# STUDY OF THYROID HORMONE DISRUPTION BY THE ENVIRONMENTAL CONTAMINANT BDE-99 IN PERINATAL RATS

**Jordi Blanco Pérez**

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# Study of thyroid hormone disruption by the environmental contaminant BDE-99 in perinatal rats

UNIVERSITAT ROVIRA I VIRGILI

Jordi Blanco Pérez

2013

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**Doctoral Thesis**

**Directed by Dr. Domingo José Sánchez Cervelló**

**Departament de Ciències Mèdiques Bàsiques**

**Unitat de Fisiologia**

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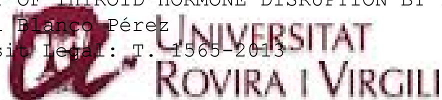

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The present work entitled "Study of Thyroid hormone disruption by the environmental contaminant BDE-99 in perinatal rats" presented by Jordi Blanco Pérez to obtain the degree of doctor by the University Rovira I Virgili, has been carried out under my supervision at the Unit of Physiology of the Department of Basic Health, and that it fulfils the requirements to obtain the European Doctorate mention.

Reus, July 15, 2013

Dr. Domingo J. Sánchez Cervelló

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# LIST OF FREQUENTLY USED ABBREVIATIONS

| | |
|---|---|
| **AhR** | Aryl hydrocarbon receptor |
| **BDNF** | Brain-derived neutrophic factor |
| **BFR** | Brominated flame retardant |
| **BW** | Body weight |
| **CaMKII** | Calmodulin-dependent protein kinase II |
| **CAR** | Constitutive androstane receptor |
| **CAT** | Catalase |
| **CGN** | Cerebellar granule neurons |
| **CYP** | Cytochrome P450 superfamily |
| **DIO** | 5'-deiodinases |
| **DMSO** | Dimethyl sulfoxide |
| **GD** | Gestational day |
| **GPx** | Gluthatione peroxidase |
| **GR** | Glutathione reductase |
| **GSSG** | Oxidised glutathione |
| **GSH** | reduced glutathione |
| **GST** | Glutathione-S-transferase |
| **HAT** | Histone acetyltransferase |
| **HRE** | Hormone responsive elements |
| **LBD** | Ligand binding domain |
| **LTP** | Long-term potentation |
| **MAPK** | Mitogen-activated protein kinases |
| **MMO** | Microsomal mono-oxygenation system |
| **MTT** | 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium |
| **NADPH** | Nicotinamide adenine dinucleotide phosphate |
| **PBDE** | Polybrominated diphenyl ethers |
| **PCB** | Polychlorinated biphenyls |
| **PCDD** | Polychlorinated dibenzo-p-dioxins |
| **PCDF** | Polychlorinated dibenzofurans |
| **PI3K** | Phosphatidyl-inositol 3-kinase |
| **PND** | Postnatal day |
| **POP** | Persistent organic pollutant |
| **PXR** | Pregnane xenobiotic receptor |
| **ROS** | Reactive oxygen species |
| **RXR** | Retinoic acid receptor |
| **SH** | Steroid hormones |
| **SOD** | Superoxid dismutase |
| **SULT** | Sulfotransferases |
| **T3** | Triiodothyronine |
| **T4** | Tetrayodotironina |
| **TGB** | Thyronine binding globulin |

| | |
|---|---|
| **TH** | Thyroid hormone |
| **THBP** | Thyroid hormone binding proteins |
| **TR** | Thyroid receptor |
| **TRE** | Thyroid hormone response elements |
| **TRH** | Thyrotropin-releasing hormone |
| **TSH** | Thyroid-stimulating hormone |
| **TTR** | Transthyretin |
| **UDP-GT** | Uridine diphosphate glucuronyltransferase |
| **XMEs** | Xenobiotic metabolizing enzymes |

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4.3 Blanco, J., Mulero, M., Heredia, L., Pujol, A., Domingo, J. L., and Sanchez, D. J. (2013). Perinatal exposure to BDE-99 causes learning disorders and decreases serum thyroid hormone levels and BDNF gene expression in hippocampus in rat offspring. *Toxicology.* 308, 122-128…… 69

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# 1. INTRODUCTION

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## 1.1 GENERAL INTRODUCTION

In the last century, there was a vertiginous development in industry based on the synthesis of chemical products from petroleum and hydrocarbon gases (polymeric materials, fuels, solvents, pesticides, pharmacological compounds, etc). Despite the improvement in the quality of life, such global industrialization has caused the release of organic chemical substances, some of which are resistant to degradation, having a long half-life in the environment. Several of these persistent substances are bio-accumulated in the food chain and cause adverse effects on ecosystems and human health. The compounds that have these characteristics are classified by the Stockholm Convention as persistent organic pollutants (POPs), and some examples are: aldrin, dioxins, furans, polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs) or polybrominated diphenyl ethers (PBDEs) (COP4, 2009).

## 1.2 PBDEs

Over the last three decades, PBDEs have been one of the most widely used brominated flame-retardants (BFRs) (Covaci et al., 2003). They are added to several flammable polymers found in electronic components, polyurethane foams, plastics, building materials, textiles, and in other applications that prevent the spread of fire (Alaee et al., 2003). When there is a fire, these chemicals are able to compete with the transmission of combustion process and mitigate the flames by forming radical species. PBDEs are not chemically bound to polymers in order to aid quick release and reduce flames in the event of fire, thus saving lives.

PBDEs were first manufactured and used in the early 1970s. Based on the 2001 production of 67,400 tons, it is estimated that total PBDEs production over the last 30

years has exceeded 2 million tons (Shaw and Kannan, 2009). Concern over the potential effects of PBDEs on the environment and on human health led to a ban on the production and usage of the penta-BDE and octa-BDE formulations in Europe in 2004 and a voluntary ban on the production of these formulations in certain American states followed shortly thereafter (Betts, 2008). Widespread distribution of penta-BDE and octa-BDE in the environment led the Stockholm Convention to include these formulations on the list of POPs (COP4, 2009). Nonetheless, large-scale production and usage of deca-BDE still continues nowadays.

### 1.2.1 *Structure and physical properties*

Diphenyl ether molecules contain 10 hydrogen atoms, any of which can be exchanged with bromine, resulting in 209 possible congeners (Figure 1.1). PBDEs have been produced extensively as three commercial mixtures with different degrees of bromination: penta-BDE, octa-BDE and deca-BDE, and classified according to their average bromine content (Table 1).

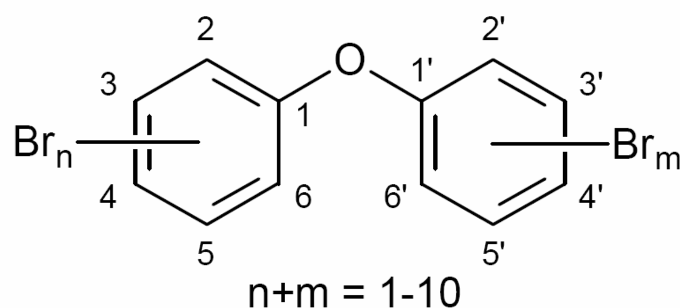


**Figure 1.1.** Basic structure of PBDEs showing various possible bromine substitutions.

PBDEs have a low vapour pressure at ambient temperature and high octanol-water partition coefficient (log *K*OW) values. Their boiling points range from 310º to 425ºC. They are poorly soluble in water and when their bromine content is increased they have more

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; Ikonomou 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 (Schecter 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 [$^{14}$C] 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; Fängström 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 potentation (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 neutrophic factor (BDNF) and the increase in the calmodulin-dependent protein kinase II (CaMKII), GAP-43, synaptophisyn 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 (17β-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 µg/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 glucorodinates 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 TRα locus encodes four proteins but only one, TRα1, is the true nuclear receptor. TRα2, TRΔα1, TRΔα2 are antagonists of TRα1 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 TRβ locus encodes 2 receptors: TRβ1 and TRβ2. TRβ1 and TRβ2 differ in length in the amino-termini. The TRβ locus in rats also encodes TRβ3 and TRΔβ3 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 TRα) and length (comparing TRβ 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: RXRα, RXRβ, and RXRγ. 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-

associated proteins and Vitamin D receptor interacting proteins, which form a mediator complex that is associated with the recruitment and activation of RNA polymerase II (Bassett et al., 2003).

### 1.5.3 *Non-genomic actions PBDEs*

THs have non-genomic action, which are largely mediated through signal transduction pathways, causing an effect without first affecting transcription. In mammalian cells, non-genomic actions are considered rapid, in the order of seconds to minutes, there is no requirement for protein synthesis to take place, and it is independent of nuclear TR (Bassett et al., 2003; Davis et al., 2005; Davis et al., 2008; Shi et al., 1996). The TH non-genomic mechanisms are equally responsive to T4 and T3 and in some cases more responsive to T4. Some of TH non-genomic actions include changes in cell morphology, respiration (mitochondrial function), and ion homeostasis. THs exert these effects through multiple pathways. THs can bind to cell surface proteins, for example the cell surface protein integrin αvβ3 binds strongly to T4 (Davis et al., 2005). Binding of T4 to integrin αvβ3 affects cell-extracellular matrix interactions and triggers intracellular signalling processes (Davis et al., 2005). TH also bind to cytosolic proteins and these proteins often have different functions, such as enzymes. Binding of TH to these cytosolic proteins may have an effect on the additional functions of these proteins. Furthermore, a small amount of the TRβ can exist in the cytoplasm and in TH-treated cells TRβ can form a complex with mitogen-activated protein kinases (MAPK). Unliganded TRβ can also interact with phosphatidyl-inositol 3-kinase (PI3K) in the cytoplasm (Davis et al., 2005; Storey et al., 2006).

Non-genomic and genomic actions of THs have been shown to crosstalk and there is evidence of the importance of interaction between kinase-signalling cascades and TH-signalling pathways in the determining the fate of cells and tissue (Skirrow et al., 2008).

### 1.5.4 *PBDEs as thyroid hormone disruptors*

Chemicals that disrupt TH can be defined more specifically as xenobiotics that alter the structure or function of the thyroid gland, alter regulatory enzymes associated with TH homeostasis, or change circulating tissue concentrations of THs (Crofton et al., 2008). The TH 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. The capacity of PBDEs to disrupt TH signalling could be largely responsible for several of the developmental disorders produced by PBDEs. Developmental exposure to low doses of BDE-47 in rats results in changes in thyroid gland histology and morphology (Talsness et al. 2008). 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 TRs. Zhou et al. (2001) reported that treatment of weanling female rats with DE-71 or DE-79 caused a reduction of serum T4 levels. In a subsequent study, Zhou et al. (2002) found that exposure of rats to DE-71 from GD 6 to PND 21 caused a significant decrease of serum T4 in the dam, and in the foetuses and pups on GD 20, PND 4 and PND 14, with a recovery on PND 36. Zhou et al. (2002) found that the decrease in T4 was associated with an induction of UDP-GT, a key phase II metabolizing enzyme involved in conjugation of T4. Such increased metabolism results in enhanced excretion and hence in lower circulating levels of T4 (Barter et al., 1992).

Moreover, certain PBDE congeners, especially their hydroxylated metabolites, OH-PBDEs, are structurally similar to TH and can compete to bind to TTR, displacing T4 from TTR, which may lead to increased glucuronidation and a consequent lower level of T4. PBDEs/HO-PBDEs were also described as antagonists of TR and can deregulate the TH-mediated gene expression (Kitamura et al., 2008; Kojima et al. 2009; Schreiber et al., 2010). 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 et al., 2008; Kojima et al., 2009).

## 1.6 OXIDATIVE STRESS

ROS can be generated from a variety of sources classified as exogenous and endogenous. UV irradiation and ozone and polyaromatic hydrocarbons (PAH) are key examples of exogenous sources. There are many sources considered endogenous, such as mitochondrial oxidative phosphorylation, xenobiotic metabolism, active peroxisomes and inflammation. Reactions catalyzed by enzymes, such as NADPH oxidases, CYP oxidases, lipoxygenases and CYP reductases will result in ROS production. It is understood that CYP-mediated xenobiotic metabolism is a significant source of ROS (Vrzal et al., 2004). In numerous studies, chemicals have induced high levels of reactive intermediates resulting in increased oxidative stress in liver and also cancer induction, in some cases.

Oxidative stress can be either direct or indirect depending on the mode of action of the toxic chemical. Chemicals such as dioxins, PCBs, phenobarbitals act indirectly to promote ROS generation, mainly through enzyme induction. These chemicals are potent inducers of xenobiotic metabolism enzymes (CYP1A, 2B, 3A, etc.) and these enzymes can generate ROS during the oxidation/reduction cycles (Schlezinger et al., 2006,

Shertzer et al., 2004). Enzyme induction increases with increasing exposure of substrate/toxic chemical, leading to an excess release of ROS. These ROS/free radicals can damage proteins (protein oxidation), lipids (lipid peroxidation) or DNA (oxidative DNA damage), which in turn release a variety of reactive species to enter a feedback loop of ROS generation.

In mammalian systems, numerous defence systems have been found to protect against accumulation of ROS. However in several abnormal/unhealthy conditions these systems fail or become ineffective. Oxidative stress (due to acute exposure of toxicant/toxic metabolites) can lead to apoptosis induction; alternatively, chronic low levels of ROS can activate specific signalling pathways or promote disease initiation and development and in some cases promote carcinogenesis (Finkel and Holbrook, 2000).

The fate of a cell greatly depends on the levels of ROS, since they act as secondary messengers in regulating specific signalling pathways. Low levels considered physiological concentrations of ROS are very important in the regulation of several cellular events including cell cycle. On the contrary, high levels result in apoptosis and cause irreversible damage to proteins, DNA and lipids, ending in massive cell death via necrosis (Droge, 2002).

Among the numerous signalling pathways activated upon exposure to toxic chemicals, genes encoding for detoxifying enzymes and antioxidant defence are the first to upregulate and induce a stress response; these are mainly regulated by transcription factor Nrf2. Other signal pathways triggered by oxidative stress include, but are not limited to, members of the MAPK, PI3K/Akt pathway, phospholipase C-g1 signalling, protein kinase C, p53 signalling, ataxia-telangiectasia-mutated kinase, nuclear factor-kappaβ signalling, and Jak/Stat pathway (Benhar et al., 2002; Martindale and Holbrook, 2002; Leonard et al., 2004; Poli et al., 2004).

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# 2. INTEREST AND OBJECTIVES

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As have been outlined in the literature review, over the last three decades, PBDEs have been one of the most widely used BFRs. Despite the bans and restrictions on the use and production of PBDEs, high amounts of these compounds have accumulated in humans. The bioaccumulation of PBDEs in pregnant women is a principal concern because of the biodisponibility of PBDEs is increased during pregnancy and lactation, being potentially transferred to the offspring. Recently epidemiological studies have correlated higher concentrations of PBDEs in cord blood and maternal milk with worse neurophysiological development and increased activity/impulsivity behaviors in toddlers. Moreover, various animal studies have attributed the primary toxic action of PBDEs to their ability to act as endocrine disruptors, but the mechanisms by which PBDEs can generate neurophysiological deficiencies are still poorly understood.

This thesis analyzes the effects of PBDEs on prenatal and perinatal development. The key hypothesis in this study is that the maternal transmission of PBDEs during pregnancy and lactation periods can induce neurophysiological alterations in the offsprings related to the TH system disruption.

To conduct the hypothesis test, one of the most prevalent congeners of PBDEs in human, BDE-99, was used in all the different experimental designs. The study was divided in three stages.

Firstly, the effect of BE-99 over cell viability and TH signalling disruption was investigated on a primary culture of rat cerebellar granule neurons (CGNs). To achieve the aim of this study the following objectives were identified:

- ✓ To determine cell viability and ROS production over time in CGNs exposed to BDE-99.
- ✓ To assess the relation between BDE-99 exposition and the possible disruption of TRs function over time in neuronal cells.

- ✓ To study the effects of BDE-99 on BDNF and Bcl-2 protein and mRNA expression over time.

Secondly, embryo/fetal toxicity of prenatal exposure to BDE-99 related with a possible disruption of TH signalling was evaluated. To achieve the aim of this study the following objectives were identified:

- ✓ To determine possible alterations during the pregnancy period in pregnant rats exposed to BDE-99.
- ✓ To determine internal and external malformations of rat fetuses whose dams were exposed to BDE-99 during the period GD 6 to GD 20.
- ✓ To determine the mRNA expression of different CYP isoforms and their correlation with different parameters of stress oxidative in rat fetus livers.

Finally, physical development and neurobehavioral postnatal assessment related to the disruption of TH signalling were assessed in rat pups perinatally exposed to BDE-99. To achieve the aim of this study the following objectives were identified:

- ✓ To measure serum levels of T3, T4 and free-T4 in perinatally exposed rat pups to BDE-99.
- ✓ To determine the cortex and hippocampal expression of the mRNA encoding the isoforms of the TRs genes and of the TH-mediated gene BDNF when rat pups were weaned.
- ✓ To evaluate learning and memory function by neurobehavioral testing.
- ✓ To determine the mRNA expression of different CYP isoforms and their correlation with different parameters of stress oxidative in rat pup livers.
- ✓ To evaluate the protein and mRNA expression of several TR isoforms, as well as the protein levels of cyclin D1 and phosphorylated form of Akt and GSK3β in rat pup livers.

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# 3. MATERIAL AND METHODS

## 3.1 *IN VITRO* STUDY IN CGNS TREATED WITH BDE-99

### 3.1.1 *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 ($DCFH_2$-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).

### 3.1.2 *Cerebellar granule cell culture*

Primary cultures of CGNs were prepared from postnatal day 7 Sprague-Dawley rat pups according to Verdaguer et al. (2004). Isolated cerebella were separated from the vessels, minced, and incubated for 15 min at 37 ºC 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 $1.2\times10^6$ cells/ml, and the cells were seeded in plates coated with poly-L-lysine (100µg/ml). Cultures were grown in DMEM supplemented with 10% heat-inactivated FBS, 0.1 mg/ml gentamicin, 2mM L-glutamate and 25mM KCl. Ara-C

(10μM) was added to the culture medium 24 h after plating to prevent the proliferation of non-neuronal cells. The cultures were maintained at 37 ºC in a humidified incubator with 5% $CO_2$ 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.

### 3.1.3 *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 50μM 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.

### 3.1.4 *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 250µM 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 595nm 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%.

### 3.1.5 *ROS production*

The levels of intracellular ROS were measured using the fluorescent probe $DCFH_2$-DA. Cells were pre-incubated for 30 min at 37 ºC with 10µM $DCFH_2$-DA (added from a 20mM stock solution in DMSO). $DCFH_2$-DA is readily taken up by cells being subsequently deesterified to 2',7'-dichlorofluorescin ($DCFH_2$). 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 (154mM NaCl, 5.6mM KCl, 3.6mM NaHCO3, 2.3mM CaCl2, 5.6mM D-glucose and 5mM HEPES, pH 7.4). The fluorescence was immediately read at an excitation wavelength of 488nm and an emission wavelength of 525nm using a microplate fluorometer (Fluoroskan Ascent® FL). Results were expressed as percentages, and the fluorescence measured in control cells was taken to be 100%.

### 3.1.6 *RNA isolation and cDNA synthesis*

Total RNA was obtained from CGNs using a Qiagen RNAeasy kit (Qiagen Inc.) according to the manufacturer's protocol. RNA was resuspended in 100µL 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 1µg 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 TRα1(NM_001017960) (forward: 5'-TGC CCT TAC TCA CCC CTA CA-3'; reverse: 5'-AAG CCA AGC CAA GCT GTC CT-3'), TRα2 (NM_031134) (forward: 5'-TGA GCA GCA GTT TGG TGA AG-3'; reverse: 5'-GAA TGG AGA ATT CCG CTT CG-3'), TRβ1 (NM_012672) (forward: 5'- AGC CAG CCA CAG CAC AGT GA-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'); 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 TTGAAGGTC TCA AA-3') with a PyroStartTM 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.

### 3.1.7 *Real-time RT-PCR*

Quantitative PCR for TRα1, TRα2, TRβ1, 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 cycler (Qiagen, Inc.). The thermal cycling comprised an initial step at 50 ºC for 2 min, followed by a polymerase activation step at 95 ºC for 15 min, and a cycling step with the following conditions: 40 cycles of denaturing at 95 ºC for 15 s, annealing at 60 ºC for 30 s, and extension at 72 ºC 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 ºC 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.

### 3.1.8 *Western blot analysis*

Aliquots of cell lysate containing 30μg of protein per sample were analyzed by western blot analysis. Briefly, samples were placed in sample buffer (0.5M 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 ºC for 5min. 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 (50mM 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.

### **3.1.9.** ***Immunocytochemistry assays***

CGNs 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 ºC 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 (Invitrogen, EU). Immuno-signal analysis was performed using a Nikon TE2000-E microscope, and digitized images were captured.

### **3.1.10** ***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±SD of

at least three independent experiments. The relationships among the examined endpoints were assessed by correlation analysis using the Pearson method.

## 3.2 ANIMAL STUDY OF MATERNAL TRANSFERENCE OF BDE-99 DURING PREGNANCY

### 3.2.1 *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 ± 2ºC and a relative humidity of 50 ± 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.

### 3.2.2 *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 ºC. 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.

### 3.2.3 *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 3 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).

- Estimation of tissue glutathione reductase (GR) activity: The GR activity is determined by the amount of NADPH consumed in the conversion of oxidised glutathione (GSSG) to reduced glutathione (GSH). The reaction is catalysed by glutathione reductase. The final concentration in 1 ml of reaction mixture were 0.1M sodium phosphate buffer (PH 7.0), EDTA 1mM, GSSG 1mM, NADPH 0.2mM. The reaction was started with the addition of homogenate (10%) and decrease in OD/min was noted and followed at every 1 min interval for 5 min at 340 nm. The results were expressed in mU of GR / mg of protein.

- Estimation of tissue gluthatione peroxidase (GPx) activity: GPx catalyses the oxidation of GSH by cumene hydroperoxide. In the presence of GR and NADPH, the GSSG is immediately converted to the reduced form with concomitant oxidation of NADPH to $NADP^{+}$. The decrease in absorbance at 340 nm for 5 min (25°C) is measured. The reaction was started with 0.1 mM cumene hydroperoxide. Values were corrected for non-enzymatic oxidation of GSH and NADPH by hydrogen peroxide. The results were expressed in mU of GPx / mg of protein.

- Estimation of tissue lipid peroxide: Malonaldehyde (MDA) produced during peroxidation can react with thiobarbituric acid (TBA) reagent to form a pink coloured product, which has an absortion maximum at 532 nm. The assay is calibrated with 1,1,3,3, tetramethoxypropane, which on hydrolysis produces

malonaldehyde. The results are expressed in terms of the amount of malonaldehyde produced during the reaction. 0.1 ml of tissue homogenate (25%) in tris buffer (PH 7) was added to a reaction mixture containing KCL (0.1 ml), ascorbic acid (0.1 ml), ferrous ammonium sulphate (0.1 ml) and tris buffer (0.1 ml) in the final volume of 0.5 ml. The reaction mixture was incubated for 1h at 100 ºC. The amount of MDA formed was expressed as nmol/ mg protein.

- Estimation of tissue superoxid dismutase (SOD) activity: SOD was determined by its ability to inhibit auto-oxidation of epinephrine which was estimated by the increase in absorbance at 480nm. The reaction mixture (3ml) contained 2.95 ml of 0.05 M sodium carbonate buffer pH 10.2, 0.02 ml of tissue homogenate, 0.03 ml of epinephrine and 0.005 N HCL was used to initiate the reaction. The reference cuvette contained 2.95 ml buffer, 0.03ml of substrate (epinephrine) and 0.02 ml of water. Enzyme activity was calculated by measuring the change in absorbance at 480nm for 5 minutes and was expressed as specific activities (U/mg protein).

- Estimation of tissue catalase activity: Catalase activity was determined by measuring the decrease in absorbance at 240nm due to the decomposition of $H_2O_2$ in a UV recording spectrophotometer. The reaction mixture (3ml) contained 0.1ml of tissue homogenate in phosphate buffer (50mM, pH 7.0) and 2.9ml of 30mM $H_2O_2$ in phosphate buffer pH 7.0. An extinction coefficient for $H_2O_2$ at 240nm of 40.0 $M^{-1}cm^{-1}$ was used for the calculation. The specific activity of catalase was expressed as micromoles of $H_2O_2$ reduced per minute per mg protein.

### 3.2.4 *RNA isolation and cDNA synthesis.*

The extraction of mRNA from fetal livers and cDNA synthesis was performed according to the same method explained above in the section 3.1.6. 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 (23) 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.

### 3.2.5 *Real-time reverse-transcription-PCR.*

Quantitative PCR for CYP1A1, CYP1A2, CYP2B1, CYP3A1, CYP3A2, and β-actin was performed according to the same manner explained above in the section 3.1.7.

### 3.2.6 *Statistics*

To evaluate 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 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 $p < 0.001$.

## 3.3 ANIMAL STUDIES OF MATERNAL TRANSFERENCE OF BDE-99 DURING GESTATION AND LACTATION

### 3.3.1 *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 ± 2ºC and a relative humidity of 50 ± 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).

**3.3.2** ***Experimental procedure***

Females with vaginal smears containing sperm were divided into 3 groups (10 animals per group) for the study in brain and neurobehavioral tests and, into 3 groups (8 animals per group) for the study of liver toxicity. 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).

In the study in brain and liver toxicity, all litters were randomly reduced to 4 pups (2 males and 2 females) on PND 1. 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 ºC. 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.

In the study of liver toxicity, all litters were randomly reduced to 4 male pups, on PND 1. Pups were weaned on PND 21 and housed in groups of 4 per cage. At that time, one pup from each litter was sacrificed by decapitation, and the liver was immediately dissected, frozen in liquid nitrogen, and stored at −80 ºC.

### 3.3.3 *Hormone analysis*

The samples serum collected on PND 21 were analyzed for T4, free T4 (FT4) andT3 concentrations with an Advia 2400 analyzer (Sakai et al., 2009).

### 3.3.4 *RNA isolation and cDNA síntesis*

The extraction of mRNA from cortex, hippocampus and liver of rat pups and cDNA synthesis was performed according to the same method explained above in the section 3.1.6. The cDNA was subsequently amplified by PCR using the same rat-specific primers for CYP1A1, CYP1A2, CYP2B1, CYP3A1, CYP3A2, TRα1, TRα2, TRβ1, BDNF and β-actin of the sections 3.1.6 and 3.2.4. In cortex and hippocampus TRα1, TRβ1, BDNF and β-actin were amplified, whereas in liver CYP1A1, CYP1A2, CYP2B1, CYP3A1, CYP3A2, TRα1, TRβ1 and β-actin were used.

### 3.3.5 *Real time reverse transcription-PCR*

PCRQuantitative PCR for CYP1A1, CYP1A2, CYP2B1, CYP3A1, CYP3A2, TRα1, TRα2, TRβ1, BDNF and β-actin was completed according to the same method explained above in the section 3.1.7.

### 3.3.6 *Neurobehavioral tests*

#### 3.3.6.1. *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 intertrial 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.

3.3.6.2. *Open-field activity*

General motor activity was measured in an open-field apparatus, consisting of a wooden 47 cm × 47 cm square surrounded by a 40-cm-high light-colored wall. A10 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).

**3.3.7** ***Oxidative stress markers*****.**

A fraction of the rat pup liver was used to assess the activity of the antioxidant enzymes SOD, and CAT, the level of TBARS, according to the same method explained above in the section 3.2.3.

**3.3.8** ***Western blot analysis*****.**

Aliquots containing 30 μg of protein per sample of liver lysate were analyzed by western blot analysis, according to the same method explained above in the section 3.1.8. Samples were then incubated overnight with primary monoclonal antibodies against TRα1, TRβ1, AKT-total, p-AKT, GSK3β-total, p-GSK3β or β-actin.

### 3.3.9 *Statistics.*

To evaluate 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). The relationships among the examined endpoints were assessed using Pearson correlations.

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# 4. RESULTS

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

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

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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 μM 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-alpha1 and TR-alpha2, 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-beta1 isoform, which is normally transcriptionally repressed by T3 in CGNs, was up-regulated. This up-regulation could compensate the down-regulation of the TR-alpha1 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.

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## 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

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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 ($DCFH_2$-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 Verdaguer 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 $1.2 \times 10^6$ cells/ml, and the cells were seeded in plates coated with poly-L-lysine (100 μg/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 μM) 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% $CO_2$ 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 μM 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 μM 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 $DCFH_2$-DA. Cells were pre-incubated for 30 min at 37 °C with 10 μM $DCFH_2$-DA (added from a 20 mM stock solution in DMSO). $DCFH_2$-DA is readily taken up by cells being subsequently deesterified to 2′,7′-dichlorofluorescin ($DCFH_2$). 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 RNAeasy kit (Qiagen Inc.) according to the manufacturer's protocol. RNA was resuspended in 100 μL 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 μg 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′-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′); 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 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 (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 μg 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 ± 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 determinate the neuroprotective effect of T3 front BDE-99 insult we here used a BDE-99 concentration close to the 50% of cell death (25 μM, Fig. 1). Time course of cell viability was assessed after exposure of CGNs to BDE-99 (25 μM) (Fig. 2 A). A significant ($P < 0.0001$) decrease was observed relative to control cells at 3 (14 ± 4%), 6 (24 ± 4%), 12 (48 ± 3%) and 24 h (58 ± 3%). At those times, ROS production was quantified by the fluorescence intensity of the oxidized product of $DCFH_2$-DA. A significant ($P < 0.0001$) increase in the fluorescein signal generated by intracellular oxidative stress was seen at 12 (2322 ± 471%) and 24 h (3350 ± 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 ($R = 0.9410$, $P = 0.0062$).

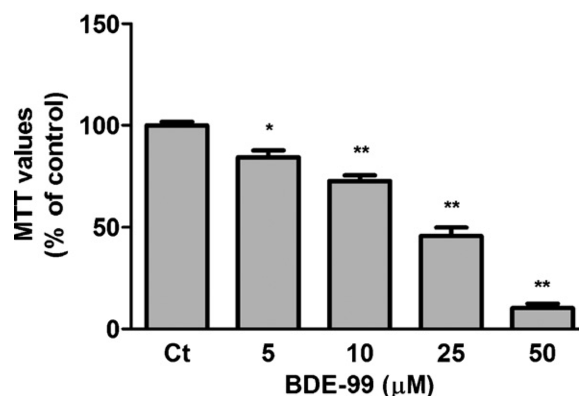


**Fig. 1.** Evaluation on cerebellar granular cells viability after 24 h of exposure to BDE-99 at 5 μM, 10 μM, 25 μM and 50 μM. Data are expressed as the mean ± SD of four independent experiments. Statistical differences relative to the control (0 h) were analyzed by one-way ANOVA followed by Bonferroni's 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

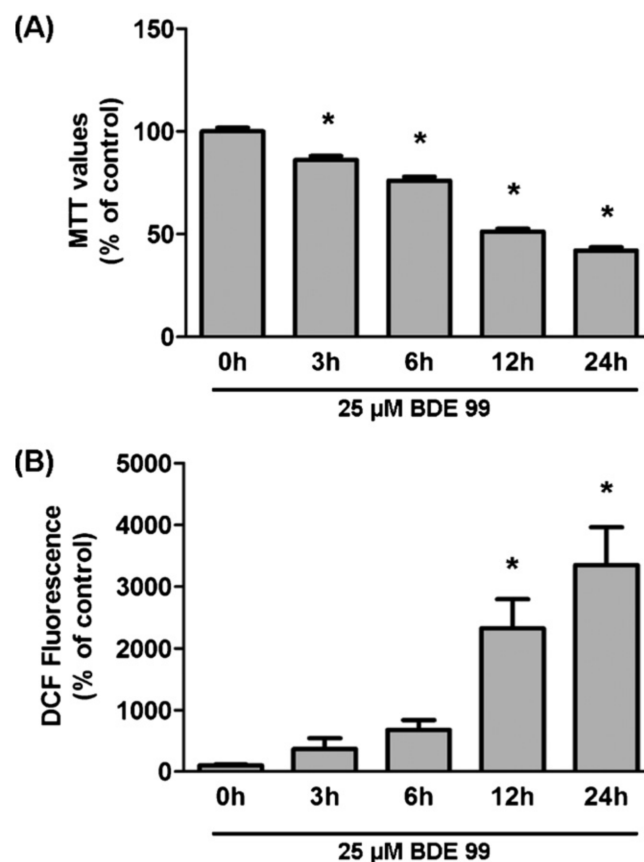


**Fig. 2.** Time-dependent effects of BDE-99 on cell viability and ROS production. CGN cells were exposed to 25 μM BDE-99 for 3, 6, 12 and 24 h. (A) Cell viability and (B) the production of ROS were determined by the MTT method and by DCF fluorescent signal intensity, respectively. Data are expressed as the mean ± 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.

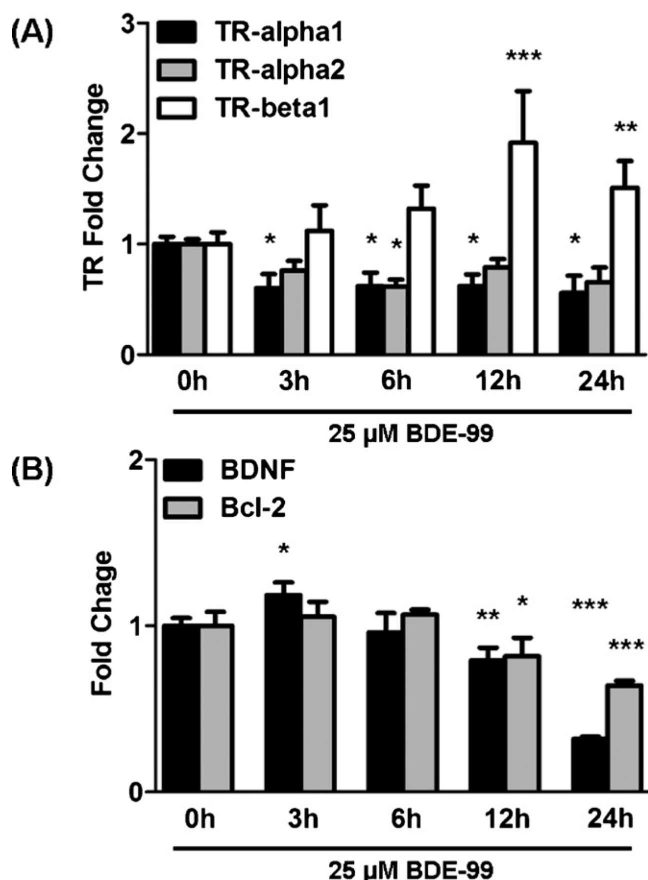


**Fig. 3.** BDE-99 deregulates the expression of the TR gene products and decreases the expression of BDNF and Bcl-2. CGN cells were exposed to 25 μM BDE-99 for 3 h, 6 h, 12 h and 24 h. The relative expression levels of the mRNAs encoding (A) the TR-alpha1, TR-alpha2 and TR-beta1 isoforms, and (B) BDNF 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 ± 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 μM) 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 ± 6%). In contrast to TR-alpha1 and TR-alpha2, the expression of TR-beta1 at 12 h (92 ± 46%, $P<0.0001$) and 24 h (51 ± 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 ± 7%, $P<0.001$) and 24 h (68 ± 12%, $P<0.001$), and Bcl-2 relative concentrations at 12 (21 ± 11%, $P<0.05$) and 24 h (36 ± 3%, $P<0.01$) were significantly down-regulated in CGNs after treatment with BDE-99 (25 μM) relative to the control. Surprisingly, in comparison to control we observed a significant up-regulation of BDNF at 3 h (18 ± 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. 4 A), being protein levels determined by the chemiluminescent intensities of their bands. Significant decreases relative to the control were observed for BDNF at 12 (44 ± 16%, $P<0.001$) and 24 h (65 ± 12%, $P<0.0001$), and for Bcl-2 at 24 h (35 ± 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 μM) 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 μM), and the viability of the CGNs after 24 h of exposure using the MTT method (Fig. 5 A) was evaluated. Treatment with T3 did not show significant difference with the control. The viability of CGNs decreased (59 ± 6%) after BDE-99 exposure. However, simultaneous treatment with BDE-99 and $10^{-6}$ M (21 ± 5%, $P<0.05$) or $10^{-5}$ M (28 ± 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 μM) 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

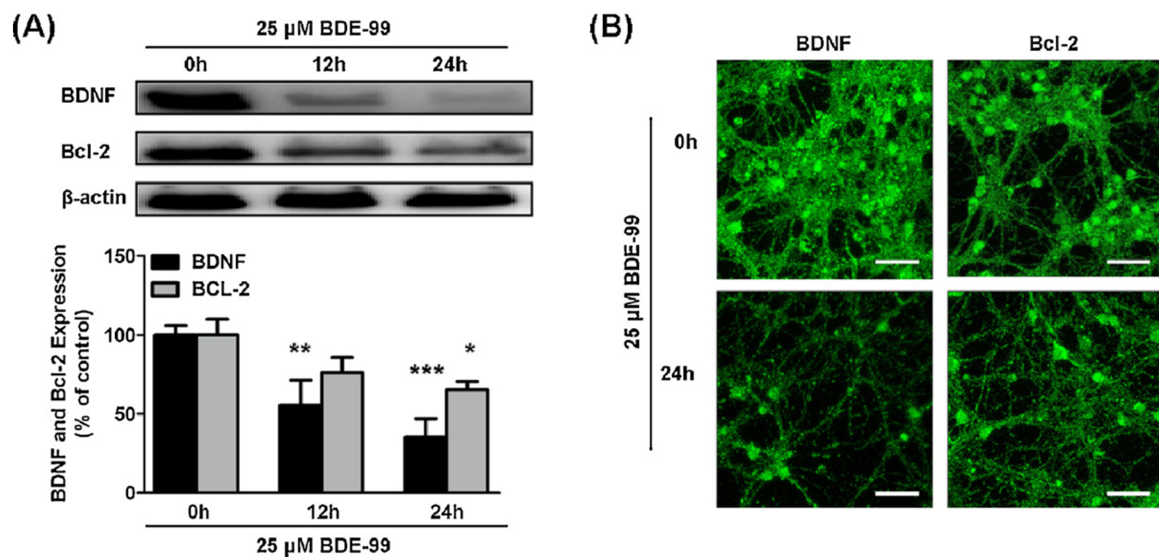


**Fig. 4.** BDE-99 decreases the expression of the BDNF and Bcl-2 proteins. (A) CGN cells were exposed to 25 μM BDE-99 for 12 h and 24 h, and the relative levels of BDNF and Bcl-2 protein expression were measured by Western blot analysis. The intensity of the bands was determined by densitometric analysis, 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 ± 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 μm.

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-alpha gene. The expression levels of TR-alpha1, the predominant TR isoform in CGNs, and TR-alpha2, 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-alpha 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-alpha in females, as well as a decrease in TR-beta 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-alpha2, may play an important role in BDE-99 neuronal toxicity. The splice variant of TR-alpha1 appears to exert a dominant negative activity on the T3-dependent trans-activation of TR target genes. A decrease in TR-alpha2 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-90, 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-beta1, 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,

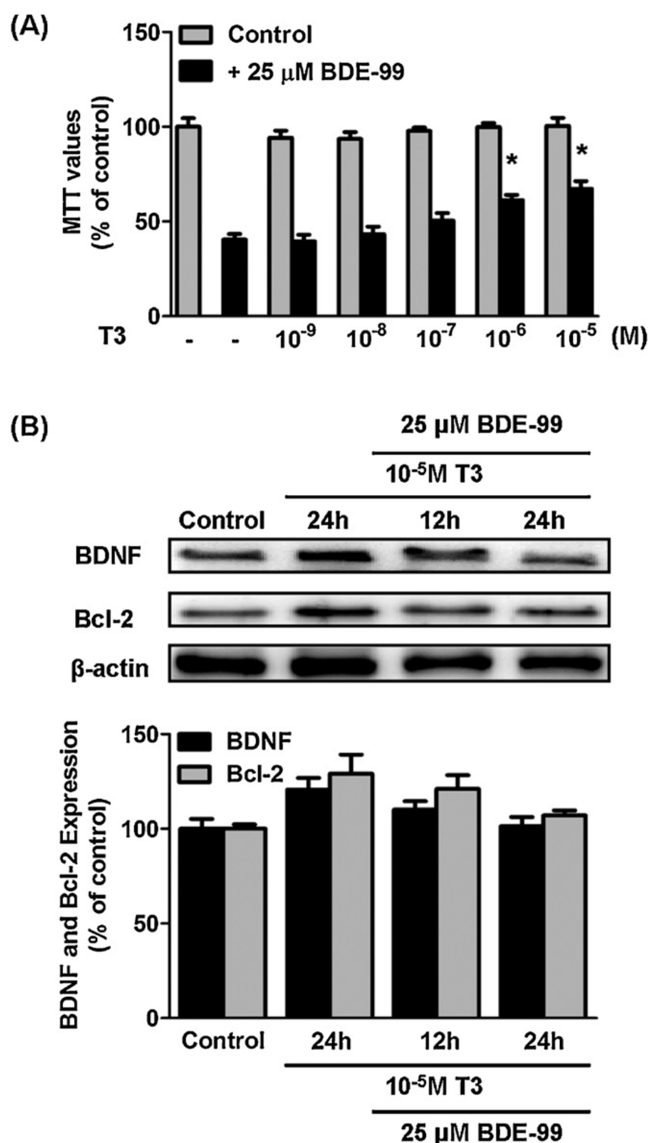


**Fig. 5.** T3 treatment partially rescues the cell death induced by BDE-99 and resets the BDNF and Bcl-2 protein levels. (A) CGNs were exposed to 25 μM BDE-99 for 24 h, either alone or with a range of concentrations ($10^{-9}$ to $10^{-5}$ M) of T3, and the cell viability was determined by the MTT method. 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.

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UNIVERSITAT ROVIRA I VIRGILI
STUDY OF THYROID HORMONE DISRUPTION BY THE ENVIRONMENTAL CONTAMINANT BDE-99 IN PERINATAL RATS
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## 4.2 Gestational exposure to BDE-99 produces toxicity through upregulation of CYP isoforms and ROS production in the fetal rat liver.

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

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**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 (Schecter *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

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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 examined 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 ± 2°C and a relative humidity of 50 ± 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 × 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×) 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

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**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 |

[a]Corrected 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.

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**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 skeletaly (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 |
| Palietal 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 ± 53%, $p = 0.011$), CYP1A2 (257 ± 89%, $p = 0.005$), and CYP2B1 (649 ± 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 ± 43%, $p = 0.017$) and BDE 2 (401 ± 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 (μmol/min/mg protein) | $237 \pm 51^{a}$ | $300 \pm 60^{ab}$ | $354 \pm 67^{bc}$ | $446 \pm 48^{c}$ |
| 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) | $11.3 \pm 4.3^{a}$ | $17.6 \pm 4.1^{ab}$ | $20.3 \pm 3.4^{b}$ | $23.5 \pm 4.3^{b}$ |

*Note*. Results are expressed as mean values ± 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 $p < 0.05$.

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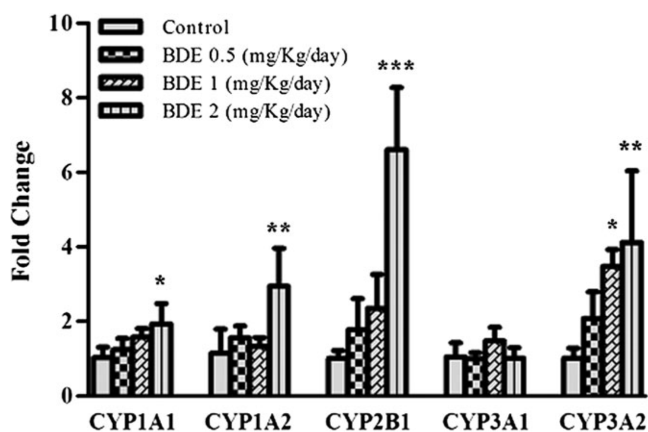


**FIG. 1.** BDE-99 increases the mRNA expression of CYP1A1, CYP1A2, CYP2B1, and CYP3A1 in fetal rat liver. The mRNA was extracted from the liver of rat fetuses whose dams were orally exposed to 0, 0.5, 1, and 2 mg/kg/day of BDE-99 from GD 6 to GD 20. The relative expression levels of the mRNAs encoding 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 ± 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 transplacentary 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 (17β-estradiol and testosterone) and tetraiodothyroxine (T4) in offspring of rats. The excessive metabolization 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

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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.

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## 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.

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

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A B S T R A C T

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), tetraiiodothyronine (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.

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## 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 biomagnification 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

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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 CaMII, 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 (220–240 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 ± 2 °C and a relative humidity of 50 ± 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 TRα1 (NM_001017960) (forward: 5′-TGC CCT TAC TCA CCC CTA CA-3′; reverse: 5′-AAG CCA AGC CAA GCT GTC CT-3′), TRα2 (NM_031134) (forward: 5′-TGA GCA GCA GTT TGG TGA AG-3′; reverse: 5′-GAA TGG AGA ATT CCG CTT CG-3′), TRβ1 (NM_012672) (forward: 5′-AGC CAG CCA CAG CAC AGT GA-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×) 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 TRα1, TRα2, TRβ1, 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 × 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 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.

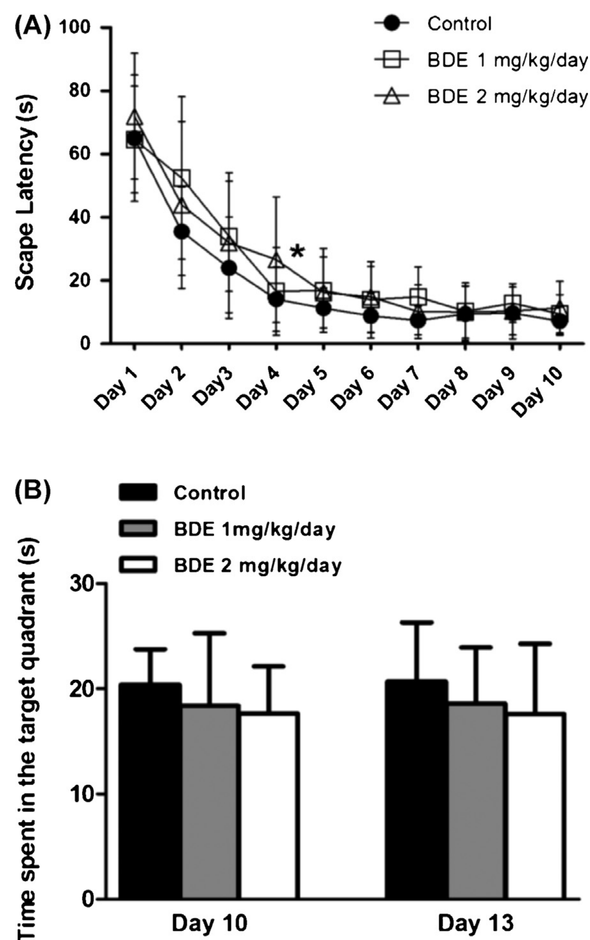


**Fig. 1.** Perinatal exposure to BDE-99 produces a delay in the spatial learning task. Rat offspring of dams that were exposed to 0, 1 and 2 mg/kg/day of BDE-99 from GD 6 to PND 21 were tested in the water maze. (A) An initial, longer latency in reaching the platform was produced at the highest BDE-99 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 ± SD. Significant differences were analyzed with an ANOVA followed by Bonferroni's post hoc test. *$P < 0.05$.

### 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.

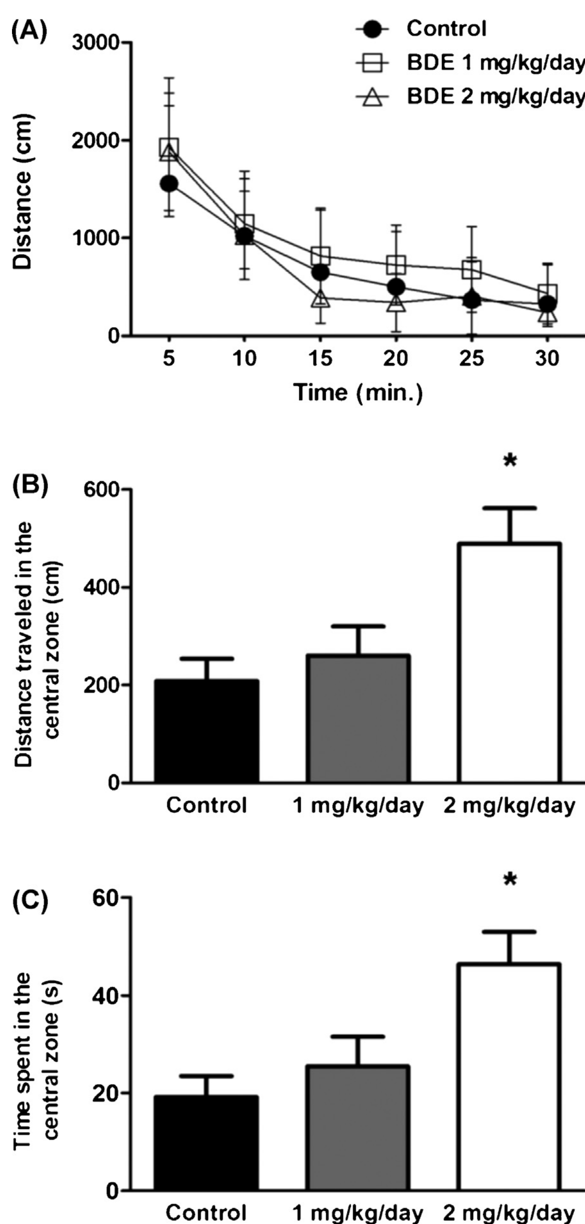


**Fig. 2.** Perinatal exposure to BDE-99 reduces anxiety. Rat offspring of dams that were exposed to 0, 1, 2 mg/kg/day of BDE-99 from GD 6 to PND 21 were tested in the open-field. (A) No significant differences were observed in the total distance traveled. Significant differences were observed in the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

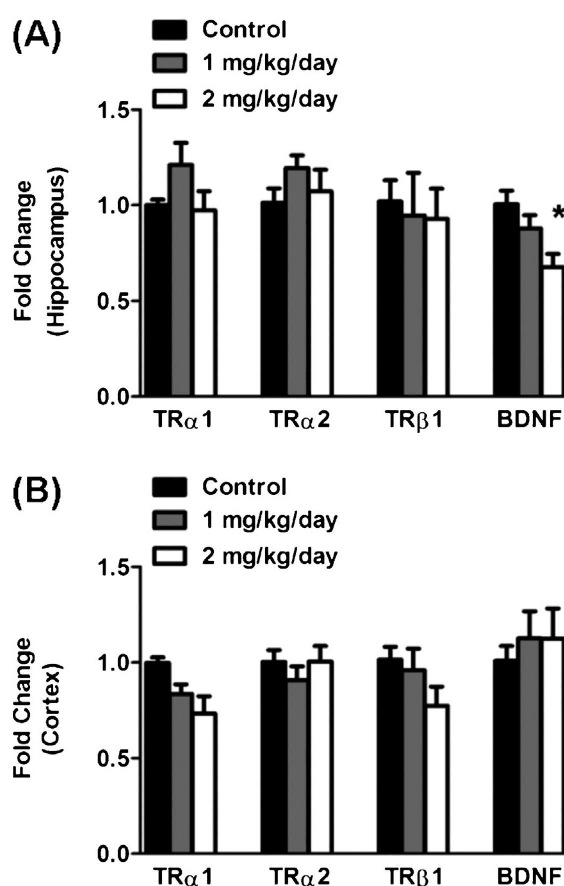


**Fig. 3.** Perinatal exposure to BDE-99 down-regulate the hippocampus gene expression of BDNF. The (A) hippocampus and (B) cortex of rat offspring of dams that were exposed to 0, 1, 2 mg/kg/day of BDE-99 from GD 6 to PND 21 were dissected, and the relative expression levels of the mRNAs encoding the TRα1, TRα2, TRβ1 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 ± 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 ± 9% in the 2 mg/kg/day group with respect to control group. Similarly, the total T4 (25 ± 13%, $P = 0.003$) and T3 (13 ± 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 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 ± 17%, $P = 0.199$), being statistically significant in the group exposed to 2 mg BDE-99/kg/day (32 ± 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, TRα1 and TRβ1 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) | $1.91 \pm 0.18^{a}$ | $1.84 \pm 0.16^{ab}$ | $1.65 \pm 0.18^{b}$ |
| T4 (nmol/L) | $39.8 \pm 6.2^{a}$ | $32.3 \pm 5.3^{ab}$ | $29.8 \pm 4.2^{b}$ |
| FT4 (pmol/L) | $32.1 \pm 2.8^{a}$ | $28.7 \pm 2.1^{ab}$ | $26.4 \pm 3.2^{b}$ |

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 phosphatidyl-inositol 3 kinase, phospholipase C gamma, and intracellular signal-regulated kinase 1/2 (Liao et al., 2007; Schratt 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.

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UNIVERSITAT ROVIRA I VIRGILI
STUDY OF THYROID HORMONE DISRUPTION BY THE ENVIRONMENTAL CONTAMINANT BDE-99 IN PERINATAL RATS
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# Perinatal Exposure to BDE-99 Causes Decreased Protein Levels of Cyclin D1 via GSK3β Activation and Increased ROS Production in Rat Pup Livers

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**We here examined the potential liver toxicity in rat pups from dams exposed during the gestational and lactation periods to 2,2',4,4',5-pentaBDE (BDE-99). Dams were exposed to 0, 1, and 2 mg/kg/day of BDE-99 from gestation day 6 to post-natal day 21. When the pups were weaning, the liver from one pup of each litter was excised to evaluate oxidative stress markers and the mRNA expression of multiple cytochrome P450 (CYP) isoforms. To determine whether thyroid hormone (TH) was disrupted, the proteins and mRNA expression of several TH receptors (TRs) isoforms were evaluated, as well as the protein levels of cyclin D1 and the phosphorylated protein kinases Akt and GSK3β. Perinatal exposure to BDE-99 produced decreased levels of cyclin D1 in rat pup livers. A decrease in the active form of Akt and an increase in the active form of GSK3β were observed. The decreased Akt pathway may be due to a potential disruption of the non-genomic actions of TH by BDE 99 and its metabolites. This possible TH disruption was noted as a decrease in TR isoforms expression. By contrast, we observed an up-regulation of CYP2B1 gene expression, which is correlated with an increase in ROS production. This outcome indicates activation of the nuclear constitutive androstane receptor, which could induce the expression of other enzymes capable of metabolizing TH. The present findings support the hypothesis that perinatal exposure to PBDEs, at levels found in humans, may have serious implications for metabolic processes in rat pup livers.**

***Key Words*: BDE-99; reactive oxygen species (ROS); CYP enzyme system; thyroid hormone receptors; Akt; GSK3β; cyclinD1.**

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

(PBDEs). The lipophilic nature and resistance to degradation of PBDEs have allowed their biomagnification in trophic chains, being these compounds currently present in all ecosystems and human food (Costa *et al*., 2008; Darnerud, 2003; Domingo, 2004, 2012; Klosterhaus *et al*., 2012; Na *et al*., 2013).

Despite the bans and restrictions on the use and production of PBDEs (COP4, 2009), high amounts of these compounds have accumulated in humans (Costa *et al*., 2008). These levels are particularly harmful in women because of the biodisponibility of PBDEs is increased during pregnancy and lactation, being potentially transferred to the offspring (Gomara *et al*., 2007; Schuhmacher *et al*., 2009; Shen *et al*., 2013). Epidemiological studies have correlated higher concentrations of PBDEs in cord blood and maternal milk with worse neurophysiological development and increased activity/impulsivity behaviors in toddlers (Eskenazi *et al*., 2013; Gascon *et al*., 2012; Herstman *et al*., 2010; Hoffman *et al*., 2012; Roze *et al*., 2009; Shy *et al*., 2011). Moreover, various animal studies have attributed the primary neurotoxic action of PBDEs to their ability to act as disruptors of the thyroid hormone (TH) signaling (Blanco *et al*., 2013; Kuriyama *et al*., 2005, 2007; Ta *et al*., 2011; Talsness *et al*., 2005; Zhou *et al*., 2002).

The liver is a key organ implicated in the TH disruption caused by PBDE exposure. PBDEs induce increased hepatic gene expression of enzymes involved in the detoxification of xenobiotic compounds (Blanco *et al*., 2012; Sanders *et al*., 2005; Szabo *et al*., 2009). These enzymes catalyze the addition of hydrophilic groups to the lipophilic structures of PBDEs, resulting in metabolites that are easily removed from the body. However, these metabolites may act as stronger endocrine disrupters than the parent compounds. In vitro studies have identified the mechanism by which several hydroxylated PBDEs (HO-PBDEs) bind antagonistically to TH transport proteins (Yang

75 *et al*., 2011) and nuclear TH receptors (TRs) (Kojima *et al*., 2009; Kitamura *et al*.,
76 2008), thereby deregulating the expression of genes that are fundamental for normal cell
77 growth and survival.

78 Triiodothyronine (T3), the biologically active form of TH, has been shown to be a
79 powerful inducer of cell proliferation. Among other mechanisms, T3 increases the
80 levels of cyclin D1 via non-genomic actions, leading to cell cycle transition from G1 to
81 S phase. T3 stimulates activation of Akt via phosphatidylinositol-3-kinase (PI3K) non-
82 genomic signaling (Cao *et al*., 2005; Hiroi *et al*., 2006 ; Pibiri *et al*., 2001 ; Radenne *et*
83 *al*., 2008). The active protein kinase Akt restrains the activity of glycogen synthase
84 kinase 3 beta (GSK3β), avoiding the exclusion of cyclin D1 from the nucleus and its
85 proteasomal degradation (Takahashi *et al*., 2008). Dunnick and Nyska (2009) and Lee *et*
86 *al*. (2010) reported that long-term exposure to PBDEs produced hepatocyte
87 hypertrophy, necrosis and increased vacuolization in rodents, which suggests
88 deregulation of the cell cycle. Recently, we observed liver enlargement, increased
89 hepatic production of reactive oxygen substances (ROS), and gene induction of
90 detoxifying enzymes in the livers from rat fetuses whose dams were orally exposed to
91 PBDEs on gestation days 6-19 (Blanco *et al*., 2012). High levels of ROS are known to
92 produce DNA damage, programmed cell death or can activate specific signalling
93 pathways (Finkel *and* Holbrook, 2000).

94 The main goal of this study was to determine whether liver toxicity occurred in rat
95 pups from dams exposed, during the gestational and lactation periods, to one of the
96 most prevalent PBDE congeners in human samples, 2,2',4,4',5-pentaBDE (BDE-99).
97 Pup livers were excised when pups were weaned. We assessed the hepatic mRNA
98 expression of several detoxifying isoforms of the cytochrome P450 (CYP) family
99 enzymes and their correlation with the antioxidant enzyme activity of catalase (CAT),

superoxide dismutase (SOD) and the total levels of thiobarbituric acid reactive substances (TBARS). To evaluate possible TH disruption, the protein and mRNA expression of several TR isoforms was evaluated, as well as the protein levels of cyclin D1 and phosphorylated protein kinases Akt and GSK3β.

## 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 ± 2ºC and a relative humidity of 50 ± 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).

***Experimental procedures.*** Rat females with vaginal smears containing sperm were divided into 3 groups, with 8 animals per group. Rats in each group received daily doses of BDE-99 (gavage) at 0, 1, and 2 mg/kg body weight from GD 6 to postnatal day (PND) 21, except for PND 0 when the dams were left untreated. Animals in the control group received 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 of the tissue distribution of BDE-99 in rats (Cheng *et al.*, 2009). On PND 1, all litters were randomly reduced to 4 male pups. Pups were weaned on PND 21 and housed in groups of 4 per cage. At that time, one pup from each litter was sacrificed by decapitation, and the liver was immediately dissected, frozen in liquid nitrogen, and stored at −80ºC.

***Oxidative stress markers.*** A fraction of the rat pup liver was used to assess the activity of the antioxidant enzymes SOD, 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 X 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 and CAT 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 rat pup 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

150 Reverse Transcription Kit (Qiagen Inc., Hilden, Germany) according to the
151 manufacturer's protocol. An identical reaction, without reverse transcriptase, was
152 performed to verify the absence of genomic DNA. The cDNA was subsequently
153 amplified by PCR using rat-specific primers for CYP1A1 (NM_012540; forward: 5'-
154 CTG CAG AAA ACA GTC CAG GA-3'; reverse: 5'-CAG GAG GCT GGA CGA
155 GAA TGC-3'), CYP1A2 (NM_012541; forward: 5'-CCA AGC CGT CCA CGA GAC
156 TT-3'; reverse: 5'-GAG GGA TGA GAC CAC CGT TG-3'), CYP2B1
157 (NM_001134844; forward: 5'-CCA AGC CGT CCA CGA GAC TT- 3'; reverse: 5'-
158 TTG GGA AGC AGG TAC CCT C-3'), CYP3A1 (NM_013105; forward: 5'-CCG
159 CCT GGA TTC TGT GCA GA-3'; reverse: 5'-TGG GAG GTG CCT TAT TGG GC-
160 3'), CYP3A2 (NM_153312; forward: 5'-TTG ATC CGT TGC TCT TGT CA-3';
161 reverse: 5'-GGC CAG GAA ATA CAA GAC AA-3'), TR α1 (NM_001017960;
162 forward: 5'-TGC CCT TAC TCACCC CTA CA-3'; reverse: 5'-AAG CCA AGC CAA
163 GCT GTC CT-3'), TRβ1 (NM_012672; forward: 5'-AGC CAG CCA CAG CAC AGT
164 GA-3'; reverse: 5'-CGC CAG AAG ACT GAA GCT TGC-3') and β-actin
165 (NM_031144; forward: 5'-TGT CAC CAA CTG GGA CGA TA-3'; reverse: 5'-GGG
166 GTG TTG AAG GTC TCA AA-3') with a PyroStart Fast PCR Master mix (2X) kit
167 (Fermentas, Burlington, Canada) according to the manufacturer's protocol. The PCR
168 products were separated on a 1% agarose gel, and only specific bands were detected.
169 The non reactivity of the primers with contaminant genomic DNA was tested by the
170 inclusion of controls that omitted reverse transcriptase from the cDNA synthesis
171 reaction.
172
173 ***Real-time reverse-transcription-PCR.*** Quantitative PCR for CYP1A1, CYP1A2,
174 CYP2B1, CYP3A1, CYP3A2, TRα1, TRβ1 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 50ºC for 2 min, followed by a polymerase activation step at 95ºC for 15 min and a cycling step with the following conditions: 40 cycles of denaturing at 95ºC for 15 s, annealing at 60ºC for 30 s, and extension at 72ºC for 30 s. As oligonucleotides of varying lengths produce dissociation peaks at different melting temperatures, 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 72ºC 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.

***Western blot analysis.*** Aliquots containing 30 μg of protein per sample of liver lysate were analyzed by western blot analysis. Briefly, samples were placed in sample buffer (0.5M 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 (50mM Tris, 1.5% NaCl, 0.05% Tween 20, pH 7.5). They were then incubated overnight with primary monoclonal antibodies against TRα1, TRβ1, AKT-total, AKT-p, GSK3β-total, GSK3β-p 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.

***Statistics.*** To evaluate 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). The relationships among the examined endpoints were assessed using Pearson correlations.

## RESULTS

*BDE-99 Increases the Body Weight of Rat Pup*

Body weight of the pups was significantly increased ($p = 0.033$) in the BDE 2 group compared to the control group (Table 1). Although the pup liver weight was increased in a dose-dependent manner, no significant differences were observed (Table 1). However, this trend disappeared when the liver weight was corrected with the pup body weight.

*BDE-99 Increases Oxidative Stress Markers in the Rat Pup Liver*

The production of ROS in the rat pup liver was indirectly evaluated by measuring different oxidative stress markers. The activity of the antioxidant enzymes SOD and CAT and the total level of TBARS were measured in the rat pup liver (Table 1). A BDE-99 dose-dependent increase was observed in the levels of the evaluated oxidative stress markers. The CAT activity was significantly increased in the BDE 2 group ($p = 0.003$) compared to the control group. The SOD activity was also significantly increased in both, the BDE 1 ($p = 0.014$) and BDE 2 ($p < 0.001$) groups, compared to the control group. These results suggest that ROS production in pup liver is proportional to the level of BDE-99 exposure in pregnant dams.

*The BDE-99-Induced Production of ROS in the Rat Pup Liver Is Related to Increased mRNA Expression of the CYP2B1 Isoform*

The CYP enzyme family plays a key role in the detoxification of xenobiotics, such as PBDEs. In rat pup livers, we evaluated the mRNA expression of five principal CYP isoforms involved in the biotransformation and detoxification of PBDEs (Fig. 1). Compared to the control group, a significant increase was found in the expression of the CYP2B1 isoform (714 ± 332%, $p = 0.019$) in pup livers from the BDE 2 group. Since the production of ROS may be related to the increased gene expression of the CYP2B1 isoforms, we calculated the relationship between multiple oxidative stress markers and the upregulated CYP2B1 isoform. A significant correlation between the increased CAT activity and CYP2B1 gene expression ($R = 0.445$, $p = 0.029$) was observed. A similar correlation was also noted for the total level of SOD and CYP2B1 expression ($R = 0.650$, $p = 0.001$).

*BDE-99 Deregulates Gene and Protein Expression of the TRs in the Rat Pup Liver*

The TRα and TRβ genes encode two functional isoforms of TR, TRα1 and TRβ1, which are expressed at similar levels in the liver. Because little information exists regarding the effects of PBDEs on TR expression in the liver, changes in the relative mRNA and protein levels of these two isoforms were examined by real-time qPCR (Fig. 2A) and Western blot (Fig. 2B), respectively. The mRNA expression of TRα1 was significantly down-regulated in the BDE 1(44 ± 13%, $p = 0.001$) and the BDE 2 (42 ± 4%, $p = 0.001$) groups compared to the control group. Similarly, the transcript levels of TRβ1 were also significantly down-regulated (31 ± 14%, $p = 0.033$) in the BDE 2 group compared to the control group. In contrast to the mRNA expression, the relative protein levels of the TRs were significantly decreased (36 ± 15%, $p = 0.018$) only for the TRα1 isoform in the BDE 2 group compared to the control group.

*BDE-99 Decreases the Protein Expression of Cyclin D1 and Phosphorylation of the Proteins Kinases AKT and GSK3β*

The phosphorylation of the protein kinases Akt (Fig. 3A) and GSK3β (Fig. 3B) were reduced in a dose-dependent manner, with high perinatal exposure of the pups to BDE-99. The ratios of Akt-p/Akt-total and GSK3β-p/GSK3β-total were significantly decreased (28 ± 7%, $p = 0.025$; 49 ± 9%, $p = 0.001$, respectively) in the BDE 2 group compared to the control group. The same effect was also observed for the relative protein expression of cyclin D1. Cyclin D1 was significantly decreased in the BDE 1 (22 ± 7%, $p = 0.032$) and BDE 2 groups (41 ± 9%, $p = 0.001$), compared to the control group (Fig. 3C).

## DISCUSSION

274 The results of the current study show that the maternal transmission of BDE-99 during
275 the gestation and lactation periods produced clear signs of toxicity in rat pup livers. The
276 decreased levels of the cell survival PIP3K/Akt pathway and cyclin D1, and the
277 increased ROS production, which are related to high expression of CYP2B1, indicate a
278 possible alteration in cell cycle progression and hepatocyte functions.

279 TH disruption may be partially responsible for the decreased levels of cyclin D1.
280 Several PBDE metabolites are antagonistic of TH actions. We hypothesized that these
281 metabolites might also inhibit the non-genomic actions of TH related to activation of the
282 PI3K/Akt pathway (Cao *et al*., 2005; Hiroi *et al*., 2006 ; Pibiri *et al*., 2001; Radenne *et*
283 *al*., 2008). Down-regulation of the active form of Akt, leading to decreased
284 phosphorylation and activation of GSK3β, was observed. Phosphorylation of cyclin D1
285 on $Thr^{286}$ by GSK-3β facilitates its association with CRM1, a nuclear protein that
286 mediates the nuclear export of proteins, resulting in the exclusion of cyclin D1 from the
287 nucleus to initiate its proteasomal degradation (Takahashi *et al*., 2008). Another
288 possible reason for the decrease of cyclin D1 is the increased metabolism of TH. One of
289 the earlier events in hepatocyte proliferation induced by T3 is the induction of cyclin D1
290 (Pibiri *et al*., 2001). Alisi *et al*. (2005) showed that rats treated with propylthiouracil
291 (PTU), a drug that inhibits TH synthesis in the thyroid gland, induces down-regulation
292 of cyclin D1 levels in hepatocytes. A number of studies have shown that the high
293 expression of detoxifying enzymes induced by PBDEs can promote high elimination of
294 TH in the liver, which reduces TH levels in the serum in the long term (Kuriyama et al.,
295 2007; Tseng et al., 2008; Zhou et al., 2001, 2002). In the present investigation, we noted
296 that the CYP2B1 isoform was up-regulated after perinatal exposure to BDE-99. The
297 mRNA expression of CYP2B genes is mediated by activation of the constitutive
298 androstane receptor (CAR). This nuclear receptor also encodes enzymes, such as

sulfotransferases and uridine diphosphate glucuronosyltransferases, which are capable of metabolizing TH in the liver (Szabo *et al*., 2009). Maglich *et al*. (2004) showed that TH concentrations in the serum decreased in wild-type mice treated with the CAR agonist TCPOBOP, while in $Car^{-/-}$ mice, the TH levels remained significantly higher. In a recent study, we found an increase of CYP1A, CYP2B and CYP3A genes in rat fetus livers exposed to the same conditions than those of the current study (Blanco *et al*., 2012). A possible difference in the expression of these detoxifying enzymes might be the different concentrations of BDE-99 and their metabolites or impurities transmitted during the gestation and/or lactation periods. Differences between the concentrations of PBDE congeners in maternal serum and breast milk samples have been recently reported by Jakobsson *et al*. (2012).

Other signs of possible TH disruption by BDE-99 are the decreased expression of TR isoforms, the gene regulation of which is mediated by the concentration of TH (Samuels *et al*., 1977). An increase in TH levels leads to a decrease in the predominant isoforms of TR expressed in cells, while a decrease in TH levels increases the predominant TR isoforms as an adaptive mechanism to maximize the TH response (Monden *et al*., 2006; Samuels *et al*., 1977; Shadow *et al*., 2003). PBDEs and OH-PBDEs have a similar structure to TH and may activate this auto-regulatory mechanism by creating a state that mimics hyperthyroidism (Blanco *et al*., 2011). The liver is the primary target organ for PBDEs in rats and mice, and a large hepatic concentration of these organobrominated pollutants would lead to a decrease in the expression of the TR isoforms.

Furthermore, the decreased activity of the PIP3K/Akt pathway may also affect the regulation of other proteins involved in cell survival functions or glucose and lipid metabolism. Similarly to the results of previous studies, we also observed an increased body weight for rat pups exposed to BDE-99 (Suvorov *et al*., 2009). Recently, it has

been postulated that PBDEs may contribute to the onset of diabetes in humans. It is well known that the PIP3K/Akt pathway is an obligate mediator of many of metabolic actions of insulin. Zhang *et al*. (2013) and Nash *et al*. (2013) have reported dose-related hyperglycemia, decreased insulin in the serum, increased percentage of lipids, and changes in the expression of genes related to type I diabetes mellitus in livers of rodents exposed to a commercial mixture of PBDEs, or to the congener 2,2′,3,3′,4,4′,5,5′,6,6′-decaBDE (BDE209). The decreased activity of the PIP3K/Akt pathway in other tissues may explain the negative effects of PBDEs for neurobehavioral or reproductive toxicity.

In summary, perinatal exposure to BDE-99 through gestation and ingestion of maternal breast milk may produce decreased levels of cyclin D1 in liver. In the present investigation, we also observed a decrease of the active form of Akt and a concomitant increase of the active form of GSK3β. The decrease of the PIP3K/Akt pathway may be due to disruption of the non-genomic actions of TH by BDE 99 and its metabolites. This TH disruption is noted as the decreased expression of TR isoforms. In contrast, we observed up-regulation of CYP2B1 gene expression, which is correlated with an increase in ROS production. It indicates the activation of CAR and the possible expression of other enzymes capable of metabolizing TH and decreasing its serum levels. These findings support the hypothesis that perinatal exposure to PBDEs, at levels found in humans might have serious implications on metabolic and body weight programming. Further studies on the potential health risks of these environmental pollutants are still necessary for a better understanding of these effects.

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## FIGURE LEGENDS

**FIG. 1.** BDE-99 increases the mRNA expression of CYP2B1 in rat pup livers. The mRNA was extracted of at pup livers from dams orally exposed to 0, 1, and 2 mg/kg/day of BDE-99 from GD 6 to PND 21. 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. The fold changes were calculated relative to the control. Data are expressed as the mean values ± SD of eight independent samples per group. Significance of the differences was analyzed by one-way ANOVA followed by Bonferroni's post hoc test: $*p < 0.05$ versus the control group.

**FIG. 2.** Perinatal exposure to BDE-99 down-regulates TR expression in rat pup livers. The mRNA and total protein were extracted of rat pup livers from dams orally exposed to 0, 1, and 2 mg/kg/day of BDE-99 from GD 6 to PND 21. (A) The relative expression levels of the TRα1 and TRβ1 mRNAs were determined by quantitative real-time RT-PCR. Data were normalized using the β-actin gene as an internal control. The fold changes were calculated relative to the control. (B) The relative protein expression of the TRα1 and TRβ1 isoforms was measured by Western blot analysis. The intensity of the bands was determined by densitometric analysis. Data were normalized using β-actin as an internal control. A Western blot from one representative experiment is shown in the upper panel. Data from the experiments are presented in the lower panel. All data are expressed as means ± SD. Significant differences relative to the control

group were analyzed by one-way ANOVA followed by a Bonferroni's post hoc test: $*p < 0.05$, $**p < 0.001$.

**FIG. 3.** Perinatal exposure to BDE-99 decreases the protein expression of cyclin D1 and phosphorylation of the protein kinases Akt and GSK3β. Total protein was extracted of rat pup livers from dams orally exposed to 0, 1, and 2 mg/kg/day of BDE-99 from GD 6 to PND 21. The relative protein expression levels of Akt-p (A), GSK3β-p (B) and cyclin D1 (C) were measured by Western blot analysis. The intensity of the bands was determined by densitometric analysis, and the data were normalized using Akt-total (A), GSK3β-total (B) and β-actin (C) as the internal controls. Data from the experiments are expressed as means ± SD in the lower panel. Significant differences relative to the control group were analyzed by one-way ANOVA followed by Bonferroni's post hoc test: $*p < 0.05$, $**p < 0.001$.

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3 573 **TABLES AND FIGURES**
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TABLE 1

Effects of BDE-99 Exposure on Body and Liver Weight and on Biochemical Oxidative Stress Markers of Rat Pup Livers

| Exposure group | Control, (n = 10) | BDE 1 (mg/kg/day), (n = 10) | BDE 2 (mg/kg/day), (n = 10) |
|---|---|---|---|
| Body weight (g) | 180.96 ± 10.25[a] | 194.64 ± 20.67[ab] | 200.96 ± 13.42[b] |
| Liver weight (g) | 8.24 ± 0.70 | 8.76 ± 0.87 | 9.12 ± 0.97 |
| Liver weight/Body weight (%) | 4.55 ± 0.03 | 4.50 ± 0.02 | 4.53 ± 0.03 |
| Catalase (mol/min/mg protein) | 13.47 ± 1.80[a] | 16.49 ± 1.72[b] | 18.38 ± 1.87[b] |
| SOD (U/mg protein) | 359.25 ± 81.35[a] | 477.3 ± 46.42[ab] | 542.95 ± 119.37[b] |
| TBARS (nmol/mg protein) | 19.84 ± 3.02 | 22.64 ± 3.82 | 24.26 ± 4.74 |

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

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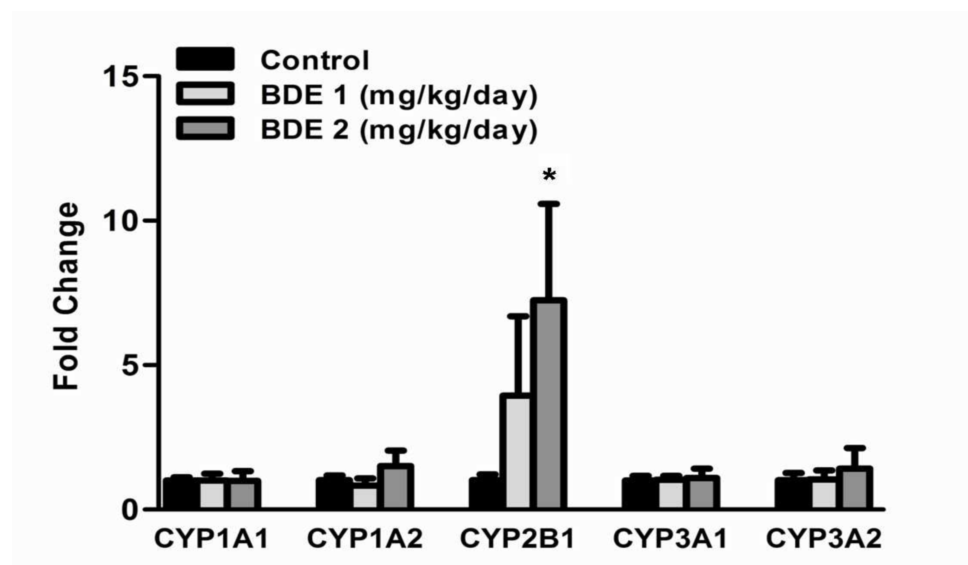


27–44
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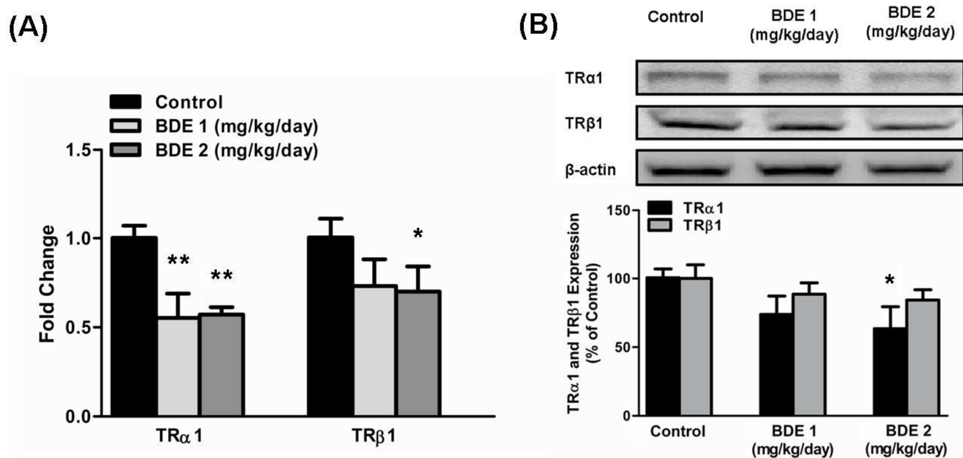


FIG. 2.

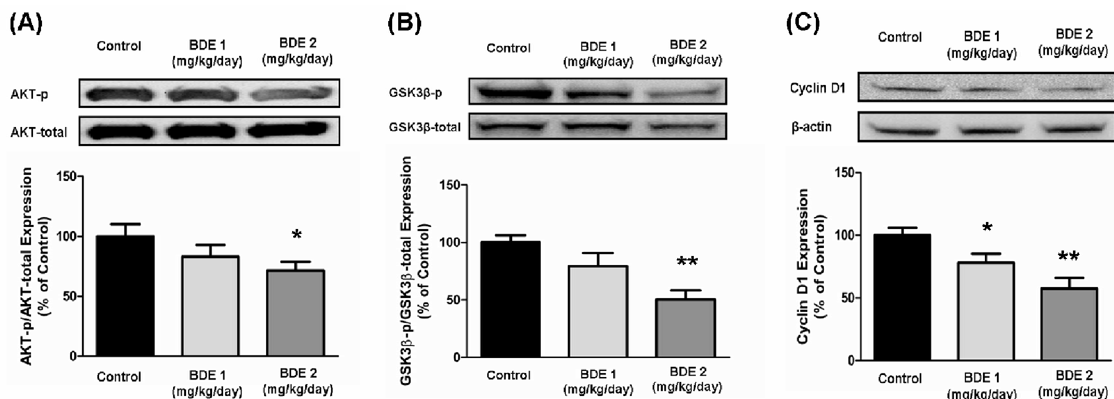


FIG. 3.

UNIVERSITAT ROVIRA I VIRGILI
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# 5. DISCUSSION

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### 5.1 *Discussion of in vitro study in CGNs treated with 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-alpha gene. The expression levels of TRα1, the predominant TR isoform in CGNs, and TRα2, 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-alpha 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-alpha in females, as well as a decrease in TR-beta 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, TRα2,

may play an important role in BDE-99 neuronal toxicity. The splice variant of TRα1 appears to exert a dominant negative activity on the T3-dependent trans-activation of TR target genes. A decrease in TRα2 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-90, 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-beta1, 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, 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 GD 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 TRα2, and act as TR antagonists.

### 5.2 *Discussion of animal study of maternal transference of BDE-99 during pregnancy*

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 transplacentary action, BDE-99 up-regulates 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 (17β-estradiol and testosterone) and tetraiodothyroxine

(T4) in offspring of rats. The excessive metabolization 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-OHBDE-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).

### 5.3 *Discussion of animal studies of maternal transference of BDE-99 during gestation and lactation*

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 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 travelled 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 serumT4 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 phosphatidyl-inositol 3 kinase, phospholipase C gamma, and intracellular signal-regulated kinase 1/2 (Liao et al., 2007; Schratt 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 perfluoro octane substances.

In the current investigation, we did not find changes in the gene expression of theTRs 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.

On the other hand, the maternal transmission of BDE-99 during the gestation and lactation periods produced clear signs of toxicity in rat pup livers. The decreased levels of the cell survival PIP3K/Akt pathway and cyclin D1, and the increased ROS production, which are related to high expression of CYP2B1, indicate a possible alteration in cell cycle progression and hepatocyte functions.

TH disruption may be partially responsible for the decreased levels of cyclin D1. Several PBDE metabolites are antagonistic of TH actions. We hypothesized that these metabolites might also inhibit the non-genomic actions of TH related to activation of the PI3K/Akt pathway (Cao et al., 2005; Hiroi et al., 2006 ; Pibiri et al., 2001; Radenne et al., 2008). Down-regulation of the active form of Akt, leading to decreased phosphorylation and activation of GSK3β, was observed. Phosphorylation of cyclin D1 on $Thr^{286}$ by GSK-3β facilitates its association with CRM1, a nuclear protein that mediates the nuclear export of proteins, resulting in the exclusion of cyclin D1 from the nucleus to initiate its proteasomal degradation (Takahashi et al., 2008). Another possible reason for the decrease of cyclin D1 is the increased metabolism of TH. One of the earlier events in hepatocyte proliferation induced by T3 is the induction of cyclin D1 (Pibiri et al., 2001). Alisi et al. (2005) showed that rats treated with propylthiouracil (PTU), a drug that inhibits TH synthesis in the thyroid gland, induces down-regulation of cyclin D1 levels in hepatocytes. A number of studies have shown that the high expression of detoxifying enzymes induced by PBDEs can promote high elimination of

TH in the liver, which reduces TH levels in the serum in the long term (Kuriyama et al., 2007; Tseng et al., 2008; Zhou et al., 2001, 2002). In the present investigation, we noted that the CYP2B1 isoform was up-regulated after perinatal exposure to BDE-99. The mRNA expression of CYP2B genes is mediated by activation of the constitutive androstane receptor (CAR). This nuclear receptor also encodes enzymes, such as sulfotransferases and uridine diphosphate glucuronosyltransferases, which are capable of metabolizing TH in the liver (Szabo et al., 2009). Maglich et al. (2004) showed that TH concentrations in the serum decreased in wild-type mice treated with the CAR agonist TCPOBOP, while in $Car^{-/-}$ mice, the TH levels remained significantly higher.

Other signs of possible TH disruption by BDE-99 are the decreased expression of TR isoforms, the gene regulation of which is mediated by the concentration of TH (Samuels et al., 1977). An increase in TH levels leads to a decrease in the predominant isoforms of TR expressed in cells, while a decrease in TH levels increases the predominant TR isoforms as an adaptive mechanism to maximize the TH response (Monden et al., 2006; Samuels et al., 1977; Sadow et al., 2003). PBDEs and OH-PBDEs have a similar structure to TH and may activate this auto-regulatory mechanism by creating a state that mimics hyperthyroidism. The liver is the primary target organ for PBDEs in rats and mice, and a large hepatic concentration of these organobrominated pollutants would lead to a decrease in the expression of the TR isoforms.

Furthermore, the decreased activity of the PIP3K/Akt pathway may also affect the regulation of other proteins involved in cell survival functions or glucose and lipid metabolism. Similarly to the results of previous studies, we also observed an increased body weight for rat pups exposed to BDE-99 (Suvorov et al., 2009). Recently, it has been postulated that PBDEs may contribute to the onset of diabetes in humans. It is well known that the PIP3K/Akt pathway is an obligate mediator of many of metabolic

actions of insulin. Zhang et al. (2013) and Nash et al. (2013) have reported dose-related hyperglycemia, decreased insulin in the serum, increased percentage of lipids, and changes in the expression of genes related to type I diabetes mellitus in livers of rodents exposed to a commercial mixture of PBDEs, or to the congener 2,2′,3,3′,4,4′,5,5′,6,6′-decaBDE (BDE209). The decreased activity of the PIP3K/Akt pathway in other tissues may explain the negative effects of PBDEs for neurobehavioral or reproductive toxicity.

## 5.4 *Summarising discussion*

Several toxic effects were observed in rat fetuses and rat pups by the maternal transference of BDE-99. Liver toxicity was a common effect observed during the pregnancy and lactation periods. We observed that BDE-99 up-regulated the mRNA expression of CYP1A1, CYP1A2, CYP2B1, and CYP3A in rat fetal liver. The changes observed in the 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. On the other hand, we found that only the mRNA expression of CYP2B1 isoform was up-regulated in rat pup livers after perinatal exposure to BDE-99. A possible difference in the expression of these detoxifying enzymes may be the different concentrations of BDE-99 and their metabolites transmitted during the gestation or lactation period. Jakobsson et al. (2012) reported differences between the concentrations of PBDEs congeners in maternal serum and breast milk samples. Another possibility could be a higher maternal transference of impurities present in the BDE-99 composition, which may increase the mRNA expression of CYP1A and CYP3A in rat fetal liver. However, both studies agree that CYP2B1 is the main hepatic detoxifying enzyme involucrate in the metabolism of BDE-99.

The positive correlation between the up-regulation of the gene expression of CYP isoforms with the increased production of oxidative stress markers was also observed in both animal studies. The CYP enzymes catalyze the oxygenation of exogenous and endogenous compounds as part of a microsomal mono-oxygenation system (MMO) localized in the membranes of the hepatic endoplasmic reticulum. The transference of electrons from NADPH to CYP enzymes is required for the mono-oxygenation reaction. The electron 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 maternal transference of BDE-99 in the MMO can lead to an increase of ROS in liver of rat fetus and rat pups, producing cell damage. ROS induced by BDE-99 exposure could be an important factor in the process of enlargement of the fetal liver and the slight fetal heart hypertrophy produced in the rat fetus. It is well known that high levels of ROS can damage DNA, leading the cells into an adaptive state of hyperplasia and/or hypertrophy.

Moreover, in the study carried out in the CGNs, we found increased ROS production correlated with a time dependent decrease in cell viability. The increase of ROS production was also correlated with the down-regulation of the gene and protein expression of Bcl-2 and BDNF. These proteins are implicated in the stability of the mitochondria membrane potential and the activation of survival pathways. The disruption of TH action by BDE-99 may explain the alterations observed in their expression. BDNF is a TH-mediated gen and their down-regulation led to a decrease in the protein Bcl-2. 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 treated with BDE-99. Furthermore, co-treatment with T3 caused a significant increase in cell viability of CGNs compared to the treatment with BDE-99 alone. 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-90, a potent TR antagonist (Kitamura et al., 2008; Kojima et al., 2009).

The synthesis of the metabolites of PBDEs is mainly produced by liver in mammals. In the studies with rats, we observed a high up-regulation of the gene expression of CYP2B1 in fetal and pup livers. The mRNA expression of CYP2B genes is mediated by activation of the constitutive androstane receptor (CAR). This nuclear receptor also encodes enzymes, such as SULT and UDP-GT, which are capable of metabolizing TH in the liver. An increase in the activity of these enzymes could alter homeostasis by enhancing the elimination of these hormones during the gestational and lactation period. Moreover, the formation of metabolites of PBDEs, as 4-OH-BDE-90, can block the regulation of TH-mediated genes during the development. The observed enhancement in the frequency of delayed ossification in rat foetuses could be due to a TH disruption induced by prenatal exposure to BDE-99. The presence of TRs in osteoblasts and osteoclasts has suggested a direct role for TH in the modulation of genes related to the differentiation, proliferation, and regulation of bone tissue.

Furthermore, other studies have reported that 4-OH-BDE-90 has a high affinity for binding to the TH transport protein transthyretin (TTR; Yang et al., 2011). The binding of 4-OHBDE-90 to TTR causes the displacement of T4 in blood. The increment in the circulating level of free T4 in serum may also stimulate the increase in the hepatic activity of some TH metabolizing enzymes in liver, which could also lead to the excessive degradation of TH (Szabo et al., 2009). In our study, we have found a significant dose-response effect of BDE-99 for TH levels in analyzed serum of rat pups

on PND21. The serum concentration of T3, T4 and free T4 decreased in perinatal exposed pups in respect to the control group. The dose-dependent decrease of TH might be responsible for a large part of the neurotoxic effects of BDE-99 observed in rat pups. We found that maternal transference of BDE-99 produced a delay in the spatial learning task in the water maze test and a dose-response anxiolytic effect in the open-field test. The neurobehavioral disturbances observed in the open-field and water maze probes suggest a possible alteration of hippocampal functioning. Symptoms of hypothyroidism include an anxiolytic pattern and difficulty learning. Similar to the results found in CGNs cell, we found that the mRNA expression of BDNF was significantly down-regulated in offspring hippocampi after perinatal exposure to BDE-99. BDNF is an important TH-regulated protein, which is implicated in neuronal processes. This neurotrophine plays a crucial role in learning and memory processes, triggering the activation of signalling pathways involving PIP3K, phospholipase C gamma, and intracellular signal-regulated kinase 1/2 (Liao et al., 2007;Schratt et al., 2004; Ying et al., 2002).

Other common signs of a possible TH disruption by BDE-99 exposure are the lower expression of TR isoforms, the gene regulation of which is mediated by the concentration of TH (Samuels et al., 1977). The mRNA expression of TRα1 and TRβ1, and the relative protein levels of TRα1 isoform were significantly down-regulated in rat pup livers by the maternal transmission of BDE-99. PBDEs and OH-PBDEs have a similar structure to TH and may activate this auto-regulatory mechanism by creating a state that mimics hyperthyroidism. The liver is the primary target organ for PBDEs in rats and mice, and a large hepatic concentration of these pollutants would lead to a decrease in the expression of the TR isoforms. In the study carried out in CGNs, we found that the gene expression of the TRα isoforms is directly deregulated by BDE-99.

This state could be transformed into a hypothetical state of TH resistance, possibly due to insufficient replacement of the TR. In contrast to these results, the mRNA expression of the different isoforms of TR did not show any significant difference in hippocampus and cortex in perinatally exposed rat pups. It was possibly due to a decrease in the TH levels in serum observed. The accumulation of PBDEs in brain is lower than in liver. 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.

On the other hand, we found that perinatal exposure to BDE-99 decreased the levels of cyclin D1 in the pup liver. We also observed a decrease in the active form of Akt and a concomitant increase in the active form of GSK3β. Several studies described that the active form of GSK3β phosphorylates cyclin D1 on the $Thr^{286}$ residue, thereby triggering translocation of cyclin D1 into the cytoplasm, followed by its ubiquitinylation and proteosomal degradation. As we have mentioned above, several PBDE metabolites are antagonistic of TH action. We hypothesize that these metabolites may also inhibit the non-genomic actions of TH related to activation of the PI3K/Akt pathway.

Furthermore, the decreased activity of the PIP3K/Akt pathway may also affect the regulation of other proteins involved in cell survival functions or glucose and lipid metabolism. Similar to other studies, we observed a higher body weight for rat fetus and rat pups exposed to BDE-99. Recently, it has been postulated that PBDEs may contribute to the onset of diabetes in humans. It is well known that the PIP3K/Akt pathway is an obligate mediator of many of metabolic actions of insulin. The lower activity of the PIP3K/Akt pathway in other tissues may explain the other negative effects of PBDEs for neurobehaviour or reproductive toxicity.

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# 6. CONCLUSIONS

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## 6.1 *In vitro* study in CGNs treated with BDE-99.

1. CGNs exposed to increasing concentrations of BDE-99 presented a dose-dependent cell death. The cytotoxicity of BDE-99 was developed gradually over time, showing a significant correlation between ROS production and cell death.

2. CGNs exposed to BDE-99 presented a down-regulation on the gene and protein expression of the TH-mediated neurotrophin BDNF and the antiapoptotic protein Bcl-2.

3. BDE-99 decreased the gene expression of the isoforms TRα1 and TRα2 in CGNs.

4. Increasing concentrations of T3 and BDE-99 co-treatment rescued cell death and decreased proteins levels of BDNF and Bcl-2 induced by BDE-99 treatment in CGNs.

## 6.2 Animal study of maternal transference of BDE-99 during pregnancy

1. Maternal transference of BDE-99 during pregnancy provoked a delayed ossification, induced a slight hypertrophy of the heart and induced the enlargement of the rat fetus liver.

2. Maternal transference of BDE-99 to rat fetus during pregnancy induced the upregulation of the mRNA of CYP1A1, CYP1A2, CYP2B1 and CYP3A2 genes in the fetal liver, which was correlated with a significant increase of the oxidative stress markers CAT and TBARS.

## 6.3 Animal studies of maternal transference of BDE-99 during gestation and lactation

1. Maternal transference of BDE-99 during gestation and lactation produced a body weight increase, and induced a decrease on the serum levels of T3, T4 and FT4 in rat offspring.

2. Perinatal exposure to BDE-99 through gestation and ingestion of maternal breast milk produced learning impairments and induced an anxiolytic pattern in the rat offspring.

3. Perinatal exposure to BDE-99 did not show effect on the gene expression of the different isoforms of TRs in cortex and hippocampus, but promoted the down-regulation of the TH-mediated gene BDNF expression in the rat pup hippocampus.

4. Perinatal exposure to BDE-99 through gestation and lactation induced an up-regulation of the CYP2B1 gene expression in rat pup liver, which was correlated with an increase of the oxidative stress markers CAT and SOD.

5. Perinatal exposure to BDE-99 through gestation and ingestion of maternal breast milk decreased protein levels of cyclin D1 and p-Akt and increased the levels of the active form of GSK3β in rat pup liver.

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