Assessment of parental benzo[a]pyrene exposure-induced cross-generational neurotoxicity and changes in offspring sperm DNA methylome in medaka fish

Wan, Teng; Au, Doris Wai-Ting; Mo, Jiezhang; Chen, Lianguo; Cheung, Kwok-Ming; Kong, Richard Yuen-Chong; Seemann, Frauke

Published in:
Environmental Epigenetics

Published: 01/01/2022

Document Version:
Final Published version, also known as Publisher’s PDF, Publisher’s Final version or Version of Record

License:
CC BY-NC

Publication details:
Wan, T., Au, D. W-T., Mo, J., Chen, L., Cheung, K-M., Kong, R. Y-C., & Seemann, F. (2022). Assessment of parental benzo[a]pyrene exposure-induced cross-generational neurotoxicity and changes in offspring sperm DNA methylome in medaka fish. Environmental Epigenetics, 8(1), Article dvac013. Advance online publication. https://doi.org/10.1093/eep/dvac013

Citing this paper
Please note that where the full-text provided on CityU Scholars is the Post-print version (also known as Accepted Author Manuscript, Peer-reviewed or Author Final version), it may differ from the Final Published version. When citing, ensure that you check and use the publisher's definitive version for pagination and other details.

General rights
Copyright for the publications made accessible via the CityU Scholars portal is retained by the author(s) and/or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights. Users may not further distribute the material or use it for any profit-making activity or commercial gain.

Publisher permission
Permission for previously published items are in accordance with publisher’s copyright policies sourced from the SHERPA RoMEO database. Links to full text versions (either Published or Post-print) are only available if corresponding publishers allow open access.

Take down policy
Contact lbscholars@cityu.edu.hk if you believe that this document breaches copyright and provide us with details. We will remove access to the work immediately and investigate your claim.

Download date: 18/12/2023
Assessment of parental benzo[a]pyrene exposure-induced cross-generational neurotoxicity and changes in offspring sperm DNA methyleme in medaka fish

Teng Wan1,2, Doris Wai-Ting Au1,2, Jiezhang Mo1,2, Lianguo Chen3, Kwok-Ming Cheung3, Richard Yuen-Chong Kong1,2,4 and Frauke Seemann1,5,*

1Department of Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong SAR, China, 2State Key Laboratory of Marine Pollution, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong SAR, China, 3State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, No. 7 Donghu South Road, Wuchang District, Wuhan 430072, China, 4South Hong Kong Branch of the Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou), The Hong Kong University of Science and Technology Clear Water Bay, Kowloon, Hong Kong SAR, China, 5Center for Coastal Studies and Department of Life Sciences, Texas A&M University-Corpus Christi, 6300 Ocean Drive, Corpus Christi, TX 78412, USA

*Correspondence address: Department of Life Sciences, College of Science and Engineering, Texas A&M University-Corpus Christi, 6300 Ocean Drive, Corpus Christi, TX 78412, USA. Tel: +1 (361) 825-2683; Fax: +1 (361) 825-2742; Email: frauke.seemann@tamucc.edu

Abstract

Previous studies have revealed that DNA methylation changes could serve as potential genomic markers for environmental benzo[a]pyrene (BaP) exposure and intergenerational inheritance of various physiological impairments (e.g. obesity and reproductive pathologies). As a typical aromatic hydrocarbon pollutant, direct BaP exposure has been shown to induce neurotoxicity. To unravel the inheritance mechanisms of the BaP-induced bone phenotype in freshwater medaka, we conducted whole-genome bisulfite sequencing of F1 sperm and identified 776 differentially methylated genes (DMGs). Ingenuity pathway analysis revealed that DMGs were significantly enriched in pathways associated with neuronal development and function. Therefore, it was hypothesized that parental BaP exposure (1 μg/L, 21 days) causes offspring neurotoxicity. Furthermore, the possibility for sperm methylation as an indicator for a neurotoxic phenotype was investigated. The F0 adult brains and F1 larvae were analyzed for BaP-induced direct and inherited toxicity. Acetylcholinesterase activity was significantly reduced in the larvae, together with decreased swimming velocity. Molecular analysis revealed that the marker genes associated with neuron development and growth (α-tubulin, mbp, syn2α, shh, and gap43) as well as brain development (dlx2, otx2, and krox-20) were universally downregulated in the F1 larvae (3 days post-hatching). While parental BaP exposure at an environmentally relevant concentration could induce neurotoxicity in the developing larvae, the brain function of the exposed F0 adults was unaffected. This indicates that developmental neurotoxicity in larvae may result from impaired neuronal development and differentiation, causing delayed brain growth. The present study demonstrates that the possible adverse health effects of BaP in the environment are more extensive than currently understood. Thus, the possibility of multigenerational BaP toxicity should be included in environmental risk assessments.

Key words: benzo[a]pyrene; neurotoxicity; locomotion; central nervous system; brain development; DNA methylation

Introduction

Polycyclic aromatic hydrocarbons (PAHs), which are organic pollutants consisting of two or more fused aromatic rings, are widely distributed in the environment and generated through both natural and anthropogenic processes, including volcanic eruptions (pyrogenic source), vehicle emissions (petrogenic source), and cigarette smoke (biological source) [1]. Petrogenic PAHs are particularly common as a result of global transportation, storage, and the heavy use of crude oil and its derivatives. The major sources of petrogenic PAHs include oceanic and freshwater oil spills, which pollute aquatic environments and threaten the health of aquatic organisms. Benzo[a]pyrene (BaP) is a typical PAH with teratogenic and carcinogenic properties. BaP is metabolically activated by xenobiotic-metabolizing enzymes, such as cytochrome P4501A1 (cyp1a1) and Urinedine 5′-diphospho-glucuronosyltransferase (ugt) [2]. These enzymes participate in phase I and II biotransformation pathways, respectively, and consequently generate a highly reactive metabolite known as benzo[a]pyrene diol epoxide [3]. Upon exposure, BaP and its metabolites are distributed to various tissues in mammals and fish [4, 5]. Parental exposure to BaP has been known to induce bone deformities in unexposed offspring [6–9]; however, no BaP has been detected in the F1 embryos [8]. BaP-induced changes in germ cell DNA methylation were proposed as one of the epigenetic mechanisms responsible for cross- and
transgenerational phenotype transmission (reviewed in 10), which has been corroborated by reduced DNA methyltransferase activity upon BaP exposure in zebrafish (F0) and mice (F1), as well as altered offspring methylation profiles [11, 12]. Furthermore, DNA methylation profiles were investigated as potential genomic markers of environmental BaP exposures and may indicate the intergenerational inheritance of phenotype impairments [13, 14]. Therefore, the sperm methylome was assessed in the F1 generation to test the hypothesis that the sperm methylome may be predictive of phenotype impacts in the same generation and may serve as an indicator for population exposure history.

A growing body of evidence is suggesting that parental BaP exposure is inducing systemic impairment manifested as reproductive toxicity, cardiotoxicity, osteotoxicity, and neurotoxicity (reviewed in 10). Although several studies have covered multiple biological levels to understand ancestral BaP-induced osteotoxicity, the impacts of cross-generational neurotoxicity have only been reported at the organism level. Thus, the present study aimed to shed light on the underlying molecular mechanisms involved in neuronal impairment and the potential for epigenome modification in the germ line upon parental BaP exposure.

A timely and appropriate behavioral reaction to the environment depends on neurotransmitter functions for nerve signal transmission. Acetylcholine (ACh) is the major excitatory neurotransmitter present in critical regions of the central nervous system (CNS) and somatic nervous system in vertebrates [15]. ACh signal transmission is dependent on acetylcholinesterase (AChE) activity, which is responsible for the synaptic clearing of ACh. Thus, AChE gene expression and activity are well-characterized biomarkers for various neurotoxics and neurotoxic impacts [16]. The primary role of AChE is to hydrolyze ACh to choline, which terminates the synaptic transmission and signaling, thereby preventing continuous nerve activation and subsequent receptor desensitization at the synapse [17].

The development of the nervous system is a complex process consisting of multiple events, including proliferation, migration, differentiation, synaptogenesis, myelination, and apoptosis [18]. The correct development of the brain and nervous system during early life stages is particularly important since it is more vulnerable to environmental BaP exposures [19]. Interference with the developmental processes of brain neurons may alter fundamentally brain structures and functions, resulting in deleterious effects on behavior and health [20]. Several genes have been proposed as markers for neuron and brain development during the embryonic and early larval stages of medaka. For the development and differentiation of the forebrain, midbrain, and hindbrain, distal-less homeobox 2 (dlx2), orthodenticle homeobox 2 (otx2), and early growth response 2 (egr2/krox-20) have been reported as key genes [21–23]. Notably, myelin basic protein (mbp), growth-associated protein (gap43), synapsin II (syn2a), alpha-tubulin, sonic hedgehog (shh), gliarial fibrillary acidic protein (gfap), and ache are involved in neuron and CNS development and are biomarkers of developmental neurotoxicity, as well as neurochemical and behavioral changes [24].

The extent to which changes in somatic cell gene expression may be reflected in the methylation patterns of germ cells and thus indicative of a potential transgenerational transmission of the phenotype currently remains unclear [25–27]. Environmental pollutants such as vinclozolin and Aroclor 1221 (polychlorinated biphenyls mixture) have been found to cause differential methylation in sperm and the brain, while a subset of differentially methylated regions was retained from the F1 to F3 generations. This suggests that DNA methylation in sperm may embody the future phenotypes of neurobehavioral disorders caused by direct or indirect exposure [28]. BaP has been demonstrated to induce epigenetic modifications and was found to change the DNA methylation level globally and in a gene-specific manner [11, 29–31]. Knecht et al. (2017) [12] demonstrated that the developmental exposure of F0 zebrafish embryos to BaP (concentrations: 1262 μg/l and 2514 μg/l) could cause the transgenerational inheritance of neurobehavioral and physiological deficits in subsequent generations (i.e. increased locomotor activity, decreased heart-beat, and reduced mitochondrial function in F0 and F2 juveniles). Moreover, it was observed that reduced global DNA methylation was coupled with the downregulated expression of dmnt genes in F0 embryos, which suggests that DNA methylation may serve a role in the transgenerational neurophysiological effects resulting from BaP exposure. However, methylation data for the F1 and F2 generations were not investigated.

Using the Japanese medaka as a model organism, the present study aimed to shed light on the molecular mechanisms and cross-generational transmission of neurotoxic effects induced by preconceptional BaP exposure by assessing F0 detoxification enzyme and AChE activity as well as F1 larval swimming behavior, AChE activity, and the expression of key genes involved in brain development and function. Using developmental observations, behavioral testing, biochemical assays, and quantitative polymerase chain reaction (qPCR), we tested the hypothesis that the parental exposure of sexually mature medaka to an environmentally relevant concentration of BaP induces neurotoxicity in both adults and developing larvae. While both osteo- and neurotoxicity have been reported beyond the F1 generation, it currently remains unclear whether ancestral BaP exposure is affecting F1 germ cell methylation (e.g. sperm methylation). The comparison of differentially methylated genes (DMGs) in the F1 sperm and the expression of these genes in somatic cells will forward possible candidates to monitor the risk of neuronal impairments in offspring. It is hypothesized that sperm DNA methylation changes associated with brain development and neuronal function are reflected in larval somatic cell gene expression.

Materials and Methods

Benzo[a]pyrene

BaP (Chemical Abstracts Service no. 50-32-8; purity >97%) was purchased from Sigma-Aldrich. A stock solution (20 000 μg/l) was prepared by dissolving BaP powder in ethanol–dimethyl sulfoxide solvent (EtOH:DMSO= 4:1), which was stored at −20°C in aliquots. The ethanol and DMSO used were analytical or high-performance liquid chromatography grade.

Medaka Model

The Japanese medaka (Cab strain) used in this study originated from Prof. Christoph Winkler’s laboratory at the Department of Biological Sciences, National University of Singapore, and have been maintained in our laboratory since 2015. The breeding pairs (~6 months old) used in this experiment were raised in charcoal-dechlorinated tap water at a constant temperature (26 ± 1°C) with a 14:10 h light:dark cycle.

BaP Exposure

The BaP exposure experiment was conducted as previously described [6]. In brief, 10 pairs of fish were kept in aquarium tanks containing 20 l of water, with a total of three and four replicates
for the solvent control and BaP exposure groups, respectively. Adults were exposed to BaP at 1 μg/l with a final concentration of EtOH:DMSO at 0.0005% (vol/vol). A group exposed to 0.0005% EtOH:DMSO and no BaP was included as the solvent control.

BaP exposure was continued for 21 days, with 75% of the water being renewed every second day. Then, 100 μl of the BaP stock solution or solvent were added after each water renewal. The fish were maintained at a constant temperature (26 ± 1°C) with a 14:10 h light-dark cycle. Adult fish were fed twice daily with dry food and once daily with brine shrimp. To minimize embryos being directly exposed to BaP, 75% of the water was changed twice on Day 22 and three times on Day 23. Egg collection began on Day 24 and hatched embryos were reared following a standard protocol, and these were subsequently used for different endpoint measurements. F1 embryos were reared in charcoal-dechlorinated tap water containing methylene blue (0.5 p.p.m.) until hatching. All experimental procedures were conducted in accordance with the Animal Ethics Committee of the City University of Hong Kong.

DNA Library Preparation for Whole-Genome Bisulfite Sequencing

The testes of adult medaka (F1) were dissected out. Whole testes were then squeezed in cold phosphate-buffered saline (PBS) using forceps to release mature sperm. Sperm-containing PBS was then centrifuged at 500 g for 5 minutes. After the supernatant was discarded, sperm samples were stored at −80°C. Before extracting genomic DNA (gDNA), sperm samples were collected from 10 fish. The gDNA was extracted for whole-genome bisulfite sequencing (WGBS) using the phenol-chloroform-isoamyl alcohol method. DNA quantity, integrity, and purity were determined using 1% agarose gel electrophoresis and a Qubit DNA Assay on a Qubit 3.0 fluorometer (Life Technologies, CA, USA). For library generation, equal amounts of gDNA from the three biological replicates were combined in equal amounts (N = 1; 30 fish). The gDNA (2.5 μg) was fragmented into 200-300 bp fragments by sonication. Terminal repair, A-ligation, and methylation sequencing adapter ligation were then performed on the DNA fragments. DNA library bisulfite treatment was conducted using the EZ DNA Methylation Gold Kit (Zymo Research Corp, Irvine, CA, USA). The resulting single-stranded DNA was amplified using a HiFi Hot-Start Uracil + ReadyMix polymerase chain reaction (PCR) Kit (KAPA Biosystems, Boston, MA, USA). Thereafter, the library concentration and quality were determined using a Qubit 3.0 Fluorometer and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. Libraries were sequenced using the HiSeq X Ten System at Novogene Co., Ltd (Beijing, China) to generate 150-bp paired-end reads delivered in FASTQ format, which provided sufficient genome coverage for differential methylation analysis.

WGBS Analysis

The sequencing files generated by the sequencer first underwent initial quality control (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and were then subjected to quality and adaptor trimming by using Trim Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with the following parameters: (i) removal of base calls with a Phred score <20; (ii) removal of reads <20 bp; and (iii) removal of reads with an adaptor sequence. The remaining sequences were mapped to the medaka ASM223467v1 genome using Bismark [32], and CG methylation calls were extracted which excluded any duplicate calls.

For differential methylation assessment, the bioconductor package DSS (Dispersion Shrinkage for Sequencing data) [33] used a Bayesian hierarchical model to estimate and shrink CpG-site-specific dispersions. Wald tests were performed for the detection of differential methylation. Since only one biological sample in each treatment was sequenced, the method used to analyze the differentially methylated loci (DMLs) or regions (DMRs) was limited. However, DSS considers neighboring CpG sites as pseudo-replicates due to smoothing, which facilitates a reasonably precise dispersion calculation, despite the lack of biological replicates. Data for DSS input contained the following information: chromosome number, genomic coordinates, the total number of reads, and the number of reads with methylation. The callDML and callDMR functions of the DSS package were used to extract DMLs and DMRs with methylation differences >10% (DMLs: P < 0.001 and DMRs: P < 0.01). DMRs were filtered based on their length (≥50 bp) and number of CpG sites (≥4). The neighboring DMRs were merged if the distance between them was <50 bp. Regions containing at least four significantly methylated CpG sites were identified as DMRs (≥50 bp). Additionally, an absolute cut-off of 10% methylation difference between the treatment and control used to filter these results. A DMR was considered hypomethylated if its average methylation level was lower than the control. Moreover, it was considered hypermethylated if its average methylation level was higher than the control. Sperm from BaP-exposed males was compared to that of corresponding control males in the same generation.

Gene Ontology Enrichment Analysis

Genes that had DMRs in their promoter regions [4 kb upstream and 200 bp downstream of the transcription start site (TSS)] and gene body (from the TSS to transcription end site) were considered DMGs. These DMGs were used in the Gene Ontology (GO) (biological process) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis in Metascape [34] and Ingenuity pathway analysis (IPA, version: 60.467.501, Qiagen, USA). The complete pipeline for WGBS data analysis is presented in Fig. 1.

Gene Transcription

Total RNA was extracted from the brain of adult fish (two fish/sex/rePLICATE, three replicates in the solvent control and four replicates in BaP treatment group) and 3 days post-hatching (d.p.h.) medaka larvae (10 larvae/replicate; three replicates in the solvent control and four replicates in BaP treatment group) using TRIzol reagent (Life Technologies, CA, USA) according to the manufacturer’s instructions. RNA quality and quantity were assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, DE, USA) and gel electrophoresis. cDNA was synthesized using a PrimeScript RT Reagent Kit (Perfect Real Time, Takara Bio USA, Inc., CA, USA) according to the manufacturer’s instructions. The primer sequences of selected genes (see Table S1) were designed using National Center for Biotechnology Information (NCBI)’s Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and their efficiency was validated before calculating expression levels. The rpl7 gene was used as a reference gene [35]. Quantitative real-time PCR with SYBR green detection was performed using the QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, CA, USA) based on the manufacturer’s protocol (Luna Universal qPCR Master Mix, New England Biolabs, MA, USA). Briefly, denaturation was performed for 30 s at 94°C, followed by 40 cycles of 5 s at 94°C, 30 s at 60°C, and 10 s at 72°C, with a final dissociation step of 60–94°C. The relative quantification of target gene expression of the treatment group was calculated using the 2−ΔΔCT method [36].
Acetylcholinesterase Activity Assay

Adult fish (F0) were anesthetized in ice water on Exposure Day 21, at which point the brains of male and female medaka were dissected out, pooled (two gender-matched brains/pool), and stored at −80°C. For medaka larvae (F1), 30 larvae (3 d.p.h.) in each replicate were anesthetized in ice water and rinsed with cold PBS before storage at −80°C. For enzyme extraction, the brains of adult medaka and larvae from each replicate (three replicates in the control group and four replicates in the BaP group) were homogenized on ice in 10 vol of Tris–citrate buffer (50 mM Tris, 2 mM Ethylenediaminetetraacetic acid, and 2 mM ethylene glycol tetraacetic acid, pH 7.4). Following centrifugation, the supernatant was transferred into a new tube, and enzyme activity was measured with a commercial AChE assay kit according to the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Optical density was recorded at 412 nm using a microplate reader (Powerwave; Biotek, VT, USA), and protein concentrations were measured with a Bradford assay using Coomassie brilliant blue G-250.

Larval Locomotor Behavior Assay

The locomotor activity of F1 larvae (3 d.p.h.) was tracked and analyzed using an EthoVision XT video tracking system (Noldus, Wageningen, the Netherlands) as previously described [37]. Briefly, larvae were placed in a 24-well flat-bottom plate (Falcon, NY, USA) with one larva per well in 2 ml of water. Each treatment group included 24 larvae (8 larvae per replicate in the control group and 6 larvae per replicate in the BaP group). After 10 min of acclimation inside the chamber, free-swimming activity under continuous visible light (15 min) and response to a dark-to-light photoperiod stimulation (three cycles of 5 min dark and 5 min light) were recorded [20].

Data Analysis

Statistical analysis was performed using R 4.0.0 [38]. Assumptions of the normality and homogeneity of variance were verified by the Kolmogorov–Smirnov test and Levene’s test, respectively, before applying either parametric (Student’s t-test) or non-parametric tests (Chi-squared test; used in the malformation rate comparison in Table S2), depending on whether normality was confirmed. Values were represented as the mean ± standard deviation (SD). A P-value < 0.05 was considered statistically significant.

Results

BaP-induced Modifications of the F1 Sperm Methylome

The qualitative assessment of the sperm methylome in the F1 generation resulted in a total of 1997 DMRs with 1010 hypomethylated and 987 hypermethylated regions in response to parental BaP exposure. Overall, 42.3% of the DMRs were located in gene bodies, while 41.2% were identified in intergenic regions and 16.5% were found in the promoter region. DMR analysis led to the identification of 776 DMCs located on all chromosomes in the F1 sperm following parental BaP exposure (Fig. 2A).

IPA revealed that DMRs were significantly enriched in pathways associated with neuronal development and function, especially the netrin signaling pathway (positive Z-score) and synaptogenesis pathway (negative Z-score), which are strongly associated with synaptic development and axon guidance, respectively (Fig. 2B) [39]. Seven pathways seemed to be repressed (negative Z-score), while the netrin signaling pathway, the endocannabinoid neuronal synapse pathway, and the calcium signaling appeared to be activated (positive Z-score) (Fig. 2B). Key DMCs associated with neuronal development and function, as well as major components of gamma-aminobutyric acid (GABA) receptor signaling, netrin signaling, axonal guidance signaling, and the synaptogenesis signaling pathway, can be separated into three groups (see Table 1), as follows: (i) hypomethylation of the promoter region in sperm from parentally exposed individuals was found for apolipoprotein E (apoE); (ii) hypomethylation of the gene body in the sperm upon parental BaP exposure was measured for actin binding LIEM protein family member 3 (ablim3), rho guanine nucleotide exchange factor 7 (aro7g7), microtubule associated protein tau (mapt), adenylate cyclase 8 (adcy8), discs large MAGUK scaffold protein 4 (dlg4), and neuroligin 2 (nlgn2); (iii) hypermethylation in the gene body of the F1 sperm upon ancestral BaP exposure was observed for gamma-aminobutyric acid type B receptor subunit 1 (gabbr1), DCC netrin 1 receptor (dcc), and patched 1 (ptch1) (Table 1).

Neuronal and Brain Developmental Marker Gene Expression in Medaka Larvae

All assessed genes were involved in brain development and differentiation. Distal-less homeobox 2 (dlx2), orthodontic...
homeobox 2 (otx2), and early growth response 2 (egr2/krox-20) were significantly downregulated 2–3 fold in parentally BaP-exposed F1 larvae when compared to the control group ($P = 0.046$, 0.026, and 0.007, respectively; Fig. 3A). The transcript levels of genes associated with neuron and CNS development, differentiation, and growth—i.e. alpha1-tubulin, gap43, mbp, shh, and syn2a—were reduced by at least 50% within the BaP group when compared to the control ($P = 0.005$, 0.005378, 0.01072, 0.00498, and 0.00142, respectively; Fig. 3B). Only the $gfap$ expression level remained unchanged between the control and BaP groups.

Overall, 8 of the 10 genes that were found to be differently methylated in adult sperm tended to be downregulated in the larvae samples following parental BaP exposure (Fig. 3C and D). The downregulation of $adcy8$, $nlgn2$, and $arhgef7$ was statistically significant ($P = 0.0039$, 0.014, and 0.0446, respectively; Fig. 3C and D). With the exception of $apoE$, $gabbr1$, and $ablim3$, the direction of methylation observed in the adult sperm was consistent with the gene expression measured in larvae for all genes involved in pathways for synaptic signaling and axon guidance.

### Table 1: IPA of DMGs of sperm of adult medaka following BaP exposure

| Pathway                                      | Human gene name                  | Abbreviation | Region   | Differential methylation |
|----------------------------------------------|----------------------------------|--------------|----------|--------------------------|
| GABA receptor signaling                      | Gamma-aminobutyric acid type B   | $gabbr1$     | Gene body| 0.4405                   |
|                                              | receptor subunit 1                |              |          |                          |
|                                              | DCC netrin 1 receptor             | $dcc$        | Gene body| 0.2691                   |
| Netrin signaling Axonal guidance signaling   | Actin binding LIM protein family  | $ablim3$     | Gene body| −0.2978                  |
|                                              | member 3                          |              |          |                          |
|                                              | Rho guanine nucleotide exchange factor 7 | $arhgef7$   | Gene body| −0.3600                  |
| Synaptogenesis signaling pathway             | Patched 1                         | $ptch1$      | Gene body| 0.3850                   |
|                                              | Microtubule associated protein Tau| $mapt$       | Gene body| −0.1526                  |
|                                              | Adenylate cyclase 8               | $adcy8$      | Gene body| −0.3244                  |
|                                              | Discs large MAGUK scaffold protein 4 | $dlg4$      | Gene body| −0.3533                  |
|                                              | Neurolign 2                       | $nlgn2$      | Gene body| −0.4596                  |

AChE Activity and Gene Expression in F0 Brains and F1 Larvae

AChE activity and gene expression levels were measured in the brain of adult fish and F1 larvae. In the directly BaP-exposed adults, no appreciable change in AChE enzyme activity or gene expression was observed (Fig. 4A, B). Moreover, the total AChE activity was inhibited by 31.7% in larvae from the BaP group when compared to the control group ($P = 0.02771$) (Fig. 4C). Consistent with the reduced enzyme activity, the level of $ache$ gene expression in the larvae was reduced by 75% in the BaP treatment group when compared to the control group ($P = 0.02127$; Fig. 4D).

Locomotor Activity of Medaka Larvae

The swimming speed of larvae derived from the parental BaP exposure group was significantly reduced during the continuous light period trial ($P = 0.03443$; Fig. 5A). Assessing swimming speed during dark–light transition revealed that the average speed of larvae from the BaP group was significantly reduced in the first light period when compared to the solvent control group ($P = 0.03252$; Fig. 5B). This difference was the most apparent at the beginning of
of the first dark–light transition, where the average swimming speed of the larvae from the BaP group in the first and second minutes was significantly decreased \( (P = 0.0058\) and \( P = 0.0117\) respectively, Fig. 5C).

Discussion

This is the first study to demonstrate that parental preconception exposure to BaP is affecting neurodevelopment and behavior in the F1 offspring, which further indicates that the environmental toxicant BaP has far-reaching impacts on fish health. The neurotoxic effect of parental BaP exposure is evidenced by reduced swimming performance among offspring, which is potentially caused by the delay or disturbance of neuronal, brain, and CNS development at multiple biological levels (i.e. gene transcription and enzyme activity). A qualitative assessment of the sperm DNA methylation profiles of adult F1 offspring revealed a pattern consistent with somatic cell gene expression in the F1 larvae for genes associated with neuronal and brain development as well as synaptic signaling. Notably, further research is required to confirm the methylation status of these genes in neurons and neuronal progenitors. The presence of DMGs in adult F1 sperm indicates the potential for neurotoxic phenotype transmission into the F2 generation. Sperm harbors a repertoire of methylation markers that are associated with different physiological functions, especially neurobehavioral activity. Sperm-borne differential DNA methylation in genes that regulate the development and function of the CNS in offspring has been demonstrated for methylmercury in zebrafish [40] and in obese men whose sperm DMGs were enriched for the term "nervous system development" [41]. Moreover, a preliminary analysis of the F2 sperm methylome revealed conserved DMGs associated with neurologic function (unpublished data).

Qualitative Analysis of the F1 Sperm Methylome Indicates Neurotoxicity of BaP

The present results reveal that the methylation profiles and expression levels of adcy8, nlgn2, and arhgef7 warrant particular attention due to their potential contribution to cross-generational BaP-induced neurotoxicity in medaka. Adenylate cyclase adcy8, which is crucial for cellular secondary messenger transmission, has been associated with axon pathfinding defects in zebrafish and is involved in long-term potentiation and synaptic plasticity in mammals [42–44]. Moreover, the deregulation of adcy8 entailed modified avoidance behaviors and anxiety response in mice [45, 46]. A male-biased response of adcy8 expression has been demonstrated in hypoxia-induced neurotoxicity [47]. The cell adhesion molecule nlgn2 is expressed in inhibitory, dopaminergic, and cholinergic synapses [48–50]. Thus, it is strongly involved in brain circuit functioning and behavior [51, 52]. Arhgef7 (synonyms: \( \beta \)Pix and cool1) is a crucial regulator of excitatory and inhibitory synapse formation and neural circuit establishment and has been associated with microcephaly in humans [53, 54]. Besides impaired neuron and synapsis morphogenesis, Arhgef7 heterozygous mice displayed impacted social behavior [53]. Thus, all three genes differentially methylated in the F1 sperm and downregulated in the F1 larvae upon parental BaP exposure could be associated with altered behavioral phenotypes.

It should be noted that the majority of the investigated genes did not show a correlation between gene expression in the larval
brain and methylation changes in the sperm. Limited correlation between the transcription level and methylation changes due to environmental stress including increased temperature [55] and chemical exposure [56] even in the same tissue were observed previously. The involvement of other epigenetic or regulatory effects may provide an explanation for the poor correlation. In this study, sperm and whole larvae were used for methylome and gene expression analysis, respectively, which may increase the disparity of the correlation due to cell diversity in the larval analysis. Future studies may take advantage of single cell sequencing to analyze the gene expression and methylation changes in specific cell types, which could provide a more accurate and comprehensive understanding of methylome regulation of transcription in somatic cells and gametes.

Parental Preconception Exposure to BaP Induces Larval Neurotoxicity

The assessment of selected neurotoxic marker genes indicative of brain differentiation and CNS development further corroborated the cross-generational nature of BaP-induced neurotoxicity. The transcription levels of *gap43* and *syn2a* were decreased in medaka larvae following parental BaP exposure, which may affect axonal growth regulation and neuronal network establishment. Similar expression changes were observed in zebrafish and rockfish upon direct exposure to various neurotoxicants, including pyrene, methamidophos, and triphenyl phosphate [57–59]. As a neuro-specific phosphoprotein, *gap43* plays an important role in nerve regeneration, axon development, and neuronal network formation [60]. Additionally, the upregulation of *gap43* is an indicator of nerve regeneration after damage from toxin exposure [20, 61]. Thus, parental BaP exposure may impact neuronal repair mechanisms. Furthermore, *syn2a* encodes a neuronal phosphoprotein involved in neurotransmitter release, synaptic plasticity, transmission, and synaptogenesis, which is critical for neuronal network establishment [62, 63]. Given the role of *syn2a* in orchestrating neurotransmitter release, the change in AChE activity and its gene expression in larvae from the BaP group could be attributed to the downregulation of *syn2a* gene expression.

The protein encoded by *alpha1-tubulin* participates in the formation and polymerization of microtubules, which are important part of the neuron cytoskeleton and serve as a backbone to developing axons and dendrites [64]. Moreover, *alpha1-tubulin* gene expression is enriched in the developing and regenerating CNS of vertebrates [65]. In our study, *alpha1-tubulin* expression was significantly downregulated in medaka larvae derived from BaP-exposed parents when compared to the control group. Other studies have reported similar expression patterns when investigating neurotoxicity caused by flame retardants [20, 59, 66]. Considering the structural and functional importance of microtubules for neurodevelopment, the decreased gene expression of *alpha1-tubulin* may impact the cytoskeleton structure and subsequently impair brain and CNS function.

Studies in mammals have reported BaP exposure interfering with myelination [18, 67]. Notably, myelination is critical for microtubule stability and requires myelin basic protein (*mbp*), an oligodendrocyte-specific protein essential for oligodendrocyte morphogenesis at the late stages of cell differentiation that leads to the formation of myelin sheaths [68–70]. The myelin sheath serves as a protective layer for neurons in the CNS and enhances the transmission of electrical impulses. In our results, we observed a significant decrease in *mbp* mRNA in larvae from the parental

**Figure 4:** AChE activity and *ache* gene expression in response to direct BaP exposure (1 μg/l). AChE activity and gene expression in the brain of adult fish (A, B) and larvae (C, D) was measured following parental exposure to the solvent control (blue bars) and BaP (1 μg/l; pink bars). All data are expressed as mean ± SD. Asterisks indicate statistically significant differences between the BaP-exposed group and treatment group (*P* < 0.05), Student’s t-test.
Figure 5: Locomotor behavior of medaka larvae after parental exposure to BaP. Locomotor behavior was assessed in medaka larvae (3 d.p.h.) with parental BaP exposure. The following parameters were measured: (A) average swimming velocity during a continuous light test; (B) average swimming velocity during a light (white bars)–dark (black bars) photoperiod stimulation test; (C) locomotor traces. Data are displayed as mean ± SD from three or four replicates in the control and BaP group, respectively (8 larvae/replicate in the control group and 6 larvae/replicate in the BaP group), in 1-min intervals. Asterisks indicate statistically significant differences between the BaP-exposed group and treatment group (\( P < 0.05 \), \( **P < 0.01 \)), Student’s t-test.

BaP exposure group when compared to the control group. This inhibited mhp expression may also impair the myelination process in the CNS, thereby further perturbing the signaling transmission of neurons beneath the myelin sheath.

Our results also suggest that parental BaP exposure suppressed the expression of the neural regulatory gene, shh, in the developing larvae. Shh encodes a protein called sonic hedgehog, an essential chemical messenger for embryonic development that plays multiple roles during organ patterning in vertebrates, including the CNS [71]. In medaka, the forebrain develops under the influence of myriad signaling pathways, including shh signaling [72]. In zebrafish, embryonic BaP exposure caused developmental brain toxicity, which was attributed to reduced shh expression in the mid-diencephalic organizer, the basal plate, and the hypothalamus [73]. Significant downregulation of the shh gene in larvae from the parental BaP exposure group is a further indicator of the potential inhibition of CNS and brain development and differentiation in the parentally exposed F1 generation.

Preconceptional Exposure to BaP Delays Brain Development and Affects Swimming Behavior in Larvae

To evaluate the possible inhibitory effects of BaP on larval brain development, we assessed the expression levels of marker genes for brain development and differentiation. We observed the decreased expression of dlx2, otx2, and krox-20 (forebrain, midbrain, and hindbrain markers, respectively) in the larvae derived from parental BaP exposure. Since dlx2 is involved in neuron migration and differentiation during development in fish [74], these results suggest that brain development is hindered. Moreover, dlx2 downregulation may indicate growth impairments of the forebrain, which can be corroborated by the decrease in shh expression. Given the central role of the forebrain in information processing and its association with cognitive activities, voluntary motor activities, and sensory perception [75, 76], the parental BaP exposure-induced reduction of dlx2 expression may be associated with the affected F1 larvae motor activity (reduced swimming velocity). A similar inhibition of dlx2 was reported in zebrafish upon embryonic exposure to fenvalerate—a common insecticide—at 25–100μg/l. In this study, the exposed zebrafish exhibited decreased swimming ability and apoptosis in the larval brain [77, 78]. Together with the decreased expression of otx2 and krox-20, our data suggest that parental BaP exposure leads to the potentially impaired development of the midbrain and patterning of the hindbrain in F1 larvae [79]. Interestingly, sublethal exposure to deep water horizon crude oil (4.74μg/l ∑PAH50) of larvae of red drum (Sciaenops ocellatus) significantly reduced the brain size which was in agreement with the corresponding transcriptomic changes [80]. However, no morphological abnormality in brain size and larval size was observed in this or prior BaP-exposure experiments conducted in our laboratory. In comparison to the study by Xu et al. [80], the present study used a relatively lower concentration of BaP. The absence of any drastic morphological brain abnormality may suggest that the BaP-induced expression changes of marker genes related to brain development and differentiation precede tissue level effects and are not a consequence thereof. Subsequent mechanistic studies are required to address
the effect of altered gene expression patterns observed in the F1 larvae on brain tissue morphology.

The slower average swimming velocity of larvae from the parental BaP exposure group was associated with the significant inhibition of AChE activity and gene expression, a common neurotoxicity biomarker [81]. The reduced swimming velocity may be attributed to reduced cholinergic neuron transmission [82], as reported upon flame retardant and pesticide exposure in different fish species [20, 83–85]. Reductions in AChE activity and enzyme transcript levels support the assumption that ACh is accumulating at the neuromuscular junctions due to reduced hydrolysis of the transmitter in parentally BaP-exposed medaka larvae [86]. Several studies have demonstrated the inhibition of AChE activity in brain of tilapia [87] and electric eel [88] following direct exposure to PAHs that contain three or more aromatic rings (including BaP) [89]. In fish, inhibited AChE activity generally alters behavioral responses, resulting in changes such as decreased swimming speed and distance [90, 91].

The observed developmental neurotoxicity in F1 larvae upon parental preconception BaP exposure is potentially linked to disrupted neuronal, CNS, and brain development [92]. Neurogenesis and gliogenesis are crucial to the CNS function since neurogenesis modifies neuronal connectivity in specific brain areas and gliogenesis is involved in myelination and the generation of new supporting cells (i.e. astrocytes and oligodendrocytes) [93]. Investigating the expression of genes related to neuronal, CNS, and brain development at early life stages will help to understand the damaged pathways and mechanisms that may be responsible for the observed developmental neurotoxicity in larvae [20, 22].

BaP exposure (waterborne, 1 μg/l, 21 days) did not impact the AChE activity of F0 adult brains. This result indicates that the BaP concentration used here does not result in measurable neurotoxicity in adult individuals; however, it affects the developing brains of F1 offspring. Acute BaP neurotoxicity in the adult brain manifested as anxiolytic-like behavioral responses in adult zebrafish (waterborne, 7 days; 4 μg/l) [94] and as increased AChE activity in adult catfish (Ram这边的text needs to be filled in as there is a placeholder) and adult pale chub (Zacco platypus; waterborne, 5 μg/l; 14 days) [95, 96]. The aforementioned results indicate that the exposure protocol used in our study is below the threshold to impact neuronal and brain homeostasis.

Notably, the present study is the first to demonstrate that F1 offspring are more sensitive to CNS impairment than the exposed parent generation. Cross-generational impacts were observed at both molecular and cellular levels. Both ache gene expression and AChE activity were reduced in medaka larvae after parental BaP exposure, which was associated with the downregulation of marker genes associated with CNS development (mbp, gap43, syn2a, alpha1-tubulin, and shh) as well as brain development and patterning (dix2, otx2, and krox-20). The comparison of larval gene expression and sperm DNA methylation revealed three potential markers (adcy8, nlg2, and arhgef7) for BaP-induced cross-generational neurotoxicity and behavioral impairment. Collectively, the altered gene expression may explain how parental BaP exposure led to swimming speed reduction in the F1 generation.

The data presented here provide a potential connection between ancestral PAH exposure and the risk of behavioral and psychiatric disorders in vertebrates. Together with research investigating cross- and transgenerational bone toxicity and potential cardiac toxicity, our results demonstrate that BaP is a cross-generational, multi-organ system toxicant that induces adverse health effects that are more extensive than currently addressed in (eco)toxicity assessments.

Supplementary Data

Supplementary data are available at EnvEpig online.

Acknowledgements

The authors would like to acknowledge the generosity of Prof. Christoph Winkler of the National University of Singapore in providing us with the transgenic medaka fish line used in this study; Dr Jiarrui Gu, Ms Xian Qin, and Mr Nathan Yi-Kan Tam for their assistance with data analysis; and Ms Iris Chau-Fong Tsang for primer validation.

Conflict of interest statement. None declared.

Data Availability

Raw reads of the methylome have been deposited in NCBI's BioProject accession number PRJNA801412.

Author contributions

T.W., J.Z.M., and K.M.C. conceived the study. T.W. and J.Z.M. performed the experiments. T.W. acquired and analyzed the data. L.G.C. contributed reagents. T.W. wrote the manuscript, and R.Y.C.K. and F.S. edited and approved the manuscript.

Funding

This work was supported by funding from the Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou) (SMSEG1205002) and the National Natural Science Foundation of China (No. 41977371). Dr F.S. was supported by the National Institute of Environmental Health Sciences of the National Institutes of Health under award number 1R15ES032936-01.

References

1. Mumtaz MM et al. ATSDR evaluation of health effects of chemicals. IV. Polycyclic aromatic hydrocarbons (PAHs): understanding a complex problem. Toxicol Ind Health 1996;12:742–971.
2. Whitelaw M et al. Ligand-dependent recruitment of the Arnt coregulator determines DNA recognition by the dioxin receptor. Mol Cell Biol 1993;13:2504–14.
3. Schrenk D. Impact of dioxin-type induction of drug-metabolizing enzymes on the metabolism of endo- and xenobiotics. Biochem Pharmacol 1998;55:1155–62.
4. Lemaire F et al. The uptake metabolism and biological half-life of benzo[a]pyrene in different tissues of sea bass, Dicentrarchus labrax. Ecotoxicol Environ Saf 1990;20:223–33.
5. Boysen G, Hecht SS. Analysis of DNA and protein adducts of benzo[a]pyrene in human tissues using structure-specific methods. Mutat Res 2003;543:17–30.
6. Seemann F et al. Insight into the transgenerational effect of benzo[a]pyrene on bone formation in a teleost fish (Oryzias latipes). Comp Biochem Physiol Toxicol Pharmacol 2015;178:60–7.
7. Seemann F et al. Ancestral benzo[a]pyrene exposure affects bone integrity in F3 adult fish (Oryzias latipes). Aquat Toxicology (Amsterdam, Netherlands) 2017;183:127–34.
8. Mo J et al. Multigenerational impacts of benzo[a]pyrene on bone modeling and remodeling in medaka (Oryzias latipes). Environ Sci Technol 2020;54:12271–84.
9. Corrales J et al. Multigenerational effects of benzo[a]pyrene exposure on survival and developmental deformities in zebrafish...
Mo J et al. Benzo[a]pyrene osteotoxicity and the regulatory roles of genetic and epigenetic factors: a review. Crit Rev Environ Sci Technol 2021;1–39.

Zhang W et al. Paternal benzo[a]pyrene exposure alters the sperm DNA methylation levels of imprinting genes in F0 generation mice and their unexposed F1-2 male offspring. Chemosphere 2019;228:586–94.

Knecht AL et al. Transgenerational inheritance of neurobehavioral and physiological deficits from developmental exposure to benzo[a]pyrene in zebrafish. Toxicol Appl Pharmacol 2017;329:148–57.

Meehan RR et al. DNA methylation as a genomic marker of exposure to chemical and environmental agents. Curr Opin Chem Biol 2018;45:48–56.

Oluwayeiso OA et al. Paternal preconception phthalate exposure alters sperm methylome and embryonic programming. Environ Int 2021;155:106693.

Mineau P. Cholinesterase-inhibiting Insecticides: Their Impact on Wildlife and the Environment. Chemicals in Agriculture (Netherlands): Elsevier, 1991.

Payne JF et al. Acetylcholinesterase, an old biomarker with a new future? Field trials in association with two urban rivers and a paper mill in Newfoundland. Mar Pollut Bull 1996;32:225–31.

Loosli F et al. Six3, a medaka homologue of the Drosophila homeobox gene sine oculis is expressed in the anterior embryonic shield and the developing eye. Mech Dev 1998;74:159–64.

Rice D, Barone S Jr. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect 2000;108:511–33.

Daston G et al. A framework for assessing risks to children from exposure to environmental agents. Environ Health Perspect 2004;112:238–56.

Chen L et al. Prenatal transfer of polybrominated diphenyl ethers (PBDEs) results in developmental neurotoxicity in zebrafish larvae. Environ Sci Technol 2012;46:9727–34.

Kage T et al. Morphogenesis and regionalization of the medaka embryonic brain. J Comp Neurol 2004;467:219–39.

Chen X et al. Molecular staging of marine medaka: a model organism for marine ecotoxicity study Mar Pollut Bull 2011;63:309–17.

Chen X et al. Rapid adaptation of molecular resources from zebrafish and medaka to develop an estuarine/ marine model. Comp Biochem Physiol Toxicol Pharmacol 2009;149:647–55.

Rico EF et al. Chronic ethanol treatment alters purine nucleotide hydrolysis and nucleotide gene expression pattern in zebrafish brain. Neurotoxicology 2011;32:871–8.

Anway MD et al. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science(New York, N Y) 2005;308:1466–9.

Skinner MK et al. Epigenetic transgenerational actions of endocrine disruptors. Reprod Toxicol 2011;31:337–43.

Wang SY et al. Hypoxia causes transgenerational impairments in reproduction of fish. Nat Commun 2016;7:1–9.

Gillette R et al. Passing experiences on to future generations: endocrine disruptors and transgenerational inheritance of epimutations in brain and sperm. Epigenetics 2018;13:1106–26.

Fang X et al. Benzo[a]pyrene decreases global and gene specific DNA methylation during zebrafish development. Environ Toxicol Pharmacol 2013;36:40–50.

Corrales J et al. Effects on specific promoter DNA methylation in zebrafish embryos and larvae following benzo[a]pyrene exposure. Comp Biochem Physiol Toxicol Pharmacol 2014;163:37–46.
51. Katzman A, Alberini CM. NLGN1 and NLGN2 in the prefrontal cortex: their role in memory consolidation and strengthening. Curr Opin Neurobiol 2018;48:122–30.

52. Babaev O et al. Neurolgin 2 deletion alters inhibitory synapse function and anxiety-associated neuronal activation in the amygdala. Neuropharmacology 2016;100:56–65.

53. Kwon Y et al. βPix heterozygous mice have defects in neuronal morphology and social interaction. Biochem Biophys Res Commun 2019;516:1204–10.

54. Walczak-Sztulpa J et al. Chromosome deletions in 13aq33-34: report of four patients and review of the literature. Am J Med Genet A 2008;146A:337–42.

55. Anastasiadi D et al. Footprints of global change in marine life: Inferring past environment based on DNA methylation and gene expression marks. Mol Ecol 2021;30:747–60.

56. Aluru N et al. Role of DNA methylation in altered gene expression patterns in adult zebrafish (Danio rerio) exposed to 3, 3′, 4, 4′, 5-pentachlorobiphenyl (PCB 126). Environ Epigenet 2018;4:dvy005.

57. He C et al. Exposure of Sebastiscus marinus embryos to pyrene results in neurodevelopmental defects and disturbs related mechanisms. Aquatic Toxicology (Amsterdam, Netherlands) 2012;116–117:109–15.

58. He X et al. Developmental neurotoxicity of methamidophos in the embryo-larval stages of zebrafish. Int J Environ Res Public Health 2016;14:23.

59. Sun L et al. Developmental neurotoxicity of organophosphate flame retardants in early life stages of Japanese medaka (Oryzias latipes). Environ Toxicol Chem 2016;35:2931–40.

60. Basi GS et al. Primary structure and transcriptional regulation of GAP-43, a protein associated with nerve growth. Cell 1987;49:785–91.

61. Alm H et al. Exposure to brominated flame retardant PBDE-99 affects cytосkeletal protein expression in the neonatal mouse cerebral cortex. Neurotoxicology 2008;29:628–37.

62. Kao HT et al. A third member of the synapsin gene family. Proc Natl Acad Sci USA 1998;95:4667–72.

63. Cohen-Cory S. The developing synapse: construction and modulation of synaptic structures and circuits. Science (New York, N Y) 2002;298:770–6.

64. Lin S et al. Oxidative stress and apoptosis in benzo[a]pyrene-induced neural tube defects. Free Radic Biol Med 2018;116:149–58.

65. Miller FD et al. Isotypes of alpha-tubulin are differentially regulated during neuronal maturation. J Cell Biol 1987;105:3055–73.

66. Shi Q et al. Developmental neurotoxicity of triphenyl phosphate in zebrafish larvae. Aquatic Toxicology (Amsterdam, Netherlands) 2018;203:80–7.

67. Dutta K et al. A common carcinogen benzo[a]pyrene causes neuronal death in mouse via microglial activation. PloS one 2010;5:e9984.

68. Gillespie CS et al. Characterization of a cytосkeletal matrix associated with myelin from rat brain. Biochem J 1989;260:689–96.

69. Wilson R, Brophy PJ. Role for the oligodendrocyte cytосkeletal in myelination. J Neurosci Res 1989;22:439–48.

70. Piroil et al. Ca(2+)/calmodulin regulated effectors of microtubule stability in neuronal tissues. Biochim Biophys Acta 1992;1160:113–9.

71. Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. Genes Dev 2001;15:3059–87.

72. Ohkubo Y et al. Coordinate regulation and synergistic actions of BMP4, SHH and FGF8 in the rostral prosencephalon regulate morphogenesis of the telencephalic and optic vesicles. Neuroscience 2002;111:1–17.

73. Lin Y-C et al. Integrated hypoxia signaling and oxidative stress in developmental neurotoxicity of benzo[a]pyrene in zebrafish embryos. Antioxidants (Basel, Switzerland) 2020;9:731.

74. Sperber SM et al. Zebrafish dlx2a contributes to hindbrain neural crest survival, is necessary for differentiation of sensory ganglia and functions with dlx1a in maturation of the arch cartilage elements. Dev Biol 2008;314:59–70.

75. Preilowski BF. Possible contribution of the anterior forebrain commissures to bilateral motor coordination. Neuropsychologia 1972;10:267–77.

76. Baxter MG, Chiba AA. Cognitive functions of the basal forebrain. Curr Opin Neurobiol 1999;9:178–83.

77. Gu A et al. Exposure to fenvalerate causes brain impairment during zebrafish development. Toxicol Lett 2010;197:188–92.

78. Han J et al. Mechanisms underlying melatonin-mediated prevention of fenvalerate-induced behavioral and oxidative toxicity in zebrafish. J Toxicol Environ Health A 2017;80:1331–41.

79. Voiculescu O et al. Hindbrain patterning. Krox20 couples segmentation and specification of regional identity. Development (Cambridge, England) 2001;128:4967–78.

80. Xu EG et al. Larval red drum (Sciaenops ocellatus) sublethal exposure to weathered deepwater horizon crude oil: developmental and transcriptomic consequences. Environ Sci Technol 2017;51:10362–72.

81. Payne JF et al. Acetylcholinesterase, an old biomarker with a new future? Field trials in association with two urban rivers and a paper mill in Newfoundland. Mar Pollut Bull 1996;32:225–31.

82. Ren Q et al. Integrative characterization of toxic response of zebra fish (Danio rerio) to deltamethrin based on AChe activity and behavior strength. Biomed Res Int 2016;2016:7309184.

83. Beauvais SL et al. Physiological measures of neurotoxicity of diazinon and malathion to larval rainbow trout (Oncorhynchus mykiss) and their correlation with behavioral measures. Environ Toxicol Chem 2000;19:1875–80.

84. Sandahl JF et al. Comparative thresholds for acetylcholinesterase inhibition and behavioral impairment in coho salmon exposed to chlorpyrifos. Environ Toxicol Chem 2005;24:136–45.

85. Kavitha P, Rao JV. Toxic effects of chlorpyrifos on antioxidant enzymes and target enzyme acetylcholinesterase interaction in mosquito fish, Gambusia affinis. Environ Toxicol Pharmacal 2008;26:192–8.

86. Smallman BN, Mansingh A. The cholinergic system in insect development. Annu Rev Entomol 1969;14:387–408.

87. Dange AD, Masurekar VB. Toluen toxicity: effects of sublethal levels on enzyme activities in seawater adapted tilapia (Satrorodon mossambicus Peters). J Biosci 1981;3:129–34.

88. Kang JJ, Fang HW. Polycyclic aromatic hydrocarbons inhibit the activity of acetylcholinesterase purified from electric eel. Biochem Biophys Res Commun 1997;238:367–9.

89. Jett DA et al. Additive inhibitory action of chlorpyrifos and polycyclic aromatic hydrocarbons on acetylcholinesterase activity in vitro. Toxicol Lett 1999;105:223–9.

90. Beauvais SL et al. Physiological measures of neurotoxicity of diazinon and malathion to larval rainbow trout (Oncorhynchus mykiss) and their correlation with behavioral measures. Environ Toxicol Chem 2000;19:1875–80.

91. Bisson M, Hontela A. Cytotoxic and endocrine-disrupting potential of atrazine, diazinon, endosulfan, and mancozeb in...
adrenocortical steroidogenic cells of rainbow trout exposed in vitro. Toxicol Appl Pharmacol 2002;180:110–7.

92. Masuo Y, Ishido M. Neurotoxicity of endocrine disruptors: possible involvement in brain development and neurodegeneration. J Toxicol Environ Health B Crit Rev 2011;14:346–69.

93. Bergmann O, Jonas F. Neuroscience. Why adults need new brain cells. Science (New York, N.Y) 2013;340:695–6.

94. Mohanty R et al. Modulation of benzo[a]pyrene induced anxiolytic-like behavior by retinoic acid in zebrafish: involvement of oxidative stress and antioxidant defense system. Neurotox Res 2017;31:493–504.

95. Kim W-K et al. Integrative assessment of biomarker responses in pale chub (Zacco platypus) exposed to copper and benzo[a]pyrene. Ecotoxicol Environ Saf 2013;92:71–8.

96. Oliveira HHP et al. Mixtures of benzo(a)pyrene, dichlorodiphenyl-trichloroethane and tributyltin are more toxic to neotropical fish Rhamdia quelen than isolated exposures. Ecotoxicol Environ Saf 2015;122:106–15.