Effect of Early Life Exposure of Polystyrene Microplastics on Behavior and DNA Methylation in Later Life Stage of Zebrafish

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Research Article

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Abstract

Microplastic contamination has received increasing attention in recent years, and concern regarding the toxicity of microplastics to the environment and humans has increased. In this study, we investigated the neurodevelopmental toxicity of polystyrene microplastics (PSMPs) in the zebrafish *Danio rerio* under different exposure scenario. We investigated the effect of early life exposure to PSMPs on responses later in life, under different exposure scenarios. Zebrafish were exposed to PSMPs during embryonic stage, then allowed the fish to recover. The neurodevelopmental toxic responses were investigated using fish behavior and behavior-related gene expression. Early life exposure to PSMPs did not alter fish behavior at the early stage, however, it led to hyperactivity later life-stage. Generally, oxidative stress (i.e. *sod2* and *nrf2a*) and nervous system (i.e. *slc6a4b*, *npv* and *nrbf2*)-related gene expression increased in all PSMPs-exposed fish. DNA hypomethylation was observed in fish challenged for a second time using the same PSMPs. Taken together, the current results imply that PSMPs have neurodevelopmental toxic potential when introduced early in life.

1. Introduction

In recent decades, the distribution of microplastics has increased markedly in the aquatic ecosystem, the terrestrial environment and the atmosphere (Ng et al. 2018). Approximately 155 – 265 million tons of plastic are expected to accumulate in the natural environment by 2060, with microplastics accounting for 13.2% of this weight (Bergmann et al., 2019; Geyer et al., 2017). Invertebrates and various species of fish have been reported to ingest microplastics at concentrations of one to hundreds of particles per organism (Vandermeersch et al., 2015; Wesch et al., 2016). Plastic toxicity is thought to be connected to stress related to ingestion of the plastic itself as well as leakage of additives in the plastics and adsorbed organic pollutants on the surface of microplastics (Anderson et al., 2016). Recent studies using polystyrene particles as a model suggest that plastic particles accumulate in various fish species, then lead to behavior alteration in the fish. For example, polystyrene particles were shown to accumulate in the brain tissue of adult crucian carp that was exposed to ~130 mg of particles per feeding, leading to morphological changes in the brain and behavioral changes (hypoactivity) (Mattsson et al. 2017). Also, polystyrene particles (1 – 50 mg/L) were found to accumulate in zebrafish (*Danio rerio*) embryos/larvae (Chen et al., 2017; van Pomeroy et al., 2017), and this accumulation was linked to behavioral alterations, oxidative stress, and a reduction in acetylcholinesterase activity (Chen et al. 2017).

The zebrafish has become one of the most popular alternative toxicological models due to its rapid growth, high egg yield, short generation time, and well-documented genetics and developmental biology (Bahl et al., 2020; Lam et al., 2019). The key characteristics that make zebrafish an excellent model system for developmental toxicity testing include external development of transparent embryos, which facilitates morphological assessment during development, and its significant physiological and genetic homology with humans (Gomes et al., 2020; Cavalieri and Spinelli, 2017; Dach et al., 2019). Recently, zebrafish embryos exposed to various chemicals have been used as a platform to screen for DNA methylation modifications (Bouwmeester et al. 2016).

Exposure to environmental chemicals in early life stages could increase the susceptibility to an adverse outcome or disease burden in later life or disrupt the normal pattern of development because the early lifetime period (embryo or fetus) is more sensitive to chemicals (Schug et al. 2011; Boekelheide et al. 2012). Recently, we examined the toxicity mechanisms and susceptible developmental stage of the biocide CMIT/MIT by analyzing several endpoints (morphology, heart rate, behavior, global DNA methylation, and protein profile) in zebrafish embryos (Chatterjee et al. 2021). In that study, the life stage windows with the greatest sensitivity to biocide exposure were identified through a zebrafish embryo test.

Maternal exposure to neurotoxic substances and stress in the prenatal period is likely to affect brain development, increasing the risk of neurological disorders in later stages of life (Harris and Seckl, 2011, Kundakovic and Jaric, 2017, Perera and Herbstman, 2011, Tran and Miyake, 2017). The epigenome is particularly sensitive to environmental exposure during prenatal development because epigenetic reprogramming and programming events occur extensively, establishing cell- and tissue-specific gene expression. (Perera and Herbstman, 2011). During neurodevelopment, epigenetic programming processes prompt the expression of specific factors, promoting neuronal or glial gene transcription to regulate neural stem cell (NSC) commitment to neuronal or glial fate (Hirabayashi and Gotoh, 2010). Therefore, in the developmental process, the epigenome is a major target because external stress can disrupt normal neurodevelopment and gene expression patterns, resulting in long-term effects on brain structure and function.

In this study, we investigated the effect of early exposure to polystyrene microplastics (PSMPs) on the later life stage of zebrafish. We used apical endpoints (mortality and behavior) and an anchored molecular approach (gene expression and DNA methylation) to elucidate the underlying mechanisms of toxicity after developmental exposure of zebrafish to PSMPs. Initially, the embryos were continuously exposed to PSMPs for 4 days post-fertilization, and mortality, behavior, gene expression, and global DNA methylation were examined. Subsequently, the fish were maintained in clean media for 4 months, followed by a second challenge for 3 days. Fish that were exposed to PSMPs only during adulthood were also tested. The same endpoints were examined after the cessation of the second exposure.

Finally, the multi-level results were combined to explore the effects of PSMPs exposure during the early developmental stages. Part 1. The effect of early exposure to PSMPs on adult zebrafish. Part 2. The effect of re-exposure to adult stage of zebrafish early exposed to PSMPs. Given that microplastics are widely distributed in the environment, humans are expected to be exposed to them throughout all life stages; this study sheds light on the adverse developmental effects related to microplastic exposure.

2. Materials And Methods

2.1 Maintenance of zebrafish

Wild-type zebrafish (*D. rerio*) were provided by the Zebrafish Center for Disease Modeling (ZCDM), Korea. Zebrafish were maintained in an automated circulatory system with a 14:10 h light/dark cycle. The water

was maintained at a temperature of 28°C and pH 7.0 – 8.0. Before beginning the experiments, the fish were fed twice daily, with fish food flakes (Tetramin; Tetrawerke) in the morning and brine shrimp (INVE SEP Art Artemia, USA) in the afternoon. Before egg collection, each tank contained 15 adult zebrafish with a female to male ratio of 1:2. Spawning was induced in the morning when the lights came on after the 10 h dark period. At 6 hours post-fertilization (6 hpf), embryos that developed normally and reached the blastula stage were selected for subsequent experiments under a dissection microscope. All experiments using zebrafish were conducted according to approved guidelines (OECD 1992).

2.2 Polystyrene microplastics characterization and preparation

Polystyrene microplastics (PSMPs, size 9.5-11.5 µm) were purchased from Cospheric LLC (Korea). According to the manufacturer, the particles are spherical and the density is 1.07 g/ cm³. Red color dyed polystyrene microplastics (average diameter 10 µm, density 1.06g/cm³) were purchased from Phosphorex Inc. The shape and structure of PSMPs were determined using a field-emission scanning electron microscope (SEM, HITACHI, SU8010). The size distribution and zeta potential of PSMPs in the water were measured using dynamic light scattering (DLS) (Zetasizer Nano, Malvern Instruments Ltd., Malvern, UK).

2.3 Embryo toxicity assay

300 embryos were transferred into six petri dishes (SPL, 90x15mm) containing 30 mL of 50 mg/L PSMPs solution. And 300 embryos were transferred into six petri dishes (SPL, 90x15mm) containing 30 mL of clean water as the control group. From 4 to 96 hpf (from 0 to 4 dpf), three groups of embryos were subjected to the locomotor assay at the end of exposure. The other three groups of embryos were gently transferred to a 1 L beaker containing 500 mL of clean water for adults test.

2.4 Adults fish toxicity assay

The zebrafish larvae (96 hpf) were cultured 1 L beaker containing 500 mL of clean water for 10 days, then transferred to a 3 L automated circulatory system tank for 4 months. After 4 months, 12 adult fish were randomly chosen for the later life stage PSMPs exposure. Six of these 12 were transferred in a 3 L beaker containing clean water as a control group, while the other 6 adult fish were exposed to PSMPs 50 mg/L solution in a 3 L beaker for 3 days with food. After the 3 days, the 6 adult fish were transferred to 6-well plate (one fish/well) for the larvae locomotor activity assay and then stored at -80°C for molecular work (global DNA methylation and gene expression).

2.5 Exposure scenario

Exposure to chemicals in early life stage could increase susceptibility to chemical toxicity or disease burden in later life stage (Duan et al. 2020; Jakubowska et al. 2020). To investigate the effect of early life exposure to PSMPs on responses later in life, we studied the toxic response underlying four different exposure scenarios (Fig. 2). 1. The effect of early exposure to PSMPs on the adult stage of zebrafish was investigated using fish exposed to PSMPs during the embryonic stage and then allowed to recover (PS-

C). 2. The response was compared with that of unexposed fish (C-C). 3. Effect of re-exposure to PSMPs on the adult stage of zebrafish was investigated using fish exposed to PSMPs at the embryonic stage and re-exposed at the adult stage (PS-PS). 4. The response was compared with that of the fish exposed only in adulthood (C-PS). The toxic responses were compared across different exposure scenarios (C-C vs. PS-C and C-PS vs. PS-PS) on the basis of fish behavior, behavior-related gene expression, and DNA methylation.

2.6 Locomotor activity assay

At the early life stage, 96 hpf (4 dpf) larvae were gently transferred into a 96-well plate (one larva per well with 200 µL of clean water) from the three 90 pi petri dishes. At the later life stage, 122 dpf fish were gently transferred into a 6-well plate (one fish per well with 5mL of clean water) from the PSMPs exposure groups. We allowed enough time (2 – 3 hours) for the larva and adult fish in the incubator to adapt to the behavior plate (96-well or 6-well plate) before they were subjected to the locomotor activity assay. Locomotive behavior was measured using a ZebraBox (Viewpoint, France). Locomotor activity was quantified using VideoTrack for Zebrafish™ (Version 3.5 with background subtraction, Viewpoint, France) based on a previous study with some modifications (Winter et al. 2008). Data (number of movements) were collected every 30 min and analyzed. The parameters of the videotracking system needed were set as follows: detection threshold, 20; duration, 30 min; movement threshold, inact/small, 4.0 mm/s, small/large, 20 mm/s.

2.7 Global DNA methylation

Total DNA was extracted from the PSMPs-treated samples using a DNA extraction kit (NucleoSpin, Macherey-Nagel). We used a Nanodrop (ASP-2680, ACTGene, Piscataway, USA) to detect DNA quantity and quality. Then, global DNA methylation assays were conducted in accordance with the manufacturer's instructions (MethylFlash Methylated DNA 5-mC Quantification Kit (Colorimetric), Epigentek Group Inc. NY, USA). DNA (100 ng) and other supplied standards were added to a 5-Methylcytosine (5-mc)-coated well and incubated for another 90 min at 37°C. Subsequently, after addition of the antibodies (anti-5mc and secondary antibody in the supplied enzyme-linked immunosorbent assay buffer), enhancers and stop solutions, absorbance at OD450 nm was measured and 5-mc % was calculated from the standard curve. Standard curves were prepared using the appropriate negative and positive controls (Chatterjee et al. 2016).

2.8 Gene expression using qRT-PCR

Zebrafish brains were isolated to analyze gene expression. Only the zebrafish larvae were used the whole body for analysis. The gene expression analysis of exposed zebrafish was performed at 4 and 122 dpf. Total RNA was extracted from the zebrafish using an RNA extraction kit (NucleoSpin, Macherey-Nagel). The quantity and purity of the RNA were assessed using a Nanodrop. cDNA was synthesized using an oligo (dT) primer (Bio-Rad Laboratories, Hercules, CA). Quantitative real time-PCR (qRT-PCR) analysis was carried out with a CFX manager (Bio-Rad) using an IQ™ SYBR Green SuperMix (Bio-Rad Laboratories, Hercules, CA). The qPCR reaction was performed using the two-step RT-PCR method with IQ™ SYBR Green

SuperMix (Bio-Rad) and the data were analyzed according to the $\Delta\Delta CT$ normalized expression method. Control samples and housekeeping gene (*ef1a*) was used for normalization. Two general oxidative stress genes were selected, superoxide dismutase (*sod2*) and nuclear respiratory factor 2 alpha (*nrf2a*). The expression profiles of four genes known to encode enzymes involved in the zebrafish nervous system, solute carrier family 6 member 4b (*slc6a4b*), oxytocin receptor like (*oxtl*), neuropeptide Y (*npv*), and nuclear receptor binding factor 2 (*nrbf2*) were investigated. The primers were constructed based on the gene sequences available at NCBI.

2.9 Statistical analysis

The results are presented as mean $\pm$ standard error of the mean. Data relating to mortality and locomotor activity are presented compared to the control, with statistical differences determined by the t-test. For gene expression, data are presented as the mean compared to control, and statistical analysis was done by one-way ANOVA. All statistical tests were performed with the Statistical Package for Social Sciences 12.0 KO (SPSS Inc., Chicago, IL, USA).

3. Results & Discussion

3.1 Characterization of Polystyrene microplastics (PSMPs)

The polystyrene microplastics (PSMPs) used in this study were characterized by SEM and DLS. SEM image clearly showed the characteristic shape and structure of PSMPs, and the primary particle sizes matched closely (Fig. 1A). Hydrodynamic diameter (HDD) and zeta potential (ζ) were measured by DLS (Fig. 1B). The DLS results showed that the PSMPs were $11.7 \pm 0.4 \mu m$ in size and had a zeta potential of $-40.5 mV$ in clean water. The HDD results indicated that the PSMPs were evenly dispersed in the water. We counted the number of particles depending on the PSMPs concentration (from 0 to 100 mg/L) using a hemocytometer (Fig. 1C). A linear relationship was observed between the number of particles and the nominal concentration of PSMPs in the test media. The 50 mg/L of PSMPs exposed in this study had 1.1×10^5 particles/L. Embryo toxicity assay exposed approximately 60 particles per embryo and adult fish toxicity assay exposed approximately 50000 particles per fish. The number of plastic particles found in the oceans or freshwater could be known from previous studies. Song et al. 2015 reported that a maximum of 162 particles/L was observed with a mesh of $0.75 \mu m$ in the South-west Sea of Korea, and Di Mauro et al. 2017 reported that a maximum of 157 particles/L was observed with a mesh of $0.7 \mu m$ in freshwater of Mexico. It seems that the number of plastic particles exposed in this study is large compared to the number of plastic particles found in nature. However, in this study, the exposure concentration was set in consideration of the bioconcentration concentration through the food chain occurring in nature because it is a short-term exposure.

3.2. The effect of early exposure to PSMPs on adult zebrafish (C-C vs PS-C)

To investigate the effect of PSMPs exposure during the early life stage of zebrafish, developmental toxicity was investigated from 6 hpf to 96 hpf (hours post fertilization).

Coated microplastics ingested in the digestive tract of zebra fish larva (96 hpf) were investigated through a microscope (Fig. S1). Increased mortality was evident in zebrafish exposed to more than 500 mg/L PSMPs (Fig. S2A), whereas no effect on locomotor activity (Fig. S2B) and global DNA methylation (Fig. S2C) was found. Similarly, Mattsson et al., (2017) reported no changes during the first half of a 2-month experiment in which fish were exposed to amino-modified polystyrene particles through the food chain. After confirming that embryonic exposure to PSMPs did not lead to significant toxicity, we investigated the effect of further exposure in adult fish.

Behavioral analysis in animal models has been suggested as a sensitive endpoint for developmental toxicity screening of various environmental chemicals (Dach et al. 2019). Behavioral analysis of microplastic exposure has previously been studied in various fish models (Yin et al., 2019; Yang et al., 2020; Pitt et al., 2018; Chen et al., 2017). In this study, behavioral activity was measured in PSMPs-exposed adult zebrafish by quantifying fish movements (described in detail in the Materials and Methods). Interestingly, adult-stage zebrafish that were exposed to PSMPs during the embryonic stage (PS-C) showed hyperactivity (Fig. 3A) even after the exposure ceased, suggesting that exposure to PSMPs early in life may show an effect in adulthood.

Our results contradict the findings of Yin et al. (2019) who showed that behavioral changes were recovered when black rockfish exposed to PSMPs for 14 days underwent a 7-day depuration period. They suggested that this was due to the elimination of the PS beads from organs/tissues (e.g., gill, skin, and intestine) and the consequent reduction in stress and damage. In line with our results, Limonta et al. (2019) reported hyperactivity in zebrafish exposed to high-density polyethylene (PE) and PS for 20 days. In the same study, the researchers also observed alterations in the expression of genes related to immune and metabolic pathways due to MP exposure that may relate to hyperactivity.

To investigate the underlying mechanism of hyperactivity due to PS exposure, regardless of life stage, we next examined the expression of genes related to oxidative stress and the nervous system to assess whether the increase in locomotive activity is related to the expression of these genes. Numerous studies have suggested that exposure to MPs increases the levels of reactive oxygen species (ROS), thereby activating the cellular antioxidant defense system to combat oxidative stress (Qiao et al., 2019; Wang et al., 2019).

Therefore, we examined the expression of representative oxidative stress defense genes (i.e., *sod2* and *nrf2a*) in an attempt to relate the findings to the change in behavioral indicators due to PSMPs exposure. The expression of the *sod2* and *nrf2a* genes increased in PSMPs exposure groups (Fig. S3). Consistent with our results, many other studies have also demonstrated increased expression of antioxidant enzyme genes such as *sod*, *cat*, *gpx*, and *gst* following PSMPs exposure (J. Wang et al., 2019; Yu et al., 2018).

A few previous studies have reported the neurotoxic effects of MPs in several organisms. For example, PE adversely affect the neuronal functioning of ray-finned fish and gobies (Luís et al. 2015), and PS inhibit acetylcholinesterase (AChE) activity in freshwater fish red tilapia (Ding et al. 2018). The serotonergic system and neuropeptides play modulatory roles in a variety of behaviors. In zebrafish, the solute carrier family includes two serotonin transporter genes (*slc6a4a* and *slc6a4b*) that mediate serotonin reuptake (Theodoridi et al. 2017). Reichmann et al. (2016) reported that stress has a potent effect on neuropeptide Y (*npy*) expression in the brain, with the type and duration of stress, time point of *npy* measurement, and specific brain region all impacting the magnitude and direction of stress-induced *npy* alterations. Many previous studies have demonstrated that *npy* transcription increases after exposure to acute stressors (Chigr et al., 2010, Chigr et al., 2014). Mehr et al. (2020) showed that isotocin, an oxytocin homolog, is involved in social behavior. They found that guppies injected with isotocin spent 29% more time associating with conspecifics than those injected with an isotocin antagonist. Here, we investigated serotonin transporter-related genes (Parolini 2019) to identify the neurotoxic effects of PSMPs and subsequently elucidated the observed alterations in locomotive activity. These genes are solute carrier family 6 members 4b (*slc6a4b*), oxytocin receptor-like (*oxtl*), neuropeptide Y (*npy*), and nuclear receptor binding factor 2 (*nrbf2*).

The expression pattern of serotonin transporter-related genes was consistent with the behavioral results of PS-C (Fig. 3). The expression of *npy*, *slc6a4b*, and *nrbf2* genes was significantly increased by PSMPs exposure (Fig. 3B, C and E). It seems that the expression of serotonin transporter-related genes is significantly upregulated, increasing locomotive activity as the neurotransmitter stays at the synapse for a longer time (Wong et al. 2013). In Wong et al. (2013), when fish showing depressive behavior were selected and treated with fluoxetine (selective serotonin reuptake inhibitor), locomotive activity increased along with a significantly increased expression of solute carrier family genes *oxtl*, *npy*, and *nrbf2*.

Several environmental chemicals are known to modify the developmental epigenome; zebrafish embryos have been used as a platform for screening DNA methylation modifications caused by exposure to various chemicals (Bouwmeester et al., 2016). To investigate the potential of MPs to induce DNA methylation changes, global DNA methylation was assessed in four exposure scenarios (C-C, C-PS, PS-C, and PS-PS). Global DNA methylation showed little change in adults exposed to PSMPs early in life (Fig. 5A). The absence of changes in global DNA methylation does not imply that methylation did not occur in the gene, but it was not possible to observe the significant expression of neuronal-related genes and their association with global DNA methylation.

3.3. The effect of re-exposure to adult stage of zebrafish early exposed to PSMPs (C-PS vs PS-PS)

The second PSMPs challenge was analyzed in 4-month-old fish that were either unexposed or exposed during the embryonic stage (i.e., embryonic stage control-adult stage exposure [C-PS] vs. embryonic stage exposure-adult stage exposure [PS-PS]).

There was no significant difference in locomotive activity (Fig. 4A) when fish were re-exposed to the same PSMPs in adults with and without exposure early in life (C-PS vs. PS-PS). However, their locomotive

activity was significantly higher than that of the control group (C-C), and the results were similar to those of the adults exposed to PSMPs early in life (PS-C).

To investigate the underlying mechanism of hyperactivity due to PS re-exposure regardless of exposure during the early life stage (Fig. 4A), we examined the expression of genes related to oxidative stress and the nervous system. Key events at the molecular level of increased behavior were explored. The expression levels of *slck6ab*, *oxtl*, *nrbf2* and increased (Fig. 4C, D and E) when adults exposed in the early stages of life were re-exposed, whereas the expression levels of *npv* decreased (Fig. 4B). Although the locomotive activity between the two groups (C-PS vs. PS-PS) was similar (Fig. 4A), there were clear changes in the expression levels of neural-related genes (Fig. 4B, C, D and E). The expression of the *nrf2a* gene increased but, the expression of *sod2* gene has not significant change in PS-PS group (Fig. S4). Given that the differences were not consistent, exposure to PSMPs appears to act as a nervous system confounding factor.

Interestingly, a significant trend towards DNA hypomethylation was evident in the PS-PS group (Fig. 5B). DNA methylation results are also related to gene expression results. Although the results of global DNA methylation are insufficient, it can be hypothesized that the demethylation of genes confuses the nervous system when adults exposed to PSMPs in the early stages of life are re-exposed (PS-PS). However, the underlying epigenetic mechanisms require further investigation. There was no difference in DNA methylation in the adults exposed to PSMPs in the early stages of life (Fig. 5A), but when re-exposed at the adult stage (PS-PS), DNA was significantly demethylated compared to the adults (Fig. 5B) who were not exposed to the PSMPs in the early stages of life. These results provide evidence that exposure to PSMPs early in life also affects susceptibility to PSMPs in adulthood.

4 Conclusion

This study examined the behavioral impairments caused by early life stage exposure to PSMPs using molecular markers (i.e., gene expression and DNA methylation) in a zebrafish model. Although there is a limitation in the study that there is only one exposure concentration, the results confirmed that early life stage exposure to PSMPs can change neurobehavior (locomotor activity) and alter epigenetic biomarkers (global DNA methylation) at the adult stage after recovery. Given that zebrafish is a surrogate model for human toxicity, this study supports further risk assessment and human extrapolation studies on PSMPs exposure, specifically regarding neurodevelopmental toxicity.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Jeongeun Im was a major contributor to the experiment and analyzed the results and wrote the manuscript. Hyun-Jung Eom contributed to interpreting gene expression data and writing the manuscript. Jinhee Choi designed the exposure experiment and did the final review of the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Characterization of PSMPs used to test for their toxicity towards zebrafish. (A) SEM image of PSMPs. (B) Size distribution of PSMPs in clean water measured by DLS. (C) Relationship between the number of PSMPs and their concentration in the media.

Part 1 The effect of early exposure to PSMPs on adult zebrafish

Part 2 The effect of re-exposure to adult stage of zebrafish early exposed to PSMPs

Figure 2

Experimental scheme of part 1 and part 2 PSMPs exposure scenarios.

Figure 3

Behavioral changes (A) and nervous system-related (npv; B, slc6a4b; C, oxtl; D, and nrbf2; E) gene expression of adult zebrafish exposed to PSMPs in the early life. Locomotive behavior is expressed as the amount of activity (4 – 20 mm/s) exhibited under dark conditions for 30 min. The results are expressed as the mean value compared to the C-C group (n = 3; mean ± standard error of the mean; *p < 0.05, **p < 0.01, ***p < 0.001).

Figure 4

Behavioral changes (A) and nervous system-related (*npy*; B, *slc6a4b*; C, *oxtl*; D, and *nrbf2*; E) gene expression of adult zebrafish exposed to PSMPs at the embryonic stage and re-exposed at the adult stage. Locomotive behavior is expressed as the amount of activity (4 – 20 mm/s) exhibited under dark conditions for 30 min. The results are expressed as the mean value compared to the C-PS group ($n = 3$; mean $\pm$ standard error of the mean; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 5

Global DNA methylation level of adult zebrafish's brain following the PSMPs exposure scenarios. Part 1. Global DNA methylation level change of early exposure to PSMPs on adult zebrafish (C-C vs. PS-C); A. Part 2. Global DNA methylation level change of re-exposure to adult stage of zebrafish early exposed to PSMPs (C-PS vs. PS-PS); B. The results are expressed as the mean value compared to C-C ($n = 3$; mean $\pm$ standard error of the mean). The statistical differences between each exposure scenarios were also analyzed (* $p < 0.05$).

Supplementary Files

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