated in the used *in vitro* systems, to investigate possible confounding on the observed effects of brominated flame retardants (BFRs) by cytotoxicity. Cell viability has been assessed by measuring cell density, and by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and a combination of Alamar Blue (AB) and Neutral Red (NR) assays.

Cell density was determined merely by measuring the proportion of the surface of the cell culture dish occupied by living PC12 cells (identified by trypan blue exclusion) after exposure to BFRs.

The MTT assay is based on the conversion of MTT to the blue-colored formazan (Denizot and Lang 1986). After exposure to BFRs in multi-wells plates, cells were incubated with MTT, after which the formazan was extracted by lysing the cells. The concentration of formazan formed, recorded photospectrometrically, is a measure for the amount of cells.

Also, a combination of AB and NR uptake assays was used (Magnani and Bettini 2000; Repetto et al. 2008). After exposure to BFRs in multi-wells plates, cells were incubated with AB. When processed by mitochondria, AB becomes fluorescent. The fluorescence was recorded as a measure of cell viability. Subsequently, the cells were incubated in NR, after which the NR was extracted. The concentration of NR, which is taken up passively in lysosomes, was also used as a measure of cell viability.

Because effects on mitochondria were detected for hydroxylated PBDEs, assays based on mitochondrial activity, such as MTT and AB assays, are less useful for detecting effects on cell viability. Therefore, passive assays such as determination of cell density of NR uptake are preferable.

2.6 Ca^{2+} homeostasis

Ca^{2+} has been shown to be involved in various cellular (muscle contraction, fertilization, cell motility, cell adhesion, action potential propagation, exocytosis and cell death) as well as subcellular processes (mitochondrial function, protein translocation, gene transcription). Maintenance of Ca^{2+} homeostasis by buffering, compartmentalization and extrusion mechanisms is essential for cell viability, as high concentrations of Ca^{2+} are associated with oxidative stress and could even precipitate with phosphates (reviewed in Clapham 2007). Compartmentalization involves mainly sarcoendoplasmic reticular Ca^{2+}/ATPases (SERCA pumps) but also the mitochondrial Ca^{2+} uniporter. Extrusion occurs mainly via high-affinity, low-capacity plasma membrane Ca^{2+}/ATPases (PMCA pumps) and low-affinity, high-capacity Na^{+}/Ca^{2+} exchangers (NCXs; Duman et al. 2008). Release of Ca^{2+} from endoplasmic reticulum induces replenishing mechanisms: store-operated Ca^{2+} entry (Parekh and Putney 2005).

In neuronal cells, Ca^{2+} is also the major trigger of vesicular neurotransmitter release through activation of the exocytotic release machinery (Barclay et al. 2005). When neuronal communication is required, rapid increases in Ca^{2+} are achieved by the opening of voltage-gated Ca^{2+} channels (VGCCs) by depolarization of the presynaptic membrane. Ca^{2+} influx through ligand-gated ion channels also regulates $[Ca^{2+}]_i$ (Burnashev 1998). Compartmentalization in endoplasmic reticulum (ER) and mitochondria shapes the Ca^{2+} increase and consequently the exocytotic response (reviewed in García et al. 2006). Subtypes of VGCCs with differences in activation, inactivation, voltage range and conductance are present in different tissues (reviewed in García et al. 2006). In PC12 cells, L-, N- and P/Q-type VGCCs are mainly contributing to the depolarization-evoked influx of Ca^{2+}, although the presence of R- and T-type VGCCs has also been demonstrated (Del Toro et al. 2003; Greene and Tischler 1976; Liu et al. 1996; Shafer and Atchison 1991).

2.7 Intracellular Ca^{2+} imaging with Fura-2 (for more details: chapter 3 and 7)

Average free cytosolic Ca^{2+} levels were determined by measuring Ca^{2+}-dependent fluorescence of the high-affinity Ca^{2+}-responsive fluorescent dye Fura-2 (Grynkiewicz et al. 1985; Molecular Probes, Invitrogen).

Because of the presence of its acetoxymethyl (AM) ester-group, Fura-2-AM is membrane permeable. Cells could therefore be loaded non-invasively with Fura-2-AM by incubation at room temperature in saline containing Fura-2-AM. Subsequently, the Fura-2-AM medium was removed from the cells because Fura-2-AM is fluorescent, but not sensitive to Ca^{2+}. Incubation in saline allowed de-esterification of Fura-2-AM by endogenous enzymes, releasing the Ca^{2+}-sensitive Fura-2 into the cytosol.

It should be taken into account that, as Fura-2 binds Ca^{2+}, it is an additional Ca^{2+} buffer which may influence the $[Ca^{2+}]_i$. When using Fura-2-AM for Ca^{2+} imaging, translocation to

intracellular compartments could also occur (fluorescence can still be measured but is not sensitive to cytosolic free Ca^{2+}). Also, Fura-2 could bind to cellular proteins (reviewed in Takahashi et al. 1999).

A suitable region of cells was selected by using an inverted microscope. Fluorescence was evoked by 340 and 380 nm excitation wavelengths (F_{340} and F_{380}) using a polychromator and collected at 510 nm with a digital camera. Because of the spectral shift upon binding of Ca^{2+}, fluorescence evoked by F_{340} is positively associated with free $[Ca^{2+}]_i$, while fluorescence evoked by F_{380} is negatively associated with free $[Ca^{2+}]_i$. After subtraction of background fluorescence, the F_{340}/F_{380} ratio (R) was calculated as the qualitative measure for intracellular Ca^{2+} levels. A major advantage of these ratiometric measurements is a relative independence of parameters involving amounts of Fura-2; Fura-2 loading, cell volume (thickness), photobleaching and Fura-2 leakage.

From the experimental $[Ca^{2+}]_i$ data, different parameters were investigated. Firstly, average and amplitude of $[Ca^{2+}]_i$ within the entire experiment duration were investigated. When transient processes were observed, amplitudes were determined within defined exposure durations. Moreover, to investigate more subtle effects on Ca^{2+} homeostasis, fluctuations in $[Ca^{2+}]_i$ were analyzed. Fluctuations were defined as periods in which $[Ca^{2+}]_i$ was higher than a defined threshold. Frequencies of fluctuating cells and fluctuation frequency per cell were investigated, as well as the fluctuation amplitude and duration.

When Ca^{2+} increases were observed, the involvement of Ca^{2+} sources was determined by exposing the cells in Ca^{2+}-free medium (to identify the contribution of influx of extracellular Ca^{2+}). To identify involvement of intracellular Ca^{2+} sources, cells were exposed in Ca^{2+}-free medium after pre-exposure to thapsigargin (TG) and/or carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP; to identify involvement of endoplasmic and mitochondrial Ca^{2+} stores). Involvement of specific VGCCs in the inhibition of depolarization-evoked $[Ca^{2+}]_i$ increase was investigated by selective pharmacological blocking using nifedipine (L-type VGCCs), ω-conotoxin GVIA (N-type VGCCs), and ω-conotoxin MVIIC (P/Q-type VGCCs).

The sensitivity of Fura-2 for Ca^{2+} decreases non-linearly with increasing $[Ca^{2+}]_i$. It is therefore important, especially when investigating $[Ca^{2+}]_i$ at the top range of Fura-2 sensitivity (during influx of Ca^{2+} through VGCCs by depolarization), to correct for this non-linear sensitivity of Fura-2 by using Grynkiewicz's equation to calculate $[Ca^{2+}]_i$ (Deitmer and Schild 2000; Grynkiewicz et al. 1985):

$$[Ca^{2+}]_i = K_{d*} \times (R - R_{min}) / (R_{max} - R).$$

In this equation, K_{d*} is the dissociation constant of Fura-2 determined for the experimental set-up. Minimum and maximum ratios (R_{min} and R_{max}) are determined by adding ionomycin (5 µM) and ethylenediaminetetraacetic acid (EDTA; 17 mM), respectively, to the cells at the end of experiments.

2.8 Vesicular neurotransmitter release

Secretory vesicles from chromaffin and PC12 cells are large dense-core vesicles (LDCVs), which are also present in other neuroendocrine cells and sympathetic neurons (Winkler 1993). PC12 cells also accumulate acetylcholine (ACh) in vesicles which are morphologically and biochemically similar to small synaptic vesicles which can be found throughout the brain (Schubert and Klier 1977). In LDCVs, a pH gradient, maintained by a specific H^+-ATPase, is used by the vesicular monoamine transporter (VMAT) to accumulate catecholamines (Masson et al. 1999). These vesicles contain catecholamines, as well as adenosine triphosphate (ATP), ascorbate and a dense matrix formed by peptides and chromogranins (reviewed in Machado et al. 2009). Vesicular Ca^{2+} accumulation occurs mainly via a SERCA-type Ca^{2+}-ATPase, but also via the H^+/Ca^{2+} antiport (Santodomingo et al. 2008). In this study, vesicular neurotransmitter release was measured by using amperometry, meaning that only vesicular release of the oxidizable neurotransmitters from LDCVs is measured.

Neurotransmitter-loaded vesicles are docked to plasma membrane release sites, after which they are prepared (‘primed’) for fusion with the plasma membrane (reviewed in Becherer and Rettig 2006). The increased presence of Ca^{2+} is translated to the exocytotic release machinery by binding to the Ca^{2+} sensor protein synaptotagmin (reviewed in Chapman 2008; Xu et al. 2007). The binding of Ca^{2+} induces the interaction of synaptotagmins with the phospholipids in the cell membrane and the exocytotic release machinery, which is required for fusion.

The exocytotic release machinery is composed of soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNARE) proteins, combined with regulatory proteins (Südhof 2008). SNARE proteins are membrane proteins present on opposing membranes that may fuse; the vesicular and cell plasma membranes. Formation of a complex of SNARE proteins on opposing membranes connects vesicles with the cell membrane (Hanson et al. 1997). The very rapid exocytotic response to increases in Ca^{2+} is mediated by complexin proteins. In the absence of Ca^{2+}, complexin proteins bind to the SNARE protein complex, thereby creating an activated but stationary state. When Ca^{2+} levels increase, Ca^{2+}-activated synaptotagmins couple phospholipids with the SNARE proteins and displace the complexin ‘lock’, thereby rapidly forming and opening the fusion pore (reviewed in Chapman 2008; Südhof 2008). After fusion, vesicles are retrieved into the cell membrane by endocytosis, after which they can be reloaded with neurotransmitters (Royle and Lagnado 2003).

2.9 Detection of catecholamines by amperometry (for more details: chapter 3 and 7)

Basal and depolarization-evoked catecholamine release was measured using carbon fiber microelectrode amperometry from isolated chromaffin cells and dexamethasone-differentiated PC12 cells as described previously (Westerink et al. 2000).

Amperometric recordings were performed with KCl-filled carbon fiber microelectrodes at room temperature under continuous superfusion. The carbon fiber (at 700 mV) was placed gently on the cell surface. Released catecholamine molecules are rapidly oxidized on the surface of the carbon fiber. The electrons liberated from the catecholamine molecules by oxidation (2 electrons per catecholamine molecule) form the measurable current, which was measured and amplified using an EPC-7 patch clamp amplifier. The association of exocytosis and vesicular neurotransmitter release was previously demonstrated by simultaneous capacitance and amperometry measurements (Albillos et al. 1997).

Because of the large heterogeneity in catecholamine release frequencies (reviewed in Westerink and Ewing 2008), cells were selected for inclusion in data-analysis based on basal and depolarization-evoked release frequencies prior to the experimental exposure to BFRs. Hence, non-responsive or extraordinary cells with high basal release frequency, low depolarization-evoked release frequency or poor recovery to basal frequency after depolarization were excluded from data-analysis.

Amperometric traces were used to investigate effects of BFRs on basal and depolarization-evoked catecholamine release frequencies, as well as on vesicular release parameters of the amperometric release events. Effects of BFRs on amplitude, $t_{1/2}$ (half-width) and time integral (Q, used to calculate vesicular content according to Faraday's Law) were investigated to reveal possible effects on the exocytotic process.

Chapter 3

Neonatal exposure to brominated flame retardant BDE-47 reduces long-term potentiation and postsynaptic protein levels in mouse hippocampus

Milou M.L. Dingemans[1], Geert M.J. Ramakers[2], Fabrizio Gardoni[3], Regina G.D.M. van Kleef[1], Åke Bergman[4], Monica Di Luca[3], Martin van den Berg[1], Remco H.S. Westerink[1] and Henk P.M. Vijverberg[1]

1. Institute for Risk Assessment Sciences, Utrecht University, Utrecht, the Netherlands.
2. Rudolf Magnus Institute of Neuroscience, University Medical Centre, Utrecht, the Netherlands.
3. Department of Pharmacological Sciences and Center of Excellence on Neurodegenerative Diseases, University of Milan, Milan, Italy.
4. Department of Environmental Chemistry, Stockholm University, Stockholm, Sweden.

Environmental Health Perspectives **115**, 865 - 870.

Abstract

Background: Increasing environmental levels of brominated flame retardants raise concern about possible adverse effects, particularly through early developmental exposure.
Objective: The objective of this research was to investigate neurodevelopmental mechanisms underlying previously observed behavioral impairments observed after neonatal exposure to polybrominated diphenyl ethers (PBDEs).

Methods: C57Bl/6 mice received a single oral dose of 2,2´,4,4´-tetrabromodiphenyl ether (BDE-47) on postnatal day (PND) 10 (i.e., during the brain growth spurt). On PND 17 - 19, effects on synaptic plasticity, levels of postsynaptic proteins involved in long-term potentiation (LTP), and vesicular release mechanisms were studied *ex vivo*. We investigated possible acute *in vitro* effects of BDE-47 on vesicular catecholamine release and intracellular Ca^{2+} in rat pheochromocytoma (PC12) cells.

Results: Field-excitatory postsynaptic potential (*f*-EPSP) recordings in the hippocampal CA1 area demonstrated reduced LTP after exposure to 6.8 mg (14 µmol)/kg body weight (bw) BDE-47, whereas paired-pulse facilitation was not affected. Western blotting of proteins in the postsynaptic, triton-insoluble fraction of hippocampal tissue revealed a reduction of glutamate receptor subunits NR2B and GluR1 and autophosphorylated-active Ca^{2+}/calmodulin dependent protein kinase II (αCaMKII), whereas other proteins tested appeared unaffected. Amperometric recordings in chromaffin cells from mice exposed to 68 mg (140 µmol)/kg bw BDE-47 did not reveal changes in catecholamine release parameters. Modest effects on vesicular release and intracellular Ca^{2+} in PC12 cells were seen following acute exposure to 20 µM BDE-47. The combined results suggest a postsynaptic mechanism *in vivo*.

Conclusion: Early neonatal exposure to a single high dose of BDE-47 causes a reduction of LTP together with changes in postsynaptic proteins involved in synaptic plasticity in the mouse hippocampus.

Introduction

Fetal and neonatal exposure to neurotoxicants have adverse effects on neurodevelopment. Early (small) effects of xenobiotics on the brain could aggravate these effects during development, creating a critical window for neurotoxicity. However, the underlying mechanisms are not well understood (Szpir 2006). Recently, a range of behavioral and neurochemical effects have been described for polychlorinated biphenyls (PCBs; reviewed in Fonnum et al. 2006; Mariussen and Fonnum 2006). Nowadays, the increasing concentrations of the structurally related polybrominated diphenyl ethers (PBDEs) in the environment, human food chain, and human tissues (Hites 2004) raise concern about possible neurotoxic effects. In most samples, 2,2´,4,4´-tetrabromodiphenyl ether (BDE-47) is the predominant congener. PBDEs are used as flame retardants in a range of products, including electronic equipment, furniture, construction materials, and textiles.

Of concern is that children, at the age of early brain development, accumulate BDE-47 more rapidly than adults because of their diet (breast-feeding/relatively large intake) and behavior (contact with house-dust; Jones-Otazu et al. 2005). Distribution studies show that developing mice reach higher tissue concentrations of BDE-47 compared with adult mice after identical dosing regimens (Staskal et al. 2006a). Behavioral studies have demonstrated adverse neurodevelopmental effects on learning and memory after neonatal BDE-47 exposure. Habituation capability in mice, studied by scoring spontaneous behavior after placement in a new environment, is reduced and this effect is long-lasting and increases with age (Eriksson et al. 2001b).

Recently, a proteomics approach was used to investigate the effect of a single oral dose of 12 mg (21.2 µmol)/kg body weight (bw) 2,2´,4,4´,5-pentabromodiphenyl ether (BDE-99) on brain protein levels in mice, 24 hr after exposure. Levels of striatal proteins associated with neurodegeneration and neuroplasticity and of hippocampal proteins associated with metabolism and energy production were found to be changed (Alm et al. 2006). It is unclear whether such changes occur after exposure to other congeners, and whether these protein changes have functional consequences.

The main objective of our study was to gain insight in the mechanisms underlying the observed effects of BDE-47 on learning and memory (Eriksson et al. 2001b). To this purpose we have investigated N-methyl-D-aspartate (NMDA)-dependent long-term potentiation (LTP) in hippocampal slices from animals exposed to a dose of BDE-47 known to induce behavioral aberrations. NMDA-dependent LTP has been used as an electrophysiologic substrate for learning and memories for many years. This form of LTP is induced by tetanic stimulation, strong depolarization, and a large increase in intracellular Ca^{2+} level (reviewed in Lynch 2004; Malenka and Nicoll 1999; Soderling and Derkach 2000). Paired pulse facilitation (PPF), a form of short-lasting plasticity that presumably reflects presynaptic function (Xu-Friedman and Regehr 2004), was investigated to reveal possible presynaptic effects of BDE-47. In additional *ex vivo* experiments, we investigated protein expression levels in the postsynaptic density

(PSD) and catecholamine release from chromaffin cells to further reveal underlying mechanisms. Acute effects of BDE-47 on intracellular Ca^{2+} and catecholamine release of PC12 cells have been studied *in vitro* to assess the involvement of transient acute effects on potential presynaptic targets. Our findings provide a functional basis for previously observed neurobehavioral changes (Eriksson et al. 2001b).

Materials and methods

Animals and chemicals. Male C57Bl/6 mice pups (litters culled to 5 pups each) with mother (Harlan, Horst, the Netherlands) were housed in a standard animal facility on a 12-hr light/dark cycle with food and water *ad libitum*. Animals were treated humanely and with regard for alleviation of suffering. All experimental procedures were performed according to Dutch law and approved by the Ethical Committee for Animal Experimentation of Utrecht University. Male C57Bl/6 mice received a single oral dose of vehicle [1:10 (wt/wt) mixture of egg lecithin (Sigma-Aldrich, Zwijndrecht, the Netherlands) and peanut oil (*Oleum arachidis;* Sigma-Aldrich), sonicated with water to obtain a 20% (wt/wt) fat:water emulsion] or 6.8 mg (14 µmol)/kg bw BDE-47 via a metal gastric tube on postnatal day (PND) 10 for field-excitatory postsynaptic potential (*f*-EPSP) recordings and brain protein analysis, or 68 mg (140 µmol)/kg bw BDE-47 for amperometric recordings of chromaffin cells (to investigate presynaptic effects) at PND 17 - 19. Experimental groups consisted of mice from different nests. BDE-47 was synthesized and purified (~99%) at the Wallenberg laboratory of Stockholm University. For oral dosing, BDE-47 was dissolved in the egg lecithin/peanut oil mixture and sonicated with water to obtain a 20% (wt/wt) fat:water emulsion.

Hippocampal slice preparation. On PND 17 - 19 (directly after brain growth spurt), the animals were killed by decapitation after inhalation anesthesia (isoflurane), and the brain was rapidly dissected on ice. Hippocampal slices were prepared as described previously (van der Heide et al. 2005). Briefly, transverse hippocampal slices (450 µm) were cut in ice-cold carbogenated Mg^{2+}-enriched artificial cerebrospinal fluid (ACSF) [containing NaCl (124 mM), KCl (3.3 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (2.6 mM), $CaCl_2$ (2.5 mM), $NaHCO_3$ (20 mM), and glucose (10 mM)] using a Leica VT1000 S vibrotome (Leica Microsystems, Wetzlar, Germany). The slices were allowed to stabilize at room temperature in carbogenated ACSF ($MgSO_4$: 1.3 mM) for at least 1.5 hr.

Extracellular recording of field potentials. We recorded *f*-EPSPs in the CA1 region of hippocampal slices as previously described by van der Heide et al. (2005), with minor modifications. Slices were superfused with carbogenated ACSF (~2 mL/min) in a recording chamber at 30°C. A bipolar stainless steel stimulation electrode (Ø 0.1 mm) was placed on the afferent fibers of the stratum radiatum of the hippocampal CA1 region, as shown in a Nissl-

stained hippocampal slice in Figure 3.1A. *f*-EPSPs were recorded with ACSF-filled glass microelectrodes using an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA, USA). Data were digitized and stored using "Spike2" software (Cambridge Electronic Design, Cambridge, UK). Stimulation intensities for threshold and maximum *f*-EPSPs were determined. Slices with a maximum response amplitude of ≥ 1 mV were included in the experiment. During baseline recording, half-maximum *f*-EPSPs were evoked every 30 s. After 15 min baseline recording, LTP was induced with a single tetanic stimulation (100 Hz, 1 sec) and *f*-EPSPs were recorded for another 30 min. PPF, with interstimulus intervals of 50, 100, 200, 500, and 1000 ms, was recorded under identical conditions as for LTP. For data analysis, we determined initial slopes of the *f*-EPSPs (Figure 3.1B). For quantification of LTP, the slope was normalized against the average *f*-EPSP slope during baseline. Average relative increase of the slope was determined 20 - 30 min after tetanic stimulation as a measure for LTP and 0 - 7.5 min after tetanic stimulation as a measure for posttetanic potentiation (PTP) in the individual animals. To determine PPF, paired-pulse ratio (PPR) was determined by dividing the slope of the second average *f*-EPSP by the slope of the first average *f*-EPSP (n = 10).

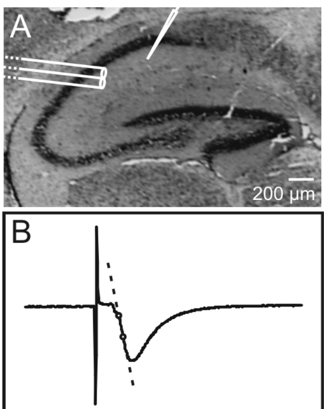


Figure 3.1. *f*-EPSP recordings in the CA1 region of hippocampal slices. A. Placement of bipolar stimulation electrode and ACS*F*-filled microelectrode in CA1 region in a Nissl-stained hippocampal slice; bar = 200 µm. B. Representative *f*-EPSP recording. For data analysis, the initial slope is determined between dots.

Western blotting analysis. We performed Western blotting analysis as described previously by Gardoni et al. (2006a), with minor modifications. The triton-insoluble fraction (TIF) was purified from blind samples of single cortices and hippocampi of control (n = 4) and BDE-47-exposed animals (6.8 mg (14 µmol)/kg bw; n = 4) using a previously validated biochemical fractionating method (Gardoni et al. 2006a), in the presence of protease inhibitors (CompleteTM; Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitors (Sigma,

St. Louis, MO, USA). Similar protein yield was obtained in TIF purified from cortex (~200 µg) and hippocampi (~50 µg) of both groups. Protein composition of this preparation was tested for the absence of presynaptic marker synaptophysin (Gardoni et al. 2001) and enrichment in the PSD proteins (Gardoni et al. 2006a). Samples (3 µg) were applied to SDS-PAGE and electroblotted. For each TIF preparation three independent western blotting experiments were run. After blocking nonspecific protein interactions with 10% albumin in Tris-buffered saline (TBS), the nitrocellulose papers were incubated for 2 hr at room temperature with the primary antibodies: NR1 (1:1000; Pharmingen, San Diego, CA, USA), NR2A (1:1000; Zymed, San Francisco, CA, USA), NR2B (1:1000; Zymed), GluR1 (1:1500; Chemicon, Temecula, CA, USA), PSD-95 (1:2000; Affinity BioReagents, Golden, CO, USA), SAP97 (1:1000; StressGen, San Diego, CA, USA), Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII; 1:3000; Chemicon), and p286-αCaMKII (1:1000; Promega, San Luis Obispo, CA, USA) in 3% albumin in TBS. After extensive rinsing in TBS/0.1% Tween 20, the nitrocellulose papers were incubated with horseradish peroxidase-conjugated secondary antibodies. Finally, the antigen-antibody complex was revealed by enhanced chemiluminescence (ECL; Amersham Biosciences, Little Chalfont, UK). Quantification was performed by means of a Quantity-One computer-assisted imaging system (Bio-Rad, Hercules, CA, USA).

Intracellular Ca^{2+} imaging. We investigated acute effects of BDE-47 *in vitro* in PC12 cells. PC12 cells were subcultured in poly-L-lysine-coated glass-bottom cell culture dishes (MatTek, Ashland MA, USA) at 37°C, 5% CO_2 as described previously (Westerink et al. 2000). We used the high-affinity Ca^{2+}-responsive fluorescent dye Fura-2-AM (Molecular Probes; Invitrogen, Breda, the Netherlands) to measure the intracellular Ca^{2+} concentration. PC12 cells were incubated with Fura-2-AM (5 µM, 20 min at room temperature) in saline containing $CaCl_2$ (1.8 mM), glucose (24 mM), Hepes (10 mM), KCl (5.5 mM), $MgCl_2$ (0.8 mM), NaCl (125 mM), and sucrose (36.5 mM) at pH 7.3 (adjusted with NaOH). After incubation, the cells were washed with saline and left at room temperature for 15 min to allow intracellular de-esterification of Fura-2-AM. After de-esterification, the cells were placed on the stage of an Axiovert 35M inverted microscope (Zeiss, Göttingen, Germany) equipped with a TILL Photonics Polychrome IV (TILL Photonics GmBH, Gräfelfing, Germany). Fluorescence evoked by 340 and 380 nm excitation wavelengths (F_{340} and F_{380}) was collected at 510 nm with an Image SensiCam digital camera (TILL Photonics GmBH). The digital camera and polychromator were controlled by imaging software (TILLvisION, version 4.01), which was also used for data collection and processing. The F_{340}/F_{380} ratio, which is a qualitative measure for intracellular Ca^{2+} concentration, was measured every 20 s during baseline. After 5 min baseline recording, BDE-47 was bath-applied to obtain final concentrations of 2 and 20 µM, and ratios were collected every 6 s. Maximum and minimum ratios were determined after 25 min recording by addition of ionomycin (5 µM) and EDTA (17 mM) as a control for experimental conditions.

Amperometry. We measured spontaneous and K^+-evoked catecholamine release using carbon fiber microelectrode amperometry from isolated chromaffin cells and PC12 cells as described previously (Westerink and Vijverberg 2002). Chromaffin cells from mice exposed to vehicle or 68 mg (140 µmol)/kg bw BDE-47 were isolated and cultured as described previously (Westerink et al. 2006). PC12 cells were superfused with BDE-47 for 15 min to investigate acute effects on vesicular catecholamine release. Recordings were performed at room temperature. PC12 cells with high basal release (> 5 events/min) or low evoked release (< 16 events/min) were excluded for data analysis (3/25 cells).

Statistical analysis. All data are presented as mean ± SE. PC12 data were compared using Student's paired *t*-test. We first compared the LTP data using a two-way analysis of variance (ANOVA) with *post hoc* Bonferroni testing (Sigmastat software; Systat Software Inc, Erkrath, Germany), followed by additional unpaired *t*-tests to specify the effects on PTP and LTP. We used unpaired Students' *t*-test for all other data.

Results

Pups exposed to BDE-47 did not differ in body weight and relative thymus weight compared with their unexposed littermates (data not shown), indicating the absence of general toxicity, treatment-dependent food competition, extensive immune suppression, and stress. Additionally, visual inspection of the brain slices of exposed pups did not show any changes of general hippocampus morphology (data not shown).

Figure 3.2 shows the results from *f*-EPSP recordings in the CA1 region of mouse hippocampus for control and BDE-47-exposed groups. No differences in stimulus-response relation were seen. No effects were observed on half-maximum *f*-EPSP slopes before LTP induction (control: 682 ± 138 V/sec; BDE-47 exposed animals: 679 ± 92 V/sec).

After tetanic stimulation, an immediate large increase of the *f*-EPSP is apparent, although the increase is significantly lower in the BDE-47-exposed group than in the control group. The increase of the *f*-EPSP during the first 7.5 min post-tetanus is classified as PTP. In the BDE-47-exposed mice, there was significantly less PTP (135 ± 9%) than in the control mice (190 ± 17%; $p < 0.01$; Figure 3.2). After PTP the *f*-EPSP size decreases but stabilizes at a higher level than baseline. This level of LTP is maintained for at least 30 min. In the BDE-47-exposed mice, LTP was significantly lower (130 ± 7%) than in the control group (165 ± 16%; $p < 0.05$). The significance of these findings was confirmed by two-way ANOVA with *post hoc* Bonferroni testing. The trace inset illustrates the enhancement of *f*-EPSPs after tetanic stimulation. The cumulative probability curve of LTP in the individual experiments (Figure 3.2) indicates a shift to lower LTP values in the BDE-47 group.

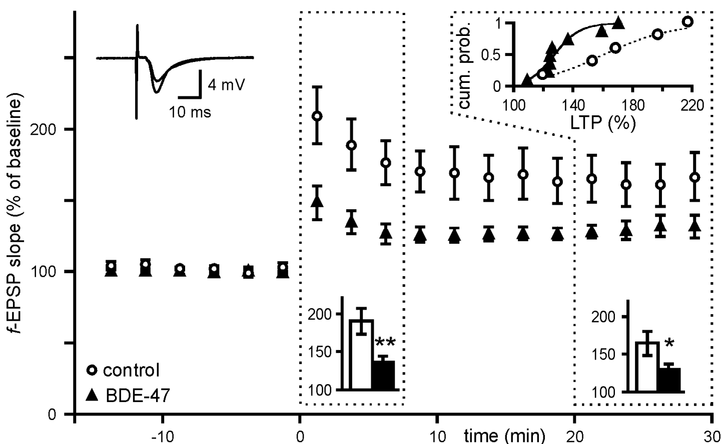


Figure 3.2. Exposure to 6.8 mg (14 µmol)/kg bw BDE-47 reduces PTP (0 - 7.5 min after tetanus at t = 0) and LTP (20 - 30 min) in hippocampal neurons in BDE-47-exposed mice (n = 8) compared with control mice (n = 5). The upper left inset shows superimposed traces illustrating the enhancement of the *f*-EPSP by LTP induction. The bar diagram insets in the dashed frames show averages of data. The upper right inset shows the cumulative probability (cum. prob.) curve of LTP in the individual experiments.
*$p < 0.05$; **$p < 0.01$.

Figure 3.3 shows the effect of BDE-47 on PPF at different interstimulus intervals. For the 50-msec interstimulus interval, the PPR was 1.98 ± 0.11% in the control group and 1.87 ± 0.15% in the BDE-47 group. For the 1000-msec interstimulus interval, the PPR was decreased to 1.16 ± 0.03% in the control group and 1.08 ± 0.03% in the BDE-47 group. Insets show representative recordings of PPF. No effects of BDE-47 on PPR were detected.

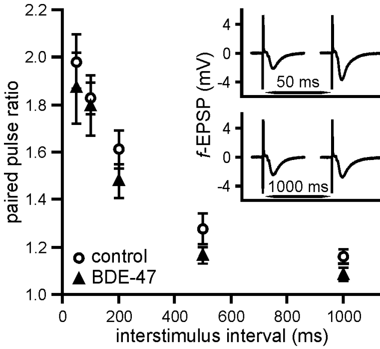


Figure 3.3. Exposure to 6.8 mg (14 µmol)/kg bw BDE-47 has no effects on PPF; PPR was calculated from paired pulses (interstimulus interval 50 - 1000 msec) in control (n = 12) and BDE-47-exposed (n = 12) mice. Inset shows representative *f*-EPSP traces of paired pulses.

Because activation of NMDA receptors is required for LTP, the reduction of LTP in BDE-47-treated mice could reflect an alteration of NMDA receptor-associated signaling elements. Because the NMDA receptor complex is enriched in the PSD, we used Western blot analysis to measure protein levels of NMDA receptor subunits and other PSD-associated signaling proteins in total homogenate and TIF, representing the PSD compartment by Western blot analysis (Gardoni et al. 2001). Protein composition of this preparation was carefully tested for the absence of presynaptic markers and enrichment in PSD proteins (Figure 3.4A; Gardoni et al. 2001). Representative Western blots for all investigated proteins in hippocampal homogenate and TIF are also shown in Figure 3.4B. BDE-47 had no effects on protein levels in cortical homogenate and TIF (data not shown) and hippocampal homogenate (Figure 3.4C). Figure 3.4C shows amounts of the proteins in TIF of the hippocampus of BDE-47-exposed mice compared with control mice. Significant changes in protein levels of NMDA receptor subunits NR1 and NR2A, and NMDA receptor interacting proteins PSD-95 and SAP97 were not detected. However, protein levels of NMDA receptor subunit NR2B (75 ± 2%) and α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) receptor subunit GluR1 (71 ± 4%) were significantly reduced ($p < 0.01$). There was a significant decrease in the autophosphorylated-active form of αCaMKII (p286-αCaMKII) to 65 ± 8% of control level ($p < 0.05$), although total αCaMKII was not changed.

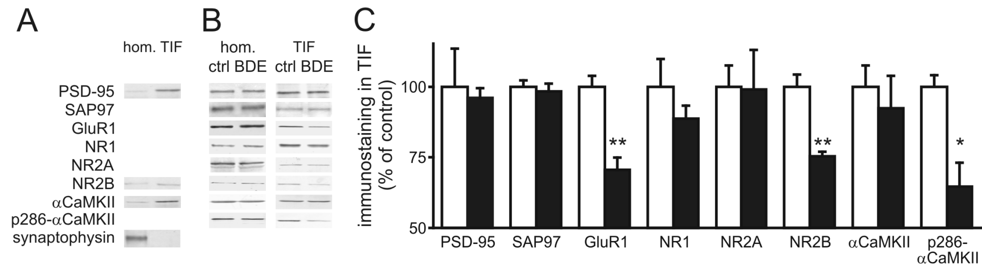


Figure 3.4. Effects of 6.8 mg (14 µmol)/kg bw BDE-47 on levels of postsynaptic proteins in the hippocampus of control (Ctrl; $n = 4$) and BDE-47-exposed ($n = 4$) mice. A. Western blotting for PSD-95, NR2B, αCaMKII, and synaptophysin in homogenate (Hom.) and TIF from hippocampus. B. Representative Western blots of the investigated postsynaptic proteins in hippocampal homogenate (Hom.) and TIF. C. Relative amount of postsynaptic proteins in hippocampal TIF (representing the PSD). $*p < 0.05$. $**p < 0.01$.

In the experiments described above, postsynaptic effects of BDE-47 are observed, whereas presynaptic functional effects are not detected. However, possible effects on presynaptic mechanisms might remain undetected at a dose of 6.8 mg (14 µmol)/kg bw BDE-47. To ascertain the apparent absence of presynaptic effects of BDE-47, we investigated

effects on catecholamine release in chromaffin cells obtained from mice exposed to vehicle or to a higher dose [68 mg (140 µmol)/kg bw] of BDE-47. No changes were detected in the different parameters of vesicular catecholamine release; that is, basal and high-K^+ evoked release frequency and vesicular release parameters like quantal size (vesicle content), spike amplitude, and 50 - 90% rise time (data not shown).

Additional *in vitro* experiments were performed in PC12 cells to investigate acute effects of BDE-47 exposure on calcium homeostasis and release mechanisms. Figure 3.5A shows the average F_{340}/F_{380} ratio in PC12 cells during bath application of DMSO, 2 µM BDE-47, and 20 µM BDE-47 normalized to baseline (first 5 min). The higher concentration of BDE-47 induced an increase in normalized F_{340}/F_{380} ratio (t = 12 - 24 min, $p < 0.01$). To investigate whether the increase in intracellular Ca^{2+} has functional consequences, vesicular catecholamine release was also investigated (Figure 3.5B). The average number of amperometrically recorded events of vesicular release amounted to 1.9 ± 0.7 events/min ($n = 9$) in control experiments. During superfusion with 20 µM BDE-47, the release frequency was enhanced to 6.0 ± 1.7 events/min ($n = 6$; $p < 0.05$), whereas superfusion with 2 µM BDE-47 caused no detectable effect (1.2 ± 0.5 events/min; $n = 7$). BDE-47 had no effect on release evoked by high-K^+ depolarization of the cells. Differences in vesicular release parameters could not be detected (data not shown).

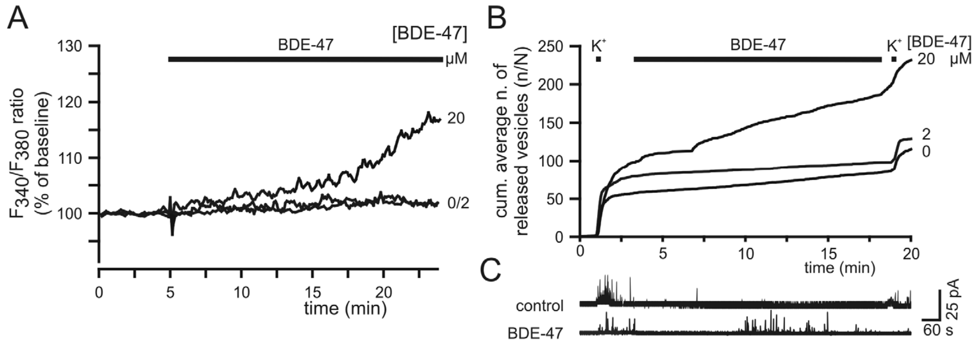


Figure 3.5. Acute effects of BDE-47 on Ca^{2+} and vesicular catecholamine release in PC12 cells. A. Intracellular free Ca^{2+} (normalized F_{340}/F_{380}) in cells exposed to DMSO ($n = 79$), 2 µM BDE-47 ($n = 32$), or 20 µM BDE-47 ($n = 27$); base level (t = 2 - 4 min) and effect (t = 12 - 14 min) differed significantly for 20 µM BDE-47. B. Cumulative (Cum) average number of amperometrically recorded vesicles from PC12 cells exposed to DMSO ($n = 9$), 2 µM BDE-47 ($n = 7$), or 20 µM BDE-47 ($n = 6$). C. Representative amperometric traces of PC12 cells exposed to DMSO (control) or 20 µM BDE-47.

Discussion

A broad spectrum of neurotoxicants (e.g., environmental pollutants such as metals, pesticides, and PCBs) has been shown to cause a reduction of habituation after neonatal exposure (Eriksson et al. 1990, 1991; Eriksson and Fredriksson 1991; Fredriksson et al. 1992). However, from the behavioral effects it is difficult to deduce information about underlying mechanisms.

In the present study, we found that neonatal exposure to BDE-47 causes developmental effects consisting of a reduction of PTP and LTP, as well as specific reductions of key postsynaptic proteins involved in glutamate receptor signaling. Presynaptic parameters were not affected *ex vivo*. *In vitro* experiments on PC12 cells show an increase in intracellular Ca^{2+} and spontaneous vesicular release, only at the highest concentration BDE-47 (20 μM). The combined results suggest that presynaptic changes do not directly contribute to the observed defect in synaptic plasticity.

The exposure to BDE-47 took place during a period of rapid brain growth, which in mice takes place during the first 3 - 4 weeks of life, reaching its peak around PND 10 (Davison and Dobbing 1968). The multitude and complexity of processes during this rapid development makes the developing brain particularly vulnerable to the effects of xenobiotics, like the adverse effect of BDE-47 on spontaneous behavior and habituation (Eriksson et al. 2001b). Interestingly, exposure to BDE-47 does not affect performance in the Morris water maze test (Eriksson et al. 2001b), commonly used as a learning task to detect effects in the hippocampus. This suggests that habituation is a more sensitive parameter for BDE-47 effects in the hippocampus.

We observed a specific reduction of key proteins in the PSD (i.e., GluR1, NR2B, and p286-αCaMKII). Because no changes were observed in total hippocampus homogenate, the specific decrease in the PSD is therefore attributed to changes in glutamate receptor subunit trafficking or clustering in the PSD instead of a reduced protein translation.

A study in GluR1-knockout mice showed that approximately 10% of the normal amount of GluR1 is sufficient for LTP (Mack et al. 2001). Also, a GluR1-independent form of LTP has been observed in juvenile GluR1-knockout mice (Jensen et al. 2003). Therefore, major effects on LTP as a consequence of the observed reduction of AMPA subunit GluR1 by approximately 30% are not expected.

The observed reduction of NR2B subunits results in an increased NR2A/NR2B ratio. The majority of NMDA receptors consist of 2 NR1 and 2 NR2A or 2 NR2B subunits. NR2A-NMDA receptors gate smaller Ca^{2+} currents, have a lower affinity for glutamate, and desensitize faster than NR2B-NMDA receptors (Kutsuwada et al. 1992). Therefore, an increased NR2A/NR2B ratio is likely to result in a higher threshold for LTP induction, which could explain the reduction of PTP and LTP.

In mice exposed to BDE-47, the autophosphorylated-active form of αCaMKII was significantly reduced. Because CamKII autophosphorylation is essential for hippocampal

NMDA-dependent LTP (Giese et al. 1998), this specific effect may lead to reduced synaptic plasticity resulting in behavioral impairments.

To ascertain the absence of presynaptic effects, we investigated neurotransmitter release from chromaffin cells from BDE-47-exposed [68 mg (140 µmol)/kg bw] mice. Because PPR and chromaffin neurotransmitter release remained unchanged after developmental exposure to BDE-47, and because modest acute effects on free intracellular Ca^{2+} and spontaneous vesicular catecholamine release in PC12 cells were only detected at a concentration of 20 µM BDE-47, we propose that presynaptic changes do not contribute considerably to the observed functional defect in synaptic plasticity. Based on tissue distribution data for 1 mg/kg bw ^{14}C-BDE-47 orally given to C57Bl/6 mice on PND 10 (Staskal et al. 2006a), brain concentration at sacrifice after exposure to 6.8 mg (14 µmol)/kg bw BDE-47 is estimated to be 0.43 - 0.81 µM and the peak brain concentration, reached 8 hr after exposure, is estimated to be 1.1 µM. These estimated concentrations are at least one order of magnitude lower than the lowest effective concentration in the *in vitro* experiments described here.

As with PCBs (reviewed in Fonnum et al. 2006), *in vitro* exposure to the commercial penta-BDE mixture DE-71, which contains (on a weight basis) 31.8% BDE-47 (Reistad and Mariussen 2005), affects several other transmitter systems. Previous studies reported cell death of cerebellar granule cells, alterations of Ca^{2+} homeostasis in human neutrophils and brain microsomes, and arachidonic acid release and protein kinase C translocation in cerebellar granule cells; inhibition of dopamine reuptake in rat brain synaptic vesicles has been reported after *in vitro* exposure to DE-71 in the micromolar range (2 - 20 µM; Kodavanti and Ward 2005; Mariussen and Fonnum 2003; Reistad et al. 2002; Reistad and Mariussen 2005). Interestingly, addition of the NMDA receptor antagonist MK801 protects cerebellar granule cells against DE-71-induced cell death (Reistad et al. 2006). No other effects of PBDEs on glutamate receptors have yet been published.

Pure (~99%) BDE-47, which has been used in only a few experiments, has revealed formation of reactive oxygen species in human neutrophils and increased ^{3}H-phorbol ester binding in primary rat cerebellar granule neurons, also at micromolar concentrations (Kodavanti et al. 2005; Reistad and Mariussen 2005). The effects of BDE-47 in PC12 cells reported here occur at concentrations in the same order of magnitude.

Effects on spontaneous motor activity and habituation in mice have been described for several lower and higher brominated diphenyl ethers after a single oral dose of maximally 21 µmol/kg bw (Branchi et al. 2002, 2003; Eriksson et al. 2001b, 2002; Viberg et al. 2003a, 2003b, 2006). In rats, effects on behavior have been observed after maternal exposure to 10 mg (18 µmol)/kg bw BDE-99 at gestational days 10 - 18 and after oral exposure to 30 mg/kg bw DE-71 at PND 6 - 12 (Dufault et al. 2005; Lilienthal et al. 2006).

In the 1990s, an association between delayed human neurodevelopment and prenatal or early exposure to PCBs was reported by cohort studies. These results were corroborated by experiments demonstrating the developmental neurotoxicity of PCBs. The observed

interaction with the thyroid hormone system is usually considered part of the underlying mechanism (reviewed in Winneke et al. 2002). For hazard characterization of PCBs and the structurally related PBDEs, it is relevant to investigate whether they induce similar effects through similar mechanisms. This is of particular importance because, in neonatal mice, the effects of a combined dose of PCB-52 and BDE-99 on spontaneous motor behavior and habituation capability appear to be additive or perhaps even synergistic (Eriksson et al. 2006a).

High human serum concentrations of BDE-47 were measured in female inhabitants of California by Petreas et al. (2003); the concentration of BDE-47 in serum ranged from 5 to 510 ng/g lipid weight, with a median of 16.5 ng/g lipid weight. High concentrations (> 100 ng/g lipid weight) have also been reported in Californian children (Fisher et al. 2006). The highest and median values correspond (using average physiologic values) to blood concentrations of approximately 11.5 nM and approximately 0.37 nM. Using the tissue distribution data for 1 mg/kg bw ^{14}C-BDE-47 (Staskal et al. 2006a), the dose used in the current study corresponds to an estimated blood concentration of approximately 2.6 µM after 3 hr and to approximately 0.6 µM after 10 days (i.e., ~50 - 200 times higher than in the worst, and ~1600 - 7,000 times higher than in the median human situation described above). For risk assessment, the difference between the animal dose level causing an adverse effect and the highest human dose levels is relatively small, considering safety factors for species extrapolation and intraspecies variability. Additional uncertainty comes from the fact that humans are exposed to multiple flame retardants over a lifetime. Accumulation of BDE-47, as demonstrated in primary rat cerebellar granule neurons and primary rat neocortical cells (Kodavanti et al. 2005; Mundy et al. 2004), is another reason for concern about the neurotoxic potential of PBDEs.

No tolerable daily intake is assigned to PBDEs because sufficient data are not available. However, the limited toxicity data suggest that adverse effects induced by exposure to the more toxic congeners in rodents occur at doses of at least 100 µg/kg bw per day [Joint FAO/WHO Expert Committee on Food Additives (JECFA) 2005]. The combination of quantitative molecular data with functional neurophysiologic effects reported here provides strong functional support for the previously reported neurobehavioral effects (Eriksson et al. 2001b) and is essential for characterization of the neurotoxic hazard of brominated flame retardants, particularly for rational risk assessment, which is required in response to the general concern about the vulnerability of the developing brain.

Acknowledgements

The authors thank A. de Groot for excellent technical assistance and L. van Halewijn for hippocampal Nissl staining.

Chapter 4

Hydroxylation increases the neurotoxic potential of BDE-47 to affect exocytosis and calcium homeostasis in PC12 cells

Milou M.L. Dingemans[1], Aart de Groot[1], Regina G.D.M. van Kleef[1], Åke Bergman[2], Martin van den Berg[1], Henk P.M. Vijverberg[1] and Remco H.S. Westerink[1]

1. Institute for Risk Assessment Sciences, Utrecht University, Utrecht, the Netherlands.
2. Department of Environmental Chemistry, Stockholm University, Stockholm, Sweden.

Environmental Health Perspectives **116**, 637 - 643.

Abstract

Background: Oxidative metabolism, resulting in the formation of hydroxylated polybrominated diphenyl ether (PBDE) metabolites, may enhance the neurotoxic potential of brominated flame retardants.

Objective: Our objective was to investigate the effects of a hydroxylated metabolite of 2,2´,4,4´-tetrabromodiphenyl ether (BDE-47; 6-OH-BDE-47) on changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and vesicular catecholamine release in PC12 cells.

Methods: We measured vesicular catecholamine release and $[Ca^{2+}]_i$ using amperometry and imaging of the fluorescent Ca^{2+}-sensitive dye Fura-2, respectively.

Results: Acute exposure of PC12 cells to 6-OH-BDE-47 (5 µM) induced vesicular catecholamine release. Catecholamine release coincided with a transient increase in $[Ca^{2+}]_i$, which was observed shortly after the onset of exposure to 6-OH-BDE-47 (1 - 20 µM). An additional late increase in $[Ca^{2+}]_i$ was often observed at ≥ 1 µM 6-OH-BDE-47. The initial transient increase was absent in cells exposed to the parent compound BDE-47, whereas the late increase was observed only at 20 µM. Using the mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and thapsigargin to empty intracellular Ca^{2+} stores, we found that the initial increase originates from emptying of the endoplasmic reticulum and consequent influx of extracellular Ca^{2+}, whereas the late increase originates primarily from mitochondria.

Conclusion: The hydroxylated metabolite 6-OH-BDE-47 is more potent in disturbing Ca^{2+} homeostasis and neurotransmitter release than the parent compound BDE-47. The present findings indicate that bioactivation by oxidative metabolism adds considerably to the neurotoxic potential of PBDEs. Additionally, based on the observed mechanism of action, a cumulative neurotoxic effect of PBDEs and *ortho*-substituted polychlorinated biphenyls on $[Ca^{2+}]_i$ cannot be ruled out.

Introduction

Increasing concentrations of brominated flame retardants, in particular polybrominated diphenyl ethers (PBDEs), in the environment, human food chain, and human tissues raise concern about possible neurotoxic effects. High concentrations of PBDEs and the structurally related polychlorinated biphenyls (PCBs) increase intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in cultured neuronal cells, likely through mobilizing Ca^{2+} from intracellular stores (reviewed in Mariussen and Fonnum 2006).

Such a xenobiotic-induced increase in $[Ca^{2+}]_i$ is of particular concern because this increase, in addition to being essential for multiple physiologic and pathologic processes, is the trigger for vesicular release of neurotransmitters (exocytosis). This correlation between increased $[Ca^{2+}]_i$ and the occurrence of exocytosis has been widely studied in neurons (reviewed in Barclay et al. 2005) and neuroendocrine cells, including rat pheochromocytoma PC12 cells (reviewed in García et al. 2006).

Evidence of oxidative metabolism of PBDEs is accumulating, but the neurotoxic potential of hydroxylated PBDE metabolites and their ability to affect Ca^{2+} homeostasis is still unknown. In most biotic samples, 2,2´,4,4´-tetrabromodiphenyl ether (BDE-47) is the predominant PBDE congener (Hites 2004). Neonatal exposure to this PBDE congener induces neurobehavioral changes (Eriksson et al. 2001b) and reduces long-term potentiation (LTP) in mouse hippocampal slices (Dingemans et al. 2007). Analysis of brain tissue from BDE-47-exposed mice revealed that alterations in the composition of postsynaptic density proteins and kinase activity might play a role in the reduction of synaptic plasticity (Dingemans et al. 2007).

The doses of BDE-47 resulting in impaired learning and memory and reduced LTP measured in hippocampal slices were estimated (using a distribution study; Staskal et al. 2006a) to result in peak brain concentrations of approximately 1 µM, whereas acute toxic effects of BDE-47 were seen *in vitro* only at concentrations ranging from 3 to 20 µM (Coburn et al. 2008; Dingemans et al. 2007).

The results of *in vitro* endocrine studies (focusing mostly on 6-hydroxy-2,2´,4,4´-tetrabromodiphenyl ether; 6-OH-BDE-47) on interactions with the estrogen and thyroid hormone receptor systems indicate that hydroxylated metabolites of PBDEs are more potent than the parent compounds (Cantón et al. 2005, 2006; Harju et al. 2007; Meerts et al. 2001).

The conversion of PBDEs to hydroxylated metabolites was confirmed by recent toxicokinetics studies (Huwe et al. 2007; Malmberg et al. 2005; Marsh et al. 2005; Staskal et al. 2006b). Additionally, marine sponges can produce *ortho*-OH-PBDEs (Hakk and Letcher 2003). Hydroxylated metabolites have been detected in blood from wildlife and humans (reviewed in Hakk and Letcher 2003). Therefore, we investigated the effects of 6-OH-BDE-47, a hydroxylated metabolite of the environmentally relevant PBDE congener BDE-47, on Ca^{2+} homeostasis and vesicular catecholamine release in PC12 cells to compare its neurotoxic potential with that of the parent compound.

Methods

Chemicals. BDE-47 and 6-OH-BDE-47 were synthesized and purified (~99% purity) at the Wallenberg Laboratory of Stockholm University as described by Marsh et al. (1999). Dibenzo-*p*-dioxins and dibenzofurans were removed from the PBDEs with a charcoal column as described by Örn et al. (1996). All other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands).

Cell culture. Rat pheochromocytoma (PC12) cells (Greene and Tischler 1976) obtained from the ATCC (American Type Culture Collection, Manassas, VA, USA) were cultured for up to 15 passages in RPMI 1640 medium (Invitrogen, Breda, the Netherlands) supplemented with 5% fetal calf serum and 10% horse serum (ICN Biomedicals, Zoetermeer, the Netherlands). For Ca^{2+} imaging experiments, we subcultured undifferentiated PC12 cells in poly-L-lysine-coated glass-bottom dishes (MatTek, Ashland, MA, USA) as described previously (Dingemans et al. 2007). For amperometric recordings, the cells were differentiated for 3 - 5 days with 5 µM dexamethasone to enhance exocytosis, as described previously by Westerink and Vijverberg (2002).

Cell viability assay. We used cell density as an indicator of cell viability. After 20 min of exposure to dimethylsulfoxide (DMSO) or 20 µM 6-OH-BDE-47, cells were cultured in fresh cell culture medium for another 24 hr. After replacing the culture medium, which washes away most dead, detached cells, and trypan blue inclusion, which stains the remaining dead cells, we determined the proportion of the surface of the cell culture dish occupied by living PC12 cells in triplicate for three dishes per experimental condition.

***Amperometry*.** Amperometric recordings of K^+-evoked and spontaneous vesicular catecholamine release from dexamethasone-differentiated PC12 cells using carbon fiber microelectrodes were made as described previously (Dingemans et al. 2007; Westerink and Vijverberg 2002). Following 1 min of baseline recording, we superfused PC12 cells for 15 sec with high K^+-containing saline (K^+ increased to 125 mM and Na^+ lowered to 5.5 mM) to determine their responsiveness. Cells were allowed to recover for 2 min before a 15-min exposure to BDE-47 or 6-OH-BDE-47 to investigate acute effects on vesicular catecholamine release. Recordings were performed at room temperature. To ensure exclusion of nonresponsive or extraordinary cells, we determined basal release frequency for 22 cells. Cells that showed a basal release frequency larger than the average + 2 standard deviations were considered to have an extraordinary high release frequency. Based on these findings, we excluded cells with a basal release frequency > 5/min. Similarly, cells with an evoked release frequency < 16/min were excluded. We used the resulting 20 cells for further data analysis.

Intracellular Ca^{2+} imaging. We measured changes in $[Ca^{2+}]_i$ using the Ca^{2+}-responsive fluorescent ratio dye Fura-2 as described previously (Dingemans et al. 2007). Briefly, cells were loaded with 5 µM Fura-2-AM (Molecular Probes; Invitrogen) in external saline (containing 1.8 mM $CaCl_2$, 24 mM glucose, 10 mM HEPES, 5.5 mM KCl, 0.8 mM $MgCl_2$, 125 mM NaCl, and 36.5 mM sucrose, adjusted to pH 7.3 with NaOH) for 20 min at room temperature; this was followed by 15 min de-esterification in external saline. The cells were then placed on the stage of an Axiovert 35M inverted microscope (Zeiss, Göttingen, Germany) equipped with a TILL Photonics Polychrome IV (TILL Photonics GmBH, Gräfelfing, Germany). Fluorescence evoked by 340 and 380 nm excitation wavelengths (F_{340} and F_{380}) was recorded every 12 sec at 510 nm with an Image SensiCam digital camera (TILL Photonics GmBH). The digital camera and polychromator were controlled by imaging software (TILLvisION, version 4.01), which was also used for data collection and processing. We further analyzed changes in the F_{340}/F_{380} ratio, reflecting changes in $[Ca^{2+}]_i$, using custom-made Excel macros (Microsoft Corp., Redmond, WA, USA). After 5 min baseline recording, cells were exposed to 0.2 - 20 µM BDE-47 or 6-OH-BDE-47. Maximum and minimum ratios were determined after 25 min recording (20 min exposure) by addition of ionomycin (5 µM) and ethylenediamine tetraacetic acid (EDTA; 17 mM) as a control for experimental conditions. Where applicable, cells were washed with Ca^{2+}-free external saline (containing 10 µM EDTA to remove residual extra-cellular Ca^{2+}) just before the imaging experiments. In specific experiments, intracellular Ca^{2+} stores were emptied by incubation with 1 µM thapsigargin (TG) and 1 µM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) in Ca^{2+}-free external saline for 10 min. FCCP depolarizes the mitochondrial membranes, resulting in the uncoupling of oxidative phosphorylation and subsequent Ca^{2+} release from mitochondria (Taylor et al. 2000). TG is a high-affinity inhibitor of sarcoplasmic/endoplasmic reticulum (ER) Ca^{2+} ATPase (SERCA). These compounds are commonly used under experimental conditions to empty intracellular Ca^{2+} stores (Toyoshima and Inesi 2004). To further distinguish between direct effects on ER and Ca^{2+} influx pathways, we used dantrolene as an inhibitor of Ca^{2+} release from the ER.

Data analysis and statistics. To determine effects on $[Ca^{2+}]_i$, we used the normalized F_{340}/F_{380} ratio. Any change in the normalized ratios to ≥ 1.1 was considered an increase and was used for further data analysis. We refer to a transient increase in $[Ca^{2+}]_i$ reaching its peak value (amplitude) between 0 and 4.5 min after application as an initial increase. We consider an additional increase after cessation of the initial transient increase to be a late increase. In a number of experiments (4/33), the initial fast transient was absent, and instead, a slower transient increase was observed. Because it is unclear whether this was a delayed initial transient increase or a transient form of the late increase, we excluded these experiments from further analysis. All data are presented as mean ± SE from the number of cells indicated. Statistical analyses were performed using SPSS 12.0.1 (SPSS, Chicago, IL, USA). Categorical data were compared using Fisher's exact and chi-square tests. We compared continuous data using Student's *t*-test, paired or unpaired where applicable. Analysis of variance (ANOVA) and

post hoc t-tests (corrected for multiple comparisons) were performed to investigate possible dose-response relationships. A *p*-value < 0.05 was considered statistically significant.

Results

6-OH-BDE-47 increases catecholamine release in PC12 cells. Exposure of PC12 cells to a high concentration (20 µM) of the brominated flame retardant BDE-47 was previously shown to induce vesicular catecholamine release, coinciding with a gradual increase in $[Ca^{2+}]_i$ (Dingemans et al. 2007). To investigate whether oxidative metabolism changes the ability of PBDEs to affect vesicular catecholamine release, we measured the effects of 6-OH-BDE-47, a hydroxylated metabolite of BDE-47. Although cytotoxicity has been reported after subchronic exposure (24 hr) to 2.5 µM 6-OH-BDE-47 (Cantón et al. 2005), 20 min of exposure to 20 µM 6-OH-BDE-47 did not have any effects on cell viability determined 24 hr later, suggesting the absence of acute cell toxicity (data not shown).

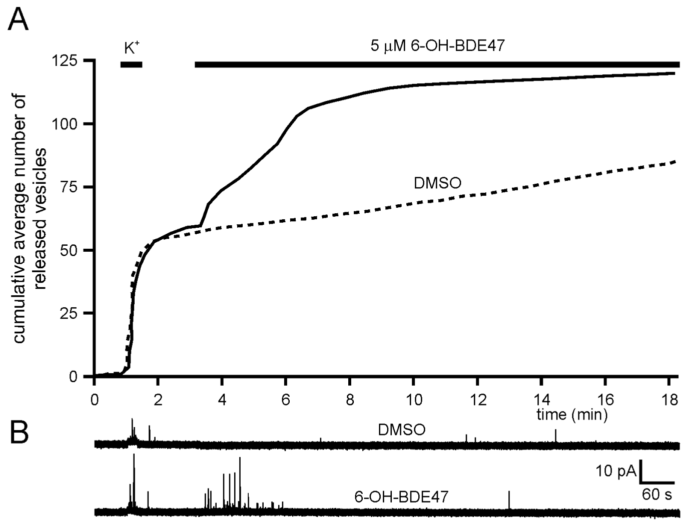


Figure 4.1. Catecholamine release in PC12 cells exposed to DMSO (*n* = 9) or 5 µM 6-OH-BDE-47 (*n* = 11) shown as the cumulative average number of released vesicles (A). Results clearly demonstrate that 6-OH-BDE-47 induced exocytosis. B. Representative amperometric traces recorded from cells exposed to DMSO or 5 µM 6-OH-BDE-47.

To investigate whether exposure to 6-OH-BDE-47 has functional consequences for neuronal communication, we measured vesicular catecholamine release (Figure 4.1). First, cells were challenged for 15 sec with high K^+-containing saline to determine their

responsiveness. Responsive cells displayed depolarization-evoked release (at least 16 released vesicles/min), after which the release frequency returned to baseline values. During the first 2.5 min of a subsequent exposure to 5 μM 6-OH-BDE-47 ($n = 9$), the release frequency was enhanced from 1.0 ± 0.3 to 13 ± 5.3 events/min ($p < 0.05$). This enhancement did not occur in cells exposed to DMSO only (from 1.8 ± 0.5 to 1.7 ± 0.7 events/min, not significant; $n = 11$). After the burst of exocytotic activity, the release frequency in 6-OH-BDE-47-exposed cells declined to a value not significantly different from basal release.

6-OH-BDE-47 causes a biphasic increase in $[Ca^{2+}]_i$ in PC12 cells. To investigate whether the observed changes in neurotransmitter release are caused by a disruption of calcium homeostasis, we measured the effects of 6-OH-BDE-47 on the $[Ca^{2+}]_i$. Exposure of PC12 cells to 6-OH-BDE-47 (≥ 1 μM) resulted in a dose-dependent increase in $[Ca^{2+}]_i$ in PC12 cells (Figures 4.2 and 4.3), whereas exposure to similar concentrations of the parent compound had no effects on $[Ca^{2+}]_i$ (Figure 4.2; Dingemans et al. 2007). The parent compound BDE-47 caused a gradual increase of $[Ca^{2+}]_i$ only at 20 μM (data not shown; Dingemans et al. 2007), whereas exposure to 1 μM of the hydroxylated metabolite resulted in an initial transient increase in $[Ca^{2+}]_i$ (Figures 4.2 and 4.3). At concentrations ≥ 1 μM, 6-OH-BDE-47 also caused an additional late increase in $[Ca^{2+}]_i$ (Figures 4.2 and 4.3). The relative occurrences (percentages of cells showing an effect) of initial transient and late increases in $[Ca^{2+}]_i$ increased with increasing concentrations of 6-OH-BDE-47 (Figure 4.3). Exposure to vehicle or 0.1 or 0.2 μM 6-OH-BDE-47 had no significant effect on $[Ca^{2+}]_i$ (Figure 4.3).

Exposure to 20 μM 6-OH-BDE-47 caused a large initial transient increase in $[Ca^{2+}]_i$ (1.7 ± 0.1), and an even larger late increase (3.2 ± 0.4) compared to the normalized baseline. At this high concentration, nonspecific effects are likely to occur. Therefore, we investigated the concentration dependence of the amplitude of the two types of increases in $[Ca^{2+}]_i$ within the range of 1 μM (the lowest concentration where the effects occur) to 5 μM. ANOVA analysis indicated no relationship between the applied 6-OH-BDE-47 concentration and the amplitude of the initial Ca^{2+} transient (Figure 4.4A). Analysis of the late increase indicated that the amplitude of this increase is concentration dependent (Figure 4.4B), although the biologic relevance of this small change remains to be determined. The distinct temporal aspects combined with this observation on the concentration dependence suggest that distinct mechanisms underlie both phases of increasing $[Ca^{2+}]_i$.

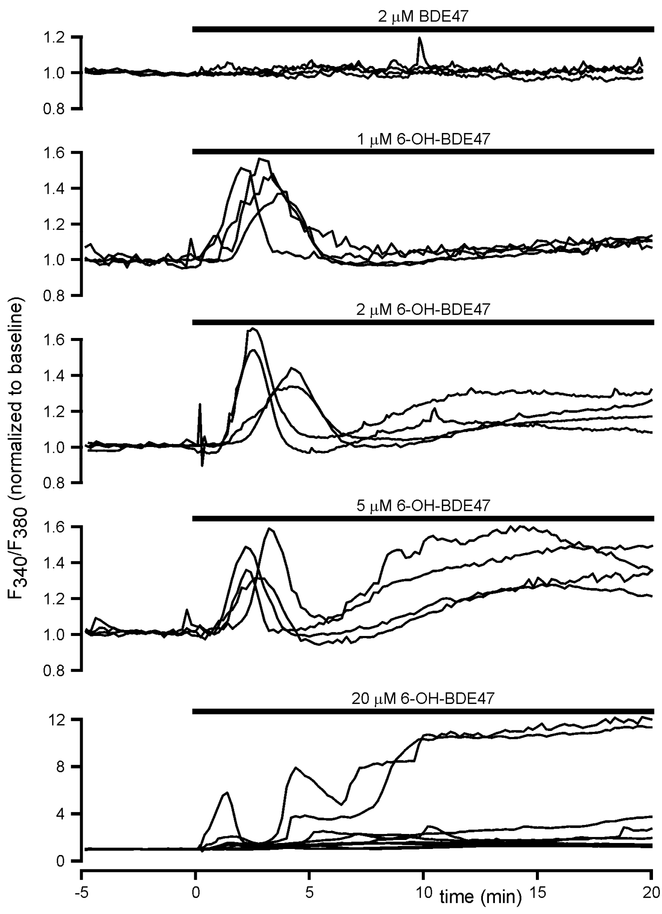


Figure 4.2. Biphasic increase in $[Ca^{2+}]_i$ in PC12 cells after exposure to 6-OH-BDE-47. Results are shown as representative traces of normalized F_{340}/F_{380} (reflecting $[Ca^{2+}]_i$) from individual PC12 cells exposed to 2 µM BDE-47 and 1, 2, 5, and 20 µM 6-OH-BDE-47 for 20 min, applied at t = 0, as indicated. Note the difference in scaling for 2 µM BDE-47 and 20 µM 6-OH-BDE-47.

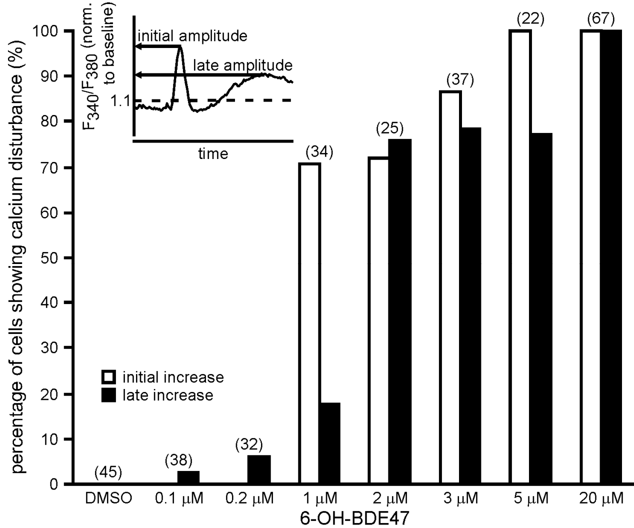


Figure 4.3. Concentration dependence of the occurrence of different types of $[Ca^{2+}]_i$ disturbances during exposure to 6-OH-BDE-47. Bars indicate the percentage of cells showing an initial transient increase in $[Ca^{2+}]_i$ or those showing a late increase in $[Ca^{2+}]_i$. For both processes, the percentage of cells displaying an increase is significantly higher than in control at concentrations ≥ 1 μM (initial: $p < 0.001$; late: $p < 0.01$). Data are shown from three to eight experiments per concentration; numbers above each bar indicate the number of cells used for data analysis. Inset: representative recording with the characteristics of the increase in $[Ca^{2+}]_i$ used in this article (i.e., amplitude of the initial and late increase in $[Ca^{2+}]_i$).

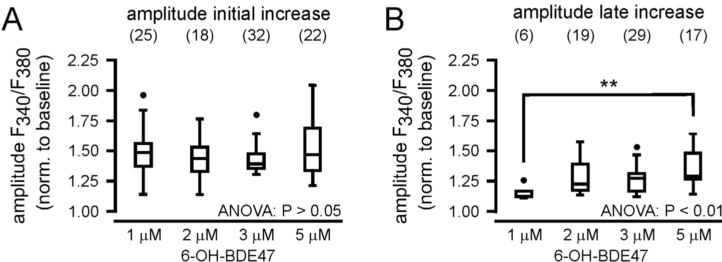


Figure 4.4. Amplitude of the initial and late increase in $[Ca^{2+}]_i$ after exposure to 1 - 5 μM 6-OH-BDE-47 shown as boxplots of amplitudes reached during the initial transient (A) and late increase (B). Upper and lower borders of the box represent upper and lower quartiles; the line within the box is the median; whiskers represent lowest and highest values, and circles represent outliers. ANOVA analysis indicates no differences in the amplitude of the initial increase at different concentrations ($p > 0.05$). ANOVA analysis and *post hoc* *t*-tests indicate that the amplitude of the late increase in $[Ca^{2+}]_i$ increases with increasing concentration ($p < 0.01$). Data shown are from three to eight experiments per concentration; numbers above each box indicate the number of cells used for data analysis.

6-OH-BDE-47-induced increase in [Ca^{2+}]$_i$ mainly originates from intracellular stores. To investigate the mechanisms underlying the observed increase in [Ca^{2+}]$_i$, we performed Ca^{2+} imaging experiments under Ca^{2+}-free conditions to reveal whether extracellular Ca^{2+} is required. Both the initial transient and the additional late increase in [Ca^{2+}]$_i$ were still present under Ca^{2+}-free conditions. However, the occurrence and amplitude of the initial increase were significantly higher under physiologic Ca^{2+} conditions (1.8 mM; Figures 4.5 and 4.6). The occurrence and amplitude of late increases were not altered under Ca^{2+}-free conditions (Figures 4.5 and 4.6). From these data, we conclude that the initial increase depends only partially on extracellular Ca^{2+}, whereas the late increase is independent of external Ca^{2+}, indicating that the 6-OH-BDE-47-induced increase in [Ca^{2+}]$_i$ largely relies on the release of Ca^{2+} from intracellular stores.

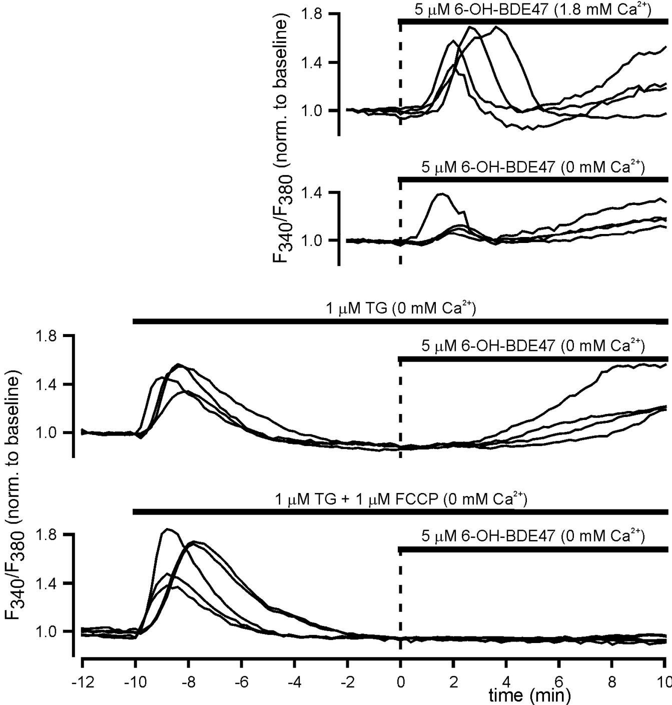


Figure 4.5. Release from intracellular Ca^{2+} stores in PC12 cells after exposure to 6-OH-BDE-47. Results are shown as representative traces of [Ca^{2+}]$_i$ measurements of individual PC12 cells exposed to 5 µM 6-OH-BDE-47 (applied at t = 0; dashed line) in external saline (containing 1.8 mM Ca^{2+}) and under Ca^{2+}-free conditions as indicated for each panel. The initial transient increase is smaller under Ca^{2+}-free conditions (note the different scale). When cells were pretreated with TG or TG + FCCP, an immediate increase in [Ca^{2+}]$_i$, corresponding to the emptying of intracellular stores, can be seen. Upon subsequent exposure to 6-OH-BDE-47, the initial transient increase, as observed under control conditions, is absent. Only in cells pretreated with TG and FCCP is the 6-OH-BDE-47-induced late increase also absent.

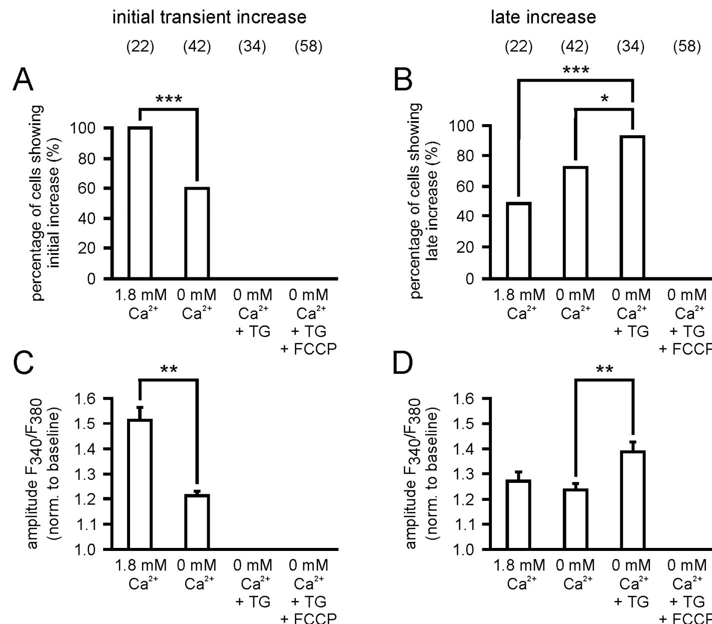


Figure 4.6. Occurrence and amplitudes of initial and late 6-OH-BDE-47-induced increases at different experimental conditions. The effects of 5 µM 6-OH-BDE-47 on $[Ca^{2+}]_i$ were measured in external saline (1.8 mM Ca^{2+}), Ca^{2+}-free saline (0 mM Ca^{2+}), Ca^{2+}-free saline after pretreatment with TG (0 mM Ca^{2+} + TG), and Ca^{2+}-free saline after pretreatment with both TG and FCCP (0 mM Ca^{2+} + TG + FCCP). Occurrence (A) and amplitude (C) of the initial increase in $[Ca^{2+}]_i$. Occurrence (B) and amplitude (D) of the late increase in $[Ca^{2+}]_i$. Data are from four experiments per treatment. Numbers above bars indicate the number of cells used for data analysis; values shown are mean ± SE for the number of cells indicated. *$p < 0.05$; **$p < 0.01$; #$p < 0.001$.

To identify the intracellular stores responsible for the observed increase in $[Ca^{2+}]_i$, we performed additional Ca^{2+} imaging experiments using PC12 cells in which mitochondrial and TG-sensitive intracellular Ca^{2+} stores were depleted by pretreatment with FCCP and TG, respectively. After depletion of ER Ca^{2+} stores with TG under Ca^{2+}-free conditions, 5 µM 6-OH-BDE-47 was no longer able to evoke the initial transient increase in $[Ca^{2+}]_i$, but the late increase was still present (Figure 4.5). Both the occurrence and the amplitude of the late increase were larger after TG pretreatment compared with normal and Ca^{2+}-free conditions (Figure 4.6), indicating a tight coupling between intracellular Ca^{2+} stores. After depletion of both mitochondrial and ER Ca^{2+} stores with FCCP and TG under Ca^{2+}-free conditions, both the initial transient and the late increase in $[Ca^{2+}]_i$ were completely absent. These combined data indicate that the initial transient increase in $[Ca^{2+}]_i$, which depends only partly on Ca^{2+} influx, is

mainly caused by intracellular Ca^{2+} release from the ER, whereas the late increase is mainly due to Ca^{2+} release from mitochondria.

To investigate whether the influx of extracellular Ca^{2+} in the initial transient increase was related to emptying of the ER, we exposed dantrolene (100 µM)-pretreated PC12 cells to 5 µM 6-OH-BDE-47 under physiologic Ca^{2+} conditions. The occurrence and amplitude of the initial transient increase were markedly reduced under these conditions (data not shown), suggesting that store-operated Ca^{2+} entry (SOCE) largely accounts for the influx of extracellular Ca^{2+}.

Discussion

The results of the present study demonstrate that both the abundant PBDE congener BDE-47 and its hydroxylated metabolite 6-OH-BDE-47 increase $[Ca^{2+}]_i$ in PC12 cells, although the hydroxylated metabolite does so at much lower concentrations. The initial transient and the late increase in $[Ca^{2+}]_i$ are due to release of Ca^{2+} from endoplasmic and mitochondrial Ca^{2+} stores, respectively; extracellular Ca^{2+} also plays a role in the observed initial increase in $[Ca^{2+}]_i$ during exposure to 6-OH-BDE-47. Interestingly, the initial increase in $[Ca^{2+}]_i$ is temporally linked with vesicular catecholamine release, raising concern about effects of BDE exposure on neurotransmission.

The increase in $[Ca^{2+}]_i$ is mainly caused by release of Ca^{2+} from intracellular stores, which are involved in controlling intracellular Ca^{2+} homeostasis and neurotransmitter release (reviewed in García et al. 2006). It is noteworthy that disruption of intracellular Ca^{2+} homeostasis by release of Ca^{2+} from intracellular stores and influx of extracellular Ca^{2+} is also considered an important factor in the neurotoxicity of PCBs (reviewed in Fonnum et al. 2006; Kodavanti 2005).

The effects of 6-OH-BDE-47 on exocytosis and $[Ca^{2+}]_i$ have been investigated in PC12 cells, which are widely used as an *in vitro* neuroendocrine model to study neurotransmitter secretion (reviewed in Westerink and Ewing 2008). Possible origins for the 6-OH-BDE-47-induced increase in $[Ca^{2+}]_i$ are influx of extracellular Ca^{2+} or release from intracellular Ca^{2+} stores. In adrenal chromaffin and PC12 cells, intracellular Ca^{2+} stores are ER, mitochondria, nucleus, and secretory vesicles. Influx via voltage-gated Ca^{2+} channels and SOCE channels, and efflux from the ER and the mitochondria, are tightly coupled and locally control the $[Ca^{2+}]_i$ that regulates exocytosis (reviewed in García et al. 2006; Parekh and Putney 2005). The increase in $[Ca^{2+}]_i$ following exposure to 6-OH-BDE-47 is also associated with an increase in vesicular catecholamine release in PC12 cells. The increase in catecholamine release was most apparent during the first 2.5 min of exposure, whereas release frequencies were no longer different between control cells and cells exposed to 6-OH-BDE-47 after 5 min of exposure (Figure 4.1). The strong temporal link between the 6-OH-BDE-47-induced initial transient increase in $[Ca^{2+}]_i$ (by emptying of the ER and subsequent SOCE) and the 6-OH-BDE-47-

induced burst of exocytotic activity strongly suggests a causal relationship. Because the observed late increase in $[Ca^{2+}]_i$ (by Ca^{2+} release from mitochondria) has a smaller effect on $[Ca^{2+}]_i$ than the emptying of the ER and subsequent SOCE (Figure 4.4) at concentrations < 5 µM, the association between this late increase in $[Ca^{2+}]_i$ and neurotransmitter release is likely to be of less toxicologic concern.

Because the initial peak is completely absent in TG-treated cells, we concluded that this increase in $[Ca^{2+}]_i$ primarily originates from the ER. Another brominated flame retardant (tetrabromobisphenol A) has recently been shown to be a potent inhibitor of the SERCA Ca^{2+} pump (Ogunbayo and Michelangeli 2007). The reduced amplitude of the initial transient $[Ca^{2+}]_i$ increase under Ca^{2+}-free conditions (without TG pretreatment) indicates that both an intracellular and extracellular Ca^{2+} component contribute to this transient increase. The extracellular Ca^{2+} component could be caused by a direct effect of 6-OH-BDE-47. However, the TG experiments suggest it is more likely that SOCE, through SOCE channels, in response to 6-OH-BDE-47-induced emptying of the ER, accounts for the involvement of extracellular Ca^{2+}. SOCE is commonly observed in PC12 cells after depletion of Ca^{2+} stores (Bennett et al. 1998; Taylor and Peers 1999). When Ca^{2+} release from the ER is inhibited by dantrolene during exposure to 6-OH-BDE-47, the initial transient increase is markedly reduced, suggesting that a large part of the extracellular component is indeed an indirect effect of 6-OH-BDE-47 associated with SOCE. However, as a small initial transient increase can still be observed, it is not possible at present to exclude a direct effect of 6-OH-BDE-47 on other Ca^{2+} influx pathways.

In cells treated with TG and the mitochondrial uncoupler FCCP, both the initial and the late increase no longer occur after application of 6-OH-BDE-47. As the initial increase was already abolished by TG, these results indicate that the late increase in $[Ca^{2+}]_i$ mainly originates from mitochondria. In TG-treated cells, the amplitude of the late Ca^{2+} increase is larger (Figure 4.6). A possible explanation for this enhancement of the late Ca^{2+} increase is the tight coupling between Ca^{2+} influx via voltage-gated and SOCE channels and efflux from intracellular stores (reviewed in García et al. 2006; Parekh and Putney 2005). This coupling predicts that mitochondria take up Ca^{2+} from the emptied TG-sensitive stores and will thus be filled to a larger extent. As a consequence, emptying of mitochondrial stores by 6-OH-BDE-47 will result in an enhancement of the late Ca^{2+} increase (Figure 4.6).

The present findings add to previous studies demonstrating that *ortho*-substituted (nonplanar) PCBs increase $[Ca^{2+}]_i$ in cultured neuronal cells and brain preparations (Howard et al. 2003; Inglefield and Shafer 2000; Kang et al. 2004; Kodavanti et al. 1993; Magi et al. 2005; Voie and Fonnum 1998; Wong et al. 1997). Inhibition of endoplasmic and mitochondrial Ca^{2+}-ATPases, mobilization of Ca^{2+} from the ER through interaction with the inositol triphosphate (IP_3)- and ryanodine receptors, and disruption of plasma, mitochondrial, and endoplasmic membranes have all been proposed as possible mechanisms. Furthermore, the commercial PBDE mixture DE-71, which contains 31.8% BDE-47, has also been shown to disrupt microsomal Ca^{2+} homeostasis (Kodavanti and Ward 2005). More recently, a gradual increase

in $[Ca^{2+}]_i$ in PC12 cells has also been reported for the environmentally relevant BDE-47, although only at 20 µM (Dingemans et al. 2007). Also, BDE-47, as well as 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99), result in a reduced net Ca^{2+} uptake by microsomes and mitochondria isolated from frontal cortex, cerebellum, hippocampus, and hypothalamus of adult male rats measured after 20 min exposure to 3 - 30 µM (Coburn et al. 2008).

The underlying mechanisms of the (hydroxylated) PBDE-induced disruption of Ca^{2+} homeostasis should be investigated in more detail. Increasing evidence suggests that formation of reactive oxygen species (ROS) could be responsible for the observed effects on $[Ca^{2+}]_i$. In rat hippocampal neurons as well as in human SH-SY5Y cells and neutrophil granulocytes, BDE-47 has been shown to induce ROS formation at exposure concentrations of 41, 4, and 6 µM, respectively (He et al. 2008a, 2008b; Reistad and Mariussen 2005). However, the effect of hydroxylated BDE-47 on ROS formation remains to be determined. Mechanisms usually associated with increased ROS formation include activation of tyrosine kinase, IP_3-kinase, protein kinase C, phospholipase C, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; release of arachidonic acid; and disturbed and increased $[Ca^{2+}]_i$ (Kodavanti and Derr-Yellin 2002; Kodavanti and Ward 2005; Reistad and Mariussen 2005). Because of the ether group connecting the phenyl rings in PBDEs, these compounds display a structural resemblance with *ortho*-substituted PCBs. It is therefore likely that disruption of Ca^{2+} homeostasis (reviewed in Mariussen and Fonnum 2006), primarily caused by Ca^{2+} release from intracellular stores, is a common feature underlying the neurotoxicity of both *ortho*-PCBs and PBDEs.

Determination of the relative potency of 6-OH-BDE-47 and BDE-47 based on half maximal effective concentrations requires full dose-response curves with similar slopes and efficacy, which is not realistic due to the occurrence of nonspecific effects at high concentrations of 6-OH-BDE-47 and the relatively low potency of the parent compound and consequent solubility problems. Nonetheless, comparison of lowest observed effect concentrations clearly reveals that 6-OH-BDE-47 has a potency at least one order of magnitude higher than the parent compound BDE-47.

In the 1990s, an association between delayed human neurodevelopment and prenatal or neonatal exposure to PCBs was reported in cohort studies, which were corroborated by experiments demonstrating developmental neurotoxicity of PCBs (reviewed in Winneke et al. 2002). Although epidemiologic evidence for a similar association of PBDEs is yet lacking, it has been established that exposure of mice to these environmental pollutants during brain development can cause toxic effects at doses much lower than those affecting adult brain function (Eriksson et al. 2001a). It is thus of particular concern that young children at critical stages of brain development are exposed to higher concentrations of PBDEs than adults. This high exposure is mainly associated with an increased exposure of children to house dust, which is an important source of PBDEs (Jones-Otazu et al. 2005). Additionally, global differences in PBDE body burden are observed, with average levels in North America being approximately 10 times higher than in Europe and Asia (Birnbaum and Cohen Hubal

2006). Very high serum concentrations of BDE-47 (as well as other PBDE congeners) have recently been measured in children working and living on a waste dumpsite in Nicaragua (Athanasiadou et al. 2008). These samples also have shown that hydroxylated PBDE metabolites bioaccumulate in human serum. The highest concentration of 6-OH-BDE-47 measured was 13 pmol/g lipid weight, corresponding to approximately 0.14 nM in blood (calculated using average physiologic values). These *in vivo* values are still orders of magnitude lower than those that exert effects in the present *in vitro* study. However, particular concern about neurotoxicity arises from the fact that comparable or even higher levels were observed for several other hydroxylated PBDE metabolites, for which even fewer toxicity data are available than for 6-OH-BDE-47.

In summary, exposure of PC12 cells to ≥ 1 µM 6-OH-BDE-47 increases exocytosis and $[Ca^{2+}]_i$, mainly via release from ER and mitochondria, whereas its parent compound BDE-47 causes comparable effects only at 20 µM. Furthermore, recent *in vivo* findings demonstrated that neonatal exposure of mice to BDE-47 causes permanent effects on neurobehavior (Eriksson et al. 2001b) and synaptic plasticity (Dingemans et al. 2007). Human exposure to hydroxylated PBDE metabolites results from uptake from natural sources and from internal oxidative metabolism of PBDEs (Hakk and Letcher 2003). In this respect, it should also be noted that exposure to PBDEs in children at the age of rapid brain development is disproportionally high (Jones-Otazu et al. 2005). The stronger Ca^{2+} homeostasis-disrupting effect of these hydroxylated metabolites is therefore a critical factor that should be taken into account in human PBDE risk assessment, in particular in relation to neurotoxicity and neurodevelopment. Based on the mechanism of action observed in the present study with PBDEs and those reported earlier for *ortho*-substituted PCBs (i.e., disruption of Ca^{2+} homeostasis), a cumulative neurotoxic effect (on $[Ca^{2+}]_i$) of both groups of compounds can not be ruled out. Further research should determine whether combined exposure to PBDEs and *ortho*-PCBs is of neurotoxicologic relevance in humans.

Chapter 5

Bromination pattern of hydroxylated metabolites of BDE-47 affects their potency to release calcium from intracellular stores in PC12 cells

Milou M.L. Dingemans[1], Harm J. Heusinkveld[1], Åke Bergman[2], Martin van den Berg[1] and Remco H.S. Westerink[1]

1. Institute for Risk Assessment Sciences, Utrecht University, Utrecht, the Netherlands.
2. Department of Environmental Chemistry, Stockholm University, Stockholm, Sweden.

Environmental Health Perspectives doi:10.1289/ehp.0901339.

Abstract

Background: Brominated flame retardants, including the widely used polybrominated diphenyl ethers (PBDEs), have been detected in humans, raising concern about possible neurotoxicity. Recent research demonstrated that the hydroxylated metabolite 6-OH-BDE-47 increases neurotransmitter release by releasing Ca^{2+} from intracellular stores at much lower concentrations than its environmentally relevant parent congener BDE-47. Recently, several other hydroxylated BDE-47-metabolites, besides 6-OH-BDE-47, have been detected in human serum and cord blood.

Objective and methods: To investigate the neurotoxic potential of other environmentally relevant PBDEs and their metabolites, we investigated and compared the acute effects of BDE-47, BDE-49, BDE-99, BDE-100, BDE-153 and several metabolites of BDE-47, i.e., 6-OH-BDE-47 (and its methoxylated analogue 6-MeO-BDE-47), 6'-OH-BDE-49, 5-OH-BDE-47, 3-OH-BDE-47 and 4'-OH-BDE-49, on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), measured using the Ca^{2+}-responsive dye Fura-2 in neuroendocrine pheochromocytoma (PC12) cells.

Results: In contrast with the parent PBDEs and 6-MeO-BDE-47, all hydroxylated metabolites induced Ca^{2+} release from intracellular stores, although with different lowest-observed-effect concentrations (LOECs). The major intracellular Ca^{2+} sources were either endoplasmic reticulum (ER; 5-OH-BDE-47 and 6'-OH-BDE-49) or both ER and mitochondria (6-OH-BDE-47, 3-OH-BDE-47 and 4'-OH-BDE-49). When investigating fluctuations in $[Ca^{2+}]_i$, a more subtle endpoint, lower LOECs were observed for 6-OH-BDE-47 and 4'-OH-BDE-49, whereas also BDE-47 increased the number of fluctuations.

Conclusions: The present findings demonstrate that hydroxylated metabolites of BDE-47 cause disturbance of the $[Ca^{2+}]_i$. Importantly, shielding of the OH-group on both sides with Br-atoms and/or the ether-bond to the other phenyl ring lowers the potency of hydroxylated PBDE-metabolites.

Introduction

Brominated flame retardants (BFRs) are added to a wide array of consumer products. Adverse effects of these compounds on the developing nervous system give cause for concern (reviewed in Costa and Giordano 2007). Long-lasting neurobehavioral changes following neonatal exposure have been detected in rodents after exposure to polybrominated diphenyl ethers (PBDEs; Branchi et al. 2003; Eriksson et al. 2001; Lilienthal et al. 2006; Rice et al. 2007; Viberg et al. 2003, 2006) as well as various functional and structural alterations in the brain (Dingemans et al. 2007; Viberg 2009; Xing et al. 2009).

In particular during development, acute effects on neuronal activity may result in the observed long-lasting changes in proteins, brain function and behavior. A key regulator for neuronal function is the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which regulates many cellular processes, including the release of neurotransmitter at the presynaptic terminal (reviewed in Clapham 2007; Laude and Simpson 2009).

PBDEs have been shown to affect Ca^{2+} homeostasis in microsomes (Kodavanti and Ward 2005) and PC12 cells (Dingemans et al. 2007) and to reduce the Ca^{2+} uptake by brain microsomes and mitochondria (Coburn et al. 2008) at relatively high concentrations. PBDEs have also been shown to induce oxidative stress in neuronal cells (He et al. 2008; Kodavanti and Ward 2005). Recently, it was demonstrated that 6-OH-BDE-47, a hydroxylated metabolite of BDE-47, increases vesicular neurotransmitter release and $[Ca^{2+}]_i$ by releasing Ca^{2+} from intracellular stores in PC12 cells. The increase in vesicular neurotransmitter release was induced by 6-OH-BDE-47 at much lower concentrations than by the parent PBDE (Dingemans et al. 2008). Therefore, hydroxylated PBDEs (OH-PBDEs) may be more important than the parent compounds for human risk assessment for neurotoxicity.

It has recently been found that not only PBDEs but also various hydroxylated metabolites of PBDEs bioaccumulate in humans (Athanasiadou et al. 2008; Qiu et al. 2009). Therefore, the aim of this study was to determine whether mono-hydroxylated metabolites of the abundant BDE-47 affect $[Ca^{2+}]_i$ and to compare this with the effects of a methoxylated analogue and several other environmentally relevant PBDE-congeners.

Materials and Methods

Chemicals. In the present study, the effects of BDE-47, BDE-99, BDE-100, BDE-153 (main constituents of the commercial DE-71 pentaBDE mixtures; La Guardia et al. 2006), as well as several metabolites of BDE-47 (6-OH-BDE-47, 6’-OH-BDE-49, 5-OH-BDE-47, 3-OH-BDE-47, 4’-OH-BDE-49 and methoxylated analogue 6-MeO-BDE-47) were investigated. During formation of 6’-OH-BDE-49 and 4’-OH-BDE-49 a bromine-shift takes place. Consequently, BDE-49 is included as a control for possible influences of this change in bromination pattern.

PBDEs (Figure 5.1, for full names see Supplemental Material, Table 5.S1) were synthesized and purified (~99% purity) at the Department of Environmental Chemistry of Stockholm University (Marsh et al. 1999). Dibenzo-*p*-dioxins and dibenzofurans were removed from the PBDEs with a charcoal column as described by Örn et al. (1996). All other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands).

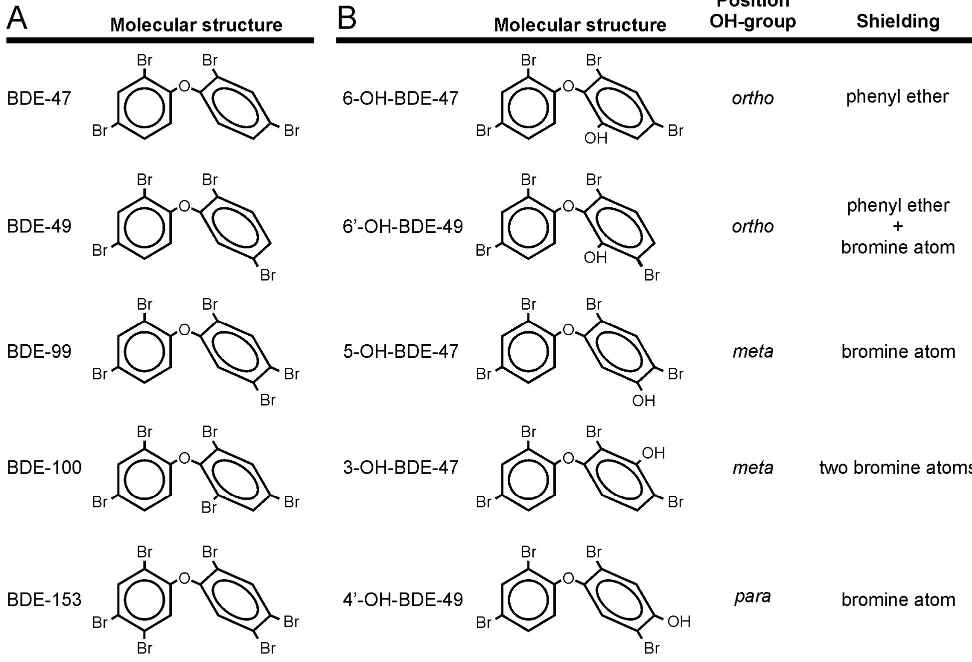


Figure 5.1. Molecular structures of the PBDEs (A; BDE-47, BDE-49, BDE-99, BDE-100 and BDE-153) and metabolites of BDE-47 (B; 6-OH-BDE-47, 6'-OH-BDE-49, 5-OH-BDE-47, 3-OH-BDE-47, 4'-OH-BDE-49 and methoxylated analogue 6-MeO-BDE-47) investigated in this study. For the metabolites, also the position of the OH- or MeO-group is indicated, as well as the shielding of this group by occupancy of the adjacent C-atoms by either phenyl ether and/or bromine atoms.

Cell culture. Rat pheochromocytoma (PC12) cells (Greene and Tischler 1976), obtained from ATCC (American Type Culture Collection, Manassas, VA, USA), were cultured as described previously (Dingemans et al. 2008; for more details, see Supplemental Material).

Cell viability assay. To investigate possible acute effects of the PBDEs on cell viability, the Alamar Blue (AB) and neutral red (NR) uptake assays were used with minor modifications (Magnani and Bettini 2000; Repetto et al. 2008). For more details, see Supplemental Material.

Intracellular Ca^{2+} imaging. Changes in $[Ca^{2+}]_i$ were measured using the Ca^{2+}-sensitive fluorescent ratio dye Fura-2 as described previously (Dingemans et al. 2007, 2008). For more detailed information on experimental conditions and calculation of $[Ca^{2+}]_i$ see Supplemental Material.

The average and amplitude of $[Ca^{2+}]_i$ during exposure were determined per cell to investigate effects of PBDEs on Ca^{2+} homeostasis. The standard deviation during baseline $[Ca^{2+}]_i$ recording ranged from 2 - 74% of average $[Ca^{2+}]_i$ (n = 1538; see Supplemental Material, Figure 5.S1). To prevent registration of false positive effects, an increase in $[Ca^{2+}]_i$ to > 175% of baseline was therefore used to determine no-observed-effect concentrations (NOECs) and lowest-observed-effect concentrations (LOECs). A transient increase within 0 - 10 min from the start of exposure is referred to as an 'initial increase', while additional increases are referred to as 'late increases' (Dingemans et al. 2008). At NOEC levels, based on average and amplitude of $[Ca^{2+}]_i$ levels, increases in $[Ca^{2+}]_i$ levels to > 175% of baseline during exposure were scored as fluctuations to investigate more subtle effects on the Ca^{2+} homeostasis. The number of cells showing fluctuations in $[Ca^{2+}]_i$ as well as frequency, amplitude and duration of these fluctuations, were determined.

Data analysis and statistics. All data are presented as mean ± SE from the number of cells (n) or fluctuations in $[Ca^{2+}]_i$ (N) indicated. Statistical analyses were performed using SPSS 16 (SPSS, Chicago, IL, USA). Categorical and continuous data were compared using respectively Fisher's exact test and Student's *t*-test, paired or unpaired where applicable. Analysis of variance (ANOVA) and *post hoc t*-tests were performed to investigate possible dose-response relationships. To investigate structure-activity relationships, the possible influence of hydroxylation position and/or shielding of the OH-group by adjacent atomic groups was investigated on the efficacy to increase $[Ca^{2+}]_i$. To this aim, a multifactorial ANOVA was performed using the mean increase in basal $[Ca^{2+}]_i$ at 20 µM as the dependent variable. Hydroxylation position (*ortho*, *meta* or *para*) and the presence of either one or two shielding atomic groups (phenyl ring and/or bromine atom) adjacent to the OH-group were used as fixed variables (Figure 5.1). A p-value < 0.05 is considered statistically significant.

Results

Effects of parent PBDEs on $[Ca^{2+}]_i$. Exposure to solvent control DMSO, 20 µM BDE-47, BDE-49, BDE-99, BDE-100 or BDE-153 did not decrease cell viability (not shown), increase the average or amplitude of $[Ca^{2+}]_i$ or increase the percentage of cells showing fluctuations in $[Ca^{2+}]_i$ (see

Supplemental Material, Figure 5.S2). However, the frequency and duration of fluctuations in $[Ca^{2+}]_i$ increased during exposure to 20 µM BDE-47. At 2 µM BDE-47, similar effects were detected (NOEC: 1 µM). No effects could be detected on the frequency, duration or amplitude of fluctuations during exposure to 20 µM BDE-49, BDE-99, BDE-100 or BDE-153 (see Supplemental Material, Table 5.S3).

Hydroxylated BDE-47 metabolites increase $[Ca^{2+}]_i$. After 20 min exposure to 6-OH-BDE-47, 5-OH-BDE-47 or 4'-OH-BDE-49, only at 20 µM a decrease in cell viability measured with NR was observed to respectively 86 ± 0.3%, 86 ± 1.1% and 93 ± 0.8% of control. No significant decreases in cell viability measured by NR assay were observed for 6'-OH-BDE-49 and 3-OH-BDE-47. In the AB assay, exposure to 6-OH-BDE-47, 6'-OH-BDE-49 or 3-OH-BDE-47 increases the relative fluorescence intensity dose-dependently above the control level (not shown).

Initial increases in $[Ca^{2+}]_i$ were observed during exposure to 6-OH-BDE-47, 6'-OH-BDE-49, 5-OH-BDE-47, 3-OH-BDE-47 or 4'-OH-BDE-49 (see Supplemental Material, Figure 5.S3 and Table 5.S2). Exposure to OH-PBDEs resulted in a dose-dependent increase in average and amplitude of $[Ca^{2+}]_i$ with varying LOECs. LOECs are determined by the amplitude of the increase in $[Ca^{2+}]_i$ (Figure 5.2) and the percentage of cells showing initial and late increases in $[Ca^{2+}]_i$ (Figure 5.3). No effects on the average and amplitude of $[Ca^{2+}]_i$ were detected during exposure to 20 µM 6-MeO-BDE-47. Effects on cell viability, the percentage of cells showing increases in $[Ca^{2+}]_i$ to > 175% of baseline or on the average frequency, duration and amplitude of fluctuations could also not be detected for 6-MeO-BDE-47.

For 6'-OH-BDE-49, 3-OH-BDE-47 and 4'-OH-BDE-49, the LOEC for increased $[Ca^{2+}]_i$ is 20 µM. Exposure to 20 µM 6'-OH-BDE-49 results in an initial increase in 50% of the cells. Late increases are observed less frequently and with lower amplitude. The shapes of the increases in $[Ca^{2+}]_i$ during exposure to 20 µM 6'-OH-BDE-49 vary widely (Figure 5.4B). Initial increases are observed during exposure to 20 µM 3-OH-BDE-47 in 48% of the cells. Exposure to 20 µM 4'-OH-BDE-49 results in a modest initial increase compared to baseline in 83% of the cells, after which a larger late increase is observed.

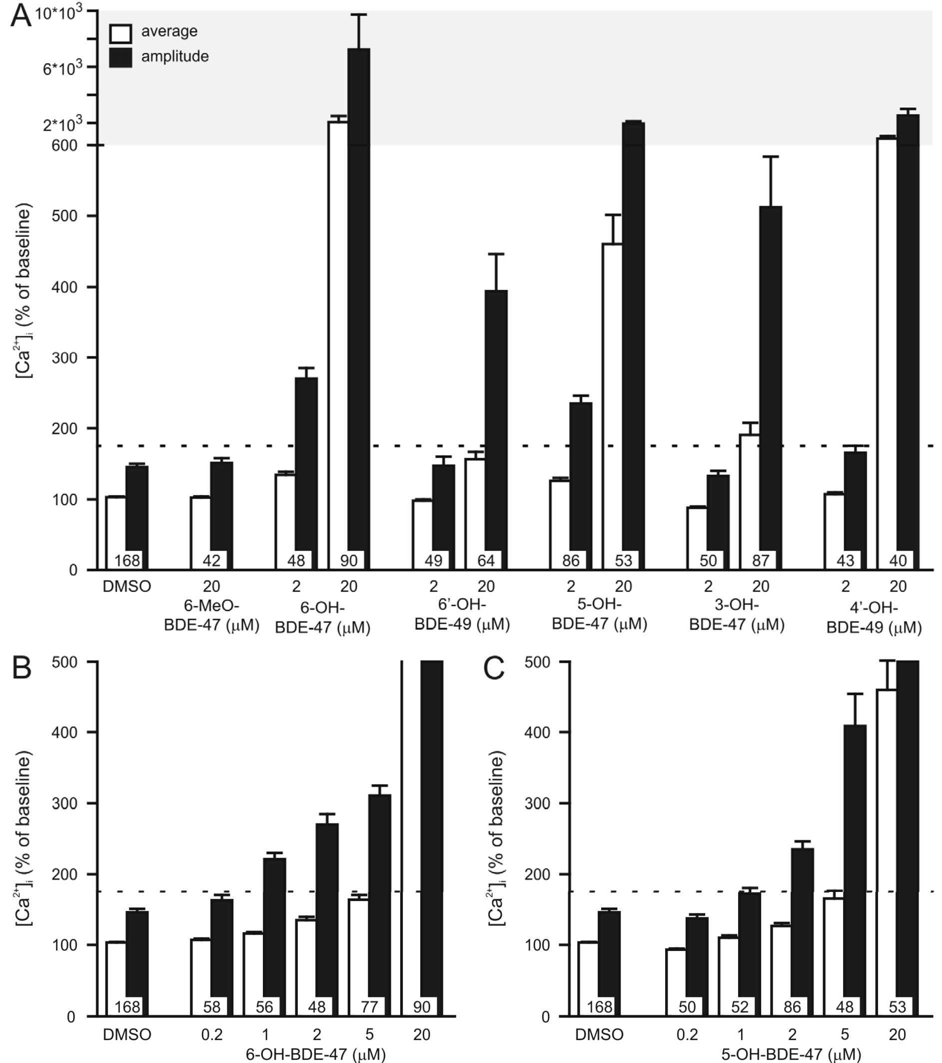


Figure 5.2. Hydroxylated metabolites of BDE-47 increase $[Ca^{2+}]_i$. Bars (A) represent average (open bars) and amplitude (closed bars) of $[Ca^{2+}]_i$ during exposure to 2 or 20 μM of 6-MeO-BDE-47, 6-OH-BDE-47, 6'-OH-BDE-49, 5-OH-BDE-47, 3-OH-BDE-47 or 4'-OH-BDE-49. For 6-OH-BDE-47 (B) and 5-OH-BDE-47 (C), which show increase in $[Ca^{2+}]_i$ to > 175% of baseline (as indicated with dashed line) at 2 μM, more concentrations are tested. Note the difference in scaling as indicated by the grey plot area. Data are shown from 4 to 19 experiments per concentration; numbers inside the bars indicate the number of cells used for data analysis.

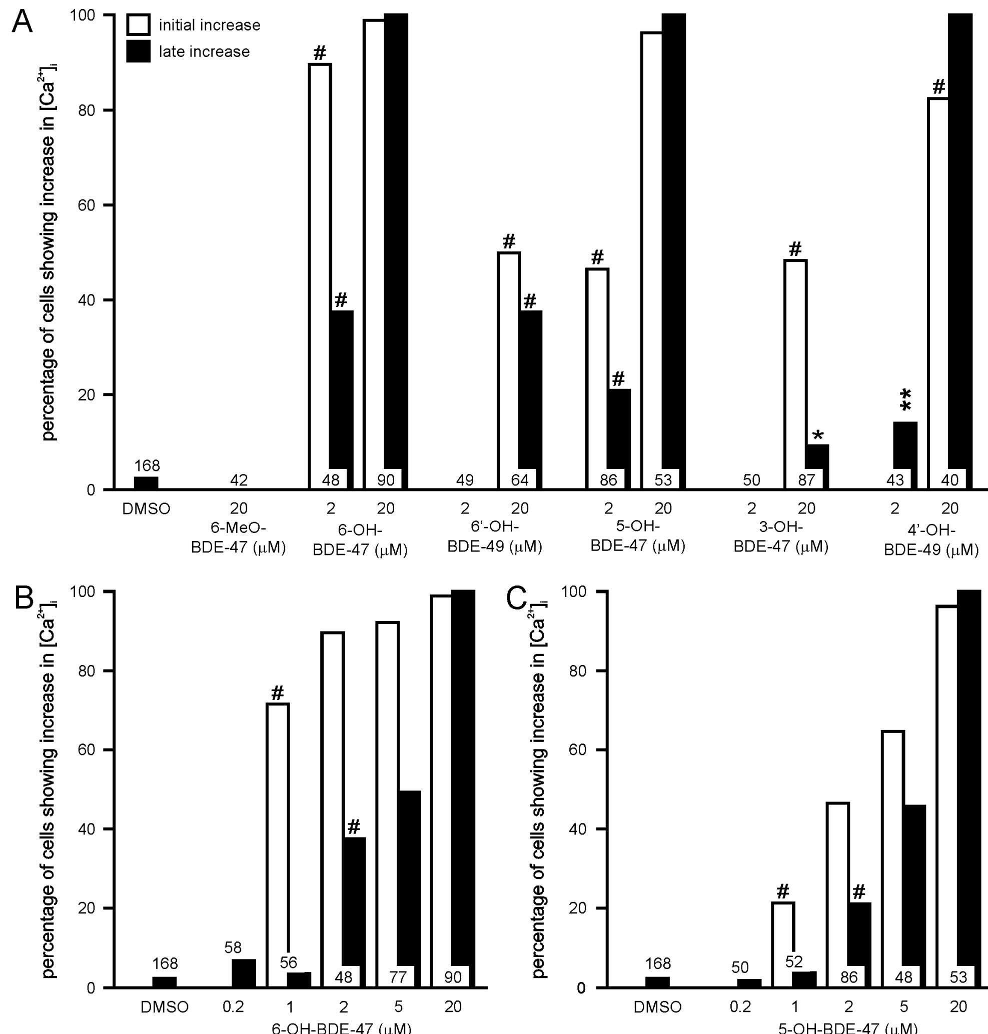


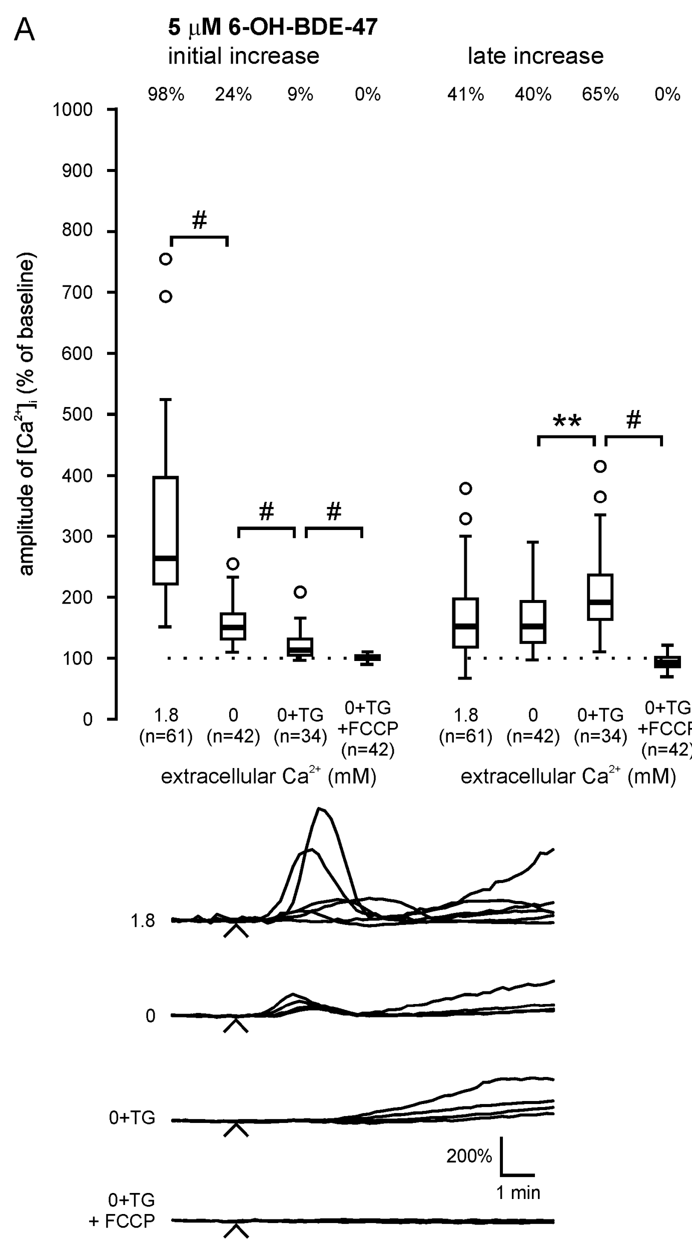
Figure 5.3. Concentration dependence of the occurrence of different types of $[Ca^{2+}]_i$ disturbances during exposure to 2 or 20 µM of 6-MeO-BDE-47, 6-OH-BDE-47, 6'-OH-BDE-49, 5-OH-BDE-47, 3-OH-BDE-47 or 4'-OH-BDE-49. Bars indicate the percentage of cells showing an initial transient increase in $[Ca^{2+}]_i$ (open bars) and those showing a late increase in $[Ca^{2+}]_i$ (closed bars). For 6-OH-BDE-47 (B) and 5-OH-BDE-47 (C), which show initial and late increases of $[Ca^{2+}]_i$ at 2 µM, more concentrations are tested. Significant increases in the percentage of cells showing initial or late increases in $[Ca^{2+}]_i$ are indicated at the lowest-observed-effect concentration (*$p < 0.05$; **$p < 0.01$; #$p < 0.001$). Data are shown from 4 to 19 experiments per concentration; numbers inside the bars indicate the number of cells used for data analysis.

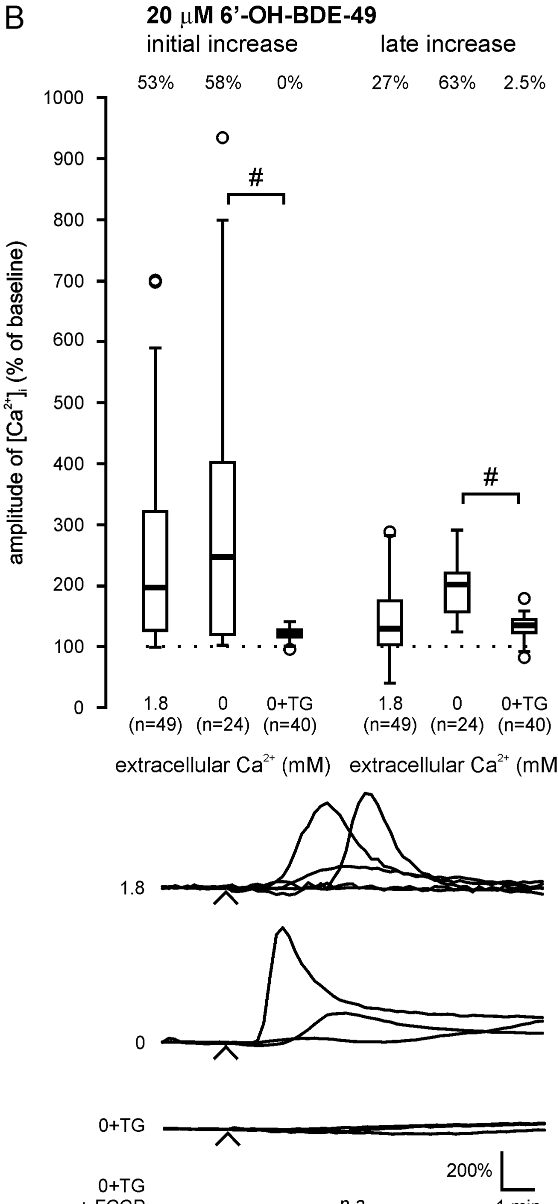
For the OH-PBDEs with an effect at 2 µM (6-OH-BDE-47 and 5-OH-BDE-47), lower concentrations were also tested (Figure 5.2B, 5.2C, 5.3B and 5.3C), showing that the LOECs for these OH-PBDEs are 1 µM. Late increases are observed only at concentrations $\geq$ 2 µM. At concentrations $\leq$ 5 µM, this late increase is of comparable amplitude as the initial increase (~200 - 400% of baseline). At 20 µM, the late increase induced by 6-OH-BDE-47 or 5-OH-BDE-47 is much larger (see Supplemental Material, Table 5.S2), as was also observed for 20 µM 4'-OH-BDE-49. The amplitudes of the initial and late increases observed during exposure to 6-OH-BDE-47 or 5-OH-BDE-47 are dose-dependent (ANOVAs: 6-OH-BDE-47: initial $p < 0.0001$, late $p < 0.0001$; 5-OH-BDE-47: initial: $p < 0.01$, late $p < 0.0001$).

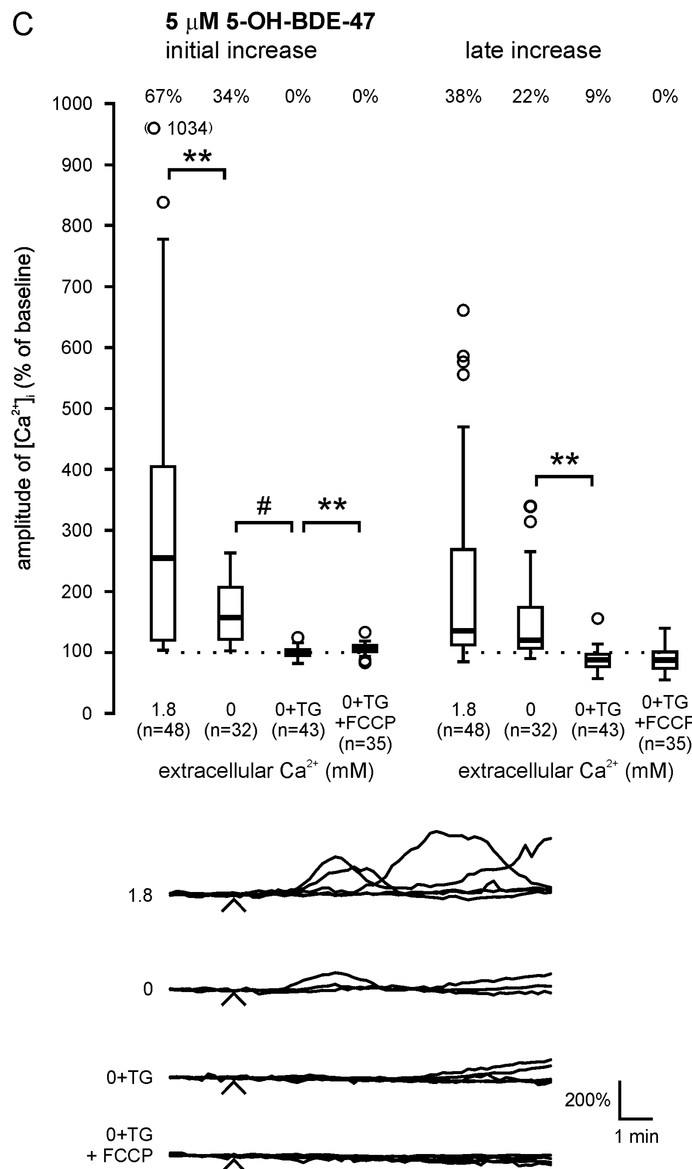
The mean amplitude of $[Ca^{2+}]_i$ during exposure to 20 µM of the hydroxylated metabolites was independent of the position (*ortho*, *meta* or *para*) of the OH-group on the PBDE-molecule (ANOVA: n.s.). However, OH-PBDEs in which the OH-group was shielded on only one side, with either the other phenyl ring or a bromine atom, induce significantly higher increases in $[Ca^{2+}]_i$ compared to OH-PBDEs in which the OH-group was shielded on both sides (ANOVA: $p < 0.01$). However, this influence of the shielding of the OH-group (on one compared to two sides) is independent of its position (*ortho*, *meta* or *para*) on the PBDE-molecule (ANOVA: n.s.).

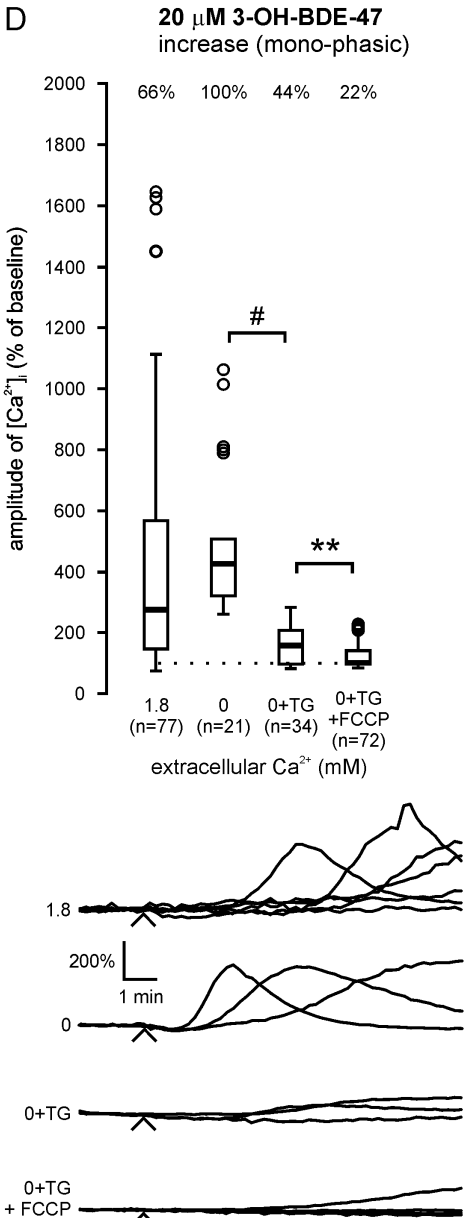
Hydroxylated BDE-47 metabolites increase $[Ca^{2+}]_i$ by release of Ca^{2+} from intracellular stores. Though initial increases in $[Ca^{2+}]_i$ induced by 6-OH-BDE-47, 5-OH-BDE-47, and to a lesser extent by 4'-OH-BDE-49, were reduced in Ca^{2+}-free conditions, increases were still observed for all of the OH-PBDEs (Figure 5.4). To identify the responsible Ca^{2+} stores, endoplasmic reticulum (ER) or both ER and mitochondrial Ca^{2+} stores were depleted by either 1 µM thapsigargin (TG) or 1 µM TG and 1 µM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP). TG and TG/FCCP pretreatment causes a transient increase in $[Ca^{2+}]_i$ of respectively 321 ± 11 ($n = 191$) and 393 ± 11% of baseline ($n = 195$), after which $[Ca^{2+}]_i$ stabilizes and the OH-PBDE is applied.

When depleting ER Ca^{2+} stores in Ca^{2+}-free conditions, the initial increase induced by 5 µM 6-OH-BDE-47 is largely diminished (Figure 5.4A), Although the amplitude of the late increase is not different in Ca^{2+}-free conditions, it increased after depletion of the ER Ca^{2+} stores, but greatly diminished after depletion of both endoplasmic and mitochondrial Ca^{2+} stores.


A
5 μM 6-OH-BDE-47
initial increase
late increase
98%
24%
9%
0%
41%
40%
65%
0%
1000
900
800
700
600
500
400
300
200
100
0
amplitude of [Ca2+]i (% of baseline)
#
#
#
**
#
1.8 (n=61)
0 (n=42)
0+TG (n=34)
0+TG +FCCP (n=42)
1.8 (n=61)
0 (n=42)
0+TG (n=34)
0+TG +FCCP (n=42)
extracellular Ca2+ (mM)
extracellular Ca2+ (mM)
1.8
0
0+TG
200%
1 min
0+TG + FCCP


B
20 μM 6'-OH-BDE-49
initial increase
late increase
53%
58%
0%
27%
63%
2.5%
amplitude of [Ca2+]i (% of baseline)
1000
900
800
700
600
500
400
300
200
100
0
#
#
1.8
(n=49)
0
(n=24)
0+TG
(n=40)
1.8
(n=49)
0
(n=24)
0+TG
(n=40)
extracellular Ca2+ (mM)
extracellular Ca2+ (mM)
1.8
0
0+TG
0+TG
+ FCCP
n.a.
200%
1 min


C
5 μM 5-OH-BDE-47
initial increase
late increase
67% 34% 0% 0%
38% 22% 9% 0%
(O 1034)
**
#
**
**
amplitude of [Ca2+]i (% of baseline)
0
100
200
300
400
500
600
700
800
900
1000
1.8 (n=48)
0 (n=32)
0+TG (n=43)
0+TG +FCCP (n=35)
1.8 (n=48)
0 (n=32)
0+TG (n=43)
0+TG +FCCP (n=35)
extracellular Ca2+ (mM)
extracellular Ca2+ (mM)
1.8
0
0+TG
0+TG + FCCP
200%
1 min


D
20 μM 3-OH-BDE-47
increase (mono-phasic)
66%
100%
44%
22%
2000
1800
1600
1400
1200
1000
800
600
400
200
0
amplitude of [Ca2+]i (% of baseline)
#
**
1.8
(n=77)
0
(n=21)
0+TG
(n=34)
0+TG
+FCCP
(n=72)
extracellular Ca2+ (mM)
1.8
200%
1 min
0
0+TG
0+TG
+ FCCP

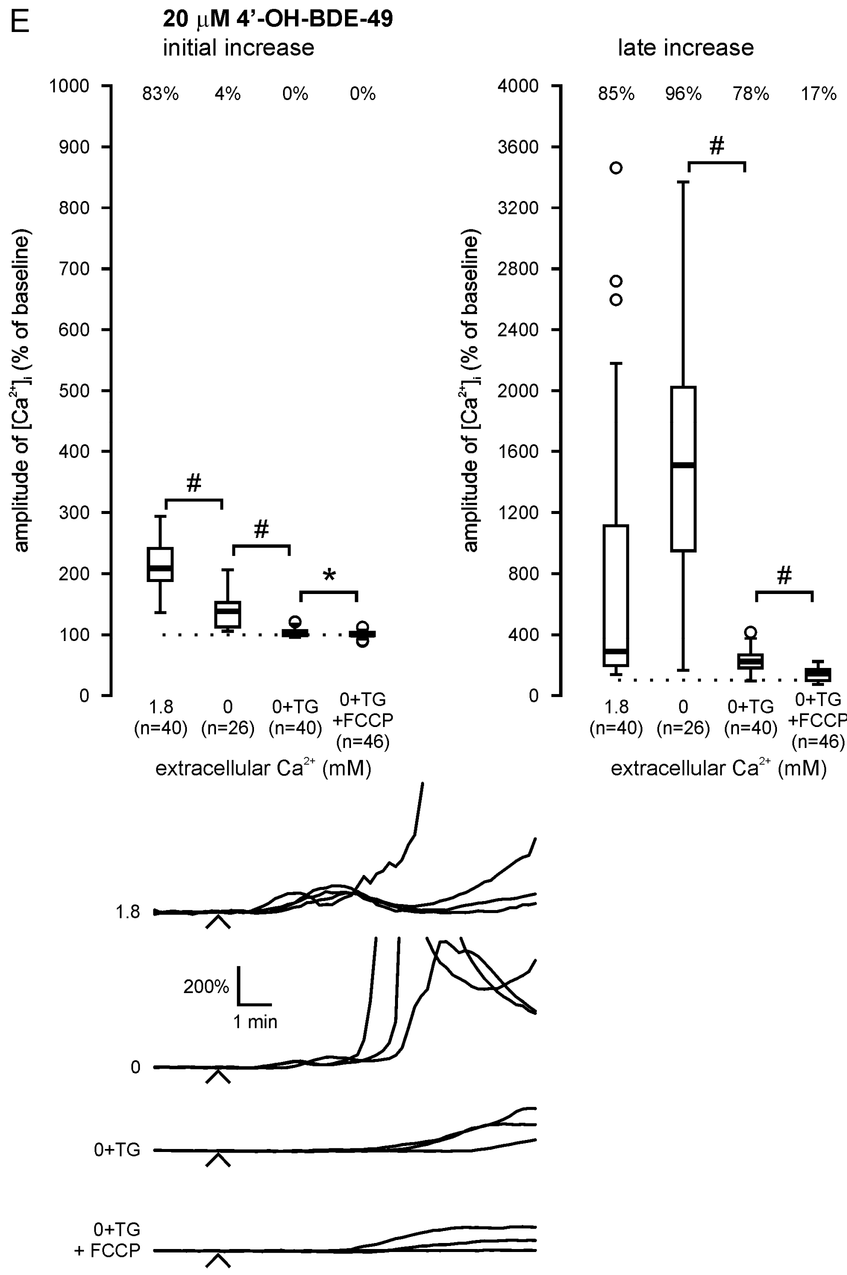

E
20 μM 4'-OH-BDE-49
initial increase
late increase
83%
4%
0%
0%
85%
96%
78%
17%
amplitude of [Ca2+]i (% of baseline)
1000
900
800
700
600
500
400
300
200
100
0
4000
3600
3200
2800
2400
2000
1600
1200
800
400
#
*
1.8 (n=40)
0 (n=26)
0+TG (n=40)
0+TG +FCCP (n=46)
extracellular Ca2+ (mM)
1.8
0
0+TG
0+TG + FCCP
200%
1 min

Figure 5.4. Increase in $[Ca^{2+}]_i$ by OH-PBDEs results from the release of Ca^{2+} from intracellular stores. Boxes represent amplitudes of $[Ca^{2+}]_i$ in PC12 cells during exposure to 6-OH-BDE-47 (5 µM, A), 6'-OH-BDE-49 (20 µM; B), 5-OH-BDE-47 (5 µM; C), 3-OH-BDE-47 (20 µM; D) and 4'-OH-BDE-49 (20 µM; E) measured in external saline (1.8 mM Ca^{2+}), Ca^{2+}-free saline (0 mM Ca^{2+}), Ca^{2+}-free saline after pretreatment with TG (0 mM Ca^{2+} + TG), and Ca^{2+}-free saline after pretreatment with both TG and FCCP (0 mM Ca^{2+} + TG + FCCP). Cells were pre-treated with TG and FCCP to deplete respectively ER and mitochondrial Ca^{2+} stores. Upper and lower borders of the box represent upper and lower quartiles, the line within the box is the median, whiskers represent lowest and highest values, and circles represent outliers (outliers more than 3 interquartile ranges from the box not shown for clarity). Data shown are from 3 to 7 experiments per treatment; numbers below each box indicate the number of cells used for data analysis. The percentage of responding cells (with increase in $[Ca^{2+}]_i$ to > 175% of baseline) are denoted above each box. * $p < 0.05$; ** $p < 0.01$; # $p < 0.001$. Representative traces of $[Ca^{2+}]_i$ measurements of individual PC12 cells exposed to OH-PBDE for 10 min (applied as indicated by arrowheads) in external saline (containing 1.8 mM Ca^{2+}) and in Ca^{2+}-free conditions are shown below.

The variety in $[Ca^{2+}]_i$ responses during exposure to 20 µM 6'-OH-BDE-49 was also observed in Ca^{2+}-free conditions (Figure 5.4B). After depletion of ER Ca^{2+} stores, both initial and late transient increases in $[Ca^{2+}]_i$ were no longer observed. When depleting the ER Ca^{2+} stores, the amplitudes of both initial and late increases induced by 5 µM 5-OH-BDE-47 are largely diminished (Figure 5.4C). After depletion of both endoplasmic and mitochondrial Ca^{2+} stores, the late increase is further reduced. Monophasic increases during exposure to 20 µM 3-OH-BDE-47 are observed in Ca^{2+}-free conditions, with similar amplitude (Figure 5.4D). After depletion of the ER Ca^{2+} stores the amplitude is significantly decreased, and even further after depletion of endoplasmic and mitochondrial Ca^{2+} stores. During exposure to 20 µM 4'-OH-BDE-49 after depletion of the ER Ca^{2+} stores, the amplitude of the initial increase is largely diminished (Figure 5.4E). The amplitude of the late increase is not significantly changed in Ca^{2+}-free conditions. After depletion of the ER Ca^{2+} stores, the amplitude is significantly decreased, and even further after depletion of endoplasmic and mitochondrial Ca^{2+} stores.

Lower LOECs are identified for hydroxylated metabolites when investigating fluctuations. At LOECs and NOECs based on the amplitude of $[Ca^{2+}]_i$, fluctuations in $[Ca^{2+}]_i$ were investigated (Table 5.1, see also Supplemental Material, Table 5.S4). No effects could be detected on the percentage of cells showing fluctuations or the frequency, duration and amplitude of these fluctuations during exposure to 2 µM 6'-OH-BDE-49 or 2 µM 3-OH-BDE-47. During exposure to 0.2 µM 6-OH-BDE-47, an increase in the percentage of cells showing fluctuations was observed. The average duration of fluctuations was increased, without effects on the frequency or amplitude (NOEC: 0.1 µM 6-OH-BDE-47). At 1 µM 5-OH-BDE-47, the number of cells showing fluctuations was increased. While the duration increased, the amplitude and frequency were not affected (NOEC: 0.2 µM 5-OH-BDE-47). At 2 µM 4'-OH-BDE-49, the number of cells showing fluctuations was increased, as well as the average fluctuation frequency, duration and amplitude (NOEC: 1 µM 4'-OH-BDE-49).

Table 5.1. Table summarizes the LOECs of BDE-47 and hydroxylated metabolites on cell viability and different parameters of $[Ca^{2+}]_i$ investigated in PC12 cells in the present research. A: Below the mechanisms responsible for effects on Ca^{2+} homeostasis, LOEC values of effects causing a decrease of > 25% in the specific Ca^{2+}-free experiments (Figure 5.4) are indicated, as well as resulting NOEC levels. B: Below the investigated parameters of fluctuations in $[Ca^{2+}]_i$, LOEC values are indicated, as well as resulting NOEC levels. When NOEC levels for effects on fluctuations in $[Ca^{2+}]_i$, are lower compared to NOEC values for effects on Ca^{2+} homeostasis related processes, this is indicated by (<). For values of $[Ca^{2+}]_i$ and fluctuation parameters, see Supplemental Material, Tables 5.S2 and 5.S3. For table including all PBDEs and MeO/OH-PBDEs investigated in this study, see Supplemental Material, Table 5.S4.

	decreased cell viability	A: Ca^{2+} homeostasis				B: fluctuations in $[Ca^{2+}]_i$				
		Ca^{2+} influx (extracellular)	Ca^{2+} release (ER)	Ca^{2+} release (mitochondria)	NOEC	% of cells showing fluctuations	fluctuation frequency	fluctuation duration	fluctuation amplitude	NOEC
BDE-47	-	-	-	-	20 µM	-	2 µM	2 µM	-	1 µM (<)
6-OH-BDE-47	20 µM	1 µM	1 µM	2 µM	0.2 µM	0.2 µM	-	0.2 µM	-	0.1 µM (<)
6'-OH-BDE-49	-	-	20 µM	-	2 µM	20 µM[a]	20 µM[a]	20 µM[a]	20 µM[a]	2 µM[b]
5-OH-BDE-47	20 µM	1 µM	1 µM	-	0.2 µM	1 µM	-	1 µM	-	0.2 µM[b]
3-OH-BDE-47	-	-	20 µM	20 µM	2 µM	20 µM[a]	20 µM[a]	20 µM[a]	20 µM[a]	2 µM
4'-OH-BDE-49	20 µM	20 µM	20 µM	20 µM	2 µM	2 µM	2 µM	2 µM	2 µM	1 µM (<)

Discussion

Hydroxylated PBDE metabolite 6-OH-BDE-47 has previously been shown to disrupt $[Ca^{2+}]_i$ in PC12 cells by releasing Ca^{2+} from intracellular stores at lower concentrations than its parent compound BDE-47 (Dingemans et al. 2008). The results presented here demonstrate that also other hydroxylated metabolites of BDE-47 induce Ca^{2+} release from intracellular stores, while the investigated methoxylated analogue and parent compounds lack this effect (Figure 5.2). From experiments in Ca^{2+}-free conditions (Figure 5.4) it can be concluded that the initial increases induced by 6-OH-BDE-47, 5-OH-BDE-47 and 4'-OH-BDE-49 are partly caused by influx of extracellular Ca^{2+}. The initial and late increases induced by 6-OH-BDE-47 are caused by release of Ca^{2+} from respectively ER and mitochondria. The widely varying increase induced by 6'-OH-BDE-49 is mainly caused by release from endoplasmic Ca^{2+} stores. Both initial and late increases induced by 5-OH-BDE-47 and the increase induced by 3-OH-BDE-47 are caused by release of Ca^{2+} mainly from ER, but also from mitochondria. Both initial and late increases induced by 4'-OH-BDE-49 are caused by release of Ca^{2+} from ER, but the late increase also from mitochondria. When investigating fluctuations in $[Ca^{2+}]_i$, subtle effects of BDE-47 on the Ca^{2+} homeostasis are detected and lower LOECs are observed for several OH-PBDEs (Table 5.1).

No or mild effects on cell viability were detected for the investigated PBDEs and MeO/OH-PBDEs, indicating that the observed effects on $[Ca^{2+}]_i$ are not confounded by cytotoxicity. The AB assay, which is based on mitochondrial activity, appeared to be less useful to determine cell viability, as for 6-OH-BDE-47, 6'-OH-BDE-49 and 3-OH-BDE-47 the relative fluorescence intensity was increasing dose-dependently above the control level, suggesting induction of mitochondrial activity. This may be related to mitochondrial uncoupling, which was previously demonstrated for 6-OH-BDE-47 in isolated zebrafish mitochondria (van Boxtel et al. 2008).

The low basal $[Ca^{2+}]_i$ of PC12 cells is maintained by the removal of Ca^{2+} ions by the plasma membrane Ca^{2+} ATPases and Na^{2+}-Ca^{2+} exchanger (Duman et al. 2008, reviewed by Westerink 2006). Additionally, Ca^{2+} can be sequestered into organelles, mainly ER and mitochondria. 3-OH-BDE-47 and 4'-OH-BDE-49 induce increases in $[Ca^{2+}]_i$ even after depletion of both ER and mitochondria by TG and FCCP. It has been shown that Ca^{2+} also accumulates in endosomes, lysosomes, secretory granules, the Golgi-apparatus and nucleus (reviewed by Laude and Simpson 2009). The Golgi-apparatus stores Ca^{2+} via sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase pumps (Missiaen et al. 2007), which are inhibited by TG. Therefore, it is unlikely that release of Ca^{2+} from the Golgi-apparatus is causing the additional increase, while this remains unclear for the other mentioned organelles.

At concentrations not affecting the average and amplitude of increases in $[Ca^{2+}]_i$, BDE-47, 6-OH-BDE-47 and 4'-OH-BDE-49 caused an increase in the frequency, amplitude and/or duration of fluctuations in $[Ca^{2+}]_{i.}$ These subtle effects on the Ca^{2+} homeostasis resulted in lower NOECs for most of these BFRs, particularly BDE-47 (Table 5.1). Ca^{2+} signals

vary from microdomains to global across the cell and from milliseconds to many hours (Laude and Simpson 2009). As the measured $[Ca^{2+}]_i$ is an average value for the entire cytosol, underestimation of membrane- or store-associated high $[Ca^{2+}]_i$, or high $[Ca^{2+}]_i$ microdomains (reviewed in Cheng and Lederer 2008) can be expected. The timing of Ca^{2+} signals, including frequency and duration, impacts how external stimuli cause (patho-)physiological results (Boulware and Marchant 2008). Moreover, $[Ca^{2+}]_i$ transients trigger activity-dependent developmental events in neurons, either by activating gene expression, cytoskeletal elements or neurotransmitter release, while the characteristics of these responses are determined by amplitude, frequency, source and spatial location of Ca^{2+} signals (reviewed in Moody and Bosma 2005). Therefore, the observed effects of OH-PBDEs at low concentrations can be of relevance for the development of the nervous system.

None of the parent PBDEs, except BDE-47, showed any effects on the Ca^{2+} homeostasis in PC12 cells. Nonetheless, neurotoxic effects of BDE-47, BDE-99, BDE-100 as well as BDE-153 have been detected at different biological levels (Costa and Giordano 2007). Because of the lack of effects in this study, no effects of bromination pattern could be investigated. The activity of the OH-PBDE was confirmed to depend on the presence of the OH-group, as no effects were observed during exposure to the methoxylated analogue of 6-OH-BDE-47. The higher activity of 6-OH-BDE-47 compared to its methoxylated analogue is in line with other studies, mostly on endocrine effects (van Boxtel et al. 2008; Cantón et al. 2008; Kojima et al 2009). The mean amplitude of increases in $[Ca^{2+}]_i$ could not be related to the location of the OH-group on the PBDE-molecule. However, it appeared that when the OH-group is shielded on both sides by either the other phenyl ring and/or Br-atoms (as in 6’-OH-BDE-49 and 3-OH-BDE-47), the OH-PBDE increased $[Ca^{2+}]_i$ less than when the OH-group is less shielded (as in 6-OH-BDE-47, 5-OH-BDE-47 and 4’-OH-BDE-49). Also, the OH-PBDEs with only one shielded side of the OH-group induce release of Ca^{2+} from ER at the lowest concentrations (6-OH-BDE-47 and 5-OH-BDE-47) or with the highest amplitude in Ca^{2+}-free conditions (4’-OH-BDE-49). Thus, the toxicity of OH-PBDEs appears attenuated by shielding of the OH-group on both sides by either the other phenyl ring and/or Br-atoms.

Several animal studies confirmed the generation of hydroxylated metabolites of PBDEs *in vivo* (Hakk et al. 2009; Malmberg et al. 2005; Marsh et al. 2006) and OH-PBDEs were also formed in human liver cells exposed to BDE-99 (Stapleton et al. 2009). Interesting, marine organisms have also been shown to produce hydroxylated as well as methoxylated PBDE metabolites (Hakk and Letcher 2003).

Only very recently, the occurrence and accumulation of hydroxylated metabolites was confirmed in humans (Athanasiadou et al. 2008), with sum OH-PBDE serum concentrations up to 120 pmol/g lipids. Another study also detected OH-PBDEs in US fetal serum samples and confirmed the bioaccumulation of these metabolites (Qiu et al. 2009). Moreover, they demonstrated that concentrations of OH-PBDEs were similar or sometimes even higher than the concentration of PBDEs. Fetal sum OH-PBDE serum concentrations ranged from 2.01 to 899.1 ng/g lipids (median: 21.96 ng/g lipids). The most abundant BDE-47 metabolites in fetal

blood were 5-OH-BDE-47 and 6-OH-BDE-47 (Qiu et al. 2009). It is concerning that these metabolites caused an increase of $[Ca^{2+}]_i$ at much lower concentrations than the other metabolites of BDE-47 investigated.

All five OH-PBDEs investigated in our study caused Ca^{2+} release from intracellular Ca^{2+} stores, although with different LOECs. Likely, depending on the position the OH-group and adjacent phenyl ether and/or Br-atoms, other hydroxylated metabolites of tetra- and penta-PBDEs have a similar effect on cellular calcium homeostasis. The median (21.96 ng/g lipids) and highest concentrations (899.1 ng/g lipids) of sum OH-PBDEs observed in fetal plasma correspond to respectively ~0.4 and ~17.4 nM in blood (calculated with average physiological parameters). Thus, the highest concentration observed in human blood is only two orders of magnitude lower than the LOEC for Ca^{2+} release from intracellular stores by OH-PBDEs (1 µM). Moreover, the LOEC for increased Ca^{2+} fluctuations is even lower (0.2 µM), meaning that the margin of exposure is insufficient in some individual exposure situations. Also, since OH-PBDEs are not associated with lipids, as are the parent PBDEs, but have a high affinity for plasma proteins (Verreault et al. 2005), the estimated blood concentration calculated from exposure values at a lipid weight adjusted basis (ng/g lipids) may be underestimated. However, the LOEC (1 µM) used to calculate the margin of exposure is higher for other metabolites, and it remains to be determined whether the observed effects on fluctuations in $[Ca^{2+}]_i$ could result in functional or even adverse effects *in vivo*.

As it has been shown that exposure to organohalogen compounds within the time frame of rapid brain development can result in behavioral defects in mice (reviewed in Costa and Giordano 2007), it is concerning that children are exposed to these environmental pollutants pre- as well as postnatally. Moreover, several studies have observed interactions between environmental pollutants to enhance (neuro-)toxicity. Additive and synergistic neurotoxic effects of polychlorinated biphenyls (PCBs) and PBDEs have been detected *in vivo* (Eriksson et al. 2006) and *in vitro* (Gao et al. 2009). Concern about possible effects on the developing brain arises from the fact that an increase in $[Ca^{2+}]_i$ by release from intracellular stores appears to be a common mechanism for OH-PBDEs and *ortho*-PCBs (reviewed by Mariussen and Fonnum 2006). Therefore, a possible additive effect of these environmental pollutants with respect to increases in cytosolic $[Ca^{2+}]_i$, which is not only a trigger for neurotransmitter release but affects many cellular processes (Clapham 2007), is not unlikely.

As very high concentrations of PBDEs are occasionally measured in humans, the voluntary and legislative measures to reduce the release of PBDEs into the environment appear justified. Also, hydroxylated metabolites of PBDEs, which were recently found to bioaccumulate in humans (Athanasiadou et al. 2008; Qiu et al. 2009), either from man-made PBDEs or of natural origin, are currently not taken into account in regulatory human risk assessment. The results presented here revealed a structure-activity relationship for metabolites of PBDEs (more shielding of the OH-group reduces the potency of OH-PBDEs) and reinforce that oxidative metabolism should be included in human risk assessment of persistent organic pollutants.

Materials and Methods supplemental

Cell culture. Rat pheochromocytoma (PC12) cells (Greene and Tischler 1976), obtained from ATCC (American Type Culture Collection, Manassas, VA, USA), were cultured as described previously (Dingemans et al. 2008). Briefly, PC12 cells were cultured for up to 15 passages in RPMI 1640 (Invitrogen, Breda, The Netherlands) supplemented with 5% fetal calf serum and 10% horse serum (ICN Biomedicals, Zoetermeer, The Netherlands). For Ca^{2+} imaging experiments, PC12 cells were subcultured in poly-L-lysine coated glass-bottom dishes (MatTek, Ashland MA, USA).

Cell viability assay. To investigate possible acute effects of the PBDEs on cell viability, the Alamar Blue (AB) and neutral red (NR) uptake assays were used with minor modifications (Magnani and Bettini 2000; Repetto et al. 2008). Briefly, PC12 cells were plated at a density of $2*10^5$ cells/well 24 h before exposure in 24-wells plates. Cells were exposed for 20 min to 20 µM polybrominated diphenyl ether (PBDE) or 0.2 - 20 µM hydroxylated PBDE (OH-PBDE) in saline (containing 1.8 mM $CaCl_2$, 24 mM glucose, 10 mM HEPES, 5.5 mM KCl, 0.8 mM $MgCl_2$, 125 mM NaCl, and 36.5 mM sucrose, adjusted to pH 7.3 with NaOH). Cells were subsequently incubated with 400 µl AB (6.25 µM) in saline for 30 min at 37°C in the dark. Fluorescence was measured, with excitation at 530 nm and emission at 590 nm using a FLUOstar Galaxy V4.30-0 platereader (BMG Labtechnologies, Offenburg, Germany). The same plate was then incubated with 800 µl NR (33 µg/ml) in saline for 60 min at 37°C in the dark. The cells were lysed using 400 µl extraction solution (50% ethanol absolute, 49% MilliQ water, 1% acetic acid). After 10 min shaking, fluorescence was measured, with excitation at 530 nm and emission at 645 nm using a FLUOstar Galaxy V4.30-0.

Intracellular Ca^{2+} imaging. Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured using the Ca^{2+}-sensitive fluorescent ratio dye Fura-2 as described previously (Dingemans et al. 2007, 2008). Briefly, cells were loaded with 5 µM Fura-2-AM (Molecular Probes; Invitrogen, Breda, the Netherlands) in saline for 20 min at room temperature; this was followed by 15 min de-esterification in saline. The cells were then placed on the stage of an Axiovert 35M inverted microscope (Zeiss, Göttingen, Germany) equipped with a TILL Photonics Polychrome IV (TILL Photonics GmbH, Gräfelfing, Germany). Fluorescence, evoked by 340 and 380 nm excitation wavelengths (F_{340} and F_{380}), was recorded every 12 sec at 510 nm with an Image SensiCam digital camera (TILL Photonics GmBH). The digital camera and polychromator were controlled by imaging software (TILLvisION, version 4.01), which was also used for data collection and processing. After 5 min baseline recording, cells were exposed to 0.1 - 20 µM of the PBDE(-metabolites). Maximum and minimum ratios (R_{max}/R_{min}) were determined after 25 min recording (20 min exposure) by addition of ionomycin (5 µM) and EDTA (17 mM), respectively.

Where applicable, cells were washed with Ca^{2+}-free saline (containing 10 µM EDTA to remove residual extracellular Ca^{2+}) just before the imaging experiments. In specific experiments, thapsigargin (TG)-responsive endoplasmic and mitochondrial Ca^{2+} stores were emptied by incubation with respectively 1 µM TG and 1 µM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) in Ca^{2+}-free saline for 10 min (Dingemans et al. 2008). TG is a high-affinity inhibitor of sarcoplasmic/endoplasmic reticulum (ER) Ca^{2+} ATPase (SERCA; Toyoshima and Inesi 2004). FCCP depolarizes the mitochondrial membranes, resulting in the uncoupling of oxidative phosphorylation and subsequent Ca^{2+} release from mitochondria (Taylor et al. 2000). These compounds are commonly used under experimental conditions to empty intracellular Ca^{2+} stores.

Free cytosolic $[Ca^{2+}]_i$ was calculated using Grynkiewicz's equation

$$[Ca^{2+}]_i = K_{d*} \times (R - R_{min})/(R_{max} - R)$$

(as described in Deitmer and Schild, 2000), where K_{d*} is the dissociation constant of Fura-2 determined in the experimental set-up used for the fluorescence measurements using Fura-2 Ca^{2+} imaging calibration buffers (Molecular Probes; Invitrogen). For Ca^{2+}-free experiments, in which baseline $[Ca^{2+}]_i$ is lower, a correction factor is applied to allow for comparison with experiments in normal (1.8 mM) Ca^{2+}-conditions.

Table 5.S1. Full names of the PBDEs and hydroxylated PBDEs discussed in this paper.

3-OH-BDE-47	3-hydroxy-2,2',4,4'-tetrabromodiphenyl ether
4'-OH-BDE-49	4'-hydroxy-2,2',4,5'-tetrabromodiphenyl ether
5-OH-BDE-47	5-hydroxy-2,2',4,4'-tetrabromodiphenyl ether
6-MeO-BDE-47	6-methoxy-2,2',4,4'-tetrabromodiphenyl ether
6-OH-BDE-47	6-hydroxy-2,2',4,4'-tetrabromodiphenyl ether
6'-OH-BDE-49	6'-hydroxy-2,2',4,5'-tetrabromodiphenyl ether
BDE-47	2,2',4,4'-tetrabromodiphenyl ether
BDE-49	2,2',4,5'-tetrabromodiphenyl ether
BDE-99	2,2',4,4',5-pentabromodiphenyl ether
BDE-100	2,2',4,4',6-pentabromodiphenyl ether
BDE-153	2,2',4,4',5,5'-hexabromodiphenyl ether
BDE-209	2,2',3,3',4,4',5,5',6,6'-decabromodiphenyl ether

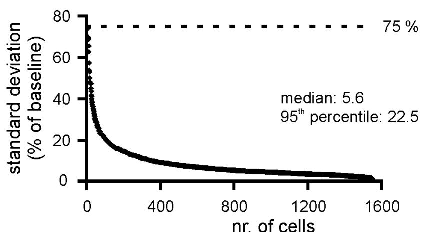


Figure 5.S1. Distribution of standard deviations (sorted descending) calculated from 5 min baseline Ca^{2+} recordings (*n* = 1538, 160 experiments) in normal Ca^{2+} conditions (1.8 mM Ca^{2+}), to determine the minimum change in $[Ca^{2+}]_i$. To prevent false positive results, increases of $[Ca^{2+}]_i$ to > 175% of baseline are considered relevant.

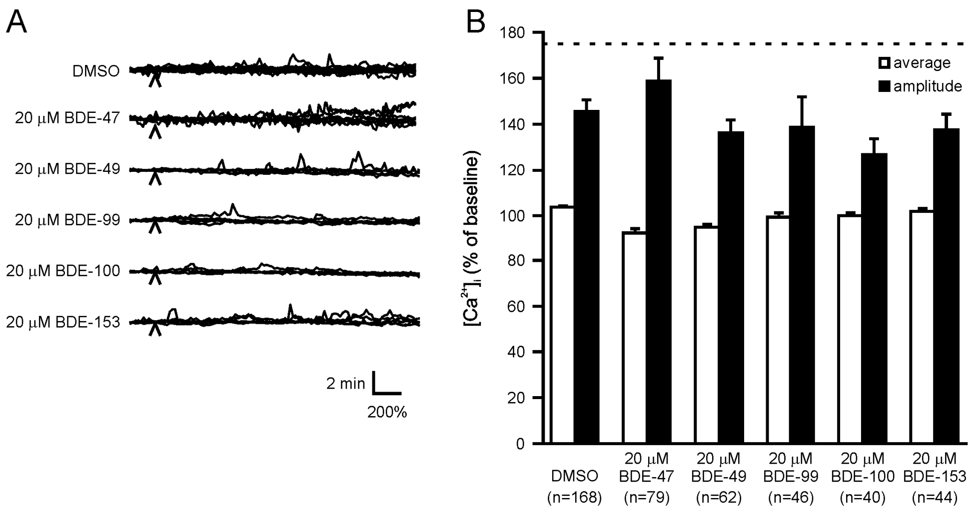


Figure 5.S2. Lack of increase in $[Ca^{2+}]_i$ in PC12 cells during exposure to PBDEs. Results are shown as representative traces (A) of normalized $[Ca^{2+}]_i$ from individual PC12 cells exposed to DMSO, 20 µM BDE-47, BDE-49, BDE-99, BDE-100 and BDE-153 for 20 min (applied at t = 0 min as indicated by the arrowheads) and as average (B, open bars) and amplitude (closed bars) of $[Ca^{2+}]_i$ during exposure to the PBDEs. None of the PBDEs increases $[Ca^{2+}]_i$ > 175% of baseline (as indicated with dashed line). Data are shown from 5 to 19 experiments per concentration; numbers below each bar indicate the number of cells used for data analysis.

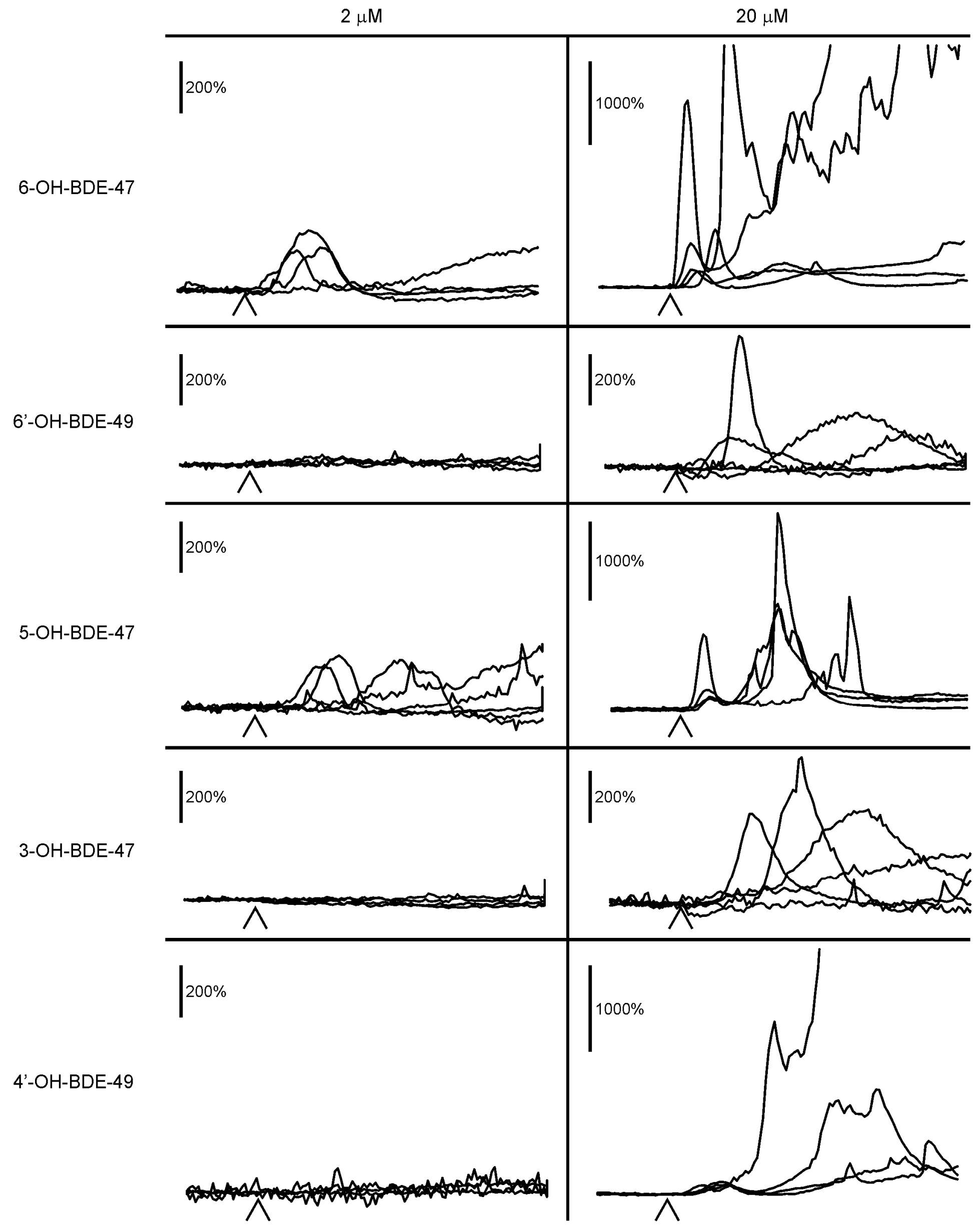


Figure 5.S3. Increase in $[Ca^{2+}]_i$ in PC12 cells during exposure to hydroxylated metabolites of BDE-47. Results are shown as representative traces of normalized $[Ca^{2+}]_i$ from individual PC12 cells exposed to 2 or 20 μM 6-OH-BDE-47, 6'-OH-BDE-49, 5-OH-BDE-47, 3-OH-BDE-47 or 4'-OH-BDE-49 for 20 min, applied as indicated by arrowheads. Note the difference in scaling for 20 μM 6-OH-BDE-47, 5-OH-BDE-47 and 4'-OH-BDE-49 compared to the other traces.

Table 5.S2. Effects of OH-PBDEs on the amplitude of initial and late increases in $[Ca^{2+}]_i$ in PC12 cells during 20 min exposure. No initial or late increases were observed during exposure to DMSO, the parent PBDE congeners (BDE-47, BDE-49, BDE-99, BDE-100 and BDE-153) or 6-MeO-BDE-47. Data are expressed as mean ± SE from the number of cells (*n*) indicated.

	concentration (µM)	$[Ca^{2+}]_i$ amplitude of initial increase (% of baseline)	$[Ca^{2+}]_i$ amplitude of late increase (% of baseline)
6-OH-BDE-47	0.2	n.a.	n.a.
	1	249 ± 9 (*n* = 40)	n.a.
	2	280 ± 15 (*n* = 43)	247 ± 15 (*n* = 18)
	5	316 ± 14 (*n* = 71)	301 ± 17 (*n* = 38)
	20	481 ± 40 (*n* = 89)	2485 ± 408 (*n* = 83)
6’-OH-BDE-49	2	n.a.	n.a.
	20	566 ± 95 (*n* = 32)	235 ± 9 (*n* = 24)
5-OH-BDE-47	0.2	n.a.	n.a.
	1	248 ± 22 (*n* = 11)	n.a.
	2	273 ± 13 (*n* = 40)	306 ± 24 (*n* = 18)
	5	443 ± 56 (*n* = 31)	302 ± 32 (*n* = 22)
	20	553 ± 70 (*n* = 51)	1803 ± 155 (*n* = 52)
3-OH-BDE-47	2	n.a.	n.a.
	20	868 ± 127 (*n* = 42)	236 ± 15 (*n* = 8)
4’-OH-BDE-49	1	n.a.	n.a.
	2	n.a.	231 ± 27 (*n* = 6)
	20	224 ± 5 (*n* = 33)	2518 ± 486 (*n* = 40)

Table 5.S3. Effects of (OH-)PBDEs, at NOECs based on the amplitude of increase of $[Ca^{2+}]_i$, on the percentage of cells showing fluctuations and the frequency, duration and amplitude of these fluctuations. Data are expressed as mean ± SE from the number of cells (*n*) or fluctuations (*N*) indicated. Statistical significance compared to DMSO control is indicated by: n.s. not significant; * $p < 0.05$; ** $p < 0.01$; # $p < 0.001$.

	concentration (μM)	% of cells showing fluctuations	fluctuation frequency (fluctuations/h)	fluctuation duration (min)	fluctuation amplitude (% of baseline)
DMSO	n.a.	14 (*n* = 168)	1.2 ± 0.3 (*n* = 168)	0.4 ± 0.0 (*N* = 65)	205 ± 4 (*N* = 65)
BDE-47	1	23 (*n* = 26)[n.s.]	2.4 ± 1.1 (*n* = 26)[n.s.]	0.4 ± 0.1 (*N* = 18)[n.s.]	194 ± 4 (*N* = 18)[n.s.]
	2	31 (*n* = 48)[#]	2.8 ± 0.9 (*n* = 48)[*]	0.7 ± 0.1 (*N* = 140)[*]	212 ± 50 (*N* = 140)[n.s.]
	20	23 (*n* = 79)[n.s.]	2.3 ± 0.6 (*n* = 79)[*]	0.8 ± 0.2 (*N* = 60)[*]	202 ± 8 (*N* = 60)[n.s.]
BDE-49	20	16 (*n* = 62)[n.s.]	1.0 ± 0.4 (*n* = 62)[n.s.]	0.5 ± 0.2 (*N* = 21)[n.s.]	203 ± 6 (*N* = 21)[n.s.]
BDE-99	20	7 (*n* = 46)[n.s.]	0.5 ± 0.4 (*n* = 46)[n.s.]	-[a]	-[a]
BDE-100	20	5 (*n* = 40)[n.s.]	0.2 ± 0.1 (*n* = 40)[n.s.]	-[a]	-[a]
BDE-153	20	11 (*n* = 44)[n.s.]	0.6 ± 0.2 (*n* = 44)[n.s.]	-[a]	-[a]
6-MeO-BDE-47	20	24 (*n* = 42)[n.s.]	1.6 ± 0.5 (*n* = 42)[n.s.]	0.3 ± 0.0 (*N* = 23)[n.s.]	193 ± 4 (*N* = 23)[n.s.]
6-OH-BDE-47	0.1	20 (*n* = 35)[n.s.]	1.5 ± 0.7 (*n* = 35)[n.s.]	1.1 ± 0.4 (*N* = 18)[n.s.]	209 ± 11 (*N* = 18)[n.s.]
	0.2	31 (*n* = 58)[**]	1.7 ± 0.4 (*n* = 58)[n.s.]	1.3 ± 0.4 (*N* = 33)[**]	223 ± 19 (*N* = 33)[n.s.]
6'-OH-BDE-49	2	10 (*n* = 49)[n.s.]	0.3 ± 0.2 (*n* = 49)[n.s.]	no data (*N* = 5)	no data (*N* = 5)
5-OH-BDE-47	0.2	14 (*n* = 50)[n.s.]	0.5 ± 0.2 (*n* = 50)[n.s.]	no data (*N* = 7)	no data (*N* = 7)
	1	33 (*n* = 52)[**]	1.6 ± 0.4 (*n* = 52)[n.s.]	2.4 ± 0.5 (*N* = 28)[#]	203 ± 4 (*N* = 28)[n.s.]
3-OH-BDE-47	2	10 (*n* = 49)[n.s.]	2.0 ± 0.8 (*n* = 49)[n.s.]	0.4 ± 0.0 (*N* = 50)[n.s.]	238 ± 24 (*N* = 50)[n.s.]
4'-OH-BDE-49	1	14 (*n* = 22)[n.s.]	1.2 ± 0.8 (*n* = 22)[n.s.]	no data (*N* = 9)	no data (*N* = 9)
	2	35 (*n* = 43)[**]	4.3 ± 1.3 (*n* = 43)[#]	0.8 ± 0.1 (*N* = 88)[*]	288 ± 20 (*N* = 88)[#]

a. The number of data points to investigate duration and amplitude of the fluctuations in $[Ca^{2+}]_i$ is insufficient.

Table 5.S4. Table summarizes the LOECs of BDE-47 and hydroxylated metabolites on cell viability and different parameters of $[Ca^{2+}]_i$ investigated in PC12 cells in the present research. A: Below the mechanisms responsible for effects on Ca^{2+} homeostasis, LOEC values of effects causing a decrease of > 25% in the specific Ca^{2+}-free experiments (Figure 5.5) are indicated, as well as resulting NOEC levels. B: Below the investigated parameters of fluctuations in $[Ca^{2+}]_i$, LOEC values are indicated, as well as resulting NOEC levels. When NOEC levels for effects on fluctuations in $[Ca^{2+}]_i$, are lower compared to NOEC values for effects on Ca^{2+} homeostasis related processes, this is indicated by (<). Values of $[Ca^{2+}]_i$ and fluctuation parameters are shown in Tables 5.S2 and 5.S3.

		A: Ca^{2+} homeostasis				B: fluctuations in $[Ca^{2+}]_i$				
	decreased cell viability	Ca^{2+} influx (extracellular)	Ca^{2+} release (ER)	Ca^{2+} release (mitochondria)	NOEC	% of cells showing fluctuations	fluctuatio n frequency	fluctuation duration	fluctuation amplitude	NOEC
BDE-47	-	-	-	-	20 µM	-	2 µM	2 µM	-	1 µM (<)
BDE-49	-	-	-	-	20 µM	-	-	-	-	20 µM
BDE-99	-	-	-	-	20 µM	-	-	-	-	20 µM[b]
BDE-100	-	-	-	-	20 µM	-	-	-	-	20 µM[b]
BDE-153	-	-	-	-	20 µM	-	-	-	-	20 µM[b]
6-MeO-BDE-47	-	-	-	-	20 µM	-	-	-	-	20 µM
6-OH-BDE-47	20 µM	1 µM	1 µM	2 µM	0.2 µM	0.2 µM	-	0.2 µM	-	0.1 µM (<)
6'-OH-BDE-49	-	-	20 µM	-	2 µM	20 µM[a]	20 µM[a]	20 µM[a]	20 µM[a]	2 µM[b]
5-OH-BDE-47	20 µM	1 µM	1 µM	-	0.2 µM	1 µM	-	1 µM	-	0.2 µM[b]
3-OH-BDE-47	-	-	20 µM	20 µM	2 µM	20 µM[a]	20 µM[a]	20 µM[a]	20 µM[a]	2 µM
4'-OH-BDE-49	20 µM	20 µM	20 µM	20 µM	2 µM	2 µM	2 µM	2 µM	2 µM	1 µM (<)

b. Possible effects on parameters of fluctuations are obscured by release of Ca^{2+} from intracellular stores.
c. The number of data points to investigate duration and amplitude of the fluctuations in $[Ca^{2+}]_i$ is insufficient to ensure the NOEL.

Acknowledgement

The authors thank Ing. Aart de Groot for excellent technical assistance.

Chapter 6

Calcium-related processes involved in the inhibition of depolarization-induced calcium increase by hydroxylated PBDEs in PC12 cells

Milou M.L. Dingemans[1], Martin van den Berg[1], Åke Bergman[2], Remco H.S. Westerink[1]

1. Institute for Risk Assessment Sciences, Utrecht University, Utrecht, the Netherlands.
2. Department of Environmental Chemistry, Stockholm University, Stockholm, Sweden.

Toxicological Sciences doi:10.1093/toxsci/kfp310.

Abstract

In vitro studies indicated that hydroxylated polybrominated diphenyl ethers (OH-PBDEs) have an increased toxic potential compared to their parent congeners. An example is the OH-PBDE-induced increase of basal intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by release of Ca^{2+} from endoplasmic reticulum (ER) and mitochondria, and/or influx of extracellular Ca^{2+}.

ER and mitochondria regulate Ca^{2+} homeostasis in close association with voltage-gated Ca^{2+} channels (VGCCs). We therefore investigated whether (OH-)PBDEs also affect the depolarization-evoked (100 mM K^+) net increase in $[Ca^{2+}]_i$ (depolarization-evoked $[Ca^{2+}]_i$), measured in neuroendocrine pheochromocytoma (PC12) cells using the Ca^{2+}-responsive dye Fura-2.

PBDEs and methoxylated BDE-47 neither affected basal nor depolarization-evoked $[Ca^{2+}]_i$, except for BDE-47, which moderately increased fluctuations in basal $[Ca^{2+}]_i$ and depolarization-evoked $[Ca^{2+}]_i$. OH-PBDEs dose-dependently inhibited depolarization-evoked $[Ca^{2+}]_i$. This inhibition was potentiated by preceding increase in basal $[Ca^{2+}]_i$. Especially at higher concentrations of OH-PBDEs (5 - 20 µM), high increases in basal $[Ca^{2+}]_i$ strongly inhibited depolarization-evoked $[Ca^{2+}]_i$. The inhibition appeared more sensitive to increases in basal $[Ca^{2+}]_i$ by Ca^{2+} release from intracellular stores (by 3-OH-BDE-47 or 6'-OH-BDE-49) compared to increases by influx of extracellular Ca^{2+} (by 6-OH-BDE-47 or 5-OH-BDE-47). The expected $[Ca^{2+}]_i$ difference close to the membrane suggests involvement of Ca^{2+}-dependent regulatory processes close to the VGCCs. When co-applied with depolarization, some OH-PBDEs induced also moderate direct inhibition of depolarization-evoked $[Ca^{2+}]_i$.

These findings demonstrate that OH-PBDEs inhibit depolarization-evoked $[Ca^{2+}]_i$, depending on preceding basal $[Ca^{2+}]_i$. Related environmental pollutants that affect Ca^{2+} homeostasis (e.g. polychlorinated biphenyls) may also inhibit depolarization-evoked $[Ca^{2+}]_i$. Further investigation of possible mixture effects of environmental pollutants is therefore justified.

Introduction

Polybrominated diphenyl ethers (PBDEs), a group of brominated flame retardants (BFRs), have been shown to affect learning and spontaneous behavior in rodents (reviewed in Costa and Giordano 2007; Fonnum and Mariussen 2009). PBDEs have been detected, occasionally at high concentrations, in humans and particularly in young children (reviewed in Frederiksen et al. 2009).

In vitro neurotoxicity and endocrine studies have revealed that oxidative metabolism, resulting in hydroxylated PBDEs (OH-PBDEs), increases the potency of PBDEs (e.g. Cantón et al. 2008; Dingemans et al. 2008; Kojima et al. 2009). Recently, OH-PBDEs have also been detected in human serum at concentrations similar to those of parent PBDE congeners (Athanasiadou et al. 2008; Qiu et al. 2009), giving rise to concern about possible neurotoxic effects in humans.

Possible mechanisms underlying the neurobehavioral effects of PBDEs or their metabolites have been partly revealed at different biological levels, ranging from structural and functional effects in the brain, to cellular and molecular effects measured *in vitro* (reviewed in Costa and Giordano 2007; Fonnum and Mariussen 2009). These include among others the effects of (OH-)PBDEs on Ca^{2+} homeostasis (Coburn et al. 2008; Dingemans et al. 2008; Kodavanti and Ward 2005).

During basal conditions, average cytosolic Ca^{2+} levels in chromaffin and PC12 cells are maintained around 100 nM by Ca^{2+} buffering and extrusion mechanisms (Duman et al. 2008; García et al. 2006). However, neuronal signaling also requires rapid, transient increases in $[Ca^{2+}]_i$, triggering various intracellular processes including neurotransmitter release (Barclay et al. 2005; Clapham 2007). In PC12 cells, rapid increases in $[Ca^{2+}]_i$ in response to depolarization mainly originate from influx of Ca^{2+} via voltage-gated Ca^{2+} channels (VGCCs) located in the cell membrane. VGCCs expressed in undifferentiated PC12 cells include L-, N-, P/Q-, R- and T-type Ca^{2+} channels (Del Toro et al. 2003; Liu et al. 1996; Shafer and Atchison 1991). High voltage-gated L-, N-, and P/Q-type VGCCs account for the majority of the depolarization-evoked increase in $[Ca^{2+}]_i$; it has been shown previously that blocking L-, N-, and P/Q-type VGCCs reduces the depolarization-evoked increase in $[Ca^{2+}]_i$ to ~15% of control (Dingemans et al. 2009a).

Previously, the effects of parent PBDE and OH-PBDEs as well as a methoxylated analogue on basal $[Ca^{2+}]_i$ have been investigated in PC12 cells. While the parent PBDEs and methoxylated 6-MeO-BDE-47 do not affect the average or amplitude of basal $[Ca^{2+}]_i$ during 20-min exposure, all investigated OH-PBDEs increase basal $[Ca^{2+}]_i$ by release of Ca^{2+} from endoplasmic reticulum (ER), in some cases combined with release of Ca^{2+} from mitochondria and/or influx of extracellular Ca^{2+}. The potency of OH-PBDEs for affecting basal intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is at least partly determined by shielding of the OH-group by Br-atoms or aromatic rings (Dingemans et al. 2009b).

Because of the close functional associations of ER and mitochondria with VGCCs for regulating Ca^{2+} homeostasis (García et al. 2006), the aim of this study was to investigate in a neuroendocrine *in vitro* model (PC12 cells) whether OH-PBDEs also affect depolarization-evoked $[Ca^{2+}]_i$.

Methods

Chemicals. PBDEs (BDE-47, BDE-49, BDE-99, BDE-100 and BDE-153) and OH-PBDEs (3-OH-BDE-47, 4'-OH-BDE-49, 5-OH-BDE-47, 6'-OH-BDE-49, 6-OH-BDE-47 and methoxylated analogue 6-MeO-BDE-47; full names in table 6.1) were synthesized and purified (~99% purity) at the Department of Environmental Chemistry of Stockholm University as described earlier (Dingemans et al. 2008, Dingemans et al. 2009b). All other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands).

Table 6.1. Full names of the PBDEs and hydroxylated/methoxylated PBDEs discussed in this paper.

6-MeO-BDE-47	6-methoxy-2,2',4,4'-tetrabromodiphenyl ether
3-OH-BDE-47	3-hydroxy-2,2',4,4'-tetrabromodiphenyl ether
4'-OH-BDE-49	4'-hydroxy-2,2',4,5'-tetrabromodiphenyl ether
5-OH-BDE-47	5-hydroxy-2,2',4,4'-tetrabromodiphenyl ether
6-OH-BDE-47	6-hydroxy-2,2',4,4'-tetrabromodiphenyl ether
6'-OH-BDE-49	6'-hydroxy-2,2',4,5'-tetrabromodiphenyl ether
BDE-47	2,2',4,4'-tetrabromodiphenyl ether
BDE-49	2,2',4,5'-tetrabromodiphenyl ether
BDE-99	2,2',4,4',5-pentabromodiphenyl ether
BDE-100	2,2',4,4',6-pentabromodiphenyl ether
BDE-153	2,2',4,4',5,5'-hexabromodiphenyl ether

PC12 cell culture. Rat pheochromocytoma (PC12) cells were cultured as described previously (Dingemans et al. 2008).

Ca^{2+} imaging. Changes in $[Ca^{2+}]_i$ were measured using the Ca^{2+}-sensitive fluorescent ratio dye Fura-2 as described previously (Dingemans et al. 2008). Membrane depolarization by 100 mM K^+ was used to investigate effects of the (MeO/OH-)PBDEs on the depolarization-evoked increase in $[Ca^{2+}]_i$. The amplitude of $[Ca^{2+}]_i$ within a minute of the start of depolarization was determined per cell and the net increase (amplitude $[Ca^{2+}]_i$ - preceding $[Ca^{2+}]_i$ last min prior to depolarization) was used to investigate effects of PBDEs on depolarization-evoked $[Ca^{2+}]_i$, both following 20-min pre-exposure to (MeO/OH-)PBDEs (Figure 6.1) and during co-application of 100 mM K^+ and (MeO/OH-)PBDE to investigate direct effects of the (MeO/OH-)PBDEs.

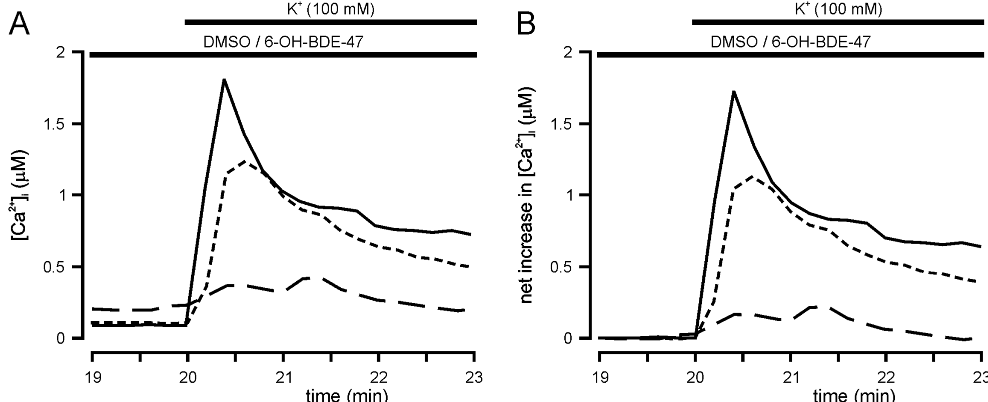


Figure 6.1. Exposure of PC12 cells to OH-PBDEs inhibits the depolarization-evoked increase in $[Ca^{2+}]_i$. Representative traces of cytosolic $[Ca^{2+}]_i$ measurements of individual PC12 cells exposed to different concentrations 6-OH-BDE-47 are shown, illustrating the reduction of the depolarization-evoked increase in $[Ca^{2+}]_i$ by exposure to OH-PBDEs (control: solid line; 2 µM 6-OH-BDE-47: small dash; 20 µM 6-OH-BDE-47: large dash). During 20 min pre-exposure to OH-PBDEs, an increase in basal $[Ca^{2+}]_i$ is observed (A). The difference between the amplitude of $[Ca^{2+}]_i$ (measured within 1 min from the start of depolarization) and basal $[Ca^{2+}]_i$ (measured within 1 min before depolarization) is therefore used as the measure of depolarization-evoked $[Ca^{2+}]_i$ (depolarization-evoked net increase in $[Ca^{2+}]_i$; B).

Data analysis and statistics. All data are presented as mean ± SE from the number of cells (*n*) indicated. Statistical analyses were performed using SPSS 16 (SPSS, Chicago, IL, USA). Categorical and continuous data were compared using respectively Fisher's exact test and Student's *t*-test, paired or unpaired where applicable. A *p*-value < 0.05 is considered statistically significant.

Results

OH-PBDEs dose-dependently inhibit depolarization-evoked net increase in $[Ca^{2+}]_i$**.** In control cells (20 min exposed to 0.1% DMSO), a robust depolarization-evoked net increase in $[Ca^{2+}]_i$ (1.82 ± 0.09 µM, $n = 168$) was observed in the majority of cells. Exposing PC12 cells for 20 min to BDE-49, BDE-99, BDE-100 or BDE-153 or methoxylated PBDE 6-MeO-BDE-47 did neither affect basal $[Ca^{2+}]_i$, nor the subsequent depolarization-evoked net increase in $[Ca^{2+}]_i$. However, cells exposed to 20 µM BDE-47 showed more fluctuations in basal $[Ca^{2+}]_i$, as well as a larger depolarization-evoked net increase in $[Ca^{2+}]_i$ (2.46 ± 0.23 µM, $n = 37$, $p < 0.01$) compared to control cells (Figure 6.2).

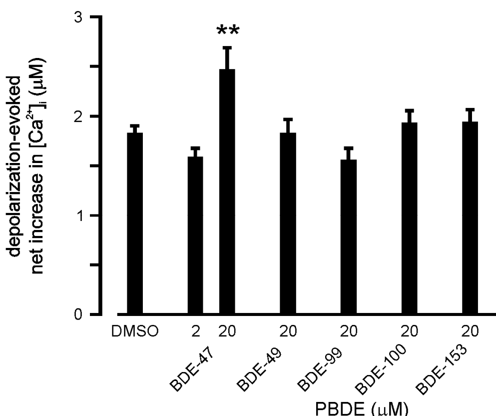


Figure 6.2. BDE-47 (20 μM) increases depolarization-evoked net increase in $[Ca^{2+}]_i$, while the other parent PBDEs have no effect. Bar graph shows the amplitudes of depolarization-evoked net increase in $[Ca^{2+}]_i$ in PC12 cells 20-min pre-exposed to PBDEs. Bars display data from 3 - 5 experiments per PBDE-treatment (*n* = 37 - 62; average: 46) and 19 control experiments (*n* = 168). Difference from control: ** $p < 0.01$.

Previously, it was shown that exposing PC12 cells for 20 min to OH-PBDEs resulted in increases in basal $[Ca^{2+}]_i$ by release of Ca^{2+} from intracellular stores and/or influx of extracellular Ca^{2+} (Dingemans et al. 2009b). A dose-dependent increase in basal $[Ca^{2+}]_i$ was observed during exposure to 6-OH-BDE-47 or 5-OH-BDE-47 (lowest-observed effect concentration; LOEC: 1 μM). 6-OH-BDE-47 and 5-OH-BDE-47 also dose-dependently inhibited the subsequent depolarization-evoked net increase in $[Ca^{2+}]_i$ with a LOEC of respectively 2 μM and 5 μM and near complete inhibition at 20 μM. During 20-min pre-exposure to 20 μM 6'-OH-BDE-49, 3-OH-BDE-47 or 4'-OH-BDE-49, basal $[Ca^{2+}]_i$ increased and the subsequent depolarization-evoked net increase in $[Ca^{2+}]_i$ was largely inhibited (Figure 6.3; LOEC: 20 μM).

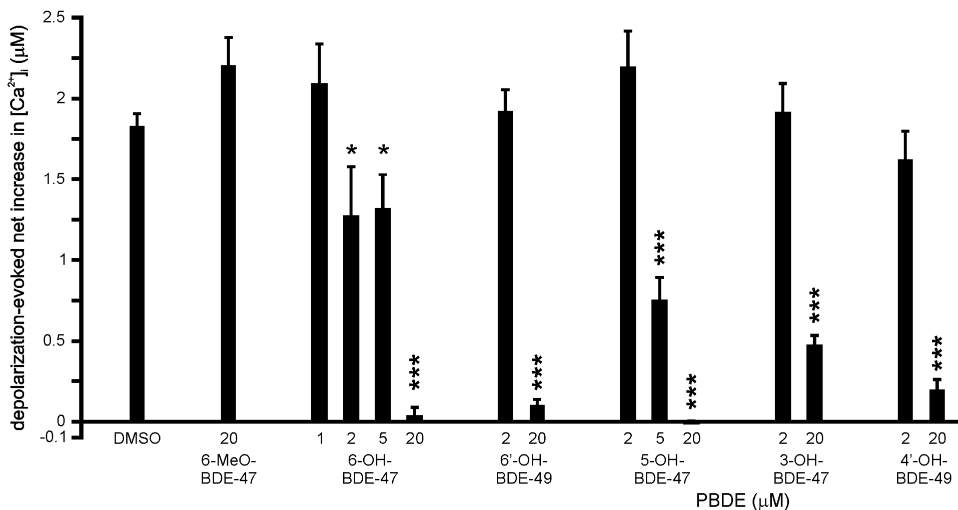


Figure 6.3. OH-PBDEs inhibit depolarization-evoked net increase in $[Ca^{2+}]_i$ at similar LOECs as for increase in basal $[Ca^{2+}]_i$. Bar graph shows the dose-dependent reduction of the depolarization-evoked net increase in $[Ca^{2+}]_i$ by OH-PBDEs. Bars display data from 4 - 8 experiments per MeO/OH-PBDE-treatment ($n = 31 - 86$; average 54) and 19 control experiments ($n = 168$). Difference from control: * $p < 0.05$; *** $p < 0.001$.

Increase in basal $[Ca^{2+}]_i$ potentiates inhibition of depolarization-evoked net increase in $[Ca^{2+}]_i$. During exposure to OH-PBDEs, transient (initial) and/or late increases in basal $[Ca^{2+}]_i$ are observed (Dingemans et al. 2009b). When comparing the depolarization-evoked net increase in $[Ca^{2+}]_i$ in cells with only a transient or only a late increase in basal $[Ca^{2+}]_i$, the depolarization-evoked net increase in $[Ca^{2+}]_i$ in cells with a late increase is within the same range as in cells with a transient increase in basal $[Ca^{2+}]_i$ (data not shown). Therefore, the average net increase in basal $[Ca^{2+}]_i$ measured during 20-min pre-exposure was used as a measure for basal $[Ca^{2+}]_i$ disruption to investigate correlations between net increases in basal and depolarization-evoked $[Ca^{2+}]_i$.

A negative association exists between average net increases in basal $[Ca^{2+}]_i$ and the depolarization-evoked net increase in $[Ca^{2+}]_i$ when taking in account all different OH-PBDE treatments. Plotting the depolarization-evoked net increase in $[Ca^{2+}]_i$ only from individual cells exposed to 6-OH-BDE-47 (at different concentrations) against their preceding net increase in basal $[Ca^{2+}]_i$ revealed that in cells of which the average basal $[Ca^{2+}]_i$ increased with > 55 nM, depolarization-evoked net increase in $[Ca^{2+}]_i$ was near-completely inhibited (Figure 6.4A). This was also observed for the other OH-PBDEs, although with varying threshold values of net increase in basal $[Ca^{2+}]_i$ for near-complete inhibition of the depolarization-evoked net increase in $[Ca^{2+}]_i$ (full-inhibition-thresholds): 6'-OH-BDE-49: 20 nM; 5-OH-BDE-47:

55 nM; 3-OH-BDE-47: 25 nM; 4'-OH-BDE-49: 25-50 nM. These full-inhibition-threshold values were determined from frequency distribution of near-completely inhibited depolarization-evoked net increases in $[Ca^{2+}]_i$ (< 100 nM) for net increase in basal $[Ca^{2+}]_i$ values (5 nM intervals). The full-inhibition-threshold was identified by a rapid increase (to 100%) in the percentage of cells with inhibited depolarization-evoked net increases in $[Ca^{2+}]_i$. The full-inhibition-threshold for 4'-OH-BDE-49 (25-50 nM) could not be specified further due to a lack of data-points in this range of net increase in basal $[Ca^{2+}]_i$.

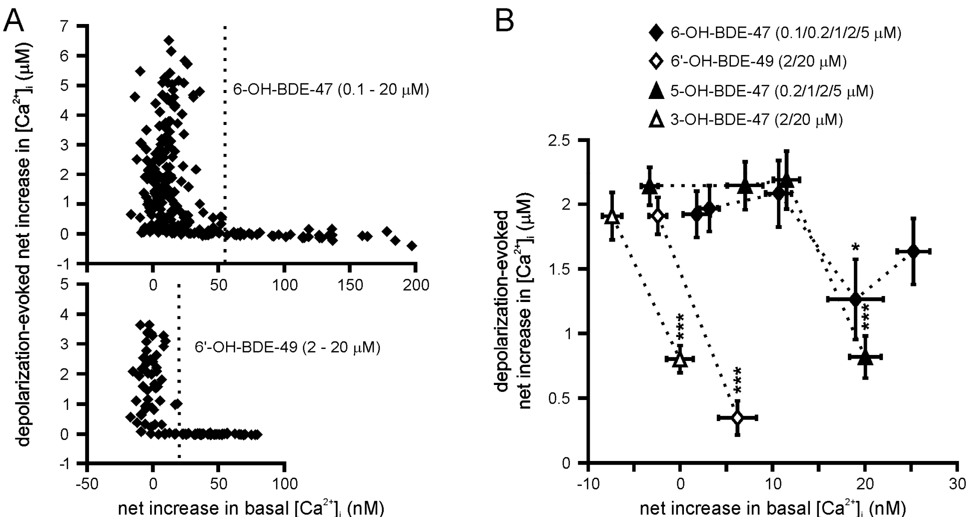


Figure 6.4. Net increases in basal $[Ca^{2+}]_i$ higher than the 'full-inhibition-threshold' completely inhibits depolarization-evoked net increase in $[Ca^{2+}]_i$. A. Examples of associations of net increase in basal versus depolarization-evoked net increase in $[Ca^{2+}]_i$. Determined 'full-inhibition-thresholds' are indicated by dashed lines. B. When cells with net increases in basal $[Ca^{2+}]_i$ > full-inhibition-threshold are removed, the inhibition of depolarization-evoked net increase in $[Ca^{2+}]_i$ is still dependent on net increases in basal $[Ca^{2+}]_i$. Different sensitivities of the depolarization-evoked net increase in $[Ca^{2+}]_i$ for net increases in basal $[Ca^{2+}]_i$ are observed for 6'-OH-BDE-49 and 3-OH-BDE-47 versus 6-OH-BDE-47 and 5-OH-BDE-47. Scatter display data from 3 - 8 experiments per OH-PBDE-treatment (n = 21 - 86; average 44) and 19 control experiments (n = 168). Each data point represents depolarization-evoked net increase in $[Ca^{2+}]_i$ in PC12 cells exposed to a OH-PBDEs at a single exposure concentration (as indicated in figure, identifiable from left to right by increasing net increases in basal $[Ca^{2+}]_i$). Difference from control: * $p < 0.05$; *** $p < 0.001$.

Moderate OH-PBDE-induced net increases in basal [Ca^{2+}]$_i$ inhibit depolarization-evoked net increases in [Ca^{2+}]$_i$. In all cells with net increases in basal [Ca^{2+}]$_i$ above the full-inhibition-threshold, near-complete inhibition of depolarization-evoked net increase in [Ca^{2+}]$_i$ is observed (5 µM 6-OH-BDE-47: 0.01 ± 0.01 µM, n = 19; 20 µM 6'-OH-BDE-49: 0.00 ± 0.00 µM, n = 46, 5 µM 5-OH-BDE-47: 0.21 ± 0.16 µM, n = 7; 20 µM 3-OH-BDE-47: 0.10 ± 0.03 µM, n = 41). To remove the influence of high net increase in basal [Ca^{2+}]$_i$, cells with a net increase in basal [Ca^{2+}]$_i$ higher than the full-inhibition-threshold were excluded from the following analysis. Subsets of cells with a net increase in basal [Ca^{2+}]$_i$ below the full-inhibition-threshold could only be identified within the groups of cells exposed to 5 µM 6-OH-BDE-47, 20 µM 6'-OH-BDE-49, 5 µM 5-OH-BDE-47 or 20 µM 3-OH-BDE-47. When cells with a high net increase in basal [Ca^{2+}]$_i$ are removed, the inhibition of depolarization-evoked net increases in [Ca^{2+}]$_i$ is still associated with preceding net increases in basal [Ca^{2+}]$_i$. In figure 6.4B, a difference in sensitivity of inhibition of depolarization-evoked net increase in [Ca^{2+}]$_i$ for net increase in basal [Ca^{2+}]$_i$ can be seen for 6'-OH-BDE-49 and 3-OH-BDE-47 versus 6-OH-BDE-47 and 5-OH-BDE-47.

OH-PBDE directly inhibit depolarization-evoked net increase in [Ca^{2+}]$_i$. To investigate whether the effects of the OH-PBDEs depend on pre-exposure, in separate experiments 20 µM of OH-PBDE was applied only during depolarization (Figure 6.5).

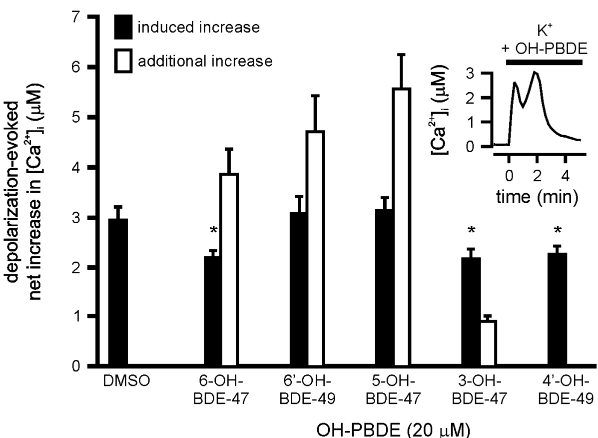


Figure 6.5. Several OH-PBDEs directly inhibit depolarization-evoked net increase in [Ca^{2+}]$_i$. An additional increase (representative trace in inset) is also observed in all cells during exposure to 20 µM 6-OH-BDE-47, 20 µM 6'-OH-BDE-49, 20 µM 5-OH-BDE-47 and in 76% of cells exposed to 20 µM 3-OH-BDE-47 (n = 19). Bars display depolarization-evoked (closed bars) and additional (open bars) net increases in [Ca^{2+}]$_i$ data from 3 - 4 experiments per treatment (n = 25 - 39; average 31). Difference from control: * $p < 0.05$.

In control cells, depolarization-evoked net increase in $[Ca^{2+}]_i$ increased with 2.95 ± 0.28 µM. During exposure to 20 µM 6-OH-BDE-47, 3-OH-BDE-47 or 4'-OH-BDE-49, the depolarization-evoked net increase in $[Ca^{2+}]_i$ was inhibited to approximately 75% of control. Interestingly, during exposure to 6-OH-BDE-47, 6'-OH-BDE-49, 5-OH-BDE-47 and 3-OH-BDE-47, an additional increase is observed (Figure 6.5, inset). For 6-OH-BDE-47, 6'-OH-BDE-49 and 5-OH-BDE-47, the amplitude of this additional increase is larger than the depolarization-evoked net increase in $[Ca^{2+}]_i$ (approximately 4 - 6 µM; Figure 6.5). During exposure to 3-OH-BDE-47, the additional increase in $[Ca^{2+}]_i$ is smaller than the depolarization-evoked net increase in $[Ca^{2+}]_i$, and is observed in only 76% of the cells. Additional increases last approximately 5 min from the start of depolarization, and amplitudes of the additional increase in $[Ca^{2+}]_i$ are reached 1 - 4 min after the start of depolarization (data not shown). During exposure to 4'-OH-BDE-49, an additional increase is not observed.

Discussion

OH-PBDEs dose-dependently inhibit the depolarization-evoked net increase in $[Ca^{2+}]_i$ after 20-min pre-exposure. This inhibition at least partly depends on net increase in basal $[Ca^{2+}]_i$ during pre-exposure to OH-PBDEs. Especially at high concentrations, OH-PBDEs induce high net increases in basal $[Ca^{2+}]_i$ that near-completely inhibit depolarization-evoked net increases in $[Ca^{2+}]_i$ ('Ca^{2+}-induced inhibition', see below). When cells with a high net increase in basal $[Ca^{2+}]_i$ are excluded from data-analysis, inhibition associated with a net increase in basal $[Ca^{2+}]_i$ is still observed ('Ca^{2+}-mediated inhibition', see below). Moderate inhibition of the depolarization-evoked net increase in $[Ca^{2+}]_i$ was also observed for some OH-PBDEs when co-applied with depolarization ('direct inhibition', see below). No or subtle effects were observed on basal and depolarization-evoked net increases in $[Ca^{2+}]_i$ during exposure to parent PBDEs and 6-MeO-BDE-47.

From the combined data it can be concluded, that when the average net increase in basal $[Ca^{2+}]_i$ is larger than a certain value (full-inhibition-threshold), the depolarization-evoked net increase in $[Ca^{2+}]_i$ is near-completely inhibited (Ca^{2+}-induced inhibition). During depolarization, Ca^{2+}- and voltage-dependent processes desensitize VGCCs (Catterall 2000). Membrane-depolarization should have been similar in all experimental exposure conditions, as no evidence exists for direct depolarization induced by (OH-)PBDEs. Therefore, the inhibitory effect on depolarization-evoked net increase in $[Ca^{2+}]_i$ observed in cells with high net increases in basal $[Ca^{2+}]_i$ (Ca^{2+}-induced inhibition) is likely mediated by Ca^{2+}-induced desensitization of VGCCs (reviewed in Budde et al. 2002).

Interestingly, the full-inhibition-threshold for Ca^{2+}-induced desensitization of VGCCs varied between OH-PBDEs. When considering different sources of Ca^{2+} responsible for preceding net increases in basal $[Ca^{2+}]_i$ (Dingemans et al. 2009b), it is apparent that for those OH-PBDEs that induce influx of extracellular Ca^{2+} besides Ca^{2+} release from intracellular stores

(6-OH-BDE-47 and 5-OH-BDE-47), the threshold for Ca^{2+}-induced desensitization of VGCCs is higher compared to the threshold observed for those OH-PBDEs that mainly induce Ca^{2+} release from intracellular stores (6'-OH-BDE-49 and 3-OH-BDE-47; Dingemans et al. 2009b). Because of spatial differences between these processes, higher concentrations of $[Ca^{2+}]_i$ are expected in local membrane-associated Ca^{2+} microdomains during pre-exposure to 6-OH-BDE-47 and 5-OH-BDE-47, compared to 3-OH-BDE-47 and 6'-OH-BDE-49. It is noteworthy that this difference is larger than when comparing measured increases in basal $[Ca^{2+}]_i$, because a cytosolic average is measured. Therefore, increases in $[Ca^{2+}]_i$ close to the membrane are overestimated when release of Ca^{2+} from intracellular stores is involved, but underestimated when influx of extracellular Ca^{2+} is involved. The presumed difference in membrane-associated $[Ca^{2+}]_i$ suggests Ca^{2+}-dependent regulation of the observed Ca^{2+}-induced desensitization of VGCCs (higher full-inhibition-thresholds). As Ca^{2+}-induced desensitization involves calmodulin as Ca^{2+} sensor in complex with VGCCs (Kim et al. 2004), this is a potential target for the regulatory processes caused by preceding increase in basal $[Ca^{2+}]_i$ induced by OH-PBDEs.

LOECs for inhibition of depolarization-evoked net increase in $[Ca^{2+}]_i$, are comparable with LOECs for preceding increases in basal $[Ca^{2+}]_i$ (Dingemans et al. 2009b). However, the observed effects on depolarization-evoked net increase in $[Ca^{2+}]_i$ are confounded by large preceding net increases in basal $[Ca^{2+}]_i$ at high concentrations of OH-PBDEs. When cells with a high net increase in basal $[Ca^{2+}]_i$ are excluded, inhibition of depolarization-evoked net increase $[Ca^{2+}]_i$ still depends on preceding net increases in basal $[Ca^{2+}]_i$ (Ca^{2+}-mediated inhibition; Figure 6.4B). Interestingly, inhibition of depolarization-evoked net increases in $[Ca^{2+}]_i$ appeared more sensitive to increases in basal $[Ca^{2+}]_i$ by 3-OH-BDE-47 and 6'-OH-BDE-49 than to increases in basal $[Ca^{2+}]_i$ by 6-OH-BDE-47 and 5-OH-BDE-47. Since higher sensitivities to increases in basal $[Ca^{2+}]_i$ coincide (Figure 6.4B) with lower thresholds for Ca^{2+}-induced desensitization (Figure 6.4A), these processes appear associated and dependent on membrane-associated $[Ca^{2+}]_i$ (as discussed above).

When co-applied with depolarization, 20 µM 6-OH-BDE-47, 20 µM 3-OH-BDE-47 and 20 µM 4'-OH-BDE-49 inhibit the depolarization-evoked net increase in $[Ca^{2+}]_i$, suggesting direct inhibition of VGCCs by these OH-PBDEs (direct inhibition; Figure 6.5). For 6-OH-BDE-49, 5-OH-BDE-47 and 3-OH-BDE-47, the relative inhibition of the depolarization-evoked net increase in $[Ca^{2+}]_i$ is larger after 20-min pre-exposure compared to co-application, indicating pre-exposure-dependent effects, which affirms the hypothesis on regulatory processes.

Only after exposure to BDE-47, the depolarization-evoked net increase in $[Ca^{2+}]_i$ is enhanced to approximately 135% of control. At concentrations of OH-PBDEs that induce an increase in the frequency, amplitude or duration of fluctuations in $[Ca^{2+}]_i$ (0.2 µM 6-OH-BDE-47, 1 µM 5-OH-BDE-47, 2 µM 4'-OH-BDE-49; Dingemans et al. 2009b), a small (not significant) increase in the depolarization-evoked net increase in $[Ca^{2+}]_i$ is also observed (not for 2 µM 4'-OH-BDE-49). This may be due to facilitation of VGCC by the increased number of preceding fluctuations in basal $[Ca^{2+}]_i$. Also, release of Ca^{2+} from intracellular stores is involved

in the depolarization-evoked increase in $[Ca^{2+}]_i$ by Ca^{2+}-induced Ca^{2+} release (reviewed in García et al. 2006). Possibly, intracellular stores have compartmentalized more Ca^{2+} because of the increase in fluctuations by BDE-47. This could moderately increase the depolarization-evoked net increase in $[Ca^{2+}]_i$.

Based on the temporal characteristics of the additional increase in $[Ca^{2+}]_i$ observed during co-application of K^+ and 20 µM of 6-OH-BDE-47, 6'-OH-BDE-49, 5-OH-BDE-47 and to a lesser extent 3-OH-BDE-47, this is likely caused by the previously described release of Ca^{2+} from intracellular Ca^{2+} stores (mainly ER; Dingemans et al. 2009b), although with much higher amplitude. Possibly, this higher amplitude occurs because involved Ca^{2+} stores are charged by Ca^{2+} originating from influx through VGCCs.

As discussed above, thresholds for Ca^{2+}-induced desensitization of VGCCs and sensitivity of depolarization-evoked net increase in $[Ca^{2+}]_i$ to preceding net increase in basal $[Ca^{2+}]_i$ appear associated with OH-PBDE-induced changes in membrane-associated $[Ca^{2+}]_i$, i.e., in close proximity to VGCCs. Ca^{2+} currents through VGCCs are regulated by protein kinases, mainly protein kinase C (PKC), protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and phosphatases (reviewed in Catterall 2000; Dai et al. 2009). These kinases and phosphatases are also involved in regulation of inositol triphosphate (IP_3) receptor-mediated and ryanodine receptor-mediated Ca^{2+} release from ER (reviewed in Vanderheyden et al. 2009; Zalk et al. 2007).

Some evidence exists for interaction of PBDEs with PKC. Influx of extracellular Ca^{2+} by PBDEs and polychlorinated biphenyls (PCBs) was caused by the release of arachonidic acid, a second messenger involved in, among other things, PKA and PKC activation (Kodavanti and Derr-Yellin 2002). BDE-99 activates PKC (Madia et al. 2004), while PCBs and PBDEs also increase PKC translocation (Dorn and Mochly-Rosen 2002) and affect its binding to IP_3-receptors (Kodavanti et al. 1994; Kodavanti and Ward 2005; Shafer et al. 1996). PKC is also known to phosphorylate IP_3-receptors causing increased IP_3-mediated Ca^{2+} release from ER (Matter et al. 1993). PCBs and PBDEs have also been demonstrated to activate IP_3 kinase (Reistad and Mariussen 2005; Voie and Fonnum 2000). Additionally, several studies revealed effects of PBDEs on (phosphorylated-activated-)CaMKII levels in mice exposed to PBDEs (Dingemans et al. 2007; Viberg 2009a; Viberg et al. 2008). Phosphorylation of VGCCs by CaMKII facilitates Ca^{2+} currents (Lee et al. 2006). Like PKC, CaMKII also phosphorylates IP_3 receptors, thereby controlling IP_3-mediated Ca^{2+} release from ER (Zhang et al. 1993). Therefore, effects of OH-PBDEs on PKC- and/or CamKII-mediated processes are possibly involved in the observed release of Ca^{2+} from intracellular stores by OH-PBDEs (Dingemans et al. 2009b).

For the first time, it is demonstrated here that OH-PBDEs not only increase basal $[Ca^{2+}]_i$, but also inhibit depolarization-evoked $[Ca^{2+}]_i$. Because of the abovementioned similar effects of PBDEs and PCBs on $[Ca^{2+}]_i$ and related protein kinases, the observed inhibition of depolarization-evoked $[Ca^{2+}]_i$, as well as the potentiation of inhibition of depolarization-evoked $[Ca^{2+}]_i$ by preceding increases in basal $[Ca^{2+}]_i$ might be not specific for

OH-PBDEs. It is not unlikely that the observed inhibition also has functional consequences for depolarization-evoked neurotransmitter release in neuronal cells, but possibly also for other more general Ca^{2+}-related processes, such as apoptosis and gene transcription (Clapham 2007).

In summary, OH-PBDEs inhibit the depolarization-evoked net increase in $[Ca^{2+}]_i$. Regulatory mechanisms, possibly related to protein kinases, likely play a role in the basal $[Ca^{2+}]_i$ dependent inhibition of depolarization-evoked net increase in $[Ca^{2+}]_i$ (Ca^{2+}-induced inhibition and Ca^{2+}-mediated inhibition), but also moderate direct inhibition is observed. The observed inhibition might also occur in the *in vivo* situation, in which resting and stimulated states of neurons alternate, with varying temporal characteristics. As (spontaneous) neuronal activity is essential in early brain development and brain function (Spitzer 2006; Moody and Bosma 2005), an imbalance in neuronal activity by the observed increase in basal $[Ca^{2+}]_i$ and inhibition of depolarization-evoked $[Ca^{2+}]_i$ could play a role in the observed neurobehavioral effects of PBDEs and possibly PCBs. Further investigation of possible mixture effects of environmental pollutants is therefore justified.

Acknowledgement

The authors thank Dr. Alfons B.A. Kroese for helpful discussions.

Chapter 7

BDE-47 and its hydroxylated metabolite 6-OH-BDE-47 modulate calcium homeostasis in primary human neural progenitor cells

Kathrin Gassmann[1*], Guido Krause[2*], Milou M.L. Dingemans[3*], Timm Schreiber[1*], Josef Abel[1], Åke Bergman[4], Hans F. Merk[5], Michaela Moors[1], Christine R. Rose[2], Remco H.S. Westerink[3], Ellen Fritsche[1,5]

1. Institut für umweltmedizinische Forschung gGmbH, Heinrich-Heine-University, Düsseldorf, Germany.
2. Institut für Neurobiologie, Heinrich-Heine-University, Düsseldorf, Germany.
3. Institute for Risk Assessment Sciences, Utrecht University, Utrecht, the Netherlands.
4. Department of Environmental Chemistry, Stockholm University, Stockholm, Sweden.
5. Universitätshautklinik, RWTH Aachen, Aachen , Germany.

*. These authors contributed equally.

In preparation.

Abstract

Polybrominated diphenyl ethers (PBDEs), which are bioaccumulating in human tissues, raise concern for developmental neurotoxicity. Hydroxylated metabolites of 2,2´,4,4´-tetrabromodiphenyl ether (BDE-47), which have also been detected in humans, have been demonstrated to increase intracellular calcium levels $[Ca^{2+}]_i$ in rat pheochromocytoma (PC12) cells, at lower concentrations than their parent congener BDE-47. In this study, possible effects of BDE-47 and the hydroxylated metabolite 6-OH-BDE-47 on $[Ca^{2+}]_i$ were investigated in a heterogeneous *in vitro* model for neurodevelopment, i.e., human neural progenitor cells (hNPCs), by using the fluorescent Ca^{2+}-sensitive dye Fura-2.

Acute exposure of hNPCs to BDE-47 ($\geq$ 2 µM) or 6-OH-BDE-47 ($\geq$ 0.2 µM) resulted in a significant increase in $[Ca^{2+}]_i$. Additional experiments under Ca^{2+}-free conditions revealed that BDE-47 induced influx of extracellular Ca^{2+}. 6-OH-BDE-47 induced an early persistent increase combined with a transient increase in $[Ca^{2+}]_i$ as well as a late increase in $[Ca^{2+}]_i$. Emptying intracellular mitochondrial and endoplasmic Ca^{2+} stores, by respectively FCCP and thapsigargin under Ca^{2+}-free conditions revealed that the transient increase depends mainly on Ca^{2+} release from the endoplasmic reticulum, whereas the early persistent increase in $[Ca^{2+}]_i$ is mainly due to Ca^{2+} release from mitochondria. The late increase in $[Ca^{2+}]_i$ is caused by influx of extracellular Ca^{2+}.

Both parent BDE-47 and the hydroxylated metabolite 6-OH-BDE-47 disturb Ca^{2+} homeostasis in hNPCs. As previously observed in PC12 cells, the hydroxylated metabolite has a higher potency than the parent compound. However, the lowest-observed-effect concentrations of both BDE-47 and 6-OH-BDE-47 are an order of magnitude lower in the hNPCs compared to in PC12 cells, suggesting a higher sensitivity for disturbance of Ca^{2+} homeostasis in human neural progenitor cells. These findings affirm that oxidative metabolism should be included in the risk assessment for developmental toxicity of PBDEs in humans.

Introduction

A number of studies have established the almost ubiquitous presence of polybrominated diphenyl ethers (PBDEs) in the environment, in animals and in humans (reviewed in Frederiksen et al. 2009; Law et al. 2003). 2,2´,4,4´-tetrabromodiphenyl ether (BDE-47) is the predominant PBDE congener in human blood samples (Hites 2004). The greatest concern for potential adverse health effects of PBDEs relates to their developmental neurotoxicity (DNT; reviewed in Costa and Giordano 2007). Previous data demonstrated that PBDEs administered during pregnancy and/or within the first weeks after birth alter neurobehavior of the offspring in rodents ranging from altered motor activity over changes in sweet preference to impairment of cognitive functions (Cheng et al. 2009; Eriksson et al. 2002; Lilienthal et al. 2006; Viberg et al. 2003a). Additional research demonstrated that these behavioral effects are possibly related to decreased hippocampal synaptic plasticity: decreased long-term potentiation (LTP) was measured after developmental exposure to BDE-47 or BDE-209 (Dingemans et al. 2008; Xing et al. 2009a).

In a rat pheochromocytoma (PC12) cell line, disruption of calcium homeostasis by BDE-47 was demonstrated (Dingemans et al. 2008, 2009b). Recent studies demonstrated that PBDEs and structurally related *ortho*-substituted polychlorinated biphenyls (PCBs) increase $[Ca^{2+}]_i$ in different neural and neuroendocrine cell types and disrupt Ca^{2+} homeostasis in rodent microsomes isolated from different brain regions (reviewed in Fonnum and Mariussen 2009).

It was also demonstrated in PC12 cells that the effects on calcium homeostasis are more pronounced for hydroxylated PBDEs (OH-PBDEs) compared to their parent compound BDE-47 (Dingemans et al. 2008, 2009b). Thus, oxidative metabolism adds to the neurotoxic potential of PBDEs. Also in *in vitro* studies on endocrine parameters, higher activity of hydroxylated PBDE metabolites compared to their parent congeners was observed (Cantón et al. 2005; Harju et al. 2007; Meerts et al. 2001). *In vivo* conversion of PBDEs to hydroxylated metabolites was confirmed by toxicokinetics studies (Huwe et al. 2007; Malmberg et al. 2005; Staskal et al. 2006b) and high amounts of hydroxylated metabolites have been detected in blood of humans (Athanasiadou et al. 2008), especially in fetal blood samples (Qiu et al. 2009).

The early-response second messenger Ca^{2+} plays a key role in a number of physiological processes, including cell proliferation, differentiation and apoptosis (Berridge et al. 2000; Clapham 2007). The complex spatial and temporal properties of intracellular Ca^{2+} signals also encode a wide variety of specific neuronal processes, including dendritic spine growth, synaptic plasticity and neurotransmission (Boulware and Marchant 2008). Ca^{2+} influx as well as Ca^{2+} release from intracellular Ca^{2+} stores, such as endoplasmic reticulum (ER) or mitochondria, define the magnitude, time course and spatial spread of the Ca^{2+} signal (Berridge et al. 2000; Laude and Simpson 2009).

As PBDEs have been shown affect neurobehavior after developmental exposure in rodents, PBDEs are of concern to cause DNT in humans. However, no data exists on the impact of PBDEs on human brain development, neither from epidemiological studies nor from investigations in human cells *in vitro*. An *in vitro* neurosphere system with human neural progenitor cells (hNPCs) was developed, in which basic processes of brain development, namely proliferation, migration, differentiation and apoptosis, can be investigated. This cellular model has previously been used to investigate DNT of PCBs, methylmercury and mercury (Fritsche et al. 2005, Moors et al. 2009). Recently, it was demonstrated that BDE-47 and BDE-99 affect migration and differentiation of hNPCs via endocrine disruption of cellular thyroid hormone signaling after one week of exposure (Schreiber et al. 2009). In the present study, it was investigated whether acute effects on $[Ca^{2+}]_i$ could be involved in the previously observed effects of PBDEs in hNPCs.

Materials and Methods

Chemicals. BDE-47 and 6-OH-BDE-47 were synthesized and purified (~99% purity) at the Department of Environmental Chemistry of Stockholm University as described by Marsh et al. (1999). Dibenzo-*p*-dioxins and dibenzofurans were removed from the PBDEs with a charcoal column as described by Örn et al. (1996). All additional chemicals used (unless otherwise noted) were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands).

Cell culture. The hNPCs used in this study were prepared from a single donor (Lonza Verviers SPRL, Belgium). hNPCs were cultured in proliferation medium (Dulbecco´s modified Eagle medium and Hams F12 (3:1) supplemented with B27 (Invitrogen GmBH, Karlsruhe, Germany), 20 ng/ml epidermal growth factor (Biosource, Karlsruhe, Germany) and 20 ng/ml recombinant human fibroblast growth factor (R&D Systems, Wiesbaden-Nordenstadt, Germany) in suspension culture as previously described (Moors et al. 2007, 2009). For Ca^{2+} imaging experiments, hNPCs were differentiated by growth factor withdrawal in differentiation medium (Dulbecco´s modified Eagle medium and Hams F12; 3:1; supplemented with N2; Invitrogen) and plated onto poly-D-lysine/laminin coated cover glasses (MatTek, Ashland MA, USA).

Calcium imaging. Ratiometric calcium imaging was performed as described previously (Dingemans et al. 2008). Briefly, cells were loaded with 5 µM Fura-2-AM (Molecular Probes; Invitrogen) in saline for 20 min at room temperature. Subsequently, the cells were washed with external saline and placed on the stage of a microscope equipped with a TILL Photonics Polychrome IV (TILL Photonics GmbH, Gräfelfing, Germany). Fluorescence (emission wavelength: 510 nm) was recorded every 6 sec (excitation wavelengths at 340 and 380 nm; F_{340} and F_{380}) with a CCD camera (TILL Photonics GmbH). Data collection and digital camera

and polychromator control were performed by imaging software (TILLvisION, version 4.01). F_{340}/F_{380} was calculated and data were analyzed using custom-made Excel macros (Microsoft Corp., Redmond, WA, USA).

Cells were exposed to 0.2 - 20 µM BDE-47 or 0.02 - 20 µM 6-OH-BDE-47 for 20 min, after 5 min baseline recording. In specific experiments, in which intracellular Ca^{2+} stores were emptied by 10 min pre-incubation with 1 µM thapsigargin (TG) and 1 µM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), cells were exposed to 20 µM BDE-47 or 6-OH-BDE-47 for 15 min. For the experiments under Ca^{2+}-free conditions, Fura-2-loaded cells were washed with Ca^{2+}-free external saline (containing 10 µM EDTA) just before the imaging experiments. Maximum and minimum ratios were determined after 25 min recording by addition of ionomycin (5 µM) and ethylenediamine tetraacetic acid (EDTA; 17 mM) as a control for experimental conditions.

LDH assay. Cell viability was measured using a lactate-dehydrogenase (LDH) assay (CytoTox-One, Promega) as previously described (Moors et al. 2009). Briefly, supernatants from cells that were exposed to 0.02 - 20 µM BDE-47 or 6-OH-BDE-47 for 20 min were collected and incubated 1:1 with the CytoTox-One reagent for 4 h prior to detection of fluorescence (excitation wavelength: 540 nm; emission wavelength: 590 nm). Complete lysis of cells with the included lysis buffer for 2 h at room temperature serves as positive control.

Data analysis and statistics. To determine effects on $[Ca^{2+}]_i$, F_{340}/F_{380} values were normalized to the average F_{340}/F_{380} during 5 min baseline recording. An increase in the normalized fluorescence ratio of more than 1.2 was considered as an increase in $[Ca^{2+}]_i$ and was used for further data analysis. All data are presented as mean ± SE from the number of cells (*n*) indicated. Categorical data were compared using Fisher´s exact and chi-square tests. We compared continuous data using paired Student's *t*-test. A *p*-value < 0.05 was considered statistically significant.

Results

BDE-47 as well as 6-OH-BDE-47 increase $[Ca^{2+}]_i$ in human neural progenitor cells. The acute effects of BDE-47 and 6-OH-BDE-47 were investigated in cells that had grown out from the hNPC neurosphere during differentiation.

BDE-47 induced a concentration-dependent increase in $[Ca^{2+}]_i$ with a lowest-observed-effect concentration (LOEC) of 2 µM (Figure 7.1A and 7.2A). At an exposure concentration of 20 µM BDE-47, the amplitude of $[Ca^{2+}]_i$ was increased to 2.1 ± 0.2 F_{340}/F_{380} (normalized to baseline). Multiple transient increases in $[Ca^{2+}]_i$ were observed starting 3 - 5 min after application of BDE-47 (2 - 20 µM).

Exposure of differentiated hNPCs to 6-OH-BDE-47 also induced a concentration-dependent increase in $[Ca^{2+}]_i$, with a LOEC of 0.2 µM (Figure 7.1B and 7.2B). At an exposure concentration of 20 µM 6-OH-BDE-47, the amplitude of $[Ca^{2+}]_i$ was increased to 5.7 ± 0.5 F_{340}/F_{380} (normalized to baseline). 6-OH-BDE-47 caused a persistent $[Ca^{2+}]_i$ elevation starting 0 - 1 min after 6-OH-BDE-47 application (early persistent increase) in concentrations ≥ 0.2 µM. At 20 µM 6-OH-BDE-47, the early persistent increase in $[Ca^{2+}]_i$ was combined with a transient $[Ca^{2+}]_i$ increase within the first 5 min after application. Also, a late (> 10 min) increase in $[Ca^{2+}]_i$ was observed. Some cells showed a very high $[Ca^{2+}]_i$ increase without recovering (Figure 7.1B).

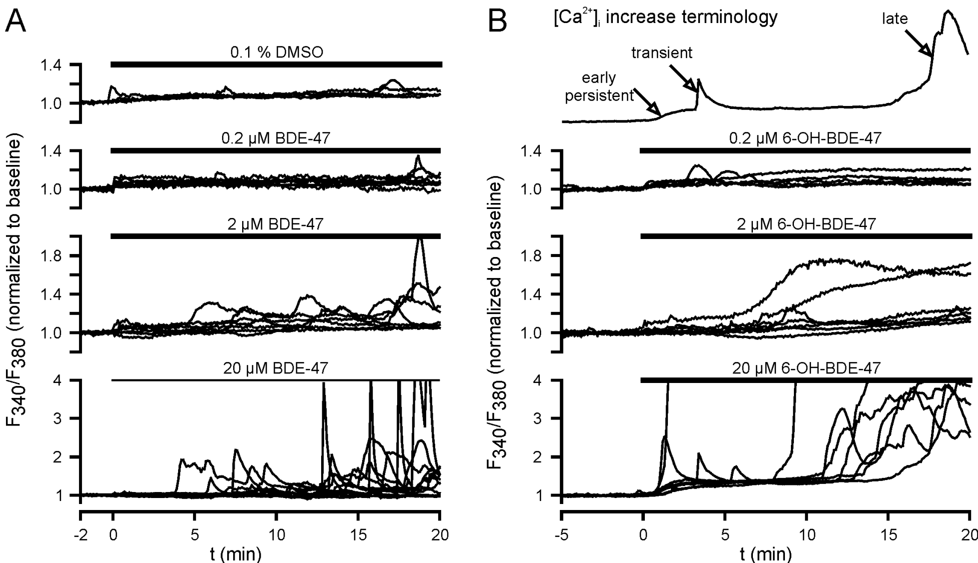


Figure 7.1: Increase in $[Ca^{2+}]_i$ in human neurospheres during exposure to BDE-47 or 6-OH-BDE-47. Results are shown as representative traces of normalized F_{340}/F_{380} ratio, from individual cells exposed to 0.2 to 20 µM BDE-47 (A) or 0.2 to 20 µM 6-OH-BDE-47 (B) for 20 min, applied at t = 0 min, as indicated. Note the difference in scaling for 20 µM. On top of the 6-OH-BDE-47 example traces, a legend of the terminology for different increases in $[Ca^{2+}]_i$ (early persistent, transient and late) is shown.

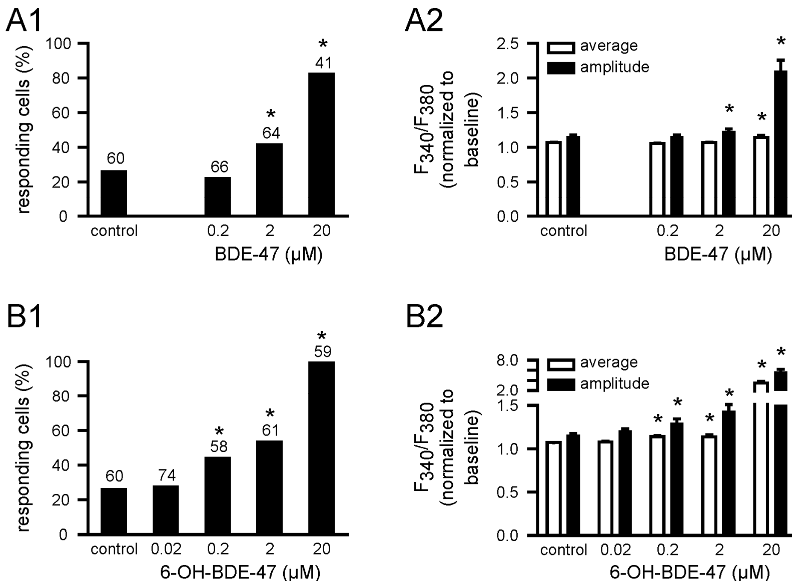


Figure 7.2: Occurrence of $[Ca^{2+}]_i$ disturbances and amplitude of $[Ca^{2+}]_i$ during exposure to BDE-47 or 6-OH-BDE-47. A1 and B1. Bars indicate the percentage of cells showing an increase in $[Ca^{2+}]_i$ within 20 min after application. A2 and B2. Bars indicate average and amplitudes of increase in $[Ca^{2+}]_i$ after application. Data shown are from 3 - 4 independent experiments (mean ± SE). Numbers above the bars (A1 and B1) indicate the number of cells using for data analysis (both for percentage of responding cells and normalized F_{340}/F_{380}). * Significances compared to control ($p < 0.05$).

PBDE-induced increases in $[Ca^{2+}]_i$ originate from extracellular and intracellular stores. To investigate the underlying mechanisms of the observed increases in $[Ca^{2+}]_i$, additional Ca^{2+}-imaging experiments were performed with BDE-47 and 6-OH-BDE-47 (20 µM) in differentiated hNPCs under Ca^{2+}-free conditions. To identify the intracellular stores responsible for the observed increases in $[Ca^{2+}]_i$ additional Ca^{2+} imaging experiments were performed. In these experiments, mitochondrial and TG-sensitive ER Ca^{2+} stores were depleted by pretreatment with TG and FCCP, respectively, under Ca^{2+}-free conditions. TG and TG/FCCP pretreatments increase $[Ca^{2+}]_i$ transiently, after which the baseline stabilizes.

In these experiments, BDE-47 induced only very few fast transient $[Ca^{2+}]_i$ increases. Compared to BDE-47 exposure under physiological (1.8 mM) Ca^{2+}-conditions, where up to 4 transients/cell were recorded, only 1 transient/cell was measured under Ca^{2+}-free conditions in only ~25% of the cells (Figure 7.3A). Moreover, both the number of responsive cells and the amplitude of the increase in $[Ca^{2+}]_i$ were decreased under Ca^{2+}-free conditions from 83%

to 26% and the amplitude of the $[Ca^{2+}]_i$ increase was significantly smaller (1.2 ± 0.1; 1.7 ± 0.1, Figure 7.3C and 7.3D). In cells in which ER Ca^{2+} stores were depleted with TG, increases in $[Ca^{2+}]_i$ were completely absent during exposure to BDE-47 (data not shown).

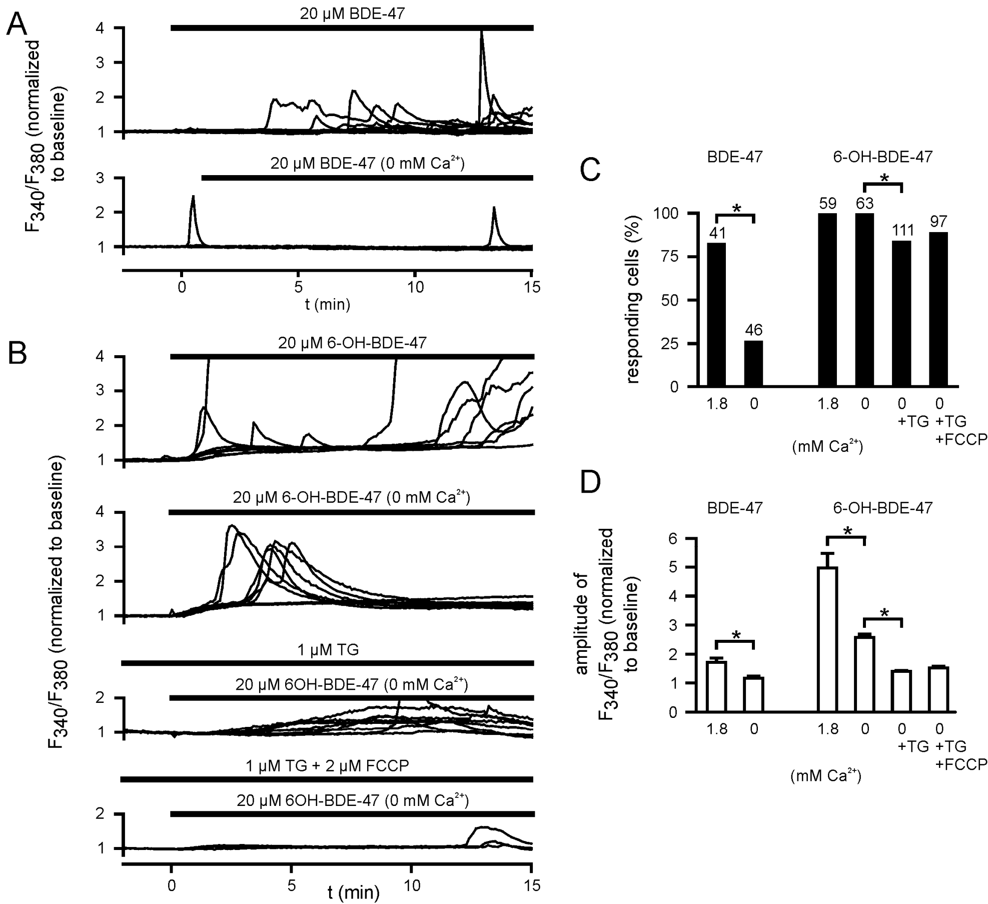


Figure 7.3: Release from intracellular Ca^{2+} stores in human neurospheres during exposure to BDE-47 or 6-OH-BDE-47. Results are shown as representative traces of $[Ca^{2+}]_i$ measurements of individual cells exposed to 20 µM 6-OH-BDE-47 (A) or BDE-47 (B) for 15 min, applied at t = 0 min. The diagrams show percentage of responding cells (C) and the amplitude of increase in $[Ca^{2+}]_i$ (D). Data shown are from 3 - 4 independent experiments (mean ± SE). Numbers above the bars (C) indicate the number of cells using for data analysis (both for percentage of responding cells and normalized F_{340}/F_{380}). * Significances compared as indicated ($p < 0.05$).

During exposure to 6-OH-BDE-47, the number of responding cells did not change under Ca^{2+}-free conditions (Figure 7.3C). The early persistent and the transient increases in $[Ca^{2+}]_i$ were still present, while the late increase was not observed (Figure 7.3B). The average and the amplitude of $[Ca^{2+}]_i$ were lower under Ca^{2+}-free conditions compared to physiological Ca^{2+} conditions (Figure 7.3D). During exposure to 6-OH-BDE-47 after depletion of endoplasmic Ca^{2+} stores, the number of responding cells and amplitude of $[Ca^{2+}]_i$ were decreased (Figure 7.3C and 7.3D). The early persistent 6-OH-BDE-47-induced increase in $[Ca^{2+}]_i$ still remained in presence of TG, although delayed by several minutes. When mitochondrial Ca^{2+} stores were also depleted under Ca^{2+}-free conditions, number of responding cells and amplitude of $[Ca^{2+}]_i$ were not decreased (Figure 7.3C and 7.3D). However, the early persistent increase in $[Ca^{2+}]_i$ was no longer observed, although a small, late increase in $[Ca^{2+}]_i$ remained (Figure 7.3B).

BDE-47 and 6-OH-BDE-47 are not cytotoxic in hNPCs. Possible acute cytotoxicity of BDE-47 or 6-OH-BDE-47 in differentiated hNPCs was investigated using a LDH assay. hNPCs were exposed to different concentrations of BDE-47 or 6-OH-BDE-47 for 20 min, after which the supernatant was collected and LDH release was analyzed. An exposure up to 20 µM BDE-47 or 6-OH-BDE-47 did not increase LDH release from the cells compared to solvent control (data not shown).

Discussion

This study demonstrated that BDE-47 and 6-OH-BDE-47 acutely increase $[Ca^{2+}]_i$ in differentiated hNPCs. PBDEs have been shown to affect Ca^{2+} homeostasis in microsomes (Kodavanti and Ward 2005) and to reduce Ca^{2+} uptake by brain microsomes and mitochondria (Coburn et al. 2008) at relatively high concentrations. Increase in $[Ca^{2+}]_i$ was previously also observed in PC12 cells during exposure to BDE-47 (Dingemans et al. 2007). Disruption of Ca^{2+} homeostasis by PBDEs could be involved in the previously observed induction of reactive oxygen species production, neurotransmitter release and re-uptake, activation of protein kinases and related processes (reviewed in Fonnum and Mariussen 2009).

The effects on calcium homeostasis in PC12 cells were more pronounced for the hydroxylated metabolite 6-OH-BDE-47 compared to the parent compound BDE-47 (Dingemans et al. 2008, 2009b). This was also observed in this study in differentiated hNPCs, in which different increases were observed: early persistent, transient and late increases in $[Ca^{2+}]_i$. As Ca^{2+} is essential in cell proliferation and differentiation (Berridge et al. 2000), the observed acute increases in $[Ca^{2+}]_i$ are possibly involved in the previously observed effects of BDE-47 on differentiation and migration of hNPCs (as described in Schreiber et al. 2009).

The removal of Ca^{2+} from external saline results in a dramatic decrease in number of responding cells during exposure to BDE-47. This indicated that the transient increases

induced by BDE-47 are caused by influx of extracellular Ca^{2+}. After depletion of ER under Ca^{2+}-free conditions, transient increases are completely absent, indicating that a small number of the transient increases are originating from Ca^{2+} release from the ER.

During exposure to 6-OH-BDE-47 under Ca^{2+}-free conditions, the late increase is completely absent. This indicates that the late increase induced by 6-OH-BDE-47 originates mainly from influx of extracellular Ca^{2+}. Possible routes for Ca^{2+}-influx are influx of extracellular Ca^{2+} through voltage-gated Ca^{2+}-channels (Catterall 2000) or store-operated Ca^{2+} entry (SOCE) channels (Parekh and Putney 2005). Furthermore, the loss of membrane integrity can result in an unspecific increase in $[Ca^{2+}]_{i.}$ However, it is unlikely that BDE-47 or 6-OH-BDE-47 disrupt membrane integrity, as extracellular LDH was not detected after exposure to 20 µM BDE-47 or 6-OH-BDE-47. As the release of Ca^{2+} from intracellular stores was induced by 6-OH-BDE-47, replenishing of Ca^{2+} by SOCE is probably involved in the observed influx of extracellular Ca^{2+} (Parekh and Putney 2005).

The transient increase induced by 6-OH-BDE-47 that remains under Ca^{2+}-free conditions is completely absent after depletion of ER by TG, indicating that these increases in $[Ca^{2+}]_i$ primarily originate from Ca^{2+} release from the ER. After depletion of ER Ca^{2+} stores, the early persistent increase in $[Ca^{2+}]_i$ caused by exposure to 20 µM 6-OH-BDE-47 could still be observed. As this increase is not observed in cells in which mitochondrial and endoplasmic stores were depleted with TG and FCCP, it can be concluded that this early persistent increase induced by 6-OH-BDE-47 originates primarily from Ca^{2+} release from mitochondrial Ca^{2+} stores. In most cells, a late increase in $[Ca^{2+}]_i$ is observed after depletion of ER and mitochondrial Ca^{2+} stores. Possible sources involved in the remaining increase in $[Ca^{2+}]_i$ are endosomes, lysosomes, secretory granules, the Golgi-apparatus and nucleus (reviewed in Laude and Simpson 2009).

6-OH-BDE-47 increases $[Ca^{2+}]_i$ at lower concentrations than parent compound BDE-47 in differentiated hNPCs. This potency difference was previously shown in rat PC12 cells (Dingemans et al. 2008). However, in the primary differentiated hNPCs, the effects of BDE-47 and its hydroxylated metabolite appear stronger than in PC12 cells. Acute exposure of hNPCs to $\geq$ 2 µM BDE-47 or $\geq$ 0.2 µM 6-OH-BDE-47 results in a significant increase in $[Ca^{2+}]_{i,}$ whereas in PC12 cells higher concentrations of BDE-47 (20 µM) and 6-OH-BDE-47 ($\geq$ 1 µM) are required to induce increases in $[Ca^{2+}]_i$ (Dingemans et al. 2008, 2009b).

Moreover, different kinetics of Ca^{2+} store-release and influx induced by 6-OH-BDE-47 were observed in the two models. In PC12 cells, Ca^{2+} release from ER in combination with influx of extracellular Ca^{2+} could be observed as a transient increase in $[Ca^{2+}]_i$ within approximately the first 5 min following application of 6-OH-BDE-47. A late increase in $[Ca^{2+}]_i$ in PC12 cells was mainly due to release of Ca^{2+} from mitochondria. In differentiated hNPCs, the Ca^{2+} release from mitochondria starts immediately upon application of 6-OH-BDE-47. Based on cell size and cell morphology, the cells that were used for $[Ca^{2+}]_i$ measurement are most likely glial progenitor cells (Fritsche et al. 2005). Possibly, this difference in timing could be related to the particular sensitivity of glial cells to oxidative stress (Andersen 2004). The

timing of ER release is similar in the hNPCs as in PC12 cells. However, this is not combined with influx of extracellular Ca^{2+}, which is, in hNPCs, observed as a late increase in $[Ca^{2+}]_i$. As different kinetics of processes involved in the increases in $[Ca^{2+}]_i$ are observed in PC12 and hNPCs, the difference in sensitivity is possibly due to differences in regulatory mechanisms for maintaining Ca^{2+} homeostasis in these two cell types.

Release of Ca^{2+} from ER is a major effect of 6-OH-BDE-47, both in PC12 as in differentiated hNPCs (Dingemans et al. 2008, 2009b, this study) The principle mechanisms affecting release of Ca^{2+} from ER stores involves activation of inositol 1,4,5-triphosphate (IP_3) and/or ryanodine (Ry) receptors (reviewed in Vanderheyden et al. 2009; Zalk et al. 2007). It is unknown whether (OH-)PBDEs affect either of these receptors to induce Ca^{2+} release from ER. Nonetheless, *ortho*-PCBs were demonstrated to affect both IP_3- (Inglefield et al. 2000; Kang et al. 2004) and Ry-receptor-mediated Ca^{2+} release (Wong et al. 1997, 2001; Gafni et al. 2004). Interaction of 6-OH-BDE-47 with these endoplasmic receptors is therefore not unlikely.

In summary, exposure to BDE-47 or 6-OH-BDE-47 causes an increase in $[Ca^{2+}]_i$ in hNPCs. The combined data indicate that the transient increase in $[Ca^{2+}]_i$ depends on intracellular Ca^{2+} release from the ER, whereas the early persistent increase in $[Ca^{2+}]_i$ is mainly due to Ca^{2+} release from mitochondria. The late increase in $[Ca^{2+}]_i$ is caused by influx of extracellular Ca^{2+}, since it is absent under Ca^{2+}-free conditions. Increase in $[Ca^{2+}]_i$ occurs at concentrations ≥ 2 µM (BDE-47) and 0.2 µM (6-OH-BDE-47). The observed increase in $[Ca^{2+}]_i$ by release from intracellular stores and influx of extracellular Ca^{2+} occurs at lower concentrations compared to in PC12 cells. 6-OH-BDE-47 has a potency approximately one order of magnitude higher than the parent compound BDE-47 in both PC12 as differentiated hNPCs. The Ca^{2+} homeostasis-disrupting effect of hydroxylated metabolites of PBDEs is therefore a critical factor that should be taken into account in risk assessment for PBDE-induced DNT in humans.

Chapter 8

Hexabromocyclododecane inhibits depolarization-induced increase in intracellular calcium levels and neurotransmitter release in PC12 cells

Milou M.L. Dingemans[1*], Harm J. Heusinkveld[1*], Aart de Groot[1], Åke Bergman[2], Martin van den Berg[1] and Remco H.S. Westerink[1]

1. Institute for Risk Assessment Sciences, Utrecht University, Utrecht, the Netherlands.
2. Department of Environmental Chemistry, Stockholm University, Stockholm, Sweden.

*. These authors contributed equally.

Toxicological Sciences **107**, 490 - 497.

Abstract

Environmental levels of the brominated flame retardant (BFR) hexabromocyclododecane (HBCD) have been increasing. HBCD has been shown to cause adverse effects on learning and behavior in mice, as well as on dopamine uptake in rat synaptosomes and synaptic vesicles. For other BFRs, alterations in the intracellular Ca^{2+} homeostasis have been observed.

Therefore, the aim of this study was to investigate whether the technical HBCD mixture and individual stereoisomers affect the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in a neuroendocrine *in vitro* model (PC12 cells). $[Ca^{2+}]_i$ and vesicular catecholamine release were measured using respectively single-cell Fura-2 imaging and amperometry.

Exposure of PC12 cells to the technical HBCD mixture or individual stereoisomers did neither affect basal $[Ca^{2+}]_i$, nor the frequency of basal neurotransmitter release. However, exposure to HBCD (0 - 20 µM) did cause a dose-dependent reduction of a subsequent depolarization-evoked increase in $[Ca^{2+}]_i$. This effect was apparent only when HBCD was applied at least 5 min before depolarization (maximum effect after 20 min exposure). The effects of α- and β-HBCD were comparable to that of the technical mixture, whereas the inhibitory effect of γ-HBCD was larger. Using specific blockers of L-, N- or P/Q-type voltage-gated Ca^{2+} channels (VGCCs) it was shown that the inhibitory effect of HBCD is not VGCC-specific. Additionally, the number of cells showing depolarization-evoked neurotransmitter release was markedly reduced following HBCD exposure.

Summarizing, HBCD inhibits depolarization-evoked $[Ca^{2+}]_i$ and neurotransmitter release. As increasing HBCD levels should be anticipated, these findings justify additional efforts to establish an adequate exposure, hazard and risk assessment.

Introduction

Brominated flame retardants (BFRs) are a structurally diverse class of compounds added to consumer products for fire prevention. Increasing concentrations of these compounds in the environment, human food chain and in human tissues raise concern about possible neurotoxic effects, particularly for the developing nervous system (reviewed in Costa and Giordano 2007). This concern has led to voluntary action and legislative measures to eliminate the penta- and octabrominated diphenyl ethers from the market in Europe and North America. After cessation of their use in consumer products, a sharp rise in environmental levels of the BFR hexabromocyclododecane (HBCD) was recently observed (reviewed in Law et al. 2008b). This is possibly due to an increased use of HBCD as a replacement of the banned polybrominated diphenyl ethers (PBDEs), mainly in polystyrene products. The technical mixture of HBCD consists of three stereoisomers, denoted α-, β-, and γ-HBCD. HBCD has been shown to biomagnify in marine food chains (Jenssen et al. 2007). Interestingly, biotic samples are in general dominated by the α-stereoisomer although the technical mixture consists for 70 - 90% of γ-HBCD (Covaci et al. 2006). This is probably due to isomer composition changes during heating of the technical mixture during industrial application (Heeb et al. 2008), and/or cytochrome P450 mediated biotransformation of β- and γ-HBCD in biota (Zegers et al. 2005).

Acute *in vivo* toxicity of HBCD appears limited (Darnerud 2003). So far, studies on the toxic potential of HBCD have mainly focused on endocrine disruption. Adverse effects of HBCD on the steroid and thyroid hormone system as well as antagonistic effects on the androgen, progesterone and aryl hydrocarbon receptors have been described (Ema et al. 2008; Hamers et al. 2006; Palace et al. 2008; van der Ven et al. 2006). Additionally, effects on hepatic gene expression and hepatic enzymes have been observed (Cantón et al. 2008a; Germer et al. 2006; Ronisz et al. 2004; Zhang et al. 2008c).

Although an adverse effect on learning and behavior in mice (1.4 µmol/kg bw HBCD; Eriksson et al. 2006b), as well as on oxidative stress and the cellular antioxidant defense systems in fish brain (28 days exposure to 500 µg/l HBCD; Zhang et al. 2008c) have been observed, *in vitro* neurotoxicity data on HBCD are limited. So far, only inhibition of dopamine uptake in rat synaptosomes (IC_{50}: 4 µM) and synaptic vesicles (IC_{50}: 3 µM; Mariussen and Fonnum 2003) and necrotic cell death in cerebellar granule cells after 24 h exposure (LC_{50}: 3 µM; Reistad et al. 2006) have been reported.

Alterations in the intracellular Ca^{2+} homeostasis, as observed for PBDEs and *ortho*-substituted (noncoplanar) polychlorinated biphenyls (*ortho*-PCBs; reviewed in Mariussen and Fonnum 2006), may play a role in the observed cytotoxicity and oxidative stress (Orrenius et al. 2003). In neuronal cell-types, the main influx route of Ca^{2+} is via voltage-gated Ca^{2+} channels (VGCCs). VGCCs are located in the cell membrane and open when the membrane depolarizes. The subsequent rapid influx of Ca^{2+} can trigger various intracellular processes including neurotransmitter release (Barclay et al. 2005; Clapham 2007). Therefore, the aim of

this study was to investigate the possible effects of the technical HBCD mixture and the individual HBCD stereoisomers on basal and depolarization-evoked cytosolic Ca^{2+} levels as well as on vesicular catecholamine release in a neuroendocrine *in vitro* model (PC12 cells), using respectively Fura-2 imaging and amperometry.

Methods

Chemicals. The technical HBCD mixture was synthesized and the stereoisomers α-, β-, and γ-HBCD isolated and purified (~99%) at the Stockholm University (Sweden) as described previously (Fång 2007). Unless otherwise noted, PC12 cells were exposed to the technical HBCD mixture. Before dilution to the desired final concentration, HBCD stock solutions in dimethyl sulfoxide (DMSO) were sonicated for at least 15 min. All other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich, Zwijndrecht, the Netherlands.

PC12 cell culture. Rat pheochromocytoma (PC12) cells (Greene and Tischler 1976) were cultured in RPMI 1640 (Invitrogen, Breda, The Netherlands) supplemented with 5% fetal calf serum and 10% horse serum (ICN Biomedicals, Zoetermeer, The Netherlands). For Ca^{2+} imaging experiments, undifferentiated PC12 cells were subcultured in poly-L-lysine coated glass-bottom dishes (MatTek, Ashland, MA) as described previously (Dingemans et al. 2007). For amperometric recordings, cells were differentiated for 3 - 5 days with 5 µM dexamethasone to enhance vesicular catecholamine release as described previously (Westerink and Vijverberg 2002).

Cell viability assay. Effects of HBCD on cell viability were determined by measuring the capacity of undifferentiated PC12 cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Denizot and Lang 1986). One day before the cell viability test, PC12 cells were seeded in a 96-wells plate at a density of $5*10^4$ cells per well. The cells were exposed to different concentrations of HBCD for 30 min, after which the cells were incubated for 30 min in 200 µl MTT (1 mg/ml) in serum-free medium. After washing the cells with phosphate buffered saline, the formazan was extracted in 100 µl of isopropanol. This isopropanol fraction was measured spectrophotometrically at 595 nm (FLUOstar Galaxy V4.30-0, BMG Labtechnologies, Offenburg, Germany) to determine the quantity of the blue formazan, which is used as a measure for cell viability.

Ca^{2+} imaging. Changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured using the Ca^{2+}-sensitive fluorescent ratio dye Fura-2 as described previously (Dingemans et al. 2007). Briefly, cells were loaded with 5 µM Fura-2-AM (Molecular Probes; Invitrogen) in external saline (containing in mM: 1.8 $CaCl_2$, 24 glucose, 10 HEPES, 5.5 KCl, 0.8 $MgCl_2$, 125 NaCl, and 36.5 sucrose at pH 7.3, adjusted with NaOH) for 20 min at room temperature,

followed by 15-min de-esterification in external saline. After de-esterification, cells were placed on the stage of an Axiovert 35M inverted microscope (Zeiss, Göttingen, Germany) equipped with a TILL Photonics Polychrome IV (TILL Photonics GmBH, Gräfelfing, Germany). Fluorescence evoked by 340 and 380 nm excitation wavelengths (F_{340} and F_{380}) was collected every 12 s at 510 nm with an Image SensiCam digital camera (TILL Photonics GmBH). The digital camera and polychromator were controlled by imaging software (TILLvisION, version 4.01), which was also used for data collection and processing. Changes in F_{340}/F_{380} ratio (R), reflecting changes in $[Ca^{2+}]_i$, were analyzed using custom-made MS-Excel macros. Following 5 min baseline recording, cells were exposed to DMSO, HBCD (0.2 - 20 µM), or α-, β-, or γ-HBCD (0.2 - 2 µM). Membrane depolarization by 100 mM K^+ was used to investigate effects of HBCD on the depolarization-evoked increase in $[Ca^{2+}]_i$, caused by influx of Ca^{2+} through VGCCs. Maximum and minimum ratios (R_{max} and R_{min}) were determined after the recording by addition of ionomycin (5 µM) and ethylenediamine tetraacetic acid (EDTA; 17 mM) as a control for experimental conditions. Free cytosolic $[Ca^{2+}]_i$ was calculated using Grynkiewicz's equation:

$$[Ca^{2+}]_i = K_{d*} \times (R - R_{min})/(R_{max} - R)$$

(as described in Deitmer and Schild 2000), where K_{d*} is the dissociation constant of Fura-2 determined in the experimental set-up used for the fluorescence measurements using Fura-2 Ca^{2+} imaging calibration buffers (Molecular Probes; Invitrogen). Because the standard deviation during baseline $[Ca^{2+}]_i$ recording ranged from 1 - 21 nM (1 - 20 % of average $[Ca^{2+}]_i$), $[Ca^{2+}]_i$ levels > 125% of baseline during exposure were scored as spontaneous fluctuations. The average basal $[Ca^{2+}]_i$ and the number of cells showing spontaneous fluctuations in basal $[Ca^{2+}]_i$, as well as amplitude and duration of these fluctuations, were determined to investigate possible effects of HBCD on basal $[Ca^{2+}]_i$. The amplitude of $[Ca^{2+}]_i$ observed during depolarization was used to investigate possible effects of HBCD on the depolarization-induced increase in $[Ca^{2+}]_i$. The involvement of specific VGCCs was investigated by selective pharmacological blocking using 2 µM nifedipine (L-type VGCCs), 2 µM ω-conotoxin GVIA (N-type VGCCs; Biotrend, Wangen, Switzerland), and 2 µM ω-conotoxin MVIIC (P/Q-type VGCCs; Biotrend).

Amperometry. Amperometric recordings of spontaneous and K^+-evoked vesicular catecholamine release from dexamethasone-differentiated PC12 cells using carbon fiber microelectrodes were made as described previously (Dingemans et al. 2007; Westerink and Vijverberg 2002). Following 150 s of baseline recording, PC12 cells were superfused for 15 s with high K^+ containing saline (K^+ increased to 125 mM and Na^+ lowered to 5.5 mM) to determine their responsiveness. Cells were allowed to recover for 135 s prior to a 20-min exposure to HBCD to investigate acute effects on vesicular catecholamine release. Recordings were performed at room temperature. As described previously (Dingemans et al. 2007, 2008),

PC12 cells with high basal release (> 2 events/min), low evoked release (< 20 events/min) or poor recovery after depolarization ('recovered basal release'; > 14 events/min) were excluded from data analysis (5/34 cells). The remaining 29 cells were used to investigate effects of HBCD on the frequency of vesicular catecholamine release and vesicular release parameters, that is, amplitude, $t_{1/2}$ (half-width) and time integral (Q, vesicular content) of the amperometric release events.

Data analysis and statistics. All data are presented as mean ± SEM from the number of cells indicated. Statistical analyses were performed using SPSS 12.0.1 (SPSS, Chicago, IL). Continuous data were compared using Student's *t*-test, paired or unpaired where applicable. ANOVA and *post hoc t*-tests were performed to investigate possible dose-response relationships. Categorical data were compared using Fisher's exact tests. A p value < 0.05 is considered statistically significant; n.s. indicates the absence of a statistically significant effect.

Results

Cell viability. PC12 cells, cultured in a 96-wells plate, were exposed to 0 - 20 µM technical HBCD mixture or the individual stereoisomers for 30 min before incubation in 1 mg/ml MTT. Exposure to 0 - 2 µM HBCD (30 min) had no effects on cell viability, whereas exposure to 20 µM HBCD (30 min) resulted in a limited reduction of cell viability to 88 ± 3% of control ($p < 0.05$). After 30 min exposure to 0 - 20 µM α-, β-, or γ-HBCD, no effects on cell viability were observed (Figure 8.1).

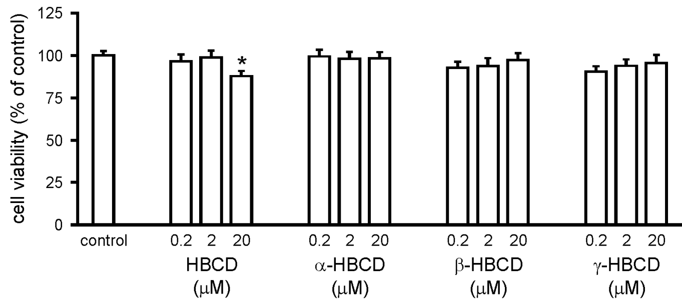


Figure 8.1. Exposure of PC12 cells to the technical HBCD mixture has a small effect on cell viability only at 20 µM, whereas no significant effects were observed after exposure to the individual stereoisomers. Bars represent average cell viability as measured by mitochondrial MTT reduction, normalized to DMSO control wells per plate. Bars display data from 4 experiments, number of wells used for data analysis: control: $n = 36$; all HBCD treatments: $n = 12$. *$p < 0.05$.

Cytosolic [Ca^{2+}]$_i$. To investigate whether HBCD affects basal [Ca^{2+}]$_i$, Fura-2 loaded PC12 cells were exposed for 20 min to HBCD (0 - 20 µM). Basal [Ca^{2+}]$_i$ before exposure amounted to 106 ± 1 nM (*n* = 440). In control experiments, 30% of the cells showed spontaneous fluctuation in [Ca^{2+}]$_i$. The average basal [Ca^{2+}]$_i$ (20 µM HBCD: 100 ± 3 nM; n.s.) as well as the percentage of cells showing spontaneous fluctuations in [Ca^{2+}]$_i$ (20 µM HBCD: 35%; n.s.) were not affected by exposure to HBCD (Figure 8.2). The amplitude and duration of these spontaneous fluctuations in [Ca^{2+}]$_i$ were also not affected during exposure to 20 µM HBCD (data not shown). Exposure to the separate α-, β-, or γ-stereoisomers (0 - 2 µM) did neither affect the average basal [Ca^{2+}]$_i$ nor the percentage of cells showing spontaneous fluctuations in [Ca^{2+}]$_i$, during exposure (data not shown).

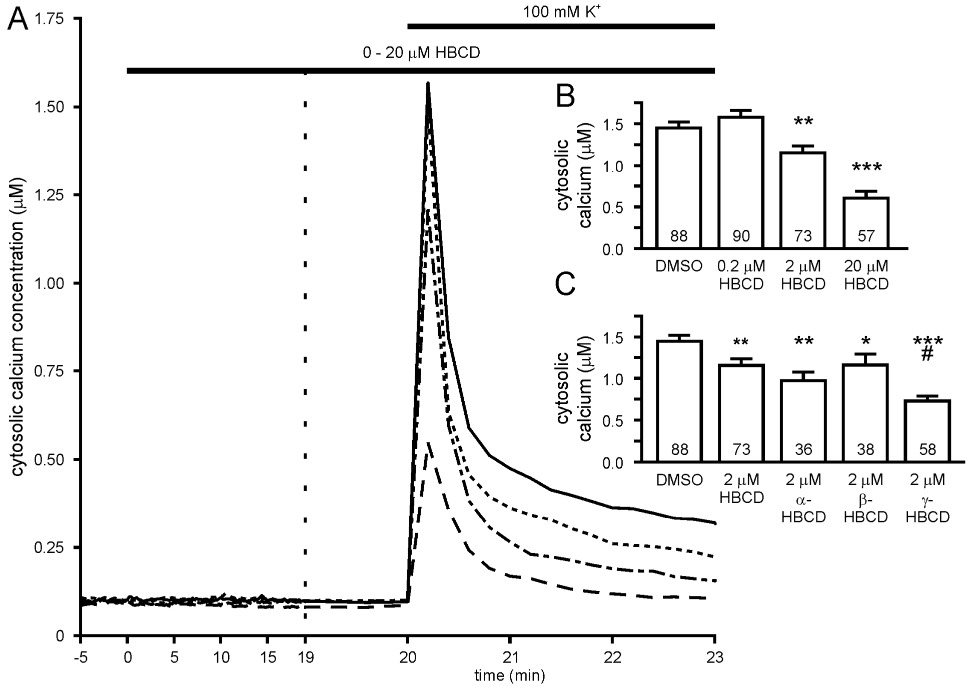


Figure 8.2. Exposure of PC12 cells to HBCD has no effects on basal [Ca^{2+}]$_i$, but inhibits the depolarization-evoked increase in [Ca^{2+}]$_i$. A. Representative traces of cytosolic [Ca^{2+}]$_i$ measurements of individual PC12 cells are shown, illustrating the reduction of the depolarization-evoked increase in [Ca^{2+}]$_i$ by exposure to 0 - 20 µM HBCD (control: solid line; 0.2 µM HBCD: small dash; 2 µM HBCD: dash-dot-dash; 20 µM HBCD: large dash; note the difference in time scaling from t = 19 min). B. Bar graph shows the dose-dependent reduction of the depolarization-evoked increase in [Ca^{2+}]$_i$ by HBCD. C. Bar graph shows the reduction of the evoked [Ca^{2+}]$_i$ by the technical mixture and separate stereoisomers of HBCD. 2 µM γ-HBCD significantly differs from all other HBCD treatments (# $p < 0.05$). Bars display data from three to nine experiments per treatment. The number of cells used for data analysis is indicated inside each bar. Difference from control: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

VGCCs expressed in undifferentiated PC12 cells include L-, N-, P/Q-, R-, and T-type Ca^{2+} channels (Del Toro et al. 2003; Greene and Tischler 1976; Liu et al. 1996; Shafer and Atchison 1991), with L-, N-, and P/Q-type VGCCs accounting for the majority of the depolarization-induced increase in $[Ca^{2+}]_i$ (blocking L-, N-, and P/Q-type VGCCs reduced the depolarization-evoked increase in $[Ca^{2+}]_i$ to ~15% of control, data not shown). To investigate whether HBCD has an effect on the depolarization-evoked increase in $[Ca^{2+}]_i$, PC12 cells were exposed to different concentrations of HBCD before and during depolarization by a high K^+-containing saline.

When applying 20 μM HBCD immediately before the depolarization, no effects were observed on the depolarization-evoked increase in $[Ca^{2+}]_i$, making direct effects of HBCD on VGCCs less likely. When the exposure time is prolonged, the inhibitory effect on the depolarization-evoked increase in $[Ca^{2+}]_i$ aggravated. Increasing the exposure time from 20 to 40 min did not further reduce the depolarization-evoked increase in $[Ca^{2+}]_i$ (data not shown). Therefore, in all following $[Ca^{2+}]_i$ measurements, cells were exposed to HBCD for 20 min prior to depolarization.

Exposing PC12 cells for 20 min to HBCD technical mixture (0 - 20 μM) caused a dose-dependent reduction of the subsequent depolarization-evoked increase in $[Ca^{2+}]_i$ (ANOVA: $p < 0.0001$; Figure 8.2). In control cells, $[Ca^{2+}]_i$ increased to 1.45 ± 0.07 μM ($n = 88$) during depolarization (basal $[Ca^{2+}]_i$: 107 ± 3 nM). No effects on evoked $[Ca^{2+}]_i$ were observed when exposing the cells to 0.2 μM HBCD. Increasing the concentration of HBCD resulted in a reduction of evoked $[Ca^{2+}]_i$ with a lowest observed effect level (LOEL) of 2 μM HBCD (1.15 ± 0.08 μM, $n = 73$; $p < 0.01$). Exposing the cells to 20 μM HBCD inhibited the evoked $[Ca^{2+}]_i$ to 0.61 ± 0.08 μM ($n = 57$; $p < 0.0001$), which is approximately 40% of the control value.

Although the technical mixture mainly consists of γ-HBCD, it has been observed that in biotic samples, α-HBCD is most abundant. To investigate possible differences in activity between the different stereoisomers of HBCD, PC12 cells were exposed to 2 μM of the different stereoisomers (α-, β-, and γ-HBCD). All three stereoisomers inhibited the depolarization-evoked increase in $[Ca^{2+}]_i$. The inhibitory effects of α- and β-HBCD were comparable with that of the technical mixture (HBCD mixture: 1.15 ± 0.08 μM, $n = 73$; α-HBCD: 0.97 ± 0.10 μM, $n = 36$; β-HBCD: 1.16 ± 0.13 μM, $n = 38$), whereas the inhibitory effect of γ-HBCD was larger (0.73 ± 0.06 μM, $n = 58$; Figure 8.2).

To investigate whether specific VGCCs were involved in the inhibitory effects of HBCD on the depolarization-evoked increase in $[Ca^{2+}]_i$, L-, N-, or P/Q-type VGCCs were pharmacologically blocked using 2 μM of, respectively, nifedipine, GVIA, or MVIIC ω-conotoxins (Figure 8.3). Though all VGCC blockers decreased the depolarization-evoked increase in $[Ca^{2+}]_i$, nifedipine had the largest effect (0.80 ± 0.05 μM). Exposure to HBCD (20 μM) while blocking L-type VGCCs with nifedipine, resulted in a further decrease of the depolarization-induced $[Ca^{2+}]_i$ (0.64 ± 0.07 μM). Comparable results were seen when blocking either N- or P/Q-type VGCCs (Figure 8.3).

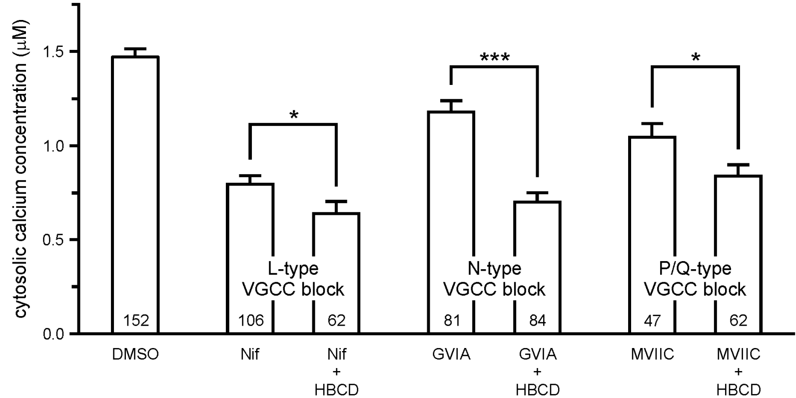


Figure 8.3. HBCD a-specifically inhibits Ca^{2+} influx through all major VGCC-types in PC12 cells. Nifedipine (Nif; 2 μM), ω-conotoxin GVIA (2 μM), and ω-conotoxin MVIIC (2 μM) were used to block L-, N-, and P/Q-type VGCCs, respectively. All treatments significantly differ from the DMSO control. Co-exposure to HBCD (20 μM) results in a further reduction of the depolarization-evoked increase in $[Ca^{2+}]_i$ compared with the corresponding 'blocker alone' treatments. Bars display data from 4 to 13 experiments per treatment. The number of cells used for data analysis is indicated inside each bar. $*p < 0.05$; $***p < 0.001$.

Vesicular neurotransmitter release. To investigate whether the observed robust reduction in depolarization-evoked $[Ca^{2+}]_i$ after exposure to 20 μM HBCD has functional consequences for neurotransmitter release, depolarization-evoked release of catecholamines was measured following 20-min exposure to 20 μM HBCD. Analysis of the release frequencies showed that the average basal release frequency amounted to 0.4 ± 0.1 events/min (n = 29). Upon depolarization the release frequency increased to 97 ± 12 events/min (n = 29), which slowly declined to basal release levels (recovered basal release: 2.5 ± 0.5 events/min, n = 29) after cessation of depolarization. Basal release during exposure to 20 μM HBCD (1.1 ± 0.3 events/min, n = 14) did not significantly differ from basal release in control cells (0.5 ± 0.1 events/min, n = 15; Figure 8.4). However, following 20-min exposure to HBCD, ~70% of the cells (10 out of 14) no longer showed evoked release (> 20 events/min), whereas in control experiments, ~85% of the cells (13 out of 15) still showed evoked release following 20-min exposure to 0.1% DMSO (p < 0.01). Release frequencies of the four cells that still show evoked release following exposure to HBCD were within the range of evoked release in control cells (20 - 216 events/min) and the vesicular release parameters (i.e., vesicle content, amplitude and $t_{1/2}$) were unaffected (data not shown).

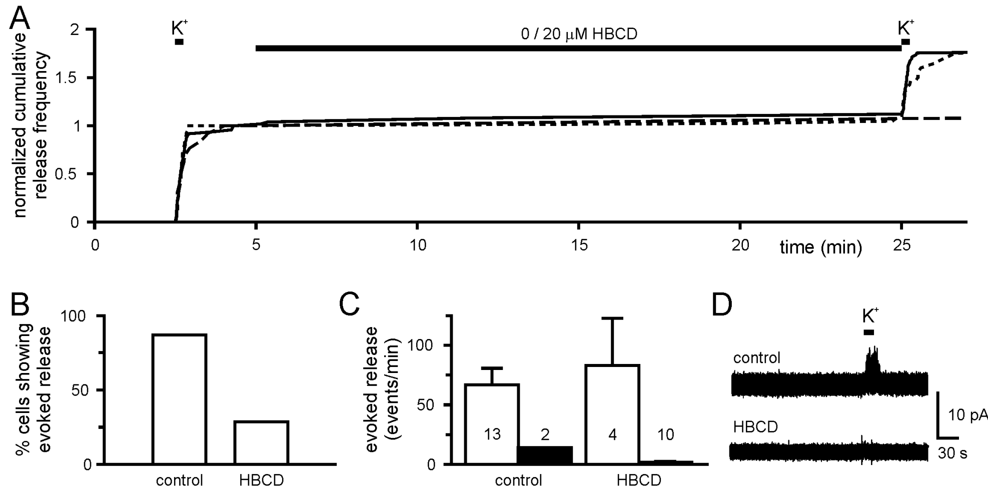


Figure 8.4. Exposure to HBCD inhibits depolarization-evoked catecholamine release. A. Representative cumulative event frequencies, normalized to the number of events observed before the onset of exposure, of a control cell (solid line) and two cells exposed to 20 µM HBCD, one of which shows evoked release after exposure (small dash) and one which does not (large dash). B. Percentage of cells showing evoked release after exposure to vehicle (13 out of 15) or 20 µM HBCD (4 out of 14). C. The average evoked release frequencies of control and HBCD-exposed cells. Although the number of cells lacking evoked release (closed bars) was strongly increased after exposure to HBCD, release frequencies in cells that still exhibit vesicular catecholamine release after exposure to HBCD (open bars) are within the range of control, DMSO-exposed cells. D. Representative amperometric traces recorded from individual PC12 cells. The majority of cells show evoked release after exposure to vehicle (control; top), whereas the majority of cells lacks evoked release after exposure to 20 µM HBCD (bottom).

Discussion

The results presented here demonstrate that the BFR HBCD does not affect the basal $[Ca^{2+}]_i$, which is in accordance with previous observations in rat cerebellar granule cells (Reistad et al. 2006). Additionally, HBCD exposure does not affect the frequency of basal catecholamine release (Figure 8.4) or the parameters of the vesicular release events. However, HBCD exposure does exert an inhibitory effect on the depolarization-evoked increase in $[Ca^{2+}]_i$ via VGCCs with a LOEL of 2 µM (Figures 8.2 and 8.3) as well as on depolarization-evoked vesicular catecholamine release (Figure 8.4).

A small cytotoxic effect (measured via mitochondrial activity) was observed after exposing PC12 cells to 20 µM technical HBCD mixture for only 30 min, whereas exposure to the individual stereoisomers or lower concentrations of HBCD did not result in reduction of

cell viability (Figure 8.1). In cerebellar granule cells, 24 h exposure to 10 μM HBCD resulted in ~90% cell death, whereas 24 h exposure to 2 μM HBCD (the LOEL in the current study) caused ~35% cell death (Reistad et al. 2006). A recent study (Zhang et al. 2008b) describes stereoisomer- and enantiomer-specific cytotoxicity of HBCD in HepG2 cells, showing that γ-HBCD has the strongest cytotoxic effect, and that (+)-enantiomers are significantly more toxic than the corresponding (-)-enantiomers. From the current cell viability results and other cell viability studies it can be concluded that the LOEL for effects in Ca^{2+} homeostasis in the current study (2 μM HBCD) is not confounded by cytotoxicity.

To investigate whether HBCD specifically blocks one subtype of VGCCs, the depolarization-induced increase in $[Ca^{2+}]_i$ was investigated in the presence of specific blockers of the different VGCC-subtypes. Though all VGCC blockers decrease the depolarization-induced increase in $[Ca^{2+}]_i$, nifedipine has the largest effect indicating that L-type VGCCs are the most abundant VGCC-subtype in undifferentiated PC12 cells. If HBCD would specifically block one subtype of VGCCs, a combined exposure to HBCD and a specific blocker should inhibit the depolarization-induced increase in $[Ca^{2+}]_i$ to a similar extent as the specific blocker alone. A further decrease of the depolarization-induced $[Ca^{2+}]_i$ was observed after exposure to HBCD (20 μM) while blocking either the L-, N-, or P/Q-type VGCCs (Figure 8.3). These combined data therefore indicate that the effect of HBCD is not VGCC-specific. Additionally, effects on other Ca^{2+}-influx pathways involved in the depolarization-evoked increase in $[Ca^{2+}]_i$ cannot be excluded.

Exposing the PC12 cells to HBCD immediately before depolarization did not affect the depolarization-evoked increase in $[Ca^{2+}]_i$. Hence, HBCD apparently has no direct effect on the VGCCs. Interestingly, the inhibitory effect of HBCD aggravates when the exposure is prolonged. The maximal effect is reached after 20 min of exposure and increasing the exposure time to 40 min did not further increase the inhibitory effect of HBCD. Although the underlying cause of the exposure-time dependency of the inhibitory effect of HBCD remains as yet unclear, a possible explanation could be the targeting of voltage-independent intracellular signaling pathways by HBCD which need some minutes to take effect. VGCCs are regulated by a range of signal transduction pathways, including phosphorylation and G-protein modulation. Nevertheless, few signaling pathways inhibit L-, N-, and P/Q-type VGCCs, and potentiation has been observed as well (reviewed in Westerink 2006). Another possible explanation could reside in the physico-chemical properties of HBCD. The high *n*-octanolwater-partition coefficient (log K_{ow} ≈ 5; Hayward et al. 2006) indicates the potential for HBCD to accumulate in cell membranes. Possibly, the inhibitory effect of HBCD depends on the amount of HBCD partitioned into the cell membrane, and is limited (as the effect no longer aggravated after 20 min exposure) by the available quantity of HBCD and the absolute quantity of cell membrane. If so, effects on other membrane-anchored proteins, including ion channels, cannot be excluded.

The percentage of cells that do not show evoked release following HBCD exposure is significantly increased. However, cells that still exhibit depolarization-evoked release

following HBCD exposure display a release frequency that is comparable to control cells, suggesting that the inhibition of the depolarization-evoked release is related to the inhibition of depolarization-evoked increase in $[Ca^{2+}]_i$, whereas exocytosis and its underlying processes appear unaffected. Thus, due to the inhibition of the depolarization-evoked $[Ca^{2+}]_i$ a larger proportion of the cells probably does not reach the Ca^{2+} threshold for release after exposure to HBCD.

HBCD has previously been shown to inhibit the plasma membrane uptake of dopamine (IC_{50}: 4 µM, measured after 15 min exposure to HBCD), as well as the vesicular uptake of dopamine (IC_{50}: 3 µM, measured after 15 min exposure to HBCD; Mariussen and Fonnum 2003). Inhibition of uptake of dopamine in rat brain synaptic vesicles has also been demonstrated for the commercial penta-PBDE mixture DE-71 and *ortho*-PCBs (reviewed in Mariussen and Fonnum 2006). However, changes in vesicle content were not observed following exposure to HBCD. This indicates that the functional consequences for quantal release per se are rather limited, or are obscured because of the relatively short time span of the amperometric experiments compared with the slow rate of vesicle cycling in PC12 cells (Westerink et al. 2000).

Human HBCD serum levels are based on only a few observations, which so far have been limited to European countries and the United States. HBCD intake related to oily fish consumption was estimated to be 0.3 ng/kg bw/day (Knutsen et al. 2008). The highest concentration of HBCD measured in human serum in an occupational setting (856 ng/g lipids; Thomsen et al. 2007) corresponds to ~15 nM HBCD in blood (calculated using average physiologic values), which is approximately two orders of magnitude lower than the LOEL in this study. For risk assessment this difference is relatively small, especially considering safety factors for species extrapolation and intraspecies variability, as well as the lipophilic and bioaccumulative properties of HBCD. Considering the current findings on neuronal signaling, additional safety regulations in an occupational setting should be considered.

The highest background level of HBCD measured in humans is 7.4 ng/g lipids (Meijer et al. 2008), which corresponds to ~0.13 nM HBCD in blood (calculated using average physiologic values). This is approximately four orders of magnitude lower than the LOEL in this study, thus well below the concentrations at which adverse effects have been observed. Nevertheless, *in vivo* HBCD exposure is expected to be of a longer or maybe even lifelong duration, whereas the LOEL obtained in this *in vitro* study was based on only 20-min exposure. Additional concern arises from the fact that organohalogen compounds, including PCBs, PBDEs and HBCD, transfer across the placenta (Meijer et al. 2008). Also, HBCD has been detected in human breast milk, up to 5 ng/g lipids (Antignac et al. 2008). As it has been shown that exposure to HBCD within the time frame of rapid brain development results in behavioral defects in mice (Eriksson et al. 2006b), it is concerning that children are exposed to HBCD pre- as well as postnatally.

The major constituent of the technical mixture, γ-HBCD, has a larger effect on the depolarization-evoked increase in $[Ca^{2+}]_i$ than α- and β-HBCD, which have a comparable effect

as seen with the technical mixture. Presently, very few toxicity data is available to compare the activity of the three stereoisomers present in the technical HBCD mixture. All three stereoisomers affect the thyroid hormone system, but γ-HBCD to the largest extent (Palace et al. 2008). Nevertheless, biotic samples are generally dominated by α-HBCD (Covaci et al. 2006), although for humans this has been demonstrated only once (Antignac et al. 2008). This shortage in knowledge on the potency of the individual stereoisomers and (tissue-specific) stereoisomer distribution in humans hampers proper effect and risk assessment. In this light it is noteworthy that as α-HBCD is less potent but more common in biological samples, neurotoxic effects might be overestimated when the technical mixture, which mostly contains γ-HBCD, is used in experimental studies. In view of the differences observed in response between the individual stereoisomers, future toxicity studies should include these stereoisomers to improve the relevance of experimental studies for the human exposure situation.

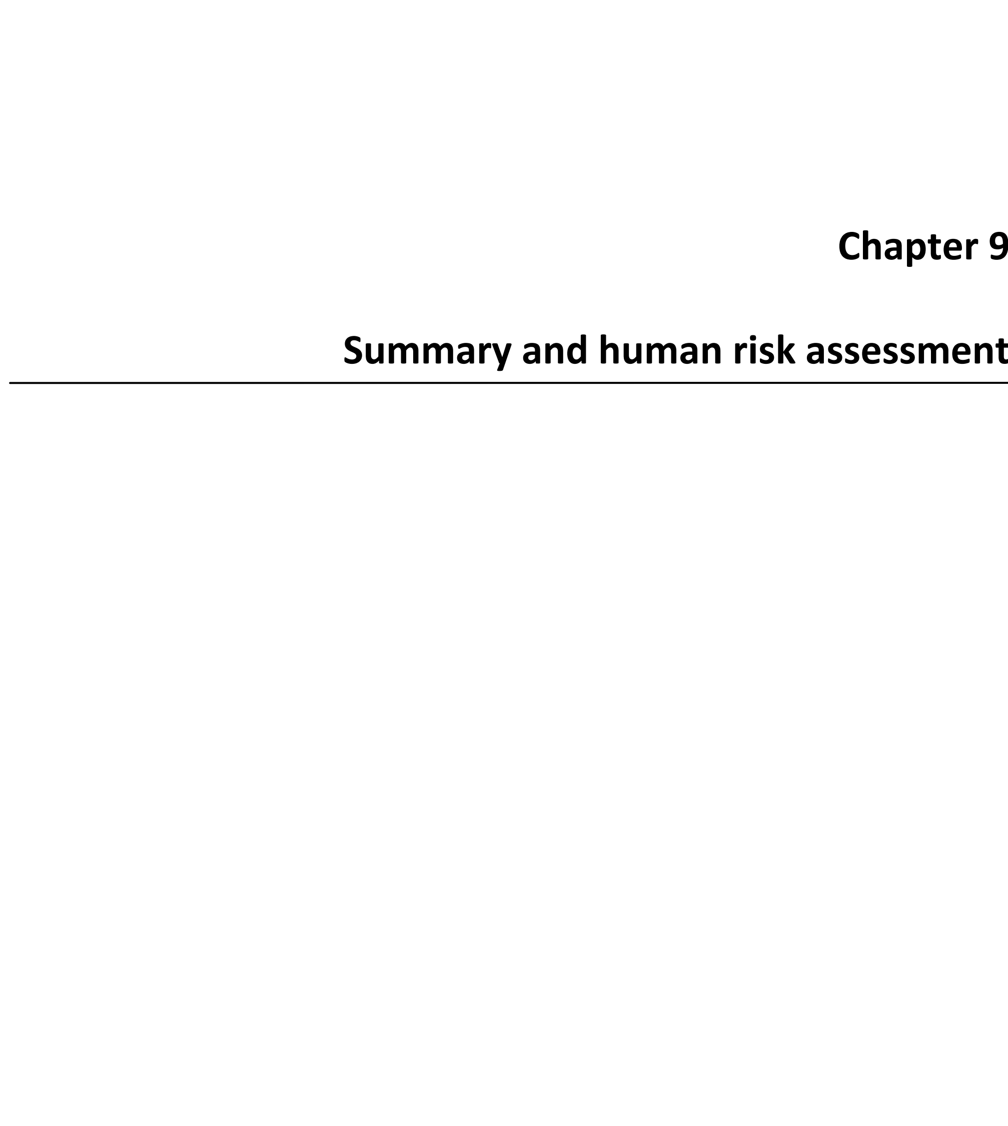
The cessation of the use of the Penta- and OctaPBDE, and in the EU also the use of the DecaBDE, could result in increased use of HBCD in polystyrene materials as an alternative for the banned PBDEs. Consequently, it is not unlikely that human exposure levels increase in the future. Considering this potential increase as well as the current findings on neuroendocrine functioning, additional efforts to establish an adequate exposure, hazard, and risk assessment are justified.

Acknowledgement

The authors gratefully thank Dr. Henk P.M. Vijverberg for helpful discussions.

Chapter 9

Summary and human risk assessment



9.1 Introduction

Polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD) affect neurobehavior in rodents (reviewed in Costa and Giordano 2007; Fonnum and Mariussen 2009). The aim of this PhD-research was to investigate whether underlying mechanisms could be identified in brain function and/or neuronal signaling. Endocrine and hepatic effects are toxic endpoints of these brominated flame retardants (BFRs), as well as effects on neurobehavior (chapter 1). These effects appear to be the most sensitive. Although not discussed in this thesis, also immunotoxic (e.g. Fowles et al. 1994) and genotoxic effects (e.g. He et al. 2008a) of PBDEs have been described. To the aim of human risk assessment, the lowest observed-effect concentrations obtained in this research (chapter 3 - 8) are calculated to corresponding lipid-adjusted serum concentrations by using average physiological blood parameters of blood, plasma and lipid content from the Handbook of Toxicology (Deleranko and Hollinger 2001). This allows for comparison with human serum levels, which are commonly expressed in ng/g lipids. Only in the case of the neonatal exposure to BDE-47, brain concentrations could be rather accurately estimated by using data from a toxicokinetics study. In this study, organ distribution of 1 mg/kg bw ^{14}C-BDE-47 was investigated in neonatal mice (after a single oral dose at postnatal day; PND 10; Staskal et al. 2006a).

Human serum levels of parent PBDEs, hydroxylated PBDEs (OH-PBDEs) or HBCD and the lowest observed-effect concentrations (LOECs) and their corresponding estimated serum concentration in ng/g lipids, are compared. To this aim, only standard (traditional) safety factors of inter- and intraspecies variability are used (10x10; Renwick and Lazarus 1998). Following the risk assessment of the observed effects of (OH-)PBDEs and HBCD on human health, several issues are discussed that may influence the safety factors, and hence margin of exposure/safety.

9.2 BDE-47 decreases LTP: observed effects and human risk assessment

As described in chapter 3, neonatal exposure to BDE-47 impairs long-term potentiation (LTP), a form of synaptic plasticity, measured *ex vivo* in brain slices. The same exposure regiment was previously shown to affect neurobehavior (Eriksson et al. 2001b). While presynaptic parameters measured in the brain slices and isolated chromaffin cells were not affected, a number of proteins involved in LTP were decreased in the postsynaptic density (PSD; chapter 3). No effects were detected on PSD scaffolding proteins PSD-95 and SAP97 (a protein involved in α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor trafficking), unphosphorylated α- Ca^{2+}/calmodulin kinase II (CaMKII) and *N*-methyl-D-aspartic acid (NMDA) receptor subunits NR1 and NR2A. Amounts of AMPA receptor subunit GluR1, NMDA receptor subunit NR2B, and phosphorylated-active αCaMKII were decreased in the

PSD after exposure to BDE-47. Thus, the observed impaired synaptic plasticity induced by BDE-47 appears related to alterations in postsynaptic proteins.

Recently, a study by Xing et al. (2009a) revealed that also the fully brominated BDE-209 affects LTP, measured *in vivo* in the dentate gyrus of the hippocampus in rats. Moreover, rats were exposed during different developmental periods: pregnancy, lactation, after weaning, or from pregnancy through weaning (in which brain concentration of BDE-209 reached 200 ng/g wet weight). The data indicated that the lactation period (PND 0 - 20) is most sensitive to effects of BDE-209, causing impairment of LTP (Xing et al. 2009a). This finding demonstrates that exposure during the brain growth spurt is also important for impaired LTP induced by BDE-209.

Although a no observed-effect level (NOEL) was not established in the study on possible effects of neonatal BDE-47 exposure on LTP (chapter 3), the impairment of LTP occurred after a single oral dose of 6.8 mg (14 µmol)/kg bw. By using data from a toxicokinetics study of ^{14}C-BDE-47 in neonatal mice (Staskal et al. 2006a), it was estimated that concentrations in the brain could reach a peak value of 1.1 µM (8 h after administration after which levels decreased).

Table 9.1. Estimated serum concentrations corresponding to LOECs in this research (A) and a selection of human PBDE serum concentrations (B; for a more extensive overview, particularly on regional differences, see Frederiksen et al. 2009).

A. Estimated serum concentrations corresponding to *in vivo* and *in vitro* treatments in this research.

sample	PBDEs	estimation (ng/g lipids)	median (ng/g lipids)	reference
mouse, 8h after oral administration	2.6 µM BDE-47	$110*10^3$	n.a.	Staskal et al. 2006a
mouse, 10 days after oral administration	0.6 µM BDE-47	$25*10^3$	n.a.	Staskal et al. 2006a
LOEC in PC12 cells	20 µM BDE-47 1 µM 6-OH-BDE-47	$850*10^3$ $10*10^3$	n.a. n.a.	this research
LOEC in hNPCs	2 µM BDE-47 0.2 µM 6-OH-BDE-47	$85*10^3$ $1*10^3$	n.a. n.a.	this research

B. Human exposure to PBDEs and PBDE metabolites.

sample	PBDEs	estimation (ng/g lipids)	median (ng/g lipids)	reference
PBDE exposure in children				
18-months old, US	BDE-47 ΣPBDE[a]	245 651	n.a. n.a.	Fischer et al. 2006
2 - 6 year olds, Australia	ΣPBDE	33 - 49	41	Toms et al. 2009
PBDE exposure in adults				
adults, the Netherlands	BDE-47 ΣPBDE 6-OH-BDE-47	0.1 - 6.1 0.5 - 33.1 n.d.	0.8 3.3 n.d.	Meijer et al. 2008

adults, Sweden	BDE-47 ΣPBDE[a]	n.d. - 8.3 8.6 - 50.5	3.5 20.3	Karlsson et al. 2007
adults, Spain	BDE-47 ΣPBDE[a]	0.3 - 9 5.5 - 43	2.4 12	Ramos et al. 2007
adults, South-China	BDE-47 ΣPBDE	0.4 - 3.6 1.6 - 17	1.0 4.4	Bi et al. 2006
adults, US	BDE-47	< 10 - 511	10	Petreas et al. 2003
adults, US	BDE-47 ΣPBDE	9.2 - 310 15 - 580	28 37	Mazdai et al. 2003
adults, US	BDE-47 ΣPBDE 6-OH-BDE-47 ΣOH-PBDE	8.0 - 28.9 17.9 - 50.8 0.1 - 0.5 3.8 - 11.3	15.2 34.0 0.3 6.3	Qiu et al. 2009
fetal PBDE exposure				
cord blood, South-China	BDE-47 ΣPBDE	0.1 - 4.9 1.5 - 12	1.4 3.9	Bi et al. 2006
cord blood, Spain	BDE-47 ΣPBDE[a]	< 0.1 - 35 6.3 - 82	3.3 17	Gómara et al. 2007
cord blood, Sweden	BDE-47 ΣPBDE	0.3 - 3.3 0.5 - 4.3	0.98 1.7	Guvenius et al. 2003
cord blood, the Netherlands	6-OH-BDE-47	n.d.	n.d.	Meijer et al. 2008
cord blood, US	BDE-47 ΣPBDE	8.4 - 210 14 - 460	25 39	Mazdai et al. 2003
cord blood, US	BDE-47 ΣPBDE 6-OH-BDE-47 ΣOH-PBDE	2.6 - 550.9 4.7 - 797.6 0.1 - 62.1 2.0 - 899.1	13.45 30.9 1.0 22.0	Qiu et al. 2009
occupational PBDE exposure				
electronics dismantlers, US	BDE-47 ΣPBDE[a]	< 0.5 - 23.4 7.5 - 37.3	4.8 26	Sjödin et al. 1999
electronics dismantlers, Norway	BDE-47 ΣPBDE	0.9 - 15 3.8 - 24	4.0 (mean) 8.8 (mean)	Thomsen et al. 2001
computer technicians, Sweden	BDE-47	< 1.0 - 13.6	1.3	Jakobsson et al. 2002
e-waste dismantlers, South-China	BDE-47 ΣPBDE[a]	n.d. - 180 140 - 8500	9.5 600	Bi et al. 2007
foam workers, US	BDE-47 ΣPBDEs	19.5 - 540 67 - 973	77.8 160	Stapleton et al. 2008b
14 year-olds working on waste-dump site (pooled samples), Nicaragua	BDE-47 ΣPBDEs[a] 6-OH-BDE-47 ΣOH-PBDE	330.5 656.5 6.2 61.5	n.a. n.a. n.a. n.a.	Athanasiadou et al. 2008

n.d.: not detected; n.a.: not applicable; a.: including BDE-209

Blood concentrations were calculated to approximate 2.6 and 0.6 µM at respectively 3h and 10 days after administration. A blood concentration of 2.6 µM BDE-47 corresponds to a serum concentration of approximately 110 µg/g lipids, while 0.6 µM corresponds to approximately 25 µg/g lipids.

Although very high concentrations of BDE-47 have occasionally been measured in humans, in particular in the US (Table 9.1), the very high serum concentration of 110 mg/kg lipids is unlikely to be met in human serum. Based on the estimated concentrations reached in the mice, the used exposure regiment is therefore not relevant for most human background exposure situations. Therefore, the risk of decreased synaptic plasticity in adult humans by exposure to BDE-47 appears very low based on the results of this study (chapter 3). Nonetheless, it was demonstrated here for the first time that BDE-47 also impairs LTP, which is commonly used as a neurophysiological *ex vivo* model of learning (Lynch 2004). Therefore, the data presented in this thesis strongly support the previously observed neurobehavioral effects of BDE-47 (Eriksson et al. 2001b).

9.3 Acute effects of parent PBDEs, OH-PBDEs and HBCD on $[Ca^{2+}]_i$ and vesicular neurotransmitter release: observed effects and human risk assessment

PBDEs. Investigated presynaptic parameters were not affected in mice at the time when LTP was measured and shown to be impaired (7 - 9 days after exposure to BDE-47; chapter 3). However, other studies reported effects of PBDEs either on presynaptic processes or effects not specific for either pre- or postsynaptic cells (see §1.3). Moreover, the modification of neural circuits in the developing brain depends on neuronal activity (Spitzer 2006). Temporary alterations of neuronal activity during critical periods in brain development could be underlying the observed effects on behavior and hippocampal synaptic plasticity. Therefore, it was also investigated whether presynaptic effects on neuronal signaling could be expected. As proper neurotransmission critically depends on strict regulation of Ca^{2+} homeostasis as a trigger for neurotransmitter release (Barclay et al. 2005), acute effects of BDE-47 on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and vesicular neurotransmitter release were investigated in a neuroendocrine *in vitro* model (PC12 cells) by respectively imaging of the fluorescent Ca^{2+}-sensitive dye Fura-2 and amperometry.

As described in chapter 5, no effects at all were observed on $[Ca^{2+}]_i$ for BDE-49, BDE-99, BDE-100 and BDE-153. However, BDE-47 increased both $[Ca^{2+}]_i$ and vesicular neurotransmitter release, but only at 20 µM BDE-47 (chapter 3). Nonetheless, already at 2 µM, BDE-47 induced subtle changes in Ca^{2+} homeostasis, that manifested as increased frequency and duration of fluctuations in $[Ca^{2+}]_i$ (chapter 5). BDE-47 was also the only parent PBDE that affected depolarization-evoked $[Ca^{2+}]_i$ (chapter 6). The investigation of possible effects of tetra- to hexabrominated parent PBDEs demonstrated that only the tetrabrominated BDE-47 affected the intracellular Ca^{2+} homeostasis. As the estimated brain concentration of BDE-47 amounts at most to 1.1 µM, presynaptic changes might possibly contribute to the observed functional defect in synaptic plasticity. Possible presynaptic effects of PBDEs *in vivo* (as demonstrated *in vitro*; chapter 4 - 7) were probably transient, and therefore no longer detectable at 7 - 9 days after administration, when LTP was measured.

Influence of transient alterations in $[Ca^{2+}]_i$ on brain development is not unlikely, since Ca^{2+} signals are essential in (early) brain development (Moody and Bosma 2005; Spitzer 2006) and the induction of synaptic plasticity (Malenka and Nicoll 1999; Soderling and Derkach 2000). Moreover, the measured values are an underestimation of local membrane- or store-associated $[Ca^{2+}]_i$, since the measured signal is an average value for the entire cytosol.

The acute effects of BDE-47 were also investigated in primary human neural progenitor cells (hNPCs; chapter 7). In hNPCs, BDE-47 robustly increased $[Ca^{2+}]_i$ already at 2 µM. Experiments under Ca^{2+}-free conditions revealed that BDE-47 increased $[Ca^{2+}]_i$ by influx of extracellular Ca^{2+}. The LOECs of BDE-47-induced alterations in $[Ca^{2+}]_i$ were the same in hNPCs and PC12 cells. Nevertheless, the increases in $[Ca^{2+}]_i$ were much larger in the hNPCs. However, it remains unclear whether the observed difference in potency of BDE-47 in PC12 and hNPCs is due to interspecies differences. Possibly, the observed potency difference results from intrinsic differences between the used pheochromocytoma cell line and primary neural progenitor cells. Investigation of the acute effects of BDE-47 on $[Ca^{2+}]_i$ in rodent neurospheres would be informative in this matter.

In this research, BDE-47 was the only parent PBDE that acutely affected neuronal signaling *in vitro*. However, neurobehavioral effects have also been detected for several higher brominated PBDEs (e.g., BDE-99 and BDE-209; see also §1.3). This demonstrates, as expected, that acute effects on $[Ca^{2+}]_i$ contribute to, but are not the major cause of the observed neurobehavioral effects *in vivo*.

The LOEC *in vitro* exposure concentration (2 µM) corresponds to a serum concentration of approximately 85 µg/g lipids. When considering only a safety factor for intraspecies variability (10), human serum levels of BDE-47 (Table 9.1) are well below the estimated safe level of 8500 ng/g lipids. However, in individual exposure situations, the serum concentrations of BDE-47 are only 1 order of magnitude lower than the estimated safe level. In European occupational settings, serum levels of PBDEs are usually similar to the general population (Table 9.1). However, in employees of an e-waste dismantling site in South-China, ΣPBDE serum levels up to up to 8500 ng/g lipids (median: 600 ng/g lipids) were measured. However, serum concentrations of BDE-47 did not account for a large part of the ΣPBDE exposure (median BDE-47: 9.5 ng/g lipids, up to 180 ng/g lipids), which was mostly accounted for by higher brominated (octa-deca) PBDEs (Bi et al. 2007). BDE-47 serum concentrations up to 540 ng/g lipids (median: 100 ng/g lipids) were recently detected in people working with flame-retarded foam (Stapleton et al. 2008b). These studies demonstrate higher exposure of people in specific occupations. As in the general population, median BDE-47 serum concentrations are below the estimated safe level, although in an increased number of individual occupational exposure situations (compared to in the general population), BDE-47 serum levels are relatively close, but still within 1 order of magnitude, to the estimated safe level.

OH-PBDEs. Endocrine studies indicated that the potency of OH-PBDEs was generally higher compared to their parent congeners (§1.4). In addition, toxicokinetics studies revealed the formation of OH-PBDEs *in vivo* and *in vitro* (§1.4). Therefore, the acute effects of OH-PBDEs on vesicular neurotransmitter release and $[Ca^{2+}]_i$ were investigated in this PhD-research.

The hydroxylated PBDE metabolite 6-OH-BDE-47 caused a robust increase in vesicular neurotransmitter release in PC12 cells at already 5 µM within several minutes after the start of exposure. While BDE-47 itself showed a moderate, slow increase in intracellular $[Ca^{2+}]_i$, 6-OH-BDE-47 caused a rapid, robust increase in $[Ca^{2+}]_i$ by release from endoplasmic reticulum (ER) and mitochondria at an exposure level of 1 µM (chapter 4). Suggestions for ER stress by two different OH-PBDEs from gene expression profiling in human adrenocortical carcinoma (H295R) cells affirm the probability that the effects of OH-PBDEs on intracellular stores are not specific for neuronal/neuroendocrine cells (Song et al. 2009).

While basal $[Ca^{2+}]_i$ was increased by the OH-PBDEs, depolarization-evoked $[Ca^{2+}]_i$ in PC12 cells was inhibited at similar LOECs (chapter 5 and 6). Increases in basal $[Ca^{2+}]_i$ prior to depolarization potentiated the direct inhibitory effect of the OH-PBDEs, probably by Ca^{2+}-dependent regulatory mechanisms.

Acute effects of 6-OH-BDE-47 on $[Ca^{2+}]_i$ were also investigated in hNPCs (chapter 7). In hNPCs, 6-OH-BDE-47 increased $[Ca^{2+}]_i$ already at 0.2 µM. Experiments in Ca^{2+}-free conditions revealed that 6-OH-BDE-47 increases $[Ca^{2+}]_i$ by releasing Ca^{2+} from the ER and mitochondria, as well as by influx of extracellular Ca^{2+}. Based on the LOEC of 6-OH-BDE-47-induced acute increase $[Ca^{2+}]_i$ in hNPCs it is plausible that OH-PBDEs may contribute to the observed impairments in LTP and neurobehavior. However, (peak) brain OH-PBDE concentrations during exposure regiments resulting in behavioral impairments are currently lacking.

Possible structure-related effects of OH-PBDEs on $[Ca^{2+}]_i$ were investigated in PC12 cells, by using a set of hydroxylated metabolites of BDE-47 with variation in hydroxylation position and bromination pattern (chapter 5). The data revealed different potencies and sources of Ca^{2+} involved in the observed OH-PBDE-induced increase in $[Ca^{2+}]_i$. Based on these results, it can be concluded that the potency of OH-PBDEs to disrupt Ca^{2+} homeostasis depends on the presence of shielding atomic groups (bromine atom or aromatic ring) adjacent to the OH, while the position of the OH (*ortho, meta* or *para*) was of lesser importance.

When using the same conversion methods as for parent PBDEs, the LOEC of 6-OH-BDE-47 (0.2 µM) in hNPCs corresponds to human serum concentrations of approximately 10 µg/g lipids. By using only a safety factor for intraspecies variability (10), this results in an estimated safe level of 6-OH-BDE-47 in human serum of 1 µg/g lipids.

Chemical oxidative transformation rates for PBDEs are slow compared to those for the OH-PBDEs, suggesting that OH-PBDEs, when released into the environment, undergo faster oxidative metabolism and excretion than the PBDEs (Moreira-Bastos et al. 2008). However, studies on human exposure to OH-PBDEs revealed that various OH-PBDEs are present in human serum, at concentrations similar to or sometimes even higher than the concentration

of PBDEs (Athanasiadou et al. 2008; Qiu et al. 2009). Nonetheless, even individual worst-case human serum levels of 6-OH-BDE-47 (Table 9.1) are still at least an order of magnitude below the estimated safe level of 1 µg/g lipids 6-OH-BDE-47 in serum.

Furthermore, it is expected that OH-PBDEs and hydroxylated polychlorinated biphenyls (OH-PCBs) are more associated to proteins than lipids. It requires further study to determine whether this property affects concentrations of OH-PBDEs measured in the serum fraction. Moreover, the binding of OH-PBDEs to circulating proteins such as transthyretin may limit its elimination (Gebbink et al. 2008a).

It was also demonstrated in this PhD-research that all investigated hydroxylated metabolites of BDE-47 increase $[Ca^{2+}]_i$ (chapter 5). Although different potencies were observed, it is not unlikely that most hydroxylated metabolites of medium brominated diphenyl ethers affect cellular calcium homeostasis. When comparing worst-case human ΣOH-PBDE levels (Table 9.1) with the worst-case LOEC of OH-PBDEs (0.2 µM, as measured in hNPCs), the margin of exposure is insufficient. However, when taking into account potency differences (chapter 5), it is possible that a higher LOEC for OH-PBDE mixtures is more realistic. When comparing the highest observed LOEC for an OH-PBDE (20 µM, corresponding to 850 µg/g lipids in serum) with the median and highest human serum levels for ΣOH-PBDEs, the margin of exposure is adequate. However, as interactions between OH-PBDEs remain unclear, the default approach for human risk assessment for OH-PBDEs should be additivity.

HBCD. Behavioral as well as *in vitro* neurotoxic effects of HBCD similar to those observed for PBDEs were found in a relatively small number of studies (Eriksson et al. 2006b; Mariussen and Fonnum 2003; Reistad et al. 2006). To study possible similarities to PBDEs considering neuronal signaling, effects of HBCD on $[Ca^{2+}]_i$ and vesicular neurotransmitter release were investigated (chapter 8). HBCD exposure did not induce any effects on basal $[Ca^{2+}]_i$ or vesicular neurotransmitter release. When vesicular neurotransmitter release was triggered by a depolarization-evoked temporary increase in $[Ca^{2+}]_i$, HBCD inhibited depolarization-evoked $[Ca^{2+}]_i$ as well as neurotransmitter release. The LOEC of the inhibition of depolarization-evoked $[Ca^{2+}]_i$ was 2 µM. The effects of α- and β-HBCD were comparable to that of the technical mixture, whereas the inhibitory effect of γ-HBCD was significantly larger. The inhibitory effect of HBCD was not specific for one type of voltage-gated Ca^{2+} channel (VGCC), and exposure for at least 5 min to HBCD prior to depolarization was required for the observed inhibition of depolarization-evoked $[Ca^{2+}]_i$.

Table 9.2. Estimated serum concentrations corresponding to LOECs in this research (A) and human HBCD serum concentrations (B).

A. Estimated serum concentrations corresponding to *in vitro* treatments in this research				
sample	HBCD	estimation (ng/g lipids)	median (ng/g lipids)	references
LOEC in PC12	2 µM HBCD	$110*10^3$	n.a.	

B. Human exposure to HBCD				
sample	HBCD	range (ng/g lipids)	median (ng/g lipids)	references
HBCD exposure in adults				
adults (m), Norway	HBCD	n.d. - 52	4.1	Thomsen et al. 2008
adults (f), Norway	HBCD	n.d. - 18	2.6	Thomsen et al. 2008
adults, the Netherlands	HBCD	n.d. - 7.4	0.7	Meijer et al. 2008
adults (elderly f), Sweden	HBCD	n.d. - 3.4	0.46	Weiss et al. 2006
fetal HBCD exposure				
cord blood, the Netherlands	HBCD	0.2 - 4.3	0.2	Meijer et al. 2008
occupational HBCD exposure				
employees in polystyrene production, Norway	HBCD	6 - 856	101	Thomsen et al. 2007

Inhibition of depolarization-evoked $[Ca^{2+}]_i$ has thus been observed in this research for both HBCD and OH-PBDEs, but not for PBDEs. The LOEC for HBCD and 6-OH-BDE-47 is the same (2 µM), while the other investigated OH-PBDEs inhibit depolarization-evoked $[Ca^{2+}]_i$ at concentrations ≥ 5 µM. The efficacy of inhibition by 2 µM 6-OH-BDE-47 or HBCD is also similar. However, OH-PBDEs near-completely inhibit depolarization-evoked $[Ca^{2+}]_i$ at 20 µM, while 20 µM HBCD inhibits depolarization-evoked $[Ca^{2+}]_i$ to approximately 50% of control. Moreover, the inhibition of depolarization-evoked $[Ca^{2+}]_i$ by OH-PBDEs coincides with increases in basal $[Ca^{2+}]_i$, while this is not the case for HBCD. Therefore, the potential of OH-PBDEs to disrupt the balance in basal and depolarization-evoked $[Ca^{2+}]_i$ is larger compared to that of HBCD (Figure 9.1). Nonetheless, *in vivo* co-exposure to (OH-)PBDEs and HBCD is to be expected. Therefore, possible additivity of OH-PBDEs and HBCD to inhibit depolarization-evoked $[Ca^{2+}]_i$ requires further study.

By applying safety factors for intra- and interspecies variability (10x10), the estimated safe level of HBCD in human serum is 110 µg/g lipids. Both in occupational settings as in the general population, human serum levels are well below this estimated safe level (Table 9.2). However, this conclusion is drawn from a relatively small number of studies. Human internal HBCD exposure has also been investigated and detected in the Belgium, Norway, Czech Republic, Spain, Russia, China, Japan and US, by measurements in adipose tissues and breast milk (Colles et al. 2008; Eljarrat et al. 2009; Johnson-Restrepo et al. 2008; Kakimoto et al. 2008; Polder et al. 2008a, 2008b; Pulkrabová et al. 2009; Shi et al. 2009).

Metabolism of HBCD has recently been investigated in toxicokinetics studies with fish as well as rodents. Debromination as well as formation of hydroxylated metabolites of HBCD was detected in rats after dietary exposure (Brandsma et al. 2009) and similar metabolic processes have been suggested to occur in the harbor seal using hepatic microsomes (Zegers et al. 2005). Considering the increased neurotoxic and endocrine potency of PBDEs resulting from oxidative metabolism and the potential increased use of HBCD, these effects need also to be investigated for hydroxylated metabolites of HBCD.

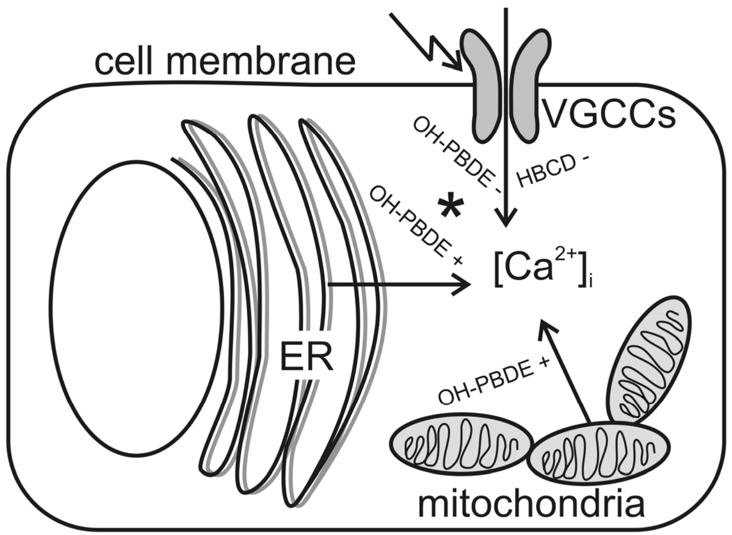


Figure 9.1. Summary of effects of (OH-)PBDEs and HBCD on intracellular Ca^{2+} homeostasis observed in this PhD-research (chapter 4 - 8). OH-PBDEs increase basal $[Ca^{2+}]_i$ mainly by inducing release of Ca^{2+} from endoplasmic reticulum (ER) and mitochondria (chapter 4 - 5). HBCD does not affect basal $[Ca^{2+}]_i$ but inhibits depolarization-evoked $[Ca^{2+}]_i$ by non-specific inhibition of voltage-gated Ca^{2+} channels (VGCCs; chapter 8). Inhibition of depolarization-evoked $[Ca^{2+}]_i$ is also observed for the OH-PBDEs (chapter 6). Moreover, preceding increases in basal $[Ca^{2+}]_i$ potentiate the subsequent inhibition in depolarization-evoked $[Ca^{2+}]_i$ by OH-PBDEs (*).

Chapter 10

Issues concerning (OH-)PBDE and HBCD neurotoxicity risk assessment

10.1 The developing brain

Based on estimated tissue concentrations during the postnatal exposure to BDE-47 resulting in impaired long-term potentiation (LTP; chapter 3) and observed-effect concentrations detected *in vitro* (chapter 4 - 7), the risk of decreased synaptic plasticity in humans resulting from acute (transient) cellular effects of environmentally relevant exposure to BDE-47 appears very low (chapter 9). Nonetheless, neurobehavioral effects have been detected at much lower exposure concentrations of polybrominated diphenyl ethers (PBDEs). After developmental exposure to BDE-99 (by administration of a single oral dose of 60 or 300 µg/kg bw to the dams), behavioral changes, mainly hyperactivity, were observed in rats (Kuriyama et al. 2005). The lowest administered dose in this study is almost 3 orders of magnitudes higher than the estimated daily intake of PBDEs by breastfed infants in the US (86.4 ng/kg bw/day; Johnson-Restrepo and Kannan 2009). However, it is within the same order of magnitude as the summed up dietary intake of PBDEs within the first year (by using a conservative approach).

These observed effects at low exposure concentrations are likely related to the higher sensitivity of the developing brain compared to the adult brain to external stimuli, including xenobiotics. This higher sensitivity is due to the multitude of strictly regulated processes in brain development (proliferation, differentiation, migration and the formation of networks) (Grandjean and Landrigan 2006). Because of the observation of behavioral impairments in rodents after PBDE exposure during postnatal brain development, PBDEs were classified as one of many possible developmental neurotoxicants (Grandjean and Landrigan 2006).

Because of the strict temporal regulation of brain development processes, time-windows particularly sensitive to neurotoxic insults occur. Although brain development in humans extends into early childhood, especially the perinatal period of brain development (‘brain growth spurt’) has been proven to be very sensitive to neurotoxic effects (Rice and Barone 2000). When reviewing human exposure to assess risk for developmental neurotoxicity by using rodent studies, interspecies differences in timing of brain development have to be considered. In rodents, usually the experimental model for developmental neurotoxicity and neurobehavioral studies, the rapid brain development occurs within the first postnatal weeks. In contrast, rapid brain development in humans occurs before and after birth (Davison and Dobbing 1968). This rapid brain growth starts in the 2nd trimester, peaks around birth and then extends into early childhood (1 - 2 years; Rice and Barone 2000; Davison and Dobbing 1968). Because of these interspecies differences in brain development timing, *in utero* exposure (primarily through placental transfer; Myren et al. 2007) is probably more relevant for developmental neurotoxicity in humans as in rodent neurodevelopment, while lactational transfer of PBDEs appears to be important for humans as well as rodents.

For PBDEs, placental transfer has been demonstrated in humans by investigating levels of PBDEs in human umbilical cord blood (Table 9.1) and fetal liver samples (Doucet et al. 2009; Schecter et al. 2007). The highest values have been detected in US samples, while in

cord blood samples from European countries, ΣPBDE levels are approximately 2 - 20 times lower (Table 9.1). Moreover, PBDEs have been detected in human extraplacental gestational membranes, although the actual fetal exposure resulting from the presence of PBDEs in these membranes remains unknown (Miller et al. 2009).

In fetal liver and placental tissue, CYP450 (CYP) enzyme activity is present (Hakkola et al. 1998). Placental transfer of hydroxylated polychlorinated biphenyls (OH-PCBs) has been demonstrated in experimental studies (Meerts et al. 2002). Although placental transfer of hydroxylated PBDEs (OH-PBDEs) has not yet been proven, it is not unlikely, due to the structural resemblance with OH-PCBs. Therefore, the internal fetal exposure may be due to internal hydroxylation and/or placental transfer from the mother.

A distribution study with radiolabeled PBDEs in mice showed that fetal uptake during gestation was relatively limited, while maternal transfer via breast milk resulted in transfer of approximately 20% of the administered dose to the offspring (Darnerud and Risberg 2006). Assuming similar toxicokinetics of PBDEs in humans during gestation and lactation, this suggests that exposure through lactation is from a quantitative point of view an important exposure route in humans. The highest median concentration of ΣPBDEs measured in human breast milk was 50.4 ng/g lipids (range: 6.3 - 321 ng/g lipids) in primipara from the Pacific Northwest of the US and Canada (She et al. 2007). In Europe and Asia, median ΣPBDE concentrations are approximately one order of magnitude lower (reviewed in Frederiksen et al. 2009). Over the last decades, European breast milk levels of PBDEs are leveling off (Guvenius et al. 2003; Lind et al. 2003) but time trends of PBDE levels in North American breast milk have not been investigated thoroughly. Very recently, methoxylated PBDEs (MeO-PBDEs; median: 16.2 pg/g lipids, range: 0 - 10041 pg/g lipids) and OH-PBDEs (median: 66.6 pg/g lipids, range: 0 - 6264 pg/g lipids) have been detected in human milk from Catalonia. In this Mediterranean region the PBDE levels are within the range of those observed in other parts of Europe and Asia (Lacorte and Ikonomou 2009). Thus, similar levels of MeO-PBDEs and OH-PBDEs in human breast milk can be expected in other parts of the world with normal background exposure to PBDEs.

Like PBDEs, hexabromocyclododecane (HBCD) has also been detected in breast milk (reviewed in Eljarrat et al. 2009). Information about the presence of specific diastereoisomers of HBCD is very limited, showing either predominance of α-HBCD or γ-HBCD (Shi et al. 2009). It is not unlikely that in the near future HBCD levels in human breast milk can rise due to increased use of this flame retardant in consumer products. However, very few observations so far can confirm this hypothesis (Eljarrat et al. 2009).

Nevertheless, a negative advice for breastfeeding has always been considered unwarranted, because of the nutritional, immunological and psychosocial benefits of breastfeeding. However, it should be recognized that a risk-benefit analysis with regard to breastfeeding and the associated exposure to xenobiotics including PBDEs and PCBs is currently lacking. Particularly in highly exposed communities, breast milk concentrations of

neurotoxic environmental pollutants, including PBDEs, should be assessed to prevent developmental neurotoxicity in humans.

Because of the complexity and strict temporal regulation of brain development, it is difficult to estimate whether gestational or lactational exposure is most relevant for developmental neurotoxicity in humans. Hence, the lower exposure during a sensitive (possibly prenatal) developmental phase may be more hazardous for the developing fetal brain than the higher exposure during the next (possibly postnatal) developmental phase via e.g. lactational transfer. Solving this issue remains difficult, as complete understanding of brain development has not been achieved yet. This is particularly true with respect to disruption by external stimuli, including xenobiotics, and their relationship with neurodevelopment and cognitive and behavioral function (Paterson et al. 2006).

For the large set of chemicals that have to be assessed for developmental neurotoxicity in humans within the REACH framework, *in vitro* screening methods for developmental neurotoxicity should be considered essential due to ethical and time considerations for animal experiments (Breier et al. 2009). The current development of *in vitro* systems is aimed to allow hazard characterization for developmental neurotoxicity in humans by comparing specific (acute) toxic effects with specific temporal vulnerabilities of processes during brain development.

10.2 Brain concentrations versus serum concentrations

For the risk assessment of neurotoxicity by (OH-)PBDEs and HBCD as described above, serum levels on a lipid weight basis were taken as a measure for human internal exposure. However, the brain is rather effectively protected from toxic insults by the blood-brain barrier (Rubin and Staddon 1999). Small lipophilic compounds are generally assumed to freely cross the blood-brain barrier by passive diffusion. Nevertheless, it remains unclear whether this is also the case for PBDEs because of their high molecular weight. Therefore, concentrations in the brain may be different from serum levels. Several environmental and animal studies, described below, have investigated accumulation of (OH-)PBDEs in the brain.

In spite of the presence of the blood-brain barrier that could reduce uptake of PBDEs from blood, significant concentrations of these compounds have been detected in the brain of wildlife species. In Swiss birds of prey and US herring gulls, brain ΣPBDEs (up to 14 ng/g wet weight and 143 ng/g wet weight, respectively) could be detected (Crump et al. 2008; Naert et al. 2007). In a study on Belgian birds of prey, approximately 5% of the PBDE body burden was detected in brain, while approximately 15% was detected in serum (Voorspoels et al. 2006). PBDEs have also been detected in the brain of mammalian top predators, albeit at lower levels than those observed in birds. In North American wild river otters, PBDEs were detected in cerebral cortex (Basu et al. 2007). In Greenland polar bears, brain ΣPBDE levels were detected up to 7.6 ng/g wet weight and approximately 2 - 3 times higher compared to blood.

In polar bears, OH-PBDEs were not detected in the brain, but in the blood, levels were found up to 13 ng/g wet weight (Gebbink et al. 2008b). In another study, PBDEs as well as OH-PBDEs were detected in cerebrospinal fluid (CSF) and cerebellar grey matter in Atlantic white-sided dolphins. Levels of PBDEs and OH-PBDEs in cerebellum were higher compared to those in CSF (Montie et al. 2009).

Partitioning of PBDEs into the brain was also investigated in toxicokinetics studies, often by using radiolabeled PBDEs. PBDEs were detected in brain after a single oral dose of BDE-47 or BDE-99 in mice (Hakk et al. 2002; Örn and Klasson-Wehler 1998). In addition, following maternal exposure to BDE-47, BDE-85 or BDE-99, PBDEs were not only detected in the dam's brain, but also in fetal brain (Darnerud and Risberg 2006). A more detailed toxicokinetics study in mice after exposure to BDE-153, administered intravenously, revealed that the percentage of the total dose present in the brain was approximately 4 times higher than in blood (Staskal et al. 2006b). In another study, neonatal mice received a single oral dose of BDE-47 on postnatal day (PND) 10, which was a similar exposure regime as used to detect neurobehavioral effects and effects on synaptic plasticity by BDE-47 (Eriksson et al. 2001b; chapter 3). Under these conditions a slower partitioning in the neonatal brain was observed compared to adult mice. In this case, BDE-47 levels in the brain reached approximately 0.6% of the administered dose 12 days post exposure (Staskal et al. 2006a). A toxicokinetics study with the commercial PBDE mixture DE-71 in pregnant mink and their offspring again demonstrated significant maternal transfer to the offspring. Moreover, a higher proportion of the PBDE body burden was present in the brain of offspring compared to the dams. While OH-PBDEs were detected in the plasma, liver and feces of offspring, OH-PBDEs were not detected in brain (Zhang et al. 2008a). After oral administration of radiolabeled BDE-209 to rats (gestation day; GD 16 - 19), only approximately 0.01% of the administered dose was detected in the maternal brain at GD 20 (Riu et al. 2008).

In Greenland polar bears, approximately 40 ng/g wet weight α-HBCD was detected in adipose tissue, but no detectable levels in serum, brain or liver (Gebbink et al. 2008a). In Glaucus gulls found dead in Norway, 0.005 to 0.5 µg/g wet weight α-HBCD was detected in brain tissue, while in contrast β- and γ-HBCD were not detected (Sagerup et al. 2009).

HBCD partitioning into the brain has also been investigated experimentally. After a peritoneal injection, HBCD was detected in rat brain, at 4% of the concentration that was found in the liver (Reistad et al. 2006). Another study investigated disposition of HBCD diastereoisomers in trout, showing gradual accumulation in and elimination from the brain over several days (Haukås et al. 2009b).

The environmental and experimental toxicokinetics studies described above demonstrate that PBDEs and HBCD partition into the brain. Therefore, it is not unlikely that these brominated flame retardants also reach the human brain. However, this appears much less the case for BDE-209. On the other hand, it has been observed in experimental and wildlife studies that OH-PBDEs are usually not detectable in brain. Therefore, high concentrations of OH-PBDEs are not likely to be found in the human brain. Although

OH-PBDEs are generally not detected in the brain, both in adults and infants, endocrine cells such as adrenal chromaffin cells and cells in the thyroid gland are not protected by the blood-brain barrier. As they are therefore exposed to organohalogen environmental pollutants at levels measured in serum, OH-PBDEs could disrupt Ca^{2+}-related processes in these and possibly other celltypes.

Nonetheless, it should be recognized that in the case of some OH-PCBs, specific accumulation in brain tissue occurs. Higher concentrations of 4-OH-PCB-109 compared to single PCB congeners was observed in fetal brain after oral administration of commercial PCB mixture Aroclor 1254 to pregnant rats from GD 10 - 16 (Morse et al. 1996). This specific accumulation of OH-PCB in the fetal brain has been suggested to be associated with binding of the OH-PCB to transthyretin, based on reduced concentrations of thyroxine in the brain (Meerts et al. 2002). OH-PBDEs also strongly bind to transthyretin as well as to thyroxine binding globulin, which is the main transporter protein for thyroid hormones in humans (Marchesini et al. 2008). Moreover, the embryonic blood-brain barrier is more permeable to small molecules (Engelhardt 2003), creating additional sensitivity to xenobiotics during development. It is of particular concern that PCBs directly disrupt blood-brain barrier integrity, as has recently been demonstrated in rats (Seelbach et al. 2009). Because of the discrepancy between observations in wildlife and toxicokinetics studies with parent PBDEs and accumulating properties demonstrated for the structurally related (OH-)PCBs, the potential of OH-PBDEs to enter the brain requires further study.

10.3 Chemical mixtures

It is evident that humans are systemically exposed to a plethora of environmental pollutants, either simultaneously or serially. Some xenobiotics detected in humans have been out of production since long, but are still present in the environment and human food chain because of their environmental persistence. While some of these xenobiotics are clearly present in concentrations far below their possible toxic levels or rapidly removed from the body, several classes of persistent environmental pollutants that are still present in the human body are associated with concerns for human health.

In relation to neurotoxicity and PBDEs the occurrence of mixtures with various congeners has to be considered for risk assessment. Although it has only just been announced that the use of DecaBDE will be phased out in North America following an earlier ban in Europe, large environmental reservoirs of BDE-209 have already been formed in sediments (Ross et al. 2009) and there is evidence arising for environmental debromination of this congener to lower brominated diphenyl ethers.

Debromination of BDE-209 by photodegradation to lower brominated congeners (tetra- to nonaBDEs) has been observed on the surface of sediment, soil and sand (Ahn et al. 2006; Söderström et al. 2004) and in solution (Bezares-Cruz et al. 2004; Christiansson et al.

2009; Eriksson et al. 2004). Interestingly, the photodegradation rate of PBDEs is positively correlated with an increasing degree of bromination (Eriksson et al. 2004). It has been estimated by environmental fate modeling that respectively 13% and 2% of penta- and tetraBDEs in the global environment originate from degradation of BDE-209 (Schenker et al. 2008). When introduced into the food chain, PBDEs can be degraded further in living organisms. Reductive debromination of BDE-209 has been observed in anaerobic bacteria (Gerecke et al. 2005; He et al. 2006) and in some fish species as well as in mammals, including humans (Huwe and Smith 2007; Kierkegaard et al. 1999, 2007; Stapleton et al. 2004a, 2006; Thuresson et al. 2005; Tomy et al. 2004b).

Until now, interactions between environmentally relevant PBDE congeners has been investigated in only one study. Dependent on the concentrations, both synergistic and antagonistic effects of BDE-47 and BDE-99 were observed with respect to reactive oxygen species (ROS) formation in human neuroblastoma SK-N-MC cells (Tagliaferri et al. 2009). At low exposure concentrations synergism was observed, indicating possibly other than additive interactions for PBDE mixtures in biota.

As OH-PBDEs should be regarded as more or at least equally toxic compared to parent PBDEs, it is essential that exposure to parent as well as OH-PBDEs is assessed. Despite the fact that DecaBDE is environmentally debrominated to lower brominated PBDEs, it remains to be seen whether hydroxylation of debromination products of DecaBDE contributes to environmental or human exposure to OH-PBDEs. It has to be kept in mind that several environmental degradation processes also occur. OH-PBDEs dissolved in water are rapidly decomposed photochemically, especially in the presence of hydrogen peroxide, which is commonly used in waste-water treatment plants (Moreiro-Bastos et al. 2009). Hydroxylated polychlorinated diphenyl ethers are methylated by micro-organisms, suggesting a possible route of degradation for OH-PBDEs (Hundt 2000).

In chapter 5, a structure-activity relationship was proposed for OH-PBDE-induced effects on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). It was demonstrated that the potency of OH-PBDEs to release Ca^{2+} from intracellular stores was decreased when the OH-group was shielded on both sides with Br-atoms or a Br-atom and an aromatic ring. It remains uncertain though plausible that OH-PBDEs, in which the C-atoms adjacent to the OH-group are unsubstituted, are even more active in disrupting Ca^{2+} homeostasis. Intriguingly, tetrabrominated bisphenol-A, in which two OH-groups are present (shielded on both sides with Br-atoms), is a potent inhibitor of Ca^{2+} pumps involved in Ca^{2+} compartmentalization in the ER (Ogunbayo and Michelangeli 2007). However, additional experimental information is required to extend the proposed structure-activity relationship to other (groups of) chemicals.

For risk assessment in the near future, it is essential to study environmental or dietary OH-PBDE exposure patterns and those originating from metabolic conversion. For the latter aspect it has also been observed that interindividual differences commonly occur (Hong and

Yang 1997). The neurotoxic potential of OH-PBDE exposure patterns for disturbance of $[Ca^{2+}]_i$, and possibly neuronal signaling, could theoretically be predicted by using structure-activity relationships.

For other toxic endpoints, mainly cytotoxicity and CYP enzyme induction, structure-activity relationships of OH-PBDEs have also been proposed. An earlier study on toxicity of OH-PBDEs in a bacterium and a fungus demonstrated that cytotoxicity was higher for the *ortho*-hydroxylated PBDEs when the adjacent C-atom was Br-substituted (Handayani et al. 1997). Cantón et al. (2005) identified significant aromatase activity inhibition in human adrenal corticocarcinoma H295R cells by *ortho*-hydroxylated PBDEs. However, in this study, *ortho*-hydroxylated PBDEs were more cytotoxic than *para*-hydroxylated PBDEs. Cytotoxicity was decreased significantly when an additional Br-atom was present that could shield the OH-group (Cantón et al. 2005). In human adrenocarcinoma H295R cells inhibition of CYP17 activity was observed by *ortho*-hydroxylated PBDEs but this was apparently not dependent on the presence of an adjacent Br-atom in the *meta*-position (Cantón et al. 2006). Inhibition of CYP17 activity was also observed for a *para*-hydroxylated PBDE, 4’-OH-BDE-49, but this was not consistent for other *para*-hydroxylated congeners tested. In both 4’-OH-BDE-49 and 4-OH-BDE-42, bromine substitution at the phenol ring is at an *ortho*- and a *meta*-position, suggesting an additional influence of the bromination pattern in itself (Cantón et al. 2006). In human placental microsomes, an extensive set of OH-PBDEs was again tested for inhibition of aromatase activity. Aromatase activity was inhibited by all tested OH-PBDEs, independent of the hydroxylation position. In this case no clear activity relationship could be deduced from IC_{50} and maximum inhibition values (Cantón et al. 2008b). Data on binding of *para*-hydroxylated PBDEs to various hormone receptors indicated also that the presence of adjacent bromine atoms causes steric hindrance for binding to the estrogen receptor (Kojima et al. 2009).

These combined studies indicate the complexity of structure-activity relationships of OH-PBDEs. Different or even opposite associations are suggested for different toxic endpoints, e.g. bromine substitution adjacent to the *ortho*-OH-group increases cytotoxicity in a fungus, but does not influence CYP17 inhibition in H295R cells while decreases disruption of Ca^{2+} homeostasis in PC12 cells. Thus, relationships between an OH-group and adjacent bromines in relation to different toxic endpoints remain until now unclear.

Another group of PBDE metabolites are the bromophenols, which have been detected in mouse plasma after exposure to commercial PBDE mixture DE-71 (Qiu et al. 2007) and human serum (Dallaire et al. 2009; Qiu et al. 2009). Formation of bromophenols was also observed in human primary hepatocytes exposed to BDE-99 or BDE-209 (Stapleton et al. 2009).

Developmental toxicity of bromophenols has been detected in zebrafish embryos (Kammann et al. 2006) and marine copepods (Wollenberger et al. 2005). Endocrine toxicity includes estrogen-like effects in human mammacarcinoma MCF-7 cells (Olsen et al. 2002) and

upregulation of gene expression of CYP enzymes involved in steroidogenesis (Ding et al. 2007). Like OH-PBDEs, bromophenols have been shown to bind to transthyretin (Ghosh et al. 2000; Hamers et al. 2006; Marchesini et al. 2008). Moreover, bromophenols also increase basal $[Ca^{2+}]_i$ and/or inhibited depolarization-evoked $[Ca^{2+}]_i$ in PC12 cells (lowest observed-effect concentration; LOEC: 300 µM; Hassenklöver et al. 2006), and directly inhibit currents through voltage-gated Ca^{2+} channels (VGCCs) in PC12 cells with a LOEC of 15 µM (Hassenklöver and Bickmeyer 2006). Although effect concentrations of bromophenols and OH-PBDEs on $[Ca^{2+}]_i$ are different, possible additive effects of these two classes of metabolites of PBDEs require further study.

Because of their structural similarity, investigation of neurotoxic endpoints of PBDEs were usually based on previous observations of effects induced by PCBs. Especially for *ortho*-substituted (*ortho*-)PCBs, neurotoxicity has been investigated thoroughly *in vivo* and *in vitro*. The main neurotoxic endpoints of *ortho*-PCBs are similar to those observed for (OH-)PBDEs. These are disruption of neurobehavior, decreased LTP, altered Ca^{2+} homeostasis, inhibition of neurotransmitter reuptake, and cytotoxicity in neuronal cell types (reviewed in Fonnum and Mariussen 2009). Furthermore, disruption of the thyroid hormone system is another important toxic endpoint of PCBs (Hallgren and Darnerud 2002).

An increase in $[Ca^{2+}]_i$ by release from intracellular stores appears to be a common effects of OH-PBDEs (chapter 4 - 5) and *ortho*-PCBs. Therefore, an additive effect of these environmental pollutants on the increase in basal $[Ca^{2+}]_i$ and possible inhibition of depolarization-evoked $[Ca^{2+}]_i$ can be expected, that could jointly affect neurotransmitter release as well as many other cellular processes (Clapham 2007).

In addition, gene expression profiling in hippocampus and cerebellum of rats that were developmentally exposed to the commercial PCB mixture Aroclor 1254 revealed effects on genes involved in Ca^{2+} homeostasis, intracellular signaling, axonal guiding, cell proliferation and differentiation. It should be noted that genes related to thyroid hormone functioning were not affected, suggesting that thyroid hormone disruption is not involved in developmental neurotoxicity of PCBs (Royland and Kodavanti 2008). Correlations between PCB-exposure and neurodevelopmental effects in humans were detected in epidemiological studies (reviewed in Boucher et al. 2009). However, in most studies other persistent organic pollutants are not investigated.

Possible interactions of *ortho*-PCBs and PBDEs have been investigated *in vitro* as well as in neurobehavioral studies. PCB-52-induced alterations in motor activity and decreased habituation were enhanced by co-exposure to BDE-99 after an oral dose at PND 10 in mice (Eriksson et al. 2006a). Co-exposure to BDE-47 with PCB-153 increases the impairment in Morris water maze performance and alterations in expression of apoptosis-related genes in the hippocampus of rats. In this study, also enhanced ultrastructural alterations of ER and mitochondria were observed in neurons from the hippocampal CA1 region (He et al. 2009a). Moreover, in human neuroblastoma SH-SY5Y cells, effects of BDE-47 on cell viability, $[Ca^{2+}]_i$,

ROS formation, apoptosis and cytogenotoxicity were enhanced by co-exposure to PCB-153 (Gao et al. 2009; He et al. 2009b, 2009c). These studies indicate that the effects of PCBs are enhanced by PBDEs, although additivity cannot be assumed.

Apart from PCBs, their hydroxylated metabolites have also been detected in the environment and human tissues. Human are also exposed prenatally to OH-PCBs via placental transfer (Park et al 2008; Sandau et al 2002; Weiss et al. 2006). As discussed above, OH-PCBs have been shown to cross the blood-brain barrier (Meerts et al. 2002).

Similar effects on neurobehavior and thyroid hormone levels have been detected in rats after developmental exposure to 4-OH-PCB-107 compared to Aroclor 1254, while 4-OH-PCB-107 and Aroclor 1254 affected brain levels of different neurotransmitters (Meerts et al. 2004). Interaction of OH-PCBs with thyroid hormone receptors has also been detected in the thyroid hormone-dependent rat pituitary GH3 cell line (Ghisari and Bonefeld-Jorgensen 2005). In addition, OH-PCBs show high affinity for human transthyretin and, to a lesser extent, thyroxine binding globulin (Marchesini et al. 2008). In rat cerebellar Purkinje cells, the thyroid hormone dependent outgrowth of dendrites was inhibited by OH-PCBs at very low concentrations (50 pM; Kimura-Kuroda et al. 2007).

After developmental exposure to 4-OH-PCB-106 in rats, gene expression of glutamate receptor subunits and thyroid hormone responsive genes were altered in cortex, hippocampus and striatum in the offspring (Takahashi et al. 2009). OH-PCBs induce ROS formation and cell death in rat cerebellar granule cells to a larger degree than their parent congeners (Dreiem et al. 2009). In PC12 cells it was detected that 4-OH-PCB-106 induces the expression of c-Jun, a protein that is activated by extra- and intracellular stress signals (including UV radiation, osmotic stress, oxidative stress, extracellular acidification, alkalinization or cytokines) with a LOEC of 10 nM. The results in this study suggest that the response may be triggered by membrane-depolarization via Na^+ influx, followed by influx of Ca^{2+} through VGCCs (Shimokawa et al. 2006). As VGCCs also play a role in the disruption of Ca^{2+} homeostasis by OH-PBDEs and HBCD (chapter 6 and 8), possible interactions of these compounds with OH-PCBs should be investigated.

Despite the relatively small number of toxicity studies of OH-PCBs, it appears that similar neurotoxic effects are observed for PCBs and their hydroxylated metabolites. However, when considering binding to thyroid hormone transport proteins, not only excretion of PCBs is facilitated, but they are also bioactivated by oxidative phase I metabolism.

Risk assessment is for most classes of chemicals, with the exception of the toxic equivalency approach for dioxins and dioxin-like compounds (van den Berg et al. 2006), based on exposure and hazard assessment of single compounds, to which humans are exposed to at presumably very low levels. Regulatory limit values are based on the toxicity threshold values for single compounds. Because of the observed similarities in effects on neurobehavior and

on acute *in vitro* effects related to neuronal signaling, additivity should be the default approach for risk assessment of mixtures of PBDEs, PCBs and their metabolites. Because of the possible additivity of these environmental pollutants at levels below effective concentrations and the higher sensitivity of the developing brain, mixture effects should be investigated and taken into account for human risk assessment for developmental neurotoxicity.

Conclusions and recommendations

In this PhD-research, it is demonstrated that observed neurobehavioral effects of polybrominated diphenyl ethers (PBDEs) are also reflected in decreased synaptic plasticity in the hippocampus (chapter 3). It was also shown that hydroxylated metabolites of BDE-47 (OH-PBDEs) cause an imbalance in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and possibly neuronal signaling in PC12 cells by increasing basal and inhibiting depolarization-evoked $[Ca^{2+}]_i$ at lower concentrations compared to their parent BDE-47 (chapter 4 - 6). A structure-activity relationship for disturbance of basal $[Ca^{2+}]_i$ has been proposed (chapter 5). Inhibition of depolarization-evoked $[Ca^{2+}]_i$ has also been observed for hexabromocyclododecane (HBCD; chapter 8), a possible replacement in additive applications of PBDEs. When OH-PBDE-induced increase in basal $[Ca^{2+}]_i$ was transient, depolarization-evoked $[Ca^{2+}]_i$ was inhibited, even if basal $[Ca^{2+}]_i$ had approached baseline levels again for approximately 10 minutes. This indicates that transient acute increases in $[Ca^{2+}]_i$ affect regulatory mechanisms in the maintenance of Ca^{2+} homeostasis. Based on this observation can be concluded that disruption of Ca^{2+} homeostasis should be investigated in real-time. A sensitivity difference was observed in primary human neuronal and primary rat neuroendocrine cells (chapter 7). This indicates that observed-effect concentrations retrieved from *in vitro* models have to be chosen carefully when used for human risk assessment, to avoid under- or overestimation of these effect concentrations.

Because of the observed analogies in disruption of $[Ca^{2+}]_i$ by (OH-)PBDEs and HBCD with *ortho*-substituted polychlorinated biphenyls (*ortho*-PCBs), it is proposed that mixture effects of these common environmental neurotoxicants should be taken into account for human risk assessment. The relevance of mixture toxicity is practically supported by a recent finding that motor, cognitive and behavior performance in 6-year old children is correlated with maternal serum levels of PBDEs and OH-PCBs, but not PCB-153, measured in the 35th week of pregnancy (Roze et al. 2009). Other persistent environmental pollutants were not investigated in this study. Since the experimental investigation of interactions of every combination of environmental pollutant is not achievable, additional research is needed to allow prediction of mixture effects.

The underlying mechanisms for developmental neurotoxicity of PBDEs is not yet solved, although it has become more clear that acute cellular neurotoxic effects as well as disruption of the thyroid hormone system can both be involved in the developmental neurotoxicity of PBDEs and HBCD (Figure 11.1).

PBDEs and HBCD are ubiquitous in the environment. Human exposure to PBDEs is mainly via food and ingestion or inhalation of house dust (Johnson-Restrepo and Kannan 2009). Exposure can therefore partly be reduced by keeping the indoor environment clean (Zhang et al. 2009b). This especially concerns indoor environments were children stay, because of child-specific hand-to-mouth behavior as well as frequent ground contact. As a result, children ingest larger amounts of dust with adsorbed PBDEs. In addition, differences in toxicokinetics compared to adults have to be taken into account for human risk assessment (Ginsberg et al. 2004).

Human serum levels of PBDE are associated with furniture flammability standards (Zota et al. 2008). One possibility to reduce exposure would therefore be to lower fire safety standards. However, it can be expected that fewer people have died in fires because of the increased escape time since the introduction of chemical flame retardants. However, it remains as yet uncertain if a decrease in fire deaths was indeed due to the application of (brominated) flame retardants. Possibly, this decrease in fire death is due to other factors, for example alterations in building methods or smoking habits. This remains unclear as a thorough risk-benefit assessment of PBDEs or brominated flame retardants is currently lacking. Such an assessment is particularly important for policymakers in the UK and the US, as fire safety standards for brominated flame retardants are possibly unnecessarily high in these countries (Law et al. 2006a).

Risk assessment for adverse effects in human and wildlife was clearly not adequate when PCBs were introduced in modern society. However, several decades later, similarities can be observed for the structurally related PBDEs. Regulatory risk assessment initially regarded commercial PBDE mixtures as safe replacements of PCBs. During the last decade, the use of PBDEs as flame retardants is now highly restricted in the EU with the exception of DecaBDE in a number of applications. As a result of concerns for human health chemical industry voluntarily withdrew the production of Penta- and OctaBDE commercial mixtures from the North American market. It has only just been announced that the use of DecaBDE will also be phased out in North America. Nevertheless, the use of these commercial PBDE mixtures is still allowed in other parts of the world, resulting in continued environmental release.

Because producers of consumer products still have to comply with fire safety regulations, alternative, less extensively studied flame retardants are being used. By addition of chemical groups, ‘new’ brominated flame retardants are constructed. Some examples are decabromodiphenyl ethane or hexachlorocyclopentadienyldibromocyclooctane, which have already been detected in the indoor environment (Stapleton et al. 2008a; Zhu et al. 2008) and wildlife (Gauthier et al. 2009). Due to their (neuro)toxic mechanisms (Figure 11.1), it is of concern that it cannot be excluded that PBDEs contribute to subclinical neurotoxic effects in some human populations. Although the use of PBDEs has been greatly reduced due to voluntary and legislative measures, humans and wildlife will be exposed to PBDEs and their metabolites for many years to come because of their environmental persistence. This underlines the necessity of implementing better hazard and risk assessment strategies for identifying human health risks of new chemicals, such as novel brominated flame retardants.

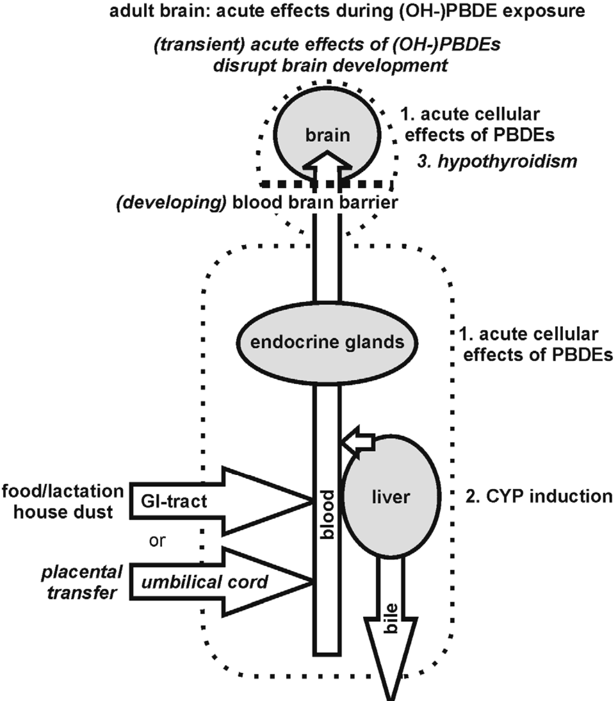


Figure 11.1. Processes involved in (developmental) neurotoxicity of (OH-)PBDEs and HBCD. Aspects that are particularly important for the developmental phase are *italic*. Relevant processes are placed on the right from the target organs (brain, endocrine glands or liver). Neurotoxicity and disruption of the thyroid hormone system are considered the main toxic endpoints for PBDEs. However, several observed toxic effects of PBDEs could contribute to the observed effects on neurobehavior. None of the acute cellular effects of PBDEs (1) has been determined as the leading cause for the observed developmental neurotoxicity. Not unlikely, all acute effects of PBDEs and HBCD on intracellular signaling (disruption of Ca^{2+} homeostasis, protein kinases, imbalance in neuronal signaling) or cell viability (oxidative stress, apoptosis) contribute directly or indirectly to the observed decreased synaptic plasticity and neurobehavioral effects. By CYP enzyme induction (2), PBDEs induce the formation of hydroxylated metabolites. In the adult brain, the duration of toxic effects are probably dependent on the exposure duration. In the developing brain, however, transient toxic effects can be maintained through neurodevelopment. Parent PBDE have been demonstrated to end up in the brain, and are therefore possibly causing acute, possibly transient, toxic effects. High levels of OH-PBDEs in the brain have not been detected, although transport over the blood-brain barrier via binding to thyroid hormone transport proteins is not unlikely and should be investigated. (OH-)PBDE induced alterations of thyroid hormone levels (3), either by induction of hepatic enzymes or competitive binding with thyroid transporter proteins, could result in alterations in developmental processes like neuronal and glial proliferation, differentiation and migration.

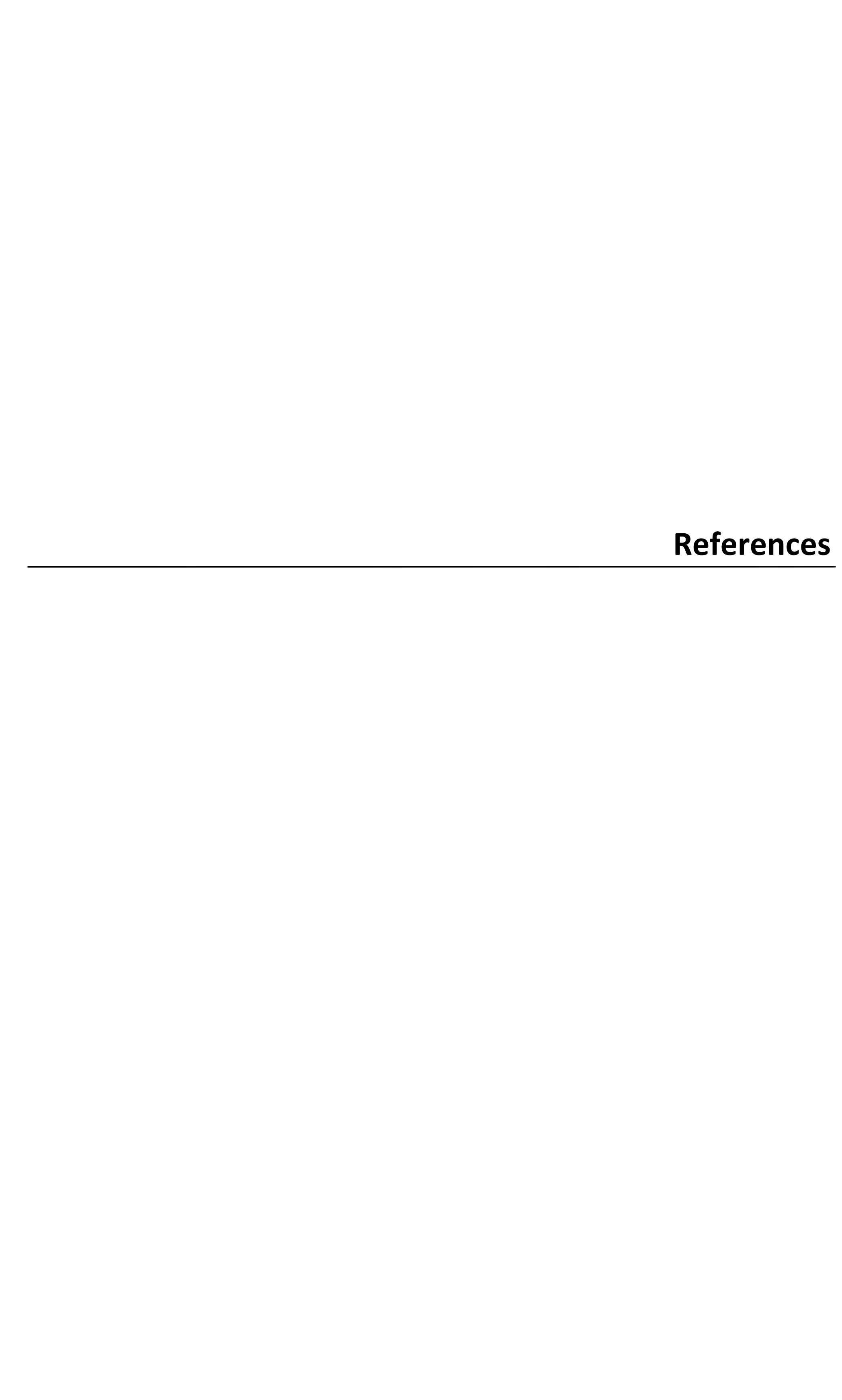
Conclusions.

1. Effects of PBDEs on learning and behavior are reflected in reduction of synaptic plasticity, possibly related to the observed reduction in LTP-related postsynaptic proteins.
2. Hydroxylated metabolites of BDE-47 (OH-PBDEs) have a higher potency to increase the basal $[Ca^{2+}]_i$ and inhibit the depolarization-evoked increase in $[Ca^{2+}]_i$ compared to their parent congener BDE-47, possibly creating an imbalance in neuronal communication.
3. Disturbance of the Ca^{2+}-homeostasis by OH-PBDEs depends on shielding of the OH-group by adjacent atomic groups.
4. BDE-47 as well as 6-OH-BDE-47 increase the basal $[Ca^{2+}]_i$ in human neural progenitor cells to a higher extent compared to in PC12 cells.
5. HBCD, a possible replacement for PBDEs, inhibits the depolarization-evoked increase in $[Ca^{2+}]_i$ and vesicular neurotransmitter release, while basal $[Ca^{2+}]_i$ or vesicular neurotransmitter release are unaffected.
6. Concentrations of PBDEs en their metabolites in human (cord)blood are lower compared to those that are expected to result in neurotoxic effects. However, because of the current shortage in knowledge on brain concentrations, brain development and mixture toxicity, this exposure to PBDEs and metabolites remains a concern for the developing brain.

Recommendations.

Neurotoxicity risk assessment of PBDEs, their metabolites and related environmental contaminants can be improved with increased knowledge on:

1. oxidative metabolism resulting in bioactivation.
2. sensitivity to neurotoxicity during specific processes of brain development.
3. concentrations of PBDEs and metabolites in brain tissues.
4. interactions between different environmental contaminants during simultaneous exposure.



References

Abdallah MA, Harrad S, Covaci A. (2008). Hexabromocyclododecanes and tetrabromobisphenol-A in indoor air and dust in Birmingham, U.K: implications for human exposure. *Environ Sci Technol* **42**, 6855-6861.

Ahn MY, Filley TR, Jafvert CT, Nies L, Hua I, Bezares-Cruz J. (2006). Photodegradation of decabromodiphenyl ether adsorbed onto clay minerals, metal oxides, and sediment. *Environ Sci Technol* **40**, 215-220.

Alaee M, Arias P, Sjödin A, Bergman Å. (2003). An overview of commercially used brominated flame retardants, their applications, their use patterns in different countries/regions and possible modes of release. *Environ Int* **29**, 683-689.

Albillos A, Dernick G, Horstmann H, Almers W, Alvarez de Toledo G, Lindau M. (1997). The exocytotic event in chromaffin cells revealed by patch amperometry. *Nature* **389**, 509-512.

Alcock RE, Sweetman AJ, Prevedouros K, Jones KC. (2003). Understanding levels and trends of BDE-47 in the UK and North America: an assessment of principal reservoirs and source inputs. *Environ Int* **29**, 691-698.

Alm H, Kultima K, Scholz B, Nilsson A, Andrén PE, Fex-Svenningsen Å, Dencker L, Stigson M. (2008). Exposure to brominated flame retardant PBDE-99 affects cytoskeletal protein expression in the neonatal mouse cerebral cortex. *Neurotoxicology* **29**, 628-637.

Alm H, Scholz B, Fischer C, Kultima K, Viberg H, Eriksson P, Dencker L, Stigson M. (2006). Proteomic evaluation of neonatal exposure to 2,2,4,4,5-pentabromodiphenyl ether. *Environ Health Perspect* **114**, 254-259.

Andersen JK. (2004). Oxidative stress in neurodegeneration: cause or consequence? *Nat Rev Neurosci* **5**, S18–S25.

Antignac JP, Cariou R, Maume D, Marchand P, Monteau F, Zalko D, Berrebi A, Cravedi JP, Andre F, Le Bizec B. (2008). Exposure assessment of fetus and newborn to brominated flame retardants in France: Preliminary data. *Mol Nutr Food Res* **52**, 258–265.

Athanasiadou M, Cuadra SN, Marsh G, Bergman Å, Jakobsson K. (2008). Polybrominated diphenyl ethers (PBDEs) and bioaccumulative hydroxylated PBDE metabolites in young humans from Managua, Nicaragua. *Environ Health Perspect* **116**, 400-408.

Barclay JW, Morgan A, Burgoyne RD. (2005). Calcium-dependent regulation of exocytosis. *Cell Calcium* **38**, 343-353.

Basu N, Scheuhammer AM, O'Brien M. (2007). Polychlorinated biphenyls, organochlorinated pesticides, and polybrominated diphenyl ethers in the cerebral cortex of wild river otters (*Lontra canadensis*). *Environ Pollut* **149**, 25-30.

Becherer U, Rettig J. (2006). Vesicle pools, docking, priming, and release. *Cell Tissue Res* **326**, 393-407.

Bennett DL, Bootman MD, Berridge MJ, Cheek TR. (1998). Ca^{2+} entry into PC12 cells initiated by ryanodine receptors or inositol 1,4,5-trisphosphate receptors. *Biochem J* **329**, 349–357.

Berridge MJ, Lipp P, Bootman MD. (2000). The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* **1**, 11-21.

Bezares-Cruz J, Jafvert CT, Hua I. (2004). Solar photodecomposition of decabromodiphenyl ether: products and quantum yield. *Environ Sci Technol* **38**, 4149-4156.

Bi X, Qu W, Sheng G, Zhang W, Mai B, Chen D, Yu L, Fu J. (2006). Polybrominated diphenyl ethers in South China maternal and fetal blood and breast milk. *Environ Pollut* **144**, 1024-1030.

Bi X, Thomas GO, Jones KC, Qu W, Sheng G, Martin FL, Fu J. (2007). Exposure of electronics dismantling workers to polybrominated diphenyl ethers, polychlorinated biphenyls, and organochlorine pesticides in South China. *Environ Sci Technol* **41**, 5647-5653.

Birnbaum LS, Cohen Hubal EA. (2006). Polybrominated diphenyl ethers: a case study for using biomonitoring data to address risk assessment questions. *Environ Health Perspect* **114**, 1770-1775.

Birnbaum LS, Staskal DF. (2004). Brominated flame retardants: cause for concern? *Environ Health Perspect* **112**, 9-17.

Birnbaum LS, Staskal DF, Diliberto JJ. (2003). Health effects of polybrominated dibenzo-*p*-dioxins (PBDDs) and dibenzofurans (PBDFs). *Environ Int* **29**, 855-860.

Bliss TVP, Lømo T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J Physiol* **232**, 331-356.

Bloom M, Spliethoff H, Vena J, Shaver S, Addink R, Eadon G. (2008). Environmental exposure to PBDEs and thyroid function among New York anglers. *Environ Toxicol Pharm* **25**, 386-392.

Blundon JA, Zakharenko SS. (2008). Dissecting the components of long-term potentiation. *Neuroscientist* **14**, 598-608.

Boucher O, Muckle G, Bastien CH. (2009). Prenatal exposure to polychlorinated biphenyls: a neuropsychologic analysis. *Environ Health Perspect* **117**, 7-16.

Boulware MJ, Marchant JS. (2008). Timing in cellular Ca^{2+} signaling. *Curr Biol* **18**, R769-R776.

Bradman A, Fenster L, Sjödin A, Jones RS, Patterson DG Jr, Eskenazi B. (2007). Polybrominated diphenyl ether levels in the blood of pregnant women living in an agricultural community in California. *Environ Health Perspect* **115**, 71-74.

Branchi I, Alleva E, Costa LG. (2002). Effects of perinatal exposure to a polybrominated diphenyl ether (PBDE-99) on mouse neurobehavioural development. *Neurotoxicology* **23**, 375-384.

Branchi I, Capone F, Alleva E, Costa LG. (2003). Polybrominated diphenyl ethers: neurobehavioral effects following developmental exposure. *Neurotoxicology* **24**, 449–462.

Branchi I, Capone F, Vitalone A, Madia F, Santucci D, Alleva E, Costa LG. (2005). Early developmental exposure to BDE-99 or Aroclor 1254 affects neurobehavioural profile: interference from the administration route. *Neurotoxicology* **26**, 183-192.

Brandsma SH, van der Ven LTM, de Boer J, Leonards PEG. (2009). Identification of hydroxylated metabolites of hexabromocyclododecane in wildlife and 28-days exposed Wistar rats. *Environ Sci Technol* **43**, 6058-6063.

Breier JM, Gassmann K, Kayser R, Stegeman H, De Groot D, Fritsche E, Shafer TJ. (2009). Neural progenitor cells as models for high-throughput screens of developmental neurotoxicity: State of the science. *Neurotoxicol Teratol* doi:10.1016/j.ntt.2009.06.005 [Online 24 June 2009].

BSEF (2009a). bsef.com (Bromine Science and Environmental Forum).

BSEF (2009b). Are brominated flame retardants (BFRs) Persistent Organic Pollutants (POPs)? bsef.com (Bromine Science and Environmental Forum).

BSEF (2009c). Factsheet Deca-BDE. bsef.com (Bromine Science and Environmental Forum).
BSEF (2009d). Factsheet HBCD. bsef.com (Bromine Science and Environmental Forum).
BSEF (2009e). Factsheet TBBPA. bsef.com (Bromine Science and Environmental Forum).
Budde T, Meuth S, Pape HC. (2002). Calcium-dependent inactivation of neuronal calcium channels. *Nat Rev Neurosci* **3**, 873-883.
Bull K, Basu N, Zhang S, Martin JW, Bursian S, Martin P, Chan LHM. (2007). Dietary and *in utero* exposure to a pentabrominated diphenyl ether mixture did not affect cholinergic parameters in the cerebral cortex of ranch mink (*Mustela vison*). *Toxicol Sci* **96**, 115-122.
Burnashev N. (1998). Calcium permeability of ligand-gated channels. *Cell Calcium* **24**, 325-332.
Burreau S, Axelman J, Broman D, Jakobsson E. (1997). Dietary uptake in pike (*Esox lucius*) of some polychlorinated biphenyls, polychlorinated naphtalenes and polybrominated diphenyl ethers administered in natural diet. *Environ Toxicol Chem* **16**, 2508-2513.
Burreau S, Broman D, Örn U. (2000). Tissue distribution of 2,2',4,4'-tetrabromo[^{14}C]diphenyl ether ([^{14}C]-PBDE-47) in pike (*Esox lucius*) after dietary exposure--a time series study using whole body autoradiography. *Chemosphere* **40**, 977-985.
Burreau S, Zebühr Y, Broman D, Ishaq R. (2004). Biomagnification of polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) studied in pike (*Esox lucius*), perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) from the Baltic Sea. *Chemosphere* **55**, 1043-1052.
Cantón RF, Peijnenburg AACM, Hoogenboom RLAP, Piersma AH, van der Ven LTM, van den Berg M, Heneweer M. (2008a). Subacute effects of hexabromocyclododecane (HBCD) on hepatic gene expression profiles in rats. *Toxicol Appl Pharmacol* **231**, 267-272.
Cantón RF, Sanderson JT, Letcher RJ, Bergman Å, van den Berg M. (2005). Inhibition and induction of aromatase (CYP19) activity by brominated flame retardants in H295R human adrenocortical carcinoma cells. *Toxicol Sci* **88**, 447-455.
Cantón RF, Sanderson JT, Nijmeijer S, Bergman Å, Letcher RJ, van den Berg M. (2006). *In vitro* effects of brominated flame retardants and metabolites on CYP17 catalytic activity: a novel mechanism of action? *Toxicol Appl Pharmacol* **216**, 274-281.
Cantón RF, Scholten DEA, Marsh G, de Jong PC, van den Berg M. (2008b). Inhibition of human placental aromatase activity by hydroxylated polybrominated diphenyl ethers (OH-PBDEs). *Toxicol Appl Pharmacol* **227**, 68-75.
Catterall WA. (2000). Structure and regulation of voltage-gated Ca^{2+} channels. *Annu Rev Cell Dev Biol* **16**, 521-555.
Chapman ER. (2008). How does synaptotagmin trigger neurotransmitter release? *Annu Rev Biochem* **77**, 615-641.
Chen G, Bunce NJ. (2003). Polybrominated diphenyl ethers as Ah receptor agonists and antagonists. *Toxicol Sci* **76**, 310-320.
Chen G, Konstantinov AD, Chittim BG, Joyce EM, Bols NC, Bunce NJ. (2001). Synthesis of polybrominated diphenyl ethers and their capacity to induce CYP1A by the Ah receptor mediated pathway. *Environ Sci Technol* **35**, 3749-3756.
Chen LJ, Lebetkin EH, Sanders JM, Burka LT. (2006). Metabolism and disposition of 2,2',4,4',5-pentabromodiphenyl ether (BDE99) following a single or repeated administration to rats or mice. *Xenobiotica* **36**, 515-534.
Chen D, Mai B, Song J, Sun Q, Luo Y, Luo X, Zeng EY, Hale RC. (2007). Polybrominated diphenyl ethers in birds of prey from Northern China. *Environ Sci Technol* **41**, 1828-1833.
Cheng J, Gu J, Ma J, Chen X, Zhang M, Wang W. (2009). Neurobehavioural effects, redox responses and tissue distribution in rat offspring developmental exposure to BDE-99. *Chemosphere* **75**, 963-968.
Cheng H, Lederer WJ. (2008). Calcium sparks. *Physiol Rev* **88**, 1491-1545.
Christiansson A, Eriksson J, Teclechiel D, Bergman Å. (2009). Identification and quantification of products formed via photolysis of decabromodiphenyl ether. *Environ Sci Pollut Res Int* **16**, 312-321.
Clapham DE. (2007). Calcium signaling. *Cell* **131**, 1047-1058.
Coburn CG, Currás-Collazo MC, Kodavanti PRS. (2008). *In vitro* effects of environmentally relevant polybrominated diphenyl ether (PBDE) congeners on calcium buffering mechanisms in rat brain. *Neurochem Res* **33**, 355-364.
Colles A, Koppen G, Hanot V, Nelen V, Dewolf MC, Noël E, Malisch R, Kotz A, Kypke K, Biot P, Vinkx C, Schoeters G. (2008). Fourth WHO-coordinated survey of human milk for persistent organic pollutants (POPs): Belgian results. *Chemosphere* **73**, 907-914.
Costa LG, Giordano G. (2007). Developmental neurotoxicity of polybrominated diphenyl ether (PBDE) flame retardants. *Neurotoxicology* **28**, 1047-1067.
Covaci A, Gerecke AC, Law RJ, Voorspoels S, Kohler M, Heeb NV, Leslie H, Allchin CR, de Boer J. (2006). Hexabromocyclododecanes (HBCDs) in the environment and humans: a review. *Environ Sci Technol* **40**, 3679-3688.
Covaci A, Voorspoels S, Ramos L, Neels H, Blust R. (2007a). Recent developments in the analysis of brominated flame retardants and brominated natural compounds. *J Chromatogr A* **1153**, 145-171.
Covaci A, Voorspoels S, Vetter W, Gelbin A, Jorens PG, Blust R, Neels H. (2007b). Anthropogenic and naturally occurring organobrominated compounds in fish oil dietary supplements. *Environ Sci Technol* **41**, 5237-5244.
Crump D, Jagla MM, Kehoe A, Kennedy SW. (2008). Detection of polybrominated diphenyl ethers in herring gull (*Larus argentatus*) brains: effects on mRNA expression in cultured neuronal cells. *Environ Sci Technol* **42**, 7715-7721.
Cui Q. (2006). Actions of neurotrophic factors and their signaling pathways in neuronal survival and axonal regeneration. *Mol Neurobiol* **33**, 155-179.
Dai S, Hall DD, Hell JW. (2009). Supramolecular assemblies and localized regulation of voltage-gated ion channels. *Physiol Rev* **89**, 411-452.
Dallaire R, Ayotte P, Pereg D, Déry S, Dumas P, Langlois E, Dewailly E. (2009). Determinants of plasma concentrations of perfluorooctanesulfonate and brominated organic compounds in Nunavik Inuit adults (Canada). *Environ Sci Technol* **43**, 5130-5136.

Dang VH, Choi KC, Jeung EB. (2007). Tetrabromodiphenyl ether (BDE-47) evokes estrogenicity and calbindin-D_{9k} expression through an estrogen receptor-mediated pathway in the uterus of immature rats. *Toxicol Sci* **97**, 504-511.

Darnerud PO. (2003). Toxic effects of brominated flame retardants in man and in wildlife. *Environ Int* **29**, 841-853.

Darnerud PO. (2008). Brominated flame retardants as possible endocrine disrupters. *Int J Androl* **31**, 152-160.

Darnerud PO, Aune M, Larsson L, Hallgren S. (2007). Plasma PBDE and thyroxine levels in rats exposed to Bromkal or BDE-47. *Chemosphere* **67**, S386-S392.

Darnerud PO, Eriksen GS, Jóhannesson T, Larsen PB, Viluksela M. (2001). Polybrominated diphenyl ethers: occurrence, dietary exposure, and toxicology. *Environ Health Perspect* **109 Suppl 1**, 49-68.

Darnerud PO, Risberg S. (2006). Tissue localisation of tetra- and pentabromodiphenyl ether congeners (BDE-47, -85 and -99) in perinatal and adult C57BL mice. *Chemosphere* **62**, 485-493.

Davison AN, Dobbing J. (1968). *Applied Neurochemistry*. Oxford, UK:Blackwell.

Deitmer JW, Schild D. (2000). Calcium—Imaging, Protocolle und Ergebnisse. In *Ca^{2+} und pH, Ionenmessungen in Zellen und Geweben* (JW Deitmer and D Schild, Eds.). Spektrum Akademischer Verleger, Heidelberg.

Deleranko MJ, Hollinger MA. (2001). Handbook of Toxicology, Second Edition. CRC Press.

Del Toro R, Levitsky KL, López-Barneo J, Chiara MD. (2003). Induction of T-type calcium channel gene expression by chronic hypoxia. *J Biol Chem* **278**, 22316-22324.

Deng J, Yu L, Liu C, Yu K, Shi X, Yeung LWY, Lam PKS, Wu RSS, Zhou B. (2009). Hexabromocyclododecane-induced developmental toxicity and apoptosis in zebrafish embryos. *Aquat Toxicol* **93**, 29-36.

Denizot F, Lang R. (1986). Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* **89**, 271-277.

Denny JB. (2006). Molecular mechanisms, biological actions, and neuropharmacology of the growth-associated protein GAP-43. *Curr Neuropharmacol* **4**, 293-304.

de Wit CA. (2002). An overview of brominated flame retardants in the environment. *Chemosphere* **46**, 583-624.

de Wit CA, Alaee M, Muir DCG. (2006). Levels and trends of brominated flame retardants in the Arctic. *Chemosphere* **64**, 209-233.

Ding L, Murphy MB, He Y, Xu Y, Yeung LWY, Wang J, Zhou B, Lam PKS, Wu RSS, Giesy JP. (2007). Effects of brominated flame retardants and brominated dioxins on steroidogenesis in H295R human adrenocortical carcinoma cell line. *Environ Toxicol Chem* **26**, 764-772.

Dingemans MML, de Groot A, van Kleef RGDM, Bergman Å, van den Berg M, Vijverberg HPM, Westerink RHS. (2008). Hydroxylation increases the neurotoxic potential of BDE-47 to affect exocytosis and calcium homeostasis in PC12 cells. *Environ Health Perspect* **116**, 637-643.

Dingemans MML, Heusinkveld HJ, de Groot A, Bergman Å, van den Berg M, Westerink RHS. (2009a). Hexabromocyclododecane inhibits depolarization-induced increase in intracellular calcium levels and neurotransmitter release in PC12 cells. *Toxicol Sci* **107**, 490-497.

Dingemans MML, Heusinkveld HJ, Bergman Å, van den Berg M, Westerink RHS. (2009b). Bromination pattern of hydroxylated metabolites of BDE-47 affects their potency to release calcium from intracellular stores in PC12 cells. *Environ Health Perspect* doi:10.1289/ehp.0901339 [Online 19 November 2009].

Dingemans MML, Ramakers GMJ, Gardoni F, van Kleef RGDM, Bergman Å, Di Luca M, van den Berg M, Westerink RHS, Vijverberg HPM. (2007). Neonatal exposure to brominated flame retardant BDE-47 reduces long-term potentiation and postsynaptic protein levels in mouse hippocampus. *Environ Health Perspect* **115**, 865–870.

Dorn GW 2nd, Mochly-Rosen D. (2002). Intracellular transport mechanisms of signal transducers. *Annu Rev Physiol* **64**, 407-429.

Doucet J, Tague B, Arnold DL, Cooke GM, Hayward S, Goodyer CG. (2009). Persistent organic pollutant residues in human fetal liver and placenta from Greater Montreal, Quebec: a longitudinal study from 1998 through 2006. *Environ Health Perspect* **117**, 605-610.

Dreiem A, Rykken S, Lehmler HJ, Robertson LW, Fonnum F. (2009). Hydroxylated polychlorinated biphenyls increase reactive oxygen species formation and induce cell death in cultured cerebellar granule cells. *Toxicol Appl Pharmacol* **240**, 306-313.

Driscoll LL, Gibson AM, Hieb A. (2009). Chronic postnatal DE-71 exposure: effects on learning, attention and thyroxine levels. *Neurotoxicol Teratol* **31**, 76-84.

Dufault C, Poles G, Driscoll LL. (2005). Brief postnatal PBDE exposure alters learning and the cholinergic modulation of attention in rats. *Toxicol Sci* **88**, 172-180.

Duman JG, Chen L, Hille B. (2008). Calcium transport mechanisms of PC12 cells. *J Gen Physiol* **131**, 307-323.

Dunnick JK, Nyska A. (2009). Characterization of liver toxicity in F344/N rats and B6C3F1 mice after exposure to a flame retardant containing lower molecular weight polybrominated diphenyl ethers. *Exp Toxicol Pathol* **61**, 1-12.

Eljarrat E, Guerra P, Martínez E, Farré M, Alvarez JG, López-Teijón M, Barceló D. (2009). Hexabromocyclododecane in human breast milk: levels and enantiomeric patterns. *Environ Sci Technol* **43**, 1940-1946.

Ema M, Fujii S, Hirata-Koizumi M, Matsumoto M. (2008). Two-generation reproductive toxicity study of the flame retardant hexabromocyclododecane in rats. *Reprod Toxicol* **25**, 335-351.

Engelhardt B. (2003). Development of the blood-brain barrier. *Cell Tissue Res* **314**, 119-129.

Eriksson P, Ankarberg E, Viberg H, Fredriksson A. (2001a). The developing cholinergic system as target for environmental toxicants, nicotine and polychlorinated biphenyls (PCBs): implications for neurotoxicological processes in mice. *Neurotox Res* **3**, 37–51.

Eriksson P, Fischer C, Fredriksson A. (2006a). Polybrominated diphenyl ethers (PBDEs), a group of brominated flame retardants, can interact with PCB in enhancing neurobehavioral defects. *Toxicol Sci* **94**, 302–309.

Eriksson P, Fischer C, Wallin M, Jakobsson E, Fredriksson A. (2006b). Impaired behaviour, learning and memory, in adult mice neonatally exposed to hexabromocyclododecane (HBCDD). *Environ Toxicol Pharmacol* **21**, 317-322.

Eriksson P, Fredriksson A. (1991). Neurotoxic effects of two different pyrethroids, bioallethrin and deltamethrin, on immature and adult mice: changes in behavioral and muscarinic receptor variables. *Toxicol Appl Pharmacol* **108**, 78–85.

Eriksson J, Green N, Marsh G, Bergman Å. (2004). Photochemical decomposition of 15 polybrominated diphenyl ether congeners in methanol/water. *Environ Sci Technol* **38**, 3119-3125.

Eriksson P, Jakobsson E, Fredriksson A. (2001b). Brominated flame retardants: a novel class of developmental neurotoxicants in our environment? *Environ Health Perspect* **109**, 903-908.

Eriksson P, Lundkvist U, Fredriksson A. (1991). Neonatal exposure to 3,3´,4,4´-tetrachlorobiphenyl: changes in spontaneous behaviour and cholinergic muscarinic receptors in the adult mouse. *Toxicology* **69**, 27–34.

Eriksson P, Nilsson-Håkansson L, Nordberg A, Aspberg A, Fredriksson A. (1990). Neonatal exposure to DDT and its fatty acid conjugate: effects on cholinergic and behavioural variables in the adult mouse. *Neurotoxicology* **11**, 345–354.

Eriksson P, Viberg H, Jakobsson E, Örn U, Fredriksson A. (2002). A brominated flame retardant, 2,2',4,4',5-pentabromodiphenyl ether: uptake, retention, and induction of neurobehavioral alterations in mice during a critical phase of neonatal brain development. *Toxicol Sci* **67**, 98-103.

Evans GJO, Cousin MA. (2005). Tyrosine phosphorylation of synaptophysin in synaptic vesicle recycling. *Biochem Soc Trans* **33**, 1350-1353.

Fång J. (2007). Separation and NMR-spectroscopy characterization of technical grade hexabromocyclododecane isomers. MSc thesis. Stockholm University, Stockholm.

Fängström B, Athanassiadis I, Odsjö T, Norén K, Bergman Å. (2008). Temporal trends of polybrominated diphenyl ethers and hexabromocyclododecane in milk from Stockholm mothers, 1980-2004. *Mol Nutr Food Res* **52**, 187-193.

Fängström B, Strid A, Grandjean P, Weihe P, Bergman Å. (2005). A retrospective study of PBDEs and PCBs in human milk from the Faroe Islands. *Environ Health* **4**, 12.

Farooqui AA, Yang HC, Rosenberger TA, Horrocks LA. (1997). Phospholipase A2 and its role in brain tissue. *J Neurochem* **69**, 889-901.

Fernie KJ, Shutt JL, Letcher RJ, Ritchie IJ, Bird DM. (2009). Environmentally relevant concentrations of DE-71 and HBCD alter eggshell thickness and reproductive success of American kestrels. *Environ Sci Technol* **43**, 2124-2130.

Fernie KJ, Shutt JL, Letcher RJ, Ritchie JI, Sullivan K, Bird DM. (2008). Changes in reproductive courtship behaviors of adult American kestrels (*Falco sparverius*) exposed to environmentally relevant levels of the polybrominated diphenyl ether mixture, DE-71. *Toxicol Sci* **102**, 171-178.

Fernie KJ, Shutt JL, Mayne G, Hoffman D, Letcher RJ, Drouillard KG, Ritchie IJ. (2005). Exposure to polybrominated diphenyl ethers (PBDEs): changes in thyroid, vitamin A, glutathione homeostasis, and oxidative stress in American kestrels (*Falco sparverius*). *Toxicol Sci* **88**, 375-383.

Fery Y, Buschauer I, Salzig C, Lang P, Schrenk D. (2009). Technical pentabromodiphenyl ether and hexabromocyclododecane as activators of the pregnane-X-receptor (PXR). *Toxicology* **264**, 45-51.

Fischer D, Hooper K, Athanasiadou M, Athanassiadis I, Bergman Å. (2006). Children show highest levels of polybrominated diphenyl ethers in a California family of four: a case study. *Environ Health Perspect* **114**, 1581-1584.

Fonnum F, Mariussen E. (2009). Mechanisms involved in the neurotoxic effects of environmental toxicants such as polychlorinated biphenyls and brominated flame retardants. *J Neurochem* doi: 10.1111/j.1471-4159.2009.06427.x [Online 10 October 2009].

Fonnum F, Mariussen E, Reistad T. (2006). Molecular mechanisms involved in the toxic effects of polychlorinated biphenyls (PCBs) and brominated flame retardants (BFRs). *J Toxicol Environ Health A* **69**, 21–35.

Fowles JR, Fairbrother A, Baecher-Steppan L, Kerkvliet NI. (1994). Immunologic and endocrine effects of the flame-retardant pentabromodiphenyl ether (DE-71) in C57BL/6J mice. *Toxicology* **86**, 49-61.

Frederiksen M, Vorkamp K, Thomsen M, Knudsen LE. (2009). Human internal and external exposure to PBDEs--a review of levels and sources. *Int J Hyg Environ Health* **212**, 109-134.

Fredriksson A, Dahlgren L, Danielsson B, Eriksson P, Dencker L, Archer T. (1992). Behavioural effects of neonatal metallic mercury exposure in rats. *Toxicology* **74**, 151–160.

Fritsche E, Cline JE, Nguyen NH, Scanlan TS, Abel J. (2005). Polychlorinated biphenyls disturb differentiation of normal human neural progenitor cells: clue for involvement of thyroid hormone receptors. *Environ Health Perspect* **113**, 871-876.

Gafni J, Wong PW, Pessah IN. (2004). Non-coplanar 2,2´,3,5´,6-pentachlorobiphenyl (PCB 95) amplifies ionotropic glutamate receptor signalling in embryonic granule neurons by a mechanism involving ryanodine receptors. *Toxicol Sci* **77**, 72-82.

Gao P, He P, Wang A, Xia T, Xu B, Xu Z, Niu Q, Guo L, Chen X. (2009). Influence of PCB153 on oxidative DNA damage and DNA repair-related gene expression induced by PBDE-47 in human neuroblastoma cells *in vitro*. *Toxicol Sci* **107**, 165-170.

García AG, García-De-Diego AM, Gandía L, Borges R, García-Sancho J. (2006). Calcium signaling and exocytosis in adrenal chromaffin cells. *Physiol Rev* **86**, 1093-1131.

Gardoni F, Picconi B, Ghiglieri V, Polli F, Bagetta V, Bernardi G, Cattabeni F, Di Luca M, Calabresi P. (2006a). A critical interaction between NR2B and MAGUK in L-DOPA induced dyskinesia. *J Neurosci* **26**, 2914–2922.

Gardoni F, Polli F, Cattabeni F, Di Luca M. (2006b). Calcium-calmodulin-dependent protein kinase II phosphorylation modulates PSD-95 binding to NMDA receptors. *Eur J Neurosci* **24**, 2694-2704.

Gardoni F, Schrama LH, Kamal A, Gispen WH, Cattabeni F, Di Luca M. (2001). Hippocampal synaptic plasticity involves competition between Ca^{2+}/calmodulin-dependent protein kinase II and postsynaptic density 95 for binding to the NR2A subunit of the NMDA receptor. *J Neurosci* **21**, 1501–1509.

Gauthier LT, Potter D, Hebert CE, Letcher RJ. (2009). Temporal trends and spatial distribution of non-polybrominated diphenyl ether flame retardants in the eggs of colonial populations of Great Lakes herring gulls. *Environ Sci Technol* **43**, 312-317.

Gebbink WA, Sonne C, Dietz R, Kirkegaard M, Born EW, Muir DCG, Letcher RJ. (2008a). Target tissue selectivity and burdens of diverse classes of brominated and chlorinated contaminants in polar bears (*Ursus maritimus*) from East Greenland. *Environ Sci Technol* **42**, 752-759.

Gebbink WA, Sonne C, Dietz R, Kirkegaard M, Riget FF, Born EW, Muir DCG, Letcher RJ. (2008b). Tissue-specific congener composition of organohalogen and metabolite contaminants in East Greenland polar bears (*Ursus maritimus*). *Environ Pollut* **152**, 621-629.

Gee JR, Hedge JM, Moser VC. (2008). Lack of alterations in thyroid hormones following exposure to polybrominated diphenyl ether 47 during a period of rapid brain development in mice. *Drug Chem Toxicol* **31**, 245-254.

Gee JR, Moser VC. (2008). Acute postnatal exposure to brominated diphenylether 47 delays neuromotor ontogeny and alters motor activity in mice. *Neurotoxicol Teratol* **30**, 79-87.

Gerecke AC, Hartmann PC, Heeb NV, Kohler HPE, Giger W, Schmid P, Zennegg M, Kohler M. (2005). Anaerobic degradation of decabromodiphenyl ether. *Environ Sci Technol* **39**, 1078-1083.

Germer S, Piersma AH, van der Ven LTM, Kamyschnikow A, Fery Y, Schmitz HJ, Schrenk D. (2006). Subacute effects of the brominated flame retardants hexabromocyclododecane and tetrabromobisphenol A on hepatic cytochrome P450 levels in rats. *Toxicology* **218**, 229-236.

Ghisari M, Bonefeld-Jorgensen EC. (2005). Impact of environmental chemicals on the thyroid hormone function in pituitary rat GH3 cells. *Mol Cell Endocrinol* **244**, 31-41.

Ghosh M, Meerts IATM, Cook A, Bergman Å, Brouwer A, Johnson LN. (2000). Structure of human transthyretin complexed with bromophenols: a new mode of binding. *Acta Crystallogr D Biol Crystallogr* **56**, 1085-1095.

Giese KP, Fedorov NB, Filipkowski RK, Silva AJ. (1998). Autophosphorylation at Thr286 of the alpha calciumcalmodulin kinase II in LTP and learning. *Science* **279**, 870–873.

Ginsberg G, Hattis D, Sonawane B. (2004). Incorporating pharmacokinetic differences between children and adults in assessing children's risks to environmental toxicants. *Toxicol Appl Pharmacol* **198**, 164-183.

Giordano G, Kavanagh TJ, Costa LG. (2008). Neurotoxicity of a polybrominated diphenyl ether mixture (DE-71) in mouse neurons and astrocytes is modulated by intracellular glutathione levels. *Toxicol Appl Pharmacol* **232**, 161-168.

Gómara B, Herrero L, Ramos JJ, Mateo JR, Fernández MA, García JF, González MJ. (2007). Distribution of polybrominated diphenyl ethers in human umbilical cord serum, paternal serum, maternal serum, placentas, and breast milk from Madrid population, Spain. *Environ Sci Technol* **41**, 6961-6968.

Grandjean P, Landrigan PJ. (2006). Developmental neurotoxicity of industrial chemicals. *Lancet* **368**, 2167-2178.

Greene LA, Tischler AS. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci U S A* **73**, 2424-2428.

Grynkiewicz G, Poenie M, Tsien RY. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**, 3440-3450.

Guvenius DM, Aronsson A, Ekman-Ordeberg G, Bergman Å, Norén K. (2003). Human prenatal and postnatal exposure to polybrominated diphenyl ethers, polychlorinated biphenyls, polychlorobiphenylols, and pentachlorophenol. *Environ Health Perspect* **111**, 1235-1241.

Hagmar L, Björk J, Sjödin A, Bergman Å, Erfurth EM. (2001). Plasma levels of persistent organohalogens and hormone levels in adult male humans. *Arch Environ Health* **56**, 138-143.

Hakk H, Huwe JK, Larsen GL. (2009). Absorption, distribution, metabolism and excretion (ADME) study with 2,2',4,4',5,6'-hexabromodiphenyl ether (BDE-154) in male Sprague-Dawley rats. *Xenobiotica* **39**, 46-56.

Hakk H, Larsen G, Klasson-Wehler E. (2002). Tissue disposition, excretion and metabolism of 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) in the male Sprague-Dawley rat. *Xenobiotica* **32**, 369-382.

Hakk H, Letcher RJ. (2003). Metabolism in the toxicokinetics and fate of brominated flame retardants--a review. *Environ Int* **29**, 801-828.

Hakkola J, Pelkonen O, Pasanen M, Raunio H. (1998). Xenobiotic-metabolizing cytochrome P450 enzymes in the human feto-placental unit: role in intrauterine toxicity. *Crit Rev Toxicol* **28**, 35-72.

Hale RC, La Guardia MJ, Harvey E, Gaylor MO, Mainor TM. (2006). Brominated flame retardant concentrations and trends in abiotic media. *Chemosphere* **64**, 181-186.

Hallgren S, Darnerud PO. (2002). Polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and chlorinated paraffins (CPs) in rats-testing interactions and mechanisms for thyroid hormone effects. *Toxicology* **177**, 227-243.

Hallgren S, Sinjari T, Håkansson H, Darnerud PO. (2001). Effects of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) on thyroid hormone and vitamin A levels in rats and mice. *Arch Toxicol* **75**, 200-208.

Hamers T, Kamstra JH, Sonneveld E, Murk AJ, Kester MHA, Andersson PL, Legler J, Brouwer A. (2006). *In vitro* profiling of the endocrine-disrupting potency of brominated flame retardants. *Toxicol Sci* **92**, 157-173.

Hamers T, Kamstra JH, Sonneveld E, Murk AJ, Visser TJ, Van Velzen MJM, Brouwer A, Bergman Å. (2008). Biotransformation of brominated flame retardants into potentially endocrine-disrupting metabolites, with special attention to 2,2',4,4'-tetrabromodiphenyl ether (BDE-47). *Mol Nutr Food Res* **52**, 284-298.

Hanari N, Kannan K, Miyake Y, Okazawa T, Kodavanti PRS, Aldous KM, Yamashita N. (2006). Occurrence of polybrominated biphenyls, polybrominated dibenzo-*p*-dioxins, and polybrominated dibenzofurans as impurities in commercial polybrominated diphenyl ether mixtures. *Environ Sci Technol* **40**, 4400-4405.

Handayani D, Edrada RA, Proksch P, Wray V, Witte L, van Soest RWM, Kunzmann A, Soedarsono (1997). Four new bioactive polybrominated diphenyl ethers of the sponge *Dysidea herbacea* from West Sumatra, Indonesia. *J Nat Prod* **60**, 1313-1316.

Hanson PI, Roth R, Morisaki H, Jahn R, Heuser JE. (1997). Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. *Cell* **90**, 523-535.

Hardy ML. (2002). The toxicology of the three commercial polybrominated diphenyl oxide (ether) flame retardants. *Chemosphere* **46**, 757-777.

Harju M, Hamers T, Kamstra JH, Sonneveld E, Boon JP, Tysklind M, Andersson PL. (2007). Quantitative structure-activity relationship modeling on *in vitro* endocrine effects and metabolic stability involving 26 selected brominated flame retardants. *Environ Toxicol Chem* **26**, 816-826.

Hassenklöver T, Bickmeyer U. (2006). The marine secondary metabolites 2,4-dibromophenol and 2,4,6-tribromophenol differentially modulate voltage dependent ion currents in neuroendocrine (PC12) cells. *Aquat Toxicol* **79**, 384-390.

Hassenklöver T, Predehl S, Pilli J, Ledwolorz J, Assmann M, Bickmeyer U. (2006). Bromophenols, both present in marine organisms and in industrial flame retardants, disturb cellular Ca^{2+} signaling in neuroendocrine cells (PC12). *Aquat Toxicol* **76**, 37-45.

Haukås M, Hylland K, Berge JA, Nygård T, Mariussen E. (2009a). Spatial diastereomer patterns of hexabromocyclododecane (HBCD) in a Norwegian fjord. *Sci Total Environ* **407**, 5907-5913.

Haukås M, Mariussen E, Ruus A, Tollefsen KE. (2009b). Accumulation and disposition of hexabromocyclododecane (HBCD) in juvenile rainbow trout (*Oncorhynchus mykiss*). *Aquat Toxicol* doi:10.1016/j.aquatox.2009.08.010 [Online 4 September 2009].

Hayward SJ, Lei YD, Wania F. (2006). Comparative evaluation of three high-performance liquid chromatography-based K_{ow} estimation methods for highly hydrophobic organic compounds: Polybrominated diphenyl ethers and hexabromocyclododecane. *Environ Toxicol Chem* **25**, 2018–2027.

He W, He P, Wang A, Xia T, Xu B, Chen X. (2008a). Effects of PBDE-47 on cytotoxicity and genotoxicity in human neuroblastoma cells *in vitro*. *Mutat Res* **649**, 62-70.

He P, He W, Wang A, Xia T, Xu B, Zhang M, Chen X. (2008b). PBDE-47-induced oxidative stress, DNA damage and apoptosis in primary cultured rat hippocampal neurons. *Neurotoxicology* **29**, 124-129.

He J, Robrock KR, Alvarez-Cohen L. (2006). Microbial reductive debromination of polybrominated diphenyl ethers (PBDEs). *Environ Sci Technol* **40**, 4429-4434.

He P, Wang AG, Xia T, Gao P, Niu Q, Guo LJ, Chen XM. (2009a). Mechanisms underlying the developmental neurotoxic effect of PBDE-47 and the enhanced toxicity associated with its combination with PCB153 in rats. *Neurotoxicology* **30**, 1088-1095.

He P, Wang AG, Xia T, Gao P, Niu Q, Guo LJ, Xu BY, Chen XM. (2009b). Mechanism of the neurotoxic effect of PBDE-47 and interaction of PBDE-47 and PCB153 in enhancing toxicity in SH-SY5Y cells. *Neurotoxicology* **30**, 10-15.

He W, Wang A, Xia T, Gao P, Xu B, Xu Z, He P, Chen X. (2009c). Cytogenotoxicity induced by PBDE-47 combined with PCB153 treatment in SH-SY5Y cells. *Environ Toxicol* 10.1002/tox.20517 [Online 26 June 2009].

Heeb NV, Schweizer WB, Kohler M, Gerecke AC. (2005). Structure elucidation of hexabromocyclododecanes--a class of compounds with a complex stereochemistry. *Chemosphere* **61**, 65-73.

Heeb NV, Schweizer WB, Mattrel P, Haag R, Kohler M, Schmid P, Zennegg M, Wolfensberger M. (2008). Regio- and stereoselective isomerization of hexabromocyclododecanes (HBCDs): kinetics and mechanism of beta-HBCD racemization. *Chemosphere* **71**, 1547-1556.

Helgason LB, Polder A, Føreid S, Bæk K, Lie E, Gabrielsen GW, Barrett RT, Skaare JU. (2009). Levels and temporal trends (1983-2003) of polybrominated diphenyl ethers and hexabromocyclododecanes in seabird eggs from north Norway. *Environ Toxicol Chem* **28**, 1096-1103.

Henny CJ, Kaiser JL, Grove RA, Johnson BL, Letcher RJ. (2009). Polybrominated diphenyl ether flame retardants in eggs may reduce reproductive success of ospreys in Oregon and Washington, USA. *Ecotoxicology* **18**, 802-813.

Hiebl J, Vetter W. (2007). Detection of hexabromocyclododecane and its metabolite pentabromocyclododecene in chicken egg and fish from the official food control. *J Agric Food Chem* **55**, 3319-3324.

Hites RA. (2004). Polybrominated diphenyl ethers in the environment and in people: a meta-analysis of concentrations. *Environ Sci Technol* **38**, 945-956.

Hong JY, Yang CS. (1997). Genetic polymorphism of cytochrome P450 as a biomarker of susceptibility to environmental toxicity. *Environ Health Perspect* **105 Suppl 4**, 759-762.

Horn S, Heuer H. (2009). Thyroid hormone action during brain development: More questions than answers. *Mol Cell Endocrinol* doi:10.1016/j.mce.2009.09.008 [Online 16 September 2009].

Houde M, Pacepavicius G, Darling C, Fair PA, Alaee M, Bossart GD, Solomon KR, Letcher RJ, Bergman Å, Marsh G, Muir DC. (2009). Polybrominated diphenyl ethers and their hydroxylated analogs in plasma of bottlenose dolphins (*Tursiops truncatus*) from the United States East Coast. *Environ Toxicol Chem* **28**, 2061-2068.

Hovander L, Malmberg T, Athanasiadou M, Athanassiadis I, Rahm S, Bergman Å, Wehler EK. (2002). Identification of hydroxylated PCB metabolites and other phenolic halogenated pollutants in human blood plasma. *Arch Environ Contam Toxicol* **42**, 105-117.

Howard AS, Fitzpatrick R, Pessah IN, Kostyniak P, Lein PJ. (2003). Polychlorinated biphenyls induce caspase-dependent cell death in cultured embryonic rat hippocampal but not cortical neurons via activation of the ryanodine receptor. *Toxicol Appl Pharmacol* **190**, 72–86.

Hu J, Liang Y, Chen M, Wang X. (2009). Assessing the toxicity of TBBPA and HBCD by zebrafish embryo toxicity assay and biomarker analysis. *Environ Toxicol* **24**, 334-342.

Hu XZ, Xu Y, Hu DC, Hui Y, Yang FX. (2007). Apoptosis induction on human hepatoma cells Hep G2 of decabrominated diphenyl ether (PBDE-209). *Toxicol Lett* **171**, 19-28.

Huang SC, Giordano G, Costa LG. (2009). Comparative cytotoxicity and intra-cellular accumulation of five polybrominated diphenyl ether (PBDE) congeners in mouse cerebellar granule neurons. *Toxicol Sci* doi:10.1093/toxsci/kfp296 [Online 9 December 2009].

Hundt K, Martin D, Hammer E, Jonas U, Kindermann MK, Schauer F. (2000). Transformation of triclosan by *Trametes versicolor* and *Pycnoporus cinnabarinus*. *Appl Environ Microbiol* **66**, 4157-4160.

Huwe J, Hakk H, Lorentzsen M. (2007). Bioavailability and mass balance studies of a commercial pentabromodiphenyl ether mixture in male Sprague-Dawley rats. *Chemosphere* **66**, 259-266.

Huwe JK, Smith DJ. (2007). Accumulation, whole-body depletion, and debromination of decabromodiphenyl ether in male sprague-dawley rats following dietary exposure. *Environ Sci Technol* **41**, 2371-2377.

Ikonomou MG, Rayne S, Addison RF. (2002). Exponential increases of the brominated flame retardants, polybrominated diphenyl ethers, in the Canadian Arctic from 1981 to 2000. *Environ Sci Technol* **36**, 1886-1892.

Inglefield JR, Shafer TJ. (2000). Polychlorinated biphenyl stimulation of Ca^{2+} oscillations in developing neocortical cells: a role for excitatory transmitters and L-type voltage-sensitive Ca^{2+} channels. *J Pharmacol Exp Ther* **295**:105–113.

Inoue K, Harada K, Takenaka K, Uehara S, Kono M, Shimizu T, Takasuga T, Senthilkumar K, Yamashita F, Koizumi A. (2006). Levels and concentration ratios of polychlorinated biphenyls and polybrominated diphenyl ethers in serum and breast milk in Japanese mothers. *Environ Health Perspect* **114**, 1179-1185.

Jakobsson K, Thuresson K, Rylander L, Sjödin A, Hagmar L, Bergman Å. (2002). Exposure to polybrominated diphenyl ethers and tetrabromobisphenol A among computer technicians. *Chemosphere* **46**, 709-716.

Janák K, Covaci A, Voorspoels S, Becher G. (2005). Hexabromocyclododecane in marine species from the Western Scheldt Estuary: diastereoisomer- and enantiomer-specific accumulation. *Environ Sci Technol* **39**, 1987-1994.

JECFA. (2005). Joint FAO/WHO Expert Committee on Food Additives, Sixty-fourth Meeting, Rome, 8–17 February 2005: Summary and Conclusions. Available: http://www.who.int/ipcs/food/jecfa/summaries/en/summary_report_64_final.pdf [accessed 20 November 2009].

Jensen V, Kaiser KMM, Borchardt T, Adelmann G, Rozov A, Burnashev N, Brix C, Frotscher M, Andersen P, Hvalby Ø, Sakmann B, Seeburg PH, Sprengel R. (2003). A juvenile form of postsynaptic hippocampal long-term potentiation in mice deficient for the AMPA receptor subunit GluR-A. *J Physiol* **553**, 843–856.

Jenssen BM, Sørmo EG, Bæk K, Bytingsvik J, Gaustad H, Ruus A, Skaare JU. (2007). Brominated flame retardants in North-East Atlantic marine ecosystems. *Environ Health Perspect* **115 Suppl 1**, 35-41.

Johansson N, Viberg H, Fredriksson A, Eriksson P. (2008). Neonatal exposure to deca-brominated diphenyl ether (PBDE 209) causes dose-response changes in spontaneous behaviour and cholinergic susceptibility in adult mice. *Neurotoxicology* **29**, 911-919.

Johnson-Restrepo B, Adams DH, Kannan K. (2008). Tetrabromobisphenol A (TBBPA) and hexabromocyclododecanes (HBCDs) in tissues of humans, dolphins, and sharks from the United States. *Chemosphere* **70**, 1935-1944.

Johnson-Restrepo B, Kannan K. (2009). An assessment of sources and pathways of human exposure to polybrominated diphenyl ethers in the United States. *Chemosphere* **76**, 542-548.

Johnson-Restrepo B, Kannan K, Addink R, Adams DH. (2005a). Polybrominated diphenyl ethers and polychlorinated biphenyls in a marine foodweb of coastal Florida. *Environ Sci Technol* **39**, 8243-8250.

Johnson-Restrepo B, Kannan K, Rapaport DP, Rodan BD. (2005b). Polybrominated diphenyl ethers and polychlorinated biphenyls in human adipose tissue from New York. *Environ Sci Technol* **39**, 5177-5182.

Jones-Otazo HA, Clarke JP, Diamond ML, Archbold JA, Ferguson G, Harner T, Richardson GM, Ryan JJ, Wilford B. (2005). Is house dust the missing exposure pathway for PBDEs? An analysis of the urban fate and human exposure to PBDEs. *Environ Sci Technol* **39**, 5121-5130.

Julander A, Karlsson M, Hagström K, Ohlson CG, Engwall M, Bryngelsson IL, Westberg H, van Bavel B. (2005). Polybrominated diphenyl ethers--plasma levels and thyroid status of workers at an electronic recycling facility. *Int Arch Occup Environ Health* **78**, 584-592.

Kajiwara N, Noma Y, Takigami H. (2008). Photolysis studies of technical decabromodiphenyl ether (DecaBDE) and ethane (DeBDethane) in plastics under natural sunlight. *Environ Sci Technol* **42**, 4404-4409.

Kakimoto K, Akutsu K, Konishi Y, Tanaka Y. (2008). Time trend of hexabromocyclododecane in the breast milk of Japanese women. *Chemosphere* **71**, 1110-1114.

Kammann U, Vobach M, Wosniok W. (2006). Toxic effects of brominated indoles and phenols on zebrafish embryos. *Arch Environ Contam Toxicol* **51**, 97-102.

Kang JH, Park IS, Oh WY, Lim HK, Wang SY, Lee SY, Choi KH, Kim JI, Jung SY, Suh CK, Kim DS. (2004). Inhibition of Aroclor 1254-induced depletion of stored calcium prevents the cell death in catecholaminergic cells. *Toxicology* **200**, 93–101.

Karlsson M, Julander A, van Bavel B, Hardell L. (2007). Levels of brominated flame retardants in blood in relation to levels in household air and dust. *Environ Int* **33**, 62-69.

Kelly BC, Ikonomou MG, Blair JD, Gobas FAPC. (2008). Hydroxylated and methoxylated polybrominated diphenyl ethers in a Canadian Arctic marine food web. *Environ Sci Technol* **42**, 7069-7077.

Kierkegaard A, Asplund L, de Wit CA, McLachlan MS, Thomas GO, Sweetman AJ, Jones KC. (2007). Fate of higher brominated PBDEs in lactating cows. *Environ Sci Technol* **41**, 417-423.

Kierkegaard A, Balk L, Tjärnlund U, de Wit CA, Jansson B. (1999). Dietary uptake and biological effects of decabromodiphenyl ether in rainbow trout (*Oncorhynchus mykiss*). *Environ Sci Technol* **33**, 1612–1617.

Kierkegaard A, Bignert A, Sellström U, Olsson M, Asplund L, Jansson B, de Wit CA. (2004). Polybrominated diphenyl ethers (PBDEs) and their methoxylated derivatives in pike from Swedish waters with emphasis on temporal trends, 1967-2000. *Environ Pollut* **130**, 187-198.

Kim J, Ghosh S, Nunziato DA, Pitt GS. (2004). Identification of the components controlling inactivation of voltage-gated Ca^{2+} channels. *Neuron* **41**, 745-754.

Kimura-Kuroda J, Nagata I, Kuroda Y. (2007). Disrupting effects of hydroxy-polychlorinated biphenyl (PCB) congeners on neuronal development of cerebellar Purkinje cells: a possible causal factor for developmental brain disorders? *Chemosphere* **67**, S412-S420.

Knutsen HK, Kvalem HE, Thomsen C, Frøshaug M, Haugen M, Becher G, Alexander J, Meltzer HM. (2008). Dietary exposure to brominated flame retardants correlates with male blood levels in a selected group of Norwegians with a wide range of seafood consumption. *Mol Nutr Food Res* **52**, 217-227.

Kodavanti PRS. (2005). Neurotoxicity of persistent organic pollutants: possible mode(s) of action and further considerations. *Dose Response* **3**, 273–305.

Kodavanti PRS, Derr-Yellin EC. (2002). Differential effects of polybrominated diphenyl ethers and polychlorinated biphenyls on [^{3}H]arachidonic acid release in rat cerebellar granule neurons. *Toxicol Sci* **68**, 451-457.

Kodavanti PRS, Shafer TJ, Ward TR, Mundy WR, Freudenrich T, Harry GJ, Tilson HA. (1994). Differential effects of polychlorinated biphenyl congeners on phosphoinositide hydrolysis and protein kinase C translocation in rat cerebellar granule cells. *Brain Res* **662**, 75-82.

Kodavanti PRS, Shin DS, Tilson HA, Harry GJ. (1993). Comparative effects of 2 polychlorinated biphenyl congeners on calcium homeostasis in rat cerebellar granule cells. *Toxicol Appl Pharmacol* **123**, 97–106.

Kodavanti PRS, Ward TR. (2005). Differential effects of commercial polybrominated diphenyl ether and polychlorinated biphenyl mixtures on intracellular signaling in rat brain *in vitro*. *Toxicol Sci* **85**, 952-962.

Kodavanti PRS, Ward TR, Ludewig G, Robertson LW, Birnbaum LS. (2005). Polybrominated diphenyl ether (PBDE) effects in rat neuronal cultures: ^{14}C-PBDE accumulation, biological effects, and structure-activity relationships. *Toxicol Sci* **88**, 181-192.

Kojima H, Takeuchi S, Uramaru N, Sugihara K, Yoshida T, Kitamura S. (2009). Nuclear hormone receptor activity of polybrominated diphenyl ethers and their hydroxylated and methoxylated metabolites in transactivation assays using Chinese hamster ovary cells. *Environ Health Perspect* **117**, 1210-1218.

Köppen R, Becker R, Jung C, Nehls I. (2008). On the thermally induced isomerisation of hexabromocyclododecane stereoisomers. *Chemosphere* **71**, 656-662.

Kuiper RV, Bergman Å, Vos JG, van den Berg M. (2004). Some polybrominated diphenyl ether (PBDE) flame retardants with wide environmental distribution inhibit TCDD-induced EROD activity in primary cultured carp (*Cyprinus carpio*) hepatocytes. *Aquat Toxicol* **68**, 129-139.

Kuiper RV, Murk AJ, Leonards PEG, Grinwis GCM, van den Berg M, Vos JG. (2006). *In vivo* and *in vitro* Ah-receptor activation by commercial and fractionated pentabromodiphenylether using zebrafish (*Danio rerio*) and the DR-CALUX assay. *Aquat Toxicol* **79**, 366-375.

Kuriyama SN, Talsness CE, Grote K, Chahoud I. (2005). Developmental exposure to low dose PBDE 99: effects on male fertility and neurobehavior in rat offspring. *Environ Health Perspect* **113**, 149-154.

Kutsuwada T, Kashiwabuchi N, Mori H, Sakimura K, Kushiya E, Araki K, Meguro H, Masaki H, Kumanishi T, Arakawa M, Mishina M. (1992). Molecular diversity of the NMDA receptor channel. *Nature* **358**, 36–41.

Lacorte S, Ikonomou MG. (2009). Occurrence and congener specific profiles of polybrominated diphenyl ethers and their hydroxylated and methoxylated derivatives in breast milk from Catalonia. *Chemosphere* **74**, 412-420.

La Guardia MJ, Hale RC, Harvey E. (2006). Detailed polybrominated diphenyl ether (PBDE) congener composition of the widely used penta-, octa-, and deca-PBDE technical flame-retardant mixtures. *Environ Sci Technol* **40**, 6247-6254.

Lam JCW, Lau RKF, Murphy MB, Lam PKS. (2009). Temporal trends of hexabromocyclododecanes (HBCDs) and polybrominated diphenyl ethers (PBDEs) and detection of two novel flame retardants in marine mammals from Hong Kong, South China. *Environ Sci Technol* **43**, 6944-6949.

Laude AJ, Simpson AWM. (2009). Compartmentalized signalling: Ca^{2+} compartments, microdomains and the many facets of Ca^{2+} signalling. *Febs J* **276**, 1800-1816.

Law RJ, Alaee M, Allchin CR, Boon JP, Lebeuf M, Lepom P, Stern GA. (2003). Levels and trends of polybrominated diphenylethers and other brominated flame retardants in wildlife. *Environ Int* **29**, 757-770.

Law RJ, Allchin CR, de Boer J, Covaci A, Herzke D, Lepom P, Morris S, Tronczynski J, de Wit CA. (2006a). Levels and trends of brominated flame retardants in the European environment. *Chemosphere* **64**, 187-208.

Law RJ, Bersuder P, Allchin CR, Barry J. (2006b). Levels of the flame retardants hexabromocyclododecane and tetrabromobisphenol A in the blubber of harbor porpoises (*Phocoena phocoena*) stranded or bycaught in the U.K., with evidence for an increase in HBCD concentrations in recent years. *Environ Sci Technol* **40**, 2177-2183.

Law RJ, Bersuder P, Barry J, Wilford BH, Allchin CR, Jepson PD. (2008a). A significant downturn in levels of hexabromocyclododecane in the blubber of harbor porpoises (*Phocoena phocoena*) stranded or bycaught in the UK: an update to 2006. *Environ Sci Technol* **42**, 9104-9109.

Law RJ, Herzke D, Harrad S, Morris S, Bersuder P, Allchin CR. (2008b). Levels and trends of HBCD and BDEs in the European and Asian environments, with some information for other BFRs. *Chemosphere* **73**, 223-241.

Law K, Palace VP, Halldorson T, Danell R, Wautier K, Evans B, Alaee M, Marvin C, Tomy GT. (2006c). Dietary accumulation of hexabromocyclododecane diastereoisomers in juvenile rainbow trout (*Oncorhynchus mykiss*) I: bioaccumulation parameters and evidence of bioisomerization. *Environ Toxicol Chem* **25**, 1757-1761.

Lee TS, Karl R, Moosmang S, Lenhardt P, Klugbauer N, Hofmann F, Kleppisch T, Welling A. (2006). Calmodulin kinase II is involved in voltage-dependent facilitation of the L-type $Ca_{v1.2}$ calcium channel: Identification of the phosphorylation sites. *J Biol Chem* **281**, 25560-25567.

Legler J. (2008). New insights into the endocrine disrupting effects of brominated flame retardants. *Chemosphere* **73**, 216-222.

Lema SC, Dickey JT, Schultz IR, Swanson P. (2008). Dietary exposure to 2,2',4,4'-tetrabromodiphenyl ether (PBDE-47) alters thyroid status and thyroid hormone-regulated gene transcription in the pituitary and brain. *Environ Health Perspect* **116**, 1694-1699.

Leonard JL. (2008). Non-genomic actions of thyroid hormone in brain development. *Steroids* **73**, 1008-1012.

Letcher RJ, Gebbink WA, Sonne C, Born EW, McKinney MA, Dietz R. (2009). Bioaccumulation and biotransformation of brominated and chlorinated contaminants and their metabolites in ringed seals (*Pusa hispida*) and polar bears (*Ursus maritimus*) from East Greenland. *Environ Int* **35**, 1118-1124.

Lilienthal H, Hack A, Roth-Härer A, Grande SW, Talsness CE. (2006). Effects of developmental exposure to 2,2 ,4,4 ,5-pentabromodiphenyl ether (PBDE-99) on sex steroids, sexual development, and sexually dimorphic behavior in rats. *Environ Health Perspect* **114**, 194-201.

Lilienthal H, van der Ven LTM, Piersma AH, Vos JG. (2009). Effects of the brominated flame retardant hexabromocyclododecane (HBCD) on dopamine-dependent behavior and brainstem auditory evoked potentials in a one-generation reproduction study in Wistar rats. *Toxicol Lett* **185**, 63-72.

Lind Y, Darnerud PO, Atuma S, Aune M, Becker W, Bjerselius R, Cnattingius S, Glynn A. (2003). Polybrominated diphenyl ethers in breast milk from Uppsala County, Sweden. *Environ Res* **93**, 186-194.

Liu H, Felix R, Gurnett CA, De Waard M, Witcher DR, Campbell KP. (1996). Expression and subunit interaction of voltage-dependent Ca^{2+} channels in PC12 cells. *J Neurosci* **16**, 7557-7565.

Llansola M, Erceg S, Monfort P, Montoliu C, Felipo V. (2007). Prenatal exposure to polybrominated diphenylether 99 enhances the function of the glutamate-nitric oxide-cGMP pathway in brain *in vivo* and in cultured neurons. *Eur J Neurosci* **25**, 373-379.

Llansola M, Hernandez-Viadel M, Erceg S, Montoliu C, Felipo V. (2009). Increasing the function of the glutamate-nitric oxide-cyclic guanosine monophosphate pathway increases the ability to learn a Y-maze task. *J Neurosci Res* **87**, 2351-2355.

Lorber M. (2008). Exposure of Americans to polybrominated diphenyl ethers. *J Expo Sci Environ Epidemiol* **18**, 2-19.

Losada S, Roach A, Roosens L, Santos FJ, Galceran MT, Vetter W, Neels H, Covaci A. (2009). Biomagnification of anthropogenic and naturally-produced organobrominated compounds in a marine food web from Sydney Harbour, Australia. *Environ Int* **35**, 1142-1149

Lynch MA. (2004). Long-term potentiation and memory. *Physiol Rev* **84**, 87-136.

Machado JD, Camacho M, Alvarez J, Borges R. (2009). On the role of intravesicular calcium in the motion and exocytosis of secretory organelles. *Commun Integr Biol* **2**, 71-73.

Mack V, Burnashev N, Kaiser KMM, Rozov A, Jensen V, Hvalby Ø, Seeburg PH, Sakmann B, Sprengel R. (2001). Conditional restoration of hippocampal synaptic potentiation in Glur-A-deficient mice. *Science* **292**, 2501–2504.

Madia F, Giordano G, Fattori V, Vitalone A, Branchi I, Capone F, Costa LG. (2004). Differential *in vitro* neurotoxicity of the flame retardant PBDE-99 and of the PCB Aroclor 1254 in human astrocytoma cells. *Toxicol Lett* **154**, 11-21.

Maervoet J, Covaci A, Schepens P, Sandau CD, Letcher RJ. (2004). A reassessment of the nomenclature of polychlorinated biphenyl (PCB) metabolites. *Environ Health Perspect* **112**, 291-294.

Magi S, Castaldo P, Carrieri G, Scorziello A, Di Renzo G, Amoroso S. (2005). Involvement of Na^{+}-Ca^{2+} exchanger in intracellular Ca^{2+} increase and neuronal injury induced by polychlorinated biphenyls in human neuroblastoma SH-SY5Y cells. *J Pharmacol Exp Ther* **315**, 291–296.

Magnani E, Bettini E. (2000). Resazurin detection of energy metabolism changes in serum-starved PC12 cells and of neuroprotective agent effect. *Brain Res Brain Res Protoc* **5**, 266-272.

Main KM, Kiviranta H, Virtanen HE, Sundqvist E, Tuomisto JT, Tuomisto J, Vartiainen T, Skakkebæk NE, Toppari J. (2007). Flame retardants in placenta and breast milk and cryptorchidism in newborn boys. *Environ Health Perspect* **115**, 1519-1526.

Malenka RC, Nicoll RA. (1999). Long-term potentiation--a decade of progress? *Science* **285**, 1870-1874.

Malmberg T, Athanasiadou M, Marsh G, Brandt I, Bergman Å. (2005). Identification of hydroxylated polybrominated diphenyl ether metabolites in blood plasma from polybrominated diphenyl ether exposed rats. *Environ Sci Technol* **39**, 5342-5348.

Malmvärn A, Marsh G, Kautsky L, Athanasiadou M, Bergman Å, Asplund L. (2005). Hydroxylated and methoxylated brominated diphenyl ethers in the red algae *Ceramium tenuicorne* and blue mussels from the Baltic Sea. *Environ Sci Technol* **39**, 2990-2997.

Malmvärn A, Zebühr Y, Kautsky L, Bergman Å, Asplund L. (2008). Hydroxylated and methoxylated polybrominated diphenyl ethers and polybrominated dibenzo-p-dioxins in red alga and cyanobacteria living in the Baltic Sea. *Chemosphere* **72**, 910-916.

Marchesini GR, Meimaridou A, Haasnoot W, Meulenberg E, Albertus F, Mizuguchi M, Takeuchi M, Irth H, Murk AJ. (2008). Biosensor discovery of thyroxine transport disrupting chemicals. *Toxicol Appl Pharmacol* **232**, 150-160.

Mariussen E, Fonnum F. (2003). The effect of brominated flame retardants on neurotransmitter uptake into rat brain synaptosomes and vesicles. *Neurochem Int* **43**, 533-542.

Mariussen E, Fonnum F. (2006). Neurochemical targets and behavioral effects of organohalogen compounds: an update. *Crit Rev Toxicol* **36**, 253–289.

Marsh G, Athanasiadou M, Athanassiadis I, Sandholm A. (2006). Identification of hydroxylated metabolites in 2,2',4,4'-tetrabromodiphenyl ether exposed rats. *Chemosphere* **63**, 690-697.

Marsh G, Athanasiadou M, Bergman Å, Asplund L. (2004). Identification of hydroxylated and methoxylated polybrominated diphenyl ethers in Baltic Sea salmon (*Salmo salar*) blood. *Environ Sci Technol* **38**, 10-18.

Marsh G, Hu J, Jakobsson E, Rahm S, Bergman Å. (1999). Synthesis and characterization of 32 polybrominated diphenyl ethers. *Environ Sci Technol* **33**, 3033-3037.

Martin M, Lam PKS, Richardson BJ. (2004). An Asian quandary: where have all of the PBDEs gone? *Mar Pollut Bull* **49**, 375-382.

Masson J, Sagné C, Hamon M, El Mestikawy S. (1999). Neurotransmitter transporters in the central nervous system. *Pharmacol Rev* **51**, 439-464.

Matter N, Ritz MF, Freyermuth S, Rogue P, Malviya AN. (1993). Stimulation of nuclear protein kinase C leads to phosphorylation of nuclear inositol 1,4,5-trisphosphate receptor and accelerated calcium release by inositol 1,4,5-trisphosphate from isolated rat liver nuclei. *J Biol Chem* **268**, 732-736.

Mazdai A, Dodder NG, Abernathy MP, Hites RA, Bigsby RM. (2003). Polybrominated diphenyl ethers in maternal and fetal blood samples. *Environ Health Perspect* **111**, 1249-1252.

McKinney MA, Cesh LS, Elliott JE, Williams TD, Garcelon DK, Letcher RJ. (2006a). Brominated flame retardants and halogenated phenolic compounds in North American west coast bald eaglet (*Haliaeetus leucocephalus*) plasma. *Environ Sci Technol* **40**, 6275-6281.

McKinney MA, De Guise S, Martineau D, Béland P, Arukwe A, Letcher RJ. (2006b). Biotransformation of polybrominated diphenyl ethers and polychlorinated biphenyls in beluga whale (*Delphinapterus leucas*) and rat mammalian model using an *in vitro* hepatic microsomal assay. *Aquat Toxicol* **77**, 87-97.

Meerts IATM, Assink Y, Cenijn PH, van den Berg JHJ, Weijers BM, Bergman Å, Koeman JH, Brouwer A. (2002). Placental transfer of a hydroxylated polychlorinated biphenyl and effects on fetal and maternal thyroid hormone homeostasis in the rat. *Toxicol Sci* **68**, 361-371.

Meerts IATM, Letcher RJ, Hoving S, Marsh G, Bergman Å, Lemmen JG, van der Burg B, Brouwer A. (2001). *In vitro* estrogenicity of polybrominated diphenyl ethers, hydroxylated PBDEs, and polybrominated bisphenol A compounds. *Environ Health Perspect* **109**, 399-407.

Meerts IATM, Lilienthal H, Hoving S, van den Berg JHJ, Weijers BM, Bergman Å, Koeman JH, Brouwer A. (2004). Developmental exposure to 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl (4-OH-CB107): long-term effects on brain development, behavior, and brain stem auditory evoked potentials in rats. *Toxicol Sci* **82**, 207-218.

Meerts IATM, van Zanden JJ, Luijks EAC, van Leeuwen-Bol I, Marsh G, Jakobsson E, Bergman Å, Brouwer A. (2000). Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin *in vitro*. *Toxicol Sci* **56**, 95-104.

Meijer L, Weiss J, van Velzen M, Brouwer A, Bergman Å, Sauer PJJ. (2008). Serum concentrations of neutral and phenolic organohalogens in pregnant women and some of their infants in The Netherlands. *Environ Sci Technol* **42**, 3428-3433.

Meironyté D, Norén K, Bergman Å. (1999). Analysis of polybrominated diphenyl ethers in Swedish human milk. A time-related trend study, 1972-1997. *J Toxicol Environ Health A* **58**, 329-341.

Mercado-Feliciano M, Bigsby RM. (2008a). The polybrominated diphenyl ether mixture DE-71 is mildly estrogenic. *Environ Health Perspect* **116**, 605-611.

Mercado-Feliciano M, Bigsby RM. (2008b). Hydroxylated metabolites of the polybrominated diphenyl ether mixture DE-71 are weak estrogen receptor-alpha ligands. *Environ Health Perspect* **116**, 1315-1321.

Miller MF, Chernyak SM, Batterman S, Loch-Caruso R. (2009). Polybrominated diphenyl ethers in human gestational membranes from women in southeast Michigan. *Environ Sci Technol* **43**, 3042-3046.

Missiaen L, Dode L, Vanoevelen J, Raeymaekers L, Wuytack F. (2007). Calcium in the Golgi apparatus. *Cell Calcium* **41**, 405-416.

Montie EW, Reddy CM, Gebbink WA, Touhey KE, Hahn ME, Letcher RJ. (2009). Organohalogen contaminants and metabolites in cerebrospinal fluid and cerebellum gray matter in short-beaked common dolphins and Atlantic white-sided dolphins from the western North Atlantic. *Environ Pollut* **157**, 2345-2358.

Moody WJ, Bosma MM. (2005). Ion channel development, spontaneous activity, and activity-dependent development in nerve and muscle cells. *Physiol Rev* **85**, 883-941.

Moors M, Cline JE, Abel J, Fritsche E. (2007). ERK-dependent and -independent pathways trigger human neural progenitor cell migration. *Toxicol Appl Pharmacol* **221**, 57-67.

Moors M, Rockel TD, Abel J, Cline JE, Gassmann K, Schreiber T, Schuwald J, Weinmann N, Fritsche E. (2009). Human neurospheres as three-dimensional cellular systems for developmental neurotoxicity testing. *Environ Health Perspect* **117**, 1131-1138.

Mörck A, Hakk H, Örn U, Klasson Wehler E. (2003). Decabromodiphenyl ether in the rat: absorption, distribution, metabolism, and excretion. *Drug Metab Dispos* **31**, 900-907.

Moreira Bastos P, Eriksson J, Bergman Å. (2009). Photochemical decomposition of dissolved hydroxylated polybrominated diphenyl ethers under various aqueous conditions. *Chemosphere* **77**, 791-797.

Moreira Bastos P, Eriksson J, Vidarson J, Bergman Å. (2008). Oxidative transformation of polybrominated diphenyl ether congeners (PBDEs) and of hydroxylated PBDEs (OH-PBDEs). *Environ Sci Pollut Res Int* **15**, 606-613.

Morris R. (1984). Developments of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods* **11**, 47-60.

Morse DC, Wehler EK, Wesseling W, Koeman JH, Brouwer A. (1996). Alterations in rat brain thyroid hormone status following pre- and postnatal exposure to polychlorinated biphenyls (Aroclor 1254). *Toxicol Appl Pharmacol* **136**, 269-279.

Mundy WR, Freudenrich TM, Crofton KM, DeVito MJ. (2004). Accumulation of PBDE-47 in primary cultures of rat neocortical cells. *Toxicol Sci* **82**, 164-169.

Myren M, Mose T, Mathiesen L, Knudsen LE. (2007). The human placenta--an alternative for studying foetal exposure. *Toxicol In Vitro* **21**, 1332-1340.

Naert C, van Peteghem C, Kupper J, Jenni L, Naegeli H. (2007). Distribution of polychlorinated biphenyls and polybrominated diphenyl ethers in birds of prey from Switzerland. *Chemosphere* **68**, 977-987.

Norén K, Meironyté D. (2000). Certain organochlorine and organobromine contaminants in Swedish human milk in perspective of past 20-30 years. *Chemosphere* **40**, 1111-1123.

Norstrom RJ, Simon M, Moisey J, Wakeford B, Weseloh DVC. (2002). Geographical distribution (2000) and temporal trends (1981-2000) of brominated diphenyl ethers in Great Lakes hewing gull eggs. *Environ Sci Technol* **36**, 4783-4789.

Nyholm JR, Norman A, Norrgren L, Haglund P, Andersson PL. (2009). Uptake and biotransformation of structurally diverse brominated flame retardants in zebrafish (*Danio rerio*) after dietary exposure. *Environ Toxicol Chem* **28**, 1035-1042.

Ogunbayo OA, Michelangeli F. (2007). The widely utilized brominated flame retardant tetrabromobisphenol a (TBBPA) is a potent inhibitor of the SERCA Ca^{2+} pump. *Biochem J* **408**, 407–415.

Olsen CM, Meussen-Elholm ETM, Holme JA, Hongslo JK. (2002). Brominated phenols: characterization of estrogen-like activity in the human breast cancer cell-line MCF-7. *Toxicol Lett* **129**, 55-63.

Olsson A, Ceder K, Bergman Å, Helander B. (2000). Nestling blood of the white-tailed sea eagle (*Haliaeetus albicilla*) as an indicator of territorial exposure to organohalogen compounds−an evaluation. *Environ Sci Technol* **34**, 2733–2740.

Örn U, Erikson L, Jakobsson E, Bergman Å. (1996). Synthesis and characterisation of polybrominated diphenyl ethers-unlabeled and radiolabelled tetra-, penta- and hexabrominated diphenyl ethers. *Acta Chem Scand* **50**, 802-807.

Örn U, Klasson-Wehler E. (1998). Metabolism of 2,2',4,4'-tetrabromodiphenyl ether in rat and mouse. *Xenobiotica* **28**, 199-211.

Orrenius S, Zhivotovsky B, Nicotera P. (2003). Regulation of cell death: The calcium-apoptosis link. *Nat Rev Mol Cell Biol* **4**, 552–565.

Pacyniak EK, Cheng X, Cunningham ML, Crofton K, Klaassen CD, Guo GL. (2007). The flame retardants, polybrominated diphenyl ethers, are pregnane X receptor activators. *Toxicol Sci* **97**, 94-102.

Palace VP, Pleskach K, Halldorson T, Danell R, Wautier K, Evans B, Alaee M, Marvin C, Tomy GT. (2008). Biotransformation enzymes and thyroid axis disruption in juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to hexabromocyclododecane diastereoisomers. *Environ Sci Technol* **42**, 1967-1972.

Parekh AB, Putney JW Jr. (2005). Store-operated calcium channels. *Physiol Rev* **85**, 757-810.

Park JS, Bergman Å, Linderholm L, Athanasiadou M, Kocan A, Petrik J, Drobna B, Trnovec T, Charles MJ, Hertz-Picciotto I. (2008). Placental transfer of polychlorinated biphenyls, their hydroxylated metabolites and pentachlorophenol in pregnant women from eastern Slovakia. *Chemosphere* **70**, 1676-1684.

Paterson SJ, Heim S, Friedman JT, Choudhury N, Benasich AA. (2006). Development of structure and function in the infant brain: implications for cognition, language and social behaviour. *Neurosci Biobehav Rev* **30**, 1087-1105.

Peck AM, Pugh RS, Moors A, Ellisor MB, Porter BJ, Becker PR, Kucklick JR. (2008). Hexabromocyclododecane in white-sided dolphins: temporal trend and stereoisomer distribution in tissues. *Environ Sci Technol* **42**, 2650-2655.

Peters AK, Nijmeijer S, Gradin K, Backlund M, Bergman Å, Poellinger L, Denison MS, van den Berg M. (2006). Interactions of polybrominated diphenyl ethers with the aryl hydrocarbon receptor pathway. *Toxicol Sci* **92**, 133-142.

Peters AK, van Londen K, Bergman Å, Bohonowych J, Denison MS, van den Berg M, Sanderson JT. (2004). Effects of polybrominated diphenyl ethers on basal and TCDD-induced ethoxyresorufin activity and cytochrome P450-1A1 expression in MCF-7, HepG2, and H4IIE cells. *Toxicol Sci* **82**, 488-496.

Petreas M, She J, Brown FR, Winkler J, Windham G, Rogers E, Zhao G, Bhatia R, Charles MJ. (2003). High body burdens of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) in California women. *Environ Health Perspect* **111**, 1175-1179.

Polder A, Gabrielsen GW, Odland JØ, Savinova TN, Tkachev A, Løken KB, Skaare JU. (2008a). Spatial and temporal changes of chlorinated pesticides, PCBs, dioxins (PCDDs/PCDFs) and brominated flame retardants in human breast milk from Northern Russia. *Sci Total Environ* **391**, 41-54.

Polder A, Thomsen C, Lindström G, Løken KB, Skaare JU. (2008b). Levels and temporal trends of chlorinated pesticides, polychlorinated biphenyls and brominated flame retardants in individual human breast milk samples from Northern and Southern Norway. *Chemosphere* **73**, 14-23.

Pop VJ, Kuijpens JL, van Baar AL, Verkerk G, van Son MM, de Vijlder JJ, Vulsma T, Wiersinga WM, Drexhage HA, Vader HL. (1999). Low maternal free thyroxine concentrations during early pregnancy are associated with impaired psychomotor development in infancy. *Clin Endocrinol (Oxf)* **50**, 149-155.

Pulkrabová J, Hrádková P, Hajšlová J, Poustka J, Nápravníková M, Poláček V. (2009). Brominated flame retardants and other organochlorine pollutants in human adipose tissue samples from the Czech Republic. *Environ Int* **35**, 63-68.

Purves D, Augustine GJ, Fitzpatrick D, Katz LC, LaManta A, McNamara JO. (1997). Plasticity in the adult nervous system. In *Neuroscience*. Sinauer, Sunderland, Massachusetts.

Qiu X, Bigsby RM, Hites RA. (2009). Hydroxylated metabolites of polybrominated diphenyl ethers in human blood samples from the United States. *Environ Health Perspect* **117**, 93-98.

Qiu X, Mercado-Feliciano M, Bigsby RM, Hites RA. (2007). Measurement of polybrominated diphenyl ethers and metabolites in mouse plasma after exposure to a commercial pentabromodiphenyl ether mixture. *Environ Health Perspect* **115**, 1052-1058.

Ramos JJ, Gómara B, Fernández MA, González MJ. (2007). A simple and fast method for the simultaneous determination of polychlorinated biphenyls and polybrominated diphenyl ethers in small volumes of human serum. *J Chromatogr A* **1152**, 124-129.

Reistad T, Fonnum F, Mariussen E. (2006). Neurotoxicity of the pentabrominated diphenyl ether mixture, DE-71, and hexabromocyclododecane (HBCD) in rat cerebellar granule cells *in vitro*. *Arch Toxicol* **80**, 785-796.

Reistad T, Mariussen E. (2005). A commercial mixture of the brominated flame retardant pentabrominated diphenyl ether (DE-71) induces respiratory burst in human neutrophil granulocytes *in vitro*. *Toxicol Sci* **87**, 57-65.

Reistad T, Mariussen E, Fonnum F. (2002). The effect of brominated flame retardants on cell death and free radical formation in cerebellar granule cells. *Organohalogen Compounds* **65**, 5–8.

Remberger M, Sternbeck J, Palm A, Kaj L, Strömberg K, Brorström-Lundén E. (2004). The environmental occurrence of hexabromocyclododecane in Sweden. *Chemosphere* **54**, 9-21.

Renwick AG, Lazarus NR. (1998). Human variability and noncancer risk assessment--an analysis of the default uncertainty factor. *Regul Toxicol Pharmacol* **27**, 3-20.

Repetto G, del Peso A, Zurita JL. (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat Protoc* **3**, 1125-1131.

Rice DC, Barone S Jr. (2000). Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environ Health Perspect* **108 Suppl 3**, 511-533.

Rice DC, Reeve EA, Herlihy A, Zoeller RT, Thompson WD, Markowski VP. (2007). Developmental delays and locomotor activity in the C57BL6/J mouse following neonatal exposure to the fully-brominated PBDE, decabromodiphenyl ether. *Neurotoxicol Teratol* **29**, 511-520.

Rice DC, Thompson WD, Reeve EA, Onos KD, Assahdollahzadeh M, Markowski VP. (2009). Behavioral changes in aging but not young mice following neonatal exposure to the polybrominated flame retardant Deca BDE. *Environ Health Perspect* **17**, 1903-1911.

Richardson VM, Staskal DF, Ross DG, Diliberto JJ, DeVito MJ, Birnbaum LS. (2008). Possible mechanisms of thyroid hormone disruption in mice by BDE-47, a major polybrominated diphenyl ether congener. *Toxicol Appl Pharmacol* **226**, 244-250.

Riu A, Cravedi JP, Debrauwer L, Garcia A, Canlet C, Jouanin I, Zalko D. (2008). Disposition and metabolic profiling of [^{14}C]-decabromodiphenyl ether in pregnant Wistar rats. *Environ Int* **34**, 318-329.

Ronisz D, Finne EF, Karlsson H, Förlin L. (2004). Effects of the brominated flame retardants hexabromocyclododecane (HBCDD), and tetrabromobisphenol A (TBBPA), on hepatic enzymes and other biomarkers in juvenile rainbow trout and feral eelpout. *Aquat Toxicol* **69**, 229-245.

Ross PS, Couillard CM, Ikonomou MG, Johannessen SC, Lebeuf M, Macdonald RW, Tomy GT. (2009). Large and growing environmental reservoirs of Deca-BDE present an emerging health risk for fish and marine mammals. *Mar Pollut Bull* **58**, 7-10.

Routti H, Letcher RJ, Chu S, van Bavel B, Gabrielsen GW. (2009). Polybrominated diphenyl ethers and their hydroxylated analogues in ringed seals (*Phoca hispida*) from Svalbard and the Baltic Sea. *Environ Sci Technol* **43**, 3494-3499.

Royland JE, Kodavanti PRS. (2008). Gene expression profiles following exposure to a developmental neurotoxicant, Aroclor 1254: pathway analysis for possible mode(s) of action. *Toxicol Appl Pharmacol* **231**, 179-196.

Royle SJ, Lagnado L. (2003). Endocytosis at the synaptic terminal. *J Physiol* **553**, 345-355.

Roze E, Meijer L, Bakker A, van Braeckel KNJA, Sauer PJJ. (2009). Prenatal exposure to organohalogens, including brominated flame retardants, influences motor, cognitive, and behavioral performance at school age. *Environ Health Perspect* **117**, 1953-1958.

Rubin LL, Staddon JM. (1999). The cell biology of the blood-brain barrier. *Annu Rev Neurosci* **22**, 11-28.

Saegusa Y, Fujimoto H, Woo GH, Inoue K, Takahashi M, Mitsumori K, Hirose M, Nishikawa A, Shibutani M. (2009). Developmental toxicity of brominated flame retardants, tetrabromobisphenol A and 1,2,5,6,9,10-hexabromocyclododecane, in rat offspring after maternal exposure from mid-gestation through lactation. *Reprod Toxicol* **28**, 456-467.

Sagerup K, Helgason LB, Polder A, Strøm H, Josefsen TD, Skåre JU, Gabrielsen GW. (2009). Persistent organic pollutants and mercury in dead and dying glaucous gulls (*Larus hyperboreus*) at Bjørnoya (Svalbard). *Sci Total Environ* **407**, 6009-6016.

Saito I, Onuki A, Seto H. (2007). Indoor organophosphate and polybrominated flame retardants in Tokyo. *Indoor Air* **17**, 28-36.

Sandanger TM, Sinotte M, Dumas P, Marchand M, Sandau CD, Pereg D, Bérubé S, Brisson J, Ayotte P. (2007). Plasma concentrations of selected organobromine compounds and polychlorinated biphenyls in postmenopausal women of Québec, Canada. *Environ Health Perspect* **115**, 1429-1434.

Sandau CD, Ayotte P, Dewailly É, Duffe J, Norstrom RJ. (2002). Pentachlorophenol and hydroxylated polychlorinated biphenyl metabolites in umbilical cord plasma of neonates from coastal populations in Québec. *Environ Health Perspect* **110**, 411-417.

Sanders JM, Burka LT, Smith CS, Black W, James R, Cunningham ML. (2005). Differential expression of *CYP1A*, *2B*, and *3A* genes in the F344 rat following exposure to a polybrominated diphenyl ether mixture or individual components. *Toxicol Sci* **88**, 127-133.

Sanders JM, Chen LJ, Lebetkin EH, Burka LT. (2006a). Metabolism and disposition of 2,2',4,4'- tetrabromodiphenyl ether following administration of single or multiple doses to rats and mice. *Xenobiotica* **36**, 103-117.

Sanders JM, Lebetkin EH, Chen LJ, Burka LT. (2006b). Disposition of 2,2',4,4',5,5'-hexabromodiphenyl ether (BDE153) and its interaction with other polybrominated diphenyl ethers (PBDEs) in rodents. *Xenobiotica* **36**, 824-837.

Sandholm A, Emanuelsson BM, Wehler EK. (2003). Bioavailability and half-life of decabromodiphenyl ether (BDE-209) in rat. *Xenobiotica* **33**, 1149-1158.

Santodomingo J, Vay L, Camacho M, Hernández-Sanmiguel E, Fonteriz RI, Lobatón CD, Montero M, Moreno A, Alvarez J. (2008). Calcium dynamics in bovine adrenal medulla chromaffin cell secretory granules. *Eur J Neurosci* **28**, 1265-1274.

Sawin S, Brodish P, Carter CS, Stanton ME, Lau C. (1998). Development of cholinergic neurons in rat brain regions: dose-dependent effects of propylthiouracil-induced hypothyroidism. *Neurotoxicol Teratol* **20**, 627-635.

Schecter A, Colacino JA, Harris TR, Shah N, Brummitt SI. (2009). A newly recognized occupational hazard for US electronic recycling facility workers: polybrominated diphenyl ethers. *J Occup Environ Med* **51**, 435-440.

Schecter A, Harris TR, Shah N, Musumba A, Päpke O. (2008). Brominated flame retardants in US food. *Mol Nutr Food Res* **52**, 266-272.

Schecter A, Johnson-Welch S, Tung KC, Harris TR, Päpke O, Rosen R. (2007). Polybrominated diphenyl ether (PBDE) levels in livers of U.S. human fetuses and newborns. *J Toxicol Environ Health A* **70**, 1-6.

Schecter A, Päpke O, Harris TR, Tung KC, Musumba A, Olson J, Birnbaum L. (2006). Polybrominated diphenyl ether (PBDE) levels in an expanded market basket survey of U.S. food and estimated PBDE dietary intake by age and sex. *Environ Health Perspect* **114**, 1515-1520.

Schecter A, Pavuk M, Päpke O, Ryan JJ, Birnbaum L, Rosen R. (2003). Polybrominated diphenyl ethers (PBDEs) in U.S. mothers' milk. *Environ Health Perspect* **111**, 1723-1729.

Schenker U, Soltermann F, Scheringer M, Hungerbühler K. (2008). Modeling the environmental fate of polybrominated diphenyl ethers (PBDEs): the importance of photolysis for the formation of lighter PBDEs. *Environ Sci Technol* **42**, 9244-9249.

Schreiber T, Gassmann K, Götz C, Hübenthal U, Moors M, Krause G, Merk HF, Crofton KM, Nguyen NH, Scanlan TS, Abel J, Rose CR, Fritsche E. (2009). Polybrominated diphenyl ethers induce developmental neurotoxicity in a human *in vitro* model: evidence for endocrine disruption. *Environ Health Perspect* doi:10.1289/ehp.0901435 [Online 7 December 2009].

Schriks M, Vrabie CM, Gutleb AC, Faassen EJ, Rietjens IMCM, Murk AJ. (2006a). T-screen to quantify functional potentiating, antagonistic and thyroid hormone-like activities of poly halogenated aromatic hydrocarbons (PHAHs). *Toxicol In Vitro* **20**, 490-498.

Schriks M, Zvinavashe E, Furlow JD, Murk AJ. (2006b). Disruption of thyroid hormone-mediated *Xenopus laevis* tadpole tail tip regression by hexabromocyclododecane (HBCD) and 2,2',3,3',4,4',5,5',6-nona brominated diphenyl ether (BDE206). *Chemosphere* **65**, 1904-1908.

Schubert D, Klier FG. (1977). Storage and release of acetylcholine by a clonal cell line. *Proc Natl Acad Sci U S A* **74**, 5184-5188.

Schubert D, LaCorbiere M, Klier FG, Steinbach JH. (1980). The modulation of neurotransmitter synthesis by steroid hormones and insulin. *Brain Res* **190**, 67-79.

Seegal RF. (1996). Epidemiological and laboratory evidence of PCB-induced neurotoxicity. *Crit Rev Toxicol* **26**, 709-737.

Seelbach M, Chen L, Powell A, Jung Choi Y, Zhang B, Hennig B, Toborek M. (2009). Polychlorinated biphenyls disrupt the blood-brain barrier integrity and promote brain metastasis formation. *Environ Health Perspect* doi:10.1289/ehp.0901334 [Online 28 October 2009].

Sellström U, Bignert A, Kierkegaard A, Häggberg L, de Wit CA, Olsson M, Jansson B. (2003). Temporal trend studies on tetra- and pentabrominated diphenyl ethers and hexabromocyclododecane in guillemot egg from the Baltic Sea. *Environ Sci Technol* **37**, 5496-5501.

Shafer TJ, Atchison WD. (1991). Transmitter, ion channel and receptor properties of pheochromocytoma (PC12) cells: a model for neurotoxicological studies. *Neurotoxicology* **12**, 473-492.

Shafer TJ, Mundy WR, Tilson HA, Kodavanti PRS. (1996). Disruption of inositol phosphate accumulation in cerebellar granule cells by polychlorinated biphenyls: a consequence of altered Ca^{2+} homeostasis. *Toxicol Appl Pharmacol* **141**, 448-455.

Shao J, Eckert ML, Lee LEJ, Gallagher EP. (2008a). Comparative oxygen radical formation and toxicity of BDE-47 in rainbow trout cell lines. *Mar Environ Res* **66**, 7-8.

Shao J, White CC, Dabrowski MJ, Kavanagh TJ, Eckert ML, Gallagher EP. (2008b). The role of mitochondrial and oxidative injury in BDE-47 toxicity to human fetal liver hematopoietic stem cells. *Toxicol Sci* **101**, 81-90.

Shaw SD, Berger ML, Brenner D, Kannan K, Lohmann N, Päpke O. (2009). Bioaccumulation of polybrominated diphenyl ethers and hexabromocyclododecane in the northwest Atlantic marine food web. *Sci Total Environ* **407**, 3323-3329.

She J, Holden A, Sharp M, Tanner M, Williams-Derry C, Hooper K. (2007). Polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) in breast milk from the Pacific Northwest. *Chemosphere* **67**, S307-S317.

Shi ZX, Wu YN, Li JG, Zhao YF, Feng JF. (2009). Dietary exposure assessment of Chinese adults and nursing infants to tetrabromobisphenol-A and hexabromocyclododecanes: occurrence measurements in foods and human milk. *Environ Sci Technol* **43**, 4314-4319.

Shimokawa N, Miyazaki W, Iwasaki T, Koibuchi N. (2006). Low dose hydroxylated PCB induces c-Jun expression in PC12 cells. *Neurotoxicology* **27**, 176-183.

Sjödin A, Hagmar L, Klasson-Wehler E, Kronholm-Diab K, Jakobsson E, Bergman Å. (1999). Flame retardant exposure: polybrominated diphenyl ethers in blood from Swedish workers. *Environ Health Perspect* **107**, 643-648.

Sjödin A, Jones RS, Focant JF, Lapeza C, Wang RY, McGahee EE 3rd, Zhang Y, Turner WE, Slazyk B, Needham LL, Patterson DG Jr. (2004). Retrospective time-trend study of polybrominated diphenyl ether and polybrominated and polychlorinated biphenyl levels in human serum from the United States. *Environ Health Perspect* **112**, 654-658.

Sjödin A, Wong LY, Jones RS, Park A, Zhang Y, Hodge C, Dipietro E, McClure C, Turner W, Needham LL, Patterson DG Jr. (2008). Serum concentrations of polybrominated diphenyl ethers (PBDEs) and polybrominated biphenyl (PBB) in the United States population: 2003-2004. *Environ Sci Technol* **42**, 1377-1384.

Soderling TR, Derkach VA. (2000). Postsynaptic protein phosphorylation and LTP. *Trends Neurosci* **23**, 75-80.

Söderström G, Sellström U, de Wit CA, Tysklind M. (2004). Photolytic debromination of decabromodiphenyl ether (BDE 209). *Environ Sci Technol* **38**, 127-132.

Song R, Duarte TL, Almeida GM, Farmer PB, Cooke MS, Zhang W, Sheng G, Fu J, Jones GDD. (2009). Cytotoxicity and gene expression profiling of two hydroxylated polybrominated diphenyl ethers in human H295R adrenocortical carcinoma cells. *Toxicol Lett* **185**, 23-31.

Song R, He Y, Murphy MB, Yeung LWY, Yu RMK, Lam MHW, Lam PKS, Hecker M, Giesy JP, Wu RSS, Zhang W, Sheng G, Fu J. (2008). Effects of fifteen PBDE metabolites, DE71, DE79 and TBBPA on steroidogenesis in the H295R cell line. *Chemosphere* **71**, 1888-1894.

Sørmo EG, Salmer MP, Jenssen BM, Hop H, Bæk K, Kovacs KM, Lydersen C, Falk-Petersen S, Gabrielsen GW, Lie E, Skaare JU. (2006). Biomagnification of polybrominated diphenyl ether and hexabromocyclododecane flame retardants in the polar bear food chain in Svalbard, Norway. *Environ Toxicol Chem* **25**, 2502-2511.

Spitzer NC. (2006). Electrical activity in early neuronal development. *Nature* **444**, 707-712.

Stapleton HM, Alaee M, Letcher RJ, Baker JE. (2004a). Debromination of the flame retardant decabromodiphenyl ether by juvenile carp (*Cyprinus carpio*) following dietary exposure. *Environ Sci Technol* **38**, 112-119.

Stapleton HM, Allen JG, Kelly SM, Konstantinov A, Klosterhaus S, Watkins D, McClean MD, Webster TF. (2008a). Alternate and new brominated flame retardants detected in U.S. house dust. *Environ Sci Technol* **42**, 6910-6916.

Stapleton HM, Brazil B, Holbrook RD, Mitchelmore CL, Benedict R, Konstantinov A, Potter D. (2006). *In vivo* and *in vitro* debromination of decabromodiphenyl ether (BDE-209) by juvenile rainbow trout and common carp. *Environ Sci Technol* **40**, 4653-4658.

Stapleton HM, Kelly SM, Pei R, Letcher RJ, Gunsch C. (2009). Metabolism of polybrominated diphenyl ethers (PBDEs) by human hepatocytes *in vitro*. *Environ Health Perspect* **117**, 197-202.

Stapleton HM, Letcher RJ, Baker JE. (2004b). Debromination of polybrominated diphenyl ether congeners BDE-99 and BDE-183 in the intestinal tract of the common carp (*Cyprinus carpio*). *Environ Sci Technol* **38**, 1054-1061.

Stapleton HM, Letcher RJ, Li J, Baker JE. (2004c). Dietary accumulation and metabolism of polybrominated diphenyl ethers by juvenile carp (*Cyprinus carpio*). *Environ Toxicol Chem* **23**, 1939-1946.

Stapleton HM, Sjödin A, Jones RS, Niehüser S, Zhang Y, Patterson DG Jr. (2008b). Serum levels of polybrominated diphenyl ethers (PBDEs) in foam recyclers and carpet installers working in the United States. *Environ Sci Technol* **42**, 3453-3458.

Staskal DF, Diliberto JJ, Birnbaum LS. (2006a). Disposition of BDE-47 in developing mice. *Toxicol Sci* **90**, 309-316.

Staskal DF, Diliberto JJ, DeVito MJ, Birnbaum LS. (2005). Toxicokinetics of BDE-47 in female mice: effect of dose, route of exposure, and time. *Toxicol Sci* **83**, 215-223.

Staskal DF, Hakk H, Bauer D, Diliberto JJ, Birnbaum LS. (2006b). Toxicokinetics of polybrominated diphenyl ether congeners 47, 99, 100, and 153 in mice. *Toxicol Sci* **94**, 28-37.

Stoker TE, Cooper RL, Lambright CS, Wilson VS, Furr J, Gray LE. (2005). *In vivo* and *in vitro* anti-androgenic effects of DE-71, a commercial polybrominated diphenyl ether (PBDE) mixture. *Toxicol Appl Pharmacol* **207**, 78-88.

Stoker TE, Laws SC, Crofton KM, Hedge JM, Ferrell JM, Cooper RL. (2004). Assessment of DE-71, a commercial polybrominated diphenyl ether (PBDE) mixture, in the EDSP male and female pubertal protocols. *Toxicol Sci* **78**, 144-155.

Südhof TC. (2008). Neurotransmitter release. *Handb Exp Pharmacol* **148**, 1-21.

Szabo DT, Richardson VM, Ross DG, Diliberto JJ., Kodavanti PRS, Birnbaum LS. (2009). Effects of perinatal PBDE exposure on hepatic phase I, phase II, phase III, and deiodinase 1 gene expression involved in thyroid hormone metabolism in male rat pups. *Toxicol Sci* **107**, 27-39.

Szpir M. (2006). New thinking on neurodevelopment. *Environ Health Perspect* **114**, A100–A107.

Tagliaferri S, Caglieri A, Goldoni M, Pinelli S, Alinovi R, Poli D, Pellacani C, Giordano G, Mutti A, Costa LG. (2009). Low concentrations of the brominated flame retardants BDE-47 and BDE-99 induce synergistic oxidative stress-mediated neurotoxicity in human neuroblastoma cells. *Toxicol In Vitro* doi:10.1016/j.tiv.2009.08.020 [Online 29 August 2009].

Takahashi A, Camacho P, Lechleiter JD, Herman B. (1999). Measurement of intracellular calcium. *Physiol Rev* **79**, 1089-1125.

Takahashi M, Negishi T, Imamura M, Sawano E, Kuroda Y, Yoshikawa Y, Tashiro T. (2009). Alterations in gene expression of glutamate receptors and exocytosis-related factors by a hydroxylated-polychlorinated biphenyl in the developing rat brain. *Toxicology* **257**, 17-24.

Talsness CE, Kuriyama SN, Sterner-Kock A, Schnitker P, Grande SW, Shakibaei M, Andrade A, Grote K, Chahoud I. (2008). *In utero* and lactational exposures to low doses of polybrominated diphenyl ether-47 alter the reproductive system and thyroid gland of female rat offspring. *Environ Health Perspect* **116**, 308-314.

Talsness CE, Shakibaei M, Kuriyama SN, Grande SW, Sterner-Kock A, Schnitker P, de Souza C, Grote K, Chahoud I. (2005). Ultrastructural changes observed in rat ovaries following *in utero* and lactational exposure to low doses of a polybrominated flame retardant. *Toxicol Lett* **157**, 189-202.

Tanabe S. (2008). Temporal trends of brominated flame retardants in coastal waters of Japan and South China: retrospective monitoring study using archived samples from *es*-Bank, Ehime University, Japan. *Mar Pollut Bull* **57**, 267-274.

Taylor SC, Peers C. (1999). Store-operated Ca^{2+} influx and voltagegated Ca^{2+} channels coupled to exocytosis in pheochromocytoma (PC12) cells. *J Neurochem* **73**, 874–880.

Taylor SC, Shaw SM, Peers C. (2000). Mitochondrial inhibitors evoke catecholamine release from pheochromocytoma cells. *Biochem Biophys Res Commun* **273**, 17–21.

Teuten EL, Xu L, Reddy CM. (2005). Two abundant bioaccumulated halogenated compounds are natural products. *Science* **307**, 917-920.

Thomsen C, Knutsen HK, Liane VH, Frøshaug M, Kvalem HE, Haugen M, Meltzer HM, Alexander J, Becher G. (2008). Consumption of fish from a contaminated lake strongly affects the concentrations of polybrominated diphenyl ethers and hexabromocyclododecane in serum. *Mol Nutr Food Res* **52**, 228-237.

Thomsen C, Lundanes E, Becher G. (2001). Brominated flame retardants in plasma samples from three different occupational groups in Norway. *J Environ Monit* **3**, 366-370.

Thomsen C, Molander P, Daae HL, Janák K, Frøshaug M, Liane VH, Thorud S, Becher G, Dybing E. (2007). Occupational exposure to hexabromocyclododecane at an industrial plant. *Environ Sci Technol* **41**, 5210-5216.

Thuresson K, Bergman Å, Jakobsson K. (2005). Occupational exposure to commercial decabromodiphenyl ether in workers manufacturing or handling flame-retarded rubber. *Environ Sci Technol* **39**, 1980-1986.

Thuresson K, Höglund P, Hagmar L, Sjödin A, Bergman Å, Jakobsson K. (2006). Apparent half-lives of hepta- to decabrominated diphenyl ethers in human serum as determined in occupationally exposed workers. *Environ Health Perspect* **114**, 176-181.

Timme-Laragy AR, Levin ED, Di Giulio RT. (2006). Developmental and behavioral effects of embryonic exposure to the polybrominated diphenylether mixture DE-71 in the killifish (*Fundulus heteroclitus*). *Chemosphere* **62**, 1097-1104.

Toms LML, Harden F, Paepke O, Hobson P, Ryan JJ, Mueller JF. (2008). Higher accumulation of polybrominated diphenyl ethers in infants than in adults. *Environ Sci Technol* **42**, 7510-7515.

Toms LML, Sjödin A, Harden F, Hobson P, Jones R, Edenfield E, Mueller JF. (2009). Serum polybrominated diphenyl ether (PBDE) levels are higher in children (2-5 years of age) than in infants and adults. *Environ Health Perspect* **117**, 1461-1465.

Tomy GT, Budakowski W, Halldorson T, Whittle DM, Keir MJ, Marvin C, MacInnis G, Alaee M. (2004a). Biomagnification of α- and γ-hexabromocyclododecane isomers in a Lake Ontario food web. *Environ Sci Technol* **38**, 2298-2303.

Tomy GT, Palace VP, Halldorson T, Braekevelt E, Danell R, Wautier K, Evans B, Brinkworth L, Fisk AT. (2004b). Bioaccumulation, biotransformation, and biochemical effects of brominated diphenyl ethers in juvenile lake trout (*Salvelinus namaycush*). *Environ Sci Technol* **38**, 1496-1504.

Tomy GT, Pleskach K, Oswald T, Halldorson T, Helm PA, MacInnis G, Marvin CH. (2008). Enantioselective bioaccumulation of hexabromocyclododecane and congener-specific accumulation of brominated diphenyl ethers in an eastern Canadian Arctic marine food web. *Environ Sci Technol* **42**, 3634-3639.

Toyoshima C, Inesi G. (2004). Structural basis of ion pumping by Ca^{2+}-ATPase of the sarcoplasmic reticulum. *Annu Rev Biochem* **73**, 269–292.

Tseng LH, Lee CW, Pan MH, Tsai SS, Li MH, Chen JR, Lay JJ, Hsu PC. (2006). Postnatal exposure of the male mouse to 2,2',3,3',4,4',5,5',6,6'-decabrominated diphenyl ether: decreased epididymal sperm functions without alterations in DNA content and histology in testis. *Toxicology* **224**, 33-43.

Tseng LH, Li MH, Tsai SS, Lee CW, Pan MH, Yao WJ, Hsu PC. (2008). Developmental exposure to decabromodiphenyl ether (PBDE 209): effects on thyroid hormone and hepatic enzyme activity in male mouse offspring. *Chemosphere* **70**, 640-647.

Turyk ME, Persky VW, Imm P, Knobeloch L, Chatterton R Jr, Anderson HA. (2008). Hormone disruption by PBDEs in adult male sport fish consumers. *Environ Health Perspect* **116**, 1635-1641.

Ucán-Marín F, Arukwe A, Mortensen A, Gabrielsen GW, Fox GA, Letcher RJ. (2009). Recombinant transthyretin purification and competitive binding with organohalogen compounds in two gull species (*Larus argentatus* and *Larus hyperboreus*). *Toxicol Sci* **107**, 440-450.

Ueno D, Darling C, Alaee M, Pacepavicius G, Teixeira C, Campbell L, Letcher RJ, Bergman Å, Marsh G, Muir D. (2008). Hydroxylated polybrominated diphenyl ethers (OH-PBDEs) in the abiotic environment: surface water and precipitation from Ontario, Canada. *Environ Sci Technol* **42**, 1657-1664.

van Boxtel AL, Kamstra JH, Cenijn PH, Pieterse B, Wagner JM, Antink M, Krab K, van der Burg B, Marsh G, Brouwer A, Legler J. (2008). Microarray analysis reveals a mechanism of phenolic polybrominated diphenylether toxicity in zebrafish. *Environ Sci Technol* **42**, 1773-1779.

van den Berg M, Birnbaum LS, Denison M, De Vito M, Farland W, Feeley M, Fiedler H, Håkansson H, Hanberg A, Haws L, Rose M, Safe S, Schrenk D, Tohyama C, Tritscher A, Tuomisto J, Tysklind M, Walker N, Peterson RE. (2006). The 2005 World Health Organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. *Toxicol Sci* **93**, 223-241.

van der Heide LP, Kamal A, Artola A, Gispen WH, Ramakers GMJ. (2005). Insulin modulates hippocampal activity-dependent synaptic plasticity in a *N*-methyl-D-aspartate receptor and phosphatidyl-inositol-3-kinase-dependent manner. *J Neurochem* **94**, 1158-1166.

Vanderheyden V, Devogelaere B, Missiaen L, De Smedt H, Bultynck G, Parys JB. (2009). Regulation of inositol 1,4,5-trisphosphate-induced Ca^{2+} release by reversible phosphorylation and dephosphorylation. *Biochim Biophys Acta* **1793**, 959-970.

van der Ven LTM, van de Kuil T, Leonards PEG, Slob W, Cantón RF, Germer S, Visser TJ, Litens S, Håkansson H, Schrenk D, van den Berg M, Piersma AH, Vos JG, Opperhuizen A. (2008a). A 28-day oral dose toxicity study in Wistar rats enhanced to detect endocrine effects of decabromodiphenyl ether (decaBDE). *Toxicol Lett* **179**, 6-14.

van der Ven LTM, van de Kuil T, Leonards PEG, Slob W, Lilienthal H, Litens S, Herlin M, Håkansson H, Cantón RF, van den Berg M, Visser TJ, van Loveren H, Vos JG, Piersma AH. (2009). Endocrine effects of hexabromocyclododecane (HBCD) in a one-generation reproduction study in Wistar rats. *Toxicol Lett* **185**, 51-62.

van der Ven LTM, van de Kuil T, Verhoef A, Leonards PEG, Slob W, Cantón RF, Germer S, Hamers T, Visser TJ, Litens S, Håkansson H, Fery Y, Schrenk D, van den Berg M, Piersma AH, Vos JG. (2008b). A 28-day oral dose toxicity study enhanced to detect endocrine effects of a purified technical pentabromodiphenyl ether (pentaBDE) mixture in Wistar rats. *Toxicology* **245**, 109-122.

van der Ven LTM, Verhoef A, van de Kuil T, Slob W, Leonards PEG, Visser TJ, Hamers T, Herlin M, Håkansson H, Olausson H, Piersma AH, Vos JG. (2006). A 28-day oral dose toxicity study enhanced to detect endocrine effects of hexabromocyclododecane in Wistar rats. *Toxicol Sci* **94**, 281-292.

van Leeuwen SPJ, de Boer J. (2008). Brominated flame retardants in fish and shellfish - levels and contribution of fish consumption to dietary exposure of Dutch citizens to HBCD. *Mol Nutr Food Res* **52**, 194-203.

Verreault J, Gabrielsen GW, Chu S, Muir DCG, Andersen M, Hamaed A, Letcher RJ. (2005). Flame retardants and methoxylated and hydroxylated polybrominated diphenyl ethers in two Norwegian Arctic top predators: glaucous gulls and polar bears. *Environ Sci Technol* **39**, 6021-6028.

Verreault J, Gebbink WA, Gauthier LT, Gabrielsen GW, Letcher RJ. (2007). Brominated flame retardants in glaucous gulls from the Norwegian Arctic: more than just an issue of polybrominated diphenyl ethers. *Environ Sci Technol* **41**, 4925-4931.

Verreault J, Maisonneuve F, Dietz R, Sonne C, Letcher RJ. (2009). Comparative hepatic activity of xenobiotic-metabolizing enzymes and concentrations of organohalogens and their hydroxylated analogues in captive Greenland sledge dogs (*Canis familiaris*). *Environ Toxicol Chem* **28**, 162-172.

Viberg H. (2009a). Exposure to polybrominated diphenyl ethers 203 and 206 during the neonatal brain growth spurt affects proteins important for normal neurodevelopment in mice. *Toxicol Sci* **109**, 306-311.

Viberg H. (2009b). Neonatal ontogeny and neurotoxic effect of decabrominated diphenyl ether (PBDE-209) on levels of synaptophysin and tau. *Int J Dev Neurosci* **27**, 423-429.

Viberg H, Fredriksson A, Eriksson P. (2002). Neonatal exposure to the brominated flame retardant 2,2',4,4',5-pentabromodiphenyl ether causes altered susceptibility in the cholinergic transmitter system in the adult mouse. *Toxicol Sci* **67**, 104-107.

Viberg H, Fredriksson A, Eriksson P. (2003a). Neonatal exposure to polybrominated diphenyl ether (PBDE-153) disrupts spontaneous behaviour, impairs learning and memory, and decreases hippocampal cholinergic receptors in adult mice. *Toxicol Appl Pharmacol* **192**, 95-106.

Viberg H, Fredriksson A, Eriksson P. (2007). Changes in spontaneous behaviour and altered response to nicotine in the adult rat, after neonatal exposure to the brominated flame retardant, decabrominated diphenyl ether (PBDE-209). *Neurotoxicology* **28**, 136-142.

Viberg H, Fredriksson A, Jakobsson E, Örn U, Eriksson P. (2003b). Neurobehavioral derangements in adult mice receiving decabrominated diphenyl ether (PBDE-209) during a defined period of neonatal brain development. *Toxicol Sci* **76**, 112-120.

Viberg H, Johansson N, Fredriksson A, Eriksson J, Marsh G, Eriksson P. (2006). Neonatal exposure to higher brominated diphenyl ethers, hepta-, octa-, or nonabromodiphenyl ether, impairs spontaneous behavior and learning and memory functions of adult mice. *Toxicol Sci* **92**, 211-218.

Viberg H, Mundy W, Eriksson P. (2008). Neonatal exposure to decabrominated diphenyl ether (PBDE-209) results in changes in BDNF, CaMKII and GAP-43, biochemical substrates of neuronal survival, growth, and synaptogenesis. *Neurotoxicology* **29**, 152-159.

Voie ØA, Fonnum F. (1998). *Ortho* substituted polychlorinated biphenyls elevate intracellular [Ca^{2+}] in human granulocytes. *Environ Toxicol Pharmacol* **5**, 105–112.

Voie ØA, Fonnum F. (2000). Effect of polychlorinated biphenyls on production of reactive oxygen species (ROS) in rat synaptosomes. *Arch Toxicol* **73**, 588-593.

von Meyerinck L, Hufnagel B, Schmoldt A, Benthe HF. (1990). Induction of rat liver microsomal cytochrome P-450 by the pentabromo diphenyl ether Bromkal 70 and half-lives of its components in the adipose tissue. *Toxicology* **61**, 259-274.

Voorspoels S, Covaci A, Lepom P, Jaspers VLB, Schepens P. (2006). Levels and distribution of polybrominated diphenyl ethers in various tissues of birds of prey. *Environ Pollut* **144**, 218-227.

Wahl M, Lahni B, Guenther R, Kuch B, Yang L, Straehle U, Strack S, Weiss C. (2008). A technical mixture of 2,2',4,4'-tetrabromo diphenyl ether (BDE47) and brominated furans triggers aryl hydrocarbon receptor (AhR) mediated gene expression and toxicity. *Chemosphere* **73**, 209-215.

Wan Y, Hu J, Zhang K, An L. (2008). Trophodynamics of polybrominated diphenyl ethers in the marine food web of Bohai Bay, North China. *Environ Sci Technol* **42**, 1078-1083.

Wan Y, Wiseman S, Chang H, Zhang X, Jones PD, Hecker M, Kannan K, Tanabe S, Hu J, Lam MHW, Giesy JP. (2009). Origin of hydroxylated brominated diphenyl ethers: natural compounds or man-made flame retardants. *Environ Sci Technol* **43**, 7536-7542.

Wang JZ, Liu F. (2008). Microtubule-associated protein tau in development, degeneration and protection of neurons. *Prog Neurobiol* **85**, 148-175.

Wayman GA, Lee YS, Tokumitsu H, Silva A, Soderling TR. (2008). Calmodulin-kinases: modulators of neuronal development and plasticity. *Neuron* **59**, 914-931.

Weiss J, Wallin E, Axmon A, Jönsson BAG, Åkesson H, Janák K, Hagmar L, Bergman Å. (2006). Hydroxy-PCBs, PBDEs, and HBCDDs in serum from an elderly population of Swedish fishermen's wives and associations with bone density. *Environ Sci Technol* **40**, 6282-6289.

Westerink RHS. (2006). Targeting exocytosis: ins and outs of the modulation of quantal dopamine release. *CNS Neurol Disord Drug Targets* **5**, 57-77.

Westerink RHS, de Groot A, Vijverberg HPM. (2000). Heterogeneity of catecholamine-containing vesicles in PC12 cells. *Biochem Biophys Res Commun* **270**, 625-630.

Westerink RHS, Ewing AG. (2008). The PC12 cell as model for neurosecretion. *Acta Physiol (Oxf)* **192**, 273-285.

Westerink RHS, Rook MB, Beekwilder JP, Wadman WJ. (2006). Dual role of calbindin-D_{28K} in vesicular catecholamine release from mouse chromaffin cells. *J Neurochem* **99**, 628-640.

Westerink RHS, Vijverberg HPM. (2002). Ca^{2+}-independent vesicular catecholamine release in PC12 cells by nanomolar concentrations of Pb^{2+}. *J Neurochem* **80**, 861-873.

Wilford BH, Harner T, Zhu J, Shoeib M, Jones KC. (2004). Passive sampling survey of polybrominated diphenyl ether flame retardants in indoor and outdoor air in Ottawa, Canada: implications for sources and exposure. *Environ Sci Technol* **38**, 5312-5318.

Wilford BH, Shoeib M, Harner T, Zhu J, Jones KC. (2005). Polybrominated diphenyl ethers in indoor dust in Ottawa, Canada: implications for sources and exposure. *Environ Sci Technol* **39**, 7027-7035.

Winkler H. (1993). The adrenal chromaffin granule: a model for large dense core vesicles of endocrine and nervous tissue. *J Anat* **183 (Pt 2)**, 237-252.

Winneke G, Walkowiak J, Lilienthal H. (2002). PCB-induced neurodevelopmental toxicity in human infants and its potential mediation by endocrine dysfunction. *Toxicology* **181–182**, 161–165.

Wolkers H, van Bavel B, Derocher AE, Wiig Ø, Kovacs KM, Lydersen C, Lindström G. (2004). Congener-specific accumulation and food chain transfer of polybrominated diphenyl ethers in two arctic food chains. *Environ Sci Technol* **38**, 1667-1674.

Wollenberger L, Dinan L, Breitholtz M. (2005). Brominated flame retardants: activities in a crustacean development test and in an ecdysteroid screening assay. *Environ Toxicol Chem* **24**, 400-407.

Wong PW, Brackney WR, Pessah IN. (1997). *Ortho*-substituted polychlorinated biphenyls alter microsomal calcium transport by direct interaction with ryanodine receptors of mammalian brain. *J Biol Chem* **272**, 15145–15153.

Wong PW, Garcia EF, Pessah IN. (2001). *Ortho*-substituted PCB95 alters intracellular calcium signaling and causes cellular acidification in PC12 cells by an immunophilin-dependent mechanism. *J Neurochem* **76**, 450-463.

Xian Q, Ramu K, Isobe T, Sudaryanto A, Liu X, Gao Z, Takahashi S, Yu H, Tanabe S. (2008). Levels and body distribution of polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecanes (HBCDs) in freshwater fishes from the Yangtze River, China. *Chemosphere* **71**, 268-276.

Xing T, Chen L, Tao Y, Wang M, Chen J, Ruan DY. (2009a). Effects of decabrominated diphenyl ether (PBDE 209) exposure at different developmental periods on synaptic plasticity in the dentate gyrus of adult rats *in vivo*. *Toxicol Sci* **110**, 401-410.

Xing TR, Yong W, Chen L, Tang ML, Wang M, Chen JT, Ruan DY. (2009b). Effects of decabrominated diphenyl ether (PBDE-209) on voltage-gated sodium channels in primary cultured rat hippocampal neurons. *Environ Toxicol* 10.1002/tox.20511 [Online 12 June 2009].

Xu J, Mashimo T, Südhof TC. (2007). Synaptotagmin-1, -2, and -9: Ca^{2+} sensors for fast release that specify distinct presynaptic properties in subsets of neurons. *Neuron* **54**, 567-581.

Xu-Friedman MA, Regehr WG. (2004). Structural contributions to short-term synaptic plasticity. *Physiol Rev* **84**, 69–85.

Yogui GT, Sericano JL. (2009). Polybrominated diphenyl ether flame retardants in the U.S. marine environment: a review. *Environ Int* **35**, 655-666.

Yu K, He Y, Yeung LWY, Lam PKS, Wu RSS, Zhou B. (2008). DE-71-induced apoptosis involving intracellular calcium and the Bax-mitochondria-caspase protease pathway in human neuroblastoma cells *in vitro*. *Toxicol Sci* **104**, 341-351.

Yuan J, Chen L, Chen D, Guo H, Bi X, Ju Y, Jiang P, Shi J, Yu Z, Yang J, Li L, Jiang Q, Sheng G, Fu J, Wu T, Chen X. (2008). Elevated serum polybrominated diphenyl ethers and thyroid-stimulating hormone associated with lymphocytic micronuclei in Chinese workers from an E-waste dismantling site. *Environ Sci Technol* **42**, 2195-2200.

Zalk R, Lehnart SE, Marks AR. (2007). Modulation of the ryanodine receptor and intracellular calcium. *Annu Rev Biochem* **76**, 367-385.

Zegers BN, Mets A, van Bommel R, Minkenberg C, Hamers T, Kamstra JH, Pierce GJ, Boon JP. (2005). Levels of hexabromocyclododecane in harbor porpoises and common dolphins from western European seas, with evidence for stereoisomer-specific biotransformation by cytochrome p450. *Environ Sci Technol* **39**, 2095-2100.

Zhang S, Bursian S, Martin PA, Chan HM, Martin JW. (2008a). Dietary accumulation, disposition, and metabolism of technical pentabrominated diphenyl ether (de-71) in pregnant mink (*Mustela vison*) and their offspring. *Environ Toxicol Chem* **27**, 1184-1193.

Zhang S, Bursian SJ, Martin PA, Chan HM, Tomy G, Palace VP, Mayne GJ, Martin JW. (2009a). Reproductive and developmental toxicity of a pentabrominated diphenyl ether mixture, DE-71, to ranch mink (*Mustela vison*) and hazard assessment for wild mink in the Great Lakes region. *Toxicol Sci* **110**, 107-116.

Zhang X, Diamond ML, Ibarra C, Harrad S. (2009b). Multimedia modeling of polybrominated diphenyl ether emissions and fate indoors. *Environ Sci Technol* **43**, 2845-2850.

Zhang X, Yang F, Xu C, Liu W, Wen S, Xu Y. (2008b). Cytotoxicity evaluation of three pairs of hexabromocyclododecane (HBCD) enantiomers on Hep G2 cell. *Toxicol In Vitro* **22**, 1520–1527.

Zhang X, Yang F, Zhang X, Xu Y, Liao T, Song S, Wang J. (2008c). Induction of hepatic enzymes and oxidative stress in Chinese rare minnow (*Gobiocypris rarus*) exposed to waterborne hexabromocyclododecane (HBCDD). *Aquat Toxicol* **86**, 4-11.

Zhang BX, Zhao H, Muallem S. (1993). Ca^{2+}-dependent kinase and phosphatase control inositol 1,4,5-trisphosphate-mediated Ca^{2+} release. Modification by agonist stimulation. *J Biol Chem* **268**, 10997-11001.

Zhou T, Ross DG, DeVito MJ, Crofton KM. (2001). Effects of short-term *in vivo* exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicol Sci* **61**, 76-82.

Zhou T, Taylor MM, DeVito MJ, Crofton KM. (2002). Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. *Toxicol Sci* **66**, 105-116.

Zhu J, Hou Y, Feng YL, Shoeib M, Harner T. (2008). Identification and determination of hexachlorocyclopentadienyl-dibromocyclooctane (HCDBCO) in residential indoor air and dust: a previously unreported halogenated flame retardant in the environment. *Environ Sci Technol* **42**, 386-391.

Zhu L, Ma B, Hites RA. (2009). Brominated flame retardants in serum from the general population in Northern China. *Environ Sci Technol* **43**, 6963-6968.

Zota AR, Rudel RA, Morello-Frosch RA, Brody JG. (2008). Elevated house dust and serum concentrations of PBDEs in California: unintended consequences of furniture flammability standards? *Environ Sci Technol* **42**, 8158-8164.

Nederlandse samenvatting

Gebromeerde vlamvertragers. In moderne samenlevingen zijn brandveiligheidseisen ingesteld om persoonlijke ongelukken en economische schade door brand te beperken. Een belangrijke maatregel daarbij is het gebruik van chemische vlamvertragers, die aan producten worden toegevoegd om ontbranding te voorkomen of vertragen. Hierdoor is meer tijd om een brand te blussen of ervan te ontsnappen.

Vanwege toenemende brandveiligheidseisen worden de laatste decennia steeds grotere hoeveelheden vlamvertragers geproduceerd en gebruikt. In Europa en Noord-Amerika worden gebromeerde vlamvertragers (brominated flame retardants; BFRs) veel gebruikt. Zoals andere halogenen vangt broom vrijkomende vrije radicalen weg waardoor vlammen niet groter kunnen worden. In de jaren ’70 van de twintigste eeuw werden BFRs geïntroduceerd als vlamvertragers in industriële en commerciële producten nadat van gechloreerde bifenylen (polychlorinated biphenyls; PCBs) negatieve effecten werden waargenomen op de gezondheid en in experimentele studies.

BFRs worden gebruikt in bijvoorbeeld elektronica, meubels, stoffering, bouwmaterialen en textielproducten. Er is een grote diversiteit aan BFRs. Gebromeerde difenyl ethers (polybrominated diphenyl ethers; PBDEs), hexabromocyclododecaan (HBCD) en tetrabrominated bisphenol-A (TBBPA) worden geproduceerd in grote hoeveelheden. De moleculaire structuur van PBDEs bestaat uit twee fenylringen, aan elkaar verbonden met een etherverbinding. De fenylringen hebben verschillende hoeveelheden en patronen van bromering, resulterend in 209 verschillende PBDEs. PBDEs zijn op de markt gebracht als commerciële mengsels van voornamelijk tetra-hexaBDEs (PentaBDE), hepta-octaBDEs (OctaBDE) en decaBDE (DecaBDE). HBCD is op de markt als een isomerisch mengsel (α-HBCD, β-HBCD and γ-HBCD) waarin vooral γ-HBCD aanwezig is. Zowel PBDEs als HBCD zijn additieve vlamvertragers, wat betekent dat deze BFRs geen chemische verbindingen hebben met de moleculen in de producten waaraan ze zijn toegevoegd. Hierdoor kunnen deze BFRs ook uit deze producten lekken.

Om blootstelling van mens en milieu te verminderen, worden BFRs in Europa gereguleerd door verschillende wetten (REACH, RoHS en WEEE), alsook door product stewardship van de betrokken industrie.

Milieucontaminatie en humane blootstelling aan PBDEs en HBCD. Tijdens de laatste decennia zijn voornamelijk PBDEs gebruikt in commerciële producten. Sinds 2004 zijn door wetgeving en vrijwillige maatregelen van de betrokken industrie Penta- en OctaBDE gefaseerd van de markt gehaald in Europa en Noord-Amerika. Recent heeft een uitspraak van het Europese Hof de uitzonderingsstatus van DecaBDE verwijderd. Dit betekent dat ook DecaBDE niet meer is toegestaan als een vlamvertrager in elektronica. Ook is zojuist (december 2009) aangekondigd dat de betrokken industrie ook in Noord-Amerika DecaBDE vrijwillig gefaseerd van de markt haalt. DecaBDE wordt echter nog steeds vrijwel ongelimiteerd gebruikt in andere delen van de wereld. Ook de lager gebromeerde PBDEs worden nog steeds in mens en milieu aangetroffen, ondanks het feit dat ze niet meer worden geproduceerd voor

commercieel gebruik. Sinds commerciële PBDE producten gefaseerd van de markt verdwijnen, is HBCD vaak aangewezen als mogelijke vervanger voor een aantal toepassingen van BFRs, hoewel dit niet is bevestigd door de betrokken industrieën. Net als PBDEs is HBCD wijd verspreid in het milieu. Nadat Penta- en OctaBDE uit gebruik waren genomen in commerciële producten werd een snelle stijging van HBCD niveaus waargenomen in zeezoogdieren. Deze toename werd toegeschreven aan een toegenomen gebruik van HBCD als vervanger voor PBDEs in polystyreen (piepschuim). Vanaf 2005 zijn deze niveaus weer gestabiliseerd, mogelijk door een verbetering in preventie van emissie van HBCD.

Blootstelling van mens en milieu aan PBDEs is afhankelijk van (historische) gebruikspatronen van de verschillende commerciële PBDE producten. PBDE komen voornamelijk het milieu in op die plaatsen waar ze worden geproduceerd of gerecycled. Voor blootstelling in het binnenmilieu is het vrijkomen van PBDEs door verdamping en slijtage van producten waarin ze zijn gebruikt ook een belangrijke bron. Mensen worden dus blootgesteld aan PBDEs via de lucht en door het inademen of inslikken van huisstof. Ook in diverse plantaardige en dierlijke voedselproducten worden PBDEs aangetroffen. Humane blootstellingsroutes aan HBCD zijn vergelijkbaar met die van PBDEs. HBCD is aangetroffen in grondmonsters, biologische monsters en voedsel. HBCD is ook al gevonden in de poolregio, wat lange-afstands en/of atmosferische transport suggereert, zoals al beschreven voor andere persistente organische verbindingen.

PBDEs en HBCD accumuleren voornamelijk in top-predatoren in voedselketens. Toxicokinetische dierstudies laten zien dat lager gebromeerde PBDEs snel worden opgenomen en langzaam geëlimineerd, en accumuleren in vetweefsel. Ook de slecht opneembare BDE-209 wordt aangetroffen in vogeleieren, vogels, vissen en zeezoogdieren, en in humane weefsels. Zorg over mogelijke ecotoxiciteit is ontstaan na de observatie van erg hoge concentraties BDE-209 in roofvogels in Noord-China, die wijst op biomagnificatie in voedselketens. Toxicokinetische studies in ratten, muizen en vissen hebben getoond dat HBCD via het dieet accumuleert en door het gehele lichaam wordt verspreid. Net als PBDEs is HBCD een lipofiele stof die accumuleert in vetrijke weefsels zoals lever en vetweefsel. De afgelopen twee decennia zijn toenemende concentraties van HBCD waargenomen in eieren van zeevogels. Voor risicobeoordeling van HBCD moet in acht genomen worden dat, hoewel het commerciële product voornamelijk γ-HBCD bevat, er in biotische monsters meestal α-HBCD wordt aangetroffen.

Zowel in mensen als wilde dieren worden voornamelijk de PBDE congeneren BDE-28, BDE-47, BDE-99, BDE-100, BDE-153 and BDE-183 aangetroffen, alsook (hoewel in lagere concentraties) BDE-209. Meestal is BDE-47 de meest voorkomende congener in biotische monsters. PBDEs zijn aangetroffen in lever, bloed, moedermelk en vetweefsel van dieren en mensen, soms met hoge concentraties. De concentratie PBDEs in dieren en mensen is in de afgelopen decennia exponentieel toegenomen (met een factor 2 in een periode van ongeveer 5 jaar). In Europa naderen humane niveaus een stabiele situatie. Echter, in Noord-Amerika zijn de humane niveaus niet alleen 1 tot 2 ordegroottes hoger vergeleken met Europa of

Japan, maar misschien zelfs nog stijgend. Een beperkt aantal studies wijst erop dat humane niveaus van HBCD lager zijn in Noord-Amerika dan die in Europa. Hogere PBDE blootstellingniveaus zijn gevonden in mensen die werken met computers, in elektronica recycling en verbrandingsovens, en hogere HBCD blootstelling in werknemers in de productie van polystyreen.

Naast PBDE congeneren, zijn ook gehydroxyleerde en gemethoxyleerde PBDEs (OH- en MeO-PBDEs) gevonden in zee- en zoetwatervissen, zeevogels, dolfijnen, zeehonden en ijsberen. De vorming van deze metabolieten *in vivo* is bevestigd in toxicokinetische studies met muizen en ratten, en in microsomen van de rattenlever. Heel recent is ook de vorming van OH-PBDEs aangetoond in primair gekweekte humane levercellen. Toxicokinetische studies met vissen lieten zien dat weinig OH-PBDEs worden gevormd, maar vooral gedebromeerde metabolieten. OH- en MeO-PBDEs zijn ook natuurlijke producten welke zijn gevonden in algen, cyanobacterien en sponzen. In 2002 werden OH-PBDEs voor het eerst gedetecteerd in humaan serum (bloed zonder bloedcellen en stollingseiwitten). De vermoede bioaccumulatie van OH-PBDEs in de mens werd recent bevestigd. Verschillende OH-PBDEs werden gevonden in kinderen wonend en werkzaam op een vuilnisbelt in Nicaragua. Ook zijn OH-PBDEs gevonden in serum van Amerikaanse moeders en in navelstrengbloed.

Meerdere studies hebben aangetoond dat jonge kinderen aan hogere concentraties PBDEs worden blootgesteld dan volwassenen. Waarschijnlijk wordt dit veroorzaakt door frequent hand-mond contact en spelen op de grond. Daardoor krijgen kinderen grotere hoeveelheden met huisstof binnen, inclusief daaraan gebonden PBDEs. Een andere bron voor PBDE én HBCD blootstelling is moedermelk, waarin voornamelijk lager gebromeerde PBDEs worden aangetroffen. Voor de geboorte vindt ook blootstelling plaats, doordat de placenta PBDEs en HBCD uit het bloed van de moeder doorlaat naar de foetus.

Toxiciteit van PBDEs en HBCD. Voor zowel PBDEs als HBCD zijn acute toxische effecten beperkt. Matige acute toxiciteit van PBDEs is aangetoond bij hoge blootstelling, voornamelijk in de lever en schildklier.

HBCD verandert genexpressie en enzymactiviteit in de lever. Aangetoonde aryl hydrocarbon receptor afhankelijke effecten van commerciële PBDE producten worden waarschijnlijk voornamelijk veroorzaakt door de aanwezigheid van kleine hoeveelheden gebromeerde dioxinen en furanen. Endocriene effecten van PBDEs, OH-PBDEs en HBCD zijn aangetoond in verschillende diersoorten, voornamelijk verstoring van het schildklierhormoonsysteem, dat ook een belangrijke rol speelt in de ontwikkeling van de hersenen. Endocriene studies hebben laten zien dat OH-PBDEs sterkere hormonale effecten veroorzaken dan PBDEs.

Vanaf 2001 werden nadelige effecten beschreven op spontaan gedrag en habituatie (gewenning aan een nieuwe omgeving) van muizen na een éénmalige postnatale (op dag 10 na de geboorte) orale dosis van PBDEs. De dosis-afhankelijke veranderingen konden zelfs enkele maanden na de blootstelling nog worden onderscheiden. Vergelijkbare langdurige

effecten zijn waargenomen na een éénmalige blootstelling op 10 dagen na de geboorte aan BDE-47, BDE-99, BDE-153, BDE-183, BDE-203, BDE-206, BDE-209 of HBCD. Door muizen bloot te stellen op verschillende tijdstippen na de geboorte bleek dat de postnatale hersenontwikkeling tijdens de eerste twee weken na de geboorte het meest gevoelig was voor effecten van PBDEs. Meerdere onderzoeksgroepen vonden vergelijkbare resultaten met verschillende blootstellingregimes en testsystemen. Uit deze studies kan worden geconcludeerd dat PBDEs leren en gedrag in knaagdieren verstoren, mits de blootstelling optreedt tijdens de vroege (postnatale) hersenontwikkeling.

Aanvullende studies wijzen erop dat zowel veranderingen in het cholinerge neurotransmitter systeem alsook verstoring van het schildklierhormoonsysteem een rol kunnen spelen in de effecten van PBDEs op leren en geheugen. Voor HBCD zijn aanwijzingen dat effecten op het dopaminerge neurotransmittersysteem een rol spelen. Ook zijn na blootstelling aan PBDEs in verschillende studies veranderingen waargenomen in eiwitniveaus, voornamelijk in de hippocampus, welke een belangrijke rol spelen in de postnatale ontwikkeling van de hersenen.

In *in vitro* neurotoxiciteitstudies zijn vooral effecten van PBDEs en HBCD op celgroei, celdood en oxidatieve stress in neurale cellen, neurotransmitter homeostase, intracellulaire signaaltransductie en ionkanalen waargenomen. Deze acute effecten, of combinaties ervan, spelen waarschijnlijk een rol in de effecten van PBDEs op leren en geheugen. Wel moet in acht worden genomen dat de concentraties van PBDEs en HBCD die neurotoxiciteit *in vitro* veroorzaken meestal niet relevant zijn voor de humane blootstellingsituatie.

Inhoud van dit proefschrift. Het bovengenoemd onderzoek wijst duidelijk op de neurotoxiciteit van PBDEs en HBCD. Oorzakelijke verbanden tussen de waargenomen *in vivo* en *in vitro* effecten zijn echter nog niet volledig duidelijk. Daarom was de doelstelling van dit PhD-project om inzicht te krijgen in de mechanismen welke betrokken zijn bij de effecten op leren en gedrag na blootstelling aan PBDEs en HBCD tijdens de hersenenontwikkeling.

In het onderzoek naar mogelijke effecten op synaptische plasticiteit is een vorm van synaptische plasticiteit in de hippocampus, NMDA-afhankelijke long-term potentiation (LTP) gebruikt als een neurofysiologisch model voor leren en geheugen. Synaptische plasticiteit is het vermogen om de efficiëntie van signaaloverdracht tussen twee zenuwcellen aan te passen afhankelijk van activiteit en stimuli. Dit proces is essentieel voor informatieopslag in de hersenen. Er is gekozen om dit proces in de hippocampus te onderzoeken, omdat dit hersengebied betrokken is bij leren en het geheugen. Een korte-termijn vorm van synaptische plasticiteit, paired-pulse facilitatie (PPF) is gebruikt om mogelijke effecten op presynaptische processen te detecteren.

Het voornaamste deel van dit proefschrift is gewijd aan acute presynaptische effecten van BFRs. Informatieoverdracht tussen twee zenuwcellen (neurotransmissie) vindt plaats in de synaps, de ruimte tussen de verzendende (presynaptische) en de ontvangende (postsynaptische) zenuwcel. De nadruk ligt in dit onderzoek op de presynaptische processen

betrokken bij de signaaloverdracht. Het voornaamste presynaptische proces is het afgeven van neurotransmitters, de chemische signaalstof, in de synaps. Neurotransmitter afgifte vindt voornamelijk plaats doordat met neurotransmitter gevulde blaasjes (vesicles) versmelten met de presynaptische celmembraan. Neurotransmitter afgifte wordt voornamelijk gereguleerd door de intracellulaire calcium concentratie ($[Ca^{2+}]_i$).

Aangezien neurotransmissie sterk afhangt van de strikte regulatie van Ca^{2+}-homeostase en neurotransmitter afgifte, zijn de acute effecten van PBDEs en HBCD op $[Ca^{2+}]_i$ en de daarmee samenhangende vesiculaire neurotransmitter afgifte gemeten in rat pheochromocytoma (PC12) cellen, een neuroendocrien *in vitro* model. Effecten van PBDEs op $[Ca^{2+}]_i$ zijn ook onderzocht in neurospheres van humane neuronale progenitor cellen, een heterogeen *in vitro* model voor hersenontwikkeling.

Intracellulair Ca^{2+} is betrokken bij verschillende cellulaire en subcellulaire processen. In neurale cellen speelt Ca^{2+} ook een belangrijke rol als signaal voor vesiculaire neurotransmitter afgifte. De trigger voor neurotransmissie is de depolarisatie van de presynaptische celmembraan, bijvoorbeeld door een actiepotentiaal. Deze depolarisatie van de celmembraan veroorzaakt de opening van spanningsafhankelijke Ca^{2+}-kanalen (voltage-gated Ca^{2+} channels; VGCCs), waardoor de $[Ca^{2+}]_i$ snel toeneemt door influx van Ca^{2+} door deze kanalen. $[Ca^{2+}]_i$ is in dit onderzoek gemeten met de Ca^{2+}-afhankelijke fluorescente kleurstof Fura-2. Onderliggende mechanismen van toenames in $[Ca^{2+}]_i$ werden onderzocht in experimenten onder extracellulair Ca^{2+}-vrije condities, en door het leegmaken van intracellulaire compartimenten waarin Ca^{2+} wordt opgeslagen (endoplasmatisch reticulum, ER en mitochondria) voorafgaand aan de blootstelling aan BFRs.

Basale en depolarisatie-geïnduceerde vesiculaire afgifte van catecholamine neurotransmitters door primaire chromaffiene cellen en dexamethason-gedifferentieerde PC12 cellen werd gemeten door middel van amperometrie met een koolstofvezel micro-elektrode.

Neonatale blootstelling aan gebromeerde vlamvertrager BDE-47 vermindert LTP en postsynaptische eiwitniveaus in de hippocampus van de muis. Als eerste is onderzocht of een afname van synaptische plasticiteit in de hippocampus een mogelijke oorzaak zou kunnen zijn voor de effecten op leren en gedrag in muizen na neonatale blootstelling aan BDE-47 (hoofdstuk 3). Muizen werden aan dezelfde hoeveelheid en op dezelfde manier blootgesteld aan BDE-47 (op dag 10 na de geboorte) als in de gedragsstudies. Op dag 7 - 9 na de geboorte werd LTP door middel van veldpotentialen gemeten. Veldpotentialen zijn neuronale signalen die extracellulair in hersenweefsel worden gemeten, en dus een gemiddeld signaal van vele omliggende zenuwcellen. De hippocampus werd opgesneden in verschillende plakjes (slices) met daarin de te bemeten structuren om verschillende metingen mogelijk te maken. Na blootstelling aan BDE-47 bleek LTP te zijn verminderd.

Mogelijke presynaptische effecten, welke een rol zouden kunnen spelen in de afname van LTP, werden onderzocht door middel van paired-pulse facilitatie (PPF), en meting van

basale en depolarisatie-geïnduceerde vesiculaire afgifte van catecholamine neurotransmitters door neuroendocriene (chromaffiene) cellen uit muizen welke werden blootgesteld aan BDE-47. Beide parameters bleken niet veranderd te zijn. Tijdelijke veranderingen van neurale activiteit tijdens kritieke periodes in hersenenontwikkeling zouden aan de waargenomen effecten op gedrag en synaptische plasticiteit ten grondslag kunnen liggen. Daarom werden ook acute presynaptische effecten van BDE-47 op $[Ca^{2+}]_i$ en neurotransmitter afgifte onderzocht. Alleen bij blootstelling aan een hoge concentratie (20 µM) BDE-47 werden milde toenames van zowel $[Ca^{2+}]_i$ als neurotransmitter afgifte waargenomen.

Ook werd de hoeveelheid van een aantal aan LTP gerelateerde eiwitten gemeten in de hippocampus door middel van Western blotting: postsynaptische eiwitten PSD-95 en SAP97, (on-)gefosforyleerd αCaMKII, AMPA receptor subunit GluR1 en NMDA receptor subunits NR1, NR2A and NR2B. Alleen in de postsynaptische fractie van hippocampi werd een afname waargenomen van glutamaat receptor subunits NR2B, GluR1 en gefosforyleerd αCaMKII. De afname in synaptische plasticiteit lijkt dus gerelateerd te zijn aan veranderingen in (relatieve) hoeveelheden van postsynaptische eiwitten.

Hoewel er soms erg hoge concentraties van BDE-47 worden gemeten in menselijke weefsels, is het onwaarschijnlijk dat een vergelijkbare concentratie als in deze dierstudie wordt bereikt in de mens. De resultaten van deze studie lieten wél voor het eerst zien dat neonatale blootstelling aan een veel voorkomende PBDE onmiskenbaar een afname van synaptische plasticiteit veroorzaakt. Daarom steunen deze resultaten sterk de eerder waargenomen effecten van BDE-47 op leren en gedrag.

Hydroxylatie vergroot de effecten van BDE-47 op calcium homeostase en vesiculaire neurotransmitter afgifte in PC12 cellen. Om te onderzoeken of gehydroxyleerde metabolieten een rol spelen in neurotoxiciteit van PBDEs, werden de acute presynaptische effecten van BDE-47 in PC12 cellen vergeleken met die van gehydroxyleerde metaboliet 6-OH-BDE-47 (hoofdstuk 4).

Terwijl bij blootstelling aan de laagste-effect concentratie 20 µM BDE-47 zowel $[Ca^{2+}]_i$ als vesiculaire neurotransmitter afgifte subtiel toenamen, veroorzaakte 6-OH-BDE-47 al bij 1 µM een transiente (voorbijgaande) toename in $[Ca^{2+}]_i$, samengaand met een toename in vesiculaire neurotransmitter afgifte. Bij hogere concentraties 6-OH-BDE-47 werd een additionele toename in $[Ca^{2+}]_i$ waargenomen. De transiente toename in $[Ca^{2+}]_i$ werd veroorzaakt door het vrijkomen van Ca^{2+} uit het endoplasmatisch reticulum (ER) en door influx van extracellulair Ca^{2+}, terwijl de additionele toename in $[Ca^{2+}]_i$ werd veroorzaakt door het vrijkomen van Ca^{2+} uit de mitochondria.

Deze resultaten laten zien dat gehydroxyleerde metaboliet 6-OH-BDE-47 grotere effecten heeft op $[Ca^{2+}]_i$ bij lagere concentraties dan BDE-47. Bioactivatie door oxidatief metabolisme zou dus bij kunnen dragen aan de neurotoxiciteit van PBDEs. Aangezien *ortho*-PCBs ook Ca^{2+} vrij kunnen maken uit intracellulaire compartimenten, kan additiviteit tussen deze twee groepen stoffen niet worden uitgesloten.

Het bromeringspatroon van gehydroxyleerde metabolieten van BDE-47 beïnvloedt hun potentie om calcium vrij te maken uit intracellulaire compartimenten in PC12 cellen. Om te onderzoeken of de waargenomen hogere activiteit van OH-PBDEs door hun moleculaire structuur zou kunnen worden voorspeld, werden de effecten op $[Ca^{2+}]_i$ onderzocht voor een reeks mono-gehydroxyleerde metabolieten van BDE-47 met variaties in hydroxylatie- en bromeringspatroon (hoofdstuk 5).

In tegenstelling tot BDE-47 en gemethoxyleerde BDE-47, veroorzaakten alle onderzochte OH-PBDEs het vrijkomen van Ca^{2+} uit intracellulaire compartimenten. De voornaamste bronnen waren ofwel ER (5-OH-BDE-47 en 6'-OH-BDE-49), of zowel ER alsook mitochondria (6-OH-BDE-47, 3-OH-BDE-47 en 4'-OH-BDE-49). Bij nader onderzoek werd voor 6-OH-BDE-47, 4'-OH-BDE-49 en BDE-47, bij nog lagere concentraties een toename van subtiele fluctuaties in $[Ca^{2+}]_i$ waargenomen. Presynaptische effecten zouden dus een rol kunnen spelen in de waargenomen functionele effecten op synaptische plasticiteit.

Deze resultaten laten zien dat alle onderzochte gehydroxyleerde metabolieten $[Ca^{2+}]_i$ verstoren door het vrijmaken van Ca^{2+} uit intracellulaire compartimenten. Afscherming van de OH-groep aan twee kanten door broomatomen ofwel een aromatische ring vermindert de effecten van OH-PBDEs op $[Ca^{2+}]_i$, terwijl de positie van de OH-groep van minder belang lijkt te zijn.

Aan calcium gerelateerde processen spelen een rol in de inhibitie van depolarisatie-geïnduceerde toename in calcium in PC12 cellen door gehydroxyleerde metabolieten van PBDEs. VGCCs spelen een belangrijke rol in de handhaving van de Ca^{2+}-homeostase en neurotransmissie. Daarom zijn, naast de effecten op basaal $[Ca^{2+}]_i$ door het vrijmaken van Ca^{2+} uit intracellulaire compartimenten, ook de effecten van een aantal PBDEs en OH-PBDEs op depolarisatie-geïnduceerde toename in $[Ca^{2+}]_i$ onderzocht in PC12 cellen (hoofdstuk 6).

PBDEs (BDE-49, BDE-99, BDE-100 and BDE-153) en gemethoxyleerd BDE-47 hadden geen effecten op basaal of depolarisatie-geïnduceerde toename in $[Ca^{2+}]_i$. BDE-47 veroorzaakte een toename in subtiele fluctuaties in basaal $[Ca^{2+}]_i$ en verhoging van depolarisatie-geïnduceerde $[Ca^{2+}]_i$. OH-PBDEs verlaagden de depolarisatie-geïnduceerde toename in $[Ca^{2+}]_i$ dosis-afhankelijk. Deze inhibitie was sterker als er een toename in basaal $[Ca^{2+}]_i$ aan vooraf was gegaan. Vooral bij hogere concentraties van OH-PBDEs (5 - 20 µM), gingen hoge toenames in basaal $[Ca^{2+}]_i$ samen met een sterke inhibitie van depolarisatie-geïnduceerde $[Ca^{2+}]_i$. Deze inhibitie bleek meer gevoelig te zijn voor voorgaande toenames in basaal $[Ca^{2+}]_i$ door het vrijmaken van Ca^{2+} uit intracellulaire compartimenten (door 3-OH-BDE-47 en 6'-OH-BDE-49) dan door influx van extracellulair Ca^{2+} (door 6-OH-BDE-47 en 5-OH-BDE-47). Het verwachte verschil in $[Ca^{2+}]_i$ in de directe nabijheid van de celmembraan suggereert dat Ca^{2+}-afhankelijke processen in de buurt van de VGCCs hier een regulerende rol in spelen. Zonder voorafgaande blootstelling aan OH-PBDEs (en dus zonder voorafgaande

verhoging van $[Ca^{2+}]_i$) werd voor een aantal OH-PBDEs ook matige directe inhibitie van de depolarisatie-geïnduceerde toename in $[Ca^{2+}]_i$ waargenomen.

Deze resultaten laten zien dat OH-PBDEs ook de depolarisatie-geïnduceerde toename in $[Ca^{2+}]_i$ inhiberen, wat versterkt wordt door voorafgaande toenames in basaal $[Ca^{2+}]_i$. Het zou kunnen dat verwante milieucontaminanten die ook de Ca^{2+}-homeostase verstoren, zoals PCBs, ook de depolarisatie-geïnduceerde toename in $[Ca^{2+}]_i$ inhiberen. Verder onderzoek naar mogelijke mengseltoxiciteit van milieucontaminanten is daarom gerechtvaardigd.

BDE-47 en 6-OH-BDE-47 moduleren calcium homeostase in primaire humane neurale progenitor cellen. In een *in vitro* model voor de ontwikkeling van de hersenen zijn eerder effecten van PCBs en PBDEs op migratie en differentiatie van humane neurale progenitor cellen (hNPCs) gevonden. Om te onderzoeken of (transiente) effecten op basaal $[Ca^{2+}]_i$ een rol zouden kunnen spelen in deze processen, zijn de acute effecten van BDE-47 en zijn gehydroxyleerde metaboliet 6-OH-BDE-47 op $[Ca^{2+}]_i$ onderzocht in deze cellen (hoofdstuk 7).

Blootstelling van de hNPCs aan BDE-47 ($\geq$ 2 µM) of 6-OH-BDE-47 ($\geq$ 0.2 µM) veroorzaakte toenames in $[Ca^{2+}]_i$. BDE-47 veroorzaakte een toename van de influx van extracellulair Ca^{2+}. 6-OH-BDE-47 veroorzaakte een snelle persistente toename door vrijmaking van Ca^{2+} uit mitochondria, gecombineerd met een transiente toename veroorzaakt door vrijmaking van Ca^{2+} uit ER en een trage toename in $[Ca^{2+}]_i$ door influx van extracellulair Ca^{2+}. Zoals eerder waargenomen in PC12 cellen, verstoort 6-OH-BDE-47 ook in hNPCs de Ca^{2+}-homeostase sterker en bij lagere concentraties dan BDE-47. De laagste effectieve concentraties voor toename van $[Ca^{2+}]_i$ liggen echter een ordegrootte lager dan in de PC12 cellen. Het is nog onduidelijk of dit verschil toe te schrijven is aan interspecies verschillen, of het verschil in celtypes.

HBCD vermindert depolarisatie-geïnduceerde toename van $[Ca^{2+}]_i$ en neurotransmitter afgifte van PC12 cellen. Aangezien andere BFRs de Ca^{2+}-homeostase verstoren, zijn mogelijke effecten van het commerciële HBCD product en HBCD stereoisomeren op $[Ca^{2+}]_i$ onderzocht in PC12 cellen (hoofdstuk 8). Blootstelling van PC12 cellen aan HBCD of aan de individuele stereoisomeren had geen effect op basaal $[Ca^{2+}]_i$ of basale vesiculaire neurotransmitter afgifte. De depolarisatie-geïnduceerde toename van $[Ca^{2+}]_i$ verminderde dosis-afhankelijk na voorafgaande blootstelling aan HBCD. Voor dit effect moest er minstens 5 min blootstelling hebben plaatsgevonden voor de depolarisatie, terwijl de maximale inhibitie optrad na 20 min blootstelling. De effecten van α- en β-HBCD waren vergelijkbaar met dat van het technische mengsel, terwijl het inhiberende effect van γ-HBCD groter was. Met behulp van specifieke blockers van L-, N- of P/Q-type VGCCs kon worden aangetoond dat het inhiberende effect van HBCD niet specifiek is voor een subtype VGCC. Ook was er een sterke afname te zien in het aantal cellen waarin vesiculaire neurotransmitter afgifte kon worden getriggerd met depolarisatie.

Deze resultaten laten zien dat HBCD de depolarisatie-geïnduceerde toename in $[Ca^{2+}]_i$ en neurotransmitter afgifte remt. Aangezien concentraties van HBCD in mens en milieu toe zouden kunnen nemen, rechtvaardigen deze bevindingen extra inspanning voor adequate risicobeoordeling van HBCD.

Risicobeoordeling. Om uitspraken te kunnen doen over mogelijke risico's voor de mens, zijn de laagste effect-concentraties (uit dit onderzoek) omgerekend naar de bijbehorende serum concentraties met behulp van gemiddelde fysiologische parameters van bloed en bloedsamenstelling. Deze laagste effect-concentraties werden vergeleken met humane blootstelling. Om in te kunnen schatten of er wel of niet een risico voor de mens is, is in dit onderzoek alleen gebruik gemaakt van de traditionele veiligheidsfactoren voor inter- en intraspecies variatie (10x10). Concentraties in de mens moeten dus minstens 100 keer lager zijn dan de laagste-effect concentraties om als veilig te worden beschouwd.

Voor de schatting van bloed- en orgaan concentraties van BDE-47 in neonatale muizen werd gebruik gemaakt van de resultaten van een toxicokinetische studie. Vergelijking met waardes van humane blootstelling wijst erop dat het risico voor verstoring van $[Ca^{2+}]_i$ in neurale en endocriene cellen door blootstelling aan BDE-47 laag is in de algemene populatie. Echter, in individuele gevallen van beroepsmatige blootstelling worden serum concentraties van BDE-47 gevonden welke de ingeschatte laagste veilige concentratie benaderen.

De aanwezigheid van OH-PBDEs in humaan serum, met vergelijkbare of zelfs hogere concentraties dan van PBDEs, is in een beperkt aantal studies aangetoond. Zelfs de hoogste gemeten concentratie van 6-OH-BDE-47 is een ordegrootte lager dan de ingeschatte veilige concentratie. In dit onderzoek is ook aangetoond dat, naast 6-OH-BDE-47, andere gehydroxyleerde metabolieten van BDE-47 de Ca^{2+}-homeostase verstoren. Deze bevinding suggereert dat de meeste gehydroxyleerde metabolieten van lager gebromeerde PBDEs de Ca^{2+}-homeostase beïnvloeden. Het verschil tussen de hoogst gemeten ΣOH-PBDE (alle gemeten OH-PBDEs samen) concentratie in humaan serum en de laagste-effect concentratie van een OH-PBDE (*in vitro*) is onvoldoende om als veilig te kunnen worden beschouwd. De hier aangenomen additiviteit van OH-PBDEs, dus het kunnen optellen van blootstellingconcentraties, is echter nog niet experimenteel aangetoond. Ook zijn mogelijke verschillen in potentie tussen verschillende OH-PBDEs niet in acht genomen. Mogelijk ligt de laagste-effect concentratie in de realiteit hoger voor mengsels van OH-PBDEs dan vergeleken met de meest potente OH-PBDEs. Het verschil tussen de hoogste of mediaan humane serum concentratie van ΣOH-PBDEs en de hoogste laagste-effect concentratie uit dit onderzoek kan als veilig beschouwd worden. Omdat de interacties tussen OH-PBDEs nog onduidelijk zijn, moet de standaardbenadering voor risicobeoordeling voor blootstelling aan OH-PBDEs additiviteit zijn.

Humane serum niveaus van HBCD, zowel in de algemene humane populatie als bij beroepsmatige blootstelling, liggen lager dan de geschatte veilige concentratie, en kunnen dus als veilig worden beschouwd. Inhibitie van de depolarisatie-geïnduceerde toename in

$[Ca^{2+}]_i$ en vesiculaire neurotransmitter afgifte door HBCD blootstelling wordt dus niet verwacht in de humane situatie. Deze conclusie moet echter worden getrokken uit een beperkt aantal blootstellingstudies, waardoor voorzichtigheid op zijn plaats is.

Inhibitie van depolarisatie-geïnduceerde toename in $[Ca^{2+}]_i$ is in dit onderzoek waargenomen voor zowel HBCD als OH-PBDEs, maar niet voor PBDEs. De laagste-effect concentraties zijn gelijk voor HBCD en 6-OH-BDE-47 in PC12 cellen (2 µM), terwijl de andere onderzochte OH-PBDEs pas inhiberen vanaf 5 µM. Bij 2 µM veroorzaken HBCD en 6-OH-BDE-47 een vergelijkbare mate van inhibitie. Bij 20 µM inhiberen de OH-PBDEs de depolarisatie-geïnduceerde toename in $[Ca^{2+}]_i$ bijna volledig, terwijl HBCD ongeveer naar 50% inhibeert. Ook wordt de inhibitie van de depolarisatie-geïnduceerde toename in $[Ca^{2+}]_i$ door OH-PBDEs minstens gedeeltelijk veroorzaakt of versterkt door voorafgaande verhoging van basaal $[Ca^{2+}]_i$, terwijl dit niet het geval is voor HBCD. De potentie om de balans tussen basaal en depolarisatie-geïnduceerde toename in $[Ca^{2+}]_i$ te verstoren is dus groter voor OH-PBDEs dan voor HBCD. Er kan echter wel worden verwacht dat blootstelling aan (OH-)PBDEs en HBCD samengaat, mogelijk resulterend in additiviteit wat betreft de inhibitie van depolarisatie-geïnduceerde toename in $[Ca^{2+}]_i$.

Wanneer concentraties van BFRs in humaan serum worden vergeleken met effectieve concentraties uit de *in vitro* studies welke in dit proefschrift zijn beschreven, blijkt er weinig tot geen risico te zijn wat betreft de acute presynaptische effecten welke mogelijk een rol spelen in de afname van synaptische plasticiteit. Een aantal aspecten moet echter in acht worden genomen voor een goede risicobeoordeling, vooral voor neurotoxische effecten in de zich ontwikkelende hersenen.

Ten eerste zijn de ontwikkelende hersenen gevoeliger dan de volwassen hersenen voor externe stimuli, inclusief chemicaliën. Deze hogere gevoeligheid wordt veroorzaakt door de veelvoud van ontwikkelingsprocessen, zoals proliferatie, differentiatie, migratie en vorming van neurale netwerken. Door de strikte temporele regulatie bestaan er periodes tijdens de hersenontwikkeling welke bijzonder gevoelig zijn voor neurotoxiciteit. Om duidelijk te krijgen welke ontwikkelingsfases van humane hersenontwikkeling het meest gevoelig zijn voor neurotoxische stoffen is meer kennis nodig van de hersenontwikkeling en in het bijzonder van de gevolgen van een verstoring daarvan voor gedrag en cognitieve functie.

Ten tweede is het onduidelijk of de serum concentratie een goede parameter is voor het beoordelen van risico's van chemische blootstelling voor het zenuwstelsel. De hersenen worden namelijk beschermd tegen ongewenste chemicaliën door de bloed-hersen barrière. Lipofiele stoffen met laag molecuulgewicht gaan waarschijnlijk vrijelijk over deze barrière door passieve diffusie. Of dit ook het geval is voor de lipofiele PBDEs, welke een hoog molecuulgewicht hebben, is nog onduidelijk. PBDEs zijn nochtans aangetroffen in de hersenen van verschillende diersoorten, en partitie in de (foetale) hersenen is ook waargenomen in toxicokinetische studies. Het is dus waarschijnlijk dat deze BFRs ook de humane hersenen kunnen bereiken, mogelijk met uitzondering van de meest lipofiele,

hoogmoleculaire BFRs zoals BDE-209. OH-PBDEs zijn slechts zeer zelden gevonden in hersenweefsel van dieren uit het wild of in toxicokinetische experimenten. Hoge concentraties van OH-PBDEs worden dus niet verwacht in de humane hersenen. Niettemin worden endocriene cellen zoals chromaffiene cellen en cellen in de schildklier niet beschermd door de bloed-hersen barrière tegen PBDEs en verwante milieucontaminanten aanwezig in serum. Ook is specifieke accumulatie van OH-PBDEs in de foetale hersenen via binding aan transporteiwitten van het schildklierhormoon gesuggereerd, hoewel dit nog niet experimenteel is aangetoond.

Ten derde is het belangrijk om effecten van mengsels van (verschillende congeneren van) PBDEs, broomfenolen, gechloreerde bifenylen (polychlorinated biphenyls; PCBs), hun gehydroxyleerde metabolieten en HBCD in acht te nemen bij risicobeoordeling voor neurotoxiciteit in mensen, aangezien al deze stoffen de Ca^{2+}-homeostase verstoren. Verstoring van de balans van basaal en depolarisatie-geïnduceerde $[Ca^{2+}]_i$ kan niet alleen neurotransmissie, maar ook veel andere cellulaire processen verstoren. Naast verstoring van de Ca^{2+}-homeostase, heeft een aantal van deze verwante milieucontaminanten ook een aantal andere neurotoxische effecten gemeen: verschillende vormen van verstoring van gedrag, afgenomen LTP, inhibitie van neurotransmitter heropname en cytotoxiciteit in neurale celtypes. Ook verstoren zowel PBDEs als PCBs en hun gehydroxyleerde metabolieten het schildklierhormoonsysteem.

In acht nemend dat ontwikkelende hersenen gevoeliger zijn voor externe stimuli, is het zorgwekkend dat de blootstelling van jonge kinderen over het algemeen hoger is dat die van volwassenen. PBDEs zijn aangetroffen in navelstrengbloed en foetale lever. Hoewel overdracht van OH-PBDEs via de navelstreng nog niet is bewezen, is het niet onwaarschijnlijk, vanwege de structurele gelijkenis met OH-PCBs. Blootstelling aan PBDEs door lactatie is, kwantitatief beschouwd, een belangrijke blootstellingsroute in mensen. Concentraties van PBDEs in moedermelk zijn (net als serum concentraties) afhankelijk van gebruikspatronen, dus hoger in Noord-Amerika dan in Europa, waar de niveaus ook stabiliseren. Recent zijn ook OH-PBDEs in moedermelk aangetroffen, samen met PBDE concentraties die vergelijkbaar zijn met achtergrondconcentraties in Europa en Azië. Ook HBCD is aangetroffen in moedermelk. Een negatief advies ten opzichte van het geven van borstvoeding is altijd als ongepast beschouwd vanwege de onbetwiste voordelen wat betreft hygiëne, voedingswaarde, immunologische effecten en psychosociale ontwikkeling. Niettemin ontbreekt enige risico-voordeel analyse met betrekking tot borstvoeding en de daarmee verbonden blootstelling aan milieucontaminanten zoals PBDEs en PCBs. In het bijzonder in de hoogst blootgestelde gemeenschappen is kennis van melkconcentraties van neurotoxische milieucontaminanten essentieel om de urgentie te beoordelen van preventieve maatregelen om neurotoxiciteit tijdens de ontwikkeling van de hersenen te voorkomen.

Het onderliggende mechanisme voor de neurotoxiciteit van BFRs, met name na blootstelling tijdens de ontwikkeling van het zenuwstelsel, is nog niet opgelost. Niettemin is het duidelijk dat zowel acute cellulaire neurotoxische effecten als verstoring van het schildklierhormoonsysteem betrokken zijn in de neurotoxiciteit van PBDEs en HBCD.

PBDEs en HBCD zijn praktisch alom aanwezig in het milieu. Humane blootstelling vindt voornamelijk plaats via voedsel en het inademen/inslikken van huisstof. Blootstelling kan dus worden verminderd door het binnenmilieu stofvrij te houden. Dit is in het bijzonder van belang voor ruimtes waar kinderen verblijven, in verband met hun hogere blootstelling via huisstof. Een alternatieve mogelijkheid om de humane blootstelling aan PBDEs te verminderen is om brandveiligheidseisen ten opzichte van chemische vlamvertragers te verlagen. Het is echter aannemelijk dat minder mensen zijn omgekomen in branden sinds de invoering van chemische vlamvertragers. Een causaal verband is echter nog nooit aangetoond. Voor humane risicobeoordeling van BFRs zou een goede kosten-baten analyse duidelijkheid scheppen. Dit is vooral belangrijk voor Groot-Brittannië en de Verenigde Staten, waar de brandveiligheidseisen betreffende hoeveelheden van BFRs relatief hoog zijn.

De risicobeoordeling van PCBs was ten tijde van hun introductie duidelijk ontoereikend. Helaas kunnen nu overeenkomsten met PBDEs worden onderscheiden. Met regulatieve risicobeoordeling kwam men tot de conclusie dat commerciële PBDE mengsels een veilig alternatief voor PCBs zouden zijn. Tijdens het afgelopen decennium is het gebruik van PBDEs als vlamvertragers sterk aan banden gelegd binnen de EU.

Vanwege zorgen over mogelijke effecten op de gezondheid heeft de betrokken chemische industrie de productie van PentaBDE en OctaBDE ook voor de Noord-Amerikaanse markt gestaakt. Aangezien producten zullen moeten blijven voldoen aan brandveiligheidseisen, is er een kans dat alternatieve, minder uitgebreid bestudeerde vlamvertragers gebruikt zullen worden. Een aantal 'nieuwe' vlamvertragers zijn zelfs al gedetecteerd in het milieu en wilde dieren. De neurotoxische effecten van PBDEs zijn een reden tot zorg voor subklinische neurotoxische effecten in sommige bevolkingsgroepen. Ondanks de vermindering van het gebruik van PBDEs door vrijwillige maatregelen en wetgeving, worden mensen nog steeds, en waarschijnlijk nog vele jaren, blootgesteld aan de moeilijk afbreekbare PBDEs en hun metabolieten. Dit geeft aan dat risicobeoordeling voor negatieve effecten op de gezondheid door nieuw ontwikkelde chemische stoffen, zoals alternatieve vlamvertragers, voor verbetering vatbaar is.

Conclusies.

1. De effecten van PBDEs op leren en gedrag worden weerspiegeld in een afname van synaptische plasticiteit, mogelijk veroorzaakt door de waargenomen afname van aan LTP gerelateerde postsynaptische eiwitten.
2. Gehydroxyleerde metabolieten van BDE-47 (OH-PBDEs) verhogen de basale $[Ca^{2+}]_i$ en verlagen de depolarisatie-geïnduceerde toename in $[Ca^{2+}]_i$ veel sterker dan BDE-47 zelf, waardoor er mogelijk een disbalans ontstaat in neurale communicatie.
3. Verstoring van de Ca^{2+}-homeostase door OH-PBDEs is afhankelijk van de afscherming van de OH-groep door omliggende atoomgroepen.
4. Zowel BDE-47 als 6-OH-BDE-47 verhogen de basale $[Ca^{2+}]_i$ in humane neurale progenitor cellen sterker dan in PC12 cellen.
5. HBCD, een mogelijke vervanging voor PBDEs, verlaagt de depolarisatie-geïnduceerde toename in $[Ca^{2+}]_i$ en vesiculaire neurotransmitter afgifte, terwijl geen effecten zijn waargenomen op basaal $[Ca^{2+}]_i$ of vesiculaire neurotransmitter afgifte.
6. Concentraties van PBDEs en metabolieten in humaan (navelstreng)bloed zijn lager dan concentraties waar neurotoxische effecten worden verwacht. Door het huidige gebrek aan kennis over hersenconcentraties, hersenontwikkeling en mengsel-toxiciteit is deze blootstelling toch een reden tot zorg voor de ontwikkelende hersenen.

Aanbevelingen.

Risicobeoordeling van PBDEs, hun metabolieten en gerelateerde milieucontaminanten kan sterk worden verbeterd met meer kennis over:

1. oxidatief metabolisme resulterend in bioactivatie.
2. gevoeligheid voor neurotoxiciteit van processen tijdens de hersenontwikkeling.
3. concentraties van PBDEs en metabolieten in hersenweefsel.
4. interacties tussen verschillende milieucontaminanten bij gelijktijdige blootstelling.

CV

Milou Maria Louisa Dingemans was born in Nijmegen, the Netherlands, on January 31, 1981. In 1999 she graduated high school at the Gymnasium Juvenaat Heilig Hart in Bergen op Zoom, and started her study Biology at Utrecht University. After an internship with Dr. Marjoke Heneweer on breast cancer models at the Institute for Risk Assessment Sciences at the Faculty of Veterinary Medicine at Utrecht University, she continued with the Master education ‘Toxicology and Environmental Health’ at the same institution. In the last half year of her undergraduate studies, she investigated phase II metabolism at the Rigshospitalet in Copenhagen, Denmark, under the supervision of Dr. Henrik Leffers. After completing the Master education in 2004, she started her PhD-research on the neurotoxicity of brominated flame retardants, under the supervision of Dr. Henk P.M. Vijverberg, Dr. Remco H.S. Westerink and Prof.dr. Martin van den Berg at the Institute for Risk Assessment Sciences at Utrecht University. In this PhD-research, of which the results are included in this thesis, insight has been gained in the mechanisms underlying the reported neurobehavioral effects of brominated flame retardants. Milou continues her research in toxicology.

Publication list

Marjoke Heneweer, Martine Muusse, Milou M.L. Dingemans, Paul C. de Jong, Martin van den Berg and J. Thomas Sanderson. 2005. Co-culture of primary human mammary fibroblasts and MCF-7 cells as an *in vitro* breast cancer model. *Toxicological Sciences* 83, 257 - 263.

Milou M.L. Dingemans, Geert M.J. Ramakers, Fabrizio Gardoni, Regina G.D.M. van Kleef, Åke Bergman, Monica Di Luca, Martin van den Berg, Remco H.S. Westerink and Henk P.M. Vijverberg. 2007. Neonatal exposure to brominated flame retardant BDE-47 reduces long-term potentiation and postsynaptic protein levels in mouse hippocampus. *Environmental Health Perspectives* 115, 865 - 870.

Milou M.L. Dingemans, Aart de Groot, Regina G.D.M. van Kleef, Åke Bergman, Martin van den Berg, Henk P.M. Vijverberg and Remco H.S. Westerink. 2008. Hydroxylation increases the neurotoxic potential of BDE-47 to affect exocytosis and calcium homeostasis in PC12 cells. *Environmental Health Perspectives* 116, 637 - 643.

Milou M.L. Dingemans, Harm J. Heusinkveld, Aart de Groot, Åke Bergman, Martin van den Berg and Remco H.S. Westerink. 2009. Hexabromocyclododecane (HBCD) inhibits depolarization-induced increase in intracellular calcium levels and neurotransmitter release in PC12 cells. *Toxicological Sciences* 107, 490 - 497.

Milou M.L. Dingemans, Harm J. Heusinkveld, Åke Bergman, Martin van den Berg and Remco H.S. Westerink. 2009. Bromination pattern of hydroxylated metabolites of BDE-47 affects their potency to release calcium from intracellular stores in PC12 cells. *Environmental Health Perspectives* doi:10.1289/ehp.0901339 [Online 19 November 2009].

Milou M.L. Dingemans, Martin van den Berg, Åke Bergman and Remco H.S. Westerink. 2009. Calcium-related processes involved in the inhibition of depolarization-evoked calcium increase by hydroxylated PBDEs in PC12 cells. *Toxicological Sciences* doi: 10.1093/toxsci/kfp310 [Online 31 December 2009].

Dankwoord

It takes a village... Op deze plaats wil ik graag iedereen bedanken die op welke manier dan ook een bijdrage heeft geleverd aan dit proefschrift.

Henk, Martin en Remco, bedankt voor alle hulp, kritische noten, begeleiding en wetenschappelijke feedback. Åke, many thanks for providing me with the pure brominated flame retardants and metabolites which have been essential for this research. Geert, bedankt dat je me *f*-EPSPs hebt leren meten in hippocampus-slices (hoofdstuk 3), en voor de gastvrijheid op je lab. Fabrizio, thank you very much for your contribution to this thesis (chapter 3). Ellen and Timm, our collaboration (chapter 7) has been a pleasure, let's get that paper out there!

Marjoke, in 2002 begon ik bij jou als stage-student bij het IRAS en ik ben er nog steeds! Bedankt dat je me kennis hebt laten maken met het toxicologisch onderzoek. Mede-stagestudenten bij Biochemische Toxicologie, Kim, Leonie, Maarten en Joost, ik vind het gezellig dat we af en toe nog bijkletsen. Succes of gefeliciteerd met jullie boekjes!

NTX-groep, bedankt voor jullie gezelligheid en collegialiteit. Vier dagen per week werken is een leuk idee, maar de celkweek moest ook op woensdag doorgaan. Bedankt dat dan er altijd iemand van jullie voor mijn cellen heeft gezorgd. De NTX-labborrels waren een aangename afwisseling op de experimenten en het typen. Remco, ik was 'part of the deal' bij je aanstelling, nochtans is onze samenwerking en je begeleiding me prima bevallen. Ik heb veel van je geleerd, en kijk ernaar uit om samen te werken binnen de neurotoxicologie. Gina, al vijf jaar zijn we kamergenoten. Bedankt voor de gezelligheid, de interessante gesprekken over maatschappelijke problemen en het aanhoren van mijn frustraties. Aart, bedankt voor je hulp bij elektronische en electrophysiologische problemen en je kritische blik op mijn statistische strategieën. Alfons, bedankt voor het meedenken over mijn experimenten en manuscripten.

Mede NTX-AIOs, *eerst was er één*... Harm, eerst stagestudent nu collega, bedankt voor je bijdrage aan dit proefschrift (hoofdstuk 8) en succes met je eigen project. Laura, koningin van het controle-experiment, veel succes! Elsa, stralende grensvlak-AIO, veel succes! Jacub, dagenlang hebben we rug-aan-rug zitten zwoegen aan papers en imaging-analyses, veel succes! Hester, ik ben erg benieuwd naar wat je gaat vinden in je onderzoek naar alternatieve vlamvertragers, veel succes!

Alle (ex-)AIOs en collega's van het IRAS, bedankt voor alle gezelligheid, tijdens de lunch, cursussen, labuitjes, op congres en buiten werktijd. Op deze plaats wil ik ook graag de collega's van secretariaat, financiën en management bedanken voor hun onmisbare ondersteuning.

Kim en Leonie, ik ben erg blij dat jullie mijn paranimfen willen zijn. Bedankt voor alle hulp en steun.

Caroline, Meike en Leonie, lieve meiden, mede-carrièrevrouwen, wanneer krijgen we het nou minder druk? Het is weer tijd voor een meidenavondje! Ook Marlieke, Miriam, Tom, Joris en Pepijn, bedankt voor de broodnodige ontspanning buiten werktijd.

Lieve familie, schoonfamilie en (aanstaande) schoonzusjes, bedankt voor jullie gezelligheid en interesse in mijn werk.

Pieter, Robert, Steffen en Sander, lieve broers, ik ben nu klaar met mijn studies. Pieter en Robert, succes met het afstuderen in het komende jaar, ik ben trots op jullie. Steffen en Sander, eerst maar eens die middelbare school, kies daarna een richting op school of in werk waar je interesse én hart ligt. Veel succes!

Pa en ma, bedankt voor alles.

Tom, de afgelopen maanden heb ik sterk op je geleund. Ik dank je. Voor alle zorg en zekerheid die je verschaft voor mij en onze zoons. Voor het onbeschaamd te kunnen aanschuiven aan het avondeten en daarna weer te verdwijnen om te typen. Voor onze gedeelde zwarte humor. Laten we klinken op nog vele jaren.

Lieve Eric, lieve Chris, mama heeft een boekje geschreven. Maar vanavond lezen we weer ‘Nijntje’, hoor.

Milou.