The neurotoxicity of DE-71: effects on neural development and impairment of serotonergic signaling in zebrafish larvae

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ABSTRACT: The underlying mechanism of polybrominated diphenyl ether (PBDE)-induced neurotoxicity is still a major concern due to its ubiquitous nature and persistence. Here, zebrafish embryos (2 h postfertilization, hpf) were exposed to different concentrations of the commercial PBDE mixture DE-71 (0–100 µg l⁻¹) until 120 hpf, and the impact on neural development and serotonergic system was investigated. The *in vivo* results revealed significantly reduced transcription of genes involved in neurogenesis (*fgf8*, *shha*, *wnt1*), and contents of proteins in neuronal morphogenesis (myelin basic protein, synapsin IIa), suggesting an impairment of neural development in zebrafish embryos. Further results demonstrated a reduction of 5-hydroxytryptamine neuron and a dose-dependent decrease of whole-body serotonin levels, as well as the transcription of genes involved in serotonergic synthesis (*tph1*, *tph2*, *trhr*) and neurotransmission (*sarta/b*, *htr1aa/b*). In addition, we predicted possible targets of PBDEs by molecular docking, and the results indicated that PBDE congeners showed high binding affinities with fibroblast growth factor 8 other than SHH and HTR1B. Taken together, this study demonstrated that PBDE exposure during embryogenesis could damage neural development and cause impairment of the serotonergic system as secondary effects in the zebrafish larvae. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: PBDEs; zebrafish larvae; neural development; serotonergic system; molecular docking

Introduction

Polybrominated diphenyl ethers (PBDEs) are among the most persistent organic pollutants, and have caused great concern during the past decades, as they have been shown to disturb thyroid hormone homeostasis due to structural similarity (Dingemans *et al.*, 2011). PBDEs have also been repeatedly reported to affect the central nervous system and cause permanent impairment in spontaneous behavior (locomotion, rearing and total activity), habituation capability as well as performance in learning tasks in rodents (Costa & Giordano, 2007; Dingemans *et al.*, 2011; Eriksson *et al.*, 2001). Some previous studies have attributed PBDE-induced neurotoxicity to indirect effects following thyroid disruption, while others have proposed direct effects of neuronal signaling, cell migration, axon formation, synaptic plasticity, ion channels, calcium homeostasis and neurotransmission (Dingemans *et al.*, 2011; Kodavanti & Ward, 2005; Mariussen & Fonnum, 2003). The mechanism of toxicity of PBDEs, however, still needs to be clarified.

To the best of our knowledge, of the key events during early neural development, three inductive signaling factors, including fibroblast growth factors (FGF8), Sonic hedgehog (SHH) and Wingless/int-1 (WNT1), have been demonstrated to collaborate in inducing the neural plate (Hynes *et al.*, 2000; Ye *et al.*, 1998), and the proliferation and differentiation of neuronal progenitors (Hirabayashi *et al.*, 2004; Muroyama *et al.*, 2004; Viti *et al.*, 2003). While in neuronal morphogenesis, the role of myelin basic protein (MBP) and synapsin IIa (SYN2a) were well studied. MBP is considered a biomarker of myelination that is required for the myelination of axons in the developing central nervous system in zebrafish (Brosamle & Halpern, 2002) under the regulation of SHH (Chen *et al.*, 2013a). SYN2a is a biomarker of synapse

formation, which plays an important role in both synaptogenesis and neurotransmitter release (Kao *et al.*, 1998); therefore, we presume that PBDEs could affect early development through these signalings to induce neurotoxicity. Although not specific, the FGF, SHH and WNT1 signalings have been proven to play critical roles in the specification and differentiation of monoamine neurons (Hynes *et al.*, 2000; Ye *et al.*, 1998). Hence, it is very likely that the monoamine serotonin (5-hydroxytryptamine, 5-HT) could also be affected. Serotonin is synthesized through the actions of different tryptophan hydroxylases, Tph1, Tph2 and TphR. The serotonin transporter (SERT) and serotonin receptors play important roles in synaptic 5-HT neurotransmission: SERT acts as both sym- and antiporter in the presynaptic membrane, responsible for removal and reuptake of serotonin from the synapse (Gershon & Tack, 2007; Wang *et al.*, 2006). Knockout of 5-HT transporter in rats could alter the brain serotonin homeostasis (Bengel *et al.*, 1998; Olivier *et al.*, 2008). The G-protein-coupled receptor, HTR1A, may act as an

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autoreceptor in a presynaptic negative-feedback loop of 5-HT, and propagate serotonergic signaling postsynaptically (Masson *et al.*, 2012; Norton *et al.*, 2008). In addition to its critical role in the modulation of behaviors (Airhart *et al.*, 2012; Schaefer *et al.*, 2009), 5-HT is of particular interest because it contributes to promote the specification, differentiation and phenotype maintenance of the hippocampal neurons via activation of its receptors (Fricker *et al.*, 2005). Therefore, it must be interesting whether PBDEs could directly interfere with genes or proteins involved in serotonergic signaling and cause corresponding effects to neural development.

Zebrafish have been shown to be a useful model for studying the mechanism of neurotoxicity by means of neural developmental, neurochemical and neurobehavioral changes (Eddins *et al.*, 2010). The definition of the molecular target as initial events for the *in vivo* observations could be another challenge. For this intention, *in silico* approaches such as molecular docking are suitable tools to fill gaps in knowledge concerning chemical and biological levels. The docking methodologies were based on the knowledge of three-dimensional structure of a receptor protein in attempt to optimize the bound ligand or a series of molecules into the active site. This approach can facilitate identification of the initial step for chemical molecular targets by estimation of receptor–ligand binding modes and binding affinities, and has been widely accepted as a potent tool in toxicological studies (Li *et al.*, 2010, 2013). Therefore, the main purpose of the present study was to evaluate the effects on neural developmental and serotonergic system in zebrafish embryo/larvae upon exposure to the PBDE mixture, DE-71, and predicted the possible targets by molecular docking.

Materials and methods

Chemicals

DE-71 (purity >99.9%) was obtained from Wellington Laboratory (Ontario, Canada). 5-HT was purchased from Sigma-Aldrich (St. Louis, MO, USA). The PBDEs standards (BDE-28, BDE-47, BDE-71, BDE-99, BDE-153, BDE-154), $^{13}\text{C}_{12}$ -labeled BDE-139 and $^{13}\text{C}_{12}$ -labeled PCB-208 were purchased from AccuStandard (St. Louis, MO, USA). Trizol reagent and PrimeScript reverse transcription (RT) reagent kits were purchased from Takara (Dalian, China). SYBR Green polymerase chain reaction (PCR) kits were purchased from GeneCopoeia (Rockville, MD, USA). All other chemicals used in the present study were analytical grade standard.

Zebrafish embryos/larvae exposure

Adult wild-type zebrafish (AB strain, 5 months old) maintenance and embryos exposure were carried out according to published protocols (Yu *et al.*, 2010). Briefly, ~2000 normal developing embryos that reached the blastula stage (2 h post-fertilization, hpf) were selected and distributed into glass beakers containing 500 ml DE-71 exposure solutions (0, 3, 10, 30 or 100 $\mu\text{g l}^{-1}$) until 120 hpf. Both the control and exposure groups received 0.005% (v/v) dimethyl sulfoxide, and the exposure solutions were renewed daily. After exposure, a subset of the larvae were used for locomotor activity measurement, whereas the others were washed five times, immediately frozen in liquid nitrogen and stored at -80°C until for analysis. The hatching, malformation and growth were also recorded.

Polybrominated diphenyl ether extraction and analysis in zebrafish larvae

For PBDE analysis, ~100 larvae (5 dpf; $n = 3$ replicates) from each group were washed five times and randomly sampled for extraction according to a previous study (Chen *et al.*, 2013b). The samples were weighed and freeze-dried, then 5 ng $^{13}\text{C}_{12}$ -labeled BDE-139 was added as recovery spike. Next, the samples were homogenized using a mixture of acetone and isooctane (1 : 1, v/v), sonicated for 30 min and extracted three times. All extracts were passed through a 0.22 μm nylon mesh filter into an autosampler vial and dried under nitrogen, the volume was added up to 500 μl with isooctane, and 4 ng $^{13}\text{C}_{12}$ -labeled PCB-208 was added as internal standard. Quantification of PBDEs was performed using a gas chromatograph (Agilent 6890; Agilent Technologies, Santa Clara, CA, USA) equipped with a mass-selective detector (Agilent 5975C). To check for interference or contamination from the solvent or glassware, procedural blanks were analyzed simultaneously in every batch of five samples. The recovery of $^{13}\text{C}_{12}$ -labeled BDE-139 ranged from 87% to 98% and corrected for the results. The limit of detection was defined as a signal/noise ratio of 3, and on average ranged from 0.01 to 0.05 ng for the PBDEs and 0.06 ng for tetra-BDE. Samples with concentrations below the detection limits were recorded as ND.

Locomotor activity measurement

Larval swimming behavior was monitored using a Video-Track system (ViewPoint Life Sciences, Montreal, Canada) following previously described methods (He *et al.*, 2011). Larvae (5 dpf) were placed into 24-well microplates (one larva per well) in water, and were allowed to acclimatize for 10 min before their swimming speed was monitored. The temperature was kept at 28°C . Larval swimming behavior was monitored under continuous light (30 min) and light/dark transition stimulation (5 min light/5 min dark/5 min light/5 min dark) to examine their swimming speed and reactions to changes of light. A total of 32 larvae of each treatment were measured, and the data (frequency of movements, distance travel and total duration of movements) were collected every 30 s and further analyzed using Open Office 2.4 software (<http://www.openoffice.org>).

Whole-mount immunofluorescence

The immunofluorescence was carried out based on a previous method (Elsalini & Rohr, 2003). Twenty larvae from each group ($n = 4$ replicates) were collected and fixed in 4% paraformaldehyde overnight at 4°C , then rinsed thoroughly in phosphate-buffered solution (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , pH 7.4) and dehydrated in a graded methanol series (25, 50, 75 and 100%), stored in methanol at -20°C . Larvae were incubated in 3 ml 10% H_2O_2 in methanol overnight at room temperature for bleaching and to block endogenous peroxidases, then 10 ml PBS-T (PBS containing 0.3% Tween-20) were added, mixed and incubated for a further 24 h at room temperature. The larvae were washed five times with PBS-T thoroughly and blocked for 2 h with 2% bovine serum albumin (BSA) in PBS-T at 37°C , then incubated in 2% BSA in PBS-T with primary antibody against HT (1 : 500) for one night at 4°C . The rabbit polyclonal anti-5HT (ImmunoStar, Hudson, WI, USA) have been verified to be reactive with zebrafish in immunofluorescence (Airhart *et al.*, 2012). After a thorough wash with PBS-T, the larvae were incubated in 2%

BSA in PBS-T containing Cy3-labeled goat antirabbit (Beyotime, Jiangsu, China) (1 : 500) for 2 h at 37 °C. After washing five times for 30 min each with PBS-T and then washed three times for 30 min each with PBS, the larvae were imaged using a two photon laser scanning system (NOL-LSM 710; Zeiss, Jena, Germany).

Serotonin (5-hydroxytryptamine) analysis

5-HT measurement was performed as described previously (Sallinen *et al.*, 2009). Fifty larvae from each group ($n = 4$ replicates) were collected and sonicated in 200 μ l of 0.1 M perchloric acid. After centrifugation for 10 min at 10 000 g at 4 °C, the supernatants were filtered with a 0.22 μ m polyvinylidene difluoride membrane. 5-HT content was determined using high-performance liquid chromatography equipped with an ESA Colocchem 5600A electrochemical detector (ECD; ESA, Chelmsford, MA, USA). Reversed phase chromatography was adopted with a C_{18} column (250 $\times$ 3 mm, 5 μ m). The mobile phase consisted of methyl alcohol, acetonitrile and phosphate buffer solution (ratio 4 : 7 : 89), and was maintained at a flow rate of 0.8 ml min⁻¹. The sample concentrations were obtained by referring to calibration curves of 5-HT standards, and expressed as ng g⁻¹ wet weight (ww).

Protein extraction and Western blot analysis

Protein extraction and Western blot analysis were performed as previously described (Chen *et al.*, 2012a). Briefly, protein from ~200 zebrafish larvae from each group ($n = 4$ replicates) was extracted using commercial kits (Key-GEN BioTECH, Nanjing, China) according to the manufacturer's instructions, and the concentration was measured by the bicinchoninic acid method. Fifty micrograms of each protein sample was loaded on to a 6% or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel, and then electrophoretically transferred to polyvinylidene difluoride membranes (Sigma-Aldrich). The membrane was blocked with 5% BSA in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 8.0) for 1 h at 37 °C and then incubated with primary antibody against the MBP (1 : 800; AnaSpec, Fremont, CA, USA), synapsin IIa (SYN2a; 1 : 1000; Synaptic Systems, Göttingen, Germany) or glyceraldehyde 3-phosphate dehydrogenase (1 : 1000) at 4 °C overnight. All primary antibodies were suitable for use in zebrafish (Chen *et al.*, 2012b). The blots were washed five times for 10 min each with Tris-buffered saline Tween-20, incubated with goat antirabbit (1 : 5000) horseradish peroxidase-conjugated affinitypure secondary antibodies

(Proteintech, Wuhan, China) at 37 °C for 1 h and then visualized with enhanced chemiluminescence using a quantitative Western blot imaging system (FluorChem Q; Alpha Innotech, San Leandro, CA, USA).

RNA isolation and quantitative real-time polymerase chain reaction

Whole-body of zebrafish larvae were homogenized (30 larvae for each replicate, $n = 4$ replicates), and total RNA was extracted using Trizol Reagents (Takara) according to the manufacturer's instructions. The quality and purity were examined by 1% agarose-formaldehyde gel electrophoresis with ethidium bromide staining and 260/280 nm ratios, and concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). First-strand cDNA was synthesized using a PrimeScript RT reagent kit (Takara) according to the manufacturer's instructions. The transcriptional levels of genes involved in neural development (*shha*, *fgf8*, *wnt1*) and genes encoding the rate-limiting enzyme for serotonin synthesis, tryptophan hydroxylase (*tph1*, *tph2*, *tphr*), for serotonin transporters (*serta* and *sertb*) as well as for 5-HT_{1A} receptors (*htr1aa*, *htr1ab*). The primer sequences were obtained using the online Primer 3 program (<http://primer3.ut.ee/>), and are listed in Table 1. The ribosomal protein L8 (*rpl8*), which did not vary upon DE-71 exposure (data not shown), was selected as the house-keeping gene. Quantitative RT-PCR was carried out using an All-in-One qPCR Mix (GeneCopoeia) and analyzed on an ABI 7300 System (PerkinElmer Applied Biosystems, Foster City, CA, USA). The change in mRNA expression was analyzed using the $2^{-\Delta\Delta C_t}$ method.

Molecular docking

Molecular docking studies were performed using the LibDock module, and binding energies were calculated for all docked compounds using Discovery Studio 4.0 (Accelrys Software, San Diego, CA, USA) with standard protocols. The crystal structure of FGF8 (2FDB), SHH (2ROT) and HTR1B (4IAQ) were obtained from the RCSB protein data bank (<http://www.pdb.org>), and used for docking modeling after preparation. For ligand preparation, the three-dimensional structures of the PBDE congeners were obtained from the PubChem Substance database (<http://www.ncbi.nlm.nih.gov/pcsubstance>) and optimized with the MMFF94 method.

Table 1. Primer sequences for the quantitative reverse transcription–polymerase chain reaction

Gene name	Sense primer (5'-3')	Antisense primer (5'-3')	GenBank accession no.
<i>rpl8</i>	TTGTTGGTGTGTGCTGGT	GGATGCTCAACAGGGTTCAT	NM_200713
<i>shha</i>	GCAAGATAACGCGCAATTCGGAGA	TGCATCTCTGTGCATGAGCCTGT	NC_007118
<i>fgf8</i>	CTATGCTCAGGTAACCATCA	TATAAGCTTGGCATGAACGTC	NC_007124
<i>wnt1</i>	ATGTAGGCTGATGCTTTG	GTCGGCTCGGAACTGCA	NC_007134
<i>tph1</i>	TCTGTGAAGTCTACGTGTGG	CACTGGGAGCATCAGACG	AF548566
<i>tph2</i>	ATCCATCCTTGCTCTCCAAC	TCTGTGAAGTCTACGTGTGG	NM_214795
<i>tphr</i>	AGATCCCATACACACGTAGAG	CGGTTCAAGAGTGTAAGAGG	AB125219
<i>serta</i>	ACCACAGAGTCTAAATGTTCCAG	CTCTTCCTTCATCTGTGTGCCTTC	NM_001039972
<i>sertb</i>	AACCTAACAGCAGTCCTCA	GGCCTCACCGTCACACAATA	NM_001177459
<i>htr1aa</i>	ATGAGGATGAGCGGGATGTAG	CAATCAGCCAGGACCACG	NM_001123321
<i>htr1ab</i>	CTGTGTCGCCTGCACITTTTC	TGATCTCCAAAGACTCGCCG	NM_001145766

Statistical analysis

All data are expressed as mean $\pm$ SEM. The normality and homogeneity of variance of data were verified using Kolmogorov–Smirnov and Levene's tests. Differences between the control and exposure groups were evaluated by one-way analysis of variance followed by Tukey's test using SPSS 13.0 software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Developmental toxicity associated with DE-71 exposure

Exposure to DE-71 did not affect embryo hatching rate, survival and growth parameters (body weight and length; data not shown). The overall hatching rates were $>90\%$, and survival rates were $>95\%$. A non-significant trend for an increase in the malformation rate was found in the exposure groups compared with the control group (data not shown).

Concentration of DE-71 in exposed zebrafish larvae

The congeners, including BDE-28, 47, 71, 99, 153 and 154, were detected in DE-71-exposed zebrafish larvae, with BDE-47 being the predominant one, followed by BDE-99. The detected total contents of PBDEs were 27.26 ± 1.03 , 94.67 ± 2.03 , 331.70 ± 12.15 and $998.40 \pm 28.23 \mu\text{g g}^{-1}$ ww in the 3, 10, 30 and $100 \mu\text{g l}^{-1}$ exposure groups, respectively (Table 2). No PBDEs were detected in the control group.

Locomotor activity is decreased in DE-71-exposed larvae

The locomotion-traced zebrafish larvae (5 dpf) were monitored during continuous light and during a light–dark transition stimulation period. Under continuous light condition, the speed was significantly decreased by 15.3% and 20.0% ($P < 0.001$) in the 30 and $100 \mu\text{g l}^{-1}$ exposure groups, respectively, compared with the control (Fig. 1A).

Figure 1(B) and (C) show the distance moved and the swimming speed of the larvae during the light–dark stimulation experiment, respectively. The total distance moved (Fig. 1B) and the average swimming speed of the larvae (Fig. 1C) during both the light and dark period were significantly decreased after exposure to 30 and $100 \mu\text{g l}^{-1}$ DE-71 ($P < 0.001$) compared with those of the control.

Exposure to DE-71 reduces larval 5-hydroxytryptamine immunoreactivity neuron and 5-hydroxytryptamine contents

Whole-mount immunofluorescence staining was performed using 5-HT antibody. The results demonstrated that 5-HT immunoreactivity neurons were distributed in the optic tectum and rhombencephalon, and strongly located laterally in the hindbrain (arrows) from zebrafish larvae in control group (Fig. 2A). In contrast, 5-HT immunoreactivity neurons declined in the optic tectum and rhombencephalon regions in zebrafish larvae exposed to $100 \mu\text{g l}^{-1}$ DE-71 for 5 days (Fig. 2B).

Moreover, the whole-body content of 5-HT was significantly reduced in a concentration-dependent manner and showed significant differences in the larvae exposed to 10, 30 and $100 \mu\text{g l}^{-1}$ DE-71 by 29.8%, 34.1% and 46.6%, respectively, compared with the solvent control (Fig. 3).

Exposure to DE-71 reduces protein expression levels of myelin basic protein and synapsin IIa

The protein expression levels of MBP and SYN2a in DE-71-exposed zebrafish were examined by Western blot. There was a concentration-dependent reduction of MBP and SYN2a (Fig. 4) in exposed embryos. Specifically, MBP was significantly reduced by 43.6% and by 52.3% in the 30 and $100 \mu\text{g l}^{-1}$ exposure groups (Fig. 4A,B), respectively, whereas SYN2a was significantly reduced by 29.8% and by 37.9% in the 30 and $100 \mu\text{g l}^{-1}$ exposure groups (Fig. 4C,D), respectively, compared with those of the control group.

Exposure to DE-71 is associated with decreased mRNA expression of genes involved in serotonergic signaling

The measured gene transcriptional profiles are shown in Fig. 5. The transcripts of *shha*, *fgf8* and *wnt1* were significantly downregulated 1.6-, 1.7- and 1.9-fold; 1.8-, 1.6- and 2.1-fold; 1.9-, 2.1- and 2.1-fold in the 10, 30 and $100 \mu\text{g l}^{-1}$ groups, respectively. The mRNA level of *tph1* was significantly downregulated 1.5-, 1.4- and 1.5-fold in the 10, 30 and $100 \mu\text{g l}^{-1}$ groups, respectively. The *tph2* gene transcription was significantly downregulated 1.2-, 1.2-, 2.1- and 2.1-fold in the 3, 10, 30 and $100 \mu\text{g l}^{-1}$ groups, respectively; the gene transcriptions of *tphr*, *serta*, *sertb* and *htr1ab* were significantly downregulated 1.4- and 1.8-fold, 1.7- and 1.7-fold, 1.8- and 2.3-fold, 2.0- and 2.1-fold in the 30 and $100 \mu\text{g l}^{-1}$ groups respectively. The *htr1aa* gene transcription was significantly downregulated 1.7-fold in the $100 \mu\text{g l}^{-1}$ group.

Table 2. PBDE congeners measured in zebrafish larvae after exposure to different concentrations of DE-71 (0, 3, 10, 30 or $100 \mu\text{g l}^{-1}$) for 5 days

PBDE concentration ($\mu\text{g l}^{-1}$)	0	3	10	30	100
BDE-28	ND	0.02 ± 0.00	0.04 ± 0.00	0.11 ± 0.01	3.19 ± 0.42
BDE-47	ND	26.46 ± 0.95	87.54 ± 1.66	288.50 ± 11.21	901.20 ± 24.75
BDE-71	ND	0.05 ± 0.00	0.06 ± 0.00	0.11 ± 0.00	0.58 ± 0.04
BDE-99	ND	0.38 ± 0.08	6.36 ± 0.50	41.79 ± 0.96	91.95 ± 3.18
BDE-153	ND	0.05 ± 0.00	0.17 ± 0.01	0.28 ± 0.00	0.41 ± 0.01
BDE-154	ND	0.31 ± 0.00	0.51 ± 0.01	0.90 ± 0.02	1.06 ± 0.00
Σ PBDE	ND	27.26 ± 1.03	94.67 ± 2.03	331.70 ± 12.15	998.40 ± 28.23

ND, below the detection limits; PBDE, polybrominated diphenyl ether.

Values (ng g^{-1} ww) are expressed as mean $\pm$ SEM of three replicate samples.

Figure 1. Abnormal locomotor behavior in zebrafish larvae exposed to DE-71. Locomotor behavior was assessed in zebrafish larvae exposed to 0, 3, 10, 30 or 100 $\mu\text{g l}^{-1}$ DE-71 for 5 days. The following parameters were measured: average swimming speed during a continuous light test (A); distance moved (B) and average swimming speed (C) of the larvae during a light-dark-light-dark photoperiod stimulation test. Data are expressed as mean $\pm$ SEM of four replicates (eight larvae per replicate) in 30 s intervals; *** $P < 0.001$ indicate significant difference between the exposed larvae and the control group.

Interactions between polybrominated diphenyl ethers and serotonergic related proteins indicated by molecular docking

No poses were generated for the PBDE congeners with the SHH protein; hence, no data for their binding energies were obtained. The binding energy of the docked PBDE congeners with FGF8 and HTR1B are shown in Table 3. All congeners detected in the present study could bind to FGF8, with binding energies

ranging from -88.81 to $-69.79 \text{ kcal mol}^{-1}$. The congeners of BDE-47, BDE-28, BDE-99 and BDE-154 were found to bind to HTR1B, with binding energies ranging from 6.72 to 25.42 kcal mol^{-1} .

The binding modes of the docked PBDE congeners were further analyzed and are shown in Fig. 6. The protein-ligand interactions depict several residues (Arg59, Arg3255 and Asn2173) and water molecules (HOH114 and HOH125) forming H-bonds with bromine atoms of BDE154, and residues (Arg3255 and Asn2173) forming the H-bond with the oxygen atom (Fig.6A). Analyses of the FGF8-BDE154 complex also showed π - π stacking interactions mediated by Phe3352 and π -cation interactions by Arg 59, Arg170 and Arg 3255. No H-bonds or π interaction pairs were found, and van der Waals force plays a major role within the HTR1B-BDE47 complex (Fig. 6B).

Discussion

The aim of this study was to validate the possible effects of PBDEs on the neural developmental and serotonergic systems by an *in vivo* zebrafish model, and to predict the molecular target through *in silico* tools. Our results demonstrated that embryonic exposure to DE-71 damaged early development and caused impairment of the genes and proteins involved in serotonergic signaling in zebrafish larvae, and the docking results suggested FGF8 as a possible target of PBDEs congeners.

First, we determined the behavioral changes as an endpoint of neurotoxicity upon DE-71 exposure, and observed hypoactivity, as indicated by reduced trace and swimming speed in continuous light and light-dark transition stimulation in zebrafish larvae exposed to 30 and 100 $\mu\text{g l}^{-1}$ DE-71. This observation is in agreement with the altered behavioral activity previously reported in PBDE-exposed zebrafish larvae (Chen *et al.*, 2012b; He *et al.*, 2011). In addition, the detected congeners, BDE-47, BDE-99 and BDE-153 have also been shown to be related to alterations of spontaneous behavior (locomotion, rearing and total activity). Importantly, although the waterborne concentrations of DE-71 used in this study were higher than those measured in the water, the body burden of total PBDEs in exposed zebrafish larvae in the 100 $\mu\text{g l}^{-1}$ group (998.40 ng g^{-1} ww) was comparable with those in fish collected from PBDE-contaminated water (1088 ng g^{-1} ww) (Luo *et al.*, 2007). Thus, our results confirmed the neurotoxicity upon DE-71 exposure and suggested a potential risk for wild fish in PBDE-contaminated water.

To analyze further the underlying mechanism, we investigated the variation of important genes and proteins regarding early neural development. The transcriptional levels of *fgf8*, *shha* and *wnt1*, showed similar, decreasing trends in zebrafish larvae exposed to DE-71 concentrations $\geq 10 \mu\text{g l}^{-1}$. These observations suggest that PBDE exposure impairs the early development procedure such as induction of the neural plate, proliferation and differentiation of neuronal progenitors. Moreover, our Western blot analysis revealed significantly reduced contents of MBP and SYN2a, both of which are involved in neuronal morphogenesis. Previous studies have reported transcriptional changes of both *mbp* and *syn2a* upon PBDE exposure in zebrafish (Chen *et al.*, 2012b), providing further support for our results. These results provided evidence for the neural development toxicity by DE-71. On this basis, we employed a docking simulation to predict the possible target among the affected modulators by evaluating their binding affinities and

Figure 2. DE-71 exposure is associated with declined 5-HT-ir cells. 5-HT-ir neurons are observed distributed in the Teo and Rh from zebrafish larvae in the control group (A). In contrast, 5-HT-ir neurons declined in Teo and Rh regions in zebrafish larvae exposed to $100 \mu\text{g l}^{-1}$ DE-71 for 5 days (B). Images were taken dorso to the ventral. Scale bar is $100 \mu\text{m}$. 5-HT-ir, 5-hydroxytryptamine (serotonin) immunoreactivity; Rh, rhombencephalon; Teo, optic tectum.

Figure 3. DE-71 exposure is associated with a reduced whole-body content of serotonin. The concentration of 5-HT was measured in zebrafish larvae exposed to 0, 3, 10, 30 and $100 \mu\text{g l}^{-1}$ DE-71 for 5 days. $n = 4$ replicate samples; each sample contained 50 larvae; data are expressed as mean $\pm$ SEM; ** $P < 0.01$ and *** $P < 0.001$ indicate significant differences between the exposed larvae and the control group. 5-HT-ir, 5-hydroxytryptamine (serotonin).

interacting modes with PBDE congeners. Owing to the availability of crystal structures, FGF8 and SHH were chosen and tested. The PBDE congeners showed poor affinities with SHH, as no docking poses were generated by docking simulation. In contrast, all congeners detected in our study could bind significantly to FGF8, as indicated by their negative binding energies. This could also be supported by observations of the H-bonds and π interaction pairs within the protein–ligand complex. Therefore, we assume that FGF8 could be the potential molecular target of PBDEs congeners. Despite the limited cognition of the complex mechanism(s) among the modulators, a functional link between FGF8 and WNT1 signaling through an autoregulatory loop (Chilov *et al.*, 2010) and an regulatory relationship between FGF8 and MBP (Chen *et al.*, 2013a) have been proved. Therefore, it was possible that the observed alterations of these genes and proteins were attributed to the alterations of *fgf8* signaling.

Considering the regulatory role of *fgf8*, *shh* and *wnt1* signaling in serotonergic neurogenesis (Teraoka *et al.*, 2004), we performed whole-mount immunofluorescence assay using 5-HT antibody, and the results demonstrated a decline in the number

of serotonin-positive neurons in the brain of zebrafish larvae. In accordance with this, 5-HT levels determined by high-performance liquid chromatography showed a dose-dependent reduction upon PBDE exposure concentrations in zebrafish larvae. These results may indicate impairment of serotonergic neurogenesis, which resulted in decreased 5-HT levels in zebrafish larvae upon DE-71 exposure. Although the specific role of the serotonergic system in the control of behavior is not clear, some correlations between the locomotion and 5-HT levels have been established. For example, 5-HT-depleted mice exhibited hypoactivity in spontaneous locomotor activity and a less anxious performance in light–dark box exploration, and in zebrafish, serotonin depletion resulted in significantly less movement (Airhart *et al.*, 2012; Schaefer *et al.*, 2009), which is similar to the behavioral observations in our study. Thus, our results indicate that the behavioral changes of zebrafish larvae upon PBDE exposure were related, at least partially, to the decreased 5-HT levels.

In addition, the transcripts of genes involved in the synthesis and neurotransmission of 5-HT were determined, and the results suggested that the genes encoding tryptophan hydroxylase (*tph1*, *tph2*, *tphr*) were significantly suppressed. Aroclor 1254, a PCB mixture, has been demonstrated to inhibit hypothalamic tryptophan hydroxylase activity and to result in decreased hypothalamic 5-HT concentrations in the Atlantic croaker (*Micropogonias undulatus*) (Khan & Thomas, 2001). Thus, inhibited transcription levels of *tph1*, *tph2* and *tphr* suggested reduced synthesis of 5-HT, and this may explain the decreased 5-HT levels in zebrafish larvae exposed to PBDEs. In a previous study, embryonic exposure to p-chlorophenylalanine, an inhibitor of TPH, resulted in decreased transcripts of *htr1a* in the brain and spinal cord, and in reduced SERT in the spinal cord of zebrafish (Airhart *et al.*, 2012). Similarly, the gene transcripts of *htr1aa/b* and *serta/b* were also found to be inhibited in addition to the decreased *tph* transcripts in DE-71-exposed zebrafish larvae. Taken together, the transcriptional suppression of 5-HT1A receptors (*htr1aa*, *htr1ab*) and serotonin transporters (*serta* and *sertb*) suggests a dysfunction in 5-HT neurotransmission caused by PBDE exposures. The results described suggest a profound toxic effect on the genes involved in serotonergic signaling upon PBDE exposures.

However, some other studies indicated a role of 5-HT in the specification, differentiation and phenotype maintenance of the hippocampal neurons, including the serotonergic

Figure 4. DE-71 exposure is associated with reduced protein expression levels of MBP and SYN2a. Western blot analysis was used to measure the protein expression level of MBP (A,B) and SYN2a (C,D) in zebrafish larvae exposed to 0, 3, 10, 30 and $100 \mu\text{g l}^{-1}$ of DE-71 for 5 days. Upper panel shows a representative Western blot (A, C) and lower panel shows quantification of four replicate samples (B, D), normalized to GAPDH expression; data are expressed as mean $\pm$ SEM; $^{**}P < 0.01$; $^{***}P < 0.001$ indicate significant differences from the control group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MBP, myelin basic protein; SYN2a, synapsin IIa.

system via activation of its receptors (Fricker *et al.*, 2005), thus raised an opposite hypothesis that PBDEs could directly affect the 5-HT signaling and result in an indirect effect on the neural development. In this regard, we evaluated the binding affinities and interacting modes between PBDE

Figure 5. Altered genes expression in zebrafish larvae exposed to DE-71. The mRNA expression levels were significantly decreased following exposure to 0, 3, 10, 30 and $100 \mu\text{g l}^{-1}$ DE-71 for 5 days in a concentration-dependent manner. Data are presented as mean $\pm$ SEM $n = 4$ replicate samples; each sample contained 30 larvae; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicate significant differences between the exposed larvae and the control group.

Table 3. Binding energies of PBDE congeners as docked ligands

Protein	PBDE congeners	Binding energy (kcal mol^{-1})
FGF8	BDE-47	-77.02
	BDE-71	-88.81
	BDE-28	-81.80
	BDE-99	-83.63
	BDE-154	-76.21
	BDE-153	-69.79
HTR1B	BDE-47	25.42
	BDE-28	13.72
	BDE-99	21.80
	BDE-154	6.72

PBDE, polybrominated diphenyl ether.

congeners and the 5-HT receptor, HTR1B. The results indicated that PBDE congeners showed weak interactions with HTR1B, as only BDE-47, BDE-28, BDE-99 and BDE-154 could be docked into generated poses with positive binding energies. The interaction diagram also showed no H-bond within the protein–ligand complex. Therefore, we suggest that PBDE congeners would probably not interact with this 5-HT receptor. In addition, these results confirmed that the impaired serotonergic signaling could be secondary effects following impaired neural development.

In conclusion, the present results demonstrate that DE-71 exposure during zebrafish embryogenesis results in the repression of certain genes and proteins involved in neural development, and resulted in secondary effects including dysfunction of serotonergic synthesis and neurotransmission, and finally the reduction of 5-HT levels. The docking simulation indicated FGF8 as a possible target of PBDE congeners. Further studies employing genetic tools would be necessary to verify and understand the role of FGF8 signaling in PBDE-induced developmental neurotoxicity.

Figure 6. Interaction map depicting protein–ligand contacts in the top hits obtained by Libdock using Discovery Studio. Ligand interaction diagram showing the protein–ligand contacts for (A) FGF8-BDE154 and (B) HTR1B-BDE47, with essential amino acid residues tagged in circles.

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Conflict of interest

The authors did not report any conflict of interest.

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