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Research article

Early-Life Benzo[a]Pyrene Exposure Causes Neurodegenerative Syndromes in Adult Zebrafish (*Danio rerio*) and the Mechanism Involved

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ABSTRACT

There is increasing recognition of the importance of early-life environmental exposures in health disorders at later-life stages. The aim of this study was to evaluate whether early-life exposure to benzo[a]pyrene (BaP) could induce neurodegenerative syndromes at later-life stages in zebrafish. Embryos were exposed to BaP at doses of 0, 0.05, 0.5, 5, and 50 nM from early embryogenesis to 96 h post-fertilization (hpf), then transferred to clean water and maintained for 365 days. We found that BaP decreased locomotor and cognitive ability, neurotransmitter levels of dopamine, 3,4-dihydroxyphenylacetic acid and norepinephrine; and induced loss of dopaminergic neurons and resulted in neurodegeneration. Additionally, BaP increased amyloid β protein and cell apoptosis in the adult zebrafish brain. Further, DNA methyltransferase 1 (DNMT1) and DNMT3a were up-regulated in 96 hpf larvae and the adult brain. MeDIP-sequencing data of the 96 hpf larvae identified 235 differentially methylated genes in promoter, with the fold change > 1.5. Guanylate cyclase 2F (*gucy2f*) and dopamine receptor D4 related sequence (*drd4-rs*) were hypermethylation promoters, whereas zinc finger C4H2 domain (*zc4h2*) was a hypomethylation promoter in 96 hpf larvae and the adult brain. The mRNA levels of *gucy2f* and *drd4-rs* were down-regulated, and *zc4h2* was up-regulated. Our findings suggested that the lasting modifications of DNA methylation were associated with neurodegenerative syndromes in adult zebrafish as a result of early-life BaP exposure.

Key words: benzo[a]pyrene; early-life stages; zebrafish; neurodegenerative disease; DNA methylation.

Early life plays a key role in the health and development of an organism. Human epidemiologic studies provide evidence that prenatal exposure to airborne polycyclic aromatic hydrocarbons (PAHs) affect the health of children. Prenatal exposure to PAHs can decrease birth length, weight, and head circumference in newborns, and affect children's IQ and behavior adversely in later life (Perera et al., 1999, 2009, 2012). Transplacental exposure to PAHs from maternal inhalation produces DNA damage in the developing fetus (Jedrychowski et al., 2013). The early child wheeze is positively associated with prenatal PAHs exposure (Jedrychowski et al., 2010). In animal studies, exposure to PAHs at early stages can cause later troubles on behavior and neurobiological in rats (Crépeaux et al., 2012, 2013), and affect the growth, reproductive ability and behaviors in zebrafish (*Danio rerio*) (Vignet et al., 2014). There are also other chemicals can have persistent effects through life (e.g., Aschengrau et al., 2015; Modgil et al., 2014). These studies indicate the importance of early-life environmental factor exposures in health disorders in later-life stages.

Whereas numerous studies are available for the long-term adverse effects of early-life exposure to environmental toxins, the mechanisms of these long-term adverse effects are not well elucidated. Epigenetic programming may be critically affected, with consequences due to certain early-life exposures being

manifested throughout the lifespan (Heijmans et al., 2008; Pinkerton and Joad, 2006). The most common observations reported in the epigenetic inheritance process are DNA methylation, histone modification, and noncoding small RNAs (Bonasio et al., 2010). DNA methylation, the hallmark of epigenetic modifications, involves the addition of a methyl group to the 5′ position of a cytosine within a cytosine-guanine dinucleotide (CpG). The process of DNA methylation is mediated by three main DNA methyltransferases (DNMTs): DNMT1, DNMT3a, and DNMT3b in mammals. DNMT1 is responsible for maintaining the global DNA methylation patterns after cell cycling, whereas DNMT3a and DNMT3b enzymes are required for *de novo* methylation and maintain methylation patterns to a lesser degree (Bestor, 2000; Hermann et al., 2004). The methyl groups are usually added to the cytosine residues which are adjacent to guanine nucleotides (CpG sites) in the genes (Syed et al., 2013). In promoters, the addition of methyl groups typically results in transcriptional silencing and, by contrast, the removal of methyl groups results in the activation of that gene (Sahu, 2012; Syed et al., 2013). Alterations in DNA methylation are the most common type of epigenetic change that is found upon prenatal exposure to hazardous environmental agents (Lo and Zhou, 2014).

Accumulating evidences indicate that environmental exposure could be the cause of neurodegenerative diseases, such as Parkinson's disease (PD) and Alzheimer's disease (AD) (Baltazar et al., 2014; Cannon and Greenamyre, 2011; Levesque et al., 2011). PD is characterized by progressive loss of dopaminergic neurons in the substantia nigra, subsequent decrease of dopamine (DA) concentrations, and motor symptom disorder (Braak et al., 2004; Forno, 1996). AD is characterized by the progressive loss of memory, the presence of neurodegeneration, and the accumulation of Aβ plaques and neurofibrillary tangles (Duyckaerts et al., 2009; Voisin and Vellas, 2009).

Benzo[a]pyrene (BaP), a representative PAH, is a known human carcinogen, and its neurotoxicity has been extensively studied (Chen et al., 2011; Gao et al., 2015; Li et al., 2012). Our previous study found that 0.05–50 nM BaP exposure during early-life stages can cause symptoms similar to cardiac hypertrophy in adult zebrafish (Huang et al., 2014a). As far as we know, there are just a few studies to describe well the possible neurotoxicity of early-life exposure to BaP on adult zebrafish and the mechanism involved (Brown et al., 2016). To expand upon our previous work, we aimed to explore whether early-life exposure to BaP may lead to neurodegenerative syndromes at later-life stages in zebrafish. Our results provide clues to the mechanism(s) by which BaP exposure during early-life stages influences fish health at later-life stages.

MATERIALS AND METHODS

Chemicals. BaP (>99% purity; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethylsulfoxide [DMSO (>99% purity)] to reach stock solutions of 0.001, 0.01, 0.1, and 1 mg/ml. The antibody of tyrosine hydroxylase (TH) was purchased from Millipore (Billerica, MA). The antibodies of beta-actin, neuronal marker (NeuN), DNMT1, DNMT3a, and DNMT3b were purchased from Abcam (Cambridge, MA). The enzyme-linked immunosorbent assay (ELISA) kits of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and norepinephrine (NE), and Protein Easy kit were purchased from Nanjing Jiancheng (Nanjing, China). The TUNEL kit was purchased from Roche Applied Science (Mannheim, Germany).

Zebrafish exposure and culture. All fish experiments in this study were approved by the Animal Ethics Committee of Xiamen University and were carried out in accordance with their ethical standards. Wild-type TU zebrafish embryos [from 0.5 to 1.5 h postfertilization (hpf)] were exposed to four concentrations of BaP: 0.05, 0.5, 5 and 50 nM. The actual concentrations were measured as 0.05 ± 0.004, 0.57 ± 0.018, 5.57 ± 0.217, and 53.93 ± 2.511 nM, following the published method (Huang et al., 2014a). About 100 embryos were cultured in 100 ml BaP solution (four replicates) in each glass petri dish with a diameter of 14.5 cm and a height of 2.5 cm, and the same number of embryos was used as controls. BaP exposure was performed in zebrafish medium (3.5 g/l NaCl, 0.05 g/l KCl, 0.05 g/l $NaHCO_3$, and 0.05 g/l $CaCl_2$). The BaP solutions were changed twice daily. Similar criteria applied to the control group, which received an equal volume of the DMSO (504 μl/l). At 96 hpf, the surviving larvae from each group were transferred to clean water (approximately 600 ml per fish) and maintained for 365 days. In each group, the four replicate dishes were accordingly transferred to four individual tanks and then maintained during adult rearing. The survival rate (%) at 365 days were 10.67 ± 0.88, 11.00 ± 0.76, 9.67 ± 1.30, 10.39 ± 0.77, and 9.00 ± 1.00 in the control and BaP-treated groups (0.05, 0.5, 5, and 50 nM), respectively. All fish were held in the water (water quality: dissolved oxygen 7–8 mg/l, pH 7.2–7.3, conductivity 500–530 Ω/cm) at $28 \pm 1\,^{\circ}C$ under a 14:10 h light:dark cycle.

Behavior tests. The novel tank test was used to analyze the locomotor activity of adult zebrafish. The T-maze test was used to assess the learning and memory ability of adult zebrafish. The detail of these two tests has been described previously (Gao et al., 2015).

In the novel tank test, the behavior of each fish was tested alone and was video-recorded using a side-view camera for 6 min ($n = 15$–20 fish per group) in a 1.5-l trapezoidal tank (28 cm top × 23 cm bottom × 15 cm height × 7 cm width; Aquatic Habitats, Apopka, FL). The total moving distance and velocity were analyzed by Ethovision XT7 software (Noldus IT, Wageningen, Netherlands). In the T-maze test, each fish was placed at the beginning of the 68 cm long arm (6 cm wide and 15 cm deep), and then had a choice of two 47 cm short arms (6 cm wide and 15 cm deep), one of which opens into a large reservoir (576 cm^2) which was 20 cm deep. This reservoir contains artificial grass and cobblestones that offers a favorable habitat for zebrafish, and most of the tested fish spent the majority of their time in this compartment once they had found it. The time taken by zebrafish to first encounter the reservoir and stay for at least 20 s was recorded ($n = 15$–20 fish per group). The fish were given a second trial after 3 h and a third trial at 24 h.

Immunohistochemical procedure. The brains were fixed, paraffin-embedded, and cut into 5 μm sections and mounted on slides. The immunohistochemical procedure was performed, as previously described (Gao et al., 2015). The antibodies used were mouse anti-TH (1:400) and rabbit anti-NeuN (1:100). The sections were incubated with diaminobenzidine, which resulted in a brown immunoreaction, and then counterstained with Harris hematoxylin. Next, the sections were examined using a light microscope (Olympus BX41). IPwin software was used to quantify the immunoreactive cells from images of the sections ($n = 3$–4 fish per group, 2–3 sections per fish).

TUNEL assay. TUNEL staining was performed based on previous studies with minor modifications (Gavrieli et al., 1992). The

sections were incubated with the TUNEL reaction mix containing fluorescein-labeled dUTP and terminal deoxynucleotide transferase enzyme. The slides were incubated with diaminobenzidine to produce brown staining, and then lightly counterstained with Harris hematoxylin. Sections held with DNase I (grade I, 0.5 mg/ml in 50 mmol/l Tris-HCl, pH 7.4, 1 mg/ml BSA) for 10 min at room temperature were used as the positive controls and the TUNEL mixture was omitted in the negative controls. Sections were examined using a light microscope (Olympus BX41). IPwin software was used to quantify the TUNEL-positive apoptotic cells from images of the sections ($n = 3$–4 fish per group, 2–3 sections per fish).

Aβ42, DA, DOPAC, and NE assay. The zebrafish brains were homogenized in 0.9% ice-cold NaCl with a mass-to-volume ratio of 1:9. The homogenate was centrifuged and the levels of amyloid β (Aβ42), DA, DOPAC, and NE in the supernatant were determined using ELISA kits, according to the protocol of the manufacturer. Aβ42, DA, DOPAC, and NE are markers of several neurodegenerative diseases such as AD and PD (Dunnett and Björklund, 1999; Duyckaerts et al., 2009; Goldstein et al., 2012; Rommelfanger and Weinshenker, 2007). The amount of protein in the supernatant was measured using the Protein Easy kit, according to instructions of the manufacturer ($n = 4$–5 per treatment).

Western blotting assay. Total protein extracted from larvae and dissected brains [$n = 3$–4 adult brains per treatment and $n = 3$–4 tubes larvae (30 larvae per tube) per treatment] with SDS-sample buffer were applied for Western blotting using the method of previous studies (Cai et al., 2009). The protein band, specifically bound to the primary antibodies, was detected using an anti-rabbit/mouse IgG-AP-linked antibody.

Real-time quantitative PCR. Total RNA was extracted from zebrafish larvae and brains [$n = 4$–6 adult brains per treatment and $n = 4$–6 tubes larvae (30 larvae per tube) per treatment] using TRIzol (Invitrogen, Carlbad, CA), following the protocols of Weston et al. (1989). The Brilliant SYBR Green real-time quantitative PCR (qPCR) reagent kit (Stratagene) was used for the qPCR on an Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA), following the protocol of the manufacturer. The primers were designed and are listed in Table 1. The standard curves of the glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), guanylate cyclase 2F (*gucy2f*), zinc finger C4H2 domain (*zc4h2*), and dopamine receptor D4-related sequence (*drd4-rs*) mRNA in the present study gave PCR efficiencies of 91.3%, 88.3%, 72.8%, and 97.0%. Our previous study suggests that BaP exposure cannot alter the expression of *gapdh* (Huang et al., 2014b). Therefore, in this study, the gene expression levels were normalized to zebrafish *gapdh*. The $2^{-\Delta\Delta CT}$ method was further used to calculate the relative expression of target gene mRNA (Livak and Schmittgen, 2001).

Analyses of MeDIP-sequencing data. The 96-hpf larvae DNA samples from the control and 50 nM groups were sequenced using the Illumina platform. Three biological replicates were sequenced for each group. With MeDIP-sequencing analysis, statistically significant MeDIP-enriched regions (peaks) detected by MACS v2 (Model-based Analysis of ChIP-Seq) were identified by comparison to input background. We selected a minimum absolute fold difference of 1.5 between the controls and exposed data sets (*P* value threshold of 0.001). Promoter-associated differentially methylated regions were annotated with UCSC RefSeq database. The clean reads were aligned to zebrafish genome (UCSC danRer7). The MeDIP-sequencing data have been submitted to GEO and the accession number is GSE87192.

TABLE 1. List of Primers Used for Gene Expression and DNA Methylation

Target Gene	PCR Step	Primer Sequence
gapdh	qPCR	F: 5′-GACGCTGGTGCTGGTATTGCT-3′
		R: 5′-CTACTCCTTGGAGGCCATGTGT-3′
drd4-rs	qPCR	F: 5′-CTCTCATCCTTATTATCATTTTGGG-3′
		R: 5′-ACATCCATAGTCATCAGGGCAT-3′
gucy2f	qPCR	F: 5′-GAAACCATTGGAGATGCCTATA-3′
		R: 5′-TCCATACGAGAGGCGGTG-3′
zc4h2	qPCR	F: 5′-GACCCTCGGCTTACAGAGACTT-3′
		R: 5′-CCGGTGGATTTGTTGGTGAC-3′
drd4-rs	BSP	F: 5′-TTAATTAATTTAGAATAATTTGTGGTTGAA-3′
		R: 5′-TTAAAAAAACATACAAACTCCACACA-3′
gucy2f	BSP	F: 5′-GTTTTATGGTAGTTTTTTAGTTATTTTT-3′
		R: 5′-TAATACTACTACATTACAATATCCTAAATT-3′
zc4h2	BSP	F: 5′-AATTTATTTATAGTATATGTTTTTGAATT-3′
		R: 5′-AACTCCTAAATTTTTCTTTCTTAAATAATA-3′

Bisulfite sequencing. Genomic DNA was extracted from larvae and dissected brains using the EasyPure Genomic DNA Kit (TransGen, Beijing, China), following the protocols given in the kit. Bisulfite treatment was carried out using the EZ DNA Methylation-Direct™ kit (Zymo Research, USA), following the instructions from the manufacturer. Modified DNA was amplified, and PCR products were gel-purified and sub-cloned into a pMD19-T vector system (TaKaRa, Dalian, China). Ten colonies were sequenced to assess the degree of methylation at each differential methylated gene (Cui et al., 2015). The primers of bisulfite treatment are listed in Table 1.

Statistical analyses. The data were statistically analyzed with one-way of variance (ANOVA) followed by the Duncan test via SPSS 17.0 software (SPSS Inc., Chicago, IL). Data were presented as means ± SE. Statistical differences were considered significant at the $P < .05$ level.

RESULTS

BaP Impaired Locomotor Activity in Adult Zebrafish

BaP exposure during the early-life stage affected the swimming behavior of adult zebrafish. The total distance travelled and the velocity of adult zebrafish were significantly decreased in 5 and 50 nM groups (1.35- and 1.41-folds), compared with the control (Figure 1). There were no significant differences among the five groups in anxiety-related behaviors including immobility duration, latency to top and the percent of time spent at the bottom (Supplementary Figure 1).

BaP Reduced Dopaminergic Neurons in Adult Zebrafish

Dopaminergic neurons were detected in the adult zebrafish brain using immunostained with TH antibody, which has specificity in zebrafish brain (Kaslin and Panula, 2001). TH is the rate-limiting enzyme in the synthesis of dopamine, and TH antibody is usually used to detect the dopaminergic neurons (Hökfelt et al., 1976; Romeo et al., 2013; Tönges et al., 2012). As shown in the representative pictures, BaP reduced the number of TH-positive neurons in the telencephalon (Figure 2A), and

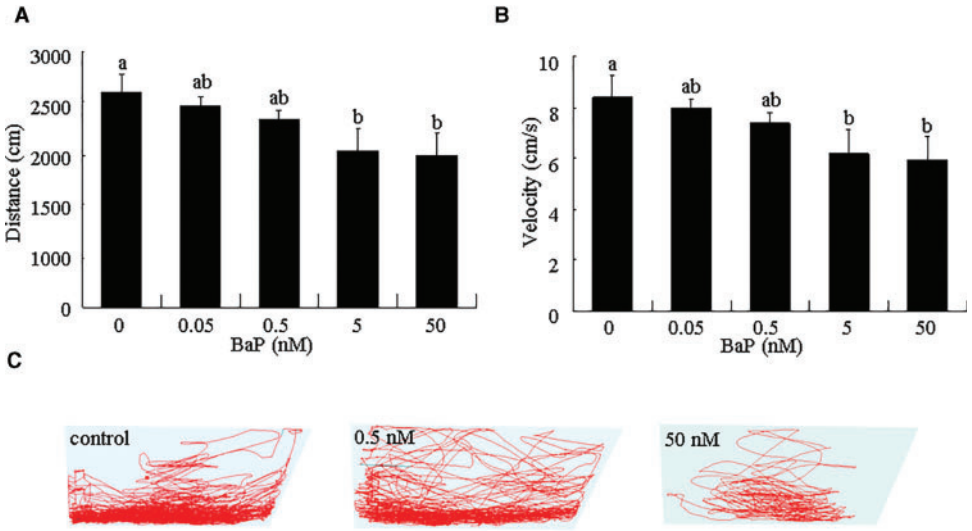

FIG. 1. BaP decreased the locomotor activity in adult zebrafish. The total distance (A) and mean velocity (B) of the zebrafish. Representative traces of zebrafish showed in (C). Data (mean ± SE) are analyzed by one-way ANOVA followed by the Duncan test ($n = 15–20$). Treatments not sharing a common letter are significantly different at $P < .05$.

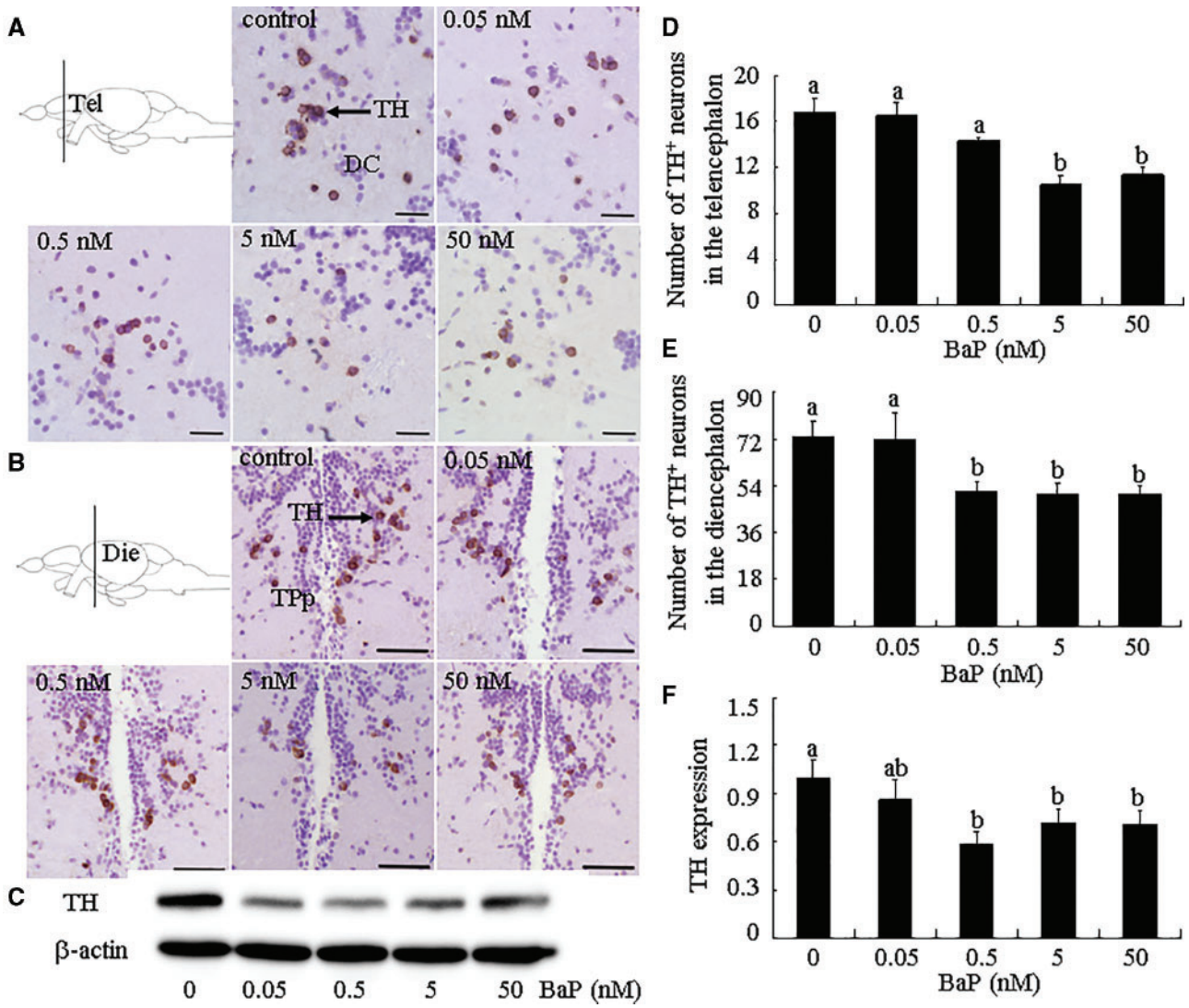

FIG. 2. BaP induced the reduction of dopaminergic neurons in the adult zebrafish brain. Representative sections of TH-immunoreactivity showed in the telencephalon (A, D) and diencephalon (B, E). Changes in protein level of TH (C, F). Tel, telencephalon; Dc, central zone of dorsal telencephalic area; Die, diencephalon; TPp, periventricular nucleus of posterior tuberculum. TH-positive neurons are indicated by the arrows. Data (mean ± SE) are analyzed by one-way ANOVA followed by the Duncan test ($n = 3–4$ zebrafish per group, 2–3 sections per zebrafish). Treatments not sharing a common letter are significantly different at $P < .05$. Scale bar: 50 μm.

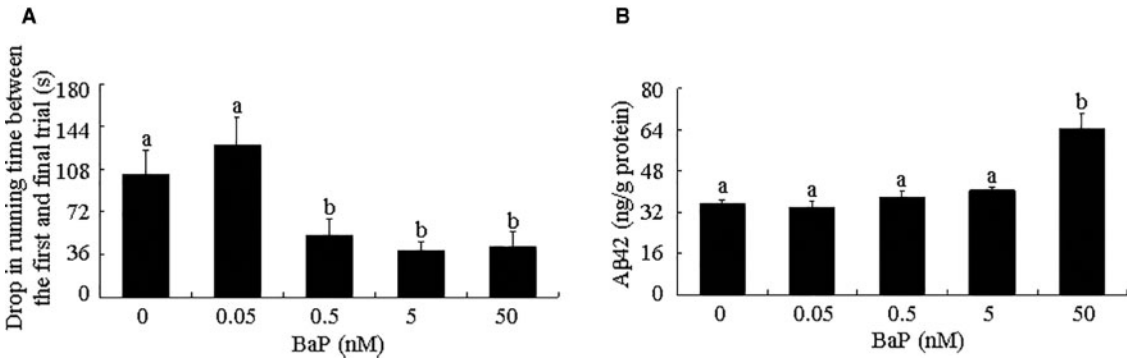

FIG. 3. BaP impaired cognitive ability and elevated Aβ42 in the adult zebrafish brain. The cognitive ability of adult zebrafish was tested using T-maze (A, $n = 15–20$). The level of Aβ42 protein in whole brain was measured using ELISA (B, $n = 4–5$). Data (mean ± SE) are analyzed by one-way ANOVA followed by the Duncan's test. Treatments not sharing a common letter are significantly different at $P < .05$.

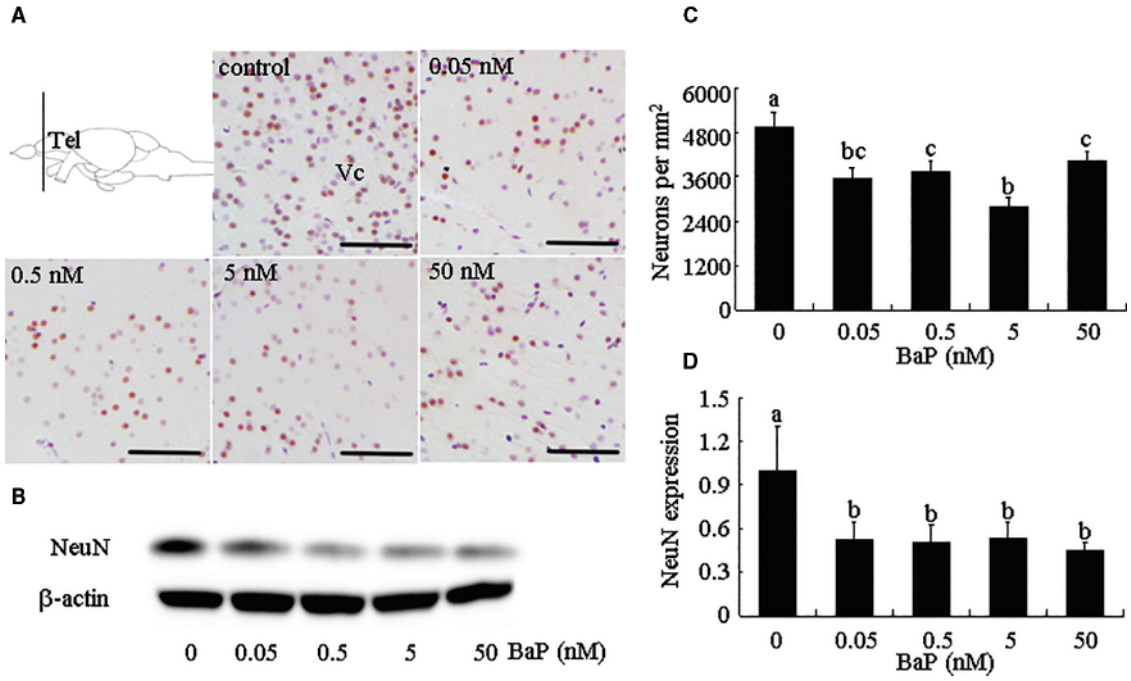

FIG. 4. BaP induced neurodegeneration in adult zebrafish. Representative sections of neuronal density (number of neurons per mm^2) showed in the telencephalon after NeuN staining (A, C). Changes in protein level of NeuN (B, D). Tel, telencephalon; Vc, central nucleus of ventral telencephalic area. Data (mean ± SE) are analyzed by one-way ANOVA followed by the Duncan test ($n = 3–4$ zebrafish per group, 2–3 sections per zebrafish). Treatments not sharing a common letter are significantly different at $P < .05$. Scale bar: 50 μm.

diencephalon (Figure 2B) of adult zebrafish. In the telencephalon, a significant reduction in the number of TH-positive neurons was observed in the 5 and 50 nM groups (1.59- and 1.48-folds) compared with the control (Figure 2D). In the diencephalon, the number of TH-positive neurons was decreased significantly in the 0.5, 5, and 50 nM groups (1.41-, 1.43-, and 1.43-folds) compared with the control (Figure 2E). We also examined the protein expression level of TH in the whole brain of adult zebrafish. Our results showed that a significant decrease of TH expression was observed in the 0.5, 5, and 50 nM groups (1.71-, 1.38-, and 1.40-folds) compared with the control (Figs. 2C and F).

BaP Impaired Cognitive Ability and Elevated Accumulation of Aβ42 in Adult Zebrafish

In the T-maze test, the learning and memory ability of adult zebrafish was reduced after BaP exposure. The drop in time required to reach the reservoir between the initial trial and final trial was decreased significantly in the 0.5, 5, and 50 nM groups (1.96-, 2.64-, and 2.42-folds) compared with the control (Figure 3A).

The amount of Aβ42 accumulation in the whole brain of adult zebrafish was significantly increased in the 50 nM BaP group (1.80-fold) compared with the control (Figure 3B).

BaP Induced Neurodegeneration in Adult Zebrafish

The number of neurons was quantified to analyze the neuronal loss in the adult zebrafish telencephalon after NeuN staining. Zebrafish treated with BaP during the early-life stage caused significant decreases of the neuronal density (number of neurons per mm^2) in the 0.05, 0.5, 5, and 50 nM groups (1.38-, 1.31-, 1.76-, and 1.22-folds) compared with the control (Figs. 4A and C). We also detected the protein expression level of NeuN in the

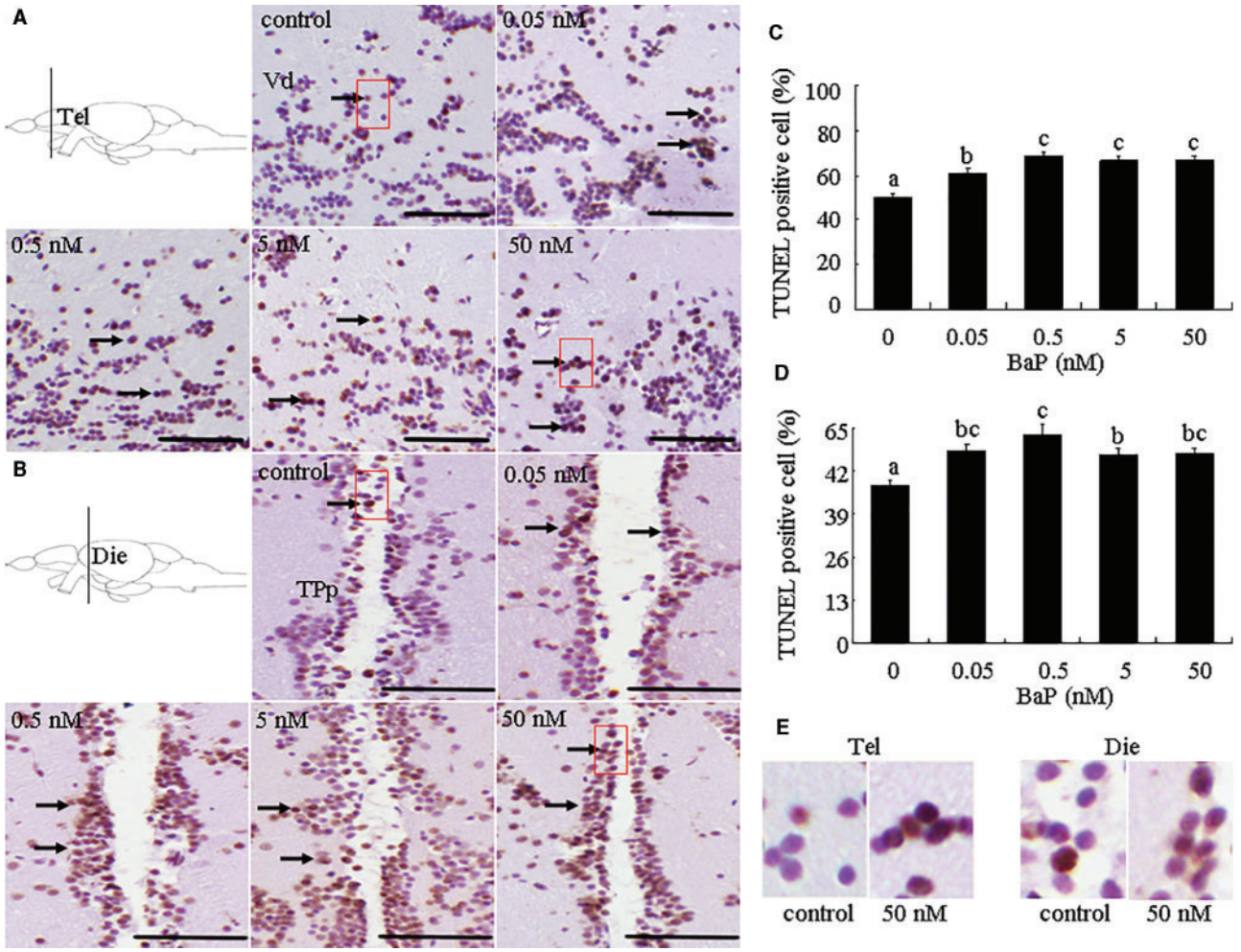

FIG. 5. BaP increased apoptosis in the adult zebrafish brain. Representative sections of TUNEL staining showed in the telencephalon (A, C) and diencephalon (B, D). Partial enlarged views of the *box* from both control and 50 nM groups showed in the telencephalon and diencephalon (E). The percentage of positive cells was calculated as apoptotic cells/total cells × 100% in each section. TUNEL positive cells are indicated by the arrows. Tel, telencephalon; Vd, dorsal nucleus of ventral telencephalic area; Die, diencephalon; TPp, periventricular nucleus of posterior tuberculum. Data (mean ± SE) are analyzed by one-way ANOVA followed by the Duncan test ($n = 3$–4 zebrafish per group, 2–3 sections per zebrafish). Treatments not sharing a common letter are significantly different at $P < .05$. Scale bar: A, 50 μm; B, 20 μm.

whole brain of adult zebrafish. Our results showed that a significant decrease of NeuN expression was observed in the 0.05, 0.5, 5, and 50 nM groups (1.89-, 1.96-, 1.85-, and 2.21-folds) compared with the control (Figs. 4B and D).

BaP Increased Cell Apoptosis in the Adult Zebrafish Brain

We detected apoptotic cells by TUNEL staining in the brain of adult zebrafish. The number of TUNEL positive cells was elevated in the telencephalon and diencephalon (Figs. 5A and B). The percentage of TUNEL-positive cells was significantly elevated in the 0.05, 0.5, 5 and 50 nM groups in the telencephalon (1.22-, 1.37-, 1.33-, and 1.35-folds) and diencephalon (1.22-, 1.32-, 1.19-, and 1.20-folds) compared with the control (Figs. 5C and D).

BaP Reduced the Concentrations of DA, DOPAC, and NE in Adult Zebrafish Brain

The concentrations of DA and its metabolites, DOPAC and NE, were analyzed in the whole brain of adult zebrafish. Our results showed that the levels of DA (1.20-, 1.15-, 1.26-, and 1.26-folds) and DOPAC (1.22-, 1.28-, 1.24-, and 1.43-folds) were significantly decreased in the 0.05, 0.5, 5, and 50 nM groups compared with the control (Figure 6). However, there was no significant change

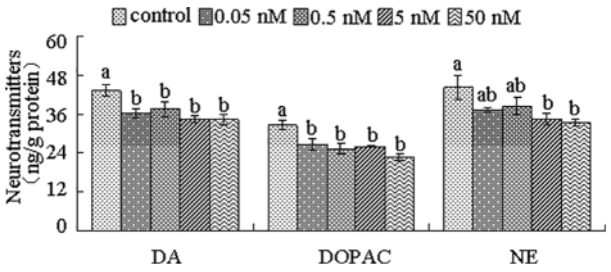

FIG. 6. BaP induced the reduction of neurotransmitters in the adult zebrafish brain. Data (mean ± SE) are analyzed by one-way ANOVA followed by the Duncan test ($n = 4$–5). Treatments not sharing a common letter are significantly different at $P < .05$.

in the ratio of DOPAC/DA (Supplementary Fig. 2). The level of NE was significantly decreased in the 5 and 50 nM groups (1.28- and 1.33-folds) compared with the control (Figure 6).

BaP Elevated DNMTs in 96 Hpf Larvae and the Adult Zebrafish Brain

Western blotting analysis showed that the expression of DNMT1 was significantly increased in the 0.05, 0.5, 5, and 50 nM groups

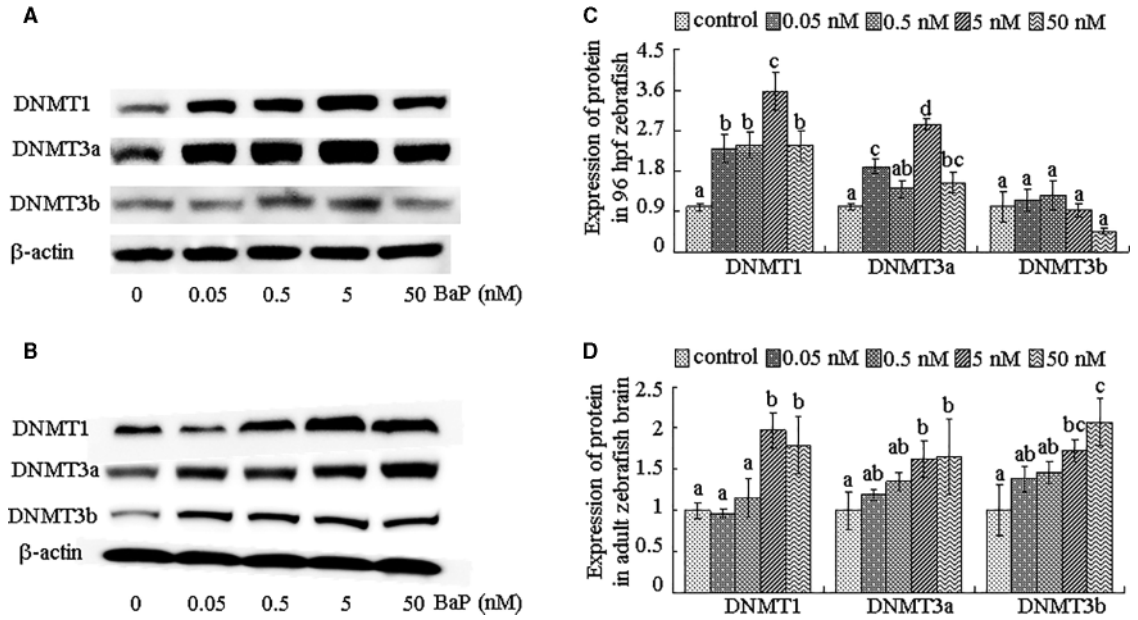

FIG. 7. BaP affected the expression of DNMTs in zebrafish. The protein levels of DNMTs in 96 hpf larvae (A, C) and in adult zebrafish (B, D). Data (mean ± SE) are analyzed by one-way ANOVA followed by the Duncan test ($n = 3$–4). Treatments not sharing a common letter are significantly different at $P < .05$.

(2.30-, 2.39-, 3.58-, and 2.36-folds) in 96 hpf larvae compared with the control (Figs. 7A and C). Similarly, the expression of DNMT3a was significantly increased in the 0.05, 5, and 50 nM groups (1.89-, 2.84-, and 1.54-folds) (Figs. 7A and C). However, there was no significant change in DNMT3b in 96 hpf larvae between BaP exposure groups and the control group (Figs. 7A and C). In the adult zebrafish brain, the expression of DNMT1 (1.97- and 1.79-folds), DNMT3a (1.62- and 1.65-folds), and DNMT3b (1.73- and 2.07-folds) were significantly increased in the 5 and 50 nM groups compared with the control (Figs. 7B and D).

BaP Changed Methylation and mRNA Levels of Nervous System Development-Related Genes

The MeDIP-sequencing data of the 96 hpf larvae identified 235 differentially methylated genes in promoter, with the fold change > 1.5 (Supplementary Table 1). Further, *zc4h2* was chosen from the Gene Ontology (GO) analysis of both central nervous system development and nervous system development, *gucy2f* was chosen from the GO analysis of central nervous system development, *drd4-rs* was chosen from the GO analysis of dopamine receptor signaling pathway. Their function predicted and fold change are shown in Table 2. In 96 hpf larvae, the methylation levels of both *gucy2f* (1.24-, 1.34-, 1.37-, and 1.30-folds) and *drd4-rs* (1.07-fold in all four) were significantly increased in the 0.05, 0.5, 5, and 50 nM groups, whereas the level of *zc4h2* was decreased significantly in the 50 nM group (1.16-fold) compared with the control group (Figure 8A). In adult zebrafish brains, the methylation level of *gucy2f* was significantly increased in 0.5, 5, and 50 nM groups (1.33-, 1.26-, and 1.34-folds). The level of *drd4-rs* was significantly increased in 0.05, 0.5, 5 and 50 nM groups (1.11-fold in all four), whereas the level of *zc4h2* was decreased significantly in the 50 nM group (1.14-fold) (Figure 8B). ""Lollipop" schematic representations of *gucy2f*, *drd4-rs* and *zc4h2* are shown in Figure 8E.

In 96 hpf larvae, the mRNA level of *gucy2f* was decreased significantly in the 50 nM group (1.35-fold) compared with the control. The mRNA level of *drd4-rs* was decreased significantly in the 0.05, 0.5, and 50 nM groups (1.38-, 1.35-, and 1.45-folds), whereas the mRNA level of *zc4h2* was significantly increased in the 0.5 and 50 nM groups (2.41- and 2.44-folds) (Figure 8C). In the adult zebrafish brain, the mRNA level of *gucy2f* was significantly decreased in the 0.05, 0.5, 5, and 50 nM groups (3.95-, 1.89-, 2.44-, and 1.40-folds) compared with the control. The level of *drd4-rs* was decreased significantly in the 0.5, 5, and 50 nM groups (1.74-, 1.55-, and 1.57-folds), whereas the level of *zc4h2* was significantly increased in the 5 and 50 nM groups (1.73- and 3.45-folds) compared with the control (Figure 8D).

TABLE 2. Effects of BaP on the Promoter Methylation Level of Nervous System Genes

Gene Symbol	Fold Change	Gene Ontology
gucy2f	2.4	Central nervous system development
drd4-rs	3.6	Dopamine receptor signaling pathway
zc4h2	−6	Central nervous system development, nervous system development

DISCUSSION

Recent epidemiologic and animal studies have demonstrated that early exposure to PAHs could induce long term adverse effect (Crépeaux et al., 2012; Perera et al., 2009, 2012; Vignet et al., 2014). In this study, we found that early BaP exposure significantly decreased the velocity and distance travelled in adult zebrafish. The finding that there was no significant difference in the immobility duration among the five groups further indicated that the decrease in velocity and distance travelled was not related to the immobility duration. Early BaP exposure also significantly decreased the number of dopaminergic neurons and the concentrations of DA, DOPAC and NE transmitters. The decrease of DA suggested the degeneration of dopaminergic neurons. There was no significant change in the ratio of DOPAC/DA indicated that there is no change in DA metabolism. Furthermore, BaP decreased the cognitive ability, induced

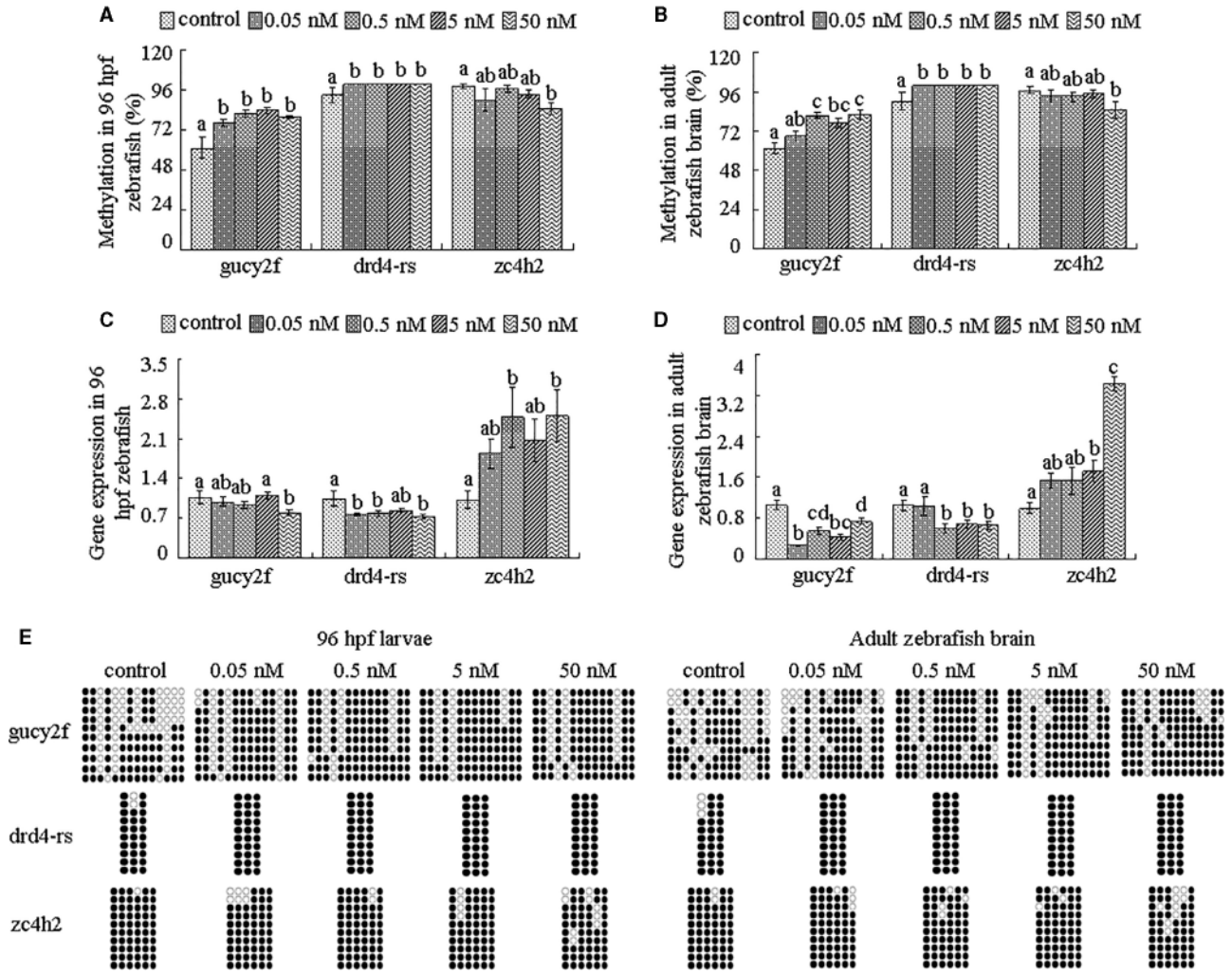

FIG. 8. BaP affected the nervous system development related genes in methylation and mRNA expression in zebrafish. The methylation levels in 96 hpf larvae (A) and the adult zebrafish brain (B), the mRNA levels in 96 hpf larvae (C) and adult zebrafish brain (D). Lollipop diagram shows the genes promoter methylation profiles of CpG sites. Unmethylated CpG sites (*hollow circle*) and methylated CpG sites (*solid circle*) are indicated (E). Data (mean ± SE) are analyzed by one-way ANOVA followed by the Duncan test (A, B: $n = 10$; C, D: $n = 4$–6). Treatments not sharing a common letter are significantly different at $P < .05$.

neurodegeneration in the telencephalon, and increased the accumulation of Aβ42. The behavioral, neuropathological, and neurochemical features are similar to the neurodegenerative diseases. BaP exposure increased the cell apoptosis in adult zebrafish. Apoptotic cells are usually increased significantly in neurodegenerative disease patients (Higami and Shimokawa, 2000). These findings indicate that early-life BaP exposure could induce neurodegenerative syndromes in adult zebrafish. Several environmental chemicals exposure to parents is found to affect neurodevelopment through alteration of epigenetics (Lo and Zhou, 2014). Previous studies find that BaP alters global and gene-specific DNA methylation patterns in cell models (Sadikovic and Rodenhiser, 2006; Sadikovic et al., 2004); and BaP decreases global DNA methylation levels and alters gene-specific methylation patterns in zebrafish embryos and larvae (Corrales et al., 2014; Fang et al., 2013). Based on these aggregate findings, we put forward a hypothesis that DNA methylation patterns may be altered by early-life exposure. In order to further prove this hypothesis, we performed several experiments.

It has been suggested that DNA methylome is the most common type of epigenetic change induced by prenatal exposure to environmental chemicals (Lo and Zhou, 2014). DNMT1, DNMT3a, and DNMT3b are three main methyltransferase enzymes. In our study, DNMT1 and DNMT3a were found to be significantly increased in both 96 hpf larvae and the adult zebrafish brain, whereas DNMT3b was increased significantly in the adult zebrafish brain. A recent study find that developmental exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin could also induce the change of DNMTs expression in zebrafish larvae (Aluru et al., 2015). The three promoter differentially methylated genes (*gucy2f*, *drd4-rs*, and *zc4h2*) were found to be associated with nervous system development in zebrafish. Gucy2f is a membrane-bound guanylate cyclase (Maddison et al., 2009). The misexpression of *gucy2f* induces neuronal degeneration in the forebrain of larval zebrafish (Maddison et al., 2009). Drd4-rs is a member of the D4 dopamine receptors, which are located in the brain region where dopamine signaling may modulate working memory and the establishment of memory fields in rat (Williams and Goldman-Rakic 1995). In zebrafish, D4 dopamine receptors can rescue the inhibitory effect of clozapine on the locomotor activity of larvae (Boehmler et al., 2007). Zc4h2, a zinc finger protein, is important for the central nervous system interneurons (May et al., 2015). Mutations of *zc4h2* cause abnormal swimming and induce neurodevelopmental disorder in

zebrafish (Hirata et al., 2013). Our results showed that both *gucy2f* and *drd4-rs* were hypermethylated in the promoter in 96 hpf larvae and the adult zebrafish brain; and *zc4h2* was hypomethylated in the promoter in 96 hpf larvae and the adult zebrafish brain. As DNMTs are responsible for maintaining and establishing DNA methylation patterns (Bestor, 2000; Hermann et al., 2004), the increase in DNMT1 and DNMT3a observed in both larvae and adult brains can be explicative of the increase of methylation observed for *gucy2f* and *drd4-rs* promoters. However, *zc4h2* was hypomethylated in both larvae and adult brains. We suspected that BaP might affect the availability of methyl donor which is another key causal alteration of DNA methylation (Lo and Zhou, 2014). Promoter DNA methylation typically inhibits/prevents gene expression, and we found that BaP-induced aberrant DNA methylation patterns also led to abnormal gene expression profiles. The mRNA levels of *gucy2f* and *drd4-rs* were down-regulated in 96 hpf larvae and the adult zebrafish brain, whereas the mRNA level of *zc4h2* was up-regulated in 96 hpf larvae and the adult zebrafish brain.

Based on these aggregate findings, we believe that BaP exposure during early-life could cause neurodegenerative syndromes in adult zebrafish, whereas the mechanism might involve the persistently altered DNA methylation patterns of *gucy2f*, *drd4-rs*, and *zc4h2*, together with the change of mRNA levels of these genes. Several studies find that prenatal exposure to cigarette smoke may induce lasting modifications of DNA methylation in the exposed offspring, and these DNA alterations can be maintained throughout life by the action of maintenance DNMTs (Faulk and Dolinoy 2011; Jurkowska et al. 2011; Lee and Pausova 2013; Lee et al., 2015). Another study proves that early-life environmental conditions can cause epigenetic changes in humans that persist throughout life (Heijmans et al., 2008) As a representative and ubiquitous PAH, BaP not only impairs fish health but also can threaten human beings (Booc et al., 2014; Perera et al., 1999, 2009, 2012; Vignet et al., 2014). Our observations provide evidence to the link between epigenetics and later-life health disorders due to early-life exposure. At the same time, our study may also provide clues to the mechanism by which environmental exposure during early-life stages affects fish health at later-life stages, and suggest new ways to prevent fish disease.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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