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Brominated and Organophosphate Flame Retardants Target Different Neurodevelopmental Stages, Characterized with Embryonic Neural Stem Cells and Neuronotypic PC12 Cells

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Abstract

In addition to their activity as endocrine disruptors, brominated and organophosphate flame retardants are suspected to be developmental neurotoxicants, although identifying their specific mechanisms for that activity has been elusive. In the current study, we evaluated the effects of several flame retardants on neurodifferentiation using two in vitro models that assess distinct “decision nodes” in neural cell development: embryonic rat neural stem cells (NSCs), which evaluate the origination of neurons and glia from precursors, and rat neuronotypic PC12 cells, which characterize a later stage where cells committed to a neuronal phenotype undergo neurite outgrowth and neurotransmitter specification. In NSCs, both brominated and organophosphate flame retardants diverted the phenotype in favor of glia and away from formation of neurons, leading to an increased glia/neuron ratio, a common hallmark of the in vivo effects of neurotoxicants. For this early decision node, the brominated flame retardants were far more potent than the organophosphates. In PC12 cells, the brominated flame retardants were far less effective, whereas tris (1,3-dichloro-2-propyl) phosphate, an organophosphate, was more effective. Thus, the two classes of flame retardants differentially impact the two distinct vulnerable periods of neurodifferentiation. Furthermore, the effects on neurodifferentiation were separable from outright cytotoxicity, an important requirement in establishing a specific effect of these agents on neural cell development. These results reinforce the likelihood that flame retardants act as developmental neurotoxicants via direct effects on neural cell differentiation, over and above other activities that can impact nervous system development, such as endocrine disruption.

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Keywords

Brominated flame retardants; Neural stem cells; Neurodifferentiation; Organophosphate flame retardants; PC12 cells

1. INTRODUCTION

The use of flame retardants in various consumer products and building materials (e.g. upholstered furniture, insulation and electronics) has led to ubiquitous human exposures to these compounds. Polybrominated flame retardants were one class of flame retardants that were phased out because of their persistence and bioaccumulation, along with evidence that they act as endocrine disruptors and developmental neurotoxicants (Dishaw et al. 2014; Hendriks and Westerink 2015). Indeed, epidemiological studies clearly point to neurodevelopmental defects in children exposed to these agents (Chen et al. 2014; Eskenazi et al. 2013; Herbstman et al. 2010). They are being replaced increasingly by organophosphate flame retardants, despite the fact that these, too, are likely to target endocrine function and brain development (Behl et al. 2015; Dishaw et al. 2014; Hendriks and Westerink 2015; Ren et al. 2016; Stapleton et al. 2011, 2012; van der Veen and de Boer 2012). In zebrafish, both brominated and organophosphate flame retardants impair neurobehavioral function after developmental exposures in the nM to μ M range (Noyes et al. 2015) and parallel effects have been demonstrated in mammalian species (Costa and Giordano 2007; Dingemans et al. 2011; Viberg and Eriksson 2011; Viberg et al. 2005). In vitro models of neural cell development confirm that both classes of flame retardants have the ability to disrupt neurodifferentiation, suggesting a direct impact on nervous system development rather than indirect effects mediated on embryonic or maternal metabolism or endocrine function (Behl et al. 2015; Dishaw et al. 2011; Schreiber et al. 2010; Slotkin et al. 2013). In keeping with that interpretation, 2,2',4,4'-tetrabromodiphenyl ether (BDE47) and its bioactive metabolite, 6OH-BDE47 were found to have opposite effects on a developmental neurobehavioral battery in zebrafish, despite the fact that they both produce thyroid disruption (Oliveri et al. 2015); likewise, 2,2',4,4',5-penta-bromodiphenyl ether (BDE99) was active, but BDE47 inactive, in an in vitro model of neurodifferentiation (Dishaw et al. 2011; Slotkin et al. 2013), notwithstanding their shared endocrine properties.

If flame retardants act directly as developmental neurotoxicants, then it should be possible to demonstrate a selective effect of these agents on neural cell proliferation, survival, and/or neurodifferentiation. Studies of the emergence of neural phenotypes in embryonic stem cells readily demonstrate adverse effects, but the point-of-departure concentrations are not separable from those of outright cytotoxicity (Behl et al. 2015), and thus do not provide a convincing case for specific effects on neurodevelopment. It is important to note, though, that embryonic stem cells are not the same as neural stem cells, which have already made a commitment to a neural phenotype; indeed, toxicant effects on embryonic stem cells can differ substantially from those of neural stem cells, as shown with a standard developmental neurotoxicant, such as nicotine (Berger et al. 1998; Culbreth et al. 2012; Ishizuka et al. 2012; Liszewski et al. 2012; Slotkin et al. 2016). Furthermore, the choice of endpoints for neurodifferentiation is critically important. As just one example, neurite outgrowth, a

standard biomarker for neurodifferentiation, is a property shared by both neurons and astroglia. Toxicants that target the neuronal phenotype typically produce glial “scarring,” where neurons are replaced by glia (O’Callaghan 1993); thus, overall neurite outgrowth may be unaffected despite major shifts in the underlying cellular makeup.

A clear demonstration of direct effects of flame retardants on neurodifferentiation thus requires models where the cells are committed specifically to a neural phenotype and that distinguishes between the impact on the subsequent emergence of neurons vs. glia. We recently described the use of neural stem cells (NSCs) to examine how toxicants divert neural fate toward or away from neuronal and glial phenotypes, with effects clearly distinguishable from those of general cytotoxicity (Slotkin et al. 2016, 2017); for that purpose, we used NSCs derived from rat neuroepithelium on embryonic day 14, when phenotypic separation into neurons and glia is determined (Slotkin et al. 2016, 2017). In addition to this early “decision node,” we also examined a later node, characterized by effects on neuronotypic PC12 cells, which are derived from the same species (rat). These cells are already committed to a neuronal phenotype and their subsequent neurodifferentiation involves the switchover from growth by cell replication to cell enlargement, extension of neuritic projections and selection of the dopamine or acetylcholine neurotransmitter phenotype (Teng and Greene 1994). The PC12 model has been used in thousands of studies to evaluate many developmental neurotoxicants, including several brominated and organophosphate flame retardants (Dishaw et al. 2011, 2013). Here, we demonstrate that both classes of flame retardants have a direct effect on neurodifferentiation separable from general cytotoxicity, and further, that brominated flame retardants target primarily the early decision node (emergence of neuronal vs. glial phenotype) whereas organophosphate flame retardants preferentially target the later node (neurite outgrowth, neurotransmitter specification).

2. MATERIALS AND METHODS

2.1. NSC cultures and treatments

The techniques for NSC preparation, culturing and assays have appeared previously (Slotkin et al. 2016). Primary neural stem cells (passage zero; MTI-GlobalStem, Gaithersburg, MD) were isolated from rat cortical neuroepithelium on embryonic day 14 and were frozen in DMEM/F-12 medium with N2 supplement and 10% dimethylsulfoxide (DMSO). Cells were thawed and plated at 35,000 cells/cm² on 12 mm coverslips pre-coated with poly-L-ornithine (Sigma-Aldrich, St. Louis, MO), contained in 24-well culture plates. The culture medium consisted of DMEM/F-12/GlutaMAX™ (Thermo Fisher Scientific, Waltham, MA). with N2 Supplement and 20 ng/ml human fibroblast growth factor (both from MTI-GlobalStem), and 20 ng/ml epidermal growth factor (Sigma-Aldrich). Cultures were maintained in a humidified incubator at 37° C with 5% CO₂. Twenty-four hours later, the medium was changed to initiate spontaneous differentiation by eliminating the two growth factors, with the addition of 200 µM ascorbic acid and the test compounds: BDE47 (AccuStandard, New Haven, CT), 6OH-BDE47 (AccuStandard), BDE99 (AccuStandard), tetrabromobisphenol A (TBBPA; Sigma-Aldrich) or tris (1,3-dichloro-2-propyl) phosphate (TDCPP; Sigma-Aldrich). Structures of these compounds are shown in Figure 1. Because of

the limited water solubility of some of the flame retardants, all test substances were dissolved in DMSO (Sigma-Aldrich). The maximal final concentration of DMSO in the culture medium was 0.05%, which we found to have no effect on any of the NSC parameters (Slotkin et al. 2016). After 3 days, half the medium was replaced, including the indicated treatment agents, and the exposures were continued for another 3 days (total exposure = 6 days).

2.2. NSC assays

At the end of the exposure period, the medium was removed and the coverslips washed with Dulbecco's phosphate-buffered saline, fixed with 4% paraformaldehyde and washed three times with Dulbecco's phosphate-buffered saline containing additional Ca^{2+} and Mg^{2+} . Cells were permeabilized for 30 min in phosphate buffered saline containing 0.2% Triton X-100, washed three times with phosphate buffered saline (without Triton), followed by a 30 min incubation in BlockAid™ solution. Cells expressing a neuronal or astroglial phenotype were identified by immunocytochemistry according to manufacturers' instructions, using microtubule-associated protein 2 (MAP2) for neurons and glial fibrillary acidic protein (GFAP) for astroglia. After permeabilization, the coverslips were incubated for 1 hr at room temperature using rabbit anti-MAP2 (1:200) and rat anti-GFAP (1:20) in BlockAid™. Coverslips were rinsed four times with phosphate-buffered saline and then incubated for 1 hr at room temperature with the appropriate fluor-conjugated secondary antibodies (donkey anti-rabbit IgG Alexa Fluor 647 and goat anti-rat IgG Alexa Fluor 555) diluted 1:400 in BlockAid™. After an additional five rinses with phosphate buffered saline, coverslips were incubated for 5 min with 300 nM 4',6-diamidino-2-phenylindole (DAPI) nucleic acid stain to label individual cells. Coverslips were rinsed three times with phosphate buffered saline and mounted onto glass slides using ProLong Diamond Antifade mountant. All reagents for the NSC assays were obtained from Thermo Fisher Scientific except for rabbit anti-MAP2 (EMD Millipore, Billerica MA).

Images of 3 to 4 fields/slide (each field = $3.22 \times 10^5 \mu\text{m}^2$) were captured using a Zeiss Axio Imager widefield fluorescence microscope with 200× magnification and quantified for total cells (DAPI-positive stain for nuclei); across the multiple fields in a given culture, thousands of cells were counted. Each cell was then examined to see if it expressed a neuronal phenotype (MAP2-positive) or a glial phenotype (GFAP-positive). A cell was counted as positive only when the stain for a given phenotype coincided with a DAPI-stained nucleus. Values were averaged across the fields to render a single value for each culture.

We also included a positive control group, 10 μM dexamethasone (Slotkin et al. 2016), which evokes significant reductions in cell numbers and specifically impairs emergence of the glial phenotype. The dexamethasone results are not shown here because they were used solely to validate each NSC experiment, and because they replicated the previously published effects (Slotkin et al. 2016).

Additional studies were run to assess the effects of selected agents on cell viability using trypan blue exclusion (Song et al. 1998), which assesses the integrity of cell membrane permeability. At the end of the six-day treatment period, half the medium was removed and replaced with 0.4% trypan blue (Sigma-Aldrich) in isotonic phosphate-buffered saline, and

samples were incubated for 1–2 min. The medium was aspirated and cells were rinsed twice with phosphate-buffered saline, after which they were imaged with a Zeiss Lumar.V12 stereoscope at 120×. We counted 2000–3000 cells per well.

2.3. PC12 cell cultures and treatments

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) 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. Twenty-four hours after plating, we initiated neurodifferentiation (Jameson et al. 2006b; Slotkin et al. 2007; Teng and Greene 1994) by changing the medium to include 50 ng/ml of 2.5 S murine nerve growth factor (Promega Corporation, Madison, WI). Test substances, dissolved in DMSO, were added simultaneously so as to be present throughout neurodifferentiation. Control samples contained the corresponding final concentration of DMSO vehicle (0.1%), which has no effect on PC12 cell viability or differentiation (Qiao et al. 2001, 2003; Song et al. 1998). The medium was changed every 48 hr with the continued inclusion of nerve growth factor and test substances, and continued for 6 days, parallel to the exposure used for NSCs. At the end of the 6 day exposure period, the cultures were examined under a microscope to verify the outgrowth of neurites.

2.4. PC12 cell assays

Cells were harvested, washed, and the DNA and protein fractions were isolated and analyzed as described previously (Slotkin et al. 2007). 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). Effects on cell number were determined by measuring DNA content, since each neuronotypic cell contains only a single, diploid nucleus (Winick and Noble 1965). DNA per cell is constant, so that cell growth entails an obligatory increase in total protein per cell (protein/DNA ratio) as well as in membrane protein per cell (membrane protein/DNA ratio). If cell growth represents simply an increase in the perikaryal area, then membrane protein decreases less than total protein because of 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 increase in membrane protein larger than that predicted from this simple 2/3-power geometric relationship. 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 enzymatic activities of tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT), respectively, using established techniques (Jameson et al. 2006a, b).

For the studies with PC12 cells, the positive control included in each experiment was 10 μM benzo[a]pyrene, which again is not shown because the results simply replicated previous findings (Slotkin and Seidler 2009).

2.5. Data analysis

Each study was performed using 2–6 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 preparation, and each batch was evaluated in a separate experiment conducted on a different day with fresh reagents. Results are presented as mean $\pm$ SE, with treatment comparisons carried out by analysis of variance (ANOVA) followed by Fisher's Protected Least Significant Difference Test for post-hoc comparisons of individual treatments. The initial comparisons included factors of treatment and cell batch, and in each case, we 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.

In addition to evaluating each individual set of measurements, we performed a global repeated measures ANOVA to evaluate treatment effects across all measurements that were done on the same samples, so as to avoid an increased probability of type I errors that could arise from multiple tests of the same cultures. The data were log-transformed because of heterogeneous variance among the different types of measurements.

Significance for all tests was assumed at $p < 0.05$ (two-tailed).

3. RESULTS

3.1. BDE47

In NSCs, BDE47 reduced cell numbers with a threshold concentration of 5 μM , declining to 10% of control values at 10 μM (Figure 2A). By themselves, these reductions do not necessarily connote cytotoxicity. NSCs are still undergoing active cell replication, increasing in numbers by 7–9-fold over the six day test period (Slotkin et al. 2016); consequently, the reductions could also reflect direct antimitotic effects or promotion of neurodifferentiation at the expense of cell replication. Accordingly, we evaluated total neurodifferentiation as well as differentiation into specific neural cell phenotypes. In contrast to the 5 μM threshold for reductions in cell numbers, BDE47 impaired overall neurodifferentiation only at the higher concentration of 10 μM (Figure 2B). However, selective reductions in the glial phenotype were already apparent beginning at 5 μM (Figure 2C). Neurons appeared to be less sensitive, showing no significant impairment at 5 μM but a robust effect at the higher concentration (Figure 2D). The selectivity away from differentiation into the glial phenotype was readily apparent from the reduction in the glia/neuron ratio (Figure 2E). Since cytotoxicity involves reductions in cell numbers superimposed on impairment of all neurodifferentiation phenotypes (Slotkin et al. 2016), there were thus two phases for the effects BDE47, the initial phase in which glial cell differentiation was selectively impaired, and then at slightly higher concentrations, cytotoxicity with global impairment of cell numbers and neurodifferentiation into both phenotypes.

Repeated-measures ANOVA across all the NSC measurements identified a main effect of treatment ($p < 0.0003$) and an interaction of treatment $\times$ measurement type ($p < 0.002$), thus justifying the separate, lower-order analyses for each individual parameter.

The effects of BDE47 on neurodifferentiation in PC12 cells have appeared previously (Dishaw et al. 2011); there was little or no effect on indices of cell number or neurodifferentiation up to a concentration of 50 μM . BDE47 is thus selective for NSCs as compared to PC12 cells.

3.2. 6OH-BDE47

In contrast to BDE47, 6OH-BDE47 was much more potent in eliciting reduced cell numbers in the NSC model, with major loss at 1 μM , and $>90\%$ loss at 3 μM (Figure 3A). For this congener, the impact on overall neurodifferentiation showed the same threshold as for reduced cell numbers, but the decline was already maximal by 1 μM and did not progress further at 3 μM (Figure 3B). This dichotomy was reflected in unusual dose-response relationships for neurodifferentiation phenotypes. Formation of glia was robustly enhanced by 1 μM 6OH-BDE47 (Figure 3C), so much so that the absolute number of glial cells was increased 60% above control despite the overall decline in cell numbers (control, 33 ± 2 glial cells per field; 1 μM 6OH-BDE47, 53 ± 8 , $p < 0.02$). When the concentration was raised to 3 μM , glial cells then declined substantially. The impact on differentiation into neurons also showed a biphasic curve, with inhibition at 1 μM , but a lessening of effect at 3 μM (Figure 3D). As a consequence, the glia/neuron ratio was markedly increased at the 1 μM concentration point but not at the higher concentration (Figure 3E). These results again indicate two phases of effect, in this case, characterized by selective promotion of the glial phenotype at lower concentrations, followed by cytotoxicity (impaired cell numbers, reduced neurodifferentiation into both glial and neuronal phenotypes), but with a stimulatory component of neurodifferentiation persisting even when the cytotoxicity threshold is surpassed.

Again, repeated-measures ANOVA of all the NSC determinations verified both a main treatment effect ($p < 0.05$) and a treatment $\times$ measurement type interaction ($p < 0.0001$), validating the analyses of each individual parameter.

In the PC12 cell model, 6OH-BDE47 likewise produced a reduction in cell numbers, but with a much higher threshold than for NSCs (Figure 4A). Again, this loss did not connote cytotoxicity, since the total protein/DNA ratio, an index of cell growth, actually showed significant enhancement at the same concentration (Figure 4B). The membrane protein/DNA showed an even larger increase (Figure 4C), leading to a significant rise in the ratio of membrane protein to total protein, connoting enhanced neurite formation (Figure 4D). Repeated-measures ANOVA of all four parameters verified the presence of a main treatment effect ($p < 0.0001$) and an interaction of treatment $\times$ measurement type ($p < 0.0001$). The results in this model thus resemble those seen with NSCs, namely enhanced neurodifferentiation at concentrations short of cytotoxicity.

3.3. BDE99

The effects of BDE99 on NSCs differed from those of 6OH-BDE47 in two distinct ways. First, BDE99 exhibited lower overall potency, but more interestingly, it was far more stimulatory toward selective aspects of neurodifferentiation. With BDE99, reductions in cell numbers were first apparent at a threshold concentration of 2 μ M, declining by 90% at 10–20 μ M (Figure 5A). However, the effects on total differentiation were biphasic, with small declines at 2–5 μ M but then a marked increase above control at higher concentrations (Figure 5B), an effect not seen with either of the BDE47 congeners. For the glial phenotype, BDE99 elicited robust increases starting at 5 μ M, with further augmentation at higher concentrations (Figure 5C). Even when superimposed on overall deficits in cell numbers, these increases in glia were substantial and significant in absolute terms: 40% increase in glial cell numbers at 5 μ M (control, 43 ± 2 glial cells per field; 5 μ M BDE99, 60 ± 4 , $p < 0.04$), 85% at 10 μ M (74 ± 7 glial cells per field, $p < 0.002$). In contrast, the effects on differentiation into the neuronal phenotype was biphasic, with decrements at 2–5 μ M but then a rebound at 10–20 μ M (Figure 5D). Accordingly, the glia/neuron ratio showed clear increases at concentrations of 5 μ M and above (Figure 5E). The repeated-measures ANOVA verified the main treatment effect ($p < 0.0001$) and the interaction of treatment $\times$ measurement type ($p < 0.0001$).

The effects of BDE99 on neurodifferentiation in PC12 cells have appeared previously (Slotkin et al. 2013). Concentrations as low as 10 μ M impaired neurite formation and neurodifferentiation into dopamine or acetylcholine phenotypes, while also decreasing cell numbers. These effects did not represent cytotoxicity, since the same concentrations enhanced overall cell growth. Notably, then, the direction of effect for neurodifferentiation in PC12 cells (inhibition) was opposite to the effects seen here in NSCs (stimulation).

3.4. TBBPA

The dichotomy between effects on cell numbers and neurodifferentiation was also apparent for TBBPA. Cell numbers declined with a threshold concentration of 0.5 μ M, reaching a nadir by 5 μ M (Figure 6A). However, effects on total neurodifferentiation were much less prominent, with only minor effects up to 2 μ M and then a precipitous decline at 5 μ M indicative of cytotoxicity (Figure 6B). The biphasic nature of the dose-response relationship was readily apparent for emergence of the glial phenotype (Figure 6C). Concentrations of 1–2 μ M elicited significant enhancement of glial cell formation but differentiation was totally arrested at 5 μ M. Again, the increases at the lower concentrations reflected higher absolute numbers of glial cells even when superimposed on overall reductions in cell numbers: 32% increase at 1 μ M (control, 40 ± 2 glial cells per field; 1 μ M TBBPA, 53 ± 3 , $p < 0.0008$) and 68% increase at 2 μ M (67 ± 6 cells per field, $p < 0.0001$). In contrast, differentiation into the neuronal phenotype showed only monotonic decreases paralleling cell loss (Figure 6D). The combined impact on the two phenotypes thus produced a robust rise in the glia/neuron ratio at 1–2 μ M and then a complete collapse at 5 μ M with the onset of cytotoxicity (Figure 6E). The separation of the individual parameters was justified by a significant main treatment effect ($p < 0.0001$) and a treatment $\times$ measurement type interaction ($p < 0.0001$) in a repeated-measures ANOVA combining all parameters.

Given the robust effects of TBBPA on NSCs, we were surprised to find little or no effect of this flame retardant on nearly all aspects of PC12 cell neurodifferentiation. There was no significant impact on indices of cell numbers (Figure 7A), cell size (Figure 7B), or neurite formation (Figures 7C, 7D). The lack of effect was confirmed by the repeated-measures ANOVA across these four parameters, which did not identify significance for either a main treatment effect or an interaction of treatment $\times$ measurement type. For the neurotransmitter endpoints, differentiation into the dopamine phenotype was unaffected even up to a concentration of 50 μ M (Figure 7E) but we did find slight, but significant impairment of differentiation into the acetylcholine phenotype at the highest concentration of TBBPA (Figure 7F). These two determinations were conducted on different cell cultures from those used for DNA, total and membrane protein, so a separate repeated-measures ANOVA was run for the neurotransmitter phenotypes. This confirmed the overall significance and selectivity of treatment effects on ChAT: $p < 0.0008$ for the main treatment effect, $p < 0.0001$ for the treatment $\times$ phenotype interaction.

3.5. TDCPP

Unlike the brominated flame retardants, TDCPP exposure in NSCs had only minor effects on cell numbers (Figure 8A) or total differentiation (Figure 8B), with small, but significant impairment emerging at a threshold concentration of 20 μ M. Superimposed on this overall effect, differentiation into the glial phenotype was slightly enhanced (Figure 8C) and neuronal differentiation somewhat reduced (Figure 8D). The combined effect once again produced a robust increase in the glia/neuron ratio (Figure 8E). Repeated-measures ANOVA confirmed the selective effect of treatment on specific parameters (treatment $\times$ measurement type, $p < 0.0001$).

The effects of TDCPP and related organophosphate flame retardants on neurodifferentiation in PC12 cells have appeared previously (Dishaw et al. 2011). TDCPP enhanced neurodifferentiation into dopamine and acetylcholine phenotypes, with a threshold effect at 10 μ M. Higher concentrations also displayed antimetabolic activity, as would be expected from a switch from cell proliferation to neurodifferentiation. Thus, TDCPP showed greater potency in PC12 cells as compared to NSCs, the opposite sensitivity pattern from that of the brominated flame retardants.

3.6. Effects on NSC viability

In light of the overlap between reductions in cell number and the biphasic effects of several of the agents on NSC neurodifferentiation, we used trypan blue penetration of the cell membrane to determine whether these effects were associated with loss of viability as a potential prelude to cytotoxicity. We selected three conditions that reduced total NSC cell numbers but enhanced the absolute number of glial cells: 1 μ M 6OH-BDE47, 5 μ M BDE99, and 1 μ M TBBPA, evaluated in two separate batches of cells with 14–15 independent samples per treatment. The effects on viability were small ($\approx 5\%$) but statistically significant for two of the agents: control, $75 \pm 1\%$ unstained cells; 6OH-BDE47 $70 \pm 1\%$ ($p < 0.0002$), BDE99 $74 \pm 1\%$ (NS), TBBPA $69 \pm 1\%$ ($p < 0.0001$).

4. DISCUSSION

Our results provide a clear demonstration that both brominated and organophosphate flame retardants target key events in neurodifferentiation, providing an underlying, direct mechanism for developmental neurotoxicity. Superimposed on this main finding, we identified three key characteristics. First, the effects on neurodifferentiation were clearly separable from cytotoxicity in both the NSC and PC12 models; the coexistence of two distinct dose relationships (one for effects on neurodifferentiation, one for cytotoxicity) produced biphasic dose-response curves for many of the compounds. Second, both classes of flame retardants resulted in replacement of neurons by glia, evidenced by a rise in the glia/neuron ratio, a standard feature of the *in vivo* effects of developmental neurotoxicants (O'Callaghan 1993). Third, the brominated flame retardants primarily targeted the earlier stage of neurodifferentiation where cells commit to a neuronal or glial phenotype, whereas the organophosphate compound, TDCPP, acted primarily at the second decision node, where neurotransmitter phenotype is selected.

In the NSC model, lowered numbers of cells alone does not connote cytotoxicity, since the cells are actively dividing and increase 7–9 fold over the six day test period (Slotkin et al. 2016). Accordingly, reduced cell numbers can occur not just from outright toxicity, but also from antimitotic effects or from promotion of the switchover from cell replication to neurodifferentiation. Purely cytotoxic compounds, such as monovalent silver or tobacco smoke extract, elicit a distinct pattern where severe reductions in cell numbers are also accompanied by impairment of differentiation into both neuronal and glial phenotypes (Slotkin et al. 2016). Using that criterion, in the current study, the potencies to elicit cytotoxicity followed a distinct hierarchy: 6OH-BDE47 (3 μ M threshold) > TBBPA (5 μ M) > BDE47 (10 μ M) > BDE99 $\approx$ TDCPP (no cytotoxicity noted up to 20 μ M). With a more sensitive criterion, increased cell membrane permeability (trypan blue penetration), the detection threshold was slightly lower for two of the agents tested (6OH-BDE47 and TBBPA, both 1 μ M) but not for a third (BDE99, no effect); notably, though, the effects on permeability were extremely small ($\approx$ 5%) at these concentrations and were statistically detectable only because of the high consistency of the values. The NSC model was more sensitive to the cytotoxic effects of the flame retardants than was the PC12 model, in which we did not find any evidence for cytotoxicity at equivalent or greater concentrations, here or in our earlier work (Dishaw et al. 2011; Slotkin et al. 2013). Modest reductions in PC12 cell numbers were not accompanied by impairment of cell growth, neurite formation or differentiation into neurotransmitter phenotypes, again indicating that these effects are not a consequence purely of cytotoxicity. Our results for the cytotoxicity endpoint thus indicate three important features: (1) the early decision node (formation of neurons and glia), evaluated with NSCs, is more sensitive than the late node (neurotransmitter specification), evaluated with PC12 cells; (2) the brominated flame retardants are generally more cytotoxic than the organophosphates; and (3) the effects on neurodifferentiation in either phase cannot be explained solely by their shared property as endocrine disruptors (Hamers et al. 2006). The latter point, which will be echoed in additional findings (below), is particularly important, since it distinguishes the direct effects on neural cell development from indirect effects such as thyroid disruption.

For actions on NSC neurodifferentiation below the point of consistent cytotoxicity, the brominated flame retardants again showed overall greater potency as compared to the organophosphate, TDCPP. However, within that overall pattern, the rank order was distinct from that of cytotoxicity, reinforcing that these are entirely separable outcomes: 6OH-BDE47 $\approx$ TBBPA > BDE99 > BDE47 > TDCPP. Furthermore, within the brominated compounds, there were distinct differences in the direction of effects on gliogenesis, with BDE47 uniquely producing a decrease whereas the other agents elicited increases at concentrations short of cytotoxicity. Indeed, with the exception of BDE47, all the flame retardants studied here increased the glia/neuron ratio, a hallmark of glial “scarring” found with most developmental neurotoxicants (O’Callaghan 1993). Our findings thus entirely parallel the opposite neurobehavioral outcomes in zebrafish exposed to BDE47 as compared to 6OH-BDE47 (Oliveri et al. 2015). Although the other flame retardants all raised the glia/neuron ratio, and all increased the formation of glia, they did not share identical cellular mechanisms directed toward neuronogenesis: 6OH-BDE47, TBBPA and TDCPP suppressed the emergence of neurons, but BDE99 had a biphasic effect (suppression at lower concentrations followed by a rebound at higher concentrations). All these findings argue against a single mechanism of action underlying the developmental neurotoxicity of brominated or organophosphate flame retardants, e.g. thyroid disruption or actions at ryanodine receptors (Chen et al. 2017).

In other cell models, the effects of flame retardants on neurodifferentiation overlap the dose-response curve for outright cytotoxicity (Behl et al. 2015) and it was thus important to establish whether that was also true for the effects on NSC neurodifferentiation seen here. If effects were solely due to cytotoxicity, then we would expect parallel, monotonic declines in total cell numbers, glia and neurons for each agent. Instead, we found biphasic effects for 6OH-BDE47, BDE99 and TBBPA. For these, treatment at lower concentrations increased the absolute numbers of glial cells even in the face of a significant reduction in total cells, an effect that would seem incompatible with purely cytotoxic actions; when the concentration was raised further, then total glial cells declined in association with larger deficits in total cell numbers, as would be expected from cytotoxicity. We therefore assessed more sensitive effects on cell permeability (trypan blue permeation) as a forerunner of cytotoxicity, to see if effects could be detected at the lower concentrations that enhanced glial cell formation. We did find a statistically significant but small ($\approx 5\%$) increase in permeability for 6OH-BDE47 and TBBPA, but not for BDE99, a strong contrast to the much larger effects on total cell numbers and glial cell numbers seen for all three agents. The small effects on permeability certainly cannot explain the 30–60% promotional effects on gliogenesis at the same concentrations, and indeed, are in the wrong direction for increases in numbers of glial cells. However, our findings are consistent with the observation that effects of some of the flame retardants on neurodifferentiation are close to, or overlap, the threshold for loss of viability, a more sensitive endpoint that could lead ultimately to cytotoxicity, but that this relationship is not consistent for all of the agents that enhance gliogenesis in the face of reduced total cells.

In the PC12 model, the impact of flame retardants was entirely different from that seen with NSCs. Notably, the organophosphate, TDCPP, was far more effective relative to all but one of the brominated flame retardants, both toward reductions in cell numbers (potency rank

order 6OH-BDE47 > TDCPP > BDE99 > BDE47 $\approx$ TBBPA) and even more so for neurodifferentiation (TDCPP $\approx$ 6OH-BDE47 > BDE99 > TBBPA > BDE47). Equally important, the direction of effect differed among the various compounds, with TDCPP and 6OH-BDE47 enhancing neurite formation and/or emergence of neurotransmitter phenotypes, whereas BDE99 and TBBPA suppressed these endpoints, and BDE47 was without effect. Again, this points to multiple underlying cellular mechanisms for the effects of flame retardants on neurodifferentiation, such that, even within the brominated class, there are distinctly different potencies and directions of effect for each agent. In particular, for BDE99 and TBBPA, the effects on neurodifferentiation were opposite in the two models, with both agents showing a promotional effect in NSCs but impairment in PC12 cells. In turn, the fact that the effects of the flame retardants are highly stage-specific points out the importance of using multiple models to evaluate a wide range of developmental periods. This conclusion is reinforced by the findings for the organophosphate, TDCPP. Unlike the brominated agents, TDCPP showed greater sensitivity for the later decision node, modeled by PC12 cells, versus the early decision node (NSC model), paralleling the pattern seen with organophosphate pesticides (Dishaw et al. 2011; Slotkin et al. 2007, 2016). This is perhaps not surprising, since the chemical structure of TDCPP resembles organophosphate pesticides rather than that of the brominated flame retardants, but it reinforces the fact that the biological activities of flame retardants reflect features totally different from the shared chemical properties that make them mutually useful as industrial products.

Finally, our results emphasize the importance of selecting appropriate cell types for in vitro modeling of developmental neurotoxicity. As already discussed, the very same flame retardants shown here to be selective developmental neurotoxicants, displayed neurotoxicity only in conjunction with nonspecific cytotoxicity in previous studies (Behl et al. 2015). The main difference is that we used embryonic *neural* stem cells, i.e. cells sufficiently advanced in differentiation to be committed to a neural phenotype; in contrast, pluripotent stem cells are clearly less sensitive to these agents. Second, we found totally distinct hierarchies for type of effect and potency rank-order when going from the NSC model to the PC12 model. These mimic two separate phases of neurodifferentiation, with NSCs representing the early decision node (formation of neurons vs. glia) and PC12 cells reflecting the later decision node, where cells already committed to a neuronal phenotype select a neurotransmitter. If we are ultimately to use in vitro models as a screen for developmental neurotoxicants, we will need to consider the fact that various agents can act at totally different phases of neural cell development. Further, it is increasingly apparent that there are sex differences in vivo for the actions of developmental neurotoxicants. The NSC model has the potential ability to evaluate sex differences, since male and female embryos can be selectively used as the cell source; this will not be feasible with standardized, transformed cell models, such as the PC12 line. In any case, it is clear that no single in vitro model will be able to recapitulate all the events in neurodifferentiation that are potential targets for neurotoxicants.

5. CONCLUSIONS

In summary, our findings support the conclusion that both brominated and organophosphate flame retardants are developmental neurotoxicants that can directly affect neurodifferentiation during two critical stages of cell development. At the first decision

node, where neural stem cells commit to a neuronal or glial phenotype, most of these agents distort the phenotype in favor of glia over neurons, leading to an increased glia/neuron ratio, a hallmark effect of neurotoxicants (O'Callaghan 1993); BDE47 was the single exception, where this relationship was the reverse (decreased glia/neuron ratio). For this developmental stage, the brominated flame retardants are far more potent than TDCPP, an organophosphate. For the second decision node, where cells that are already committed to neuronal phenotype generate neurites and select a neurotransmitter, the brominated flame retardants are far less effective, whereas the organophosphate is more effective. Thus, there are two different vulnerable periods that distinguish between the two classes of compounds. Also notably, the effects on neurodifferentiation are entirely distinct from cytotoxicity, an important requirement in establishing a specific role of these agents as disruptors of neural cell development. Our results reinforce the likelihood that flame retardants act as developmental neurotoxicants via direct effects on neural cell differentiation, over and above other activities that can impact nervous system development, such as endocrine disruption.

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Abbreviations

ANOVA	analysis of variance
BDE47	2,2,4,4'-tetrabromodiphenyl ether
BDE99	2,2',4,4',5-penta-bromodiphenyl ether
ChAT	choline acetyltransferase
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethylsulfoxide
GFAP	glial fibrillary acidic protein
MAP2	microtubule-associated protein 2
NSC	neural stem cell
TBBPA	tetrabromobisphenol A
TDCPP	tris (1,3-dichloro-2-propyl) phosphate
TH	tyrosine hydroxylase

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Figure 1.
Flame retardant structures.

BDE47 - Neural Stem Cells

Figure 2.

Effects of BDE47 on neural stem cells: (A) numbers of cells, (B) percent differentiated, (C) percent glia, (D) percent neurons, (E) glia/neuron ratio. Data represent mean $\pm$ SE of the number of determinations shown in parentheses, obtained with four separate batches of cells, with each batch contributing several independent samples per treatment. ANOVA across all treatment groups appears at the top of each panel and asterisks denote individual groups that differ significantly from the control value.

Figure 3.

Effects of 6OH-BDE47 on neural stem cells: (A) numbers of cells, (B) percent differentiated, (C) percent glia, (D) percent neurons, (E) glia/neuron ratio. Data represent mean $\pm$ SE of the number of determinations shown in parentheses, obtained with three separate batches of cells, with each batch contributing several independent samples per treatment. ANOVA across all treatment groups appears at the top of each panel and asterisks denote individual groups that differ significantly from the control value.

6OH-BDE47 - PC12 Cells

Figure 4. Effects of 6OH-BDE47 on PC12 cells: (A) DNA content, (B) total protein/DNA ratio, (C) membrane protein/DNA ratio, (D) membrane protein/total protein ratio. Data represent mean $\pm$ SE of the number of determinations shown in parentheses, obtained with two separate batches of cells, with each batch contributing several independent samples per treatment. ANOVA across all treatment groups appears at the top of each panel and asterisks denote individual groups that differ significantly from the control value.

BDE99 - Neural Stem Cells

Figure 5.

Effects of BDE99 on neural stem cells: (A) numbers of cells, (B) percent differentiated, (C) percent glia, (D) percent neurons, (E) glia/neuron ratio. Data represent mean $\pm$ SE of the number of determinations shown in parentheses, obtained with four separate batches of cells, with each batch contributing several independent samples per treatment. ANOVA across all treatment groups appears at the top of each panel and asterisks denote individual groups that differ significantly from the control value.

Figure 6.

Effects of TBBPA on neural stem cells: (A) numbers of cells, (B) percent differentiated, (C) percent glia, (D) percent neurons, (E) glia/neuron ratio. Data represent mean $\pm$ SE of the number of determinations shown in parentheses, obtained with five separate batches of cells, with each batch contributing several independent samples per treatment. ANOVA across all treatment groups appears at the top of each panel and asterisks denote individual groups that differ significantly from the control value.

TBBPA - PC12 Cells

Figure 7. Effects of TBBPA on PC12 cells: (A) DNA content, (B) total protein/DNA ratio, (C) membrane protein/DNA ratio, (D) membrane protein/total protein ratio, (E) tyrosine hydroxylase activity, (F) choline acetyltransferase activity. Data represent mean $\pm$ SE of the number of determinations shown in parentheses, obtained with six separate batches of cells for panels A–D and three batches of cells for panels E and F; each batch contributed several independent samples per treatment. ANOVA across all treatment groups appears at

the top of each panel and asterisks denote individual groups that differ significantly from the control value. Abbreviation: NS, not significant.

Figure 8.

Effects of TDCPP on neural stem cells: (A) numbers of cells, (B) percent differentiated, (C) percent glia, (D) percent neurons, (E) glia/neuron ratio. Data represent mean $\pm$ SE of the number of determinations shown in parentheses, obtained with three separate batches of cells, with each batch contributing several independent samples per treatment. ANOVA across all treatment groups appears at the top of each panel and asterisks denote individual groups that differ significantly from the control value.