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In vitro neurotoxic potential of emerging flame retardants on neuroblastoma cells in an acute exposure scenario

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ABSTRACT

Since 2004, some legacy flame retardants (FRs) were restricted or removed from the European markets due to their concern on human health. Both organophosphorus FRs (OPFRs) and novel brominated FRs (NBFRs) have replaced them because they are presumably safer and less persistent emerging FRs (EFRs) and their exposure is currently occurring in indoor environments at high levels. Little is known about the neurotoxic potential risk of these EFRs in humans. The present study was aimed at assessing the acute neurotoxicity potential of Tris(1, 3-dichloro-2-propyl)phosphate (TDCPP), triphenyl phosphate (TPhP), Bis(2-ethylhexyl)tetrabromophthalate (BEH-TEBP) and 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB) on human neuroblastoma cells (SH-SY5Y). SH-SY5Y were exposed to these EFRs at low concentrations -ranging 2.5–20 μM. during 2–24 h. We investigated viability, mitochondrial function, oxidative stress, inflammatory response, as well as neural plasticity and development. The results have demonstrated that selected EFRs (TDCPP, TPhP, EH-TBB and BEH-TBP) did not impair neural function on SH-SY5Y as acute response. To the best of our knowledge, this has been the first study focused on evaluating the neural affection of TPhP on SH-SY5Y cells and of EH-TBB and BEH-TBP on neural cells. We also assessed for the first time almost all endpoints after FR exposure on neural cell lines.

1. Introduction

Flame retardants (FRs) are chemicals added to commercial products (i.e.: electronics, furniture, and textiles) in order to inhibit or delay the spread of fire. A long list of these compounds have been used since 1970. Their physicochemical properties lead to easily release and subsequent accumulation in environmental samples, house air and dust, food, animals and even in human tissues (Li et al., 2019; Ospina et al., 2018; Saillenfait et al., 2018; Yang et al., 2020; Zhong et al., 2018). Due to their widely distribution as well as to their potential adversity for human health (Feiteiro et al., 2021; Yang et al., 2019), the European Commission and the U.S. Environmental Protection Agency (US EPA) has phase out some legacy FRs (Blum et al., 2019). Recently, presumably safer and less persistent emerging FRs (EFRs), including organophosphorus FRs (OPFRs) and novel brominated FRs (NBFRs) were released onto the market despite they have not been sufficiently investigated in terms of kinetics and toxicities. In fact, there are still a lack of available data on the physico-chemical properties, environmental persistence, bioaccumulation, and toxicity of some EFRs (Klose et al., 2021). In addition, some EFRs are included in a commercialized-mixture product FM 550, being little known about the potential toxicity of this mixture and on individual FRs. Nowadays, EFRs are confirmed to be even more prevalent in environment than legacy FRs (Dong et al., 2021; Esplugas et al., 2022; Shi et al., 2018).

Various studies have reported a variety of biological toxicities of EFRs in animals and humans (Baldwin et al., 2017; Gu et al., 2018; Sun et al., 2016), being neurotoxicity of important concern due to the capacity of EFRs (including those evaluated in the current study) to enter and damage the nerve tissue, hindering the transmission of neurotransmitters (Doherty et al., 2019; Percy et al., 2020; Yao et al., 2021).

Among OPFRs, tris(1, 3-dichloro-2-propyl)phosphate (TDCPP) and triphenyl phosphate (TPhP) are particularly of a high toxicological concern, being people potentially highly exposed to them (Bajard et al., 2019). In fact, these OPFRs are two of the most frequently detected

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OPFRs in water and even in aquatic organisms (Shi et al., 2021). On one hand, TDCPP is carcinogenic, having been removed from children's pajamas in the late 1970s. However, in recent decades, the use of this FR has significantly increased for other applications (Percy et al., 2020). In fact, TDCPP replaced PentaBDE, being one of the primary FRs now found in polyurethane foam used in furniture, sofas, chairs, vehicles, and a number of baby products (Carignan et al., 2013; Van Der Veen and De Boer, 2012). On the other hand, TPhP is one of the most commonly used OPFR, which is primarily used in polyvinyl chloride (PVC) and polycarbonate/ABS alloy (PC/ABS) plastics and polyurethane foam (He et al., 2018b). TPhP is a component of the product known as Firemaster 550 and is also used as plasticizer in some personal care products (e.g., nail polish, lubricants and lacquers), providing another potential exposure route of OPFRs hazards to the environment and human health (Young et al., 2018).

Both OPFRs are universally distributed in dust, air, water, soil, sediment and biotic samples (Blum et al., 2019; He et al., 2018b; Lee et al., 2018; Li et al., 2019; Ospina et al., 2018; Van Der Veen and De Boer, 2012; Wang et al., 2020), as well as in human body fluids, including seminal plasma, breast milk, blood plasma, placenta, and urine (Christia et al., 2018; Falandysz et al., 2022; He et al., 2018b).

The available scientific literature associates TDCPP body burdens in humans with impaired neurodevelopment (He et al., 2018a; Li et al., 2017). In animals, this FR has shown to induce acute-, nerve-, developmental, reproductive, hepatic, renal, and endocrine-disrupting toxicity (Fu et al., 2013; Liu et al., 2013; Dishaw et al., 2011; McGee et al., 2012; Meeker and Stapleton, 2010). Similar toxic effects has been reported for TPhP by promoting cardiotoxicity, genotoxicity, metabolic disruption and endocrine disruption on zebrafish, mice and rats (Du et al., 2016; Mendelsohn et al., 2016; Mitchell et al., 2018; Shi et al., 2018; Wang et al., 2018; Zhang et al., 2016a), but limited research concerns to human cohorts (Estill et al., 2021). Anyhow, neurotoxicological mechanisms of both OPFRs still need to be elucidated.

With respect to NBFRs, they are found in dust, water, soils and sediment (Covaci et al., 2011; Hassan and Shoeib, 2015; Hsu et al., 2018; Ruan et al., 2009; Wu et al., 2012a; Wu et al., 2012b; Xiong et al., 2019; Yu et al., 2016), as well as in human body (Dong et al., 2021), even in brain tissue by crossing the blood–brain barrier (Barón et al., 2015; Ruan et al., 2009). Categorization for bis(2-ethylhexyl)tetrabromophthalate (BEH-TEBP) and 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB) remains uncertain taking into account that the evidence suggesting moderate hazard is mostly based on analysis of chemical mixtures such as Firemaster 550 and BZ 54 (Bajard et al., 2019). Moreover, the rapid biotransformation of both compounds difficults to establish the mechanisms of toxicity and the exact hazard of each individual compound (Dishaw et al., 2014). Although these two FRs were introduced as alternatives for PentaBDE mixture (Tao et al., 2016), various studies reported behavioral and endocrine-disrupting effects in animals, as well as impairment of adipogenesis, reproduction and thyroid system in cell culture (Patisaul et al., 2013; Pillai et al., 2014).

Considering both the continuous human exposure to TDCPP, TPhP, EH-TBB and BEH-TBP and the lack of data regarding the neurotoxic potential of these EFRs (see above for references), the present investigation was aimed at testing their neurotoxicity in vitro. The acute response on viability, mitochondrial function, oxidative stress, inflammatory response, as well as neural plasticity and development, of the widely used neuroblastoma cell model SH-SY5Y have been investigated after exposure to these 4 EFRs.

2. Material and methods

2.1. SH-SY5Ys culture and treatment

SH-SY5Y cells (EP-CL-0208, Elabscience, Houston, Texas, USA) at 18-21th passage were maintained in Dulbecco's modified Eagle medium (DMEM)/F-12 (Gibco Thermofisher, Madrid, Spain), supplemented with 10% Fetal bovine serum (FBS) (Gibco, Thermofisher, Madrid, Spain) and 1% penicillin-streptomycin (Gibco Thermofisher, Madrid, Spain), at 37 °C and 5% CO_2 in a humidified incubator. Cells were cultured at a density of 20,000cells/cm^2 and treated once a confluency of 70–80% was reached.

Cells were exposed to vehicle (control), 2.5, 5, 10 and 20 μM of TDCPP (CAS#13674–87-8, LGC, Teddington, UK), TPhP (CAS#115–86-6, LGC, Teddington, UK), EH-TBB (CAS# 183658–27-7, AccuStandard, New Haven, Connecticut, USA) and BEH-TBP (CAS# 26040–51-7, AccuStandard, New Haven, Connecticut, USA) for 2, 4, 8 and 24 h. Dimethyl sulfoxide (DMSO, CAS#67–68-5, Sigma-Aldrich, St Louis, Missouri) was used as vehicle to dilute all FRs at 0.5% (Tian et al., 2016). To confirm that DMSO did not impair cell function, a negative control without vehicle in viability and Adenosine triphosphate (ATP) quantification tests was included. A positive control using 20% DMSO was also included in these both tests (Dalberto et al., 2020). All tests were conducted in 3 independent experiments.

2.2. Cell viability assay

Viability of exposed SH-SY5Y was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were cultured in 96 wells-plate. Following exposure, 20 μL MTT were added to each well, incubating for 3 h at 37 °C. The media and excess MTT were then removed and 200 μL DMSO were added to dissolve the formazan crystals. The absorbance was recorded at 570 nm using a microplate reader (Synergy HT (Biotek), Madrid, Spain). Cell viability was expressed as a percentage of the cell survival rate compared to the negative control (without vehicle) at each time-point.

2.3. Quantification of intracellular ATP

The presence of metabolically active cells by ATP quantification was assessed using CellTiter-Blo Luminiscent Cell Viability Assay (Promega, Madison, Wisconsin, USA). Briefly, 100 μL of reactive were added onto cells cultured in 96 wells-plate, being the sample mixed on an orbital shaker for 2 min to induce cell lysis. After 10 min incubation at room temperature and light-avoided, luminescence was recorded in a Synergy HT (Biotek, Madrid, Spain). Intracellular ATP was expressed as a percentage of the levels rate compared to the negative control (without vehicle) at each time-point.

2.4. Real-time PCR

Total RNA from exposed SH-SY5Y cells was extracted using the SpeedTools Total RNA Extraction kit (Biotools, Madrid, Spain). Samples were reverse transcripted into cDNA using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, Waltham, Massachusetts, USA) by using the GeneAmp PCR System 2700 Thermal Cycler (Applied Biosystems™, Waltham, Massachusetts, USA) (60 min at 42 °C for reversing transcript, 5 min at 95 °C to inactivate the Reverse Transcriptase, and an ultimate 4 °C maintenance). Real-time PCR was carried out to detect the mRNA expression of Cytochrome-c Oxidase subunit 4 (COX4) (ID: Hs00971639_m1), Sirtuin 3 (SIRT3) (ID: Hs00953477_m1), nuclear factor erythroid 2-related factor 2 (NRF2) (ID: Hs00975961_g1), Matrix Metallopeptidase 9 (MMP-9) (ID: Hs00957562_m1), Brain Derived Neurotrophic Factor (BDNF) and Glyceraldehyde-3-Phosphate Dehydrogenasa (GAPDH) (housekeeper; ID: Hs02786624_g1) using TAqMan Assays (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the TaqMan Fast Advanced MasterMix (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Samples were tested per triplicate, being negative controls also run for each assay. 7900 HT Fast Real-Time PCR System (Applied Biosystems™, Waltham, Massachusetts, USA) was employed by activating dsDNA denaturation for 10 min at 95 °C and then amplifying samples with 40 cycles of 10 s at 95 °C followed by 1 min at 60 °C.

Results were analyzed using the 2.4 SDS Software (Applied Biosystems™, Waltham, Massachusetts, USA) and RQ Manager 1.2.1 (Applied Biosystems™, Waltham, Massachusetts, USA). The $2^{-\Delta\Delta Ct}$ method was used to compute the relative transcript abundance, with GAPDH level as an endogenous control for normalizing gene expression.

2.5. Cytokine release

To examine the inflammatory response of SH-SY5Y to the selected FRs, the Human ProcartaPlex Mix&Match 4-Plex (REF PPX-04-MXZTFJE, Invitrogen™, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used in order to measure the presence in supernatant of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1B and IL-10. Plate was read in xPONENT 4.2 for MAGPIX Software User (IVD) after 10 min shake. Samples were tested per triplicate. Curve standard controls were also performed. Concentrations of cytokines were given by the program, being fold-change in cytokines release calculated as sample release (pg/ml)/control release (pg/ml) at the respective time.

2.6. Statistics

All data were expressed as the mean ± standard deviation (SD). Gaussian distribution was assessed by Kolmogorov-Smirnov test. Then, ANOVA -followed by the Bonferroni's post-hoc test- was executed when variances were homogenous. Kruskal-Wallis -and subsequent Dunns test- was used to test unparametric variables. The level of statistical significance for all tests was established at $p < 0.05$. All data were analyzed by GraphPad Prism Statistical Analysis software (GraphPad Prism version 5.01 for Windows, San Diego, California, USA).

3. Results

3.1. Cell viability

The results show that the 4 EFRs did not impair viability of SH-SY5Y after 24 h (Fig. 1). EH-TBB was the FR decreasing the most the viability at all concentrations (~30%), but without differing significantly vs controls. We did not observe significant differences among levels of any of the EFRs.

3.2. Mitochondrial function

The results of the current investigation have shown a lack of significant modulation of ATP levels (Fig. 2) at concentrations ranging 2.5–20 μM for 2–24 h. Only TDCPP at 2.5–10 μM -but not at 20 μM- for 2 and 24 h, as well as TPhP at all concentrations showed a little decrease of 10–20%. Notwithstanding, there were not statistical significant differences vs controls.

We found some similarities in modulation of gene levels among groups (Tables 1-4). At the lowest concentration of TPhP (2.5 μM), both gene expressions were increased after 2 h about 2-times without statistical significance (Table 1). For SIRT3, TPhP also induced an increase on levels at 5 μM for 2 h, which further decreased at 24 h (from 2.32 to 0.64 values of expression) (Table 4). In contrast, SIRT3 levels were decreased by 2.5 μM EH-TBB and 20 μM TDCPP after 4 and 8 h, respectively (expression = 0.49 and 0.67) (Tables 2 and 3), while increased at 10 μM EH-TBB for 24 h (expression = 2.58)(Table 4). On the other hand, COX4 was only additionally modulated by 2.5 μM of TDCPP for 24 h (a decrease, expression = 0.68) (Table 4).

3.3. Oxidative stress and inflammatory response

The 4 EFRs here assessed little modulated NFR2 expression at all time-points, showing only some slight variations for 8 and 24 h, but without being statistically significant (Table 1-4). At 8 h, NFR2 levels were decreased by 20 μM TPhP (expression = 0.63), while were increased by 10 μM TPhP and 20 μM BEH-TBP (about 2 times). These modulations were did not find further for 24 h (Table 4). At the last time-point, both TDCPP and EH-TBB at 10 μM doubled the expression of NFR2.

Concerning to supernatant levels of TNF-α, IL-6, IL-1B nor IL-10, we did not find a release after cell exposure to selected EFR at the measured time-points.

3.4. Neural plasticity and development

The results of the present study showed no significant differences in both MMP-9 and BDNF expressions vs control and among concentrations at time-points of the 4 FRs (Tables 1-4). Both genes were the only among all measured genes that were modulated by exposure to all 4 EFR at some level and time-point, being BDNF the one exhibiting more variations in the expression.

For MMP-9, TDCPP promoted a half-decrease on expression at 10 μM for 2 and 8 h (Tables 1 and 3), as well as at 5 and 20 μM for 8 h (Table 3). By contrast, 2.5 μM of TPhP and EH-TBB highly increased the levels of MMP-9 after 2 h of exposure (almost 3 times), while less effects were promoted by 2.5 and 20 μM of BEH-TBB-TBP at this time point (expression = 2.05 and 1.91, respectively) (Table 1). The increase in MMP-9 expression due to 2.5 μM EH-TBB was further reverted by showing a decrease at 4 h (expression = 0.43) and non-differences for 8

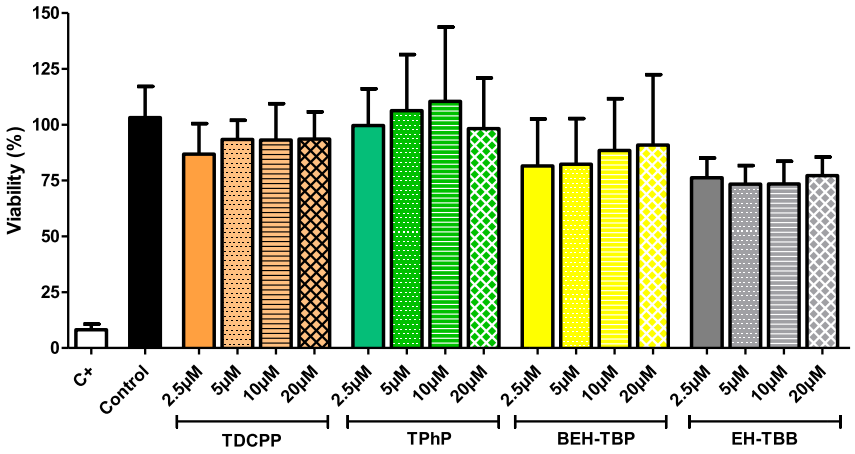

Fig. 1. Viability of SH-SY5Y exposed to TDCPP, TPhP, BEH-TBP and EH-TBB at 2.5, 5, 10 and 20 μM after 24 h. Mean ± SD of the percentage of viability calculated vs negative control (without vehicle) is shown. Control corresponded to DMSO at 0.5%. $N = 3$ independent experiments. Significant differences vs control and among levels were established at $p < 0.05$.

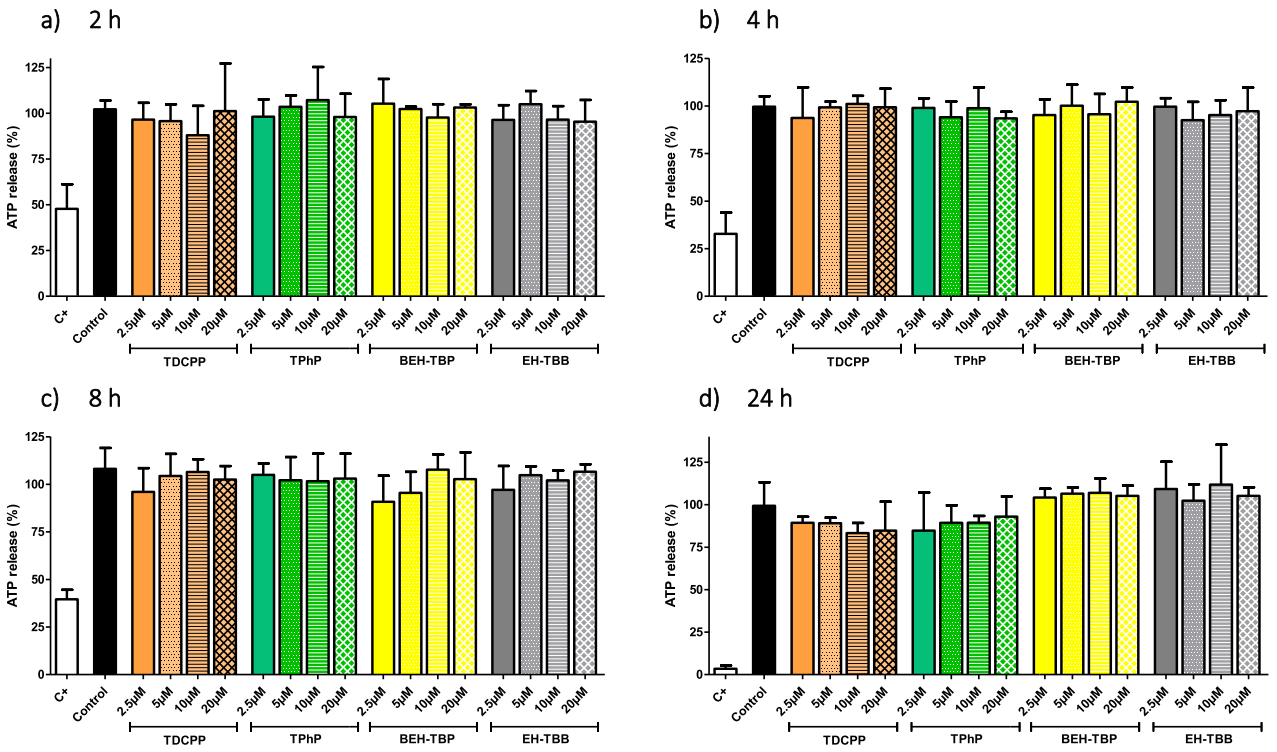

Fig. 2. ATP release of SH-SY5Y exposed to TDCPP, TPhP, BEH-TBP and EH-TBB at 2.5, 5, 10 and 20 μM after 2, 4, 8 and 24 h (a-d respectively). Mean ± SD of the percentage of intracellular ATP calculated vs negative control (without vehicle) is shown. Control corresponded to DMSO at 0.5%. N = 3 independent experiments. Significant differences vs control and among concentrations were established at $p < 0.05$.

and 24 h (Tables 2-4). After 24 h, 5 μM EH-TBB decreased MMP-9 levels, while BEH-TBP at 10 μM increased it (expression = 0.53 and 2.19, respectively) (Table 4).

With respect to BDNF, 2 h of exposure to TPhP promoted an increase at 10 μM (expression =1.91) and a decrease at 20 μM (Table 1), while after 4 and 8 h only 2.5 μM modulated BDNF levels by decreasing them about one-half (Tables 2 and 3). Interestingly, EH-TBB promoted a similar tendency at the two highest concentrations. It was observed that 10 μM for 2 and 4 h increased the levels of BDNF >2.2 times (Tables 1 and 2), which was further decreased after 8 h (expression = 0.67) (Table 3). Likewise, 20 μM EH-TBB showed a 2.09 increase at 2 h and a subsequent decrease at 8 h (expression = 0.68) (Tables 1 and 3, respectively). Furthermore, BEH-TBP highly increased the BDNF expression at 20 μM for 8 h (Table 3), and at 2.5 and 5 μM for 24 h (about 3 times) (Table 4). In contrast, TDCPP only modulated BDNF levels at 20 μM for 24 h, by a 0.67 decrease (Table 4).

4. Discussion

In this study, we tested the in vitro acute neurotoxic potential of 4 EFRs commonly used in the industry and widely distributed in environmental and human samples (see above): TDCPP, TPhP (OPFRs), EH-TBB and BEH-TBP (NBFRs). It was assessed in terms of viability, mitochondrial function, oxidative stress, inflammation, as well as neural plasticity and development. All these processes have an important role in the central nervous system (CNS) and are thought to have key functions in various pathological conditions, including mental and neurodegenerative disorders (i.e.: epilepsy, schizophrenia, alzheimer's disease) and brain tumors (De Luca et al., 2018). The neuroblastoma-cell line SH-SY5Y was selected due to its ability as a neuron model with similar susceptibility to neuroprotective agents such as primary neurons, providing ideal simulation of physiological neuron cell conditions (Dalberto et al., 2020; Li et al., 2017; Shi et al., 2020; Ríos et al., 2003). In the current study we used non- differentiated SH-SY5Y -despite differentiated cells may represent a better model because of more similarities with human adult neurons- by two main reasons. Firstly, to compare our results with those in the scientific literature focused mainly on non-differentiated SH-SY5Y (see below). Secondly, to check the ability -or not- of selected FRs to promote cell differentiation. Indeed, these cells have shown to be useful to study the neuroinflammation response (Güzel et al., 2021; Silva et al., 2021; Song et al., 2021).

4.1. Cell viability

To the best of our knowledge, this has been the first study evaluating viability of SH-SY5Y after exposure to TPhP, EH-TBB and BEH-TBB. Results show a lack of impairment of the 4 FR on cell viability.

Some variability existed with respect to the effects of TDCPP on viability of neural cells, mainly due to the differentiation stage. Li et al. (2017) reported a significant reduction of 10% viability of SH-SY5Y vs control at 12.5 μM of TDCPP at 24 h, while no effects were found at 2.5, 5 and 10 μM in differentiated SH-SY5Y. In fact, Li et al. (2017) found a concentration-dependent impairment of SH-SY5Y viability (12.5, 25, 50, 100 and 200 μM) for 24 h, in which 50% cytotoxicity was reached at 100 μM. Opposite differences in results from studies on PC12 according to differentiation, were also observed being undifferentiated more sensitive than differentiated ones (Dishaw et al., 2011; Ta et al., 2014). No adverse effect on cell viability was either found on PC12 cells after exposure to 10, 20, or 50 μM TDCPP for 24 h (Dishaw et al., 2011). In contrast, Ta et al. (2014) reported that 5 μM TDCPP significantly decreased cell viability on PC12 cells, but not at 1 μM.

The scientific literature confirms that hexabromocyclododecane (HBCD) -from NBFR family- is the highest cytotoxic FR on SH-SY5Y (Shi et al., 2018; Shi et al., 2020; Al-Mousa and Michelangeli, 2012). The diastereoisomers α-, β-, and γ- HBCD decreased the viability of SH-SY5Y in a dose-dependent manner at concentrations of 0.001, 0.01, 0.1, 1.0, 2.5, and 5.0 μM for 24 h (Shi et al., 2018). In fact, at 5 μM, cell viabilities were 44% for HBCD on this cell line (Shi et al., 2020). Cytotoxicity of

Table 1
Gene expression ($^{2-\Delta\Delta Ct}$) of SH-SY5Y exposed to TDCPP, TPhP, BEH-TBP and EH-TBB at 2.5, 5, 10 and 20 μM after 2 h.

	NFR2	BDNF	MMP-9	COX4	SIRT3
TDCPP					
2.5 μM	0.88 ± 0.14	0.77 ± 0.02	0.47 ± 0.35	0.97 ± 0.26	0.83 ± 0.28
5 μM	1.06 ± 0.25	1.01 ± 0.24	1.04 ± 0.55	1.19 ± 0.44	1.03 ± 0.19
10 μM	1.05 ± 0.22	0.97 ± 0.39	0.60 ± 0.36	1.12 ± 0.47	0.94 ± 0.27
20 μM	0.98 ± 0.18	1.20 ± 0.50	0.71 ± 0.03	1.2 6 ± 0.16	1.00 ± 0.09
TPhP					
2.5 μM	1.07 ± 0.32	0.95 ± 0.48	2.77 ± 1.79	1.84 ± 0.98	2.88 ± 1.81
5 μM	1.09 ± 0.14	1.17 ± 0.19	1.46 ± 0.71	1.57 ± 0.91	2.32 ± 0.92
10 μM	1.56 ± 0.40	1.91 ± 0.73	1.05 ± 0.48	1.06 ± 0.63	1.36 ± 0.55
20 μM	0.84 ± 0.51	0.63 ± 0.36	0.78 ± 0.27	1.07 ± 0.29	1.41 ± 0.52
BEH-TBP					
2.5 μM	1.15 ± 0.27	1.60 ± 0.67	2.05 ± 2.16	1.21 ± 0.30	1.37 ± 0.57
5 μM	1.35 ± 0.60	1.17 ± 0.33	1.20 ± 0.06	0.72 ± 0.62	0.79 ± 0.70
10 μM	1.35 ± 0.56	1.23 ± 0.34	1.56 ± 1.03	1.07 ± 0.13	1.15 ± 0.25
20 μM	1.70 ± 0.38	1.59 ± 0.41	1.91 ± 0.59	1.87 ± 0.80	1.41 ± 0.97
EH-TBB					
2.5 μM	1.26 ± 0.49	1.53 ± 0.83	2.96 ± 2.43	1.04 ± 0.35	0.94 ± 0.05
5 μM	1.33 ± 0.75	1.94 ± 1.15	1.92 ± 1.66	1.21 ± 0.42	1.07 ± 0.23
10 μM	1.14 ± 0.73	2.55 ± 2.46	1.58 ± 0.75	1.55 ± 1.02	0.83 ± 0.13
20 μM	1.07 ± 0.58	2.09 ± 1.19	1.03 ± 0.38	1.19 ± 0.39	1.08 ± 0.46

Gene expression was calculated vs control (0.5% DMSO) of each respective FR. Expression of control at value of 1 is not shown. $N = 3$ independent experiments. Significant differences vs control and among concentrations were established at $p < 0.05$.

Table 2
Gene expression ($^{2-\Delta\Delta Ct}$) of SH-SY5Y exposed to TDCPP, TPhP, BEH-TBP and EH-TBB at 2.5, 5, 10 and 20 μM after 4 h.

	Nfr2	BDNF	MMP-9	COX4	SIRT3
TDCPP					
2.5 μM	1.35 ± 0.38	1.30 ± 0.62	1.47 ± 0.58	1.37 ± 0.62	1.36 ± 0.11
5 μM	0.95 ± 0.24	0.94 ± 0.06	0.90 ± 0.04	1.21 ± 0.25	1.23 ± 0.22
10 μM	0.95 ± 0.26	0.98 ± 0.15	1.22 ± 0.44	1.3 ± 0.530	1.32 ± 0.51
20 μM	1.23 ± 0.07	0.86 ± 0.23	1.02 ± 0.08	1.19 ± 0.01	0.98 ± 0.12
TPhP					
2.5 μM	0.80 ± 0.36	0.63 ± 0.44	0.76 ± 0.82	0.73 ± 0.37	0.73 ± 0.39
5 μM	1.04 ± 0.49	0.98 ± 0.75	0.86 ± 0.63	0.85 ± 0.44	0.92 ± 0.46
10 μM	1.07 ± 0.55	0.83 ± 0.65	0.95 ± 0.98	0.73 ± 0.56	0.78 ± 0.61
20 μM	0.91 ± 0.37	0.93 ± 0.72	0.39 ± 0.40	0.78 ± 0.71	0.97 ± 0.26
BEH-TBP					
2.5 μM	1.09 ± 0.05	0.89 ± 0.50	1.22 ± 0.53	1.30 ± 0.53	1.10 ± 0.40
5 μM	0.98 ± 0.15	1.91 ± 1.89	0.96 ± 0.22	0.91 ± 0.10	0.98 ± 0.07
10 μM	1.50 ± 0.62	1.14 ± 0.75	1.56 ± 0.85	1.29 ± 0.66	1.19 ± 0.45
20 μM	0.98 ± 0.31	0.86 ± 0.36	1.59 ± 0.54	1.26 ± 0.63	1.12 ± 0.37
EH-TBB					
2.5 μM	0.73 ± 0.15	1.29 ± 0.00	0.43 ± 0.00	1.48 ± 0.74	0.49 ± 0.33
5 μM	1.03 ± 0.69	1.67 ± 0.95	1.11 ± 0.85	1.16 ± 0.55	1.11 ± 0.73
10 μM	0.98 ± 0.54	2.28 ± 1.61	1.61 ± 1.37	1.48 ± 0.20	1.14 ± 0.33
20 μM	0.98 ± 0.32	1.05 ± 0.41	0.75 ± 0.00	1.46 ± 0.48	1.10 ± 0.54

Gene expression was calculated vs control (0.5% DMSO) of each respective FR. Expression of control at value of 1 is not shown. $N = 3$ independent experiments. Significant differences vs control and among concentrations were established at $p < 0.05$.

HBCD was higher than cycloaliphatic brominated flame retardants (CBFR) on SH-SY5Y for 24 h, promoting a significant drop from 0.01 μM for HBCD, vs from 0.05 μM for TBCO and 0.2 μM for TBECH (Shi et al., 2020). HBCD was also more cytotoxic (from 2.7 μM) than tetrabromobisphenol-A (TBBPA) and decabromodiphenyl ether (DBPE) (at 15 and 28 μM, respectively) on SH-SY5Y (Al-Mousa and Michelangeli, 2012).

Variable significant concentration-effects was noted after PBDE-47 exposure on SH-SY5Y. For this FR, viability was reduced at 8, 16 and 20 μM (drop to 51% of the control) after 24 h, while it increased at lower concentrations, 2 and 4 μM (He et al., 2008). Similarly, concentrations of 5 and 10 μM of PBDE-47, but not 1 μM, induced cytotoxicity of SH-SY5Y cells at 1, 3, 6, 9, 12, 18 and 24 h (Zhang et al., 2017; Zhang et al., 2016a, 2016b). The underlying reasons of this variability must still be elucidated. On the other hand, Tris-(2,3-dibromopropyl) isocyanurate (TDBP-TAZTO) resulted less cytotoxic on SH-SY5Y by reducing viability at 25, 50 and 100 μM after 48 h, but not at concentrations of 6.25 and 12.5 μM (Dong et al., 2015).

4.2. Mitochondrial function

ATP levels are a functional and integrative endpoint of mitochondrial integrity that contributes to changes in the shape of the cell, and consequently, to its survival (Kamalian et al., 2015; Shaughnessy et al., 2015). To the best of our knowledge, the effect of selected EFRs on intracellular ATP had not been previously assessed in SH-SY5Y, but on other cell lines (see below). We found no modulation of ATP after FR exposure on SH-SY5Y, whereas exposure to TPhP and TDCPP at 10 μM significantly decreased intracellular ATP content on human hepatocellular carcinoma cells (HepG2) at high percentages (~50 and ~ 80%, respectively) for 24 h, but not at lower concentrations (Hao et al., 2019; Negi et al., 2021). As also observed in the viability test, sensitive to FR exposure may depend on cell type. Taken together, results suggest that, for 24 h, hepatocytes are more vulnerable in metabolic disorder under OPFR exposure than SH-SY5Y. On the other hand, HBCD diastereoisomers affected higher ATP production on SH-SY5Y by significantly decreased cellular ATP levels at same levels than those selected in present study: 2.5 and 5.0 μM β-HBCD (57.40 and 28.75% of ATP release compared with controls, respectively) and 5.0 μM of γ-HBCD (87.91%) (Shi et al., 2018).

In the current study, the modulation of the expression of COX4 and SIRT3 genes that participate in mitochondrial biogenesis and function, anti-oxidative defense, and energy metabolism (Song et al., 2021; Zhang et al., 2016a, 2016b) were assessed. COX4 (Cytochrome c oxidase

Table 3
Gene expression ($^{2-\Delta\Delta Ct}$) of SH-SY5Y exposed to TDCPP, TPhP, BEH-TBP and EH-TBB at 2.5, 5, 10 and 20 μM after 8 h.

	Nfr2	BDNF	MMP-9	COX4	SIRT3
TDCPP					
2.5 μM	0.94 ± 0.24	0.94 ± 0.28	1.13 ± 0.19	1.24 ± 0.12	1.07 ± 0.18
5 μM	0.72 ± 0.24	0.72 ± 0.41	0.57 ± 0.33	0.84 ± 0.43	0.85 ± 0.47
10 μM	1.11 ± 0.40	1.12 ± 0.16	0.64 ± 0.42	0.99 ± 0.31	0.91 ± 0.43
20 μM	0.93 ± 0.38	0.59 ± 0.36	0.49 ± 0.23	0.95 ± 0.41	0.67 ± 0.31
TPhP					
2.5 μM	0.91 ± 0.24	0.66 ± 0.39	1.55 ± 0.31	1.12 ± 0.23	0.90 ± 0.31
5 μM	1.06 ± 0.36	1.06 ± 0.35	1.24 ± 0.26	1.10 ± 0.35	1.09 ± 0.28
10 μM	2.02 ± 1.22	0.94 ± 0.83	0.98 ± 0.50	1.12 ± 0.40	1.12 ± 0.49
20 μM	0.63 ± 0.67	1.32 ± 0.05	1.90 ± 1.12	1.17 ± 0.17	0.99 ± 0.22
BEH-TBP					
2.5 μM	0.90 ± 0.35	1.19 ± 0.32	1.12 ± 0.56	1.04 ± 0.48	0.92 ± 0.46
5 μM	1.12 ± 0.12	1.40 ± 0.11	0.81 ± 0.14	1.43 ± 0.29	1.31 ± 0.23
10 μM	1.01 ± 0.39	1.24 ± 0.27	0.71 ± 0.32	0.76 ± 0.38	0.87 ± 0.54
20 μM	2.14 ± 2.23	3.23 ± 3.15	1.19 ± 0.15	1.62 ± 0.73	1.39 ± 0.69
EH-TBB					
2.5 μM	1.20 ± 0.38	1.20 ± 0.24	1.66 ± 1.05	1.22 ± 0.15	1.20 ± 0.13
5 μM	1.06 ± 0.07	0.87 ± 0.32	1.45 ± 1.22	1.00 ± 0.22	1.09 ± 0.04
10 μM	0.96 ± 0.09	0.67 ± 0.27	1.32 ± 1.42	0.89 ± 0.11	0.96 ± 0.09
20 μM	1.18 ± 0.70	0.62 ± 0.30	1.42 ± 0.41	1.32 ± 1.04	1.21 ± 0.59

Gene expression was calculated vs control (0.5% DMSO) of each respective FR. Expression of control at value of 1 is not shown. $N = 3$ independent experiments. Significant differences vs control and among concentrations were established at $p < 0.05$.

Table 4
Gene expression ($^{2-\Delta\Delta Ct}$) of SH-SY5Y exposed to TDCPP, TPhP, BEH-TBP and EH-TBB at 2.5, 5, 10 and 20 μM after 24 h.

	Nfr2	BDNF	MMP-9	COX4	SIRT3
TDCPP					
2.5 μM	1.28 ± 0.47	0.74 ± 0.65	1.12 ± 0.31	0.68 ± 0.47	1.20 ± 0.32
5 μM	1.97 ± 1.46	0.78 ± 0.56	1.18 ± 0.66	0.83 ± 0.23	1.77 ± 0.44
10 μM	2.08 ± 1.59	1.43 ± 0.18	1.21 ± 0.77	0.76 ± 0.44	0.98 ± 0.78
20 μM	1.24 ± 0.09	0.67 ± 0.61	0.83 ± 0.42	0.69 ± 0.50	0.97 ± 0.32
TPhP					
2.5 μM	1.34 ± 0.16	0.74 ± 0.06	1.14 ± 1.03	1.00 ± 0.20	1.05 ± 0.12
5 μM	1.04 ± 0.32	0.54 ± 0.29	1.49 ± 0.68	1.54 ± 0.31	0.64 ± 0.25
10 μM	0.97 ± 0.19	0.72 ± 0.37	0.92 ± 0.50	0.75 ± 0.39	0.82 ± 0.11
20 μM	1.32 ± 0.85	0.52 ± 0.38	1.08 ± 0.47	1.13 ± 0.31	0.99 ± 0.11
BEH-TBP					
2.5 μM	1.05 ± 0.64	2.73 ± 2.65	0.84 ± 0.17	0.97 ± 0.38	1.50 ± 0.79
5 μM	0.98 ± 0.67	2.75 ± 2.80	1.49 ± 0.95	0.82 ± 0.30	0.94 ± 0.21
10 μM	1.13 ± 0.38	1.67 ± 1.27	2.19 ± 1.82	1.64 ± 1.36	1.75 ± 1.04
20 μM	0.82 ± 0.30	1.84 ± 1.62	0.79 ± 0.20	0.85 ± 0.36	1.01 ± 0.10
EH-TBB					
2.5 μM	1.82 ± 1.57	1.28 ± 0.30	1.42 ± 1.44	1.31 ± 0.03	1.22 ± 0.52
5 μM	1.25 ± 1.45	0.84 ± 0.23	0.53 ± 0.62	0.79 ± 0.29	0.74 ± 0.66
10 μM	2.25 ± 0.97	0.74 ± 0.05	2.34 ± 1.50	1.77 ± 0.93	2.58 ± 2.87
20 μM	1.07 ± 0.56	0.71 ± 0.42	1.25 ± 0.25	0.97 ± 0.22	0.81 ± 0.38

Gene expression was calculated vs control (0.5% DMSO) of each respective FR. Expression of control at value of 1 is not shown. N = 3 independent experiments. Significant differences vs control and among concentrations were established at $p < 0.05$.

subunit 4) is the largest subunit among ten nuclear-encoded subunits, and it has been reported to be a required component of COX biogenesis. In the respiratory chain, this cytochrome cooperates to transfer electrons derived from NADH and succinate to molecular oxygen, creating an electrochemical gradient over the inner membrane that drives transmembrane transport and the ATP synthase. Currently, function and regulation of COX4 in the brain remain largely unknown (Song et al., 2021). SIRT3 is a mitochondrial NAD^+-dependent deacetylase, which is a pivotal regulator of oxidative stress by regulating the balance between ROS generation and ROS detoxification (Bause and Haigis, 2013; Sidorova-Darmos et al., 2018; Zheng et al., 2018).

In the present investigation, the link between COX4 and SIRT3 genes on mitochondrial function (Song et al., 2021; Zhang et al., 2016a, 2016b) might be supported by the significant correlation in the expression and due to some similarities in modulation of gene levels among groups (Tables 1-4). This has also been the first study measuring COX4 and SIRT3 expressions on neural cells after FR exposure. In relation to SIRT3 gene, it was downregulated by exposure of a similar concentration (10.9 μM) of TCEP on Chang liver cells (a human non-malignant liver cell line) for 24 and 48 h (Zhang et al., 2016a, 2016b). These authors also confirmed the downregulation of this gene at 43.6, 174.4 and 697.7 μM of TCEP (Zhang et al., 2016a, 2016b). It should be noted that dissimilarities may be attributed to differences in functionally of cell lines.

4.3. Oxidative stress and inflammatory response

In normal cells, reactive oxidants are produced in a controlled manner as signaling molecules to regulate processes such as cell division, inflammation, immune function, autophagy, and stress response (Ma et al., 2015). Uncontrolled production of oxidants in neural cells results in oxidative stress, which impairs cellular functions and contributes to the development of neuronal disorders and cancer, among other adverse effects (Franzoni et al., 2021). Nrf2 highlighs from its major role in resistance to oxidant stress, being a key transcription factor that regulates the expression of antioxidant, detoxifying, and defective proteins (Chen et al., 2021). Nfr2 maintains redox states, and participates in cell survival, as well as in the protection of oxidative damage, triggered by injury and inflammation (Quesada et al., 2011). The current investigation has been the first one assessing the NFR2 expression after FR exposure on cells in vitro. We observed little modulation of NFR2 expression at all time-points by exposure to the 4 EFRs without statistical significance.

The release of inflammatory cytokines in injured cells is a normal

immune response that involves extremely complex additive, synergistic, or antagonistic interactions (Güzel et al., 2021). The TNF-α is a major pro-inflammatory mediator and one of the primary stimuli to induce apoptosis, which activates phagocytes killing mechanisms (Kaur et al., 2021). This cytokine is capable of playing a dual functional role by promoting tissue regeneration/growth and destruction (Wajant, 2003). TNF-α increases the permeability of the blood-brain barrier, together with IL-6 (Abreu et al., 2018). In turn, IL-6 influences the differentiation of neurons and astrocytes (Oh et al., 2010), but it can also be neurotoxic and cause neuronal death (Conroy et al., 2004). Another pro-inflammatory messenger cytokine in the expression of ROS generation is IL-1β, whose levels positively correlate with induction of neurodegenerative diseases (Güzel et al., 2021; Song et al., 2021). In contrast, the most important function of IL-10 is to limit and eventually to terminate the inflammatory response (Li et al., 2016), which can decrease the expression of IL-6 and TNF-α (Silva et al., 2021).

Regarding supernatant levels of cytokines on SH-SY5Y, some authors confirmed the modulation on levels of TNF-α, IL-6, IL-1B nor IL-10 after exposure to some compounds as biocides (Güzel et al., 2021; Silva et al., 2021; Song et al., 2021). However, no data are related to FR exposure on SH-SY5Y. In the present study, we did not find any levels of these cytokine on supernatant samples after all EFR levels and time-points. On bone marrow-derived DCs (BMDCs), 10 μM TPhP and TDCPP at 10, 50 and 100 μM had no effect on IL-6 and IL-10 production, while higher levels of TPhP (50 and 100 μM) enhanced production of IL-6, but not IL-10 at 24 h (Canbaz et al., 2017). It should be noted that for these authors, 50 and 100 μM also decreased cell viability in these cells (Canbaz et al., 2017). In contrast, supernatant levels of IL-10 on a human monocytic leukemia cell line (THP-1) were inhibited by exposure of TPhP at 25 and 50 μM for 24 h, while no effects were found after TDCPP exposure (Li et al., 2020). When exposed at lower concentrations (0.1–20 μM), TPhP (and BDE-47) had no effect on IL-6 and TNF-α levels on 3D rat primary neural cell cultures (Hogberg et al., 2021). Variability in sensitivity to FRs, and different roles of inflammatory response among cell lines, may be the responsible of the observed differences on modulation levels of cytokine after FR exposure.

4.4. Neural plasticity and development

The release of various cytokines and chemokines are mainly processed and activated by MMP-9 in brain, controlling the immune/inflammation responses, blood–brain barrier disruption, and facilitating the extravasation of leukocytes into brain parenchyma (Vafadari et al., 2016). Neuronal MMP-9 participates in synaptic plasticity by controlling the shape of dendritic spines and function of excitatory synapses, and regulation of cell adhesion, including limited cleavage of postsynaptic components of the transsynaptic adhesive apparatus (Figiel et al., 2021). This protein plays a pivotal role in learning, memory, and cortical plasticity (Reinhard et al., 2015). Emotional and cognitive functions are importantly regulated by BDNF (Dandi et al., 2018), which is the most active growth factor in the neurotrophin family, essential to neuronal development of neurons, survival, cell death program, differentiation, and growth in the brain (Chen et al., 2018).

The results of the present study showed no significant differences in both MMP-9 and BDNF expressions vs control and among concentrations at time-points of the 4 FRs (Tables 1-4). Both genes were the only among all measured genes that were modulated by exposure to all 4 EFR at some level and time-point, being BDNF the one exhibiting more variations in the expression. In comparison to our selected EFRs, the available literature confirmed the higher potential of legacy FR on cells (including neural) to modulate both gene expressions. On one hand, BDE-47 significantly upregulated MMP-9 expression after 24 h at 10 μM on SH-SY5Y cells (Tian et al., 2016). In fact, this legacy FR and BDE-99 -at very low concentrations (0.01, 0.1, and 1.0 nM)- have shown the potential to overexpress MMP-9 on murine melanoma B16-F10 cells at this time-point, and also at chronic exposure of 15 days (Steil et al., 2021). TBBPA, another BFRs, was reported to upregulate MMP-9 on human breast carcinoma MCF-7 cell line at concentrations of 1, 5 and 10 μM for 24 h (Tian et al., 2016). On the other hand, BDNF expression decreased after 14 and 28 days of exposure, although no after 3 days on human-induced pluripotent stem cell (hiPSC)-derived neural stem cells (NSCs) by a mixture of BDE-47, BDE-99, BDE-100, BDE-153, BDE-154 and BDE-209 at concentrations equivalent to those found in Scandinavian human blood at 0.5, 1, 10, 100, 500 and 1000 times (Davidsen et al., 2021). This study (Davidsen et al., 2021) evidences the higher potential of accumulated doses of FRs on BDNF modulation.

5. Conclusions

The results of the present study demonstrate that selected EFRs (TDCPP, TPhP, EH-TBB and BEH-TBP) did not impair neural function on SH-SY5Y as acute response at widely used low-concentrations. To the best of our knowledge, this has been the first study focused on evaluating the affection of EH-TBB and BEH-TBP on neural cells, as well as of TPhP on SH-SY5Y. Cell viability, mitochondrial function, neural plasticity and development, as well as promotion of oxidative stress or inflammation, were not significantly modulated after exposure to the 4 EFRs at concentrations ranging 2.5–20 μM for 2–24 h. Measurement of selected endpoints after FR exposure were also assessed for the first time.

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Declaration of Competing Interest

The author declare that they have no known competing financial interest or personal relationship that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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