Published in final edited form as:

Neurotoxicology. 2015 May ; 48: 68–76. doi:10.1016/j.neuro.2015.03.008.

The brominated flame retardant BDE-47 causes oxidative stress and apoptotic cell death *in vitro* and *in vivo* in mice

Lucio G. Costa^{1,2}, Claudia Pellacani², Khoi Dao¹, Terrance J. Kavanagh¹, and Pamela J. Roque¹

¹Dept. of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA, USA

²Dept. Neuroscience, University of Parma, Parma, Italy

Abstract

Polybrominated diphenyl ethers (PBDEs), used for decades as flame retardants, have become widespread environmental contaminants. Exposure is believed to occur primarily through diet and dust, and infants and toddlers have the highest body burden, raising concern for potential developmental neurotoxicity. The exact mechanisms of PBDE neurotoxicity have not been elucidated, but two relevant modes of action relate to impairment of thyroid hormone homeostasis and to direct effects on brain cells causing alterations in signal transduction, oxidative stress and apoptotic cell death. The present study shows that BDE-47 (2,2',4,4'-tetrabromodiphenyl ether) induces oxidative stress and ensuing apoptotic cell death in mouse cerebellar granule neurons *in vitro*. Similarly, *in vivo* administration of BDE-47, according to an exposure protocol shown to induce behavioral and biochemical alterations (10 mg/kg, per os on post-natal day 10), induces oxidative stress and apoptosis, without altering serum levels of thyroid hormones. The effects of BDE-47 both *in vitro* and *in vivo* were more pronounced in a mouse model lacking the modifier subunit of glutamate cysteine ligase (GCLM) which results in reduced anti-oxidant capability due to low levels of GSH. Concentrations of BDE-47 in brain were in the mid-nanomolar range. These findings indicate that effects observed with BDE-47 *in vitro* are also present after *in vivo* administration, suggesting that in addition to potential endocrine effects, which were not seen here, direct interactions with brain cells should be considered as a potential mechanism of BDE-47 neurotoxicity.

Keywords

BDE-47; Oxidative stress; Glutathione; Apoptosis; Thyroid hormone; *In vivo-in vitro*

© 2015 Published by Elsevier Inc.

Correspondence: Dr. Lucio G. Costa, Dept. of Environmental and Occupational Health Sciences, University of Washington, 4225 Roosevelt Way NE, Suite 100, Seattle, WA 98105, Tel. 206-543-2831, Fax: 206-685-4696, lgcosta@u.washington.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

The family of polybrominated diphenyl ethers (PBDEs), widely used in the recent past as flame retardants, comprises 209 congeners, many of which have become persistent environmental pollutants. PBDEs have been detected in most biota, as well as in human tissues (Costa et al. 2008). Because of stricter fire regulations, the use of lower brominated mixtures (tetra-, penta-, and hexa- brominated BDEs) has been very high in North America, where levels are two orders of magnitude higher than those reported in Europe or Asia (USEPA, 2010). Five congeners (BDE-47, -99, -100, -153, -154) predominate in human tissues and account for 90% of PBDE body burden, with BDE-47 (2,2',4,4'-tetrabrominated diphenyl ether) accounting for 50% (USEPA, 2010). Body burden is 3 to 9-fold higher in infants and toddlers than in adults, because of exposure through breast milk and dust, respectively (Toms et al. 2009; USEPA, 2010), raising concerns for potential developmental toxicity and neurotoxicity of PBDEs (Eriksson et al. 2001; Costa and Giordano, 2007; Costa et al. 2014; Williams and DeSesso, 2010).

Animal studies suggest that exposure to various PBDE congeners during the prenatal and/or postnatal period causes long-lasting behavioral abnormalities, particularly in the domains of motor activity and cognition (Eriksson et al. 2001; Viberg et al. 2003; Gee and Moser, 2008; Ta et al. 2011). Increasing evidence in humans is also suggestive of neurodevelopmental effects of PBDEs, though findings are not always unequivocal. Nevertheless, effects on cognition, attention, motor coordination, activity/impulsivity, and adaptive behavior have been associated with *in utero* or early postnatal exposure to PBDEs in several cohorts (Chao et al. 2011; Herbstman et al. 2010; Hoffman et al. 2012; Eskenazi et al. 2013).

The mechanism(s) of PBDE developmental neurotoxicity have not been elucidated, but two general and not mutually exclusive modes of action have been suggested (Costa and Giordano, 2007; Costa et al. 2014). PBDEs have been shown to interfere with the homeostasis of thyroid hormones (Zhou et al. 2002; Herbstman et al. 2008; additional references in Costa and Giordano, 2007, and in Costa et al. 2008; 2014). As it is well known that hypothyroidism during gestation is associated with a large number of behavioral and morphological effects in the developing brain (Zoeller and Crofton, 2005), the effects of PBDEs on thyroid hormones may indeed be relevant to their developmental neurotoxicity. Yet, experimental studies in animals (Branchi et al. 2005; Gee et al. 2008; He et al. 2011) and observations in humans (Mazdai et al. 2003; Chevrier et al. 2011; Kim et al. 2012; Shy et al. 2012) indicate that CNS alterations may be present even in the absence of any thyroid hormone alteration.

An alternative mode of action for PBDE developmental neurotoxicity may be represented by direct effects of these compounds on brain cells. PBDEs have been shown to interfere with signal transduction pathways, such as protein kinase C (Madia et al. 2004), and calcium homeostasis (Dingemans et al. 2010). Additionally, numerous *in vitro* studies in multiple cell types have shown that PBDEs are capable of inducing oxidative stress (He et al. 2008; Giordano et al. 2008; Costa et al. 2010; Tagliaferri et al. 2010), which can lead to DNA damage (He et al. 2008; Pellacani et al. 2012), and apoptotic cell death (Madia et al. 2004; Giordano et al. 2008; He et al. 2008). We have previously shown that the neurotoxicity of

DE-71, a pentabrominated mixture, is modulated by intracellular glutathione (GSH) (Giordano et al. 2008), and that astrocytes protect neurons from DE-71 toxicity by increasing intracellular neuronal GSH levels (Giordano et al. 2009). Since evidence that some of these effects of PBDEs observed *in vitro* may occur *in vivo* is still limited, the aim of the present study was to compare specific effects of BDE-47 (the congener most abundant in human tissues) on oxidative stress and apoptotic cell death both *in vitro* and after *in vivo* administration. In order to define the contribution of cellular GSH levels in modulating the toxicity of BDE-47, we utilized *Gclm*^{+/+} mice (wild-type), and *Gclm*^{-/-} mice which lack the modifier subunit of glutamate cysteine ligase (GCLM), the first and rate-limiting enzyme in the synthesis of GSH. In the absence of GCLM, the catalytic subunit GCLC has a greatly reduced ability to promote GSH synthesis (Chen et al. 2005); as a consequence, *Gclm*^{-/-} mice have very low GSH levels (Giordano et al. 2006), and are so hypothesized to be more susceptible to BDE-47 neurotoxicity.

Materials and methods

Materials

BDE-47 (2, 2', 4, 4'-tetrabromodiphenyl ether; 99.9%) was obtained from AccuStandard Inc. (New Haven, CT). Poly-D-lysine, glutathione ethylester (GSHEE), dimethylsulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Hoechst 33342-DNA binding dye was from Molecular Probes (Eugene, OR), while 2,7'-dichlorofluorescein diacetate, neurobasal-A medium, fetal bovine serum and gentamycin were from Invitrogen (Carlsbad, CA). Melatonin was from Tocris Cookson Inc. (Elisville, MO) and the protein bicinchoninic acid (BCA) assay was from Thermo Fischer Scientific (Rockford, IL). The ELISA kit for measurement of serum thyroxine (T4) and triiodothyronine (T3) was purchased from Alpha Diagnostic International (San Antonio, TX). The ELISA kit for measurement of BDE-47 was purchased from Abraxis (Warminster, PA). The Thiobarbituric Acid Reactive Substances (TBARS) assay kit and the enzyme immunoassay (EIA) kit for measuring 8-isoprostane were purchased from Cayman Chemical Company (Ann Arbor, MI). Glass coverslips were from Fischer Scientific (Federal Way, WA) and the plastic coverslips from Nunc (Rochester, NY).

Generation of *Gclm*-null mice and genotyping

All procedures for animal use were in accordance with the National Institute of Health Guide for the Use and Care of Laboratory Animals, and were approved by the University of Washington Animal Care and Use Committee. *Gclm*-null (*Gclm*^{-/-}) mice were derived by homologous recombination techniques in mouse embryonic stem cells, as previously described in detail (Giordano et al. 2006), and pups were genotyped as described by Giordano et al. (2006).

Cultures of cerebellar granule neurons and cerebellar astrocytes

Cultures of cerebellar granule neurons (CGN) were prepared from 7 day-old mice, as described by Giordano et al. (2006). Neurons were seeded in poly-D-lysine-coated 48-well plates at the density of $5 \times 10^4/\text{cm}^2$, and cultured for 10–12 days before treatments. Primary

mouse astrocytes were obtained from PND 7 cerebellum, as previously described by Giordano et al. (2006). After counting, cells were plated at a density of 10^7 cells in 75 cm^2 tissue culture flasks pre-coated with poly-D-lysine and grown in DMEM containing 10% FBS, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin at 37°C in 5% CO_2 /95% air. After 10 days in culture, cells were plated in 24-well plates, at the density of 5×10^4 astrocytes/well.

Co-cultures of neurons and astrocytes

Astrocytes were cultured as described above. After ten days, astrocytes were dissociated with 0.25% trypsin and 0.1% DNase in Hanks' balanced salt solution and sub-cultured on permeable membranes (inserts) for 5 days. To maintain the correct hydrostatic pressure across the insert membrane, volumes in the co-cultures were regulated such that 15% of the total incubation volume was inside the insert and 85% in the well (Giordano et al. 2009).

Cell treatments

BDE-47 was dissolved in DMSO to obtain a stock solution of 12.5 mM, which was diluted appropriately in medium at the time of use. Four to five different concentrations of BDE-47, in triplicate, were used to determine IC_{50} values. Co-culture treatment was performed as described by Giordano et al. (2009). Briefly, the inserts containing cultured astrocytes were transferred to the neuronal plates, containing cultured neurons, 24 h prior to incubation with BDE-47. At time of insert transfer, half of the neuronal medium was replaced with astrocyte medium. In some experiments, GSHEE (2.5 mM) or melatonin (200 μM), were added to cell cultures one hour prior to treatments with PBDEs.

Measurement of cell viability

Cell viability was quantified using the metabolic dye MTT, as previously described (Giordano et al. 2008; 2009). Briefly, cells were treated with BDE-47 for 24 hours at 37°C . At the end of exposure, medium was removed and cells were incubated with 500 μl /well of Locke's buffer solution containing 2 mg/ml MTT for 30 minutes. MTT was then removed and reaction product dissolved in 0.25 ml DMSO/well. Absorbance was read at 570 nm, and the results expressed as the percentage of viable cells relative to the DMSO exposed controls.

Measurement of apoptosis

To visualize nuclear morphology following 24-hour BDE-47 exposure cells were grown on coverslips in 24-well plates and stained with Hoechst stain, as previously described (Giordano et al. 2007). After staining, the plastic coverslips were mounted on glass microscope slides and examined with a Labphot-2 microscope (Nikon, Japan). Cells exhibiting either classical apoptotic bodies or shrunken, rounded nuclei were scored as being apoptotic. Three fields across a diameter were photographed using a camera attached to the fluorescence microscope and counted manually using Image J software. The activation of caspase-3 in CGNs was examined using a caspase inhibitor (DEVD-FMK) conjugated to fluorescein-isothiocyanate, as described in detail by Giordano et al. (2007).

Assay of reactive oxygen species (ROS) formation

ROS formation was determined by fluorescence using 2',7'-dichlorofluorescein diacetate (DCFH₂-DA), as previously described (Giordano et al. 2006; 2008). DCFH₂-DA is readily taken up by cells and is subsequently de-esterified to DCFH₂, which can be oxidized to dichlorofluorescein (DCF) by hydrogen peroxide, peroxyxynitrite, and other ROS/reactive nitrogen species. Cells were washed with Locke's buffer and treated with BDE-47 in Locke's buffer for 1 hour at 37°C. After 40 min, DCFH₂-DA diluted from a stock solution in DMSO in Locke's solution was added to measure the level of ROS formation. After 20 min incubation in DCFH₂-DA, cells were then washed twice with Locke's buffer to remove extracellular DCFH₂-DA, and fluorescence was immediately read at excitation 488 nm, emission, 525 nm using a fluorescent microplate reader.

Measurement of Lipid Peroxidation

Lipid peroxidation, a major indicator of oxidative stress, was measured by the Thiobarbituric Acid Reactive Substances (TBARS) assay, with a commercial kit used according to manufacturer's instructions, as described by Giordano et al. (2006). TBARS measure the changes in levels of malondialdehyde (MDA), a natural byproduct of lipid peroxidation, which, upon binding to thiobarbituric acid forms a fluorescent red adduct. After exposure to BDE-47 and wash out, cells were scraped with 1% SDS lysis buffer (pH 7.4) and aliquots were removed to determine protein content. Butylhydroxytoluene (1 µM), an antioxidant, was added to the lysis buffer to prevent sample oxidation, and cells were centrifuged at 3000 × g for 10 min to remove large particles, before proceeding according to the TBARS assay protocol.

In vivo exposure of mice to BDE-47

For *in vivo* exposure to BDE-47, postnatal day (PND) 10 *Gclm*^{+/+} and *Gclm*^{-/-} male mice (previously genotyped on PND 3), were treated by gavage with BDE-47, dissolved in corn oil, at the dose of 10 mg/kg. Control animals were given corn oil only by gavage (1 ml/kg). The dose was selected on the basis of previous studies showing developmental neurotoxicity upon single postnatal exposures (Eriksson et al. 2001; Viberg et al. 2003; Dingemans et al. 2007; Gee and Moser, 2008; Gee et al. 2011). On PND 11 (24 h after exposure) mice were sacrificed and cerebellum and liver were collected for biochemical assays and measurement of BDE-47 levels. Blood was also collected for measurements of T₃ and T₄ levels and of BDE-47 levels.

Ex-vivo measurements oxidative stress and neurotoxicity

A number of measurements were carried out in cerebellum and in liver tissue after *in vivo* administration of BDE-47. The end-points tested *ex vivo* matched those studied in the *in vitro* experiments, to verify that our *in vitro* findings correlated with *in vivo* exposure. Lipid peroxidation was measured by the TBARS assay as described above. Protein carbonyls were measured using the spectrophotometric method described by Levine et al. (1994). We found this method to be more reliable than the use of the Oxyblot™ Protein Oxidation Detection Kit, particularly in *ex-vivo* tissues. 8-Isoprostane levels were quantified using an enzyme immunoassay (EIA) kit following a multistep purification protocol. In brief, following

ethanol and alkaline treatments, the homogenates were passed through a Sep-Pak C18 cartridges, and the eluate was assayed with the EIA protocol. The antibody provided in this kit was generated against 8-isoprostane, whose usefulness as a marker of oxidative damage has been reported (Milatovic et al. 2011). Caspase-3 activation was measured as an index of apoptotic cell death, as BDE-47 has been shown to cause caspase-dependent apoptosis (He et al. 2009; Zhang et al. 2013). Caspase-3 activation was determined using rabbit anti-cleaved caspase-3 and anti-pro-caspase-3 antibodies, as described in detail by Young and Olney (2006).

Measurements of serum thyroid hormone levels

Thyroid hormone levels were measured in serum. Blood was collected after decapitation and allowed to clot in 15 ml tubes at room temperature for 4 hr. Tubes were then placed at 4 °C overnight to allow the clot to retract. Serum was obtained via centrifugation of clotted samples and stored at -20 °C for later analyses. Total serum thyroxine (T4), free serum thyroxine (fT4), total serum triiodothyronine (T3) and free serum triiodothyronine (fT3) were measured using ELISA kits according to the manufacturer's instructions.

Measurement of tissue BDE-47 levels

Levels of BDE-47 were measured in cerebellum, liver and blood of *Gclm*^{+/+} mice using a magnetic particle immunoassay for PBDEs, which has been shown to exhibit high sensitivity and precision for detection of PBDEs in animal samples (Shelver et al. 2008). Unfortunately, only one measurement could be done in *Gclm*^{-/-} mice. The ELISA kit was used according to manufacturer's instructions, and tissue was prepared as described in Huang et al. (2010).

Statistical Analysis

Data are expressed as the mean ± SD of at least three independent experiments. IC₅₀ values were calculated from a concentration-response curve with 4–5 concentrations of BDE-47 (GraphPad Prism Software), using a non-linear regression with a sigmoidal fit model. *In vivo* results represent the mean (SD) of 4–5 male pups/group. In the *in vivo* experiments each animal of the same treatment group was from a different litter, and the litter was thus the statistical unit. All statistical analyses were carried out by one way ANOVA followed by Bonferroni's multiple comparison tests.

Results

In vitro effects of BDE-47

The toxicity of BDE-47 was first determined *in vitro* in CGNs and cerebellar astrocytes from *Gclm*^{+/+} and *Gclm*^{-/-} mice. IC₅₀ values were determined for two end-points, cell viability, as measured by the MTT assay, and apoptosis, using Hoechst nuclear staining. BDE-47 toxicity was higher in CGNs than in astrocytes, and in both cell types, it was higher in *Gclm*^{-/-} mice than in *Gclm*^{+/+} mice (Table 1). The IC₅₀ values for both the MTT assay and the Hoechst staining within each genotype were essentially the same, suggesting that BDE-47-induced cytotoxicity can be ascribed to an apoptotic process.

As both neurons and astrocytes are present *in vivo*, we sought to determine the effect of co-culturing CGNs with astrocytes on susceptibility to BDE-47 toxicity. As shown in Table 2, in *Gclm*^{+/+} cells, the addition of astrocytes to the CGN cultures increased the IC₅₀ by 4.1-fold (from 12.3 to 50.8 μM), significantly protecting the CGNs from BDE-47 toxicity. In contrast, co-culturing astrocytes and CGNs from *Gclm*^{-/-} mice provided only minimal protection, increasing the IC₅₀ from 2.5 to 5.7 μM, a 2.2-fold increase. The protective effect observed in *Gclm*^{+/+} CGNs after co-culture with astrocytes is likely due to the ability of astrocytes to increase GSH levels in neurons (Giordano et al. 2009). Further support for this conclusion is provided by the experiments showing that astrocytes from *Gclm*^{+/+} mice were protective for *Gclm*^{-/-} neurons, while *Gclm*^{-/-} astrocytes did not afford protection to *Gclm*^{+/+} CGNs (Table 2). In these co-culture experiments, we also measured the toxicity of BDE-47 in astrocytes. The IC₅₀ values for BDE-47 were essentially the same as in Table 1, and the presence of CGNs of either genotype had no influence on the susceptibility of astrocytes (not shown).

These initial findings support the hypothesis that the neurotoxicity of BDE-47 is modulated by intracellular GSH, and may thus involve oxidative stress (Giordano et al. 2008; Costa et al. 2010; Huang et al. 2010). To test this, we directly assessed the ability of BDE-47 to induce oxidative stress in CGNs of *Gclm*^{+/+} and *Gclm*^{-/-} mice, by measuring the generation of reactive oxygen species (ROS) and levels of lipid peroxidation. As shown in Fig. 1, BDE-47 (5 μM) increased ROS levels, and these effects were greater in CGNs from *Gclm*^{-/-} mice. Similar results were obtained when MDA levels were measured as an index of lipid peroxidation (Fig. 1). To further test an oxidative stress mode of cell death induced by BDE-47, we also determined the effects of two antioxidants, GSHEE and melatonin, on BDE-47-induced cell death, assessed by the MTT assay and by measuring caspase 3–7 activation as an index of apoptosis (Fig. 2). Both antioxidants antagonized the decrease in cell viability and the increase in caspase 3–7 induced by BDE-47 in CGNs from both *Gclm*^{+/+} and *Gclm*^{-/-} mice (Fig. 2). Altogether, these *in vitro* studies indicate that BDE-47 induced apoptotic cell death in neurons and astrocytes, which is due to oxidative stress and is modulated by intracellular GSH.

In vivo effects of BDE-47

Experiments *in vivo* confirmed these findings and provided evidence of a direct effect of BDE-47 on the CNS. Male *Gclm*^{+/+} and *Gclm*^{-/-} mice were treated by gavage with BDE-47 on PND 10, according to a dosing protocol utilized by various research groups and one that has been shown to cause long-lasting behavioral and biochemical effects (Eriksson et al. 2001; Dingemans et al. 2007; Gee and Moser, 2008; Gee et al. 2011). Mice were sacrificed after 24 h for a number of biochemical assays. Oxidative stress was assessed in the cerebellum and for comparison in liver, by measuring levels of MDA (Fig. 3A), of 8-isoprostane (Fig. 3B) and of reactive protein carbonyls (Fig. 3C). Results of all three end-points indicate that acute exposure to BDE-47 increased oxidative stress in both cerebellum and liver, and that this increase was higher in tissues from *Gclm*^{-/-} mice (Fig. 3). To confirm that apoptotic mechanisms observed *in vitro* were also seen *in vivo*, we also measured levels of cleaved caspase-3 and of pro-caspase-3 in cerebellum and liver of mice of both genotypes 24 h following an acute exposure to 10 mg/kg BDE-47. Fig. 4 shows that

administration of the flame retardant caused activation of caspase-3, and that the effect was more pronounced in *Gclm*^{-/-} mice.

BDE-47 levels were measured in cerebellum, as well as liver and blood of *Gclm*^{+/+} mice 24 h after oral BDE-47 administration (Table 3). Levels in cerebellum were 336 ng/g of tissue, equivalent to a concentration of approximately 690 nM. Levels in the liver were higher (1068 ng/g, ~2.2 μM), while levels of BDE-47 in blood were 155 ng/ml (~318 nM). A single measurement of BDE-47 in liver of *Gclm*^{-/-} mice suggests that there may be no significant differences in BDE-47 levels between mice of the two genotypes (Table 3).

As PBDEs have been shown to affect thyroid hormone homeostasis, levels of T₃ and T₄ were measured in serum. The results presented in Table 4 indicate that the exposure protocol utilized in the present *in vivo* studies did not cause any change in serum levels of thyroid hormones (total or free T₃ or T₄) 24 h after BDE-47 administration, confirming a previous observation (Gee et al. 2008); however, possible changes at later time-points cannot be ruled out.

Discussion

Results of the present study confirm and expand previous findings on the effects of PBDEs *in vitro* and show that the same effects also occur after *in vivo* administration. We confirmed that BDE-47 causes cell death in brain cells, and that apoptosis appears to be a major type of cell death. By using different cell types (neurons and astrocytes) isolated from *Gclm*^{+/+} and *Gclm*^{-/-} mice, we also showed that the potency of BDE-47 in causing cell death is associated with cellular GSH levels. Furthermore, co-culture of *Gclm*^{+/+} CGNs with cerebellar astrocytes afforded protection against BDE-47 toxicity, likely by increasing GSH levels in CGNs. Indeed, we had shown that *Gclm*^{+/+} astrocytes could increase GSH levels in CGNs of either genotype, while *Gclm*^{-/-} astrocytes could not (Giordano et al. 2009). We also directly showed that BDE-47 caused oxidative stress in CGNs, and that two different antioxidants protect neurons against apoptotic cell death. Altogether, these *in vitro* experiments indicate that BDE-47 induces apoptotic cell death in brain cell which likely results from oxidative stress. While some of these findings are confirmatory of those obtained with BDE-47 or other PBDEs (He et al. 2008; Giordano et al. 2008; 2009; Tagliaferri et al. 2010; Huang et al. 2010), altogether they serve as important background information for the *in vivo* studies.

The *in vivo* protocol involved a single oral administration of BDE-47 (10 mg/kg, per os) to mouse pups on PND 10. This protocol has been extensively utilized in the study of several PBDEs (Eriksson et al. 2001; Viberg et al. 2003; Dingemans et al. 2007; Gee and Moser, 2008; Gee et al. 2011). Administration of BDE-47 caused oxidative stress in cerebellum and liver, as evidenced by an increase in MDA, 8-isoprostane, and protein carbonyl levels 24 h after administration (Fig. 3). As predicted by the *in vitro* data, the effects of BDE-47 were more pronounced in *Gclm*^{-/-} mice, presumably because of their lower GSH content. Levels of protein carbonyls were also measured in cerebral cortex and hippocampus, and in both brain regions increases in oxidative stress markers were found; in *Gclm*^{+/+} mice protein carbonyls increased by 2.1- and 1.9-fold in cortex and hippocampus, respectively, while in

Gclm^{-/-} mice the increases were 2.9- and 3.0-fold, respectively (n=4; p<0.05).

Administration of BDE-47 also increased levels of cleaved caspase 3, an index of induction of apoptosis, while levels of pro-caspase 3 were reduced. Again as predicted, the effects of BDE-47 were more pronounced in *Gclm*^{-/-} mice (Fig. 4).

The developing brain is particularly sensitive to oxidative stress, possibly due to its richness in free iron, high levels of unsaturated lipids and limited antioxidant capacity (Sola et al. 2007). The relevant role of oxidative stress in contributing to pathological apoptosis in the developing brain, and to developmental brain disorders in general, has also been underscored (Blomgren et al. 2007; Hayashi, 2009). DNA is a cellular target of oxidation processes, and BDE-47-induced DNA damage has been shown to be mostly secondary to oxidative stress (He et al. 2008; Pellacani et al. 2012). In turn, oxidative stress-induced DNA damage may affect neurodevelopmental processes through multiple mechanisms, such as alterations in gene transcription (Wells et al. 2009; 2010). The exact molecular mechanisms by which BDE-47 (as well as other PBDEs) induces oxidative stress have not been fully defined. However, numerous findings suggest that disruption of mitochondrial function may be one potential mechanism. PBDEs have been shown to accumulate in mitochondria, and the relative order in which each congener accumulates in neuronal mitochondria has been shown to be identical to that of their relative potency in inducing oxidative stress and apoptosis (Huang et al. 2010). In the case of BDE-47, more than 30% was found in the mitochondria upon *in vitro* exposure (Huang et al. 2010), whereas only 11% of BDE-209, the congener least effective at inducing apoptosis, partitioned to the mitochondria (Huang et al. 2010). Additionally, BDE-47 and its metabolite 6-OH-BDE-47 are capable of inhibiting mitochondrial respiration (Shao et al. 2008; Marcinek et al., unpublished). Various PBDEs have been shown to uncouple and inhibit electron transport leading to disrupted energy production and increased oxidative stress (Pazin et al. 2015). In addition, BDE-47 and particularly 6-OH-BDE-47 disrupt calcium homeostasis (Dingemans et al. 2010), which may trigger mitochondrial dysfunction and oxidative stress (Orrenius et al. 2011). In this regard, it may be of interest measuring levels of 6-OH-BDE-47 in brain upon *in vivo* exposure to BDE-47.

As discussed earlier, disruption of thyroid hormone homeostasis may play a role in PBDE developmental neurotoxicity; this is based on the important role exerted by these hormones on brain development and the significant consequences of their perturbation (Zoeller and Crofton, 2005). However, a lack of correlation between PBDE exposure and T₄ or TSH blood levels was reported in several epidemiological studies (Mazdai et al. 2003; Chevrier et al. 2011; Kim et al. 2012; Shy et al. 2012), and adverse developmental effects of PBDEs in animals have been observed in the absence of significant thyroid hormone alterations (Branchi et al. 2005; Gee et al. 2008; He et al. 2011). The lack of changes in T₃ and T₄ seen in the present study is in agreement with these studies, and particularly with those of Gee et al. (2008) and Gee and Moser (2008) which utilized the same protocol of exposure to BDE-47. These findings suggest that the adverse effects of PBDEs on the developing CNS may also occur independently of disruption of thyroid hormone homeostasis.

Levels of BDE-47 were measured in various tissues of *Gclm*^{+/+} mice (Table 3). Additional measurements in cerebral cortex and hippocampus provided values (ng/g tissue) of 227 ± 41

(n=3) and 295 (n=1), respectively. Only one measurement could be done in *Gclm*^{-/-} mice (Table 3). Though limited, this result suggests that there may be no significant differences between the two mouse genotypes. Staskal et al. (2006) investigated the toxicokinetics of BDE-47 in mice upon oral administration of 1 mg/kg on PND 10. The highest BDE-47 levels were found in adipose tissue, and in all tissues levels were higher than in adult mice upon the same exposure. In whole brain, the highest levels were reached between 3 and 8 h post-dosing, and at 24 h levels of 28 ng/g tissue were found, comparable to those obtained in the present study after administration of our 10-fold higher dose. Similarly, in liver and blood Staskal et al. (2006) found levels of BDE-47 of 151 and 45 ng/g of tissue, respectively, also comparable to those found in the present study upon administration of a higher dose. In various studies, brain levels of PBDEs up to 950 nM have been reported (Cheng et al. 2009; Ta et al. 2011; Koenig et al. 2012), while in humans BDE-47 levels of 41 nM and 10–20 nM in brain and blood, respectively, have been found (Woods et al. 2012; Toms et al. 2009). Thus, the effects found in the present *in vivo* experiments were observed at PBDE levels that are one order of magnitude higher than those observed in humans (toddlers). However, the following issues should be considered: 1) the observed effects were quite severe, as oxidative stress and apoptotic cell death were increased by several fold (Figs. 3 and 4); 2) exposure to more than a single PBDE congener may lead to increased, synergistic toxicity (Tagliaferri et al. 2010); 3) certain individuals may be particularly susceptible to the developmental neurotoxicity of PBDEs because of genetic polymorphisms leading to diminished antioxidant cellular defense (Nakamura et al. 2002; Dalton et al. 2004), as evidenced by our results with *Gclm*^{-/-} mice.

Acknowledgments

The study was supported in part by grants R21ES022611 and P30ES007033 from the National Institute of Environmental Health Sciences. We thank Dr. G. Giordano for his help in these experiments.

References

- Blomgren K, Leist M, Groc L. Pathological apoptosis in the developing brain. *Apoptosis*. 2007; 12:993–1010. [PubMed: 17453164]
- Branchi I, Capone F, Vitalone A, Madia F, Santucci D, Alleva E, Costa LG. Early developmental exposure to BDE 99 or Aroclor 1254 affects neurobehavioral profile: interference from the administration route. *Neurotoxicology*. 2005; 26:183–192. [PubMed: 15713339]
- Chao HR, Tsou TC, Huang HL, Chang-Chien GP. Levels of breast milk PBDEs from Southern Taiwan and their potential impact on neurodevelopment. *Pediatr Res*. 2011; 70:596–600. [PubMed: 21857391]
- Chen Y, Shertzer HG, Schneider SN, Nebert DW, Dalton TP. Glutamate cysteine ligase catalysis: dependence on ATP and modifier subunit for regulation of tissue glutathione levels. *J Biol Chem*. 2005; 280:33766–33774. [PubMed: 16081425]
- Cheng J, Gu J, Ma J, Chen X, Zhang M, Wang W. Neurobehavioral effects, redox responses and tissue distribution in rat offspring developmental exposure to BDE-99. *Chemosphere*. 2009; 75:963–968. [PubMed: 19203780]
- Chevrier J, Harley KG, Bradman A, Sjodin A, Eskenazi B. Prenatal exposure to polybrominated diphenyl ether flame retardants and neonatal thyroid-stimulating hormone levels in the CHAMACOS study. *Am J Epidemiol*. 2011; 174:1166–1174. [PubMed: 21984658]
- Costa LG, Giordano G. Developmental neurotoxicity of polybrominated diphenyl ether (PBDE) flame retardants. *Neurotoxicology*. 2007; 28:1047–1067. [PubMed: 17904639]

- Costa LG, Giordano G, Tagliaferri S, Caglieri A, Mutti A. Polybrominated diphenylether flame retardants: environmental contamination, human body burden and potential adverse health effects. *Acta Biomed.* 2008; 79:172–183. [PubMed: 19260376]
- Costa, LG.; Huang, SC.; Giordano, G. Oxidative stress as a potential mechanism for developmental neurotoxicity of polybrominated diphenyl ether (PBDE) flame retardants. In: Merlani, PB., editor. *Flame Retardants: Functions, Properties and Safety*. Nova Science Publishers; New York: 2010. p. 101-115.
- Costa LG, de Laat R, Tagliaferri S, Pellacani C. A mechanistic view of polybrominated diphenyl ether (PBDE) developmental neurotoxicity. *Toxicol Lett.* 2014; 230:282–294. [PubMed: 24270005]
- Dalton TP, Chen Y, Schneider SN, Nebert DW, Shertzer HG. Genetically altered mice to evaluate glutathione homeostasis in health and disease. *Free Rad Med Biol.* 2004; 37:1511–1526.
- Dingemans MML, Ramakers GMJ, Gardoni F, van Kleef RGDM, Bergman A, Di Luca M, van den Berg M, Westerink RHS, Vijveberg HPM. Neonatal exposure to brominated flame retardant BDE-47 reduces long-term potentiation and postsynaptic protein levels in mouse hippocampus. *Environ Health Perspect.* 2007; 115:865–870. [PubMed: 17589592]
- Dingemans MML, van den Berg M, Bergman A, Westerink RHS. Calcium-related processes involved in the inhibition of depolarization-evoked calcium increase by hydroxylated PBDEs in PC12 cells. *Toxicol Sci.* 2010; 114:302–309. [PubMed: 20044592]
- Eriksson P, Jakobsson E, Fredriksson A. Brominated flame retardants: a novel class of developmental neurotoxicants in our environment? *Environ Health Perspect.* 2001; 109:903–908. [PubMed: 11673118]
- Eskenaazi B, Chevrier J, Rauch SA, Kogut K, Harley KG, Johnson C, Trujillo C, Sjodin A, Bradman A. *In utero* and childhood polybrominated diphenyl ether (PBDE) exposures and neurodevelopment in the CHAMACOS study. *Environ Health Perspect.* 2013; 121:257–262. [PubMed: 23154064]
- Gee JR, Moser VC. Acute postnatal exposure to brominated diphenylether 47 delays neuromotor ontogeny and alters motor activity in mice. *Neurotoxicol Teratol.* 2008; 30:79–87. [PubMed: 18166377]
- Gee JR, Hedge JM, Moser VC. Lack of hormone alterations following exposure to polybrominated diphenylether 47 during a period of rapid brain development in mice. *Drug Chem Toxicol.* 2008; 31:243–254.
- Gee JR, Moser VC, McDaniel KL, Herr DW. Neurochemical changes following a single dose of polybrominated diphenyl ether 47 in mice. *Drug Chem Toxicol.* 2011; 34:213–219. [PubMed: 21314472]
- Giordano G, White CC, MacConnachie LA, Fernandez C, Kavanagh TJ, Costa LG. Neurotoxicity of domoic acid in cerebellar granule neurons in a genetic model of glutathione deficiency. *Mol Pharmacol.* 2006; 70:2116–2126. [PubMed: 17000861]
- Giordano G, White CC, Mohar I, Kavanagh TJ, Costa LG. Glutathione levels modulate domoic acid-induced apoptosis in cerebellar granule cells. *Toxicol Sci.* 2007; 100:433–444. [PubMed: 17804861]
- Giordano G, Kavanagh TJ, Costa LG. Neurotoxicity of a polybrominated diphenyl ether mixture (DE-71) in mouse neurons and astrocytes is modulated by intracellular glutathione. *Toxicol Appl Pharmacol.* 2008; 232:161–168. [PubMed: 18656495]
- Giordano G, Kavanagh TJ, Costa LG. Mouse cerebellar astrocytes protect cerebellar granule neurons against toxicity of the polybrominated diphenyl ether (PBDE) mixture DE-71. *Neurotoxicology.* 2009; 30:326–329. [PubMed: 19150461]
- Hayashi M. Oxidative stress in developmental brain disorders. *Neuropathology.* 2009; 25:1–8. [PubMed: 19154320]
- He P, He W, Wang A, Xia T, Xu B, Chen X. PBDE-47 induced oxidative stress, DNA damage and apoptosis in primary cultured rat hippocampal neurons. *Neurotoxicology.* 2008; 29:124–129. [PubMed: 18054389]
- He P, Wang AG, Xia T, Gao P, Niu Q, Guo LJ, Xu BY, Chen XM. Mechanism of the neurotoxic effect of PBDE-47 and interaction of PBDE-47 and PCB153 in enhancing toxicity in SH-SY5Y cells. *Neurotoxicology.* 2009; 30:10–15. [PubMed: 19022289]

- He P, Wang A, Niu Q, Guo L, Xia T, Chen X. Toxic effects of PBDE-47 on thyroid development, learning and memory, and the interaction between PBDE-47 and PCB 153 that enhances toxicity in rats. *Toxicol Ind Health*. 2011; 27:279–288. [PubMed: 20947653]
- Herbstman JB, Sjodin A, Apelberg BJ, Witter FR, Halden RU, Patterson DG, Panny SR, Needham LL, Goldman LR. Birth delivery mode modifies the associations between prenatal polychlorinated biphenyl (PCB) and polybrominated diphenyl ether (PBDE) and neonatal thyroid hormone levels. *Environ Health Perspect*. 2008; 116:1376–1382. [PubMed: 18941581]
- Herbstman JB, Sjodin A, Kurzon M, Lederman SA, Jones RS, Rauh V, Needham LL, Tang D, Niedwiecki M, Wang RY, Perera F. Prenatal exposure to PBDEs and neurodevelopment. *Environ Health Perspect*. 2010; 118:712–719. [PubMed: 20056561]
- Hoffman K, Adgent M, Goldman BD, Sjodin A, Daniels JL. Lactational exposure to polybrominated diphenyl ethers and its relation to social and emotional development among toddlers. *Environ Health Perspect*. 2012; 120:1438–1442. [PubMed: 22814209]
- Huang SC, Giordano G, Costa LG. Comparative cytotoxicity and intracellular accumulation of five polybrominated diphenyl ether congeners in mouse cerebellar granule neurons. *Toxicol Sci*. 2010; 114:124–132. [PubMed: 19969594]
- Kim UJ, Kim MY, Hong YH, Lee DH, Oh JE. Assessment of impact of internal exposure to PBDEs on human thyroid function-comparison between congenital hypothyroidism and normal paired blood. *Environ Sci Technol*. 2012; 46:6261–6268. [PubMed: 22578177]
- Koenig CM, Lango J, Pessah IN, Berman RF. Maternal transfer of BDE-47 to offspring and neurobehavioral development in C57BL/6J mice. *Neurotoxicol Teratol*. 2012; 34:571–580. [PubMed: 23022914]
- Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol*. 1994; 233:346–357. [PubMed: 8015469]
- Madia F, Giordano G, Fattori V, Vitalone A, Branchi I, Capone F, Costa LG. Differential in vitro neurotoxicity of the flame retardant PBDE-99 and of the PCB Aroclor 1254 in human astrocytoma cells. *Toxicol Lett*. 2004; 154:11–21. [PubMed: 15475174]
- Mazdai A, Dodder NG, Abernathy MP, Hites RA, Bigsby RM. Polybrominated diphenyl ether in maternal and fetal blood samples. *Environ Health Perspect*. 2003; 111:1249–1252. [PubMed: 12842781]
- Milatovic D, Montine TJ, Aschner M. Measurement of isoprostanes as markers of oxidative stress. *Methods Mol Biol*. 2011; 758:195–204. [PubMed: 21815067]
- Nakamura S, Kugiyama K, Sugiyama S, Miyamoto S, Koide S, Fukushima H, et al. Polymorphism in the 5'-flanking region of human glutamate-cysteine ligase modifier subunit gene is associated with myocardial infarction. *Circulation*. 2002; 105:2968–2973. [PubMed: 12081989]
- Orrenius S, Nicotera P, Zhivotovsky B. Cell death mechanisms and their implications in toxicology. *Toxicol Sci*. 2011; 119:3–19. [PubMed: 20829425]
- Pazin M, Pereira LC, Dorta DJ. Toxicity of brominated flame retardants, BDE-47 and BDE-99 stems from impaired mitochondrial bioenergetics. *Toxicol Mech Methods*. 2015; 25:34–41. [PubMed: 25299509]
- Pellacani C, Buschini A, Galati S, Mussi F, Franzoni S, Costa LG. Evaluation of DNA damage induced by two polybrominated diphenyl ether flame retardants (BDE-47 and BDE-209) in SK-N-MC cells. *Int J Toxicol*. 2012; 31:372–379. [PubMed: 22710639]
- Shelver WL, Parrotta CD, Slavecki R, Li QX, Ikonomou MG, Barcelo D, Lacorte S, Rubio FM. Development of a magnetic particle immunoassay for polybrominated diphenyl ether and application to environmental and food matrices. *Chemosphere*. 2008; 73 (Suppl):S18–S23. [PubMed: 18471861]
- Shy CG, Huang HL, Chao HR, Chang-Chien GP. Cord blood levels of thyroid hormones and IGF-1 weakly correlated with breast milk levels of PBDEs in Taiwan. *Int J Hyg Environ Health*. 2012; 215:345–351. [PubMed: 22088798]
- Sola A, Rogido MR, Deulofeut R. Oxygen as a neonatal health hazard: call for detente in clinical practice. *Acta Paediatrica*. 2007; 96:801–812. [PubMed: 17537007]
- Staskal DF, Diliberto JJ, Birnbaum LS. Disposition of BDE 47 in developing mice. *Toxicol Sci*. 2006; 90:309–316. [PubMed: 16407092]

- Ta TA, Koenig CM, Golub MS, Pessah IN, Qi L, Aronov PA, Berman RF. Bioaccumulation and behavioral effects of 2,2',4,4',-tetrabromodiphenyl ether (BDE-47) in perinatally exposed mice. *Neurotoxicol Teratol.* 2011; 33:393–404. [PubMed: 21334437]
- Tagliaferri S, Caglieri A, Goldoni M, Pinelli S, Alinovi R, Poli D, Pellacani C, Giordano G, Mutti A, Costa LG. Low concentrations of the brominated flame retardants BDE-47 and BDE-99 induce synergistic oxidative stress-mediated neurotoxicity in human neuroblastoma cells. *Toxicol In Vitro.* 2010; 24:116–122. [PubMed: 19720130]
- Toms LM, Sjodin A, Harden F, Hobson P, Jones R, Edenfield E, Mueller JF. Serum polybrominated diphenyl ether (PBDE) levels are higher in children (2–5 years of age) than in infants and adults. *Environ Health Perspect.* 2009; 117:1461–1465. [PubMed: 19750114]
- USEPA (United States Environmental Protection Agency). An Exposure Assessment of Polybrominated Diphenyl Ethers. USEPA; Washington DC: 2010. p. 378
- Viberg H, Fredriksson A, Eriksson P. Neonatal exposure to polybrominated diphenyl ether (PBDE 153) disrupts spontaneous behavior, impairs learning and memory, and decreases hippocampal cholinergic receptors in adult mice. *Toxicol Appl Pharmacol.* 2003; 192:95–106. [PubMed: 14550744]
- Wells PG, McCallum GP, Chen CS, Henderson JT, Lee CJ, Perstin TJ, Wiley MJ, Wong AW. Oxidative stress in developmental origin of disease: teratogenesis, neurodevelopmental deficits, and cancer. *Toxicol Sci.* 2009; 108:4–18. [PubMed: 19126598]
- Wells PG, McCallum GP, Lam KC, Henderson JT, Ondovcik SL. Oxidative DNA damage and repair in teratogenesis and neurodevelopmental deficits. *Birth Def Res.* 2010; 90:103–109.
- Williams AL, DeSesso JM. The potential of selected brominated flame retardants to affect neurological development. *J Toxicol Environ Health, pt B.* 2010; 13:411–448.
- Woods R, Vallero RO, Golub MS, Suarez JK, Ta TA, Yasui DH, Chi LH, Kostyniak PJ, Pessah IN, Berman RF, LaSalle J. Long-lived epigenetic interactions between perinatal PBDE exposure and *Mecp2*³⁰² mutation. *Hum Mol Genet.* 2012; 21:2399–2411. [PubMed: 22343140]
- Young C, Olney JW. Neuroapoptosis in the infant mouse brain triggered by a transient small increase in blood alcohol concentration. *Neurobiol Dis.* 2006; 22:548–554. [PubMed: 16459096]
- Zhang S, Kuang G, Zhao G, Wu X, Zhang C, Lei R, Xia T, Chen J, Wang Z, Ma R, Li B, Yang L, Wang A. Involvement of the mitochondrial p53 pathway in PBDE-47-induced SH-SY5Y cells apoptosis and its underlying activation mechanism. *Food Chem Toxicol.* 2013; 62:699–706. [PubMed: 24140468]
- Zhou T, Taylor MM, DeVito MJ, Crofton KM. Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. *Toxicol Sci.* 2002; 66:105–116. [PubMed: 11861977]
- Zoeller RT, Crofton KM. Mode of action: developmental thyroid hormone insufficiency -Neurological abnormalities resulting from exposure to propylthiouracil. *Crit Rev Toxicol.* 2005; 35:771–781. [PubMed: 16417044]

Highlights

- The brominated flame retardant BDE-47 is shown to cause oxidative stress and ensuing apoptotic cell death of mouse cerebellar granule neurons
- The same effects are seen in cerebellum and liver after *in vivo* administration of BDE-47 to mice, in the absence of any alteration of thyroid hormone levels
- The effects of BDE-47 are seen at levels slightly higher than those found in humans, and are more pronounced in mice with low glutathione levels

Fig. 1. BDE-47 induces oxidative stress in CGNs

CGNs from *Gclm*^{+/+} or *Gclm*^{-/-} mice were treated with BDE-47 (5 μ M) for 30 min (for ROS measurements, left panel) or 12 h (for MDA measurements, right panel). Results are expressed as percent of control *Gclm*^{+/+} mice, and represent the mean ($\pm$ SD) of three separate experiments carried out in duplicate. *Significantly different from control, $p < 0.01$; ^aSignificantly different from *Gclm*^{+/+}, $p < 0.05$.

Fig. 2. Effects of antioxidants on BDE-47-induced cytotoxicity in CGNs

CGNs from *Gclm*^{+/+} and *Gclm*^{-/-} mice were treated for 24 h (Fig. 2A, top panel) or 12 h (Fig. 2B, bottom panel) with BDE-47 (5 μ M) in the absence or presence of GSHEE (2.5 mM) or melatonin (200 μ M) (both added 30 minutes prior to BDE-47). Endpoints measured were cell viability (by the MTT assay; Fig. 2A) or caspase 3–7 activation (Fig. 2B). Results represent the mean ($\pm$ SD) of three separate experiments carried out in duplicate. Significantly different from the respective BDE-47 group, * p <0.05, ** p <0.01. Effects of BDE-47 are significantly different from control (p <0.05 or <0.01).

Fig. 3. Oxidative stress induced in cerebellum and liver by *in vivo* administration of BDE-47
Male mice (one/group/litter) were dosed orally with BDE-47 (10 mg/kg) or corn oil on PND 10 and sacrificed after 24 h. Oxidative stress was measured in cerebellum or liver by assessing lipid peroxidation (Fig. 3A, top panel), levels of 8-isoprostane (Fig. 3B, center panel) or reactive protein carbonyls (Fig. 3C, bottom panel). Results represent the mean ($\pm$ SD) of 4–5 animals. *Significantly different from control, $p < 0.05$; ^aSignificantly different from *Gclm*^{+/+} mice, $p < 0.05$.

Fig. 4. Effect of in vivo BDE-47 administration on cleaved caspase-3 and pro-caspase-3 levels
Male mice (one/group/litter) were dosed orally with BDE-47 (10 mg/kg) or corn oil on PND 10 and sacrificed after 24 h. Levels of cleaved caspase-3 and of pro-caspase-3 in cerebellum and liver are shown. Results represent the mean ($\pm$ SD) of 4–5 animals. *Significantly different from control, $p < 0.05$; ^aSignificantly different from *Gclm*^{+/+} mice, $p < 0.05$.

Table 1

Neurotoxicity of BDE-47 in CGNs and in cerebellar astrocytes

	<i>Gclm</i> ^{+/+}		<i>Gclm</i> ^{-/-}	
	MTT	Hoechst	MTT	Hoechst
CGNs	11.0 ± 3.5	9.6 ± 3.4	2.2 ± 1.3	2.0 ± 1.1
GSH	12.4 ± 1.4		2.4 ± 1.1	
Cb. Astrocytes	52.6 ± 5.5*	45.9 ± 4.1*	19.7 ± 1.8*	18.5 ± 2.5*
GSH	19.3 ± 1.6		4.3 ± 1.0	

Shown are IC₅₀ values (± SD) in uM, resulting from three separate cytotoxicity (MTT) or apoptosis (Hoechst) experiments using 4–5 concentrations of BDE-47. CGNs and cerebellar astrocytes were obtained from PND-7 pups (mixed-gender) of the *Gclm*^{+/+} and *Gclm*^{-/-} genotype. Levels of GSH (in nmol/mg protein) are from Giordano et al. (2008) and were obtained from experiments obtained under the same experimental conditions as the current study (n=4).

* Significantly different from IC₅₀ in CGNs, p<0.001. All IC₅₀ values in *Gclm*^{-/-} mice are significantly different from the respective values in *Gclm*^{+/+} mice.

Table 2

Neurotoxicity of BDE-47 in CGNs co-cultured with cerebellar astrocytes

Astrocytes	CGNs	IC50 (uM)	GSH (nmol/mg protein)
None	<i>Gclm</i> ^{+/+}	12.3 ± 3.1	13.5 ± 1.0
<i>Gclm</i> ^{+/+}	<i>Gclm</i> ^{+/+}	50.8 ± 5.1 **	17.9 ± 1.1 *
<i>Gclm</i> ^{-/-}	<i>Gclm</i> ^{+/+}	18.4 ± 2.6 *	15.2 ± 1.4
None	<i>Gclm</i> ^{-/-}	2.5 ± 0.8	2.6 ± 0.7
<i>Gclm</i> ^{+/+}	<i>Gclm</i> ^{-/-}	22.9 ± 3.0 **	8.3 ± 1.1 **
<i>Gclm</i> ^{-/-}	<i>Gclm</i> ^{-/-}	5.7 ± 1.3 *	3.8 ± 0.8

Values represent IC₅₀ in the MTT assay, derived from concentration-response curves obtained with 3–4 concentrations of BDE-47, and are the means (± SD) of three separate determinations. Data for GSH levels in CGNs are from Giordano et al. (2009) and were obtained under the same experimental conditions as this study (n=3). Significantly different from the respective CGNs alone,

*
p<0.01;

**
p<0.001.

Table 3

Tissue levels of BDE-47

	Gclm^{+/+}	Gclm^{-/-}
Cerebellum	336 ± 51	N.D.
Liver	1068 ± 247	1024
Blood	155 ± 12	N.D.

Levels of BDE-47 in tissues were measured in *Gclm*^{+/+} or *Gclm*^{-/-} mice 24 h after a single oral administration of BDE-47 (10 mg/kg on PND 10), as described in Methods. Results are expressed as ng/g tissue, and represent the mean (± SD) of three animals (one animal for the *Gclm*^{-/-} mice) from separate litters. N.D. = not determined (see Methods for additional details).

Table 4

Serum thyroid hormone levels in mice treated with BDE-47

	<i>Gclm</i> ^{+/+}		<i>Gclm</i> ^{-/-}	
	Control	BDE-47	Control	BDE-47
tT ₃	4.3 ± 0.7	4.6 ± 0.6	4.0 ± 0.3	4.5 ± 0.5
fT ₃	0.2 ± 0.06	0.2 ± 0.05	0.25 ± 0.06	0.23 ± 0.04
tT ₄	84.9 ± 3.5	80.5 ± 2.6	78.3 ± 4.9	79.6 ± 2.7
fT ₄	1.6 ± 0.2	1.5 ± 0.6	1.3 ± 0.4	1.4 ± 0.1

Mice were treated with BDE-47 (10 mg/kg, per os) on PND 10 and levels of total (t) and free (f) T₃ and T₄ were measured in serum after 24 h using a commercial ELISA kit. Results are expressed in ng/ml and represent the mean (± SD) of three animals/group, each from a different litter.