Published in final edited form as:

Neurotoxicol Teratol. 2013 ; 37: 13–17. doi:10.1016/j.ntt.2013.02.001.

BDE99 (2,2',4,4',5-PENTABROMODIPHENYL ETHER) SUPPRESSES DIFFERENTIATION INTO NEUROTRANSMITTER PHENOTYPES IN PC12 CELLS

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Abstract

Early-life exposures to brominated diphenyl ethers (BDEs) lead to neurobehavioral abnormalities later in life. Although these agents are thyroid disruptors, it is not clear whether this mechanism alone accounts for the adverse effects. We evaluated the impact of 2,2',4,4',5-pentabromodiphenyl ether (BDE99) on PC12 cells undergoing neurodifferentiation, contrasting the effects with chlorpyrifos, a known developmental neurotoxicant. BDE99 elicited decrements in the number of cells, evidenced by a reduction in DNA levels, to a lesser extent than did chlorpyrifos. This did not reflect cytotoxicity from oxidative stress, since cell enlargement, monitored by the total protein/DNA ratio, was not only unimpaired by BDE99, but was actually enhanced. Importantly, BDE99 impaired neurodifferentiation into both the dopamine and acetylcholine neurotransmitter phenotypes. The cholinergic phenotype was affected to a greater extent, so that neurotransmitter fate was diverted away from acetylcholine and toward dopamine. Chlorpyrifos produced the same imbalance, but through a different underlying mechanism, promoting dopaminergic development at the expense of cholinergic development. In our earlier work, we did not find these effects with BDE47, a BDE that has greater endocrine disrupting and cytotoxic effects than BDE99. Thus, our results point to interference with neurodifferentiation by specific BDE congeners, distinct from cytotoxic or endocrine mechanisms.

Keywords

Acetylcholine; BDE99; Brominated flame retardants; Dopamine; Neurodifferentiation; PC12 cells

INTRODUCTION

There is increasing concern over the adverse developmental effects of brominated diphenyl ethers (BDEs) used commonly as flame retardants, especially in light of their bioaccumulation and persistence (Branchi et al., 2003; Dingemans et al., 2011). Animal

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Conflict of interest statement: TAS has provided expert witness testimony in the past three years at the behest of the following law firms: The Calwell Practice (Charleston WV), Finnegan Henderson Farabow Garrett & Dunner (Washington DC), Carter Law (Peoria IL), Gutglass Erickson Bonville & Larson (Madison WI), The Killino Firm (Philadelphia PA), Alexander Hawes (San Jose, CA), Pardieck Law (Seymour, IN), Tummel & Casso (Edinburg, TX) and the Shanahan Law Group (Raleigh NC).

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studies show conclusively that early-life exposure to these agents leads to persistent behavioral alterations in adulthood (Branchi et al., 2003; Viberg and Eriksson, 2011; Viberg et al., 2005); further, there is a significant correlation between prenatal BDE levels in maternal or cord blood and neurodevelopmental sequelae in children (Herbstman et al., 2010; Roze et al., 2009). Although a definitive mechanism has yet to be defined for the effects of BDEs on brain development, there are several prominent possibilities. First, these agents are known disruptors of thyroid function, including thyroid hormonal control of neurotrophic factors (Blanco et al., 2011; Branchi et al., 2005; Schreiber et al., 2010); thyroid disruption involves the same critical developmental stage over which brain development is vulnerable to BDEs (Eriksson et al., 2002). Second, the BDEs can evoke cytotoxicity via oxidative stress, both in vivo (Belles et al., 2010) and in vitro (Costa and Giordano, 2007; Huang et al., 2010). Third, they affect calcium fluxes and other cell signaling cascades that are essential both to neuronal function and development (Costa and Giordano, 2007; Dingemans et al., 2010a, b, 2011; Hendriks et al., 2010). Importantly, these effects are shared in varying degrees by multiple members of the BDE class.

In the current study, whether BDEs act directly to impair neurodifferentiation, irrespective of endocrine actions or shared properties as cytotoxic oxidative stressors. We focused on 2,2',4,4',5-penta-bromodiphenyl ether (BDE99), the congener most commonly found in human milk (Norén and Meironyté, 2000), specifically in contrast to our earlier work on 2,2',4,4'-tetra-bromodiphenyl ether (BDE47) (Dishaw et al., 2011). These two BDEs share the mechanisms of endocrine disruption, oxidative stress leading to toxicity, and cell signaling effects, in each case with actions of BDE47 or its metabolites similar to, or more prominent than, those of BDE99 (Dingemans et al., 2010a, b, 2011; Hendriks et al., 2010; Huang et al., 2010; Schreiber et al., 2010; Tagliaferri et al., 2010).

Here, we show that BDE99 directly affects neurodifferentiation in PC12 cells, completely distinct from BDE47, which is ineffective in this model (Dishaw et al., 2011). The PC12 cell line is a well-characterized model for neurodifferentiation, and we used protocols established specifically for the screening of developmental neurotoxicants (Qiao et al., 2001, 2003 2005; Slotkin et al., 2007a, b, 2008; Song et al., 1998), in keeping with recommendations by the Inspector General of the U.S. Environmental Protection Agency (U.S. Environmental Protection Agency, 2006). We evaluated indices of cell number, cell growth, neurite formation and differentiation into the dopaminergic (TH) and cholinergic (ChAT) phenotypes that are the distinctive fate of PC12 cells. To provide a perspective on the effects of BDE99, we included comparable measurements for chlorpyrifos, an organophosphate pesticide that is a known developmental neurotoxicant and that has been well-characterized in the PC12 cell line (Das and Barone, 1999; Dishaw et al., 2011; Lassiter et al., 2009; Qiao et al., 2005; Slotkin et al., 2007b; Slotkin and Seidler, 2010; Song et al., 1998).

MATERIALS AND METHODS

Cell cultures

Because of the clonal instability of the PC12 cell line (Fujita et al., 1989), the experiments were performed on cells that had undergone fewer than five passages. As described previously (Qiao et al., 2003; Song et al., 1998), PC12 cells (American Type Culture Collection CRL-1721, obtained from the Duke Comprehensive Cancer Center, Durham, NC) were seeded onto poly-D-lysine-coated plates in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% horse serum (Sigma), 5% fetal bovine serum (Sigma), and 50 µg/ml penicillin streptomycin (Invitrogen, Carlsbad, CA). Incubations were carried out with 5% CO₂ at 37°C, standard conditions for PC12 cells. To initiate neurodifferentiation (Jameson et al., 2006b; Slotkin et al., 2007b; Teng and Greene, 1994),

the medium was changed to include 50 ng/ml of 2.5 S murine nerve growth factor (NGF; Promega Corporation, Madison, WI); each culture was examined under a microscope to verify the outgrowth of neurites after NGF treatment.

Toxicant exposures were all commenced simultaneously with the addition of NGF, so as to be present throughout neurodifferentiation. There were five different treatment groups (final concentrations shown): control, BDE99 (AccuStandard Inc, New Haven, CT) in concentrations of 10, 20 and 50 μ M, and 50 μ M chlorpyrifos (Chem Service, West Chester, PA). The medium was changed every 48 hr with the continued inclusion of NGF and each toxicant for a total exposure time of 6 days. Because of their limited water solubility, the test agents were dissolved in dimethylsulfoxide (Sigma; final concentration 0.1%), which was also added to the controls; this concentration of dimethylsulfoxide has no effect on PC12 cell growth or differentiation (Qiao et al., 2001; Song et al., 1998).

Assays

Cells were harvested, washed, and the DNA and protein fractions were isolated and analyzed as described previously (Slotkin et al., 2007b). Measurements of DNA, total protein and membrane protein were used as biomarkers for cell number, cell growth, and neurite growth (Qiao et al., 2003; Song et al., 1998). Neuronotypic cells contain a single nucleus, so that the DNA content per dish provides a measure of cell number (Winick and Noble, 1965). Since the DNA per cell is constant, cell growth entails an obligatory increase in the total protein per cell (protein/DNA ratio) as well as membrane protein per cell (membrane protein/DNA ratio). If cell growth represents simply an increase in the perikaryal area, then the ratio of membrane to total protein would fall in parallel with the decline in the surface-to-volume ratio (volume increases with the cube of the perikaryal radius, whereas surface area increases with the square of the radius); however, when neurites are formed as a consequence of neurodifferentiation, this produces a specific *rise* in the ratio. Each of these biomarkers has been validated in prior studies by direct measurement of cell number (Powers et al., 2010; Roy et al., 2005), perikaryal area (Roy et al., 2005) and neurite formation (Das and Barone, 1999; Howard et al., 2005; Song et al., 1998).

To assess neurodifferentiation into dopamine and acetylcholine phenotypes, we assayed the activities of tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT), respectively (Jameson et al., 2006a, b). TH activity was measured using [14 C]tyrosine as a substrate and trapping the evolved 14 CO₂ after decarboxylation coupled to L-aromatic amino acid decarboxylase. Each assay contained 55 μ M [1- 14 C]-L-tyrosine (Moravek Biochemicals, Brea, CA; specific activity, 51 mCi/mmol, diluted to 3.3 mCi/mmol with unlabeled tyrosine) as substrate and activity was calculated as pmol synthesized per hour per μ g DNA (i.e. activity per cell). ChAT assays were utilized a substrate of 50 μ M [14 C]acetyl-coenzyme A (specific activity 60 mCi/mmol; PerkinElmer Life Sciences, Waltham, MA). Labeled acetylcholine was extracted and activity calculated on the same basis as for TH.

Data analysis

All studies were performed using 3–5 separate batches of cells, with multiple independent cultures for each treatment in each batch; each batch of cells comprised a separately prepared, frozen and thawed passage. Results are presented as mean $\pm$ SE, with treatment comparisons carried out by ANOVA (data log-transformed when variance was heterogeneous), followed by Fisher's Protected Least Significant Difference Test for post-hoc pairwise comparisons of individual treatments. First, we conducted a two-factor ANOVA (factors of treatment and cell batch) and found that the treatment effects were the same across the different batches of cells, although the absolute values differed from batch

to batch. Accordingly, we normalized the results across batches prior to combining them for presentation. Significance was assumed at $p < 0.05$.

RESULTS AND DISCUSSION

Exposure of differentiating PC12 cells to BDE99 produced a significant reduction in the number of cells, as evidenced by a decline in DNA content (Fig. 1A). The highest concentration of BDE99 (50 μ M) was less deleterious than the same concentration of chlorpyrifos. The decline in DNA was not secondary to cytotoxicity, since cell growth was unimpaired by BDE99 (Fig. 1B); in fact, the total protein/DNA ratio was significantly *increased* at all concentrations, indicating that cell enlargement was occurring simultaneously with the reduction in cell numbers. For the growth parameter, even the lowest BDE99 concentration was as effective as 50 μ M chlorpyrifos. Cell enlargement was further confirmed by measuring the membrane protein/DNA ratio (Fig. 1C). Again, all BDE99 concentrations produced a significant increment over the control value but in this case, only the highest concentration had effects that were comparable to those of chlorpyrifos. In turn, this implied that there were selective effects on membrane protein that were not shared by total protein. Since neurite formation is a major contributor to membrane protein, we then evaluated the membrane/total protein ratio. The lowest concentration of BDE99 suppressed this index, an effect that was lost at the higher concentrations; this effect was repeated in two entirely separate batches of cells conducted in independent experiments. A similar nonmonotonic relationship has been found previously for chlorpyrifos, which enhances the formation of dendritic neurites at the expense of longer-length projections at low concentrations, but suppresses neurite formation at higher concentrations (Axelrad et al., 2003; Das and Barone, 1999; Flaskos et al., 2011; Howard et al., 2005; Yang et al., 2008).

We then evaluated the effects of BDE99 on neurodifferentiation into the dopaminergic and cholinergic neurotransmitter phenotypes that are characteristic of PC12 cells. BDE99 suppressed the appearance of both TH (Fig. 2A) and ChAT (Fig. 2B) but again, the concentration-effect relationship was complex. For TH, the reduction was small, only 10%, but the effect was already maximal at the lowest BDE99 concentration. In contrast, the effect of ChAT showed a monotonic decline, eventually achieving a much larger effect, about a 30% decrement. Accordingly, at the highest concentration, there was a switch away from the cholinergic and toward the dopaminergic phenotype, as evidenced by an increase in the TH/ChAT ratio (Fig. 2C). The effects of BDE99 on TH were distinct from that of chlorpyrifos, which evoked an increase instead of a decrease (Fig. 2A), and BDE99 produced a larger decrement in ChAT than did chlorpyrifos at the same 50 μ M concentration (Fig. 2B); consequently, both BDE99 and chlorpyrifos increased the TH/ChAT ratio (Fig. 2C), but from different underlying mechanisms.

Our results indicate that BDE99 has two distinct effects on neurodifferentiation. It produces cell loss, not through cytotoxicity, but rather in association with promotion of cell growth, implying that it accelerates the transition from cell replication to cell enlargement that occurs early in neurodifferentiation (Song et al., 1998; Teng and Greene, 1994). At the same time, though, it suppresses key elements of neurodifferentiation in a complex manner. Neurite formation is reduced at low concentrations only, indicating a nonmonotonic response curve resembling that of the organophosphates. Perhaps more importantly, BDE99 suppresses the emergence of neurotransmitter phenotypes, with different concentration effects for dopamine vs. acetylcholine. The fact that the cholinergic phenotype eventually shows the largest deficits is entirely in keeping with the neurobehavioral effects of this flame retardant after early-life exposures in vivo, which likewise show strong cholinergic components (Fischer et al., 2008; Viberg et al., 2005). Equally important, in our earlier

work, we showed that BDE47 was ineffective against cell numbers, growth or neurodifferentiation in this model (Dishaw et al., 2011), so the effects of BDE99 cannot reflect their shared properties as thyroid disruptors or oxidative stressors. Indeed, the pattern of effects seen here for BDE99 also differ from those associated with known oxidative stressors in the PC12 model (Lassiter et al., 2009; Qiao et al., 2005; Slotkin et al., 2007b; Slotkin and Seidler, 2010). Accordingly, the present findings point to the likelihood that BDE99 acts as a developmental neurotoxicant by targeting neurodifferentiation *directly* in neuronal cells, independently of its other potential actions or on endocrine or other systemic effects. Since these effects are not shared by BDE47, manipulating specific structural characteristics of BDEs could enable the design of congeners with less propensity to disrupt nervous system development.

Acknowledgments

Research was supported by NIH ES010356.

Abbreviations

ANOVA	analysis of variance
BDE	brominated diphenyl ether
BDE47	2,2',4,4'-tetra-bromodiphenyl ether
BDE99	2,2',4,4',5-penta-bromodiphenyl ether
ChAT	choline acetyltransferase
NGF	nerve growth factor
TH	tyrosine hydroxylase

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Highlights

- BDE99 reduced cell numbers while enhancing cell growth in PC12 cells
- BDE99 impaired neurodifferentiation into acetylcholine and dopamine phenotypes
- Effects were not shared by BDE47, which is a greater endocrine disruptor
- Specific BDE congeners directly interfere with neurodifferentiation

Figure 1.

Effects of BDE99 and chlorpyrifos (CPF) on cell growth parameters: (A) DNA concentration, (B) total protein/DNA ratio, (C) membrane protein/DNA ratio, (D) membrane protein/total protein. Data represent means and standard errors of the number of determinations in parentheses. ANOVA across all treatments is shown at the top of each panel and pairwise comparisons are shown within the panels by asterisks (significance difference vs. control) and daggers (significant difference between 50 μM chlorpyrifos and 50 μM BDE99). Abbreviation: CPF = chlorpyrifos.

Figure 2.

Effects of BDE99 and chlorpyrifos (CPF) on neurodifferentiation into dopaminergic and cholinergic phenotypes: (A) tyrosine hydroxylase activity (TH), (B) choline acetyltransferase activity (ChAT), (C) TH/ChAT ratio. Data represent means and standard errors of the number of determinations in parentheses. ANOVA across all treatments is shown at the top of each panel and pairwise comparisons are shown within the panels by asterisks (significance difference vs. control) and daggers (significant difference between 50 µM chlorpyrifos and 50 µM BDE99). Abbreviation: CPF = chlorpyrifos.