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RESEARCH ARTICLE

Evaluation of BDE-47-induced neurodevelopmental toxicity in zebrafish embryos

Juan Zhuang[1] · Zheng-jun Pan[1] · Ying Qin[1] · Hui Liang[1] · Wen-feng Zhang[1] · Ze-yu Sun[1] · Han-bo Shi[1]

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Abstract

There are growing concerns about the neurodevelopmental toxicity of polybrominated diphenyl ethers (PBDEs), but the toxicological phenotypes and mechanisms are not well elucidated. Here, zebrafish (*Danio rerio*) were exposed to 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) from 4 to 72 h post-fertilization (hpf). The results showed that BDE-47 stimulated the production of dopamine and 5-hydroxytryptamine, but inhibited expression of Nestin, GFAP, Gap43, and PSD95 in 24 hpf embryos. Importantly, we unraveled the inhibitory effects of BDE-47 on neural crest-derived melanocyte differentiation and melanin syntheses process, evidenced by disrupted expression of *wnt1*, *wnt3*, *sox10*, *mitfa*, *tyrp1a*, *tyrp1b*, *tryp2*, and *oca2* gene in 72 hpf embryos and decreased tyrosinase activities in embryos at 48 and 72 hpf. The transcriptional activities of *myosin VAa*, *kif5ba*, *rab27a*, *mlpha*, and *cdc42* genes, which are associated with intracellular transport process, were also disturbed during zebrafish development. Ultimately, these alterations led to fast spontaneous movement and melanin accumulation deficit in zebrafish embryos upon BDE-47 exposure. Our results provide an important extension for understanding the neurodevelopmental effects of PBDEs and facilitate the comprehensive evaluation of neurotoxicity in embryos.

Keywords Polybrominated diphenyl ethers · Zebrafish · Neurotoxicity · Melanin pigmentation

Introduction

For decades, polybrominated diphenyl ethers (PBDEs) have been extensively applied as additive brominated flame retardants in commercial products including electronics, appliances, textiles, and household furnishing. Although many PBDEs have been banned or voluntarily phased out in the USA and European countries, large amounts of in-use consumer products and environmental reservoirs containing PBDEs still exist (Cai et al. 2022). It has been estimated that the total consumption of penta-, octa-, and deca-BDE was ~46,000, ~25,000, and ~380,000 tonnes, respectively, in 35 products considered in the USA and Canada from 1970 to 2020 (Abbasi et al. 2015). Indeed, high concentrations of PBDEs were widely detected in environmental media (Lin et al. 2022). In soil and road dust samples, PBDE concentrations ranged from 0.13 to 590 ng/g, dw, and 3.3–600 ng/g, dw, respectively (Li et al. 2018); the highest PBDE concentration (390,000 ng/g dw) was detected in a sample from an e-waste open-burning site (Labunska et al. 2013). Hou et al. (2019) detected the water, sediment, and fish samples from the Jinjiang River (Chengdu, China) and found the BDE-47 concentrations were 0.25 ng/L, 0.29 ng/g, dw, and 9.5~15.8 ng/g, lw, respectively. Maximum PBDE concentration (18,000 ng/g lw) has been found in the eggs of peregrine falcons (Falco peregrinus) (Lindberg et al. 2004). Labunska et al. (2014) estimated that the median PBDE exposure levels around e-waste recycling sites were 130.9 and 614.1 ng/kg bw/day for adults and children, respectively.

Prenatal or neonatal exposure to PBDEs has been associated with complex neurotoxicity outcomes in children, such as slower neuropsychological development (Drobná et al. 2019), lower levels of social and language development (Ding et al. 2015), poorer fine motor skills (Eskenazi et al. 2013), neurobehavioral abnormalities (Ji et al. 2019a), lower cognitive ability (Azar et al. 2021), and reading skills

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✉ Juan Zhuang
dajiangsky@163.com

[1] Jiangsu Key Laboratory for Eco-Agricultural Biotechnology Around Hongze Lake, Jiangsu Collaborative Innovation Center of Regional Modern Agriculture and Environmental Protection, Jiangsu Engineering Laboratory for Breeding of Special Aquatic Organisms, Huaiyin Normal University, 111 Changjiang West Road, Huaian 223300, Jiangsu, China

(Liang et al. 2019). In animal studies, neurotoxic effects of PBDEs were mainly manifested as altered spontaneous behavior and cognitive deficits (Liu et al. 2022; Zheng et al. 2021; Blanc et al. 2021; Dingemans et al. 2007), and perturbation of thyroid hormone homeostasis, oxidative stress-induced damage, interference with signal transduction, and disruption of neurotransmitter systems were suggested to be the potential mechanisms (Blanco et al. 2013; Costa et al. 2015; Chen et al. 2018; Dingemans et al. 2007). Although the neurodevelopmental effects of PBDEs have been extensively explored, the neurotoxicity phenotypes and the underlying mechanisms have not been completely elucidated.

2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) is a prominent PBDE congeners, accounting for above 50% of the total PBDEs in maternal or placental serum (Azar et al. 2021; Varshavsky et al. 2020). Zebrafish is a favorable in vivo model for studying neurotoxicity of PBDEs (Zheng et al. 2021). Zebrafish tail coiling is sensitive to chemical stimulus, which has been extensively taken as a useful behavioral endpoint of neurodevelopmental toxicity (Zheng et al. 2021). In our pilot study, BDE-47 increased tail coiling but reduced skin melanin accumulation in zebrafish embryos. Melanocytes are pigment-producing cells originated form neural crest cells, and melanocyte generating was related to the development of nervous system (Yaar and Park, 2012; Wang et al. 2020). Thereby, changes in embryonic neurobehavior and skin pigmentation following BDE-47 exposure were monitored; biochemical indicators such as monoaminergic neurotransmitter content, melamine content, and tyrosinase activity were detected; and genes or protein expression that are responsible for nervous system development, melanocyte development, melanin syntheses, and intracellular transport process were analyzed in zebrafish embryos. Our results will enrich our understanding of neurodevelopmental toxicity of PBDEs and facilitate the comprehensive evaluation of neurotoxicity in embryos.

Materials and methods

Chemicals and reagents

BDE-47 (> 99%) was purchased from ChemService, West Chester, PA. It was dissolved in DMSO (> 99.9%; Sigma-Aldrich, St. Louis, MO) and diluted to 1.250, 0.625, or 0.312 mg/L by adding zebrafish embryo culture medium (5 mM NaCl, 0.33 mM $MgSO_4$, 0.33 mM $CaCl_2$, and 0.17 mM KCl; pH7.2–7.4; Nanjing EzeRinka Biotechnology Co., Ltd., China). The TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). The reverse transcription kit and SYBR Green Master Mix were purchased from Takara (Dalian, China). Melamine, L-DOPA, and mushroom tyrosinase were from Sigma-Aldrich. Zebrafish TyR ELISA kit, Dopamine ELISA kit, and 5-HT ELISA kit were obtained from enzyme-linked Biotechnology (Shanghai, China). Antibody against Mitfa was obtained from GeneTex (North America). Antibodies against Nestin, growth-associated protein 43 (Gap43), postsynaptic density protein 95 (PSD95), glial fibrillary acidic protein (GFAP) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG), and fluorescein (FITC)-conjugated anti-rabbit IgG were obtained from Proteintech Group (China). Primer sequences were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

Fish husbandry and embryo collection

Adult AB strain zebrafish (*Danio rerio*) were purchased from Nanjing EzeRinka Biotechnology Co., Ltd. (Nanjing, China). Zebrafish were maintained in fish water (pH 7.4~7.8, conductivity 450~550 μS /cm) at 28 °C with a 14 h light and 10 h dark lighting schedule and fed with freshly hatched brine shrimp. Male and female zebrafish were placed into the spawning boxes overnight at a ratio of 1:1. Embryos were collected within 30 min after spawning and incubated in embryo medium at 28 °C.

BDE-47 exposure

BDE-47 exposure concentrations were chosen according to the previous work of Tanaka et al. (2018) and our pilot study. 6-well cell culture plates (Corning Inc. Steuben, New York, USA) were used in the experiments. The plates were incubated with the respective concentrations of BDE-47 24 h before embryo exposure. Then embryos were randomly divided into 6-well plates with about 50 embryos per well, and then exposed to 6 mL BDE-47 solutions (0, 0.325, 0.625, or 1.250 mg/L; DMSO, < 0.03%) with a minimum of three replicates. Our pilot experiment suggested that 0.03% v/v DMSO had no negative effect on embryo development. The exposure period was from 4 to 72 h post-fertilization (hpf). The incubating conditions for the embryos were the same as those for the adult fish. The exposure solution was renewed every day.

Spontaneous movement

At 24 hpf, the movements of embryos were monitored using a CCD camera (Optec TP310, China) connected to the stereomicroscope (Optec SZ780, China) as the previous report (Ramlan et al. 2017). Coiling movement is a full-body contraction that brings the tip of the tail to the head. The number of spontaneous movement and tail coiling of the zebrafish embryos without morphological malformations was counted within a 1-min period.

Morphological skin pigmentation and melanin analysis

The morphological skin pigmentation was checked using a stereomicroscope (Optec SZ780, China), and images were taken using a CCD camera (Optec TP310, China). The areas of skin melanosomes covering the dorsal regions of the zebrafish head and yolk sac and excluding the dark area covered by eyes were quantified at 72 hpf using the software image-pro plus according to the previous study (Burgoyne et al. 2015). The threshold setting in image-pro plus was used to remove all image information excluding melanosomes, allowing measurement of melanin area.

The melanin content was measured in 72 hpf embryos as described by Wang et al. (2020). Briefly, 50 zebrafish embryos of each group were anesthetized with MS-222 (0.016%) and homogenized in ice-cold phosphate buffers (PBS) and centrifuged at 10,000 rpm for 20 min at 4 °C (n = 3). After centrifugation, the melanin precipitation was resuspended with 1N NaOH/10% DMSO and vortexed. The mixture was incubated at 95 °C in dark to solubilize the melanin for 10 min. The absorbance at 405 nm was measured using a microplate reader, and the zebrafish embryonic melanin content was determined from a standard curve prepared from an authentic standard of synthetic melanin.

Tyrosinase activity assay

After anesthetization with MS-222 (0.016%), zebrafish embryos (50 embryos of each group) at 48 hpf (n = 5) or 72 hpf (n = 4~5) were homogenized in ice-cold PBS and centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatants' solution was used for zebrafish tyrosinase activity determination according to the manufacturer's instructions of the zebrafish TyR ELISA kit. Briefly, standard or samples (50 μL) and HRP conjugate (100 μL) were added to standard wells and sample wells and incubated at 37 °C for 1 h. Then, the incubation mixture was removed and washed with PBS four times. A total of 100 μL 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate was added to each well and incubated for 15 min at 37 °C. Finally, stop solution H_2SO_4 (50 μL) was added to each well, and the absorbance was measured at 450 nm. The tyrosinase activity was determined by comparing the standard curve.

To determine whether BDE-47 can directly inhibit tyrosinase activity, mushroom tyrosinase and L-DOPA were used according to the L-DOPA chrome formation method (Liu et al. 2019). Briefly, 50 μL solvent control or BDE-47 (0.325, 0.625, or 1.250 mg/L) was mixed with 50 μL mushroom tyrosinase solution (700 U/mL) and 100 μL PBS (0.1 mol/L, pH 6.8). A total of 100 μL L-DOPA (5 mM) was used as substrate and added into the mixture (n = 3). The mixture was incubated for 20 min at 37 °C, and the absorbance value of the dopachrome product was measured at 475 nm.

Measurement of monoaminergic neurotransmitters

After anesthetization with 0.016% MS-222, zebrafish embryos (50 embryos of each group, n = 4) at 24 hpf were homogenized in ice-cold PBS and centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatants of zebrafish embryos at 24 hpf were used to examine the concentrations of 5-hydroxytryptamine (5-HT) or dopamine according to the manufacturer's instructions of the zebrafish dopamine or 5-HT ELISA kit. Briefly, standard or samples (50 μL) and HRP conjugate (100 μL) were added to standard wells and sample wells of dopamine or 5-HT ELISA kit and then incubated for 1 h at 37 °C. After being washed with PBS four times, 100 μL TMB solution was added to each well and incubated for 15 min at 37 °C. Finally, H_2SO_4 (50 μL) was added to each well as a stop solution, and the absorbances at 450 nm were measured. The dopamine or 5-HT concentrations were determined from the standard curve.

Real-time PCR

Zebrafish total RNAs (30 embryos of each group, n = 3) were extracted using TRIzol Reagent. cDNA was synthesized using cDNA reverse transcription kits. SYBR-Green quantitative PCR analysis was performed using an SYBR Green Master Mix and the Bio-Rad CFX96 Real-Time System (Hercules, CA, USA). Expression of *wnt1*, *wnt3*, *sox10*, *mitfa*, *tyrp1a*, *tyrp1b*, *tryp2*, *oca2*, *myosin VAa*, *kif5ba*, *rab27a*, *mlpha*, and *cdc42* were detected. The primer sequences are presented in Supplementary Table 1.

Western blot

The protein samples (40 μg protein/sample, n = 3) were separated using 10% sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membrane. Next, the membranes were blocked with 1 × TBST with 5% nonfat dried milk for 1 h and then incubated with primary antibodies overnight at 4 °C. After being washed with TBST three times, the membranes were incubated with peroxidase-conjugated secondary antibody for 1 h at 22 °C and visualized using enhanced chemiluminescence.

Whole-mount immunofluorescence staining

Zebrafish embryos were fixed with 4% paraformaldehyde at 4 °C for 4 h. After being permeabilized by 100% methanol, embryos were incubated with Nestin antibody at 4 °C overnight. Subsequently, FITC-conjugated anti-rabbit IgG was applied after washing in PBS.

Statistical analysis

All the experiments were performed in triplicate, and the results were expressed as the mean ± standard error. Statistical analysis was performed with one-way ANOVA followed by Dunnett's Multiple Comparison Test using GraphPad Prism 5.0 software. A P value of < 0.05 was considered statistically significant.

Results

Spontaneous movement and morphological observation

Zebrafish coiling movement is regarded as a rapid and sensitive endpoint for neurotoxicity evaluation (Wang et al. 2018; Zheng et al. 2021). Previous reports demonstrated that BDE-47 stimulated zebrafish coiling activity, but the triggering concentrations were inconsistent (Usenko et al. 2011; Tanaka et al. 2018). We treated embryos with BDE-47 and observed the spontaneous movement at 24 hpf. As shown in Fig. 1, zebrafish spontaneous movement was stimulated by BDE-47 at 1.250 mg/L (Fig. 1a; $P < 0.05$, versus control group), and the coiling frequency was increased (Fig. 1b, $P < 0.01$, versus control group).

In addition to the neurobehavioral changes in 24 hpf embryos, further observation of the developing embryos revealed a novel effect of BDE-47 manifested in the suppression of skin pigmentation. As shown in Fig. 2, notable skin pigmentation appeared in the embryonic head, abdomen, and dorsolateral trunk from 32 hpf onward. Melanosomes in the skin covering the head, yolk sac, and notochord significantly were significantly suppressed in embryos starting from 32 hpf due to BDE-47 exposure concentration-dependently. The presented data indicated that BDE-47 disrupted skin pigmentation in zebrafish embryos.

Concentrations assays of 5-HT and dopamine

Dopamine and 5-TH play vital roles in zebrafish central nervous system development, behavior, and movement (Brustein et al. 2003; Thirumalai and Cline, 2008). Thus, we assessed whether the concentrations of 5-HT and dopamine changed in zebrafish exhibiting increased coiling at 24 hpf. As shown in Fig. 3a, 1.250 mg/L BDE-47 increased 5-HT levels ($P < 0.05$, versus control group), and the contents of dopamine were also elevated (Fig. 3b, $P < 0.05$, versus control group).

Melanosome area, melanin content and tyrosinase activity

To quantify the melanin presence, skin melanosome areas within the defined dorsal regions of the head and yolk sac (Fig. 4a) and melanin contents were measured in 72 hpf zebrafish. Results showed that zebrafish melanosome areas were markedly reduced in 0.625 and 1.250 mg/L BDE-47 groups (Fig. 4a, BDE-$47_{0.625\ mg/L}$: $P < 0.05$; BDE-$47_{1.250\ mg/L}$: $P < 0.01$, versus control group), and the melanin contents were accordingly decreased (Fig. 4b, BDE-$47_{0.625\ mg/L}$: $P < 0.05$; BDE-$47_{1.250\ mg/L}$: $P < 0.001$, versus control group). Furthermore, the effect of BDE-47 on tyrosinase activity was investigated, and the results suggested BDE-47 inhibited tyrosinase activities in zebrafish embryos at 48 and 72 hpf (Fig. 4c; embryos at 48 hpf: BDE-$47_{0.625\ mg/L}$: $P < 0.01$; BDE-$47_{1.250\ mg/L}$: $P < 0.001$; Fig. 4d; embryos at 72 hpf: BDE-$47_{0.625\ mg/L}$: $P < 0.05$; BDE-$47_{1.250\ mg/L}$: $P < 0.001$, versus control group). However, BDE-47 did not affect the enzymatic activities of mushroom tyrosinase at all concentrations in vitro test (Fig. 4e, $P > 0.05$, versus control group).

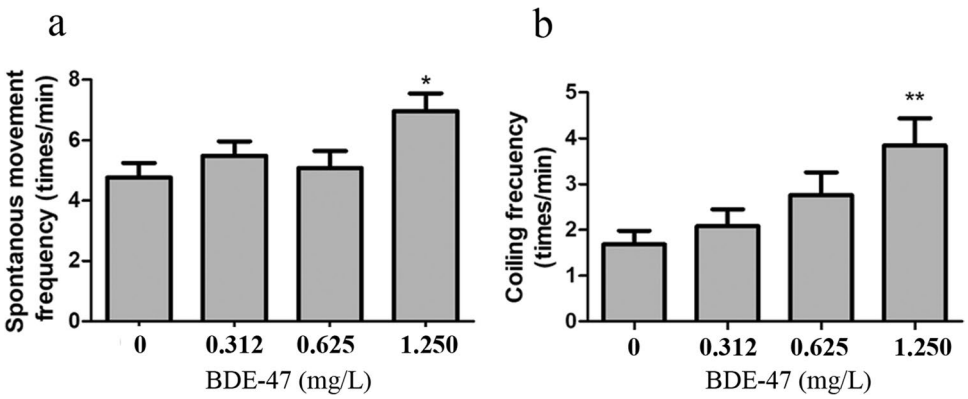

Fig. 1 BDE-47 altered spontaneous movement behavior in zebrafish embryos at 24 hpf. Zebrafish embryos were exposed to various concentrations of BDE-47 beginning at 4 hpf. **a** Zebrafish embryonic spontaneous movement. **b** Zebrafish embryonic coiling activities. ($n = 25$). * $P < 0.05$, ** $P < 0.01$ versus control group

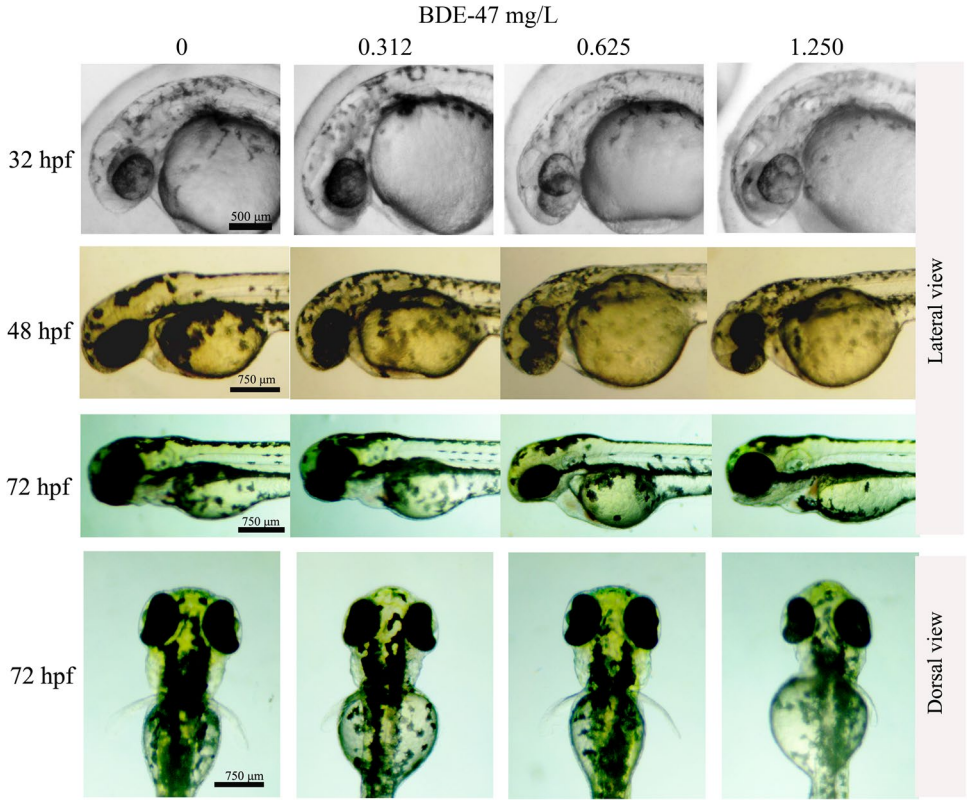

Fig. 2 Effects of BDE-47 on morphological color change of zebrafish embryos. Zebrafish embryos were exposed to various concentrations of BDE-47 beginning from 4 to 72 hpf. The morphological skin pigmentation was observed under the stereomicroscope at 32 hpf, 48 hpf, or 72 hpf

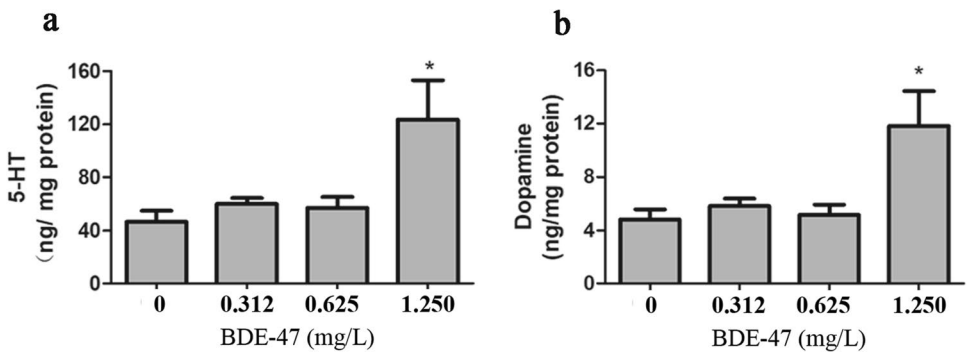

Fig. 3 Concentrations of 5-HT and dopamine were increased by BDE-47 in zebrafish embryos at 24 hpf. Zebrafish embryos were exposed to various concentrations of BDE-47 beginning at 4 hpf. **a** 5-HT concentrations. **b** Dopamine concentrations. ($n = 4$). * $P <$ 0.05 versus the control group

Proteins expression related to neural development

We further analyze the protein expression related to neural development in 24 hpf BDE-47-treated embryos. As shown in Fig. 5a and b, BDE-47 significantly inhibited the expression of Nestin, PSD95, and GFAP at concentrations of 0.625 and 1.250 mg/L (Nestin: BDE-$47_{0.625\ mg/L}$: $P <$ 0.01; BDE-$47_{1.250\ mg/L}$: $P < 0.05$; PSD95: $P < 0.05$; GFAP: BDE-$47_{0.625\ mg/L}$: $P < 0.05$; BDE-$47_{1.250\ mg/L}$: $P < 0.01$; versus control group), and decreased the protein level of Gap43 at 1.250 mg/L ($P < 0.05$, versus control group). Immunofluorescence staining results suggested that Nestin protein was widely expressed in the embryonic brain at 24 hpf, and the positive signal intensity was decreased by BDE-47 (Fig. 5c).

Expression of genes or proteins related to skin melanocyte differentiation and melanogenesis

Gene expression related to melanocyte differentiation and melanogenesis in zebrafish embryos at 72 hpf was analyzed. Gene expression of wingless-type MMTV integration site family member 1 (*wnt1*) and member 3a (*wnt3a*), which are responsible for melanocyte differentiation, were significantly inhibited at all BDE-47 tested concentrations (Fig. 6a, *wnt1*: BDE-$47_{0.312\ mg/L}$: $P < 0.05$; BDE-$47_{0.625\ mg/L}$ and BDE-$47_{1.250\ mg/L}$: $P < 0.001$; Fig. 6b, *wnt3a*: $P <$ 0.001; versus control group). The transcriptional level of SRY-Box transcription factor 10 (*sox10*) was decreased in 0.625 or 1.250 mg/L BDE-47 group (Fig. 6c, BDE-$47_{0.625\ mg/L}$: $P < 0.001$; BDE-$47_{1.250\ mg/L}$: $P < 0.01$; versus

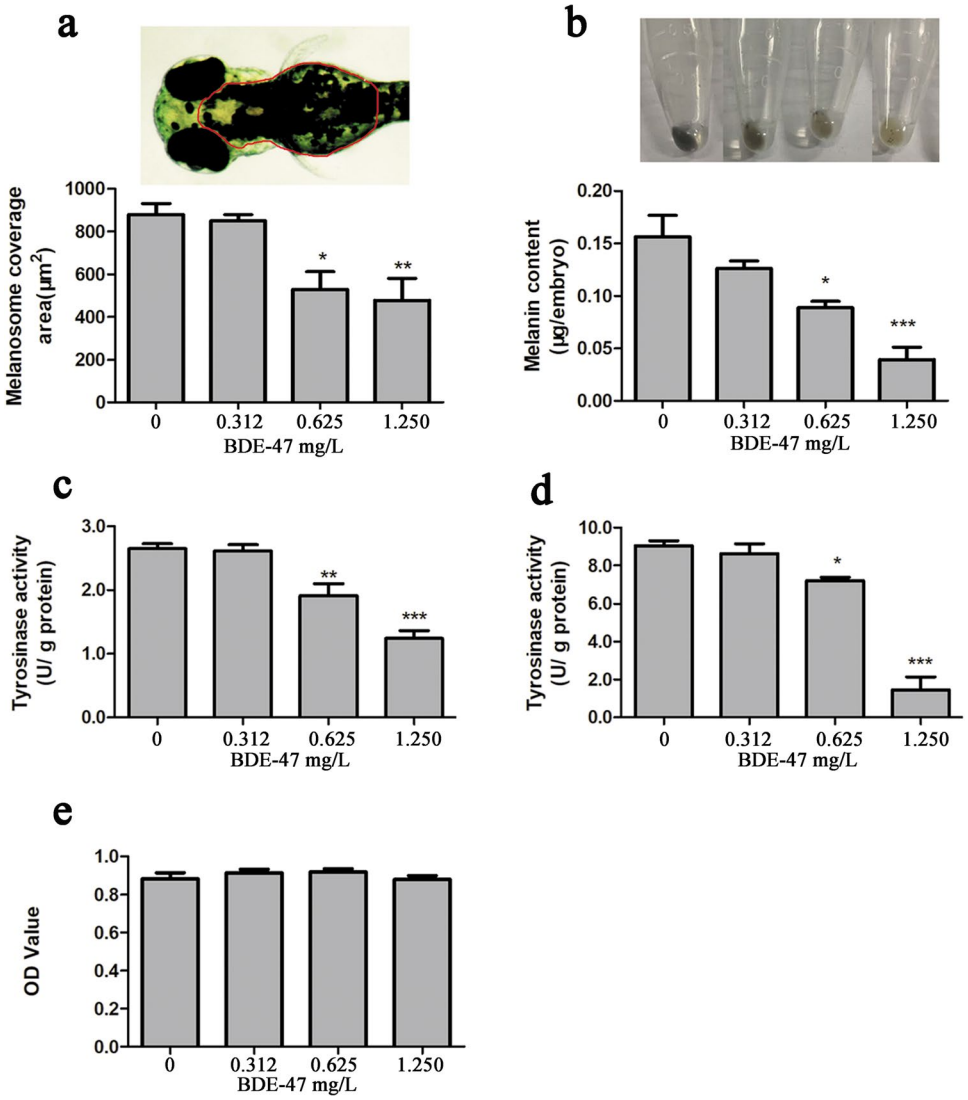

Fig. 4 Effects of BDE-47 on skin melanosome area, melanin content, and tyrosinase activity. Zebrafish embryos were exposed to various concentrations of BDE-47 beginning from 4 to 72 hpf. **a** Melanosome areas within the defined region of zebrafish embryos (indicated red-outlined area, the dorsal regions of the head and yolk sac of zebrafish) at 72 hpf were quantified using the software image-pro plus (n = 5–6). **b** A schematic representation of melanin extracted from zebrafish embryos and melanin contents of zebrafish embryos at 72 hpf (n = 3). **c** Embryonic tyrosinase activities at 48 hpf (n = 5). **d** Embryonic tyrosinase activities at 72 hpf (n = 4–5). **e** Mushroom tyrosinase activities (n = 3). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control group

control group). The expression of melanocyte-inducing transcription factor a (*mitfa*) was reduced in 0.312 mg/L or 1.250 mg/L BDE-47 group, while increased in 0.625 mg/L BDE-47 group (Fig. 6d, BDE-47$_{0.312\ mg/L}$: $P < 0.05$; BDE-47$_{0.625\ mg/L}$ and BDE-47$_{1.250\ mg/L}$: $P < 0.01$; versus control group). Tyrosinase-related protein (tyrp) and oculocutaneous albinism II (oca2) are involved in melanin biosynthesis, and *tyrp1a* was transcriptionally up-regulated in 0.625 mg/L BDE-47 group (Fig. 6e, $P < 0.01$; versus control group), while the expression of *tyrp1b*, *tryp2*, and *oca2* genes were significantly inhibited by BDE-47 (Fig. 6f, *tyrp1b*: BDE-47$_{0.312\ mg/L\sim1.250\ mg/L}$: $P < 0.001$; Fig. 6g, *tryp2*: BDE-47$_{0.312\ mg/L\sim1.250\ mg/L}$: $P < 0.001$; Fig. 6h, *oca2*: BDE-47$_{0.625\ mg/L}$ and BDE-47$_{1.250\ mg/L}$: $P < 0.01$; versus control group). Western blot analysis results showed BDE-47 decreased protein expression of mitfa in 72 hpf embryos at concentrations of 0.625 and 1.250 mg/L (Fig. 6i, $P < 0.001$; versus control group). These findings suggested that BDE-47 may disrupt the process of melanocyte differentiation and melanogenesis.

Gene expression associated with the intracellular transport process

Motor-dependent intracellular transport is required for cell polarity and function in nerve cells and melanocytes (Ultanir et al. 2014; Edgar and Bennett, 1999). Thus real-time PCR analysis was performed to determine the effects of BDE-47 on actin- and microtubule-dependent transport. At 24 hpf, BDE-47 stimulated the expression of actin-based processive motor *myosin VAa* in zebrafish embryos at concentrations of 0.312 mg/L and 1.250 mg/L (Fig. 7a, $P < 0.01$; versus control group), while the expression of microtubule-associated motor kinesin family member 5b (*kif5b*)*a* was not significantly affected (Fig. 7b, $P > 0.05$; versus control group). Besides, gene expression of *mlpha*, *rab27a*,

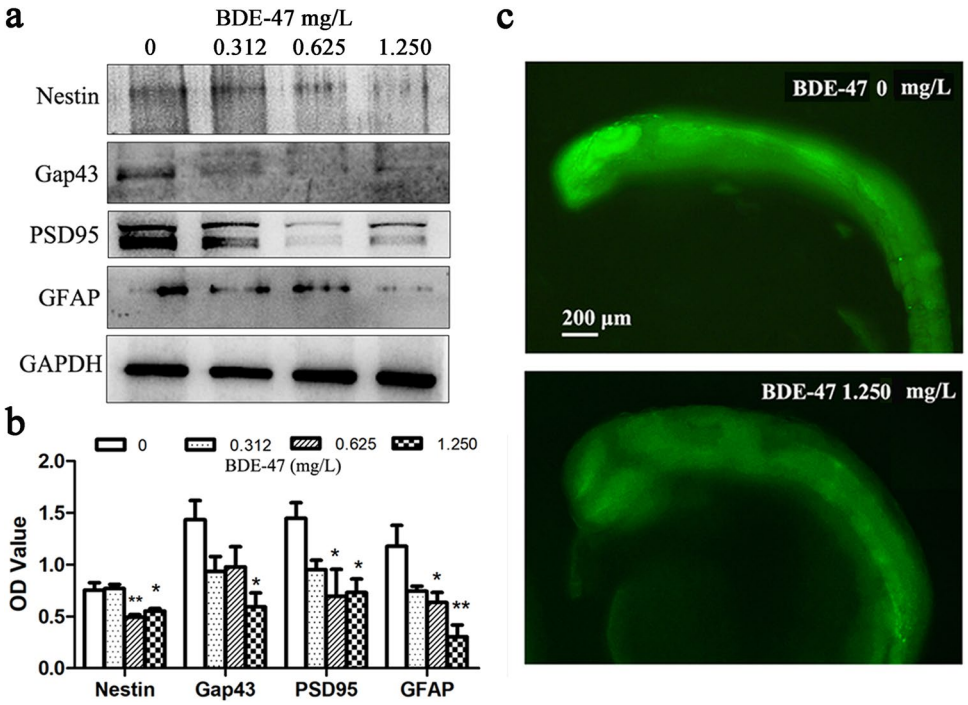

Fig. 5 Effects of BDE-47 on protein expression related to neural development at 24 hpf. Zebrafish embryos were exposed to various concentrations of BDE-47 beginning at 4 hpf. **a** Representative images of protein expression of Nestin, Gap43, PSD95, GFAP, and GAPDH. **b** The relative densitometry analysis of Nestin, GAP43, PSD95, and GFAP (normalized to GAPDH) ($n = 3$). **c** Representative images of Nestin immunofluorescence staining. FITC was used to visualize the nestin positive signal (green). * $P < 0.05$, ** $P < 0.01$ versus control group

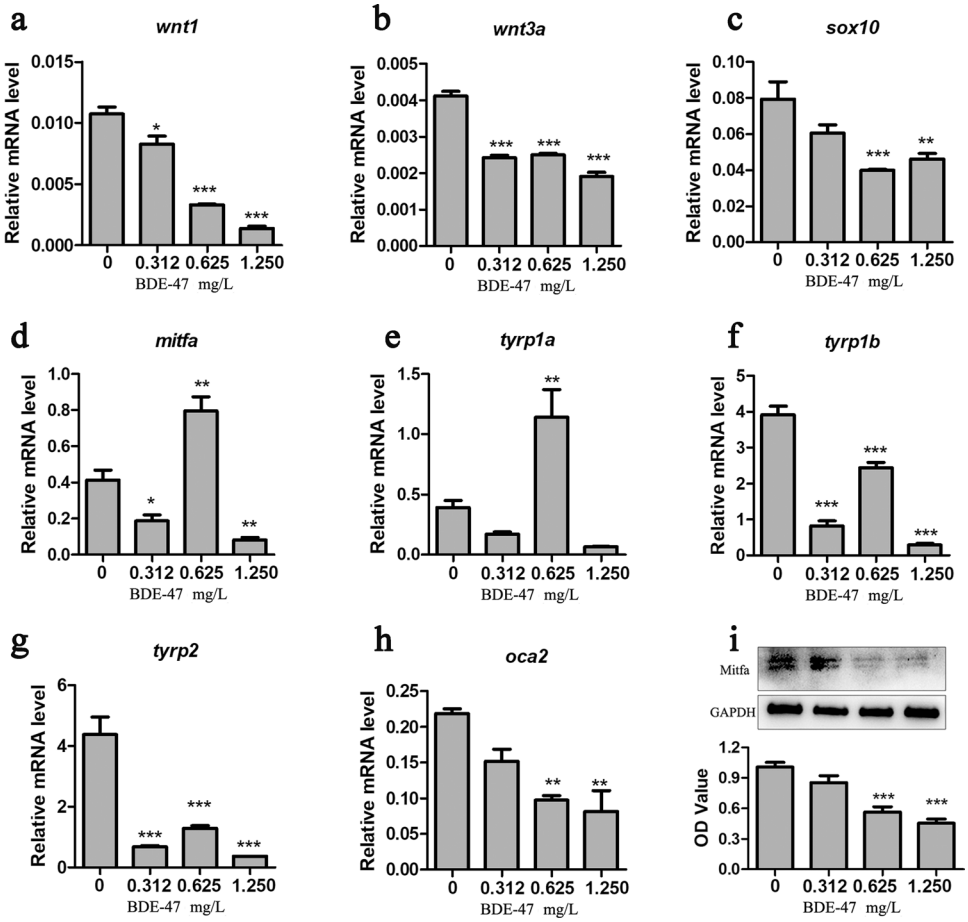

Fig. 6 Effects of BDE-47 on melanocyte differentiation and melanogenesis in zebrafish embryos at 72 hpf. Zebrafish embryos were exposed to various concentrations of BDE-47 beginning at 4 hpf. **a**–**h** Analysis of the gene expression of *wnt1*, *wnt3a*, *sox10*, *mitfa*, *tyrp1a*, *tyrp1b*, *tryp2*, and *oca2* (normalized to *β-actin*). **i** Immunoblotting and densitometry analysis of Mitfa. The relative densities were expressed as the ratio of Mitfa to GAPDH. ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control group

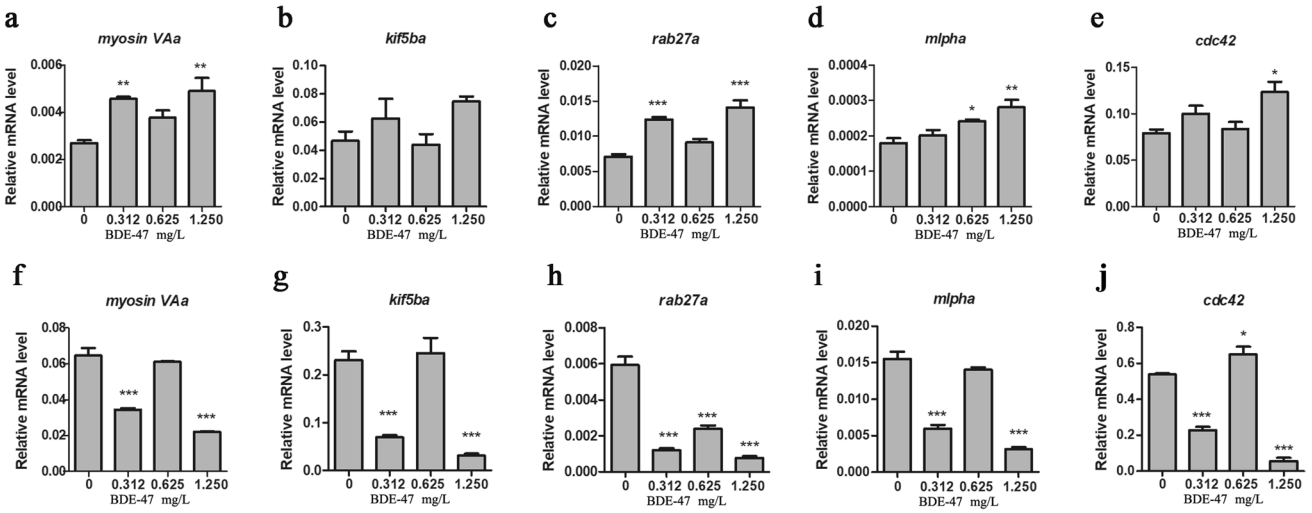

Fig. 7 Effects of BDE-47 on gene expression associated with intracellular transport process in 24 hpf and 72 hpf zebrafish embryos. Zebrafish embryos were exposed to various concentrations of BDE-47 beginning from hpf to 72 hpf. **a**–**e** Analysis of the gene expression of *myosin VAa*, *kif5ba*, *rab27a*, *mlpha*, and *cdc42* in 24 hpf zebrafish embryos (normalized to *β-actin*). **f**–**j** Analysis of the gene expression of *myosin VAa*, *kif5ba*, *rab27a*, *mlpha*, and *cdc42* in 72 hpf zebrafish embryos (normalized to *β-actin*). ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control group

and *cdc42* was increased due to BDE-47 exposure (Fig. 7c, *rab27a*: BDE-47$_{0.312\ mg/L}$ and BDE-47$_{0.625\ mg/L}$: $P < 0.001$; Fig. 7d, *mlpha*: BDE-47$_{0.625\ mg/L}$ $P < 0.05$, BDE-47$_{1.250\ mg/L}$: $P < 0.01$; Fig. 7e, *cdc42*: BDE-47$_{1.250\ mg/L}$ $P < 0.05$; versus control group). As shown in Fig. 7f-j, when the embryos were 72 hpf old, gene expression of *myosin VAa*, *kif5ba*, and *mlpha* was decreased in 0.312 and 1.250 mg/L groups ($P < 0.001$; versus control group), and the transcription levels of *rab27a* were decreased in all BDE-47 treated groups ($P < 0.001$; versus control group). The expression of cell division cycle 42 (*cdc42*) was inhibited in 0.312 mg/L or 1.250 mg/L BDE-47 group ($P < 0.001$; versus control group), but was abnormally enhanced in 0.625 mg/L BDE-47 group ($P < 0.05$; versus control group).

Discussion

This study assessed the neurotoxic effects of BDE-47 during zebrafish development (Fig. 8). Zebrafish coiling has been taken as a rapid and sensitive endpoint for neurotoxicity evaluation (Wang et al. 2018; Zheng et al. 2021). Several previous studies have established that BDE-47 exposure can stimulate zebrafish embryo coiling (Usenko et al. 2011; Chen et al. 2012; Tanaka et al. 2018). Consistently, our results in this study supported BDE-47 exposure evoked embryo coiling behavior at 1.250 mg/L by 24 hpf. Moreover, we revealed a novel inhibitory effect of BDE-47 on skin pigmentation. BDE-47 reduced melanin presence at the dorsal regions of the head and yolk sac at concentrations of 0.625 and 1.250 mg/L. PBDE concentrations in natures' water environment were generally low because they have high octanol/water partition coefficient values (Wang et al. 2021). But higher BDE-47 concentrations were required to induce phenotype changes in zebrafish during the developmental period. It has been confirmed the bioaccumulated PBDEs levels in zebrafish embryos following semi-static waterborne exposure (BDE-47 0.5-5 mg/L; DE-71 100 μg/L) fell into the same range as those environmental and human samples (Lema et al. 2007; Chen et al. 2012; Wang et al. 2022), suggesting the concentrations used in this study can generate a similar body burden relevant to environmental samples.

We then performed biochemical and protein expression assays to explore the potential mechanisms of BDE-47-induced neurobehavior changes in 24 hpf embryos. The dopamine system is an important target of PBDEs (Wang et al. 2016). In this study, dopamine level was increased by 1.250 mg/L BDE-47. Other study revealed dopamine content declined in zebrafish after exposure to 1 mg/L BDE-47 from 57 to 60 hpf (Tanaka et al. 2018). Since zebrafish dopaminergic neurons emerge at about 22 hpf, we presume the effect of BDE-47 on dopamine production is complicated and possibly related with other factors, such as exposure timing and duration. The 5-HT level was also enhanced by BDE-47 in this study, which is in line with the finding in the mice model (Ji et al. 2019b). A disrupted 5-HT system was implicated in abnormal zebrafish coiling behavior (Wang et al. 2018), thus we presume that increased 5-HT may be a cause of BDE-47-induced neurotoxicity. As a neural stem/progenitor cell marker, nestin is required for the survival, renewal, and proliferation of neural progenitor cells (Park et al. 2010). We found BDE-47 significantly decreased embryos' nestin

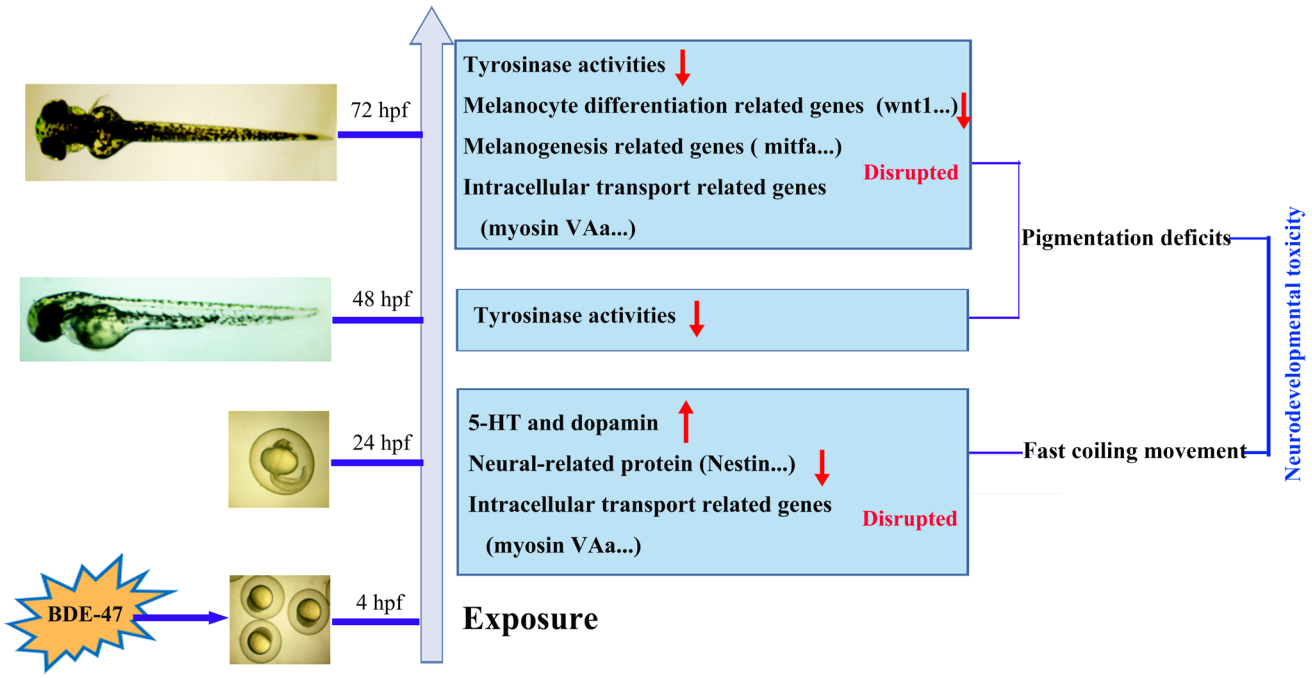

Fig. 8 Schematic diagram of neurobehavioral and morphological toxicity phenotypes and mechanisms of BDE-47 during zebrafish development

levels, indicating the early development procedure such as proliferation and differentiation of neural stem/progenitor cells may be inhibited. Gap43, PSD95, and GFAP are well-known markers for the nervous system that are critical for neurodevelopment, and they are common molecular targets of BDE-47 (Wang et al. 2011; Dingemans et al. 2007; Kodavanti et al. 2015). Similarly, we found BDE-47 descended their expression in 24 hpf embryos, suggesting the embryos' neurogenesis was also disturbed. Though previous toxicological data established BDE-47 evoked fast coiling in zebrafish before 27 hpf, the mechanism was usually investigated later (such as at 48 hpf, 72 hpf or 128 hpf) (Tanaka et al. 2018; Wang et al. 2018; Zheng et al. 2021). Here, our results obtained from the 24 hpf zebrafish embryos will provide additional information for the neurotoxicity of BDE-47.

The neural crest is a population of cells arising from the dorsal neural tube that develop initially in parallel with central nervous system precursors and then generate a variety of cell types, including neurons and glia of the peripheral nervous system and pigment cells. Neural crest has been long utilized for neurodevelopmental toxicity testing of various environmental chemicals (Zimmer et al. 2012). Zebrafish neural crest produces three distinct kinds of pigment cells, and melanocytes are the first to appear in embryos for melanin synthesis. Melanocytes share a common embryologic origin, signaling molecules, and pathways with the nervous system neurons, and the similarities make melanocytes an attractive model system for neurological disease investigation (Yaar and Park, 2012; Wang et al. 2020). Transcription factor mitf, by regulating the transcription of *tyrosinase* and *tyrp*, functions as a master regulator of multiple molecular cascades that control melanocyte survival, proliferation, and melanogenesis (Cheli et al. 2010). In zebrafish embryos, *mitf* ortholog *mitfa* is transcriptionally activated by sox10 via binding directly to its promoter in neural crest cells (Elworthy et al. 2003). Functionally, wnt signaling, especially wnt1 and wnt3 signaling, can facilitate the differentiation of neural crest cells into melanocytes through the induction of mift expression (Dorsky et al. 1998). The biosynthesis of melanin was critically regulated via a tyrosinase-dependent way, during which tyrosinase serves as a rate-limiting enzyme and acts with tyrp1 and tyrp2 to promote melanogenesis (Olivares and Solano, 2009). As a strong determinant of the eumelanin content in melanocytes, oca2 modulates the activity of tyrosinase (Manga et al. 2001). Here, BDE-47 descended zebrafish tyrosinase activity, but did not affect the mushroom tyrosinase activity, suggesting BDE-47 inhibited zebrafish tyrosinase activity in an indirect manner. Moreover, BDE-47 restrained expression of *wnt1*, *wnt3*, *sox10*, *tyrp1b*, *tyrp2*, and *oca2* genes in 72 hpf embryos. Though the effects of BDE-47 concentrations on the transcriptional activity of *mitfa* and *tyrp1a* were complicated, their protein levels were decreased by BDE-47 in zebrafish embryos (Supplementary Fig. 1). These alternations might be the potential reasons for melanin accumulation deficits in zebrafish embryos and increased gene expression of *mitfa* and *tyrp1a* in 0.625 mg/L BDE-47 group may be a compensatory mechanism for defective melanogenesis. To

date, there is little literature available concerning the effects of PBDEs on neural crest-derived melanocyte. Our results clearly indicated BDE-47 hindered melanocyte development and melanin biosynthesis, which may extend our understanding of the neurotoxicity of PBDEs. Because zebrafish melanocytes become distinguishable beginning at approximately 24 hpf (Lister, 2002), we also determined the gene expression described above in 24 hpf zebrafish. However, no significant changes were observed (except *wnt1*) (data not shown), indicating that BDE-47 affected melanocyte development and melanogenesis mainly at 72 hpf.

Myosin VA and kif5b are the two most prevalent motor proteins in nerve cells and melanocytes, responsible for actin- and microtubule-dependent transport, respectively. Myosin VA-mediated trafficking contributed to the dendritic architecture and excitatory synapse development (Ultanir et al. 2014; Lisé et al. 2009; Yoshii et al. 2013). In melanocytes, myosin VA expression inhibition resulted in fewer and shorter dendritic processes in appearance (Edgar and Bennett, 1999), suggesting the involvement of myosin VA in establishing the dendritic morphology of melanocytes. Kif5b-mediated transport is critical for neuronal developmental processes, such as neuronal polarity (Jacobson et al. 2006), presynaptic assembly and synaptogenesis (Cai et al. 2007), and synaptic plasticity (Zhao et al. 2020). It is also known that myosin VA and kif5b are essential for melanosome dispersion (Wu et al. 2002; Hara et al. 2000). Following melanin biogenesis, Kif5b regulates outward melanosome transport along microtubules, while Myosin VA, mlpha, and Rab27a form a ternary complex to drive the movement of melanosomes along actin filaments towards the cell extensions (Wu et al. 2002). Besides, Cdc42 regulates dendritic and axonal morphogenesis in neural cells (Scott et al. 2003; Schwamborn and Püschel, 2004). It also functions in melanocyte morphology and melanosome transfer (Ando et al. 2012). Our results revealed that at 24 hpf, BDE-47 markedly enhanced the expression of *myosin VAa*, *rab27a*, *mlpha*, and *cdc42*, whereas the expression of *kif5ba* was unchanged significantly. These up-regulations may be stress responses of the body to keep normal actin-based transport process to the noxious stimulation. At 72 hpf, the transcription of *myosin VAa*, *kif5ba*, *rab27a*, and *mlpha* were inhibited by BDE-47. Though the gene expression of *cdc42* was complicated, its protein level was decreased due to BDE-47 exposure (Supplementary Fig. 1). These findings suggested the intracellular transport process was disrupted by BDE-47, and the actin-based transport process was impacted prior to the microtubule-dependent transport. Together with a recent report showing the inhibitory effects of BDE-47 on the dendritic length and pine density of the prefrontal cortex (Li et al. 2021), we would like to propose that disturbed intracellular transport process might ultimately affect cell morphogenesis, synaptic plasticity, and melanosome dispersion, which may be a potential explanation for the neurobehavioral abnormalities and pigmentation deficit in zebrafish embryos during development.

In fish melanophores, melanosomes can either aggregate around the cell centre or disperse uniformly throughout the cell. Previous studies suggested the melanosomes aggregate impacts fish skin color in the developmental stage (Mueller and Neuhauss, 2014), and whether the melanosomes aggregate process was also disrupted by BDE-47 is still unknown. Although the biochemical and molecular changes in zebrafish have been investigated, the direct evidence for phenotypic changes of central or peripheral nervous system was not provided. These are limitations of this study, which are worthy of further investigation.

Conclusion

In the present study, zebrafish was utilized to explore the phenotypes and mechanisms underlying neurodevelopmental disorders of BDE-47. These results offer new insights into the neurodevelopmental toxicity of PBDE and facilitate the comprehensive evaluation of neurotoxicity in embryos.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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