

propensity for absorption in soil, sediment and air particles, rather than dissolving in water (Darnerud et al., 2001). The environmental distribution and partitioning of PBDEs varies according to their individual physicochemical properties. More highly brominated and heavier congeners, such as deca-BDE, tend to stay in soil, sediments and sewage sludge, whereas the lighter and less brominated congeners, such as penta-BDE, are deposited in plants and can become volatile again and bio-accumulate up the food chain.

Table 1.1 Substitution pattern of PBDE congeners and composition of comercial mixtures

Congener	Bromine substitution	Congener	Bromine substitution
BDE-17	2,2',4-tri BDE	BDE-183	2,2',3,4,4',5',6-hepta BDE
BDE-28	2,4,4'-tri BDE	BDE-184	2,2',3,4,4',6,6'-hepta BDE
BDE-47	2,2',4,4'-tetra BDE	BDE-191	2,3,3',4,4',5',6-hepta BDE
BDE-49	2,2',4,5'-tetra BDE	BDE-196	2,2',3,3',4,4',5,6'-octa BDE
BDE-66	2,3',4,4'-tetra BDE	BDE-197	2,2',3,3',4,4',6,6'-octa BDE
BDE-85	2,2',3,4,4'-penta BDE	BDE-203	2,2',3,4,4',5,5',6-octa BDE
BDE-99	2,2',4,4',5-penta BDE	BDE-206	2,2',3,3',4,4',5,5',6-nona BDE
BDE-100	2,2',4,4',6-penta BDE	BDE-207	2,2',3,3',4,4',5,6,6'-nona BDE
BDE-153	2,2',4,4',5,5'-hexa BDE	BDE-208	2,2',3,3',4,5,5',6,6'-nona BDE
BDE-181	2,2',3,4,4',5,6-hepta BDE	BDE-209	2,2',3,3',4,4',5,5',6,6'-deca BDE
Comercial mixtures		Composition	
DE-71	44 % BDE-99, 32 % BDE-47, 9 % BDE-100, 4 % BDE-153		
DE-79	37 % BDE-183, 22 % BDE-197; 14 % BDE-207, 9% BDE-196		
DE-83	97-98 % BDE-209, ≤ 3 % (BDE-206,BDE-207or BDE-208)		

1.2.2 Environmental sources

PBDEs enter the environment as emissions from primary production, from manufacturing plants that incorporate them into their products, and from the degradation or combustion of the products in which they are used. They are not chemically bound to the flame materials and are prone to leaching out and becoming volatile. Once released, they can be transported in the air to remote areas, far away from source points (Corsolini et al., 2006; Ikonoumou et al., 2002).

Despite the bans and restrictions on the use and production of PBDEs (COP4, 2009), elevated quantities of these compounds can be still found in the environment. The current emissions primarily come from the degradation of appliances containing PBDEs that remain functional in many homes and worksites (Jones-Otazo et al., 2005). Inadequate waste disposal and waste management of the products has also contributed as a source in the environment (Costa et al., 2008; Hites, 2004).

1.2.3 Routes of human exposure and levels

PBDEs have a hydrophobic chemical structure that allows their accumulation in lipid rich tissue and their bio-magnification across the trophic chain. Currently, there are concentrations of these pollutants present in all ecosystems, including remote regions such as the Arctic or the Antarctic (Costa et al., 2008; Darnerud, 2003).

PBDEs may enter the human body through exposure to indoor air, indoor dust and dermal uptake but the consumption of contaminated food is considered the principal route of entry (Domingo, 2004; 2012). Dietary intake of PBDEs was generally highest for fish, seafood, meat, cheese and oils.

On the other hand, the degradation of PBDE contained in products that remain functional in many homes and worksites contributes to increased concentrations of these compounds in the indoor air, which can be higher than the concentrations found outdoors (Jones-Otazo et al., 2005). The inhalation of contaminated air and dust containing these flame-retardant products could play a key role in human exposure. Several studies report a positive correlation between PBDE levels in human serum and the levels of PBDE in dust particles from homes and offices (Johnson et al., 2010; Stapleton et al., 2012).

The level of human exposure to PBDEs can be estimated by the sum of all the concentrations of the different congeners analyzed in serum (Sjödin et al., 2004; Thomsen et al., 2002) and breast milk samples (Akutsu et al., 2003; Fängström et al., 2008; Schuhmacher et al., 2009). It is interesting to note that levels in serum samples from people living in North America can be 10–70 times higher than in people living in Europe or Japan (Schechter et al., 2005). Moreover, despite the production of penta-BDEs having been stopped in 2004, the congeners present in the penta-BDEs mixtures still remain the highest in human samples than deca-BDEs. One reason could be that lower-brominated PBDEs are more bio-accumulative and persistent in the environment than the more highly brominated varieties. On the other hand, the deca-BDE mixture, which mainly contains BDE-209 has been reported to undergo degradation into lower-brominated congeners influenced by microbes or UV/sun-light, thereby increasing the environmental burden of lower-bromated congeners (Gerecke et al., 2005).

1.3 DISPOSITION AND METABOLISM OF PBDEs

Research in rodents has studied the tissue accumulation, metabolism and excretion of PBDEs. Staskal et al. (2006) administered intravenously a single dose of 1 mg/kg of different radio-labelled congeners of [¹⁴C] penta-BDEs to female C57BL/6 mice. The authors found that the measure of the residual radioactivity was highest in fat, muscle, skin and liver, while lungs, kidney, blood, and brain had less than 0.5% of the dose. Liver and fat has also been found to be the main target organs of PBDEs in other studies (Riu et al., 2008; Sanders et al., 2006). Staskal et al. (2006) observed how the urinary excretion patterns were inversely related to disposition patterns in tissues; congeners with the lowest tissue concentration had the highest urinary concentration. That study also reports that faecal excretion was higher than urinary excretion and almost 80% of

the faecally-derived radioactivity from PBDEs was identified as metabolites within the first 24 h. In addition, metabolic studies carried out in rats and mice treated with radio-labelled BDE-47, -99, and -100 resulted in detection of different hydroxylated metabolites of tetra- and tri- brominated congeners (Hakk and Letcher, 2003, Malmberg et al., 2005; Marsh et al., 2006; Sanders et al., 2006).

Liver is a primary target organ for PBDEs and is mainly responsible for their metabolism. Phase I, II and III xenobiotic metabolizing enzymes (XMEs) play a fundamental role in the metabolism and elimination of xenobiotics. XMEs can be present at the basal level and/or induced or inhibited after exposure to xenobiotics via activation of a variety of nuclear receptors including aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane xenobiotic receptor (PXR) (Tompkins and Wallace, 2007; Xu et al., 2005).

1.3.1 Nuclear receptors and xenobiotic metabolism enzymes

XMEs play a significant role in metabolism, removal and detoxification of various foreign substances, including environmental chemicals such as PBDEs from the human body. Most tissues, particularly liver tissues, contain different XMEs, known as phase I and phase II metabolizing enzymes and phase III transporters. In general, these enzymes are in an unstimulated state or at basal expression level and are activated upon exposure to xenobiotic chemicals. Phase I are an important set of enzymes involved in the hydrolysis, reduction and oxidation of xenobiotic chemicals, and most of these enzymes belong to the cytochrome P450 superfamily (CYP). CYP enzymes are mainly responsible for oxidative metabolism of a wide range of both exogenous and endogenous xenobiotics. Through the oxidative process, CYP enzymes convert these xenobiotics into hydrophilic molecules in order to excrete them from the body more

easily. Additionally, in the process of xenobiotic metabolism, some chemicals such as polyaromatic hydrocarbons (PBDEs, PCBs, and dioxins) can undergo bio-activation into highly reactive intermediates, which can cause genotoxicity and carcinogenesis.

1.3.2 Aryl hydrocarbon receptor dependent metabolism

Some *in vitro* studies conducted in rat hepatoma and human colon cells suggest that PBDEs may contain an AhR agonist (Chen et al., 2001; Hamers et al., 2006). Some rodent studies have indicated an AhR dependent activity (Chen and Bunce, 2003; Peters et al., 2004); however, studies by Sanders et al. (2005), confirmed that the agonistic activity might be due to dioxin-like impurities present in the technical mixtures such as DE-71; and PBDEs alone may not induce CYP1A enzymes. Also molecular structure calculations specify that PBDEs are non-coplanar in structure, demonstrating no binding potential to AhR (Wang et al. 2005).

1.3.3 Pregnane x receptor and constitutive androstane receptor dependent mechanism

PXR is a transcription factor that is highly expressed in liver, intestine and colon, and its main target gene is CYP3A. Due to its ambiguous and spherically-shaped ligand-binding pocket, it can bind to a wide-range of structurally different substrates, such as antibiotics, glucocorticoids, phenobarbitals and so many other pharmaceutical drugs. In mechanisms based on ligand activation/binding, PXR attaches to the 9-cis retinoic acid receptor (RXR) and forms a heterodimer, which in turn translocates to the nucleus and binds to hormone responsive elements (HREs) at the 5'-promotor region of the CYP3A genes (Sladek and Giguere, 2000). CAR has considerable similarity to PXR in ligand specificity, and needs RXR to form a heterodimer upon ligand-binding; the target genes belong to the CYP2B family. PXR and CAR are also responsible for the induction of phase II enzymes, uridine diphosphate glucuronyltransferase (UDP-GT), sulfotransferases (SULT) and

glutathione-S-transferase (GST), and transport proteins MDR1, MRP2, MRP3, and Oatp2 (Kretschmer and Baldwin 2005). In a manner similar to AhR, bioactivation of certain xenobiotic substances can lead to toxicity and disease. A high activity of both may induce effects such as liver carcinogenesis, thyroid homeostasis and thyroid neoplasia (Eriksson et al., 2001, 2002; Meerts et al., 2000). Literature to date indicates that PBDEs, predominantly the lower-brominated congeners, can induce toxicity and mainly through the PXR/CAR dependent mechanism.

1.4 POTENTIAL HEALTH RISK OF PBDEs

Over the past 20 years, concentrations of PBDEs have been increasingly contaminating human blood and breast milk samples (Sjödin et al., 2004; Thomsen et al., 2002) and breast milk samples (Akutsu et al., 2003; Fångström et al., 2008). The bioaccumulation of PBDEs in women is a particular concern because their availability increases during the period of pregnancy and lactancy and these compounds can be transferred to their infants through the placenta and breast milk (Gomara et al., 2007; Schuhmacher et al., 2009). Exposure levels in children have been calculated to be 3-6 times higher than in adults. For these reasons, PBDEs are still the focus of several studies nowadays.

1.4.1 *Animal and in vitro studies*

Animal and *in vitro* studies have described endocrine disruption, neurotoxicity, reproductive damage, foetotoxicity and hepatotoxicity as the main toxic effects of PBDEs. Different PBDEs appear to have similar toxicological profiles, with deca-BDEs being less potent than other lower-brominated congeners. Recent studies have shown that during its development the brain is vulnerable to degradation from xenobiotics

compounds like PBDEs or its metabolites. For these reasons, several studies have focussed their attention on the neurodevelopmental changes induced by PBDEs.

In rodent experiments, pre- and post-natal exposure to PBDEs has caused long-lasting changes in spontaneous behaviour, hyperactivity and has disrupted performance in learning and in memory tests (Branchi et al., 2005; Dufault et al., 2005; Eriksson et al., 2001, 2002; Kuriyama et al., 2005; Lilienthal et al., 2006; Rice et al., 2007; Viberg et al., 2002, 2004a, 2004b, 2005, 2006). The deregulation of the molecular mechanisms responsible for the normal process of neuronal neurotransmission and plasticity seems to be affected by neonatal exposure to PBDEs. Dingemans et al (2007) showed how the administration of a single dose of 6.8 mg/kg body weight (BW) of BDE-47 by gavage in mice on postnatal day (PND) 10 reduced the long-term potentiation (LTP) in the CA1 area of hippocampus. The authors also showed reduced levels of postsynaptic proteins involved in LTP as glutamate receptor subunits NR2B and GLuR1. Alternatively, the decrease in brain-derived neurotrophic factor (BDNF) and the increase in the calmodulin-dependent protein kinase II (CaMKII), GAP-43, synaptophysin and tau was observed in the hippocampus of mice treated with a single dose of 20.1 mg/kg BW of BDE-209 on PND 3 (Viberg et al., 2008; Viberg, 2009)

Several *in vitro* studies have provided a better compression of the neurotoxic action of PBDEs. Tagliaferri et al. (2010) and Yu et al. (2008) studied human neuroblastoma cells and found that they coincided with the increase in oxidative stress after the treatment of the congeners BDE-47, BDE-99 and the commercial mixture DE-71. Separately, Yu et al. (2008) reported a dose-dependent increase in lactate dehydrogenase leakage, apoptosis, DNA degradation in the cell cycle, an increase in the induction of the activity of caspase 3, 8 and 9, an increase in the intracellular concentration of calcium, the bax translocation to the mitochondria and the release of

the cytochrome c. Furthermore, an increase in reactive oxygen species (ROS) has been reported from several animal and *in vitro* studies (Cheng et al., 2009; Souza et al., 2013).

In vitro and *in vivo* investigations have described PBDEs as being able to deregulate thyroid hormone (TH) signalling homeostasis at multiple mechanistic levels (Kuriyama et al., 2007; Zhou et al., 2002) and can intercede in the biosynthesis of steroid hormones (SH) (He et al., 2008). In particular, the endocrine disruption thyroid hormone actions seem to be mainly responsible for the neurobehavioural deficiencies caused by PBDEs. Alterations in THs during the fetal or neonatal period cause striking abnormalities in neuronal migration and differentiation, and can induce neuronal cell death (Anderson, 2001). Bioactivation by oxidative metabolism adds considerably to the neurotoxic potential of PBDEs. The structure of hydroxylated congeners of PBDEs (HO-PBDEs) is more similar to the structures of THs and has a stronger disruptive action of their hormone signalling.

Contrarily, the disruption of SH seems to be responsible for alterations and damage in the reproductive system after exposure to PBDEs. Both Lilienthal et al. (2006) and Talsness et al. (2005) showed that prenatal exposure to BDE-99 decreased the levels of circulating sex steroids (17 β -estradiol and testosterone) in rat offspring. Talsness et al. (2005) also describes ultrastructural changes in the ovaries of F1 offspring whose dams (F0) were treated orally with 60 or 300 μ g/kg BW of BDE-99 on gestational day (GD) 6. The external and skeletal anomalies in offspring of F2 from two different dams of F1 were also described. Kuriyama et al. (2005) found that the exposure to low dose of PBDE-99 during development caused reduced sperm and spermatoid counts and gender-dependent alterations in sweet preference, paralleled by changes in sex

hormones have also been reported following exposure to BDE-99 (Lilienthal et al., 2006).

The liver is a target organ of PBDEs. The most common toxicity endpoints induced by PBDEs are hepatocellular hypertrophy and induction of xenobiotic metabolism enzymes (Lee et al., 2010; Sanders et al., 2005; Szabo et al., 2009). Zhou et al. (2001) treated female Long-Evans rats (PND 28) with technical mixtures DE-71, DE-79 or DE-83R for up to 4 days. DE-71 and DE-79 induced liver enlargement, hepatic ethoxyresorufin-O-deethylase, pentoxyresorufin-O-deethylase, and UDP-GT activities. Shao et al. (2008) exposed liver hematopoietic stem cells to single congener BDE-47 and observed a loss of mitochondrial membrane potential and initiation of apoptosis at low micromolar range. Similar results were observed in rainbow trout gill cells exposed to BDE47 by Shao et al. (2010). These researchers have shown that induction of apoptosis likely originates from a disruption of cellular redox status and mitochondrial oxidative injury.

1.4.2 Human studies

Despite the limitation in the collection of samples, recent epidemiological studies have correlated higher concentrations of PBDEs in cord blood and maternal milk with lower birth weight (Foster et al., 2011), damage to the reproductive system (Main et al., 2007), worse neurophysiological development and increased activity/impulsivity behaviours in toddlers (Eskenazi et al., 2013; Gascon et al., 2012; Herbstman et al., 2010; Hoffman et al., 2012; Roze et al., 2009; Shy et al., 2011).

1.5 THYROID HORMONES

THs are important throughout all stages of life and act on nearly every cell in the body. They are important for developmental processes and the maintenance of homeostasis. Their release is controlled by the neuroendocrine system and is tightly regulated through feedback mechanisms. They are released from the thyroid gland, a butterfly shaped endocrine organ located in the anterior side of the neck. The hypothalamus releases thyrotropin-releasing hormone (TRH) in humans, which stimulates the anterior pituitary gland to release thyroid-stimulating hormone (TSH), also known as thyrotropin. TSH stimulates the thyroid gland to release triiodothyronine (T3) and tetrayodotironina (T4), T4 being the main hormone produced by the thyroid gland. T4, the transport form, is converted by the deiodinase enzymes to T3, the effector or more biologically active form, in peripheral tissues. Once a TH is released, it will not only act on its target tissues but it will also signal the pituitary gland and hypothalamus to stop releasing TRH, and TSH, respectively. This negative feedback ensures that the levels of TH are tightly regulated.

THs play a critical role in maintaining homeostasis and metabolic processes. They regulate the metabolism of fats, proteins, and carbohydrates as well as body temperature. THs are important for the normal growth and development of many organs, including the brain and heart, play an important role in puberty, and are needed for the proper development of the gonads. Perhaps the most important role THs play is during embryo development and during the few critical months after birth. THs are critical for proper brain development of the foetus and a deficiency in THs can lead to irreversible neurological defects and mental retardation (de Escobar et al., 2007; Hetzel and Dunn, 1989; Hetzel and Mano, 1989; Morreale de Escobar et al., 2004; Ohara et al., 2004; Porterfield and Hendrich, 1993).

There is very little free circulating THs in the blood plasma. Most THs are bound by binding proteins (THBP). THBPs include serum albumin, thyronine binding globulin (TGB), transthyretin (TTR), and lipoproteins. THBPs play many important roles in regulating THs. They act to increase the lifespan of THs, as a buffer for TH levels, and a reserve for THs. TBG is the main THBP in humans and carries both T3 and T4, while in rodents THBPs are serum albumin and TTR, which only carry T4.

1.5.1 *Thyroid hormone metabolism*

The levels and actions of THs are regulated in three ways: 1) by synthesis and secretion of THs in the thyroid gland, 2) by proteins that bind THs and control the levels of free THs, and 3) by metabolic enzymes that act on THs to either activate or inactivate it.

T4 is the predominant hormone released from the thyroid gland although there is a small amount of T3, which is the more biologically active. About 30% of the T3 is released from the thyroid gland and the other 70% is produced in the target tissues' cells from T4 (Utiger, 1995). T4 is converted to T3 by the enzyme 5'-deiodinases, type I (DIOI) and type II (DIOII). THs are removed from the plasma by UDP-GT (DeVito et al., 1999). UDP-GT is present in the liver and it glucuronidates THs and causes them to be eliminated in bile.

1.5.2 *Thyroid hormone receptors*

The actions of TH are largely facilitated through the nuclear thyroid hormone receptor (TR), a member of the nuclear hormone superfamily (Buchholz et al., 2006; Oppenheimer et al., 1979; Yen et al., 2006). The TR is located in the nucleus and binds chromatin in both the absence and presence of TH. T3 binds TR with a 5-10 fold higher

affinity than T4. T4 is thought to only have biological effects once it is converted into T3 by DIOII, because inhibition of DIOII has been shown to also inhibit the biological effects of T4. However, there is evidence that T4 has its own unique effects independent of conversion to T3 in the brain (Helbing et al., 2007).

In humans and mice, the TR α locus encodes four proteins but only one, TR α 1, is the true nuclear receptor. TR α 2, TR $\Delta\alpha$ 1, TR $\Delta\alpha$ 2 are antagonists of TR α 1 because these isoforms bind to DNA but not to TH, which is a mechanism to suppress the expression of genes containing the thyroid hormone response element (TRE). (Plateroti et al., 2001). The TR β locus encodes 2 receptors: TR β 1 and TR β 2. TR β 1 and TR β 2 differ in length in the amino-termini. The TR β locus in rats also encodes TR β 3 and TR $\Delta\beta$ 3 isoforms (Flamant and Samarut, 2003).

The TRs have five domains, A/B, C, D, E, and F, as listed from N-terminal to C-terminal end. There is a high-degree of conservation between the TR and other members of the nuclear hormone superfamily with differences in the hormone-binding domain (Zhang and Lazar, 2000). The A/B domain, also known as the activation function (AF-1) domain, is variable in sequence (comparing TR α) and length (comparing TR β isoforms). The AF-1 can function to recruit coactivators independent of T3. The C domain is the DNA binding domain (DBD) and is the most highly conserved domain among other nuclear hormone receptors. The DBD, which also serves as the dimerization domain, has 2 adjacent zinc fingers, which in turn contain 2 histidine and 2 cysteine residues. The DBD binds to TRE in the promoter or enhancer regions of TH-responsive genes (Yen, 2001). The D domain is the variable hinge region but also carries the nuclear localization signal as well as transactivation and DNA binding functions. The E domain, along with the F domain, is the ligand-, or hormone-, binding domain (LBD) and is highly conserved among TRs in different species but has a low

level of homology among other members of the nuclear hormone receptor superfamily. The E domain functions in transcriptional activation and repression (Yen, 2001).

TRs can bind with DNA as monomers, homodimers with each other, or heterodimers with RXRs. There are three RXRs: RXR α , RXR β , and RXR γ . The TR-RXR heterodimer is considered the most stable and the facilitator of TH genomic action. TR-RXR specifically recognizes the TRE and interacts with transcription factors (Wong et al., 1995). TRs bind DNA in both the presence and absence of ligand and, unlike some other members of the steroid/TH nuclear receptor family, have a dual function in the activation and repression of genes depending upon TH status. TH can both up-regulate and down-regulate genes. Up-regulated TH responsive genes are repressed in the absence of TH, and activated upon the addition of TH. The binding of TR-RXR to TRE is independent of the presence of TH. In the absence of TH, the TR-RXR heterodimer is bound to the TRE and also interacts with co-repressors, such as silencing mediators of receptors of TH (SMRT) and nuclear receptor co-repressor (N-CoR). SMRT and N-CoR interact with the LBD of both the TR and RXR and have been shown to form a complex that binds to transcriptional repressor Sin3A, which in turn interacts with histone deacetylases (HDACs). The activity of the HDACs leads to a more compact chromatin structure, which can inhibit transcription (Yen, 2001; Yen et al., 2006).

When TH are present, they bind to the TR and induce a conformational change in the protein. Upon TH binding, co-repressors are released and co-activators are recruited, such as camp response element binding protein (CBP/p300), steroid receptor co-activators (SRC/p160), p300/CBP-associated factor (P/CAF) (Yen, 2001; Bassett et al., 2003). The AF-2 domain plays a critical role in interacting with the co-activators. These co-activators have intrinsic histone acetyltransferase (HAT) activity and can increase gene transcription. Other co-activators that do not have intrinsic HAT activity are TR-

4. RESULTS

4.1 BDE-99 deregulates BDNF, Bcl-2 and the mRNA expression of thyroid receptor isoforms in rat cerebellar granular neurons.

Blanco, J., Mulero, M., Lopez, M., Domingo, J. L., and Sanchez, D. J..

Toxicology (2011) 290, 305-311.



BDE-99 deregulates BDNF, Bcl-2 and the mRNA expression of thyroid receptor isoforms in rat cerebellar granular neurons

Jordi Blanco^{a,b}, Miquel Mulero^c, Marta López^d, José L. Domingo^a, Domènec J. Sánchez^{a,b,*}

^a Laboratory of Toxicology and Environmental Health, School of Medicine, IISPV, Universitat Rovira i Virgili, Sant Llorens 21, 43201 Reus, Spain

^b Physiology Unit, School of Medicine, IISPV, Universitat Rovira i Virgili, Sant Llorens 21, 43201 Reus, Spain

^c Nutrigenomics Group, Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Marcel·lí Domingo, s/n, Campus Sescelades, 43007 Tarragona, Spain

^d Pharmacology Unit, School of Medicine, IISPV, Universitat Rovira i Virgili, Sant Llorens 21, 43201 Reus, Spain

ARTICLE INFO

Article history:

Received 16 September 2011

Received in revised form 13 October 2011

Accepted 13 October 2011

Available online 18 October 2011

Keywords:

Polybrominated diphenyl ethers (PBDEs)

BDE-99

Thyroid receptors

Brain-derived neurotrophic factor (BDNF)

Bcl-2

Reactive oxygen species (ROS)

ABSTRACT

Although the disruption of thyroid hormone (TH) signaling can largely explain the neurotoxic effects of polybrominated diphenyl ethers (PBDEs), there are still many unknowns about how this interference occurs. In this study, we expose a primary culture of rat cerebellar granule neurons (CGNs) to a 25 μ M concentration of one of the most prevalent PBDE congeners in humans, 2,2',4,4',5-pentaBDE (BDE-99). The main goal was to investigate the time course of BDE-99 toxicity in relation to the disruption of thyroid receptor (TR) function over 24 h. In a first stage, we found that BDE-99 directly down-regulated the transcription of the isoforms TR- α 1 and TR- α 2, which may be a consequence of a hypothetical state that mimics hyperthyroidism. In a later stage, BDE-99 disrupted the expression of triiodothyronine (T3)-responsive genes, possibly as an effect of its metabolism. A down-regulation of the expression of the T3-mediated neurotrophin brain-derived neurotrophic factor (BDNF) and the anti-apoptotic Bcl-2 protein was also observed. Down-regulation of these two proteins was correlated with an increase in the production of reactive oxygen species (ROS). It was also found that expression of the TR- β 1 isoform, which is normally transcriptionally repressed by T3 in CGNs, was up-regulated. This up-regulation could compensate the down-regulation of the TR- α 1 isoform, and thus slow down cell death. The dually disruptive action of BDE-99 might provide a better understanding of the potentially neurotoxic mechanism of PBDEs.

© 2011 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Poly-brominated diphenyl ethers (PBDEs), a family of 209 flame-retardant chemicals, have been extensively used as additives in a wide range of everyday products (Alaee et al., 2003). Despite bans and regulations implemented in recent years, PBDEs are now ubiquitous in all ecosystems due to intensive industrial use and inadequate waste disposal during the last three decades (Costa et al., 2008; Hites, 2004).

The rising levels of PBDEs in human tissue samples are clearly a matter for global concern. Their presence in pregnant women is particularly concerning because these accumulated compounds can be transferred to infants through the placenta and breast milk (Gomara et al., 2007; Schuhmacher et al., 2009) and can interfere with critical stages of brain development. Neonatal exposure in rodents has been shown to cause long-lasting changes in the

cholinergic system (Johansson et al., 2008), long-term potentiation (Dingemans et al., 2007), and spontaneous behavior (Viberg et al., 2006; Branchi et al., 2002). Furthermore, epidemiological studies in humans have shown a correlation between adverse cognitive and neurodevelopment effects in toddlers and high levels of PBDEs in maternal serum and cord blood (Roze et al., 2009; Herbstman et al., 2010).

The mechanisms by which PBDE can generate neurobehavioral deficiencies are still poorly understood. However, the ability of PBDEs to disrupt thyroid hormone (TH) signaling might explain their neurotoxic effects. Pre and post-natal exposures of rodents to PBDEs have shown to reduce concentrations of serum tetraiodothyronine (T4) (van der Ven et al., 2008; Kuriyama et al., 2007; Zhou et al., 2002). Alterations to TH during the fetal or neonatal period cause striking abnormalities in neuronal migration and differentiation, and can induce neuronal cell death (Anderson, 2001).

PBDEs and especially their hydroxylated metabolites, OH-PBDEs, are structurally similar to triiodothyronine (T3), the bioactive form of T4, and can compete to bind to nuclear thyroid receptors (TRs). The strength of their binding is congener-dependent. Only a few PBDEs/OH-PBDEs have sufficient affinity to displace T3 and deregulate TR-mediated gene expression (Kitamura

* Corresponding author at: Laboratory of Toxicology and Environmental Health, School of Medicine, IISPV, Universitat Rovira i Virgili, Sant Llorens 21, 43201 Reus, Spain. Tel.: +34 977 759374; fax: +34 977 759322.

E-mail address: domingo.sanchez@urv.cat (D.J. Sánchez).

et al., 2008; Kojima et al., 2009). Recent in vitro studies have demonstrated that after incubation with different PBDE congeners, TH-induced dendrite arborization was suppressed in Purkinje cells (Ibhazehiebo et al., 2011), while migration and differentiation were decreased in neural progenitor cells (Schreiber et al., 2010). However, when these cells were treated with T3 or T4, the adverse effects were reversed.

One of the key proteins regulated by TH that could be involved in all of these changes is the brain-derived neurotrophic factor (BDNF). This neurotrophin is essential for neuronal survival, morphogenesis and plasticity (Numakawa et al., 2010). Treatment with BDNF promotes the expression of cytoprotective proteins such as Bcl-2 (Manji et al., 2001; Wu et al., 2009). Bcl-2 regulates the membrane potential of mitochondria preventing pore formation and the release of apoptotic proteins and reactive oxygen species (ROS) into the cytosol, two of the principals routes by which PBDEs can induce neuronal death (He et al., 2008). To date, few experimental studies have evaluated the relationship between PBDEs, BDNF (Jiang et al., 2008; Viberg et al., 2008; Wang et al., 2011) and the expression of the TR isoforms (Lema et al., 2008; Wang et al., 2011).

In recent years, we initiated in our laboratory a wide program focused on assessing human dietary exposure to PBDEs and biological monitoring to these environmental pollutants (Bocio et al., 2003; Domingo, 2004; Domingo et al., 2008; Schuhmacher et al., 2007, 2009). We have also studied various issues concerning 2,2',4,4',5-pentaBDE (BDE-99) exposure in rats (Albina et al., 2010; Alonso et al., 2010; Belles et al., 2010), one of the most prevalent PBDE congeners in humans (Costa et al., 2008). In order to provide a better understanding of PBDE toxicity in general and of BDE-99 in particular, the present study was designed to assess the relation between this PBDE congener and the possible disruption of TR function in neuronal cells. For it, we exposed a primary culture of rat cerebellar granule neurons (CGNs) BDE-99, and cell viability was determined over 24 h. The effects of BDE-99 on the expression of mRNA encoding the isoforms of the TR genes and examined the correlation between ROS production and the expression of the TH-mediated genes BDNF and Bcl-2 over time were also investigated.

2. Materials and methods

2.1. Chemicals

BDE-99 (99% pure) was purchased from LGC Standards S.L.U. (Barcelona). 1-(β -D-arabinofuranosyl) cytosine hydrochloride (Ara-C), 3,3',5-triiodo-L-thyronine sodium salt (T3), dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium (MTT), 2- β -mercaptoethanol, and 2,7-dichlorodihydrofluorescein diacetate (DCFH₂-DA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) for cell culture were obtained from GIBCO (Life Technologies, Paisley, U.K.). Western blot analysis was performed using primary monoclonal antibodies against BDNF, Bcl-2 and β -actin and peroxidase-conjugated IgG as a secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The Alexa Fluor 488 anti-rabbit secondary antibody was purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Cerebellar granule cell culture

Primary cultures of CGNs were prepared from postnatal day 7 Sprague-Dawley rat pups according to Verdague et al. (2004). Isolated cerebella were separated from the vessels, minced, and incubated for 15 min at 37 °C in ionic buffer containing 0.025% trypsin and 0.05% deoxyribonuclease 1. Incubation was terminated by the addition of trypsin inhibitor (0.04%) and centrifugation. Cells were dissociated by repeated pipetting and separated from non-dissociated tissue by sedimentation. The cell density was adjusted to 1.2×10^6 cells/ml, and the cells were seeded in plates coated with poly-L-lysine (100 μ g/ml). Cultures were grown in DMEM supplemented with 10% heat-inactivated FBS, 0.1 mg/ml gentamicin, 2 mM L-glutamate and 25 mM KCl. Ara-C (10 μ M) was added to the culture medium 24 h after plating to prevent the proliferation of non-neuronal cells. The cultures were maintained at 37 °C in a humidified incubator with 5% CO₂ and 95% air. Cultures were assayed at 7 days in vitro, at which point they were fully differentiated and exhibited fasciculation of the fibers that interconnect the cells. For statistical analyses, cell cultures prepared on different days were considered as single experiments.

2.3. Treatment of CGNs

BDE-99 was dissolved in DMSO and diluted in DMEM to make a stock solution, which was added to the culture medium to obtain a range of concentrations of 5, 10, 25 and 50 μ M to determinate the progression of neuronal cell death in function of BDE-99 increment insult. In all treatments, the volume of DMSO was less than 0.1% of the total volume, being control cells exposed to the same DMSO vehicle dilution as BDE-99-treated cells. A range of final concentrations (10^{-9} to 10^{-5} M) of T3 was prepared by diluting a 10^{-3} M T3 stock in sterile medium. BDE-99 and T3 solutions were prepared before use and protected from light to prevent degeneration. To assess cell viability and ROS production, cells were seeded in 96-well plates and treated with BDE-99 for 3, 6, 12, and 24 h. Cell viability was also assessed 24 h after treatment with BDE-99 in combination with a range (10^{-9} to 10^{-5} M) of concentrations of T3. For RNA isolation, cells were seeded in 6-well plates and treated with BDE-99 for 3, 6, 12, and 24 h. For Western blotting, cells were seeded in 6-well plates and treated with BDE-99 for 12 and 24 h in presence or absence of 10^{-5} M T3. For immunocytochemistry assays, cells were seeded in 24-well plates with sterile glass slides and treated with BDE-99 for 12 and 24 h.

2.4. Measurement of cell viability

Cell viability was quantified colorimetrically using the metabolic dye MTT. MTT was added to the cells at a final concentration of 250 μ M and incubated for 1 h. During that time, MTT was reduced to produce a dark blue formazan product. The medium was then removed and the cells dissolved in DMSO. Formazan production was measured by the change in absorbance at 595 nm using a microplate reader (BioTek Power Wave XS). The viability results are expressed as percentages, and the absorbance measured in the control cells was taken to be 100%.

2.5. ROS production

The levels of intracellular ROS were measured using the fluorescent probe DCFH₂-DA. Cells were pre-incubated for 30 min at 37 °C with 10 μ M DCFH₂-DA (added from a 20 mM stock solution in DMSO). DCFH₂-DA is readily taken up by cells being subsequently deesterified to 2',7'-dichlorofluorescein (DCFH₂). It is then oxidized by cytosolic ROS to dichlorofluorescein (DCF), which produces a fluorescent signal. After loading with the dye, cells were washed twice with Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO₃, 2.3 mM CaCl₂, 5.6 mM D-glucose and 5 mM HEPES, pH 7.4). The fluorescence was immediately read at an excitation wavelength of 488 nm and an emission wavelength of 525 nm using a microplate fluorometer (Fluoroskan Ascent® FL). Results were expressed as percentages, and the fluorescence measured in control cells was taken to be 100%.

2.6. RNA isolation and cDNA synthesis

Total RNA was obtained from CGNs using a Qiagen RNeasy kit (Qiagen Inc.) according to the manufacturer's protocol. RNA was resuspended in 100 μ L of RNase-free water, quantified by spectrophotometer at an absorbance of 260 nm, and tested for purity (by A260/280 ratio) and integrity (by denaturing gel electrophoresis). The first strand of cDNA was reverse transcribed from 1 μ g of total RNA from each sample using a QuantiTect Reverse Transcription Kit (Qiagen Inc.) according to the manufacturer's protocol. An identical reaction, without the reverse transcription, was performed to verify the absence of genomic DNA. The cDNA was subsequently used for PCR amplification using rat-specific primers for TR-alpha1 (NM.001017960) (forward: 5'-TGC CCT TAC TCA CCC CTA CA-3'; reverse: 5'-AAG CCA AGC CAA GCT GTC CT-3'), TR-alpha2 (NM.031134) (forward: 5'-TGA GCA GCA GTT TGG TGA AG-3'; reverse: 5'-GAA TGG AGA ATT CCG CTT CG-3'), TR-beta1 (NM.012672) (forward: 5'-AGC CAG CCA CAG CAC AGT GA-3'; reverse: 5'-CCG CAG AAG ACT GAA GCT TGC-3'); BDNF (NM.012513) (forward: 5'-CCA TAA GGA CGC GGA CTT GT-3'; reverse: 5'-GAG GCT CCA AAG GCA CTT GA-3'); Bcl-2 (NM.016993) (forward: 5'-GGA GCG TCA ACA GGG AGA TG-3'; reverse: 5'-GAT GCC GGT TCA GGT ACT CAG-3'), and β -actin (NM.031144) (forward: 5'-TGT CAC CAA CTG GGA CGA TA-3'; reverse: 5'-GGG GTG TTG AAG GTC TCA AA-3') with a PyroStart™ Fast PCR Master mix (2x) kit (Fermentas, CA) according to the manufacturer's protocol. PCR products were separated on a 1% agarose gel, and only a specific band was detected. The non-reactivity of the primers with contaminating genomic DNA was also tested by the inclusion of controls that omitted the reverse transcriptase enzyme from the cDNA synthesis reaction.

2.7. Real-time RT-PCR

Quantitative PCR for TR-alpha1, TR-alpha2, TR-beta1, BDNF, Bcl-2 and β -actin was performed using the QuantiTect SYBR Green PCR kit (Qiagen, Inc.) according to the manufacturer's protocol, being analyzed on a Rotor-Gene Q Real-Time PCR cyclers (Qiagen, Inc.). The thermal cycling comprised an initial step at 50 °C for 2 min, followed by a polymerase activation step at 95 °C for 15 min, and a cycling step with the following conditions: 40 cycles of denaturing at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Oligonucleotides of varying lengths produce dissociation peaks at different melting temperatures. Consequently, at the end of the PCR cycles, the PCR products were analyzed using a heat dissociation protocol to confirm that one single PCR product was detected by SYBR Green dye. Fluorescence

data were acquired at the 72 °C step. The threshold cycle (Ct) was calculated by the Rotor-gene Q 2.0 software to indicate significant fluorescence signals above noise during the early cycles of amplification. The software calculated copy numbers for the target samples from the Ct by interpolating from the standard curve. The relative levels of expression of the target genes were measured using β -actin mRNA as an internal control according to the $2^{-\Delta\Delta Ct}$ method.

2.8. Western blot analysis

Aliquots of cell lysate containing 30 μ g of protein per sample were analyzed by western blot analysis. Briefly, samples were placed in sample buffer (0.5 M Tris-HCl pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2- β -mercaptoethanol, 0.05% bromophenol blue), and denatured by boiling at 95–100 °C for 5 min. Samples were then separated by electrophoresis on 10% acrylamide gels. Proteins were subsequently transferred to Immobilon-P PVDF sheets (Millipore Corp., Bedford, MA) using a transblot apparatus (BioRad). The membranes were blocked for 1 h with 5% non-fat milk dissolved in TBS-T buffer (50 mM Tris, 1.5% NaCl, 0.05% Tween 20, pH 7.5). They were then incubated overnight with primary monoclonal antibodies against BDNF, Bcl-2, or β -actin. The blots were washed thoroughly in TBS-T buffer and incubated for 1 h with a peroxidase-conjugated IgG antibody. Immunoreactive proteins were visualized using an Immun-Star Chemiluminescence kit (BioRad) according to the manufacturer's instructions. Digital images were taken with a Versadoc (BioRad), which permits semi-quantification of the band intensity. The protein load was periodically monitored via the immuno-detection of actin.

2.9. Immunocytochemistry assays

B65 cells were grown on sterile glass slides. After stimulation, cells were washed twice in PBS and fixed in 4% paraformaldehyde/PBS, pH 7.4, for 1 h at room temperature. Cells were pre-incubated for 30 min at room temperature with PBS containing 0.3% Triton X-100 and 10% FBS. After blocking, the cells were incubated overnight at 4 °C with an antibody against either BDNF or Bcl-2, both of which were used at a 1:100 dilution in PBS containing 0.3% Triton X-100 and 5% FBS. The cells were then extensively washed and incubated with Alexa Fluor 488 goat anti-rabbit antibody for 2 h at room temperature. The coverslips were thoroughly washed and mounted in Fluoromount G. Immuno-signal analysis was performed using a Nikon TE2000-E microscope, and digitized images were captured.

2.10. Statistical analysis

Data were evaluated by one-way ANOVA followed by Bonferroni's post hoc test (with the exception of mRNA expression, which was evaluated by one-way ANOVA followed by Duncan's test) to identify significant differences between the controls and treatments. The Graphpad Prism 5 software was used. Differences were considered significant when *P* values were less than 0.05. Results are displayed as the mean \pm SD of at least three independent experiments. The relationships among the examined endpoints were assessed by correlation analysis using the Pearson method.

3. Results

3.1. Time course of effects of BDE-99 on cell viability and ROS production

In contrast to previous *in vitro* studies performed with neurotoxic compounds such as MPP⁺ (1-methyl-4-phenylpyridinium) (Offen et al., 2000a, b) or L-glutamate (Zhao et al., 2009), in order to determine the neuroprotective effect of T3 front BDE-99 insult we here used a BDE-99 concentration close to the 50% of cell death (25 μ M, Fig. 1). Time course of cell viability was assessed after exposure of CGNs to BDE-99 (25 μ M) (Fig. 2 A). A significant (*P* < 0.0001) decrease was observed relative to control cells at 3 (14 \pm 4%), 6 (24 \pm 4%), 12 (48 \pm 3%) and 24 h (58 \pm 3%). At those times, ROS production was quantified by the fluorescence intensity of the oxidized product of DCFH₂-DA. A significant (*P* < 0.0001) increase in the fluorescein signal generated by intracellular oxidative stress was seen at 12 (2322 \pm 471%) and 24 h (3350 \pm 612%) (Fig. 2B). These findings indicate that the cytotoxicity of BDE-99 developed gradually during the first 12 h showing a significant correlation between ROS production and cell death (*R* = 0.9410, *P* = 0.0062).

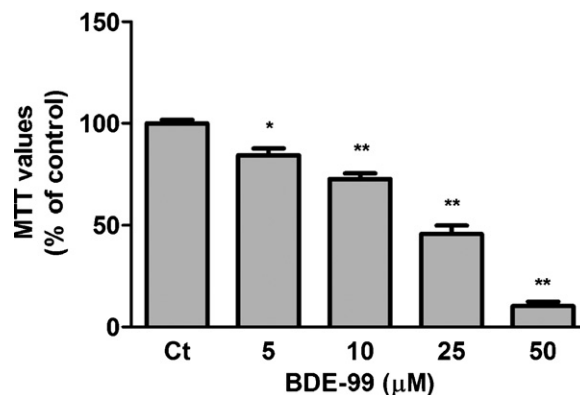


Fig. 1. Evaluation on cerebellar granular cells viability after 24 h of exposure to BDE-99 at 5 μ M, 10 μ M, 25 μ M and 50 μ M. Data are expressed as the mean \pm SD of four independent experiments. Statistical differences relative to the control (0 h) were analyzed by one-way ANOVA followed by Bonferroni's post hoc test: **P* < 0.05 and ***P* < 0.001 vs. control.

3.2. BDE-99 deregulates expression of the TR gene products

The TR-alpha and TR-beta genes encode two functional isoforms of TR that are expressed in CGNs, TR-alpha1 and TR-beta1. TR-alpha also encodes TR-alpha2, a protein that although it is abundantly expressed in the brain, it cannot be considered a TR, because it does not have any T3 binding motifs (Cook and Koenig, 1990). Since

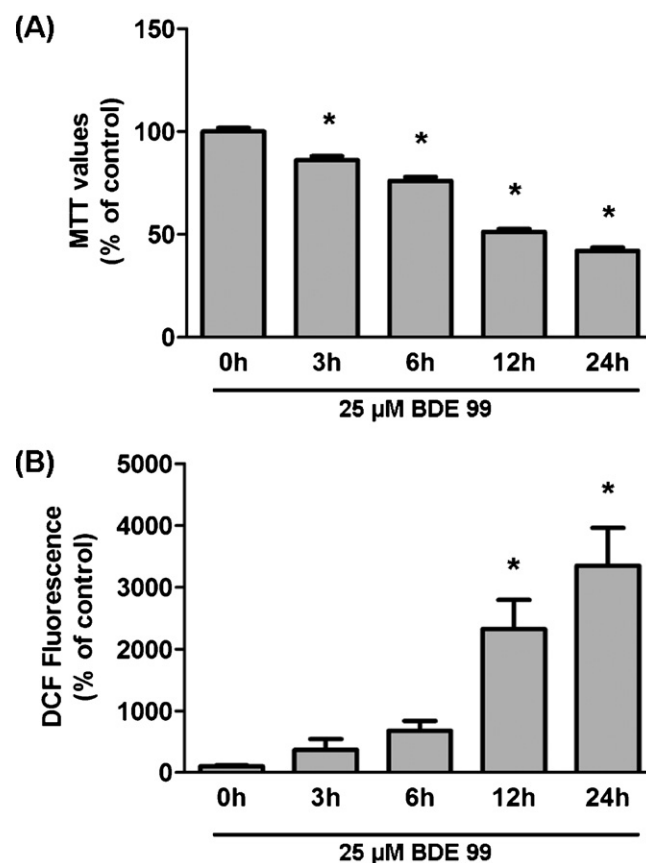


Fig. 2. Time-dependent effects of BDE-99 on cell viability and ROS production. CGN cells were exposed to 25 μ M BDE-99 for 3, 6, 12 and 24 h. (A) Cell viability and (B) the production of ROS were determined by the MTT method and by DCF fluorescent signal intensity, respectively. Data are expressed as the mean \pm SD of four independent experiments. Statistical differences relative to the control (0 h) were analyzed by one-way ANOVA followed by Bonferroni's post hoc test: **P* < 0.001 vs. control.

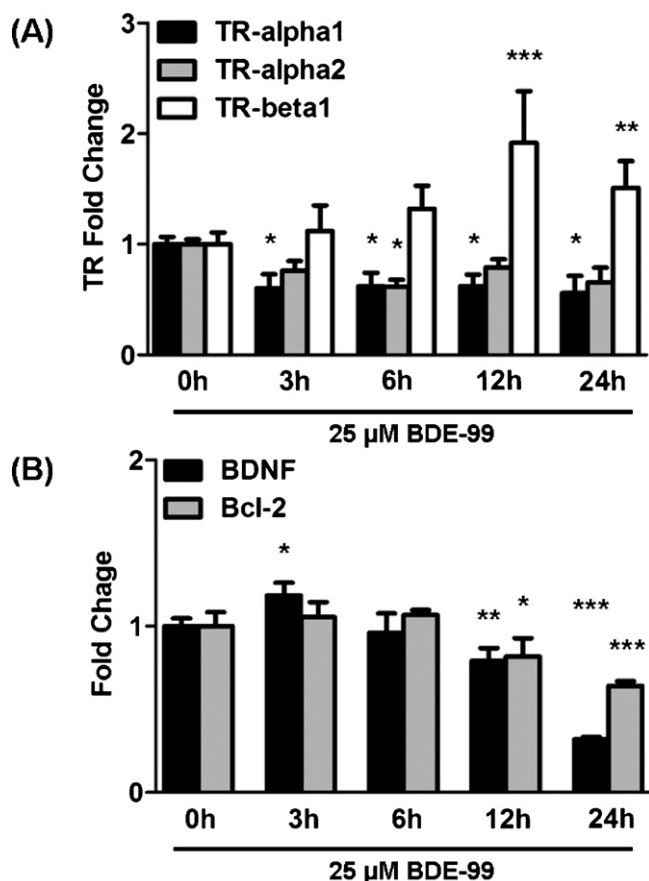


Fig. 3. BDE-99 deregulates the expression of the TR gene products and decreases the expression of BDNF and Bcl-2. CGN cells were exposed to 25 μ M BDE-99 for 3 h, 6 h, 12 h and 24 h. The relative expression levels of the mRNAs encoding (A) the TR-alpha1, TR-alpha2 and TR-beta1 isoforms, and (B) BDNF and Bcl-2 were determined by quantitative real-time RT-PCR assay. Data were normalized using the gene β -actin as an internal control. The fold changes were calculated relative to the control (0 h). Data are expressed as the mean \pm SD of four independent experiments. Statistical differences relative to the control were analyzed by one-way ANOVA followed by Duncan's post hoc test: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control.

there is little knowledge about the effects of PBDEs on TR gene expression, the time course of changes in the relative mRNA levels of these three isoforms was examined by real-time qPCR (Fig. 3A). CGN cells were exposed to BDE-99 (25 μ M) for 24 h. The expression of TR-alpha1 relative to control cells was found to be significantly ($P < 0.05$) down-regulated by approximately 40% at all time points measured. Similarly, transcript levels of TR-alpha2 were also down-regulated, but not at the same degree than TR-alpha1. A significant decrease ($P < 0.005$) was observed only at 6 h (39 \pm 6%). In contrast to TR-alpha1 and TR-alpha2, the expression of TR-beta1 at 12 h (92 \pm 46%, $P < 0.0001$) and 24 h (51 \pm 23%, $P < 0.005$) was significantly up-regulated. These results mean that the expression of the TR-alpha isoforms is directly deregulated by BDE-99 in CGNs, while TR-beta1 gene expression is affected at a later time.

3.3. BDE-99 decreases the mRNA expression of BDNF and Bcl-2

BDNF and Bcl-2 proteins are implicated in fundamental neuronal functions. Mice with these genes knocked out present severe neuronal deficiencies. Due to their biological importance, in the current study the mRNA expression of these genes (Fig. 3B) was evaluated under the same conditions than those previously used to evaluate the TR-alpha and TR-beta isoforms. The mRNA expression levels of BDNF at 12 (21 \pm 7%, $P < 0.001$) and 24 h (68 \pm 12%, $P < 0.001$), and Bcl-2 relative concentrations at 12

(21 \pm 11%, $P < 0.05$) and 24 h (36 \pm 3%, $P < 0.01$) were significantly down-regulated in CGNs after treatment with BDE-99 (25 μ M) relative to the control. Surprisingly, in comparison to control we observed a significant up-regulation of BDNF at 3 h (18 \pm 7%, $P < 0.05$). BDNF contributes to the expression levels of Bcl-2 (Manji et al., 2001). We detected a significant correlation ($R = 0.8832$, $P = 0.0176$) between the decreases in the expression levels of the two genes. We also observed that the decline in mRNA transcript levels occurred during the same time interval (6–12 h) for which increased ROS production had been previously seen. In addition, significant correlations between the time course of ROS production and the time courses of BDNF ($R = 0.9207$, $P = 0.0097$) and Bcl-2 expression ($R = 0.8561$, $P = 0.0243$) were noted. However, the correlations between the decrease in cell viability and BDNF ($R = 0.7024$, $P = 0.0763$) and Bcl-2 expression ($R = 0.7466$, $P = 0.0589$) were not statistically significant.

3.4. BDE-99 decreases the protein expression of BDNF and Bcl-2

In order to confirm that the changes in mRNA levels were relevant in CGNs, the relative protein levels under the same experimental conditions were evaluated by Western blot (Fig. 4A), being protein levels determined by the chemiluminescent intensities of their bands. Significant decreases relative to the control were observed for BDNF at 12 (44 \pm 16%, $P < 0.001$) and 24 h (65 \pm 12%, $P < 0.0001$), and for Bcl-2 at 24 h (35 \pm 5%, $P < 0.05$). These results corroborated that BDE-99 affects BDNF and Bcl-2 protein levels in CGNs. In turn, fluorescence immunocytochemistry images obtained after 24 h of treatment with BDE-99 (25 μ M) showed the global effect of these decreases (Fig. 4B).

3.5. T3 treatment partially rescues the cell death induced by BDE-99

PBDEs have been characterized as potential disruptors of TH signaling. On this basis, we treated CGN cells with a range of concentrations (10⁻⁹ to 10⁻⁵ M) of T3, with or without BDE-99 (25 μ M), and the viability of the CGNs after 24 h of exposure using the MTT method (Fig. 5A) was evaluated. Treatment with T3 did not show significant difference with the control. The viability of CGNs decreased (59 \pm 6%) after BDE-99 exposure. However, simultaneous treatment with BDE-99 and 10⁻⁶ M (21 \pm 5%, $P < 0.05$) or 10⁻⁵ M (28 \pm 6%, $P < 0.05$) T3 caused a significant increase in viability compared to treatment with BDE-99 alone. These results mean that some of the neurotoxic effects of BDE-99 can be attributed to T3 disruption.

3.6. Thyroid disruption by BDE-99 causes decreases in BDNF and Bcl-2

BDNF is regulated by TH, and a decrease in BDNF consequently leads to a decrease in Bcl-2 (Manji et al., 2001). In this case, we investigated whether treatment with T3 could restore the proteins that had previously been reduced by the action of BDE-99 to their pre-BDE-99 levels. CGN cells were exposed to BDE-99 (25 μ M) in combination with 10⁻⁵ M T3 (Fig. 5B). The additions of T3 had no significant effect when compared with the control, while the levels of BDNF and Bcl-2 at 12 and 24 h after exposure to BDE-99 coupled with T3, were not affected. It indicates that the TH-mediated gene expression of BDNF was disrupted by BDE-99.

4. Discussion

Despite the rather notable current information concerning toxic effects of PBDEs, a number of aspects on the mechanism by which these environmental pollutants can induce neuronal toxicity are

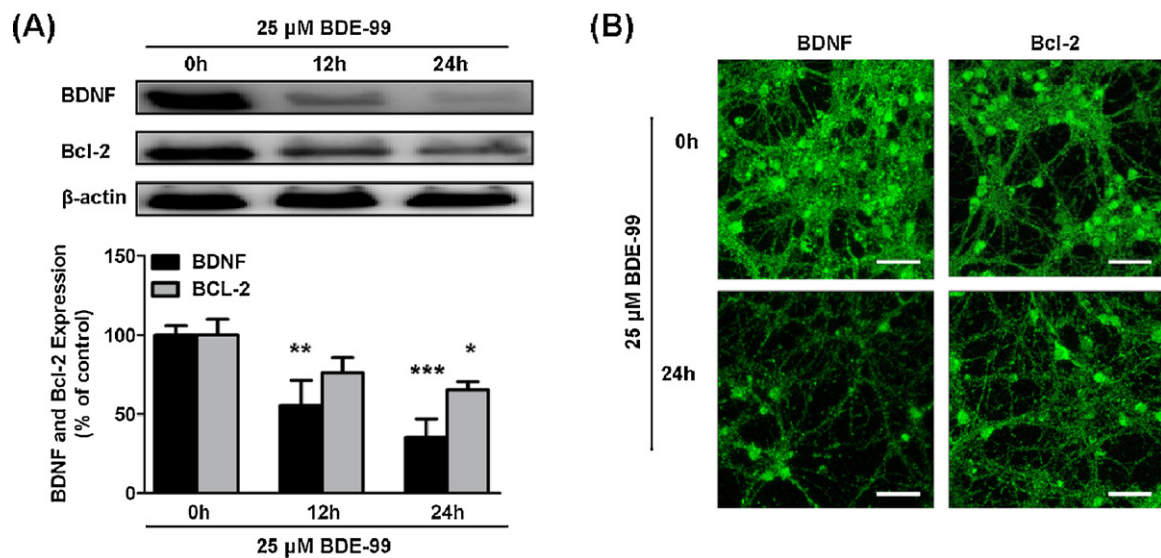


Fig. 4. BDE-99 decreases the expression of the BDNF and Bcl-2 proteins. (A) CGN cells were exposed to 25 μ M BDE-99 for 12 h and 24 h, and the relative levels of BDNF and Bcl-2 protein expression were measured by Western blot analysis. The intensity of the bands was determined by densitometric analysis, and the data were normalized using β -actin as an internal control. A Western blot from one representative experiment is shown in the upper panel. The data from three independent experiments are expressed as the mean \pm SD in the lower panel. Statistical differences relative to the control (0 h) were analyzed by one-way ANOVA followed by Bonferroni's post hoc test: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control. (B) The immunofluorescence images show the global decrease of both proteins relative to the control (0 h), confirming the results obtained from the western blot at 24 h. CGNs were treated with BDE-99 for 24 h, fixed and immunostained with rabbit anti-rat BDNF and Bcl-2 primary antibodies followed by secondary Alexa Fluor 488 goat anti-rabbit antibodies. Scale bar = 20 μ m.

still unknown. The present study was aimed at providing a better understanding of how this neurotoxic action develops over time by examining the disruption of TR activity induced by the congener BDE-99.

During the first stage of exposure, BDE-99 exerted a significant direct effect on the mRNA transcripts of the isoforms encoded by the TR- α gene. The expression levels of TR- α 1, the predominant TR isoform in CGNs, and TR- α 2, a non-hormone-binding splicing variant, were down-regulated relative to the control at all of the time points measured over 24 h of exposure to BDE-99. TRs are members of the nuclear hormone receptor family of transcription factors, and they bind to DNA at a specific sequence in the promoters of target genes called the thyroid response element (TRE) (Wu et al., 2001). The binding of T3 to a TR induces a conformational change that increases the affinity of the TR for the TRE and modulates, either positively or negatively, the basal expression of TH-mediated genes. The expression of TR isoforms is splice-, tissue-, and development-dependent, being their regulation mediated by the concentration of TH (Samuels et al., 1977) and modulated by a complex machinery of nuclear co-regulators (Smith and O'Malley, 2004). An increase in TH levels leads to a decrease in the predominant isoform of TR expressed in the cells, while a decrease in TH levels increases the predominant TR isoform as an adaptive mechanism to maximize the TH response (Monden et al., 2006; Samuels et al., 1977). PBDEs and OH-PBDEs have a similar structure to TH and may activate this auto-regulatory mechanism by creating a state that mimics hyperthyroidism, which would lead to a decrease in the expression of the TR- α gene isoforms in CGNs. A similar effect was reported in brains of fish after 21 days of treatment with the congener 2,2',4,4'-tetraBDE. In that study, Lema et al. (2008) saw an up-regulation in the expression of TR- α in females, as well as a decrease in TR- β in both sexes. However, the brain regions in which the mRNA analysis was performed were not specified. Based on the results of the current investigation, we suggest that the decrease in the non-hormone-binding isoform, TR- α 2, may play an important role in BDE-99 neuronal toxicity. The splice variant of TR- α 1 appears to exert a

dominant negative activity on the T3-dependent trans-activation of TR target genes. A decrease in TR- α 2 expression enhances the action of TH because it is replaced by functional TRs (Bolaris et al., 2005). It would explain the up-regulation of the expression of the BDNF gene observed at 3 h after BDE-99 exposure. However, it also suggests that other genes may be deregulated, altering the normal processes of neuronal cells. Recently, various epidemiological studies have suggested an association between PBDEs and subclinical hyperthyroidism (Blake et al., 2011; Chevrier et al., 2010; Lee et al., 2010). Notwithstanding, the results of none of these studies have been linked with a possible alteration to the expression of TR. This initial state could be transformed into a hypothetical state of TH resistance, possibly due to insufficient replacement of the TRs and an additional disruptive mechanism that seems to be well established following 6 h of BDE-99 exposure. The metabolism of PBDEs produces compounds that can compete with T3 to bind to TRs as an antagonist. Their binding affinities for TRs are congener-dependent, and only a few metabolites have sufficient binding strength to disrupt T3-mediated activity. One product of the metabolism of BDE-99 is the compound 4-OH-BDE-99, a TR antagonist (Kitamura et al., 2008; Kojima et al., 2009). In the present study, the gene expression of the anti-apoptotic protein Bcl-2 was significantly down-regulated. It was possibly due to a consequence of the disruption of the expression of the TH-mediated gene BDNF (Manji et al., 2001; Wu et al., 2009). In contrast, the expression of the TR isoform TR- β 1, which was usually repressed by T3 in CGNs, was being significantly up-regulated (Monden et al., 2006). These observations led to hypothesize that the metabolites of BDE-99 increase sufficiently to reach their effective concentrations in the time interval between 6 and 12 h. Further support for this hypothesis comes from the reestablishment of the diminished expression of the BDNF and Bcl-2 proteins following addition of T3 in CGNs exposed to BDE-99. Jiang et al. (2008) and Viberg et al. (2008) found a significant decrease in BDNF expression in the rodent hippocampus after exposure to the congener 2,2',3,3',4,4',5,5',6,6'-decaBDE, which has also been described as a TR antagonist (Ibhazehiebo et al., 2011). These data, in combination with our cell viability results,

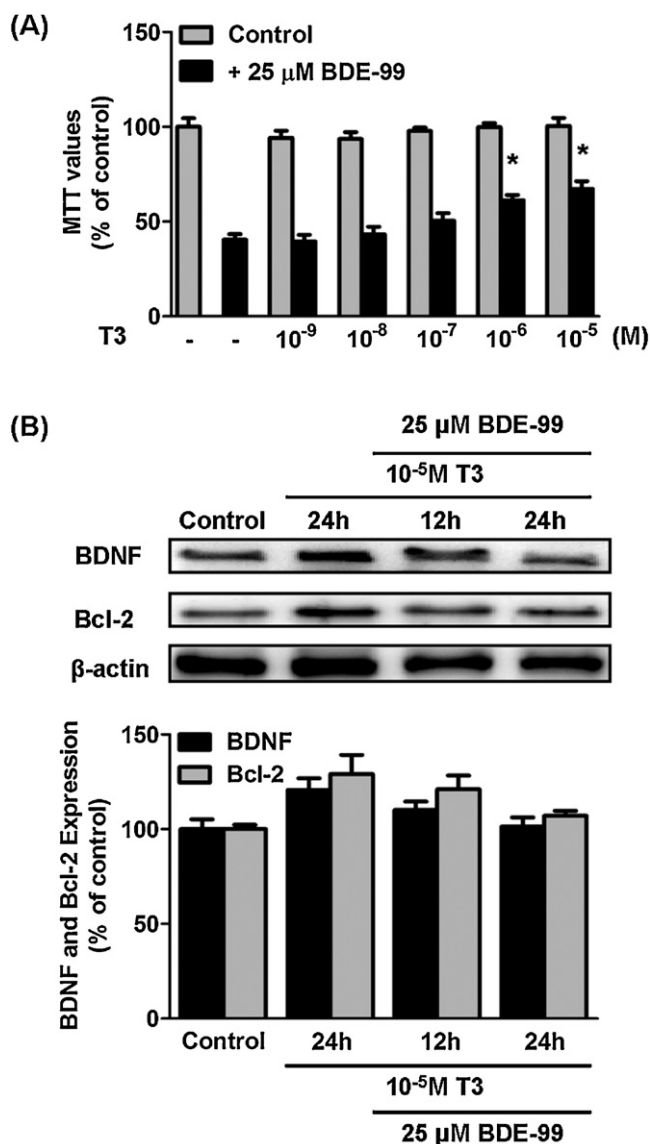


Fig. 5. T3 treatment partially rescues the cell death induced by BDE-99 and resets the BDNF and Bcl-2 protein levels. (A) CGNs were exposed to 25 μM BDE-99 for 24 h, either alone or with a range of concentrations (10⁻⁹ to 10⁻⁵ M) of T3, and the cell viability was determined by the MTT method. The data from four independent experiments are expressed as the mean ± SD. Statistical differences between CGNs treated only with T3 versus control (untreated) and CGNs treated with T3 in combination with BDE-99 versus control (treated with BDE-99 alone) were analyzed by one-way ANOVA followed by Bonferroni's post hoc test: **P* < 0.05 vs. control. (B) CGN cells were exposed to 10⁻⁵ M T3 for 24 h and to 10⁻⁵ M T3 in combination with 25 μM BDE-99 for 12 and 24 h. The relative protein expression levels of BDNF and Bcl-2 were measured by Western blot analysis. The intensity of the bands was determined by densitometric analysis, and the data were normalized using β-actin as an internal control. A Western blot from one representative experiment is shown in the upper panel. The data from three independent experiments are expressed as the mean ± SD in the lower panel. Statistical differences were analyzed by one-way ANOVA followed by Bonferroni's post hoc test.

suggest that the decrease in the TR-alpha isoforms and the disruption of TH-mediated gene expression have a greater influence on cell death during the first 12 h of BDE-99 exposure. This phenomenon is reflected by the extent of cell death induced between 0 and 12 h, which is approximately five times higher than the amount seen in the interval between 12 and 24 h. We observed that the decrease in cell viability occurred gradually during the first 12 h, after which the rate of decrease seemed to slow down. The increase of ROS production and the down-regulation expression of Bcl-2

also seemed to stabilize after 12 h. The significant up-regulation of TR-beta1 observed at 12 h could compensate the diminished function of TR-alpha1 by ameliorating the TR-mediated disruption of other fundamental proteins and partially normalizing neuronal functions. In rats, at postnatal days 1 and 7, Wang et al. (2011) found a significant up-regulation of TR-beta gene expression in the cortex of pups whose dams had been daily exposed to 2,2',4,4'-tetraBDE from gestational day 1. Although they also observed a disruption of several T3-responsive genes regulated by TRs, the change in the expression of BDNF was not significant. The current findings regarding the dual nature of the disruption of TR activity in neuronal cells may help to explain the controversial results of previous studies (Blake et al., 2011; Chevrier et al., 2010; Kuriyama et al., 2007; Lee et al., 2010; Zhou et al., 2002) in which PBDEs were shown to produce symptoms of either hypothyroidism or hyperthyroidism. These possible phenotypes may depend on the capacity of PBDE congeners to deregulate the modular expression of TRs, down-regulate the expression of TR-alpha2, and act as TR antagonists.

In summary, BDE-99 showed a direct effect on the TR-alpha isoforms TRalpha1 and TR-alpha2, decreasing their gene expression to levels that were maintained during the 24 h of exposure in CGNs, possibly as a consequence of a hypothetical state that mimics hyperthyroidism. BDE-99 caused also indirect effects that disrupted the TR-regulated T3-responsive genes, probably through the action of BDE-99 metabolites, leading the cells to enter a hypothyroid state. The expression of the TH-mediated neurotrophin BDNF was down-regulated, which in turn led to a down-regulation of Bcl-2. The decreased production of BDNF and Bcl-2 was correlated with the increased production of ROS. We also observed an up-regulation of the T3-transcriptionally repressed expression of the TR-beta1 isoform, which by replacement could compensate in part the down-regulation of the TR-alpha1 isoform, diminishing the induction of cell death. Further in vivo studies should investigate whether these relationships are affected, as well as the possible consequences over neurodevelopment.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

We thank Biochemistry, Pharmacology, Histology and Neurobiology Units of School of Medicine, Universitat Rovira i Virgili, Reus, Spain, for their technological support, especially to Dr. Francesc Sureda. The helpful methodological assistance of Dr. Javier Pizarro and Meritxeil Bordas is also gratefully appreciated. This work was financially supported by the "Fondo de Investigación Sanitaria", Ministry of Health, Spain through grant no. P107631, and FEDER funds.

References

- Alaee, M., Arias, P., Sjodin, A., Bergman, A., 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.
- Albina, M.L., Alonso, V., Linares, V., Belles, M., Sirvent, J.J., Domingo, J.L., Sanchez, D.J., 2010. Effects of exposure to BDE-99 on oxidative status of liver and kidney in adult rats. *Toxicology* 271, 51–56.
- Alonso, V., Linares, V., Belles, M., Albina, M.L., Pujol, A., Domingo, J.L., Sanchez, D.J., 2010. Effects of BDE-99 on hormone homeostasis and biochemical parameters in adult male rats. *Food Chem. Toxicol.* 48, 2206–2211.
- Anderson, G.W., 2001. Thyroid hormones and the brain. *Front. Neuroendocrinol.* 22, 1–17.
- Belles, M., Alonso, V., Linares, V., Albina, M.L., Sirvent, J.J., Domingo, J.L., Sanchez, D.J., 2010. Behavioral effects and oxidative status in brain regions of adult rats exposed to BDE-99. *Toxicol. Lett.* 194, 1–7.

- Blake, C.A., McCoy, G.L., Hui, Y.Y., LaVoie, H.A., 2011. Perinatal exposure to low-dose DE-71 increases serum thyroid hormones and gonadal osteopontin gene expression. *Exp. Biol. Med.* 236, 445–455.
- Bocio, A., Llobet, J.M., Domingo, J.L., Corbella, J., Teixido, A., Casas, C., 2003. Polybrominated diphenyl ethers (PBDEs) in foodstuffs: human exposure through the diet. *J. Agric. Food Chem.* 51, 3191–3195.
- Bolaris, S., Constantinou, C., Valcana, T., Margarity, M., 2005. Pentylentetrazole-induced convulsions affect cellular and molecular parameters of the mechanism of action of triiodothyronine in adult rat brain. *Neuropharmacology* 48, 894–902.
- Branchi, I., Alleva, E., Costa, L.G., 2002. Effects of perinatal exposure to a polybrominated diphenyl ether (PBDE 99) on mouse neurobehavioural development. *Neurotoxicology* 23, 375–384.
- Chevrier, J., Harley, K.G., Bradman, A., Gharbi, M., Sjodin, A., Eskenazi, B., 2010. Polybrominated diphenyl ether (PBDE) flame retardants and thyroid hormone during pregnancy. *Environ. Health Perspect.* 118, 1444–1449.
- Cook, C.B., Koenig, R.J., 1990. Expression of *erba* alpha and beta mRNAs in regions of adult rat brain. *Mol. Cell. Endocrinol.* 70, 13–20.
- Costa, L.G., Giordano, G., Tagliaferri, S., Caglieri, A., Mutti, A., 2008. Polybrominated diphenyl ether (PBDE) flame retardants: environmental contamination, human body burden and potential adverse health effects. *Acta Biomed.* 79, 172–183.
- Dingemans, M.M., Ramakers, G.M., Gardoni, F., van Kleef, R.G., Bergman, A., Di Luca, M., van den Berg, M., Westerink, R.H., Vijverberg, H.P., 2007. Neonatal exposure to brominated flame retardant BDE-47 reduces long-term potentiation and post-synaptic protein levels in mouse hippocampus. *Environ. Health Perspect.* 115, 865–870.
- Domingo, J.L., 2004. Human exposure to polybrominated diphenyl ethers through the diet. *J. Chromatogr. A* 1054, 321–326.
- Domingo, J.L., Martí-Cid, R., Castell, V., Llobet, J.M., 2008. Human exposure to PBDEs through the diet in Catalonia, Spain: temporal trend. A review of recent literature on dietary PBDE intake. *Toxicology* 248, 25–32.
- Gomara, B., Herrero, L., Ramos, J.J., Mateo, J.R., Fernandez, M.A., Garcia, J.F., Gonzalez, M.J., 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.
- He, P., He, W., Wang, A., Xia, T., Xu, B., Zhang, M., Chen, X., 2008. PBDE-47-induced oxidative stress, DNA damage and apoptosis in primary cultured rat hippocampal neurons. *Neurotoxicology* 29, 124–129.
- Herbstman, J.B., Sjodin, A., Kurzon, M., Lederman, S.A., Jones, R.S., Rauh, V., Needham, L.L., Tang, D., Niedzwiecki, M., Wang, R.Y., Perera, F., 2010. Prenatal exposure to PBDEs and neurodevelopment. *Environ. Health Perspect.* 118, 712–719.
- Hites, R.A., 2004. Polybrominated diphenyl ethers in the environment and in people: a meta-analysis of concentrations. *Environ. Sci. Technol.* 38, 945–956.
- Ibhazehiebo, K., Iwasaki, T., Kimura-Kuroda, J., Miyazaki, W., Shimokawa, N., Koibuchi, N., 2011. Disruption of thyroid hormone receptor-mediated transcription and thyroid hormone-induced Purkinje cell dendrite arborization by polybrominated diphenyl ethers. *Environ. Health Perspect.* 119, 168–175.
- Jiang, H.P., Yu, Y.H., Chen, D.J., Wu, Y., Xu, B., 2008. Effect of maternal BDE-209 exposure on the expression of GAP-43 and BDNF in the hippocampus of the offspring rats. *Nan Fang Yi Ke Da Xue Xue Bao* 28, 1319–1322 (in Chinese).
- 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.
- Kitamura, S., Shinohara, S., Iwase, E., Sugihara, K., Uramaru, N., Shigematsu, H., Fujimoto, N., Ohta, S., 2008. Affinity for thyroid hormone and estrogen receptors of hydroxylated polybrominated diphenyl ethers. *J. Health Sci.* 54, 607–614.
- 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.
- Kuriyama, S.N., Wanner, A., Fidalgo-Neto, A.A., Talsness, C.E., Koerner, W., Chahoud, I., 2007. Developmental exposure to low-dose PBDE-99: tissue distribution and thyroid hormone levels. *Toxicology* 242, 80–90.
- Lee, E., Kim, T.H., Choi, J.S., Nabanata, P., Kim, N.Y., Ahn, M.Y., Jung, K.K., Kang, I.H., Kim, T.S., Kwack, S.J., Park, K.L., Kim, S.H., Kang, T.S., Lee, J., Lee, B.M., Kim, H.S., 2010. Evaluation of liver and thyroid toxicity in Sprague-Dawley rats after exposure to polybrominated diphenyl ether BDE-209. *J. Toxicol. Sci.* 35, 535–545.
- Lema, S.C., Dickey, J.T., Schultz, I.R., 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.
- Manji, H.K., Drevets, W.C., Charney, D.S., 2001. The cellular neurobiology of depression. *Nat. Med.* 7, 541–547.
- Monden, T., Nakajima, Y., Hashida, T., Ishii, S., Tomaru, T., Shibusawa, N., Hashimoto, K., Satoh, T., Yamada, M., Mori, M., Kasai, K., 2006. Expression of thyroid hormone receptor isoforms down-regulated by thyroid hormone in human medulloblastoma cells. *Endocrinol. J.* 53, 181–187.
- Numakawa, T., Suzuki, S., Kumamaru, E., Adachi, N., Richards, M., Kunugi, H., 2010. BDNF function and intracellular signaling in neurons. *Histol. Histopathol.* 25, 237–258.
- Offen, D., Sherki, Y., Melamed, E., Fridkin, M., Brenneman, D.E., Gozes, I., 2000a. Vasoactive intestinal peptide (VIP) prevents neurotoxicity in neuronal cultures: relevance to neuroprotection in Parkinson's disease. *Brain Res.* 854, 257–262.
- Offen, D., Sherki, Y., Melamed, E., Fridkin, M., Brenneman, D.E., Gozes, I., 2000b. Vasoactive intestinal peptide (VIP) prevents neurotoxicity in neuronal cultures: relevance to neuroprotection in Parkinson's disease. *Brain Res.* 854, 257–262.
- Roze, E., Meijer, L., Bakker, A., Van Braeckel, K.N., Sauer, P.J., Bos, A.F., 2009. Prenatal exposure to organohalogenes, including brominated flame retardants, influences motor, cognitive, and behavioral performance at school age. *Environ. Health Perspect.* 117, 1953–1958.
- Samuels, H.H., Stanley, F., Shapiro, L.E., 1977. Modulation of thyroid hormone nuclear receptor levels by 3,5,3'-triiodo-L-thyronine in GH1 cells. Evidence for two functional components of nuclear-bound receptor and relationship to the induction of growth hormone synthesis. *J. Biol. Chem.* 252, 6052–6060.
- Schreiber, T., Gassmann, K., Gotz, C., Hubenthal, U., Moors, M., Krause, G., Merk, H.F., Nguyen, N.H., Scanlan, T.S., Abel, J., Rose, C.R., Fritsche, E., 2010. Polybrominated diphenyl ethers induce developmental neurotoxicity in a human in vitro model: evidence for endocrine disruption. *Environ. Health Perspect.* 118, 572–578.
- Schuhmacher, M., Kiviranta, H., Vartiainen, T., Domingo, J.L., 2007. Concentrations of polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) in milk of women from Catalonia, Spain. *Chemosphere* 67, S295–S300.
- Schuhmacher, M., Kiviranta, H., Ruokojarvi, P., Nadal, M., Domingo, J.L., 2009. Concentrations of PCDD/Fs, PCBs and PBDEs in breast milk of women from Catalonia, Spain: a follow-up study. *Environ. Int.* 35, 607–613.
- Smith, C.L., O'Malley, B.W., 2004. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocr. Rev.* 25, 45–71.
- van der Ven, L.T., van de Kuil, T., Verhoef, A., Leonards, P.E., Slob, W., Canton, R.F., Germer, S., Hamers, T., Visser, T.J., Litens, S., Hakansson, H., Fery, Y., Schrenk, D., van den Berg, M., Piersma, A.H., Vos, J.G., 2008. 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.
- Verdager, E., Jimenez, A., Canudas, A.M., Jorda, E.G., Sureda, F.X., Pallas, M., Camins, A., 2004. Inhibition of cell cycle pathway by flavopiridol promotes survival of cerebellar granule cells after an excitotoxic treatment. *J. Pharmacol. Exp. Ther.* 308, 609–616.
- 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.
- Wang, F., Liu, W., Jin, Y., Dai, J., Zhao, H., Xie, Q., Liu, X., Yu, W., Ma, J., 2011. Interaction of PFOS and BDE-47 co-exposure on thyroid hormone levels and TH-related gene and protein expression in developing rat brains. *Toxicol. Sci.* 121, 279–291.
- Wu, C.L., Hwang, C.S., Yang, D.I., 2009. Protective effects of brain-derived neurotrophic factor against neurotoxicity of 3-nitropropionic acid in rat cortical neurons. *Neurotoxicology* 30, 718–726.
- Wu, Y., Xu, B., Koenig, R.J., 2001. Thyroid hormone response element sequence and the recruitment of retinoid X receptors for thyroid hormone responsiveness. *J. Biol. Chem.* 276, 3929–3936.
- Zhao, L., Wei, X., Ma, Z., Feng, D., Tu, P., Johnstone, B.H., March, K.L., Du, Y., 2009. Adipose stromal cells-conditional medium protected glutamate-induced CGNs neuronal death by BDNF. *Neurosci. Lett.* 452, 238–240.
- Zhou, T., Taylor, M.M., DeVito, M.J., Crofton, K.M., 2002. Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. *Toxicol. Sci.* 66, 105–116.

4.2 Gestational exposure to BDE-99 produces toxicity through upregulation of CYP isoforms and ROS production in the fetal rat liver.

Blanco, J., Mulero, M., Domingo, J. L., and Sanchez, D. J.

Toxicol. Sci. (2012)127, 296-302.

Gestational Exposure to BDE-99 Produces Toxicity Through Upregulation of CYP Isoforms and ROS Production in the Fetal Rat Liver

Jordi Blanco,*† Miquel Mulero,‡ José L. Domingo,* and Domènec J. Sánchez*†¹

*Laboratory of Toxicology and Environmental Health and †Physiology Unit, School of Medicine, IISPV, Universitat Rovira i Virgili, 43201 Reus, Spain; and ‡Nutrigenomics Group, Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, 43007 Tarragona, Spain

¹To whom correspondence should be addressed. Fax: +34-977-759322. E-mail: domingo.sanchez@urv.cat.

Received November 16, 2011; accepted February 3, 2012

On gestation day (GD) 6 to GD 19, pregnant Sprague Dawley rats were orally exposed to 0, 0.5, 1, and 2 mg/kg/day to one of the most prevalent polybrominated diphenyl ethers congeners found in humans, 2,2',4,4',5-pentaBDE (BDE-99). All dams were euthanized on GD 20, and live fetuses were evaluated for sex, body weight, and external, internal, and skeletal malformations and developmental variations. The liver from one fetus of each litter was excised for the evaluation of oxidative stress markers and the messenger RNA expression of multiple cytochrome P450 (CYP) isoforms. Exposure to BDE-99 during the gestational period produced delayed ossification, slight hypertrophy of the heart, and enlargement of the liver in fetuses. A transplacental effect of BDE-99, evidenced by the activation of nuclear hormones receptors that induce the upregulation of CYP1A1, CYP1A2, CYP2B1, and CYP3A2 isoforms, was also found in fetal liver. These isoforms are correlated with the activity level of the enzyme catalase and the levels of thiobarbituric acid reactive substances. However, teratogenic effects from BDE-99 exposure were not observed. Clear signs of embryo/fetal toxicity, due to a possible hormonal disruption, were evidenced by a large increase in the CYP system and the production of reactive oxygen species in fetal liver.

Key Words: BDE-99; reactive oxygen species (ROS); CYP enzyme system; oxidative stress; fetal toxicity.

Polybrominated diphenyl ethers (PBDEs), a chemical family of 209 compounds, have been widely used by industries over the last three decades as flame retardants in the manufacture of polyurethane foams, electronic components, plastics, building materials, textiles, and other applications (Alaee *et al.*, 2003). The ease of release into the environment of these chemicals, the stability of their structures, and the lipophilic properties of PBDEs have contributed to their global expansion in all ecosystems and their biomagnification across different trophic levels (Costa *et al.*, 2008).

Despite the bans and restrictions on the production of PBDEs (COP4, 2009), there are currently many functional appliances that still exist in homes and worksites. The degradation of PBDE-containing polymers contributes to

increased concentrations of these compounds in the indoor air, which can be one order of magnitude higher than the concentration found outdoors (Jones-Otazo *et al.*, 2005). The ingestion, inhalation, and dermal absorption of PBDE-containing dust particles, along with their direct dietary intake, are the two main sources of human exposure to these chemical substances (Domingo *et al.*, 2008; Domingo, 2011b; Trudel *et al.*, 2011; Watkins *et al.*, 2011).

Epidemiological studies have highlighted the rising levels of PBDEs in serum samples from people living in North America, which can be 10–70 times higher than in people living in Europe or Japan (Schechter *et al.*, 2005). The bioaccumulation of PBDEs in pregnant women is a principal concern because these compounds can be transferred to the infants through the placenta and breast milk (Gomara *et al.*, 2007; Schuhmacher *et al.*, 2009). Recently, human studies have shown correlations between high levels of PBDEs in maternal serum, cord blood, and breast milk with neurobehavioral changes, cognitive deficiencies, and an increasing index of cryptorchidism in children (Gascon *et al.*, 2011; Herbstman *et al.*, 2010; Main *et al.*, 2007; Roze *et al.*, 2009). In turn, Doucet *et al.* (2009) reported that the level of these xenobiotic substances in human fetal livers increased fivefold between 1998 and 2006.

In vitro and *in vivo* investigations have described that PBDEs can deregulate thyroid hormone (TH) signaling homeostasis at multiple mechanistic levels (Kuriyama *et al.*, 2007; Zhou *et al.*, 2002) and can intercede in the biosynthesis of steroid hormones (SHs) (He *et al.*, 2008). Studies with animals exposed to PBDEs have associated these endocrine disruptions with developmental neurotoxicity (Dingemans *et al.*, 2011), damage to the reproductive system (Talsness *et al.*, 2005), fetal anomalies (Usenko *et al.*, 2011), and hepatotoxicity (Lee *et al.*, 2010).

The modulation of the expression of cytochrome P450 (CYP) isoforms by PBDEs could be in part responsible for the aforementioned alterations. The CYP system plays an important role in the synthesis and metabolism of SHs, cholesterol and vitamin D, as well as in the detoxification of endogenous

and exogenous substances. The overexpression of certain CYP isoforms during the gestational period could interfere with fetal development (Stoilov, 2001) promoting the generation of reactive oxygen species (ROS) (Zangar *et al.*, 2004). ROS production is one of the principal mechanisms by which PBDEs can induce cellular damage (Albina *et al.*, 2010; Belles *et al.*, 2010; Chen *et al.*, 2010; Hu *et al.*, 2007).

In recent years, we have investigated in our laboratory the levels of PBDEs in foodstuffs and the human exposure to these environmental pollutants through the diet (Bocio *et al.*, 2003; Domingo *et al.*, 2006, 2008; Domingo, 2011a,b; Perelló *et al.*, 2009). Moreover, we have also investigated the potential health effects of some PBDEs using animal and *in vitro* models (Albina *et al.*, 2010; Alonso *et al.*, 2010; Belles *et al.*, 2010; Blanco *et al.*, 2011). The main goal of the present study was to examine the embryo/fetal toxicity of prenatal exposure to one of the most prevalent PBDE congeners found in human samples, 2,2',4,4',5-pentaBDE (BDE-99) (Costa *et al.*, 2008). We also assessed the messenger RNA (mRNA) expression of CYP1A1, CYP1A2, CYP2B, CYP3A1, and CYP3A2 in fetal livers and their correlation with the antioxidant enzyme activity of catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), and the total levels of thiobarbituric acid reactive substances (TBARS).

MATERIALS AND METHODS

Animals and chemicals. Sexually mature male and female Sprague Dawley rats (220–240 g) were obtained from Charles River (Barcelona, Spain). After a quarantine period of 7 days, female rats were mated with males (2:1) overnight. Vaginal smears were collected the next morning to detect the presence of sperm. The day of sperm detection was considered as gestation day (GD) 0. Animals were individually housed in plastic cages in a climate-controlled facility, with a constant day-night cycle (light: 08:00 h–20:00 h) at a temperature of $22 \pm 2^\circ\text{C}$ and a relative humidity of $50 \pm 10\%$. Food (Panlab rodent chow, Barcelona, Spain) and tap water were available *ad libitum*. The use of animals and the experimental protocol were approved by the Animal Care and Use Committee of the Rovira i Virgili University (Tarragona, Catalonia, Spain). BDE-99 (99% pure) was purchased from LGC Standards S.L.U. (Barcelona). BDE-99 was administered by gavage after being dissolved in corn oil as a vehicle.

Experimental procedure. Females with vaginal smears positive for sperm were divided into four groups (8 animals per group). Rats in each group received BDE-99 doses (gavage) of 0, 0.5, 1, and 2 mg/kg of body weight (control, BDE 0.5, BDE 1, and BDE 2 groups, respectively) from GD 6 to GD 19. Animals in the control group received the vehicle only (corn oil). The doses of BDE-99 were chosen based on an estimated lowest observed adverse effect level of 1 mg/kg/day, mainly from pentaBDE data (Darnerud *et al.*, 2001) as well as from a recent report on redox responses and tissue distribution of BDE-99 (Cheng *et al.*, 2009).

During the gestation period, body weight of the dams was daily measured. On GD 20, all animals were euthanized with an overdose of ketamine-xylazine. The number of total implants, the number of resorbed fetuses, and the number of live and dead fetuses were recorded. All live fetuses were dissected from the uterus and evaluated for sex, body weight, and external malformations. One fetus of each litter was decapitated, the liver immediately excised, frozen with liquid nitrogen, and stored at -80°C . Approximately one-half of the remaining available fetuses were fixed in 95% ethanol, cleared with 1% potassium hydroxide (KOH), stained with Alizarin red S, and examined for skeletal

malformations and variations (Staples and Schnell, 1968). The remaining fetuses were fixed in Bouin's fluid, sectioned, and evaluated for internal abnormalities (Wilson, 1965). All fetuses were examined by observers who were blinded to the treatment conditions.

Oxidative stress markers. A fraction of the fetal liver was used to assess the activity of the antioxidant enzymes SOD, GR, GPx, and CAT, the level of TBARS, and the protein content. Tissue samples were thawed and washed in 0.9% saline, and homogenized in 0.2M sodium phosphate buffer (pH 6.25, 1:20, wt/vol) in a Potter-Elvehjem homogenizer fitted with a Teflon pestle (Braun, Melsungen, Germany). The supernatant was collected after centrifugation at $105,000 \times g$ for 1 h and used for biochemical analyses. The protein content was measured by the Bradford spectrophotometric method (Sigma Chemical Co., St Louis, MO) using bovine serum albumin as a standard (Merck, Darmstadt, Germany). The activities of SOD, CAT, GPx, and GR were determined according to Mulero *et al.* (2006), whereas the total TBARS levels were determined according to Zupan *et al.* (2008).

RNA isolation and cDNA synthesis. Total RNA was obtained from a fraction of the fetal livers using a Qiagen RNeasy Kit according to the manufacturer's protocol. RNA was resuspended in 100 μl of RNase-free water, quantified by a spectrophotometer at an absorbance of 260 nm, and tested for purity using an A260/280 ratio, and for integrity, using denaturing gel electrophoresis. The first strand of complementary DNA (cDNA) was reverse transcribed from 1 μg of total RNA from each sample using a QuantiTect Reverse Transcription Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's protocol. An identical reaction, without reverse transcriptase, was performed to verify the absence of genomic DNA. The cDNA was subsequently amplified by PCR using rat-specific primers for CYP1A1 (NM_012540; forward: 5'-CTG CAG AAA ACA GTC CAG GA-3'; reverse: 5'-CAG GAG GCT GGA CGA GAA TGC-3'), CYP1A2 (NM_012541; forward: 5'-CCA AGC CGT CCA CGA GAC TT-3'; reverse: 5'-GAG GGA TGA GAC CAC CGT TG-3'), CYP2B1 (NM_001134844; forward: 5'-CCA AGC CGT CCA CGA GAC TT-3'; reverse: 5'-TTG GGA AGC AGG TAC CCT C-3'), CYP3A1 (NM_013105; forward: 5'-CCG CCT GGA TTC TGT GCA GA-3'; reverse: 5'-TGG GAG GTG CCT TAT TGG GC-3'), CYP3A2 (NM_153312; forward: 5'-TTG ATC CGT TGC TCT TGT CA-3'; reverse: 5'-GGC CAG GAA ATA CAA GAC AA-3'), and β -actin (NM_031144; forward: 5'-TGT CAC CAA CTG GGA CGA TA-3'; reverse: 5'-GGG GTG TTG AAG GTC TCA AA-3') with a PyroStart Fast PCR Master mix (2 \times) kit (Fermentas, Burlington, Canada) according to the manufacturer's protocol. The PCR products were separated on a 1% agarose gel, and only specific bands were detected. The nonreactivity of the primers with contaminant genomic DNA was tested by the inclusion of controls that omitted reverse transcriptase from the cDNA synthesis reaction.

Real-time reverse-transcription-PCR. Quantitative PCR for CYP1A1, CYP1A2, CYP2B1, CYP3A1, CYP3A2, and β -actin was completed using the QuantiTect SYBR Green PCR kit (Qiagen, Inc.) according to the manufacturer's protocol and a Rotor-Gene Q Real-Time PCR cycler (Qiagen, Inc.). The thermal cycling comprised an initial step at 50°C for 2 min, followed by a polymerase activation step at 95°C for 15 min and a cycling step with the following conditions: 40 cycles of denaturing at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Oligonucleotides of varying lengths produce dissociation peaks at different melting temperatures. Consequently, at the end of the PCR cycles, the PCR products were analyzed using a heat dissociation protocol to confirm that one single PCR product was detected by SYBR Green dye. Fluorescence data were acquired at the 72°C step. The threshold cycle (C_t) was calculated by Rotor-Gene Q 2.0 software to identify significant fluorescence signals above noise during the early cycles of amplification. The software calculated copy numbers for the target samples from the C_t by interpolating from a standard curve. The relative levels of the expression of the target genes were measured using β -actin mRNA as an internal control according to the $2^{-\Delta\Delta C_t}$ method.

Statistics. To evaluate homogeneity of variances, the Levene test was used. When the variances of different treatment groups were homogeneous, an

TABLE 1
Effects of BDE-99 on Gestational Parameters in Pregnant Rats

Exposure group	Control	BDE 0.5 (mg/Kg/day)	BDE 1 (mg/Kg/day)	BDE 2 (mg/Kg/day)
Number of dams	8	8	8	8
Body weight (g) on GD 0	256.75 ± 11.35	262.12 ± 14.77	252.62 ± 4.53	248.50 ± 12.66
Body weight (g) on GD 20	386.63 ± 13.35	388.43 ± 20.95	398.55 ± 21.61	385.95 ± 28.94
Gravid uterine weight (g)	85.84 ± 10.09	77.92 ± 9.54	89.30 ± 13.73	74.67 ± 18.90
Corrected body weight gain (g) on GDs 0–20 ^a	44.04 ± 6.15 ^a	48.38 ± 11.04 ^{ab}	56.63 ± 11.30 ^{ab}	62.78 ± 13.42 ^b
Number of implants/litter	16.62 ± 3.15	14.25 ± 3.28	17.50 ± 2.20	14.12 ± 3.22
Number of viable implants/litter	14.12 ± 2.10	12.62 ± 2.50	13.87 ± 1.64	11.25 ± 2.86
Number of nonviable implants/litter (resorbed fetuses)	2.37 ± 2.19	1.62 ± 1.59	3.62 ± 1.76	2.87 ± 2.85
Sex ratio (M/F)	0.62 ± 0.16	0.57 ± 0.09	0.73 ± 0.27	0.68 ± 0.29
Average fetal body weight/litter (g)	3.94 ± 0.20	4.04 ± 0.27	4.07 ± 0.25	4.33 ± 0.73

^aCorrected body weight gain (g) on GDs 0–20 = [Body weight (g) on GD 20 – Body weight (g) on GD 0 – Gravid uterine weight (g)]. Results are expressed as mean values ± SD. The statistical differences were analyzed by ANOVA followed by a Bonferroni's *post hoc* test, except for sex ratio (M/F), and average fetal body weight/litter (g), which were analyzed by the Kruskal-Wallis test and the Mann-Whitney *U*-test. Values in the same row showing a common superscript (a and b) are not significantly different at $p < 0.05$.

ANOVA, followed by a Bonferroni *post hoc* test, was used to establish the level of significance among groups. If the variances were not homogeneous, the Kruskal-Wallis and the Mann-Whitney *U*-tests were used. The mRNA expression was evaluated by a one-way ANOVA followed by a Duncan's test in order to identify significant differences between the control and treatment groups. The level of statistical significance for all tests was set at $p < 0.05$. All data were analyzed with the SPSS 15.0 software (SPSS Sciences, Chicago, IL). The relationships among the examined endpoints were assessed using Pearson correlations. The incidence of fetal anomalies between litters was compared by means of a two-tailed Fisher exact probability test for the pairwise comparison of groups. In this case, significance was set at $p < 0.001$.

RESULTS

Effects of BDE-99 on Gestational Parameters in Pregnant Rats

No signs of gestational parameters in pregnant rats were noted in any of the treatment groups (Table 1). The number of total implants, viable implants, nonviable implants, sex ratio, and average fetal body weight did not show any significant differences between groups. In addition, BDE-99-treated rats did not show significant changes in the weight of the gravid uteri, body weight at GD 0, and body weight at GD 20, when compared with the control group. However, a significant increase in the corrected body weight gain during pregnancy was detected in the BDE 2 group when compared with the control group ($p = 0.01$).

BDE-99 Exposure Causes Delayed Ossification, Slight Hypertrophy of the Heart and Enlargement of the Liver in Rat Fetuses

PBDEs can be transferred to infants through the placenta (Gomara *et al.*, 2007). In the current study, we examined the potential teratogenic and fetotoxic effects in litters from rat dams treated from GD 6 to GD 19 at different doses of BDE-99. No

signs of external malformations in the fetuses of rats exposed to BDE-99 during pregnancy were observed. However, some bone retardations and internal variations were noted in the experimental groups (Table 2). Although the results show a clear increase of the total number of fetuses with skeletal and internal variations in the BDE 1 group, the incidence of these anomalies was statistically significant only in the BDE 2 group, compared with the control and the BDE 0.5 groups. The skeletal observations showed a significant incomplete bone deposition in the parietal and occipital bones of the skull as well as a delayed ossification of the floating ribs and caudal vertebrae bones in the BDE 2 group, when compared with the control and BDE 0.5 groups. In turn, the internal examination revealed a significant increase in the size of the ventricles of the heart and an enlargement of the liver in the fetuses of the BDE 2 group.

BDE-99 Increases Oxidative Stress Markers in the Fetal Rat Liver

As an indicator of the transplacental effects of BDE-99, the production of ROS in the fetal rat liver was indirectly evaluated by measuring different oxidative stress markers. We measured the activity of antioxidant enzymes (SOD, CAT, GPx, and GR) and the total level of TBARS in the fetal rat liver (Table 3). A BDE-99 dose-dependent increase in the level of all evaluated oxidative stress markers was found. The CAT activity was significantly increased in the BDE 1 ($p = 0.031$) and BDE 2 ($p < 0.001$) groups with respect to the activity detected in the control group as well as in the BDE 2 group ($P = 0.005$) when compared with the BDE 0.5 group. The total level of TBARS was also significantly increased in the BDE 1 ($p = 0.017$) and BDE 2 ($p = 0.001$) groups compared with the control group. These results suggest that the production of ROS in fetal rat liver was proportional to the level of BDE-99 exposure in pregnant dams.

TABLE 2
Effects of BDE-99 on Morphological Defects in Rat Fetuses

Exposure groups	Control	BDE 0.5 (mg/Kg/day)	BDE 1 (mg/Kg/day)	BDE 2 (mg/Kg/day)
Skeletal examination				
Number of fetuses examined skeletally (litters)	46 (8)	45 (8)	46 (8)	40 (8)
Total of fetuses with skeletal defects (litters)	7 ^a (6)	6 ^a (5)	18 ^{ab} (7)	21 ^b (8)
Percentage of fetuses with skeletal defects/litter	20	21	45	46
Parietal of the skull, incomplete ossification (litters)	2 ^a (1)	1 ^a (1)	7 ^{ab} (5)	15 ^b (7)
Occipital of the skull, incomplete ossification (litters)	2 ^a (1)	1 ^a (1)	7 ^{ab} (5)	15 ^b (7)
Caudal vertebrae, delayed ossification (litters)	2 ^a (2)	5 ^{ab} (4)	12 ^{ab} (7)	14 ^b (8)
Sternum, delayed ossification (litters)	5 (3)	2 (2)	5 (5)	7 (3)
Ribs, delayed ossification (litters)	1 ^a (1)	0 ^a (0)	6 ^{ab} (5)	14 ^b (7)
Xyphoid, delayed ossification (litters)	0 (0)	0 (0)	0 (0)	1 (1)
Internal examination				
Number of fetuses examined internally (litters)	47 (8)	45 (8)	47 (8)	40 (8)
Total fetuses with internal variations (litters)	1 ^a (1)	1 ^a (1)	12 ^{ab} (6)	17 ^b (8)
Percentage of fetuses with internal defects/litter	17	18	34	42
Liver, hypertrophy (litters)	1 ^a (1)	1 ^a (1)	12 ^{ab} (6)	17 ^b (8)
Heart, hypertrophy (litters)	1 ^a (1)	1 ^a (1)	9 ^{ab} (6)	12 ^b (7)
Skeletal and internal examination				
Total number of fetuses examined (litters)	93 (8)	90 (8)	93 (8)	80 (8)
Total number of skeletal and internal malformations and variations fetuses (litters)	8 ^a (6)	7 ^a (5)	25 ^{ab} (8)	38 ^b (8)
Percentage of total number of fetuses with skeletal and internal malformations and variations/litter	11	12	41	47

Note. Significant differences are indicated for comparisons between groups by the use of superscripts (a and b). Values showing a common superscript are not statistically significant at $p < 0.001$.

The BDE-99-Induced Production of ROS in the Fetal Rat Liver Is Related to an Increase in the mRNA Expression of CYP1A1, CYP1A2, CYP2B1, and CYP3A2 Isoforms

The CYP enzyme family plays a key role in the mechanism of detoxification of xenobiotics such as PBDEs. The CYP enzymes change the lipophilic structure of PBDEs into water-soluble substances by the introduction of polar groups, which can be then excreted into the urine or the bile (Szabo *et al.*, 2009). In fetal rat livers, we evaluated the mRNA expression of five of the principal CYP isoforms implicated in the biotransformation and detoxification of PBDEs (Fig. 1). Compared with the results of the control group, the data show a significant increase in the expression of CYP1A1 ($88 \pm 53\%$, $p = 0.011$), CYP1A2 ($257 \pm 89\%$, $p = 0.005$), and CYP2B1 ($649 \pm 164\%$, $p < 0.001$) isoforms in fetuses from the BDE 2

group. The mRNA expression of the isoform CYP3A2 was significantly upregulated in the BDE 1 ($339 \pm 43\%$, $p = 0.017$) and BDE 2 ($401 \pm 187\%$, $p = 0.004$) groups when compared with the control group. The production of ROS could be related to the increase in gene expression of the CYP isoforms. Thus, we calculated the relationship between multiple oxidative stress markers and the upregulated CYP isoforms. A significant correlation between the increase of CAT activity with the gene expression of CYP1A1 ($R = 0.3607$, $p = 0.0139$), CYP1A2 ($R = 0.301$, $p = 0.027$), CYP2B1 ($R = 0.441$, $p = 0.005$), and CYP3A2 ($R = 0.424$, $p = 0.006$) was found. A similar correlation was also observed between the total level of TBARS with the expression of CYP1A1 ($R = 0.367$, $p = 0.013$), CYP1A2 ($R = 0.427$, $p = 0.006$), CYP2B1 ($R = 0.462$, $p = 0.004$), and CYP3A2 ($R = 0.473$, $p = 0.003$) isoforms.

TABLE 3
Effects of Exposure to BDE-99 on Biochemical Oxidative Stress Markers of Fetal Rat Livers

Exposure group	Control, (n = 8)	BDE 0.5 (mg/Kg/day), (n = 8)	BDE 1 (mg/Kg/day), (n = 8)	BDE 2 (mg/Kg/day), (n = 8)
Catalase ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	237 ± 51^a	300 ± 60^{ab}	354 ± 67^{bc}	446 ± 48^c
SOD (U/mg protein)	4.87 ± 0.64	5.40 ± 0.73	5.99 ± 0.82	6.28 ± 1.03
GPx (mU/mg protein)	67.4 ± 7.0	80.5 ± 11.5	77.8 ± 16.4	79.4 ± 8.1
GR (mU/mg protein)	15.0 ± 1.0	15.0 ± 1.6	15.8 ± 2.2	18.4 ± 2.5
TBARS (nmol/mg protein)	11.3 ± 4.3^a	17.6 ± 4.1^{ab}	20.3 ± 3.4^b	23.5 ± 4.3^b

Note. Results are expressed as mean values \pm SD. The statistical differences were analyzed by ANOVA followed by a Bonferroni's *post hoc* test. Values in the same row showing a common superscript (a, b, and c) are not significantly different at $p < 0.05$.

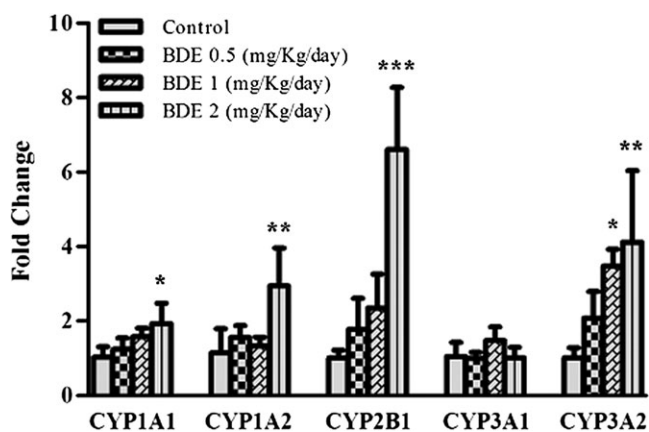


FIG. 1. BDE-99 increases the mRNA expression of CYP1A1, CYP1A2, CYP2B1, and CYP3A1 in fetal rat liver. The mRNA was extracted from the liver of rat fetuses whose dams were orally exposed to 0, 0.5, 1, and 2 mg/kg/day of BDE-99 from GD 6 to GD 20. The relative expression levels of the mRNAs encoding the CYP1A1, CYP1A2, CYP2B1, CYP3A1, and CYP3A2 isoforms were determined by a quantitative real-time (reverse-transcription-PCR) assay. Data were normalized using the β -actin gene as an internal control, and the fold changes were calculated relative to the control. Data are expressed as mean values \pm SD of eight independent samples per group. Significant differences relative to the control group were analyzed by a one-way ANOVA followed by a Duncan's *post hoc* test: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the control.

DISCUSSION

In the current study, the transplacental ability of BDE-99 caused fetotoxic effects, while the CYP system in the fetal liver could have a direct role in these anomalies. In the fetal rat liver, we observed that, as part of its transplacental action, BDE-99 upregulates the mRNA expression of CYP1A, CYP2B, and CYP3A. BDE-99-induced changes in mRNA expression of the CYP1A1 and CYP1A2 isoforms were smaller in comparison with those observed in the mRNA expression of the CYP2B1 and CYP3A2 isoforms. These results support the hypothesis of Peters *et al.* (2006), Sanders *et al.* (2005), and Szabo *et al.* (2009) that BDE-99 would be a poor activator of the aryl hydrocarbon receptor (AhR), but it would be an agonist for the pregnane X receptor (PRX) and the constitutive androstane receptor (CAR). It has been reported that the activation of AhR, PRX, and CAR by PBDEs could promote the transcription of other xenobiotic-metabolizing enzymes, such as sulfotransferase (SULT) and uridine diphosphate glucuronosyltransferase, in liver (Szabo *et al.*, 2009). These enzymes also catalyze the metabolism of circulating hormones as a homeostatic mechanism to control their concentrations in blood. An increase in the activity of the enzymes could alter homeostasis by enhancing the elimination of these hormones during the gestational period and, therefore, possess a fetotoxic effect.

The enhancement in the frequency of delayed ossification in rat fetuses could be due to a hormonal disruption induced by prenatal exposure to BDE-99. Talsness *et al.* (2005) and Breslin *et al.* (1989) observed a high incidence of incomplete

bone deposition in developing animals after prenatal exposure to low doses of PBDEs. The presence of steroid receptors and thyroid receptors (TRs) in osteoblasts and osteoclasts has suggested a direct role for SHs and THs in the modulation of genes related to the differentiation, proliferation, and regulation of bone tissue. Other authors (Lilienthal *et al.*, 2006; Talsness *et al.*, 2005) showed that prenatal exposure to BDE-99 decreased the levels of circulating sex steroids (17 β -estradiol and testosterone) and tetraiodothyroxine (T4) in offspring of rats. The excessive metabolism of these two hormones in the fetal liver could affect the process of bone formation, leading to the observed skeletal anomalies.

In the present investigation, we found an increase in maternal body weight as well as an enlargement of the fetal liver and a slight fetal heart hypertrophy, which could be attributed to a possible thyroid disruption by BDE-99. Various PBDEs studies have shown a hepatic hypertrophy, evidenced by an increase in liver weight in the rat offspring, correlated with a significant decrease of circulating T4 levels (Dunnick and Nyska, 2009; Lee *et al.*, 2010; Talsness *et al.*, 2005). However, further research on the possible cardiac effects of PBDEs is still required. To date, the only developmental studies concerning PBDEs were performed on zebrafish, reporting contradictory effects after exposure to the same tetra-BDE congener. Lema *et al.* (2007) found signs of tachycardia, which progressed into atrioventricular block arrhythmias, whereas McClain *et al.* (2011) showed a significant reduction in heart rate (both in zebrafish).

The increased production of ROS induced by BDE-99 exposure could be an important factor in the development of the internal malformations observed in the fetuses. High levels of ROS can produce DNA damage, leading the cells into an adaptive state of hyperplasia and/or hypertrophy. We found a positive correlation between the oxidative stress markers (CAT and TBARS) and the transcript levels of the CYP isoforms in fetal liver. The CYP enzymes catalyze the oxygenation of exogenous and endogenous compounds as part of a microsomal monooxygenation system (MMO) localized in the membranes of the hepatic endoplasmic reticulum. The transference of electrons from NADPH to CYP enzymes is required for the monooxygenation reaction. The electrons' carriers of MMO are inefficient in eukaryotes, causing an uncoupling of the microsomal electron-transfer chain, which can induce the generation of ROS (Zangar *et al.*, 2004). The high activity of the electron-transfer chain by BDE-99 in the MMO can lead to the increase of ROS in the fetal rat liver, producing cell damage.

The compounds generated from the MMO could also play an important role in the development of fetal anomalies. The CYP enzymes catalyze the biotransformation of PBDEs to hydroxylated (OH-PBDEs) and methylated (Me O-PBDEs) compounds. Binding assay studies have shown that several metabolites of PBDEs have a high affinity for the TRs (Kojima *et al.*, 2009; Kitamura *et al.*, 2008). One product of the

metabolism of BDE-99, 4-OH-BDE-90, is a potent antagonist of TRs, and therefore, it blocks the regulation of TH-mediated genes in the cells. Moreover, 4-OH-BDE-90 has also shown a high affinity for binding to the TH transport protein transthyretin (TTR; Yang *et al.*, 2011). The binding of 4-OH-BDE-90 to TTR causes the displacement of T4 in blood. The increment of the activity of some hormonal metabolizing enzymes in liver, stimulated by BDE-99 and the increase of the circulating level of free T4, could lead to the excessive THs degradation (Szabo *et al.*, 2009).

In conclusion, prenatal exposure of rats to BDE-99 can delay ossification and induce a slight hypertrophy of the heart and an enlargement in the liver of fetuses. The transplacental activation of the nuclear hormone receptors AhR, PXR, and primarily, CAR, by exposure to BDE-99 induces the upregulation of CYP isoforms in the fetal liver. This increase in CYP enzymes is correlated with a significant increase of the oxidative stress markers CAT and TBARS. Although teratogenic effects of BDE-99 exposure were not detected, clear signs of a possible hormonal disruption evidenced by an increase in the CYP enzyme levels could be seen. Because of the increased prevalence of PBDE levels in humans, the importance of further experimental investigations related to the potential health risks of these environmental pollutants seems to be warranted.

FUNDING

“Fondo de Investigación Sanitaria,” Ministry of Health, Spain (P107631); FEDER funds.

ACKNOWLEDGMENTS

We thank Ana Isabel Diez for her technological support. The helpful of Amparo Aguilar, Esperanza Chernizero, and Juan Valencia is also gratefully appreciated. The authors declare that there are no conflicts of interest.

REFERENCES

Alaee, M., Arias, P., Sjodin, A., and Bergman, A. (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.

Albina, M. L., Alonso, V., Linares, V., Belles, M., Sirvent, J. J., Domingo, J. L., and Sanchez, D. J. (2010). Effects of exposure to BDE-99 on oxidative status of liver and kidney in adult rats. *Toxicology* **271**, 51–56.

Alonso, V., Linares, V., Belles, M., Albina, M. L., Pujol, A., Domingo, J. L., and Sanchez, D. J. (2010). Effects of BDE-99 on hormone homeostasis and biochemical parameters in adult male rats. *Food Chem. Toxicol.* **48**, 2206–2211.

Belles, M., Alonso, V., Linares, V., Albina, M. L., Sirvent, J. J., Domingo, J. L., and Sanchez, D. J. (2010). Behavioral effects and oxidative status in brain regions of adult rats exposed to BDE-99. *Toxicol. Lett.* **194**, 1–7.

Blanco, J., Mulero, M., Lopez, M., Domingo, J. L., and Sanchez, D. J. (2011). BDE-99 deregulates BDNF, Bcl-2 and the mRNA expression of thyroid receptor isoforms in rat cerebellar granular neurons. *Toxicology* **290**, 305–311.

Bocio, A., Llobet, J. M., Domingo, J. L., Corbella, J., Teixido, A., and Casas, C. (2003). Polybrominated diphenyl ethers (PBDEs) in foodstuffs: Human exposure through the diet. *J. Agric. Food Chem.* **51**, 3191–3195.

Breslin, W. J., Kirk, H. D., and Zimmer, M. A. (1989). Teratogenic evaluation of a polybromodiphenyl oxide mixture in New Zealand white rabbits following oral exposure. *Fundam. Appl. Toxicol.* **12**, 151–157.

Chen, J., Liufu, C., Sun, W., Sun, X., and Chen, D. (2010). Assessment of the neurotoxic mechanisms of decabrominated diphenyl ether (PBDE-209) in primary cultured neonatal rat hippocampal neurons includes alterations in second messenger signaling and oxidative stress. *Toxicol. Lett.* **192**, 431–439.

Cheng, J., Gu, J., Ma, J., Chen, X., Zhang, M., and Wang, W. (2009). Neurobehavioural effects, redox responses and tissue distribution in rat offspring developmental exposure to BDE-99. *Chemosphere*. **75**, 963–968.

COP4 (2009). *Summary of the Fourth Conference of the Parties to the Stockholm Convention on Persistent Organic Pollutants: 4–8 May*. Earth Negotiations Bulletin, Vol. 15. Available at: <http://www.iisd.ca/download/pdf/enb15174e.pdf>. Accessed June 14, 2009.

Costa, L. G., Giordano, G., Tagliaferri, S., Caglieri, A., and Mutti, A. (2008). Polybrominated diphenyl ether (PBDE) flame retardants: Environmental contamination, human body burden and potential adverse health effects. *Acta Biomed.* **79**, 172–183.

Darnerud, P. O., Eriksen, G. S., Johannesson, T., Larsen, P. B., and Viluksela, M. (2001). Polybrominated diphenyl ethers: Occurrence, dietary exposure, and toxicology. *Environ. Health Perspect.* **109**(Suppl. 1), 49–68.

Dingemans, M. M., van den Berg, M., and Westerink, R. H. (2011). Neurotoxicity of brominated flame retardants: (In)direct effects of parent and hydroxylated polybrominated diphenyl ethers on the (developing) nervous system. *Environ. Health Perspect.* **119**, 900–907.

Domingo, J. L. (2011a). Influence of cooking processes on the concentrations of toxic metals and various organic environmental pollutants in food: A review of the published literature. *Crit. Rev. Food Sci. Nutr.* **51**, 29–37.

Domingo, J. L. (2011b). Polybrominated diphenyl ethers in food and human dietary exposure: A review of the recent scientific literature. *Food Chem. Toxicol.* **50**, 238–249.

Domingo, J. L., Bocio, A., Falco, G., and Llobet, J. M. (2006). Exposure to PBDEs and PCDEs associated with the consumption of edible marine species. *Environ. Sci. Technol.* **40**, 4394–4399.

Domingo, J. L., Marti-Cid, R., Castell, V., and Llobet, J. M. (2008). Human exposure to PBDEs through the diet in Catalonia, Spain: Temporal trend. A review of recent literature on dietary PBDE intake. *Toxicology* **248**, 25–32.

Doucet, J., Tague, B., Arnold, D. L., Cooke, G. M., Hayward, S., and Goodyer, C. G. (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.

Dunnick, J. K., and 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.

Gascon, M., Vrijheid, M., Martinez, D., Forn, J., Grimalt, J. O., Torrent, M., and Sunyer, J. (2011). Effects of pre and postnatal exposure to low levels of polybromodiphenyl ethers on neurodevelopment and thyroid hormone levels at 4 years of age. *Environ. Int.* **37**, 605–611.

Gomara, B., Herrero, L., Ramos, J. J., Mateo, J. R., Fernandez, M. A., Garcia, J. F., and Gonzalez, M. J. (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.
- He, Y., Murphy, M. B., Yu, R. M., Lam, M. H., Hecker, M., Giesy, J. P., Wu, R. S., and Lam, P. K. (2008). Effects of 20 PBDE metabolites on steroidogenesis in the H295R cell line. *Toxicol. Lett.* **176**, 230–238.
- Herbstman, J. B., Sjodin, A., Kurzon, M., Lederman, S. A., Jones, R. S., Rauh, V., Needham, L. L., Tang, D., Niedzwiecki, M., Wang, R. Y., et al. (2010). Prenatal exposure to PBDEs and neurodevelopment. *Environ. Health Perspect.* **118**, 712–719.
- Hu, X. Z., Xu, Y., Hu, D. C., Hui, Y., and Yang, F. X. (2007). Apoptosis induction on human hepatoma cells Hep G2 of decabrominated diphenyl ether (PBDE-209). *Toxicol. Lett.* **171**, 19–28.
- Jones-Otazo, H. A., Clarke, J. P., Diamond, M. L., Archbold, J. A., Ferguson, G., Harner, T., Richardson, G. M., Ryan, J. J., and 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.
- Kitamura, S., Shinohara, S., Iwase, E., Sugihara, K., Uramaru, N., Shigematsu, H., Fujimoto, N., and Ohta, S. (2008). Affinity for thyroid hormone and estrogen receptors of hydroxylated polybrominated diphenyl ethers. *J. Health Sci.* **54**, 607–614.
- Kojima, H., Takeuchi, S., Uramaru, N., Sugihara, K., Yoshida, T., and 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.
- Kuriyama, S. N., Wanner, A., Fidalgo-Neto, A. A., Talsness, C. E., Koerner, W., and Chahoud, I. (2007). Developmental exposure to low-dose PBDE-99: Tissue distribution and thyroid hormone levels. *Toxicology* **242**, 80–90.
- Lee, E., Kim, T. H., Choi, J. S., Nabanata, P., Kim, N. Y., Ahn, M. Y., Jung, K. K., Kang, I. H., Kim, T. S., Kwack, S. J., et al. (2010). Evaluation of liver and thyroid toxicity in Sprague-Dawley rats after exposure to polybrominated diphenyl ether BDE-209. *J. Toxicol. Sci.* **35**, 535–545.
- Lema, S. C., Schultz, I. R., Scholz, N. L., Incardona, J. P., and Swanson, P. (2007). Neural defects and cardiac arrhythmia in fish larvae following embryonic exposure to 2,2',4,4'-tetrabromodiphenyl ether (PBDE 47). *Aquat. Toxicol.* **82**, 296–307.
- Lilienthal, H., Hack, A., Roth-Harer, A., Grande, S. W., and Talsness, C. E. (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.
- Main, K. M., Kiviranta, H., Virtanen, H. E., Sundqvist, E., Tuomisto, J. T., Tuomisto, J., Vartiainen, T., Skakkebaek, N. E., and Toppari, J. (2007). Flame retardants in placenta and breast milk and cryptorchidism in newborn boys. *Environ. Health Perspect.* **115**, 1519–1526.
- McClain, V., Stapleton, H. M., Tilton, F., and Gallagher, E. P. (2011). BDE 49 and developmental toxicity in zebrafish. *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.* **155**, 253–258.
- Mulero, M., Romeu, M., Giralt, M., Folch, J., Nogues, M. R., Fortuno, A., Sureda, F. X., Linares, V., Cabre, M., Paternain, J. L., et al. (2006). Oxidative stress-related markers and Langerhans cells in a hairless rat model exposed to UV radiation. *J. Toxicol. Environ. Health Part A* **69**, 1371–1385.
- Perelló, G., Martí-Cid, R., Castell, V., Llobet, J. M., and Domingo, J. L. (2009). Concentrations of polybrominated diphenyl ethers, hexachlorobenzene and polycyclic aromatic hydrocarbons in various foodstuffs before and after cooking. *Food Chem. Toxicol.* **47**, 709–715.
- Peters, A. K., Nijmeijer, S., Gradin, K., Backlund, M., Bergman, A., Poellinger, L., Denison, M. S., and Van den Berg, M. (2006). Interactions of polybrominated diphenyl ethers with the aryl hydrocarbon receptor pathway. *Toxicol. Sci.* **92**, 133–142.
- Roze, E., Meijer, L., Bakker, A., Van Braeckel, K. N., Sauer, P. J., and Bos, A. F. (2009). Prenatal exposure to organohalogenes, including brominated flame retardants, influences motor, cognitive, and behavioral performance at school age. *Environ. Health Perspect.* **117**, 1953–1958.
- Sanders, J. M., Burka, L. T., Smith, C. S., Black, W., James, R., and Cunningham, M. L. (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.
- Schechter, A., Papke, O., Tung, K. C., Joseph, J., Harris, T. R., and Dahlgren, J. (2005). Polybrominated diphenyl ether flame retardants in the U.S. population: Current levels, temporal trends, and comparison with dioxins, dibenzofurans, and polychlorinated biphenyls. *J. Occup. Environ. Med.* **47**, 199–211.
- Schuhmacher, M., Kiviranta, H., Ruokojarvi, P., Nadal, M., and Domingo, J. L. (2009). Concentrations of PCDD/Fs, PCBs and PBDEs in breast milk of women from Catalonia, Spain: A follow-up study. *Environ. Int.* **35**, 607–613.
- Staples, R. E., and Schnell, V. L. (1968). Refinements in rapid clearing technique in the KOH-alizarin red S method for fetal bone. *Stain Technol.* **43**, 61–63.
- Stoilov, I. (2001). Cytochrome P450s: Coupling development and environment. *Trends Genet.* **17**, 629–632.
- Szabo, D. T., Richardson, V. M., Ross, D. G., Diliberto, J. J., Kodavanti, P. R., and Birbaum, L. S. (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.
- Talsness, C. E., Shakibaei, M., Kuriyama, S. N., Grande, S. W., Stermer-Kock, A., Schnitker, P., de Souza, C., Grote, K., and 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.
- Trudel, D., Scheringer, M., von Goetz, N., and Hungerbuehler, K. (2011). Total consumer exposure to polybrominated diphenyl ethers in North America and Europe. *Environ. Sci. Technol.* **45**, 2391–2397.
- Usenko, C. Y., Robinson, E. M., Usenko, S., Brooks, B. W., and Bruce, E. D. (2011). PBDE developmental effects on embryonic zebrafish. *Environ. Toxicol. Chem.* **30**, 1865–1872.
- Watkins, D. J., McClean, M. D., Fraser, A. J., Weinberg, J., Stapleton, H. M., Sjodin, A., and Webster, T. F. (2011). Exposure to PBDEs in the office environment: Evaluating the relationships between dust, handwipes, and serum. *Environ. Health Perspect.* **119**, 1247–1252.
- Wilson, J. G. (1965). Embryological considerations in teratology. In *Teratology: Principles and Techniques* (Wilson, J. G. and J. Warkany, Eds.), pp. 251–277. University of Chicago, Chicago, IL.
- Yang, W., Shen, S., Mu, L., and Yu, H. (2011). Structure-activity relationship study on the binding of PBDEs with thyroxine transport proteins. *Environ. Toxicol. Chem.* **30**, 2431–2439.
- Zangar, R. C., Davydov, D. R., and Verma, S. (2004). Mechanisms that regulate production of reactive oxygen species by cytochrome P450. *Toxicol. Appl. Pharmacol.* **199**, 316–331.
- Zhou, T., Taylor, M. M., DeVito, M. J., and Crofton, K. M. (2002). Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. *Toxicol. Sci.* **66**, 105–116.
- Zupan, G., Pilipovic, K., Hrelja, A., and Petermel, S. (2008). Oxidative stress parameters in different rat brain structures after electroconvulsive shock-induced seizures. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **32**, 771–777.

4.3 Perinatal exposure to BDE-99 causes learning disorders and decreases serum thyroid hormone levels and BDNF gene expression in hippocampus in rat offspring.

Blanco, J., Mulero, M., Heredia, L., Pujol, A., Domingo, J. L., and Sanchez, D. J. (2013)

Toxicology (2013) 308, 122-128



Perinatal exposure to BDE-99 causes learning disorders and decreases serum thyroid hormone levels and BDNF gene expression in hippocampus in rat offspring

Jordi Blanco^{a,b}, Miquel Mulero^c, Luis Heredia^{a,d}, Andreu Pujol^b, José L. Domingo^a, Domènec J. Sánchez^{a,b,*}

^a Laboratory of Toxicology and Environmental Health, School of Medicine, IISPV, Universitat Rovira i Virgili, Sant Llorens 21, 43201 Reus, Spain

^b Physiology Unit, School of Medicine, IISPV, Universitat Rovira i Virgili, Sant Llorens 21, 43201 Reus, Spain

^c Nutrigenomics Group, Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Marcel·lí Domingo, s/n, Campus Sescelades, 43007 Tarragona, Spain

^d CRAMC, Department of Psychology, Universitat Rovira i Virgili, carretera de Valls, s/n, 43007 Tarragona, Spain

ARTICLE INFO

Article history:

Received 13 March 2013

Received in revised form 25 March 2013

Accepted 25 March 2013

Available online xxx

Keywords:

Polybrominated diphenyl ethers (PBDEs)

2,2',4,4',5-pentaBDE (BDE-99)

Brain derived neurotrophic factor (BDNF)

Thyroid receptors (TRs)

Thyroid hormones (THs)

ABSTRACT

Exposure of pregnant women to polybrominated diphenyl ethers (PBDEs) may mean serious health risks. The main goal of the present study was to examine the neurobehavioral changes in rat offspring that were perinatally exposed to one of the most prevalent PBDEs congeners found in humans, 2,2',4,4',5-pentaBDE (BDE-99). Rat dams were exposed to 0, 1 and 2 mg/kg/day of BDE-99 from gestation day 6 to post-natal day 21. When pups were weaning, cortex and hippocampal gene expressions of brain-derived neurotrophic factor (BDNF) of the different isoforms of the thyroid hormone (TH) receptors (TRs) were evaluated. Serum TH levels were also determined. The remaining pups were assessed by neurobehavioral testing for learning and memory function. The results showed that maternal transference of BDE-99 produced a delay in the spatial learning task in the water maze test. Moreover, the open-field test revealed a significant dose-response anxiolytic effect. It was also found that the serum levels of triiodothyronine (T3), tetraiodothyronine (T4) and free-T4 (FT4) decreased. Although no effect on the gene expression of the different isoforms of TRs was observed, the expression of the TH-mediated gene BDNF was down-regulated in the hippocampus. These results indicate a clear signal disruption of TH and reinforce previous studies in which neurotoxic effects of PBDEs in animal research were observed at levels comparable to those found in humans.

© 2013 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

The intensive production of polymeric materials during the last century has promoted the continuous emission of xenobiotics into the environment and their consequent inclusion into the biota. Among these, polybrominated diphenyl ethers (PBDEs), a family of 209 flame retardant compounds widely used by the chemical industry as additives in the manufacture of plastics, polyurethane foams, textiles, electronic components and other applications, are included (Alaee et al., 2003; Covaci et al., 2003). The persistent hydrophobic structure of PBDEs has contributed to their bio-magnification across the trophic chain, being concentrations of

these pollutants currently present in all ecosystems (Costa et al., 2008; Darnerud, 2003).

The pollution of PBDEs is particularly harmful to people living in North America, whose exposure concentration is one or two orders of magnitude greater than that of people living in Japan or Europe (Costa et al., 2008; Johnson-Restrepo et al., 2005). Despite the bans and restrictions on the use and production of PBDEs (COP4, 2009), elevated quantities of these compounds can be still found in indoor environments. The current emissions primarily come from the degradation of appliances containing PBDEs that remain functional in many homes and worksites (Jones-Otazo et al., 2005). The ingestion, absorption and inhalation of dust particles containing PBDEs are the major sources of human exposure to these compounds, along with direct dietary intake (Domingo, 2004, 2012; Muenhor and Harrad, 2012; Quiros-Alcala et al., 2011; Vorkamp et al., 2011).

The levels of PBDEs accumulated in pregnant women present a serious health risk because these compounds can be transferred to the offspring across the placental barrier and through ingestion

* Corresponding author at: Laboratory of Toxicology and Environmental Health, School of Medicine, IISPV, Universitat Rovira i Virgili, Sant Llorens 21, 43201 Reus, Spain. Tel.: +34 977 759374; fax: +34 977 759322.

E-mail address: domingo.sanchez@urv.cat (D.J. Sánchez).

of breast milk (Gomara et al., 2007; Schuhmacher et al., 2009). A number of recent epidemiological studies have correlated pre- and post-natal exposure to high levels of PBDEs with learning and memory impairment (Herbstman et al., 2010; Roze et al., 2009), neurobehavioral changes (Chao et al., 2011; Gascon et al., 2012; Hoffman et al., 2012; Shy et al., 2011), lower birth weight (Foster et al., 2011) and damage to the reproductive system (Main et al., 2007). All these effects observed in toddlers were previously described in animal studies, several of which revealed toxic effects at lower levels than those currently detected in North American women (Kuriyama et al., 2005, 2007; Ta et al., 2011; Talsness et al., 2005).

The capacity of PBDEs to disrupt thyroid hormone (TH) signaling could be largely responsible for the neurodevelopmental disorders above described. The thyroid hormone system is of key importance for the development, establishment and survival of the nervous system (Anderson, 2001). The alteration of the TH action *in utero* or during infancy can cause more significant harm than during adulthood. Rodent studies have shown that maternal exposure to PBDEs reduces the concentration of serum tetraiodothyronine (T4) (Kuriyama et al., 2007; Zhou et al., 2002) and can alter the gene expression of different isoforms of the nuclear thyroid receptors (TRs) (Wang et al., 2011). Moreover, certain PBDEs congeners, especially their hydroxylated metabolites, OH-PBDEs, are structurally similar to triiodothyronine (T3), which is the bioactive form of T4, and can compete to bind to TRs, deregulating the expression of TH-mediated gene expression (Kitamura et al., 2008; Kojima et al., 2009; Schreiber et al., 2010).

One of the key proteins regulated by TH is the brain-derived neurotrophic factor (BDNF). This neurotrophin is involved in the process of long-term potentiation (LTP), one of the best known biochemical mechanisms that underlie learning and memory. BDNF induces a lasting form of synaptic plasticity by the increase of the synthesis of several plasticity-related proteins, including CaMKII, Arc and homer 2 (Liao et al., 2007; Schratt et al., 2004; Ying et al., 2002). The reduction of LTP was observed in rodent offspring hippocampi after acute administration of a single dose of 2,2',4,4'-tetraBDE (BDE-47) on postnatal day (PND) 10 (Dingemans et al., 2007) and after the maternal chronic administration of 2,2',3,3',4,4',5,5',6,6'-decaBDE (BDE-209) during the gestational and lactation period (Xing et al., 2009, 2010).

In recent years, we have performed in our laboratory a series of studies mainly focused on assessing human dietary exposure to PBDEs, and the concentrations accumulated in breast milk (Bocio et al., 2003; Domingo et al., 2006; Domingo, 2012; Perello et al., 2009; Schuhmacher et al., 2009). Moreover, we have also investigated the potential health effects of some PBDEs using animal and *in vitro* models (Albina et al., 2010; Alonso et al., 2010; Belles et al., 2010; Blanco et al., 2011, 2012). In the present study, we investigated the potential neurobehavioral alterations related to the disruption of the TH signaling in rat pups from dams, which were exposed during the gestational and lactation periods to one of the most prevalent PBDE congeners found in human samples, 2,2',4,4',5-pentaBDE (BDE-99) (Costa et al., 2008). When pups were weaned, serum levels of T3, T4 and free-T4 were measured in one pup from each litter, as well as the cortex and hippocampal expression of the messenger RNA (mRNA) encoding the isoforms of the TRs genes and of the TH-mediated gene BDNF. The remaining pups were subsequently evaluated by means of neurobehavioral testing for learning and memory function.

2. Materials and methods

2.1. Animals and chemicals

Sexually mature male and female Sprague Dawley rats (220–240g) were obtained from Charles River (Barcelona, Spain). After a quarantine period of 7 days,

female rats were mated with males (2:1) overnight. Vaginal smears were collected the next morning to detect the presence of sperm. The day of sperm detection was considered as gestation day (GD) 0. Animals were individually housed in plastic cages in a climate-controlled facility with a constant day-night cycle (light: 08:00h–20:00h) at a temperature of $22 \pm 2^\circ\text{C}$ and a relative humidity of $50 \pm 10\%$. Food (Panlab rodent chow, Barcelona, Spain) and tap water were available *ad libitum*. The use of animals and the experimental protocol were approved by the Animal Care and Use Committee of the Rovira i Virgili University (Tarragona, Catalonia, Spain). BDE-99 (99% pure) was purchased from LGC Standards S.L.U. (Barcelona). BDE-99 was administered by gavage after being dissolved in corn oil (vehicle).

2.2. Experimental procedure

Females with vaginal smears containing sperm were divided into 3 groups (10 animals per group). Rats in each group received daily doses of BDE-99 doses (gavage) at 0, 1 and 2 mg/kg of body weight from GD 6 to PND 21, except for PND 0 when dams were left undisturbed. Animals in the control group received the vehicle only (corn oil) during the same period. The doses of BDE-99 were chosen based on an estimated lowest observed adverse effect level (LOAEL) of 1 mg/kg/day, mainly from pentaBDE data (Darnerud et al., 2001), as well as from a recent study on tissue distribution of BDE-99 (Cheng et al., 2009).

On PND 1, all litters were randomly reduced to 4 pups (2 males and 2 females). Pups were weaned on PND 21 and housed by gender in groups of 4 per cage. To determine biochemical serum hormonal levels, one pup from each litter (a total of 5 males and 5 females per group) was anesthetized by an intraperitoneal injection of ketamine–xylazine, collecting a blood sample from the vena cava. Another pup from each litter (a total of 5 males and 5 females per group) was next sacrificed by decapitation; the hippocampus and cortex were immediately dissected on an ice-cold glass plate, frozen with liquid nitrogen and stored at -80°C . For the remaining pups, one male and one female from each litter (20 animals per group) were randomly selected to test the general motor activity on PND 22, and the spatial learning and retention memory on PND 23.

2.3. Hormone analysis

The samples serum collected on PND 21 were analyzed for T4, free T4 (FT4) and T3 concentrations with an Advia 2400 analyzer (Sakai et al., 2009).

2.4. RNA isolation and cDNA synthesis

Total RNA was obtained from a fraction of hippocampus and cortex using a Qiagen RNeasy Kit according to the manufacturer's protocol. RNA was resuspended in 100 μl of RNase-free water, quantified by a spectrophotometer at an absorbance of 260 nm and tested for purity using an A260/280 ratio, using denaturing gel electrophoresis to ensure integrity. The first strand of complementary DNA (cDNA) was reverse transcribed from 1 μg of total RNA from each sample using a QuantiTect Reverse Transcription Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's protocol. An identical reaction, without reverse transcriptase, was performed to verify the absence of genomic DNA. The cDNA was subsequently amplified by PCR using rat-specific primers for TR α 1 (NM.001017960) (forward: 5'-TGC CCT TAC TCA CCC CTA CA-3'; reverse: 5'-AAG CCA AGC CAA GCT GTC CT-3'), TR α 2 (NM.031134) (forward: 5'-TGA GCA GCA GTT TGG TGA AG-3'; reverse: 5'-GAA TGG AGA ATT CCG CTT CG-3'), TR β 1 (NM.012672) (forward: 5'-AGC CAG CCA CAG CAG CAG TA-3'; reverse: 5'-CGC CAG AAG ACT GAA GCT TGC-3'), BDNF (NM.012513) (forward: 5'-CCA TAA GGA CGC GGA CTT GT-3'; reverse: 5'-GAG GCT CCA AAG GCA CTT GA-3'); and β -actin (NM.031144) (forward: 5'-TGT CAC CAA CTG GGA CGA TA-3'; reverse: 5'-GGG GTG TTG AAG GTC TCA AA-3') with a PyroStart Fast PCR Master mix (2 \times) kit (Fermentas, Burlington, Canada) according to the manufacturer's protocol. The PCR products were separated on a 1% agarose gel and only specific bands were detected. The nonreactivity of the primers with contaminant genomic DNA was tested by the inclusion of controls that omitted reverse transcriptase from the cDNA synthesis reaction.

2.5. Real time reverse transcription-PCR

Quantitative PCR for TR α 1, TR α 2, TR β 1, BDNF, and β -actin was completed using the QuantiTect SYBR Green PCR kit (Qiagen, Inc.) according to the manufacturer's protocol and a Rotor-Gene Q Real-Time PCR cycler (Qiagen, Inc.). The thermal cycling was composed of an initial step at 50°C for 2 min followed by a polymerase activation step at 95°C for 15 min and a cycling step with the following conditions: 40 cycles of denaturing at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Oligonucleotides of varying lengths produced dissociation peaks at different melting temperatures. Consequently, at the end of the PCR cycles, PCR products were analyzed using a heat dissociation protocol to confirm that one single PCR product was detected by SYBR Green dye. Fluorescence data were acquired at the 72°C step. The threshold cycle (Ct) was calculated by Rotor-Gene Q 2.0 software to identify significant fluorescence signals above noise during the early cycles of amplification. The software calculated copy numbers for the target samples from the Ct by interpolating from a standard curve. The relative levels of the expression of

the target genes were measured using β -actin mRNA as an internal control according to the $2^{-\Delta\Delta Ct}$ method.

2.6. Neurobehavioral tests

2.6.1. Water maze test

The water maze consisted of a circular tank (diameter: 1 m; height: 60 cm) divided into four quadrants. An escape platform (10 cm in diameter) was located 1 cm below the water surface in the target quadrant (Morris, 1984). Rats performed two trials per day for 10 consecutive days. During each trial, rats were allowed 90 s to find the hidden platform and remain on it for 30 s. If the rats failed to find the platform, they were placed on it by the experimenter. The inter-trial interval was 30 min. At the start of each trial, rats were randomly placed in one of the three non-target quadrants. Animals learned where the platform is from signals external to the maze. To avoid proximal cues and to prevent egocentric learning, an internal mobile wall was added to the maze and randomly moved between trials (Ribes et al., 2008). On day 10 after the acquisition trial, animals performed a probe trial that consisted of 60 s of free navigation without the platform. Seventy-two hours after the last training session, retention of the task was tested using the same procedure. Performance was recorded with a video camera placed above the maze, being data analyzed using the video tracking program Etho-Vision® v3.0 (Noldus Information Technologies, Wageningen, The Netherlands). The latency to find the escape platform, the distance traveled, and the swim velocity during the training sessions were measured. During the probe trials, the total time spent in the target quadrant and the time spent in the other quadrants were also measured to compare the time spent searching in the target quadrant with the average time spent in the other quadrants.

2.6.2. Open-field activity

General motor activity was measured in an open-field apparatus, consisting of a wooden 47 cm \times 47 cm square surrounded by a 40-cm-high light-colored wall. A 10 cm area near the surrounding wall was delimited and considered as the periphery, while the rest of the open-field more than 10 cm far from the wall, was considered as the center area. At the start of the test, rats were released in the periphery. During the test; rats were allowed to move freely around the open-field for 30 min. The path and movements of the animals were recorded with a video camera (Sony CCD-IRIS model), which was placed above the square. The video tracking program Etho-Vision® v3.0 was used to measure the total distance traveled and the number of rearings (as a measure of vertical activity).

2.7. Statistics

To evaluate the homogeneity of variances, the Levene test was used. When the variances of different treatment groups were homogeneous, an ANOVA followed by a Bonferroni post hoc test was used to establish the level of significance among groups. If the variances were not homogeneous, the Kruskal–Wallis and the Mann–Whitney U-tests were used. The level of statistical significance for all tests was set at $P < 0.05$. All data were analyzed with the SPSS 15.0 software (SPSS Sciences, Chicago, IL).

3. Results

Because sex differences in rat offspring did not contribute significantly to any results obtained in this study, the data from males and females were reunited to study differences between treated and untreated groups.

3.1. Effects of BDE-99 on spatial learning and retention memory

The effect of BDE-99 on the correct task of spatial learning and retention memory was evaluated on young rat pups using the water maze test. During the acquisition trials (Fig. 1A), all groups showed a significant [$F(2,57) = 4.321, P = 0.18$] decrease in the time spent (over days) to locate the platform. On the second and third day, a no statistically significant increasing trend in the latency time was observed in the groups of pup rats of dams that were exposed to 1 or 2 mg/kg/day of BDE-99 with respect to those in the control group. This trend was maintained until the fourth day, when the group given 2 mg/kg/day of BDE-99 spent a significant ($P = 0.043$) longer time locating the platform compared to the control group. In subsequent days, the latency time of the treated groups was equivalent to the control group. Furthermore, there were no significant differences among the different groups in terms of time spent in the target quadrant in the retention of learning probes conducted on the tenth and thirteenth days (Fig. 1B). These results indicate

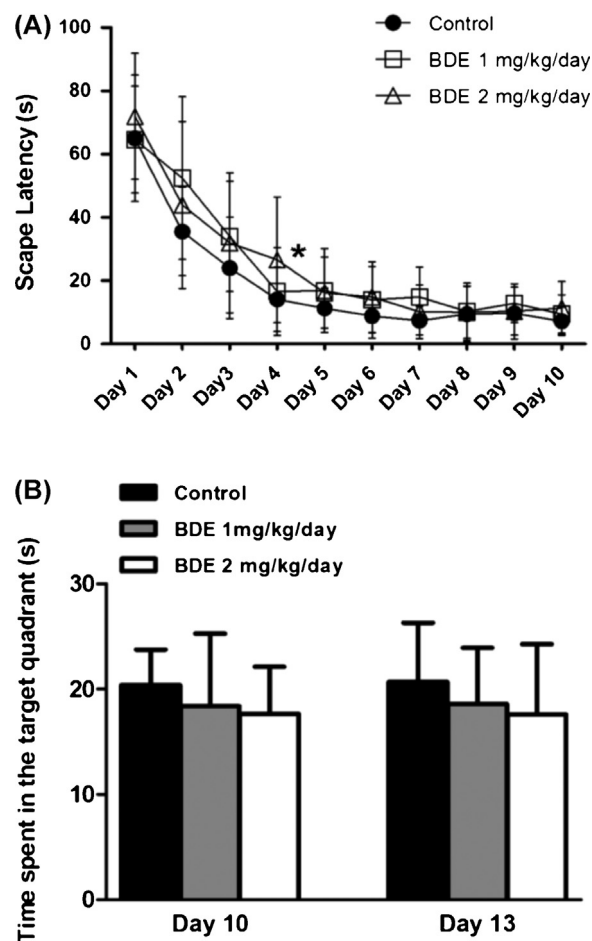


Fig. 1. Perinatal exposure to BDE-99 produces a delay in the spatial learning task. Rat offspring of dams that were exposed to 0, 1 and 2 mg/kg/day of BDE-99 from GD 6 to PND 21 were tested in the water maze. (A) An initial, longer latency in reaching the platform was produced at the highest BDE-99 dose with respect to the control group. (B) No significant differences were found in the time spent in the target quadrant (where the platform was) in the probe test. Data are expressed as means \pm SD. Significant differences were analyzed with an ANOVA followed by Bonferroni's post hoc test. * $P < 0.05$.

that the transmission of maternal accumulated BDE-99 through placenta and breast milk had an initial effect on the spatial learning task, but this effect was reduced when the days of assimilation were prolonged.

3.2. Open-field test

The possible effect of maternal transmission of BDE-99 on the behavioral activity of young rat pups was evaluated using the open-field test on PND 22. The evidence revealed habituation of all animals to the open-field environment (Fig. 2A) as reflected by a gradual decrease in their locomotor activity (Fig. 2A) with no significant differences between the groups in total distance [$F(2,57) = 3.055, P = 0.065$] and total number of rearings [$F(2,57) = 2.973, P = 0.071$]. However, the group exposed to 2 mg/kg/day of BDE-99 showed a significant increase in the time spent in the center area ($P = 0.004$) (Fig. 2B) and the distance traveled in the center area ($P = 0.006$) (Fig. 2C) compared to the control group. These findings indicate that BDE-99 could produce a significant dose-response anxiolytic effect in the treated groups, because during the open-field test rat pups tended to spend more time in the center of the open-field.

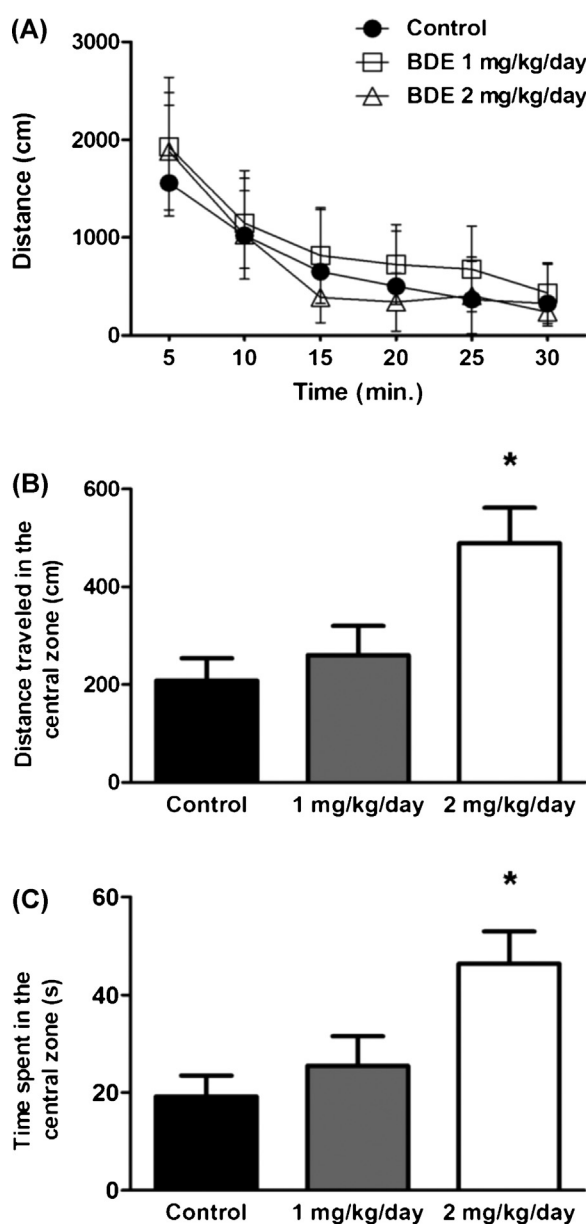


Fig. 2. Perinatal exposure to BDE-99 reduces anxiety. Rat offspring of dams that were exposed to 0, 1, 2 mg/kg/day of BDE-99 from GD 6 to PND 21 were tested in the open-field. (A) No significant differences were observed in the total distance traveled. Significant differences were observed in the (B) distance traveled in the central zone and (C) the time spent in the central zone. Data are expressed as means \pm SD. Significant differences were analyzed with an ANOVA, followed by Bonferroni's post hoc test. * $P < 0.05$.

3.3. Effect of BDE-99 over thyroid hormones levels

A significant dose response effect of BDE-99 was found for thyroid hormone levels in analyzed serum of rat pups on PND 21 (Table 1). The serum free T4 concentration was significantly ($P = 0.06$) decreased by up to $17 \pm 9\%$ in the 2 mg/kg/day group with respect to control group. Similarly, the total T4 ($25 \pm 13\%$, $P = 0.003$) and T3 ($13 \pm 9\%$, $P = 0.039$) concentrations also decreased significantly in the 2 mg/kg/day group with respect to the control group.

3.4. Thyroid receptors and BDNF gene expression in cortex and hippocampus

Due to the biological importance for the correct development and function of the nervous system, the gene expression of BDNF

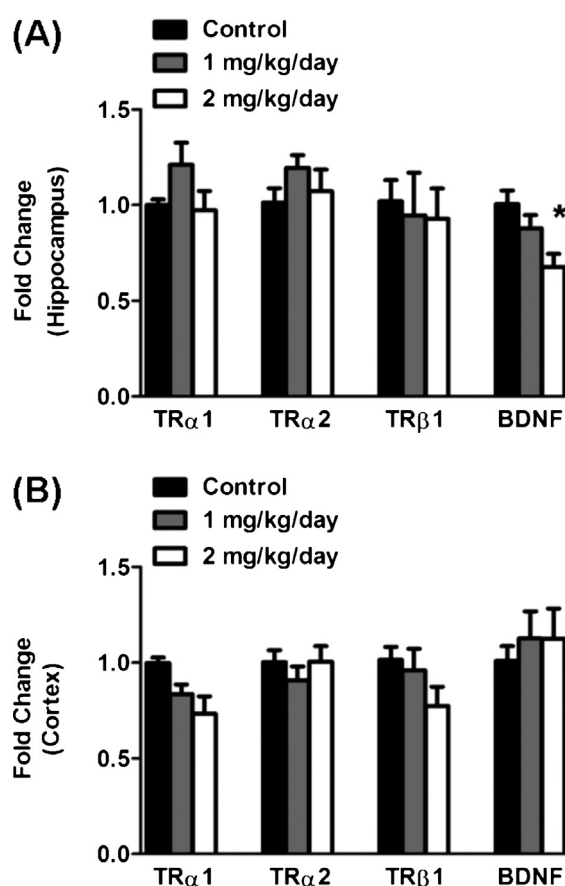


Fig. 3. Perinatal exposure to BDE-99 down-regulate the hippocampus gene expression of BDNF. The (A) hippocampus and (B) cortex of rat offspring of dams that were exposed to 0, 1, 2 mg/kg/day of BDE-99 from GD 6 to PND 21 were dissected, and the relative expression levels of the mRNAs encoding the TR α 1, TR α 2, TR β 1 and BDNF were determined by quantitative real-time RT-PCR assay. Data were normalized using the gene β -actin as an internal control. The fold changes were calculated relative to the control. Data are expressed as means \pm SD. Significant differences were analyzed with an ANOVA followed by Bonferroni's post hoc test. * $P < 0.05$.

and TRs isoforms was evaluated on PND 21 in the cortex and hippocampus of rat pups by real-time qPCR (Fig. 3). Compared with the data of the control group (Fig. 3A), the data showed a tendency to decrease of the hippocampal gene expression of BDNF in the group given BDE-99 at 1 mg/kg/day ($22 \pm 17\%$, $P = 0.199$), being statistically significant in the group exposed to 2 mg BDE-99/kg/day ($32 \pm 14\%$, $P = 0.044$). In contrast, no influence of BDE-99 (Fig. 3B) was observed in the BDNF gene expression in the cortex of the rat pups. Moreover, the mRNA expression of the different isoforms of TRs did not show any significant difference in both analyzed tissues with respect to the control. However, TR α 1 and TR β 1 tended to decrease in the cortex tissues of treated rat pups (Fig. 3B). It was also observed that BDE-99 influenced the BDNF gene expression in hippocampus, but this effect was not observed in the cortex tissues of rat pups.

4. Discussion

In this study, the maternal transmission of BDE-99 during gestational and lactation periods produced behavioral disturbances in the rat offspring. In the first four days of training in a water maze test, the BDE-99-treated groups showed a delay in the mastery of a spatial learning task with respect to the control group. This initial alteration was reduced after multiple days with the reiteration of the learning task. In recent years, a number of authors (Cheng et al., 2009; Ta et al., 2011; Woods et al., 2012) reported similar

Table 1
Effect on serum thyroid hormone levels of rat offspring after perinatal exposure to BDE-99.

Exposure group	Control (n = 10)	BDE 1 mg/kg/day (n = 10)	BDE 2 mg/kg/day (n = 10)
T3 (nmol/L)	1.91 ± 0.18 ^a	1.84 ± 0.16 ^{ab}	1.65 ± 0.18 ^b
T4 (nmol/L)	39.8 ± 6.2 ^a	32.3 ± 5.3 ^{ab}	29.8 ± 4.2 ^b
FT4 (pmol/L)	32.1 ± 2.8 ^a	28.7 ± 2.1 ^{ab}	26.4 ± 3.2 ^b

Results are expressed as mean values ± SD. Significant differences were analyzed by ANOVA followed by a Bonferroni's post hoc test. Values in the same row showing a common superscript (a, b) are not significantly different at $P < 0.05$.

neurobehavioral effects in rodent offspring. In those studies, perinatal exposure to BDE-99 (or BDE-47) caused altered performance during the execution of a water maze test; specifically, a significantly longer latency to locate the platform.

On the other hand, a various studies have shown that perinatal or neonatal exposure to BDE-99 decreased habituation and produced transient hyperactivity in mice in the open-field test (Branchi et al., 2002, 2005; Eriksson et al., 2001, 2002; Kuriyama et al., 2005; Viberg et al., 2002, 2004a,b). In the current study, the open-field test did not show significant differences in the total distance traveled and in the number of rearings between BDE-99-treated animals and those in the control group. A decrease of habituation in the highest treated group was noted on the time interval 10–25 min, but this effect was not statistically significant. However, we found a dose-response increase of the time spent in the central zone, which was significant in the group given the highest BDE-99 dose. This altered thigmotaxis might be caused by a possible anxiolytic effect of BDE-99 in the rat offspring. Branchi et al. (2002, 2005) suggested the same anxiolytic effect on the basis of the decrease of the time spent near the walls during the execution of the open-field test by mice perinatally exposed to BDE-99. Moreover, other investigations did not show a significant anxiogenic effect of PBDEs in the elevated plus maze test and in the elevated zero maze test, which are known rodent models of neurobiological anxiety research (Daubie et al., 2011; Heredia et al., 2012; Johansson et al., 2008; Ta et al., 2011). Woods et al. (2012) studied the effects of perinatal exposure to BDE-47 in a mutant mouse (Mecp2 (308)). A male mutant mouse showed social behavioral defects, such as anxiogenic phenotype (Woods et al., 2012). However, surprisingly, when the male mutant mouse was perinatally exposed to BDE-47 the anxiety level was reduced.

The neurobehavioral disturbances observed in the open-field and water maze probes suggest a possible alteration of hippocampal functioning. The dose-dependent decrease of T3, T4 and FT4 found in serum of perinatally exposed offspring might be responsible for a large part of the neurotoxic effects of BDE-99. Symptoms of hypothyroidism include an anxiolytic pattern and difficulty learning. Several animal studies have reported a decrease of the serum T4 and T3 levels in relation to PBDEs exposure (Kuriyama et al., 2007; Tseng et al., 2008; Zhou et al., 2001, 2002). A possible reason for the decrease of T4 may be that PBDEs would induce a detoxification response in the liver, which concomitantly would affect T4 (Szabo et al., 2009; Zhou et al., 2001, 2002).

The TH undertakes important actions in the processes of neuronal maturation and migration (Anderson, 2001). Dingemans et al. (2007) and Xing et al. (2009, 2010) showed that exposure to PBDEs could change the synaptic plasticity of rodent hippocampus, decreasing the voltage-gated sodium channel currents and the expression of postsynaptic proteins. BDNF is an important TH-regulated protein, which is implicated in all of these neuronal processes. This neurotrophine plays a crucial role in learning and memory processes, triggering the activation of signaling pathways involving phosphatidylinositol 3 kinase, phospholipase C gamma, and intracellular signal-regulated kinase 1/2 (Liao et al., 2007; Schrott et al., 2004; Ying et al., 2002). In the present investigation, we found that the mRNA expression of BDNF was significantly down-regulated in offspring hippocampi after perinatal exposure

to the highest dose of BDE-99. Jiang et al. (2008) and Viberg et al. (2008) also reported a decrease of BDNF in mouse hippocampus after exposure to BDE-209, while Wang et al. (2011) showed a conditional decrease of the concentration of BDNF in hippocampus, which was noted only when the rat offspring were perinatally co-exposed to both BDE-47 and perfluorooctane substances.

In a recent in vitro study, we found that BDE-99 might activate an auto-regulatory mechanism by creating a state that mimics hyperthyroidism in cells. It would lead to a decrease of the gene expression of the TRs isoforms (Blanco et al., 2011). In the current investigation, we did not find changes in the gene expression of the TRs isoforms, which was possibly due to a decrease of the thyroid hormones levels in serum. Consequently, the sum of the concentration of BDE-99 along with T3 would not be enough to activate the auto-regulatory mechanism in hippocampus and cortex.

We hypothesized that the decrease of the serum levels of TH, along with the action of hydroxylated metabolites of BDE-99, which can bind in antagonistic form to the TRs inhibiting their transcriptional action, could be responsible for the down-regulation of the gene expression of BDNF and the observed neurobehavioral alterations.

In conclusion, perinatal exposure to BDE-99 through gestation and ingestion of maternal breast milk may produce difficulties in learning and may induce an anxiolytic pattern in the rat offspring. BDE-99 also decreased the serum levels of T3, T4 and FT4, which could be partially responsible for the neurobehavioral disorders. Although no effect on the mRNA expression of the different isoforms of TRs was observed, the down-regulation of the expression of the TH-mediated gene BDNF in the hippocampus is a clear signal disruption of the TH action of BDE-99. These findings reinforce the results of previous studies where the neurotoxic effects of PBDEs were observed at levels that have been found in humans. Further investigations on the potential health risks of these pollutants are still necessary for a better understanding of these effects.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors thank Concepción Medina, Socorro Matilla, Concepción García (Hormonal Section, Laboratori Clinic ICS Camp de Tarragona, Hospital Universitari Joan XXIII) for their skillful help in the hormonal determinations. This work was financially supported by the "Fondo de Investigación Sanitaria", Ministry of Health, Spain through grant no. P107631, and FEDER funds.

References

- Alaee, M., Arias, P., Sjodin, A., Bergman, A., 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.
- Albina, M.L., Alonso, V., Linares, V., Belles, M., Sirvent, J.J., Domingo, J.L., Sanchez, D.J., 2010. Effects of exposure to BDE-99 on oxidative status of liver and kidney in adult rats. *Toxicology* 271, 51–56.

- Alonso, V., Linares, V., Belles, M., Albina, M.L., Pujol, A., Domingo, J.L., Sanchez, D.J., 2010. Effects of BDE-99 on hormone homeostasis and biochemical parameters in adult male rats. *Food Chem. Toxicol.* 48, 2206–2211.
- Anderson, G.W., 2001. Thyroid hormones and the brain. *Front. Neuroendocrinol.* 22, 1–17.
- Belles, M., Alonso, V., Linares, V., Albina, M.L., Sirvent, J.J., Domingo, J.L., Sanchez, D.J., 2010. Behavioral effects and oxidative status in brain regions of adult rats exposed to BDE-99. *Toxicol. Lett.* 194, 1–7.
- Blanco, J., Mulero, M., Domingo, J.L., Sanchez, D.J., 2012. Gestational exposure to BDE-99 produces toxicity through up-regulation of CYP isoforms and ROS production in the fetal rat liver. *Toxicol. Sci.* 127, 296–302.
- Blanco, J., Mulero, M., Lopez, M., Domingo, J.L., Sanchez, D.J., 2011. BDE-99 deregulates BDNF-Bcl-2 and the mRNA expression of thyroid receptor isoforms in rat cerebellar granular neurons. *Toxicology* 290, 305–311.
- Bocio, A., Llobet, J.M., Domingo, J.L., Corbella, J., Teixido, A., Casas, C., 2003. Polybrominated diphenyl ethers (PBDEs) in foodstuffs: human exposure through the diet. *J. Agric. Food Chem.* 51, 3191–3195.
- Branchi, I., Alleva, E., Costa, L.G., 2002. Effects of perinatal exposure to a polybrominated diphenyl ether (PBDE 99) on mouse neurobehavioural development. *Neurotoxicology* 23, 375–384.
- Branchi, I., Capone, F., Vitalone, A., Madia, F., Santucci, D., Alleva, E., Costa, L.G., 2005. Early developmental exposure to BDE 99 or Aroclor 1254 affects neurobehavioural profile: interference from the administration route. *Neurotoxicology* 26, 183–192.
- Chao, H.R., Tsou, T.C., Huang, H.L., Chang-Chien, G.P., 2011. Levels of breast milk PBDEs from southern Taiwan and their potential impact on neurodevelopment. *Pediatr. Res.* 70, 596–600.
- 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.
- COP4, 2009. Summary of the Fourth Conference of the Parties to the Stockholm Convention on Persistent Organic Pollutants: 4–8 May. *Earth Neg. Bull.* 15, Available at: <http://www.iisd.ca/download/pdf/enb15174e.pdf> (accessed 14.06.09).
- Costa, L.G., Giordano, G., Tagliiferri, S., Caglieri, A., Mutti, A., 2008. Polybrominated diphenyl ether (PBDE) flame retardants: environmental contamination, human body burden and potential adverse health effects. *Acta Biomed.* 79, 172–183.
- Covaci, A., Voorspoels, S., de Boer, J., 2003. Determination of brominated flame retardants, with emphasis on polybrominated diphenyl ethers (PBDEs) in environmental and human samples – a review. *Environ. Int.* 29, 735–756.
- Darnerud, P.O., Eriksen, G.S., Johannesson, T., Larsen, P.B., Viluksela, M., 2001. Polybrominated diphenyl ethers: occurrence, dietary exposure, and toxicology. *Environ. Health Perspect.* 109 (Suppl. 1), 49–68.
- Darnerud, P.O., 2003. Toxic effects of brominated flame retardants in man and in wildlife. *Environ. Int.* 29, 841–853.
- Daubie, S., Bisson, J.F., Lalonde, R., Schroeder, H., Rychen, G., 2011. Neurobehavioral and physiological effects of low doses of polybrominated diphenyl ether (PBDE)-99 in male adult rats. *Toxicol. Lett.* 204, 57–63.
- Dingemans, M.M., Ramakers, G.M., Gardoni, F., van Kleef, R.G., Bergman, A., Di Luca, M., van den Berg, M., Westerink, R.H., Vijverberg, H.P., 2007. Neonatal exposure to brominated flame retardant BDE-47 reduces long-term potentiation and post-synaptic protein levels in mouse hippocampus. *Environ. Health Perspect.* 115, 865–870.
- Domingo, J.L., 2004. Human exposure to polybrominated diphenyl ethers through the diet. *J. Chromatogr. A* 1054, 321–326.
- Domingo, J.L., 2012. Polybrominated diphenyl ethers in food and human dietary exposure: a review of the recent scientific literature. *Food Chem. Toxicol.* 50, 238–249.
- Domingo, J.L., Bocio, A., Falco, G., Llobet, J.M., 2006. Exposure to PBDEs and PCDEs associated with the consumption of edible marine species. *Environ. Sci. Technol.* 40, 4394–4399.
- Eriksson, P., Jakobsson, E., Fredriksson, A., 2001. Brominated flame retardants: a novel class of developmental neurotoxicants in our environment? *Environ. Health Perspect.* 109, 903–908.
- Eriksson, P., Viberg, H., Jakobsson, E., Orn, 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.
- Foster, W.G., Gregorovich, S., Morrison, K.M., Atkinson, S.A., Kubwabo, C., Stewart, B., Teo, K., 2011. Human maternal and umbilical cord blood concentrations of polybrominated diphenyl ethers. *Chemosphere* 84, 1301–1309.
- Gascon, M., Fort, M., Martinez, D., Carsin, A.E., Fornis, J., Grimalt, J.O., Santa Marina, L., Lertxundi, N., Sunyer, J., Vrijheid, M., 2012. Polybrominated diphenyl ethers (PBDEs) in breast milk and neuropsychological development in infants. *Environ. Health Perspect.* 120, 1760–1765.
- Gomara, B., Herrero, L., Ramos, J.J., Mateo, J.R., Fernandez, M.A., Garcia, J.F., Gonzalez, M.J., 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.
- Herbstman, J.B., Sjodin, A., Kurzon, M., Lederman, S.A., Jones, R.S., Rauh, V., Needham, L.L., Tang, D., Niedzwiecki, M., Wang, R.Y., Perera, F., 2010. Prenatal exposure to PBDEs and neurodevelopment. *Environ. Health Perspect.* 118, 712–719.
- Heredia, L., Torrente, M., Colomina, M.T., Domingo, J.L., 2012. Behavioral effects of oral subacute exposure to BDE-209 in young adult mice: a preliminary study. *Food Chem. Toxicol.* 50, 707–712.
- Hoffman, K., Adgent, M., Goldman, B.D., Sjodin, A., Daniels, J.L., 2012. Lactational exposure to polybrominated diphenyl ethers and its relation to social and emotional development among toddlers. *Environ. Health Perspect.* 120, 1438–1442.
- Jiang, H.P., Yu, Y.H., Chen, D.J., Wu, Y., Xu, B., 2008. Effect of maternal BDE-209 exposure on the expression of GAP-43 and BDNF in the hippocampus of the offspring rats. *Nan Fang Yi Ke Da Xue Xue Bao.* 28, 1319–1322 (in Chinese).
- 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., Kannan, K., Rapaport, D.P., Rodan, B.D., 2005. Polybrominated diphenyl ethers and polychlorinated biphenyls in human adipose tissue from New York. *Environ. Sci. Technol.* 39, 5177–5182.
- Jones-Otazo, H.A., Clarke, J.P., Diamond, M.L., Archbold, J.A., Ferguson, G., Harner, T., Richardson, G.M., Ryan, J.J., 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.
- Kitamura, S., Shinohara, S., Iwase, E., Sugihara, K., Uramaru, N., Shigematsu, H., Fujimoto, N., Ohta, S., 2008. Affinity for thyroid hormone and estrogen receptors of hydroxylated polybrominated diphenyl ethers. *J. Health Sci.* 54, 607–614.
- 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.
- Kuriyama, S.N., Talsness, C.E., 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.
- Kuriyama, S.N., Wanner, A., Fidalgo-Neto, A.A., Talsness, C.E., Koerner, W., Chahoud, I., 2007. Developmental exposure to low-dose PBDE-99: tissue distribution and thyroid hormone levels. *Toxicology* 242, 80–90.
- LiaoF L., Pilotte, J., Xu, T., Wong, C.C., Edelman, G.M., Vanderklisch, P., Yates 3rd., J.R., 2007. BDNF induces widespread changes in synaptic protein content and up-regulates components of the translation machinery: an analysis using high-throughput proteomics. *J. Proteome Res.* 6, 1059–1071.
- Main, K.M., Kiviranta, H., Virtanen, H.E., Sundqvist, E., Tuomisto, J.T., Tuomisto, J., Vartiainen, T., Skakkebaek, N.E., Toppari, J., 2007. Flame retardants in placenta and breast milk and cryptorchidism in newborn boys. *Environ. Health Perspect.* 115, 1519–1526.
- Morris, R., 1984. Developments of a water-maze procedure for studying spatial learning in the rat. *J. Neurosci. Methods* 11, 47–60.
- Muenhor, D., Harrad, S., 2012. Within-room and within-building temporal and spatial variations in concentrations of polybrominated diphenyl ethers (PBDEs) in indoor dust. *Environ. Int.* 47, 23–27.
- Perello, G., Marti-Cid, R., Castell, V., Llobet, J.M., Domingo, J.L., 2009. Concentrations of polybrominated diphenyl ethers, hexachlorobenzene and polycyclic aromatic hydrocarbons in various foodstuffs before and after cooking. *Food Chem. Toxicol.* 47, 709–715.
- Quiros-Alcala, L., Bradman, A., Nishioka, M., Harnly, M.E., Hubbard, A., McKone, T.E., Eskenazi, B., 2011. Concentrations and loadings of polybrominated diphenyl ethers in dust from low-income households in California. *Environ. Int.* 37, 592–596.
- Ribes, D., Colomina, M.T., Vicens, P., Domingo, J.L., 2008. Effects of oral aluminium exposure on behavior and neurogenesis in a transgenic mouse model of Alzheimer's disease. *Exp. Nephrol.* 214, 293–300.
- Roze, E., Meijer, L., Bakker, A., Van Braeckel, K.N., Sauer, P.J., Bos, A.F., 2009. Prenatal exposure to organohalogenes, including brominated flame retardants, influences motor, cognitive, and behavioral performance at school age. *Environ. Health Perspect.* 117, 1953–1958.
- Sakai, H., Fukuda, G., Suzuki, N., Watanabe, C., Odawara, M., 2009. Falsely elevated thyroid-stimulating hormone (TSH) level due to macro-TSH. *Endocr. J.* 56, 435–440.
- Schratt, G.M., Nigh, E.A., Chen, W.G., Hu, L., Greenberg, M.E., 2004. BDNF regulates the translation of a select group of mRNAs by a mammalian target of rapamycin-phosphatidylinositol 3-kinase-dependent pathway during neuronal development. *J. Neurosci.* 24, 7366–7377.
- Schreiber, T., Gassmann, K., Gotz, C., Hubenthal, U., Moors, M., Krause, G., Merk, H.F., Nguyen, N.H., Scanlan, T.S., Abel, J., Rose, C.R., Fritsche, E., 2010. Polybrominated diphenyl ethers induce developmental neurotoxicity in a human in vitro model: evidence for endocrine disruption. *Environ. Health Perspect.* 118, 572–578.
- Schummacher, M., Kiviranta, H., Ruokojarvi, P., Nadal, M., Domingo, J.L., 2009. Concentrations of PCDD/Fs, PCBs and PBDEs in breast milk of women from Catalonia, Spain: a follow-up study. *Environ. Int.* 35, 607–613.
- Shy, C.G., Huang, H.L., Chang-Chien, G.P., Chao, H.R., Tsou, T.C., 2011. Neurodevelopment of infants with prenatal exposure to polybrominated diphenyl ethers. *Bull. Environ. Contam. Toxicol.* 87, 643–648.
- Szabo, D.T., Richardson, V.M., Ross, D.G., Diliberto, J.J., Kodavanti, P.R., Birnbaum, L.S., 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.
- Ta, T.A., Koenig, C.M., Golub, M.S., Pessah, I.N., Qi, L., Aronov, P.A., Berman, R.F., 2011. Bioaccumulation and behavioral effects of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) in perinatally exposed mice. *Neurotoxicol. Teratol.* 33, 393–404.
- Talsness, C.E., Shakibaei, M., Kuriyama, S.N., Grande, S.W., 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.

- Tseng, L.H., Li, M.H., Tsai, S.S., Lee, C.W., Pan, M.H., Yao, W.J., Hsu, P.C., 2008. Developmental exposure to decabromodiphenyl ether (PBDE 209): effects on thyroid hormone and hepatic enzyme activity in male mouse offspring. *Chemosphere* 70, 640–647.
- 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., 2004a. Investigations of strain and/or gender differences in developmental neurotoxic effects of polybrominated diphenyl ethers in mice. *Toxicol. Sci.* 81, 344–353.
- Viberg, H., Fredriksson, A., Eriksson, P., 2004b. Neonatal exposure to the brominated flame-retardant, 2,2',4,4',5-pentabromodiphenyl ether, decreases cholinergic nicotinic receptors in hippocampus and affects spontaneous behaviour in the adult mouse. *Environ. Toxicol. Pharmacol.* 17, 61–65.
- 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.
- Vorkamp, K., Thomsen, M., Frederiksen, M., Pedersen, M., Knudsen, L.E., 2011. Polybrominated diphenyl ethers (PBDEs) in the indoor environment and associations with prenatal exposure. *Environ. Int.* 37, 1–10.
- Wang, F., Liu, W., Jin, Y., Dai, J., Zhao, H., Xie, Q., Liu, X., Yu, W., Ma, J., 2011. Interaction of PFOS and BDE-47 co-exposure on thyroid hormone levels and TH-related gene and protein expression in developing rat brains. *Toxicol. Sci.* 121, 279–291.
- Woods, R., Vallero, R.O., Golub, M.S., Suarez, J.K., Ta, T.A., Yasui, D.H., Chi, L.H., Kostyniak, P.J., Pessah, I.N., Berman, R.F., LaSalle, J.M., 2012. Long-lived epigenetic interactions between perinatal PBDE exposure and Mecp2308 mutation. *Hum. Mol. Genet.* 21, 2399–2411.
- Xing, T., Chen, L., Tao, Y., Wang, M., Chen, J., Ruan, D.Y., 2009. 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, T.R., Yong, W., Chen, L., Tang, M.L., Wang, M., Chen, J.T., Ruan, D.Y., 2010. Effects of decabrominated diphenyl ether (PBDE 209) on voltage-gated sodium channels in primary cultured rat hippocampal neurons. *Environ. Toxicol.* 25, 400–408.
- Ying, S.W., Futter, M., Rosenblum, K., Webber, M.J., Hunt, S.P., Bliss, T.V., Bramham, C.R., 2002. Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: requirement for ERK activation coupled to CREB and upregulation of Arc synthesis. *J. Neurosci.* 22, 1532–1540.
- Zhou, T., Ross, D.G., DeVito, M.J., Crofton, K.M., 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, M.M., DeVito, M.J., Crofton, K.M., 2002. Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. *Toxicol. Sci.* 66, 105–116.

4.4 Perinatal Exposure to BDE-99 Causes Decreased Protein Levels of Cyclin D1 via GSK3 β Activation and Increased ROS Production in Rat Pup Livers.

Blanco, J., Mulero, M., Domingo, J. L., and Sanchez, D. J.

In Revision in *Toxicol. Sci.* (2013)



Perinatal Exposure to BDE-99 Causes Decreased Protein Levels of Cyclin D1 via GSK3 β Activation and Increased ROS Production in Rat Pup Livers

Journal:	<i>Toxicological Sciences</i>
Manuscript ID:	Draft
Manuscript Type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Blanco, Jordi; Universidad Rovira i Virgili, Physiology Mulero, Miquel; Universitat Rovira i Virgili, Biochemistry and Biotechnology Domingo, José L; Universitat Rovira i Virgili, Laboratory of Toxicology and Environmental Health Sanchez, Domenec J; Universitat Rovira i Virgili, Cincies Mediques Basiques
Key Words:	BDE-99, reactive oxygen species (ROS), CYP enzyme system, thyroid hormone receptors, Akt, cyclinD1
Society of Toxicology Specialty Section Subject Area:	Mechanisms [116]

SCHOLARONE™
Manuscripts

1
2
3 1
4
5 2
6
7
8 3
9
10
11 4 **Perinatal Exposure to BDE-99 Causes Decreased Protein Levels of**
12
13 **Cyclin D1 via GSK3 β Activation and Increased ROS Production in Rat**
14 **Pup Livers**
15
16
17
18
19 7
20
21 8
22
23

24 9 **Jordi Blanco,^{*,†} Miquel Mulero,[‡] Jose L. Domingo,^{*} and Domènec J. Sánchez^{*,†,1}**
25
26
27 10
28
29 11

30 12 **Laboratory of Toxicology and Environmental Health, School of Medicine, IISPV,*
31
32 *Universitat Rovira i Virgili, Sant Llorens 21, 43201 Reus, Spain*
33
34

35 14 *†Physiology Unit, School of Medicine, IISPV, Universitat Rovira i Virgili, Sant Llorens*
36
37 *21, 43201 Reus, Spain*
38

39 16 *‡Nutrigenomics Group, Department of Biochemistry and Biotechnology, Universitat*
40
41 *Rovira i Virgili, Marcel·lí Domingo s/n, Campus Sescelades, 43007 Tarragona, Spain*
42
43
44 18

45
46 19
47
48 20 -----
49
50 21 ¹To whom correspondence should be addressed at Physiology Unit, School of Medicine,
51
52 IISPV, Universitat Rovira i Virgili, Sant Llorens 21, 43201 Reus, Spain. Fax: +34-099-
53
54 759322. E-mail: domingo.sanchez@urv.cat
55
56

57 24
58
59
60

1
2
3 25 We here examined the potential liver toxicity in rat pups from dams exposed
4
5 26 during the gestational and lactation periods to 2,2',4,4',5-pentaBDE (BDE-99).
6
7 27 Dams were exposed to 0, 1, and 2 mg/kg/day of BDE-99 from gestation day 6 to
8
9 28 post-natal day 21. When the pups were weaning, the liver from one pup of each
10
11 29 litter was excised to evaluate oxidative stress markers and the mRNA expression of
12
13 30 multiple cytochrome P450 (CYP) isoforms. To determine whether thyroid
14
15 31 hormone (TH) was disrupted, the proteins and mRNA expression of several TH
16
17 32 receptors (TRs) isoforms were evaluated, as well as the protein levels of cyclin D1
18
19 33 and the phosphorylated protein kinases Akt and GSK3 β . Perinatal exposure to
20
21 34 BDE-99 produced decreased levels of cyclin D1 in rat pup livers. A decrease in the
22
23 35 active form of Akt and an increase in the active form of GSK3 β were observed.
24
25 36 The decreased Akt pathway may be due to a potential disruption of the non-
26
27 37 genomic actions of TH by BDE 99 and its metabolites. This possible TH disruption
28
29 38 was noted as a decrease in TR isoforms expression. By contrast, we observed an
30
31 39 up-regulation of CYP2B1 gene expression, which is correlated with an increase in
32
33 40 ROS production. This outcome indicates activation of the nuclear constitutive
34
35 41 androstane receptor, which could induce the expression of other enzymes capable
36
37 42 of metabolizing TH. The present findings support the hypothesis that perinatal
38
39 43 exposure to PBDEs, at levels found in humans, may have serious implications for
40
41 44 metabolic processes in rat pup livers.
42
43 45

46 **Key Words:** BDE-99; reactive oxygen species (ROS); CYP enzyme system; thyroid
47 hormone receptors; Akt; GSK3 β ; cyclinD1.

48 Among the organic pollutants identified in biota samples, there is a family of
49 substances used as flame-retardants classified as polybrominated diphenyl ethers

