Journal of Hazardous Materials 409 (2021) 125016

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Microplastics impair olfactory-mediated behaviors of goldfish *Carassius auratus*

Wei Shi, Shuge Sun, Yu Han, Yu Tang, Weishang Zhou, Xueying Du, Guangxu Liu *

College of Animal Sciences, Zhejiang University, Hangzhou, PR China

ARTICLE INFO

Editor: Dr. R. Teresa

Keywords:
Polystyrene (PS)
Microplastics
Olfactory-driven behaviors
Goldfish

ABSTRACT

Recently, the ubiquitous presence of microplastics (MPs) has drawn worldwide concern over its potential threat to aquatic organisms. However, the effects of MPs on the olfactory ability of fish and the subsequent odorant evoked behaviors remain elusive. In the present study, we analyzed the potential olfactory toxicity of polystyrene (PS) MPs by assessing olfactory-driven behaviors of goldfish in response to odorants. Our results showed that the olfactory-driven behavioral responses of goldfish to L-cysteine and taurocholic acid were significantly hampered by a 28-day MP exposure. Further analysis demonstrated that exposure to MPs may suppress the expression of genes encoding olfactory G protein-coupled receptors, inhibit the enzyme activities of cation transport ATPases crucial for action potential generation, alter the *in vivo* contents of neurotransmitters as well as metabolites involved in the transduction of electrical signals, and cause olfactory bulb injury and neurotoxicity closely related to the processing of electrical signals. In conclusion, the results obtained in the present study suggest that MPs at environmentally relevant concentrations could impair the olfactory-mediated behavioral responses of goldfish, probably through hampering odorant identification, action potential generation, olfactory neural signal transduction, and olfactory information processing.

1. Introduction

Plastics are among the most commonly used artificial materials in daily life and industries, with their annual global production reaching approximately 400 million tons (Prokić et al., 2019). Inevitably, huge amounts of plastic debris enter aquatic environments during plastic production, usage, handling, and disposal (Geyer et al., 2017). It is estimated that more than 5 trillion plastic particles are floating around in the world's surface waters; this number is predicted to increase by an order of magnitude by 2025 (Van Sebille et al., 2015). Microplastics (MPs) in the environment are fragmented plastic waste pieces with diameters < 5 mm (Rochman, 2018). Currently, MPs are ubiquitous in different aquatic environments including polar areas and abyssal regions (Van Cauwenberghe et al., 2013; Lusher et al., 2015). As MPs are durable and refractory, they persist in aquatic environments, thereby posing long-term threats to aquatic organisms and ecosystems (Ma et al., 2020). MPs concentrations as high as approximately 0.25–0.697 mg/L and up to 4.5 mg/L have been detected in some polluted aquatic areas (Collignon et al., 2012; Goldstein et al., 2012; Kang et al., 2015). Owing to their small size, MPs can be easily ingested by various aquatic organisms directly from the surrounding water or indirectly through the food chain (Ma et al., 2020). For example, MPs up to 7.2 items/individual (4.0 items/g) were prevalently detected in samples of 27 fish species collected from the Chinese market (Jabeen et al., 2018). Under certain conditions, these ingested MPs can be translocated into the circulatory system and accumulate in different fish tissues, which may subsequently adversely affect many biological processes and physiological functions, such as fecundity, embryonic development, digestive performance, swimming competence, growth, immune responses, and even survival rate (Limonta et al., 2019; Naidoo and Glassom, 2019; Wang et al., 2019; Yin et al., 2019; Pannetier et al., 2020).

Olfaction, the sense of smell, is one of the most important senses through which aquatic organisms, especially those living in turbid and deep waters where visibility is greatly reduced, detect environmental signals. Behaviors including foraging, predator avoidance, navigation, and courtship that are closely linked to fish survival, rely largely on chemosensory cue detection via the olfactory system (Hamdani and Døving, 2007). Therefore, any olfactory perception and odorant response disruptions exert profound effects on fish populations and

* Corresponding author.
E-mail address: guangxu_liu@zju.edu.cn (G. Liu).

https://doi.org/10.1016/j.jhazmat.2020.125016
Received 13 November 2020; Received in revised form 15 December 2020; Accepted 29 December 2020
Available online 31 December 2020

ecosystems (Tierney et al., 2010). The peripheral olfactory fish organs are in intimate contact with the ambient environment, owing to their origin and structure; therefore, they are sensitive to environmental changes and pollutants, such as acidification, toxic metals, and pesticides (Munday et al., 2009; Tierney et al., 2010).

The occurrence of the olfactory response in fish consists of a series of consecutive events including the binding of odorants with olfactory receptors, the activation of secondary messenger systems, the action potential generation through free intracellular Na^+ and Ca^{2+} increases, the transduction and processing of electrical signals, and the behavioral responses induction (Hara, 1994). As small-sized waterborne MPs are pervasive in the water, they could, theoretically, enter the olfactory chamber of fish through the water flow and reach their sensory tissue, thereby possibly influencing their olfactory ability. However, to the best of our knowledge, the effects of MP on fish olfactory ability and subsequent odorant-evoked behaviors remain elusive.

The goldfish (*Carassius auratus*) has been widely used as a model organism in endocrinology, genetics, and sensory biology for research into olfaction, vision, and gustation (Blanco et al., 2018). Closely related to many economically and ecologically important fish species and with a variety of well-established behavioral assays, goldfish can serve as good animal models to study the behavioral responses of fish to different environmental factors and facilitate the analysis of underlying affecting mechanism (Maximino et al., 2015). The aim of this study was to improve the current understanding of the ecotoxicological effects of MPs and the potential olfactory toxicity of polystyrene (PS) MPs, one of the most abundant MPs types in the aquatic environment (Browne et al., 2011). To this end, we assessed olfactory-driven goldfish behaviors in response to odorants. Moreover, we analyzed the expression of genes encoding olfactory G protein-coupled receptors (GPCRs) for odorant identification, the Ca^{2+}-ATPase and Na^+/K^+-ATPase enzyme activities that are crucial for action potential generation, the *in vivo* contents of neurotransmitters and the expression of neurotransmitter-related genes involved in the electrical signal transduction, and the olfactory bulb injuries and neurotoxicity closely related to electrical signal processing; additionally, we performed a comparative metabolomic analysis to reveal the underlying toxicity mechanisms.

2. Materials and methods

2.1. Experimental animals and chemicals

We obtained naive (previously untested) *C. auratus* (1-year-old, wet weight 7.5 ± 1.7 g, mean ± SE) from a commercial supplier (Wenzhou, Zhejiang Province, China) in September 2019 and kept them in three 500 L tanks (approximately 200 individuals in each tank) containing 400 L freshwater (tap water that aerated continuously for 72 h to remove redundant chlorine) with constant aeration (temperature 24.2 ± 0.5 °C; pH 7.2 ± 0.3; dissolved oxygen > 9 mg/L; NO_4^+ < 0.04 mg/L; 12 h light/dark cycle; the water was filtered through a 0.45 μm membrane filter prior to use to minimize potential MP pollution). The goldfish were acclimated for four weeks before the experiment initiation. Throughout this period, the goldfish were provided with commercial food pellets (Tongwei Group, Chengdu, China) at 9:00 a.m. every day. The number of MPs in goldfish and food pellets was verified as being negligible before the experiments, following the methods reported in our previous study. All experiments of the present study were approved by the Animal Care Committee of Zhejiang University and all methods were performed in accordance with the Guidelines for the Care and Use of Animals for Research and Teaching at Zhejiang University (ETHICS CODE Permit NO. ZJU2011-1-11-009Y).

2.2. Chemicals

PS microbeads monodispersed in pure water at a concentration of 25 mg/mL with nominal sizes of 500 nm and 30 μm, the physiochemical

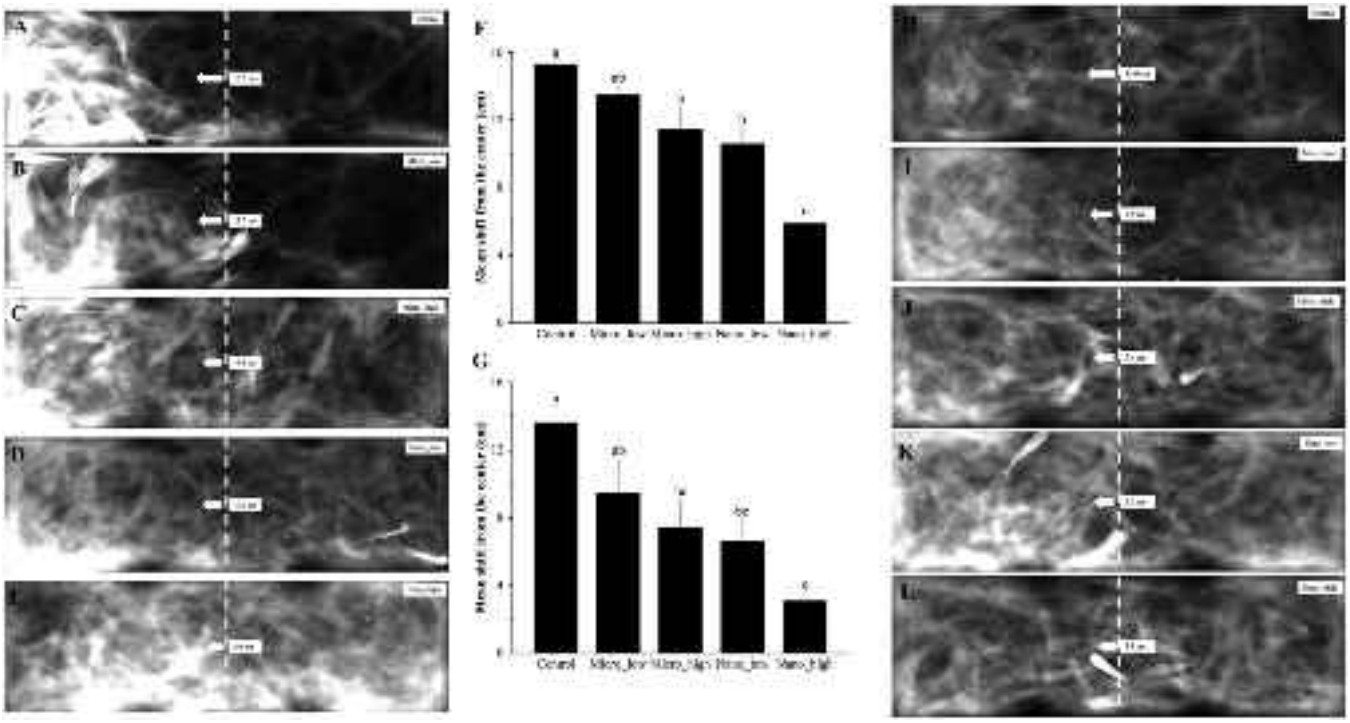

Fig. 1. Microplastic (MP) effects on the olfactory-driven behavioral response of goldfish to L-cysteine (Lys) and taurocholic acid (TCA). A–E and H–L: summed videograms showing the distribution activity of goldfish in the apparatus at the presence of Lys and TCA, respectively, after being subjected to different exposure conditions. The videograms were created from 5-min recordings of behavioral responses following divider removal. Arrows denote the mean shift from the center of the apparatus. F, G: distribution activity of goldfish from different experimental groups in response to (F) Lys and (G) TCA (Mean ± SE, n = 10). Mean values that do not share the same superscript were significantly different ($p < 0.05$).

characteristics of which have been reported in our previous studies were purchased from Aladdin Reagent Co. Ltd (Shanghai, China) (Tang et al., 2020a; Zhou et al., 2020). Taurocholic acid (TCA; powder, purity > 99%) and L-cysteine (Lys; powder, purity > 99%) were selected as experimental odorants in this study owing to their identified functions in inducing olfactory-mediated behavior in goldfish (Sorensen et al., 1987; Heffern et al., 2018). Based on previous reports, we prepared odorant solutions at the most effective concentrations to evoke olfactory-mediated responses in goldfish (10^{-8} M) by dissolving the powder of a given odorant in distilled water (Sorensen et al., 1987).

2.3. MPs exposure and sampling

One control group and four treatment groups were set up in triplicates in this study as follows: (1) Control group without the addition of MPs, (2) Nano_low group with 0.26 mg/L MPs (500 nm), (3) Nano_high group with 0.69 mg/L MPs (500 nm), (4) Micro_low group with 0.26 mg/L MPs (30 μm), and (5) Micro_high group with 0.69 mg/L MPs (30 μm). According to the prediction of global plastic waste input and mass concentration of MPs in polluted areas, 0.26 mg/L and 0.69 mg/L were selected as the MP exposure concentrations in this study to simulate realistic pollution scenarios of MP in aquatic environments (Goldstein et al., 2012; Jambeck et al., 2015). As it has been suggested that the majority of larger MPs (diameter > 20 μm) may be removed from the bodies of aquatic organisms, while smaller particles can be retained in their tissues and circulatory systems, MPs of two different critical sizes were used in this study to investigate any size dependent effects (Van Cauwenberghe and Janssen, 2014).

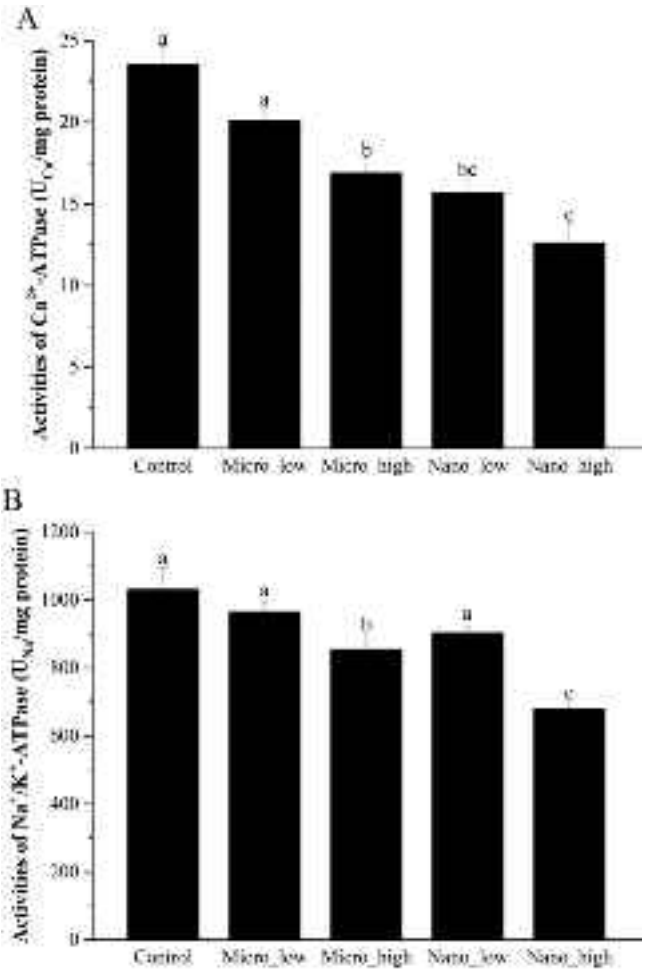

Fig. 2. Microplastic (MP) effects on the activities of (A) Ca^{2+}-ATPase and (B) Na^{+}/K^{+}-ATPase in the olfactory epithelium of goldfish (Mean ± SE, n = 6). Mean values that do not share the same superscript were significantly different ($p < 0.05$).

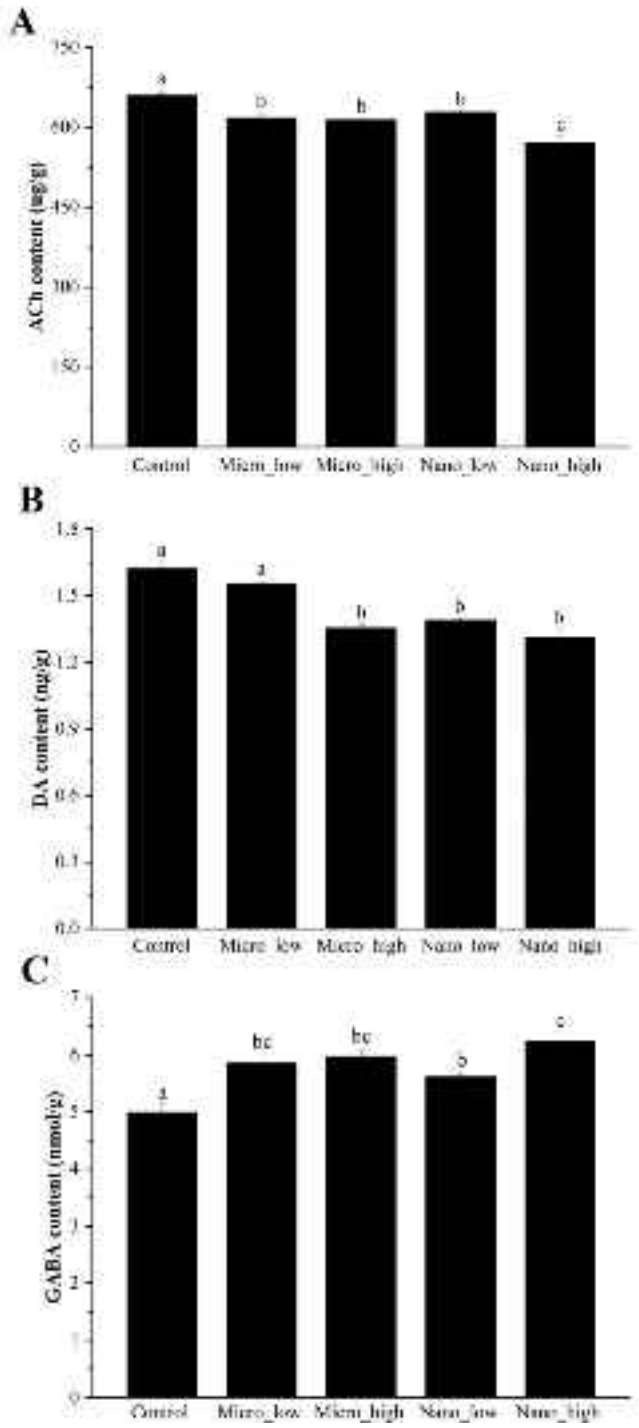

Fig. 3. The *in vivo* concentrations of (A) acetylcholine (ACh), (B) dopamine (DA), and (C) γ-aminobutyric acid (GABA) in goldfish brains after a 28-day exposure to microplastics (MPs) (Mean ± SE, n = 5). Mean values that do not share the same superscript were significantly different ($p < 0.05$).

After acclimation, 450 goldfish were randomly assigned into 15 individual tanks (5 experimental groups × 3 replicates) containing 60 L membrane filtered water with slight aeration (temperature 24.5 ± 0.3 °C, 12 h light/dark cycle). The water was refreshed daily with MPs added anew at appropriate doses and sizes to minimize the influence of food debris or fish excreta. During the experimental period, goldfish

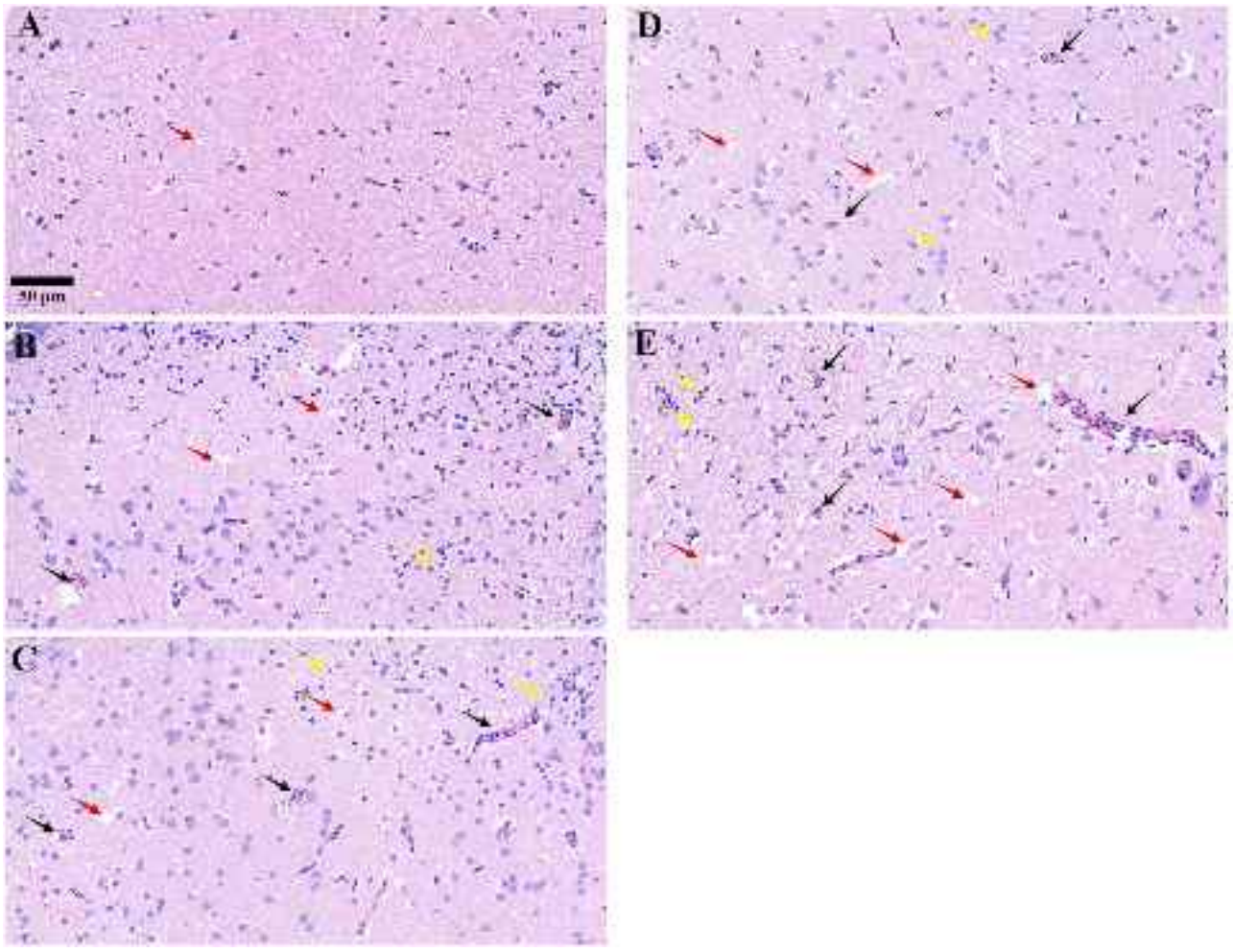

Fig. 4. Representative histological photomicrographs of goldfish brains from the (A) Control, (B) Micro_low, (C) Micro_high, (D) Nano_low, and (E) Nano_high groups [hematoxylin and eosin (H & E) ×400; scale bar = 50 μm]. Black, red, and yellow arrows indicate capillary congestion, tissue cavitation, and intercellular space around the glial cells, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were fed commercial food pellets as described above. The exposure experiment lasted four weeks and no individual mortality was observed throughout this period.

After the exposure period ended, 45 goldfish individuals were randomly sampled from each experimental group and humanely euthanized using benzocaine. Brain tissues including olfactory bulb of 15 fish were dissected on ice, brain tissues of 10 individuals were pooled in 5 samples for the neurotransmitter content determination (n = 5), and the remaining 5 brain tissues were used for histopathological and immunofluorescence observations (n = 5). Similarly, brain tissues of 15 fish were pooled into 6 samples to analyze the expression of neurotransmitter-related genes (n = 6). The olfactory epithelium tissue of 30 goldfish from each group was peeled off on ice and pooled in six samples for the measurements of the enzyme activities of Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase as well as the expression levels of GPCRs (n = 6). In addition, brain tissues of 15 goldfish from each of the Control, Nano-high, and Micro-high groups were dissected (five replicates for each experimental group, each with brain tissues pooled from three individuals) and used for the metabolomic analysis.

2.4. Behavior assays

The behavioral changes of goldfish in response to the odorants (TCA and Lys) were determined following the reported methods (Heffern et al., 2018; Shamchuk et al., 2018). In brief, a custom-built plexiglas apparatus (L 45 cm × W 15 cm × H 20 cm) with two removable inserts that divided the trough into three equally sized zones was mounted on a table with stable illumination. Membrane filtered freshwater (12 L) was added to the trough and the barriers were inserted. After a 28-day MPs exposure, five naive goldfish of each experimental group were randomly selected and transferred to the middle zone of the apparatus. Following a 10-min acclimation, a volume of 100 μL odorant solution was added into the adjacent zone and mixed thoroughly. An odorant was randomly added either to the left or the right zone of the trough and the equivalent amount of freshwater was added to the other zone. After another 10 min, which was enough for the odorant solution to diffuse equally throughout the designated side, the inserts were carefully removed and fish movement was recorded for 5 min using an overhead camera (1920 × 1080 pixels @ 30 fps; C930e; Logitech, Lausanne, Switzerland) connected to a computer. Behavioral trials were conducted between 9:00 and 17:00 to ensure light exposure occurred within the normal rearing light cycle (MacPhail et al., 2009). Ten replicates were conducted for each treatment group and each fish individual was only tested once. In addition, a negative control group was set up by adding freshwater into the apparatus, to ensure that there was no behavioral bias from goldfish toward one area of the apparatus; the results did not show any side bias.

The quantitative analysis of fish behavior was performed following the method of previous studies (Wyeth et al., 2011; Shamchuk et al., 2018). The video captured was converted into AVI format with

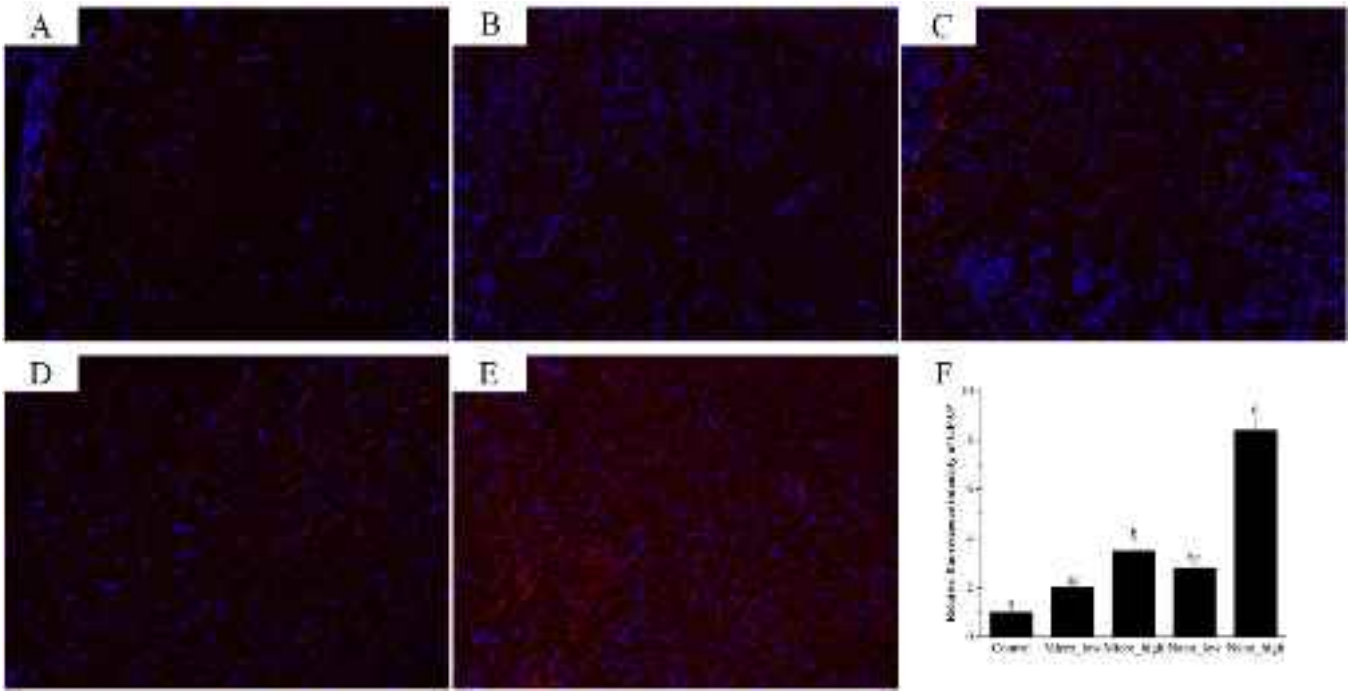

Fig. 5. Glial fibrillary acidic protein (GFAP) immunohistochemical staining of brain astrocytes after a 28-day exposure of goldfish to microplastics (MPs) (×200 magnification). The nucleus and GFAP were stained in blue and red, respectively. A, Control group; B, Micro_low group; C, Micro_high group; D, Nano_low group; E, Nano_high group; F, the relative GFAP-specific fluorescence intensity in the brain astrocytes of goldfish (Mean ± SE, n = 6). Mean values that do not share the same superscript letters were significantly different ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

VirtualDub (http://www.virtualdub.org) and a series of grayscale images were subsequently obtained using ImageJ software (https://imagej.nih.gov/ij/). In order to eliminate stationary fish and separate the moving goldfish from any background noise, the background image from each video frame was subtracted by imposing a threshold in ImageJ. These series of binary images were then summed together resulting in a videogram representing the 5-min fish movement, where the pixel intensity represented the frequency of activity in that pixel location over the entire video sequence. The movement of all five fish over time was treated together as a whole and the mean center of these fish was calculated for each videogram by the pixel intensity using ImageJ. The shift distance from the apparatus center was determined and normalized by dividing the average distance of the control, followed by conversion into percent response following the method described by Heffern et al. (2018), where 100% meant that full sensory function was retained and 0% represented the loss of olfactory-mediated behavior.

2.5. Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase activity measurements

The enzyme activities of Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase were determined based on inorganic phosphate production using commercially available kits (A070-4 and A070-2, respectively; Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer's instructions. Approximately 2 mg of the collected olfactory epithelium tissue was homogenized, mixed with a quadruple volume of ice-cold saline, and immediately centrifuged (4 °C, 2000*g*) for 10 min. The remaining supernatant was used to determine the Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase activities. The total protein concentrations of these samples were determined with a commercial kit (P0006; Beyotime Institute of Biotechnology, Nanjing, China) using the Bradford method. All the enzyme activities were expressed as U per mg protein, where U was defined as the enzyme causing the conversion of 1 mmol of substrate per min.

2.6. Quantification of neurotransmitters concentrations

The collected olfactory bulbs were homogenized in ice-cold phosphate-buffered saline (0.01 M, pH 7.4, w/v (mg/mL) = 1/1) on ice by an electric homogenizer (ART, Miccra D-1; Miccra, Heitersheim, Germany) and subsequently centrifuged (4 °C, 2000*g*) for 20 min. The precipitate was discarded, and the supernatant was used to determine the neurotransmitters concentrations. The content of neurotransmitters such as DA, ACh, and GABA that play important roles in olfactory signal transduction were measured using commercial ELISA kits (ML090244, ML095412 and ML086216, respectively; MLBIO Biotechnology, Shanghai, China) with a microplate reader (Multiskan GO; Thermo Fisher Scientific, Waltham, MA, USA) at an absorption wavelength of 450 nm following the manufacturer's instructions (Tatti et al., 2014).

2.7. Histopathological and immunofluorescence analyses

All histopathological and immunofluorescence observations were performed according to standard laboratory procedures. After being fixed in a 4% paraformaldehyde solution for 24 h, the tissues were embedded in paraffin, sliced into 5-μm thick sections, and mounted on slides. For the histopathological examination, the slides were stained with hematoxylin and eosin (H & E), and examined under an optical microscope at a magnification of 400× (Eclipse Ci-L, Nikon, Tokyo, Japan).

The immunofluorescence staining was conducted following the standard laboratory methods. In brief, brain tissue sections were treated with a heat induced antigen retrieval technique in citrate buffer (pH 6.0). Then the sections were incubated overnight with the primary anti-GFAP (1:100, GTX27260; GeneTex, Irvine, CA, USA) in 5% bovine serum albumin (A7030; Sigma-Aldrich, St. Louis, MO, USA), followed by the addition of secondary fluorescent antibodies (1:400, A0408; Beyotime, China). After a 1-h incubation, DAPI (C1005; Beyotime) was used to stain the nuclei of the sample. Images were subsequently captured

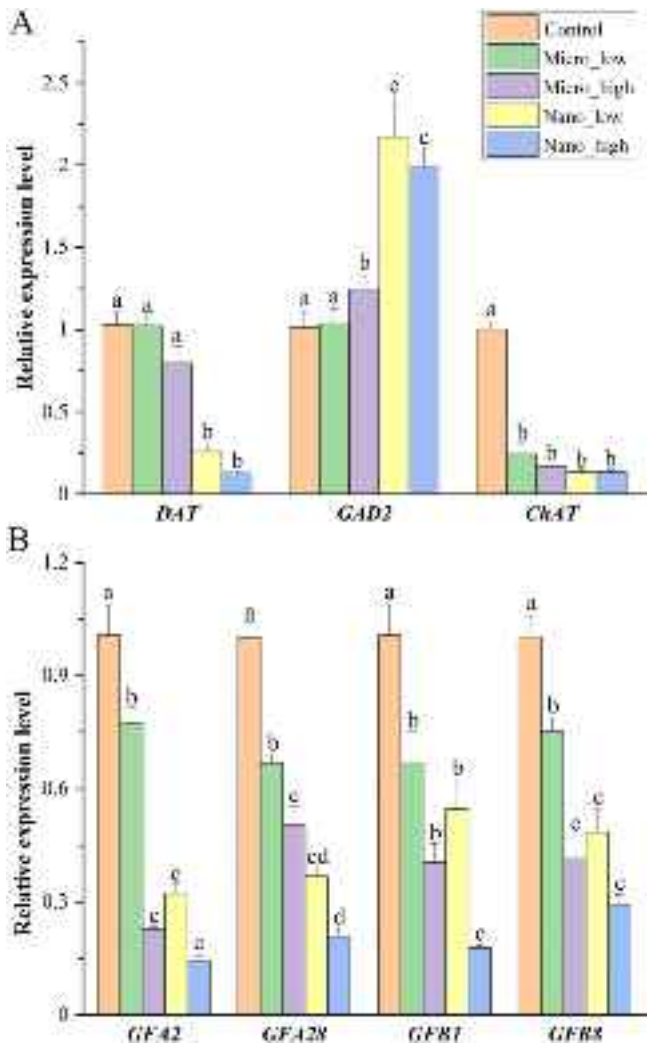

Fig. 6. Expression levels of (A) neurotransmitter related genes and (B) G protein-coupled receptors (GPCRs) after a 28-day exposure to microplastics (MPs) (Mean ± SE, n = 6). Mean values that do not share the same superscript were significantly different ($p < 0.05$).

using a confocal laser scanning microscope (BX61WI; Olympus, Tokyo, Japan) with FV-ASW 2.0 Viewer software (Olympus). The relative GFAP-specific fluorescence intensity of each sample was subsequently estimated following the method described by Gallagher (2010).

2.8. Gene expression analysis

The expression of genes encoding four GPCRs (*GFA2*, *GFA28*, *GFB1*, and *GFB8*) and three modulatory enzymes or neurotransmitter receptors (*DAT*, *GAD2*, and *ChAT*) was investigated in the olfactory epithelium and olfactory bulb, respectively (Cao et al., 1998). The total RNA of each individual was extracted from the olfactory epithelium and olfactory bulb with an EASYspin Plus RNA extraction kit (RN2802; Aidlab, Beijing, China) following the manufacturer's instructions. The RNA quality and concentrations were checked with gel electrophoresis and a NanoDrop 1000 UV/visible spectrophotometer (Thermo Fisher Scientific), respectively. High-quality RNA samples were reversely transcribed into first strand cDNA using a PrimeScript RT Reagent (RR037A; TaKaRa Bio, Beijing, China). Real-time quantitative PCR was conducted using the StepOnePlus Real-time PCR system (Applied Biosystems, Foster City, CA, USA) following reported protocols (Shi et al., 2019). The gene *β-actin* was adopted as an internal reference and the $2^{-\Delta\Delta Ct}$ method was applied to analyze the relative expression levels of genes (Livak and Schmittgen, 2001). All primers (listed in Table S1) used in this study were synthesized by Tsingke Biotech (Shanghai, China).

2.9. Metabolomics profiling for identification and analysis

After weighing, 0.1 g brain tissues collected from the Control, Nano-high, and Micro-high groups was used for the metabolomics analysis. Each replicate brain tissue was mixed with 1000 μL extract solution (acetonitrile/methanol/water = 2/2/1, containing 2 μg/L internal standard) followed by homogenization and sonication. After incubation at − 40 °C for 1 h, the sample was centrifuged (4 °C, 10,000*g*) for 15 min. The supernatant (825 μL) was transferred to a new centrifuge tube, dried in a vacuum concentrator, and reconstituted in 200 μL acetonitrile (50%). The constituent was then centrifuged (4 °C, 13,000 rpm) for 15 min and a volume of 75 μL supernatant was transferred to a fresh glass vial for the liquid chromatography-mass spectrometry (LC-MS) analysis. The sample used as a quality control (QC) was prepared by mixing equal aliquots of the supernatants of all samples. The ultra-high pressure liquid chromatography (UHPLC) separation was performed by Biomarker Technology Co. Ltd. (Beijing, China) using a 1290 Infinity series UHPLC System (Agilent Technologies, Santa Clara, CA, USA).

The raw data were converted into the mzXML format by ProteoWizard (proteowizard.sourceforge.net) and processed by the R package xcms (Version 3.2) for peak deconvolution, alignment, and integration, during which 0.5 and 0.6 were adopted as the minfrac and cut off, respectively. An in-house MS2 database was employed for the metabolite identification. Variable importance in projection (VIP) statistics from orthogonal partial least squares-discriminant analysis (OPLS-DA) modeling was used to identify the overall contribution of each variable. Metabolites with a VIP value > 1 and a p value < 0.05 were considered significantly different. The biological functions of metabolites were further inferred from the KEGG (http://www.genome.jp/kegg/).

2.10. Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's post hoc tests were conducted to compare the olfactory mediated responses, the *in vivo* concentrations of neurotransmitters, and the enzyme activities of Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase among experimental groups. The expression levels of the tested genes were compared by Duncan's multiple range tests. In all analyses, the homogeneity of data and normality of variances were verified with Levene's test and the Shapiro-Wilk's test, respectively. All analyses were performed with statistical software OriginPro 2020 (OriginLab Corporation, Northampton, MA, USA) and a p value less than 0.05 was adopted as statistically significant.

3. Results

3.1. MP effects on the olfactory-driven behavior of goldfish

The activity distribution of goldfish in the odorant supplemented zone decreased significantly compared to the control group after a 28-day exposure to MPs (Fig. 1A–L). Although we did not detect any significant differences in the distribution shift from the apparatus center between the control and Micro_low groups, exposure to higher MP concentrations (0.69 mg/L) or smaller sized MPs (500 nm) resulted in evident ($F_{4,49} = 14.8$, $p < 0.05$) alternations in the distribution of goldfish, thereby indicating a size and concentration dependent effect of MPs on goldfish (Fig. 1F,G). For example, the distribution shifts of goldfish from the Nano_high group were significantly ($p < 0.05$) suppressed to approximately 38.5% (response to TCA) and 22.7% (response to Lys) of the control group, respectively (Fig. 1F,G).

3.2. MP effects on the Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase activities

After the end of the exposure, the enzyme activities of Ca^{2+}-ATPase

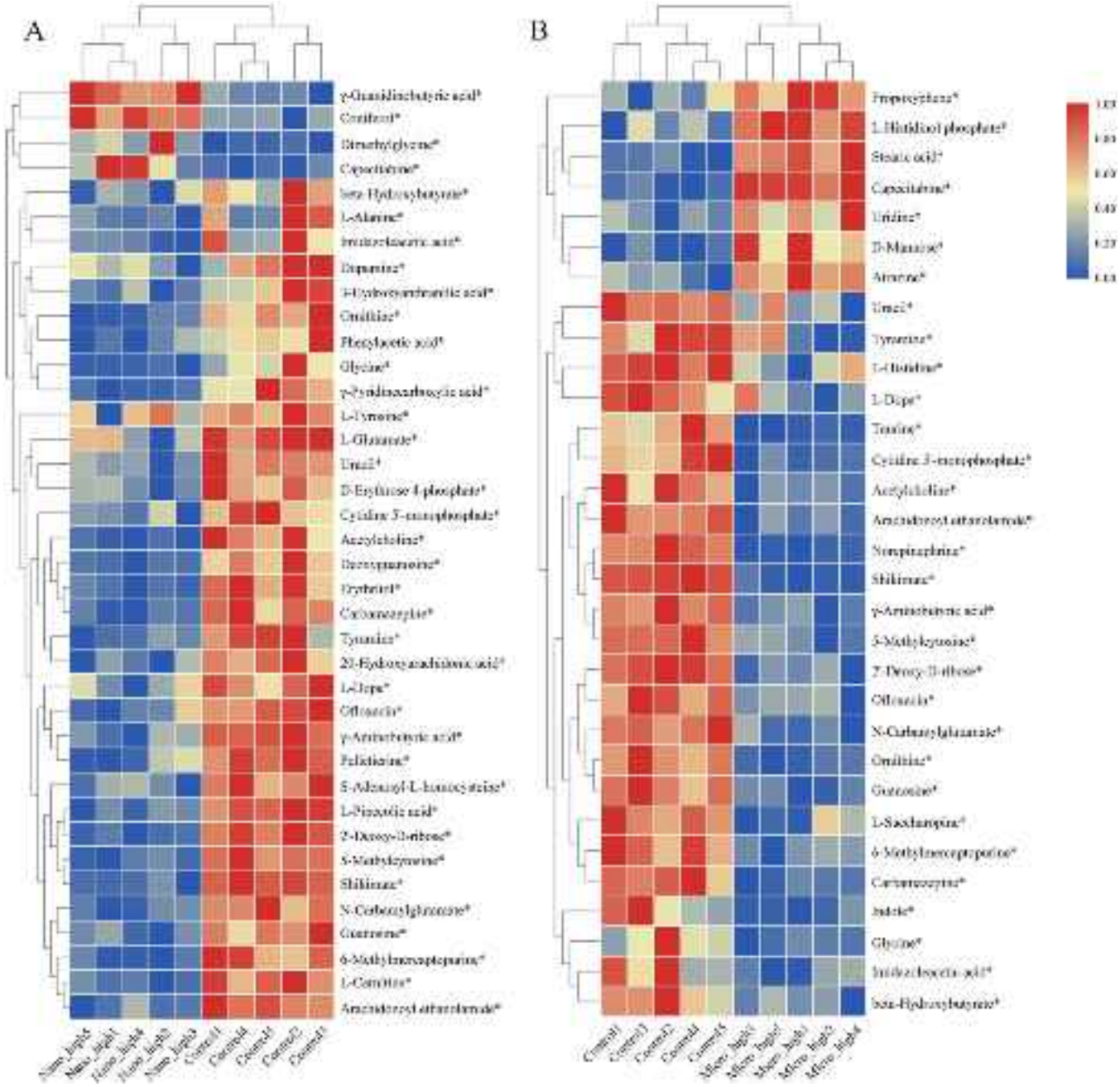

Fig. 7. Hierarchical clustering heatmap of significantly altered metabolites of (A) the Nano_high group and (B) the Micro_high group versus the control. Metabolites and samples are clustered by similarity of expression (left and top, respectively). The blue/yellow scale represents standardized abundance data. Statistically significant differences are indicated by superscript asterisks on specific metabolite names [$p < 0.05$ and variable importance in projection (VIP) > 1]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in the olfactory epithelium of goldfish reared in the Micro_high, Nano_low, and Nano_high groups were significantly reduced to only approximately 71.7%, 66.5%, and 53.5%, respectively, of those of the control (Fig. 2A, $F_{4,29} = 19.9$, $p < 0.05$). No significant difference in the activity of Ca^{2+}-ATPase was detected between the control and the Micro_low group ($p > 0.05$). The activities of Na^{+}/K^{+}-ATPase were not affected by the exposure of goldfish to MPs at the 0.26 mg/L concentration, while they declined significantly as a result of the exposure to a 0.69 mg/L MP concentration (Fig. 2B, $F_{4,29} = 11.9$, $p < 0.05$).

3.3. MP effects on the in vivo concentrations of neurotransmitters

The *in vivo* concentrations of acetylcholine (ACh) decreased significantly after the 28-day exposure of goldfish to MPs (Fig. 3A, $F_{4,24} = 18.6$, $p < 0.05$). Although no significant difference was detected between the control and Micro_low groups, the dopamine (DA) concentrations in goldfish brains were significantly suppressed by approximately 16.5%, 14.4%, and 19.1% for the Micro_high, Nano_low, and Nano_high groups, respectively (Fig. 3B, $F_{4,24} = 25.9$, $p < 0.05$). However, compared to the control, the γ-aminobutyric acid (GABA) concentrations increased significantly by approximately 17.4%, 19.7%, 12.5%, and 25.0% in the brains of goldfish from the Micro_low, Micro_high, Nano_low, and Nano_high groups, respectively (Fig. 3C, $F_{4,24} = 13.3$, $p < 0.05$).

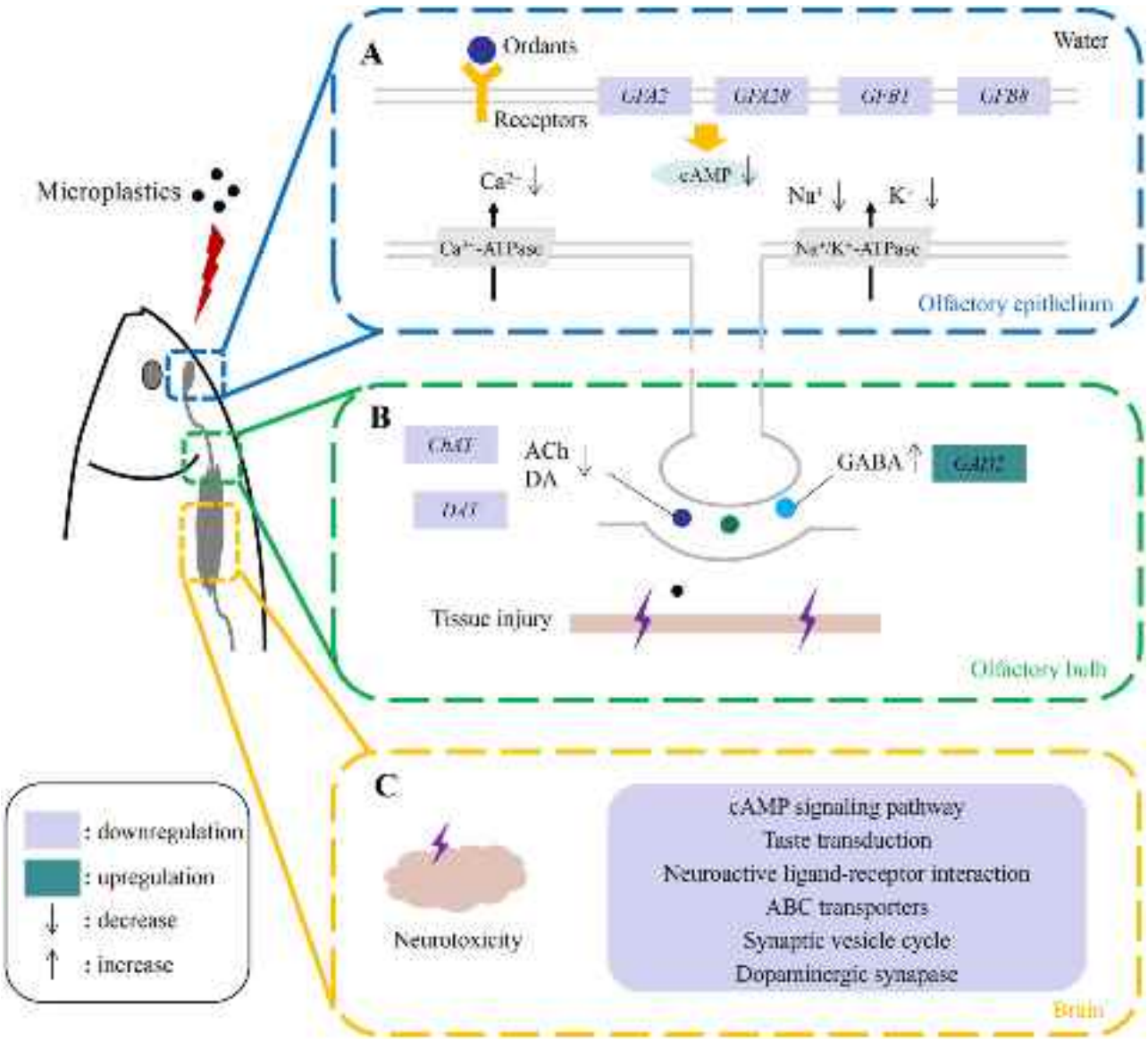

Fig. 8. Working model summarizing a tentative mechanistic pathway for microplastic (MP)-impaired, olfactory-mediated behavioral responses. (A) MPs hamper the odorant identification ability through suppressing G protein-coupled receptors (GPCRs). (B) MPs constrain the action potential generation by disturbing secondary messenger transduction pathways and ion transportation. (C) MPs interrupt the electrical signal transduction by influencing neurotransmitters and causing olfactory bulb injury. (D) MPs constrain the efficiency of olfactory information processing by inducing neurotoxicity.

3.4. Effects of MPs on the histopathology and GFAP immunoreactivity

After a 28-day exposure to MPs, goldfish had evidently congested capillaries, cavitation, and intercellular space around the glial cells in their olfactory bulbs, compared to the control group (indicated by black, red, and yellow arrows, respectively, in Fig. 4) as shown by the histopathological examination. Similarly, goldfish from the Micro_high, Nano_low, and Nano_high groups had significantly higher fluorescence intensities of glial fibrillary acidic protein (GFAP) labeling (red signals in Fig. 5, $F_{4,14} = 80.8$, $p < 0.05$) in the brain astrocytes, which were approximately 3.5, 2.8, and 8.4 times higher, respectively, than in the control group.

3.5. MP effects on the expression levels of olfactory related genes

The expression levels of genes encoding neurotransmitter modulatory enzymes and receptors were significantly altered by the exposure of goldfish to MPs ($p < 0.05$). Specifically, the expression of *DAT*, which encodes the transporter for DA, was suppressed significantly (Fig. 6A, $F_{4,14} = 37.0$, $p < 0.05$) by nano-scale, but not by micro-scale MPs. The expression of *ChAT*, a key regulatory enzyme for Ach, was significantly down-regulated ($F_{4,14} = 87.1$, $p < 0.05$) by the exposure of goldfish to the MPs tested. Although it was unaffected by the lower dose of micro-scale MPs tested (0.26 mg/mL and a diameter of 30 μm), the expression of *GAD2*, one of the key modulatory enzymes for GABA, increased significantly ($F_{4,14} = 15.0$, $p < 0.05$) by the exposure to nano-scale and micro-scale MPs at higher concentrations. All genes encoding GPCRs (*GFA2*, *GFA28*, *GFB1*, and *GFB8*) were significantly suppressed by the 28-day exposure of goldfish to the MPs tested (Fig. 6B, $p < 0.05$). In general, the effects of MP exposure on the expression of genes investigated were shown to be dose and size dependent, which is in accordance with previous studies (Jeong et al., 2016; Lei et al., 2018; Tang et al., 2020a).

3.6. MP effects on the metabolic profiles of goldfish

In total, 230 (175 down-regulated and 55 up-regulated) and 265 (153 down-regulated and 112 up-regulated) significantly different metabolites were detected in the Nano_high and Micro_high groups, respectively (Fig. S1, $p < 0.05$ and VIP > 1). The hierarchical clustering heatmap created with annotated metabolites revealed substantial differences between these experimental groups and the control (Fig. 7). The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that 27 and 30 KEGG pathways were significantly altered for the Nano_high and Micro_high groups, respectively. Most of the differentially expressed metabolites detected in the MP exposure groups were those closely related to metabolism and signal transduction (Fig. S2), including "Metabolic pathways" (ko01100), "cAMP signaling pathway" (ko04024), and "Taste transduction" (ko04742).

4. Discussion

The olfactory sensitivity of aquatic organisms is critical for their survival; however, their olfactory system could be highly susceptible to environmental pollutants (Munday et al., 2009). Although MPs are pervasive in the aquatic environment and regarded as emergent pollutants, to the best of our knowledge, the potential effects of MPs on the sensory ability of aquatic organisms remain unknown to date. The present study demonstrated that olfactory-driven behaviors of goldfish can be inhibited significantly by exposure to environmentally relevant MP concentrations. In addition, our results suggest that the observed sensory behavior impairment may be due to olfactory disruption induced by MPs (Fig. 8).

The successful binding of odorants with GPCRs is the initial step of the olfactory biochemical cascade (Hara, 1994). Therefore, any interference with this process may lead to olfactory malfunction. The presence of two different GPCR gene families, *GFA* and *GFB*, which have been suggested to assist in food and pheromone sensing, respectively, have been identified in goldfish (Cao et al., 1998). In the present study, genes encoding GPCRs including *GFA2*, *GFA28*, *GFB1*, and *GFB8* were all significantly suppressed by the exposure of goldfish to MPs. Since the down-regulation of GPCRs has been suggested to constrain the ability to induce olfactory signal and thus cause diminished olfactory-driven performance in both mammals and teleosts (Williams and Gallagher, 2013), this could be one explanation for the alteration in the olfactory-mediated behavior observed.

Once an odorant receptor binds to an agonist, the secondary messenger transduction pathways will be activated to produce cyclic adenosine monophosphate (cAMP), thereby triggering the action potential generation through the cation influx stimulation (Schild and Restrepo, 1998). However, our metabolomic analysis showed that several key metabolites including GABA, β-hydroxybutyrate, and norepinephrine in the cAMP signaling pathway were significantly suppressed by the MP exposure, which may disturb the crucial process of cAMP production (Newman and Verdin, 2017). Furthermore, following the activation of cAMP, the intracellular cation increases mediated by the Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase of the olfactory sensory neurons are essential for the action potential generation (Frings et al., 1991). As previous studies have suggested that perturbation in the activity of Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase may hamper the responses of olfactory sensory neurons to odorant signals (Menco et al., 1998), inhibited Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase enzyme activities in the olfactory epithelium of goldfish may constrain the conversion of odorant information into electrical signals by disturbing secondary messenger transduction pathways and ion transportation.

The action potential generated by olfactory neurons is transmitted to the brain through synapses at the base of the olfactory bulb and then interpreted to induce a behavioral response (Hara, 1994). The synaptic signal is known to be transmitted by modulating the release of specific neurotransmitters and the activities of corresponding receptors; therefore, interruptions in these neurotransmitter-related parameters may disrupt sensory functions as well (Rong et al., 2018). In the present study, both the *in vivo* neurotransmitter (ACh, DA, and GABA) concentrations and the expression levels of corresponding modulatory enzymes or receptors in the olfactory bulbs of goldfish were significantly altered by the exposure of the latter to MPs; this may interfere with the neural signal transduction through neurotransmitters and thereby offering another plausible explanation for the behavioral alteration detected. Besides, the histopathological and immunohistochemical results showed that exposure to MPs impaired the olfactory bulb tissues and activated astrocytes (an inflammation indicator) in goldfish brains. The brain injury induced by MPs may constrain further the efficiency of olfactory signal processing and subsequent olfactory-related behaviors.

In addition, the results of this study showed that nano-scale MPs were significantly more toxic to goldfish than micro-scale MPs. Similar size-dependent MP effect have also been documented in terms of inducing reproductive toxicity, growth inhibition, and immunotoxicity in various species (Van Cauwenberghe and Janssen, 2014; Tang et al., 2020b). As smaller sized MPs have higher surface/volume ratios and greater penetrating capability, they may have higher potential than larger MPs to be taken up by cells or during interactions with other living organisms, which may thereby cause more unintended consequences.

In conclusion, the results obtained of the present study demonstrated that MPs at environmentally relevant concentrations could impair the olfactory mediated behavioral responses of goldfish through a comprehensive mechanism that hampers odorant identification, action potential generation, olfactory neural signal transduction, and olfactory information processing. As a member of the Cyprinidae family, goldfish are closely related to many ecologically and economically important fish species. Under realistic environmental conditions, the impairment of sensory abilities may compromise the survival of these species in the long term, with potentially profound consequences for biological diversity and fisheries production.

CRediT authorship contribution statement

Shi Wei: Conceptualization, Investigation, Methodology, Writing - original draft, Writing - review & editing, Funding acquisition. **Sun Shuge**: Visualization, Investigation. **Han Yu**: Data curation, Validation, Investigation. **Tang Yu**: Resources, Investigation. **Zhou Weishang**: Formal analysis, Investigation. **Du Xueying**: Visualization, Data curation. **Liu Guangxu**: Conceptualization, Project administration, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This work was financial supported by National Key R&D Program of China (No. 2018YFD0900603), National Natural Science Foundation of China (No. 31672634), Natural Science Foundation of Zhejiang Province (LQ21C190003), and China Postdoctoral Science Foundation (2020M671743).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2020.125016.

References

Blanco, A.M., Sundarrajan, L., Bertucci, J.I., Unniappan, S., 2018. Why goldfish? Merits and challenges in employing goldfish as a model organism in comparative endocrinology research. Gen. Comp. Endocr. 257, 13–28.

Browne, M.A., Crump, P., Niven, S.J., Teuten, E., Tonkin, A., Galloway, T., Thompson, R., 2011. Accumulation of microplastic on shorelines woldwide: sources and sinks. Environ. Sci. Technol. 45, 9175–9179.

Cao, Y., Oh, B.C., Stryer, L., 1998. Cloning and localization of two multigene receptor families in goldfish olfactory epithelium. Proc. Natl. Acad. Sci. USA 95, 11987.

Van Cauwenberghe, L., Janssen, C.R., 2014. Microplastics in bivalves cultured for human consumption. Environ. Pollut. 193, 65–70.

Van Cauwenberghe, L., Vanreusel, A., Mees, J., Janssen, C.R., 2013. Microplastic pollution in deep-sea sediments. Environ. Pollut. 182, 495–499.

Collignon, A., Hecq, J.H., Glagani, F., Voisin, P., Collard, F., Goffart, A., 2012. Neustonic microplastic and zooplankton in the North Western Mediterranean Sea. Mar. Pollut. Bull. 64, 861–864.

Frings, S., Benz, S., Lindemann, B., 1991. Current recording from sensory cilia of olfactory receptor cells in situ. II. Role of mucosal Na^+, K^+, and Ca^{2+} ions. J. Gen. Physiol. 97, 725–747.

Gallagher, S.R., 2010. Digital image processing and analysis with Image. J. Curr. Protoc. Essent. Lab. Tech. 3. A.3C.1-A.3C.24.

Geyer, R., Jambeck, J.R., Law, K.L., 2017. Production, use, and fate of all plastics ever made. Sci. Adv. 3, e1700782.

Goldstein, M.C., Rosenberg, M., Cheng, L., 2012. Increased oceanic microplastic debris enhances oviposition in an endemic pelagic insect. Biol. Lett. 8, 817–820.

Hamdani, E.H., Døving, K.B., 2007. The functional organization of the fish olfactory system. Prog. Neurobiol. 82, 80–86.

Hara, T.J., 1994. Olfaction and gustation in fish: an overview. Acta Physiol. Scand. 152, 207–217.

Heffern, K., Tierney, K., Gallagher, E.P., 2018. Comparative effects of cadmium, zinc, arsenic and chromium on olfactory-mediated neurobehavior and gene expression in larval zebrafish (*Danio rerio*). Aquat. Toxicol. 201, 83–90.

Jabeen, K., Li, B., Chen, Q., Su, L., Wu, C., Hollert, H., Shi, H., 2018. Effects of virgin microplastics on goldfish (*Carassius auratus*). Chemosphere 213, 323–332.

Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R., Law, K.L., 2015. Plastic waste inputs from land into the ocean. Science 347, 768–771.

Jeong, C.B., Won, E.J., Kang, H.M., Lee, M.C., Hwang, D.S., Hwang, U.K., Zhou, B., Souissi, S., Lee, S.J., Lee, J.S., 2016. Microplastic size-dependent toxicity, oxidative stress induction, and p-JNK and p-p38 activation in the Monogonont Rotifer (*Brachionus koreanus*). Environ. Sci. Technol. 50, 8849–8857.

Kang, J.H., Kwon, O.Y., Shim, W.J., 2015. Potential threat of microplastics to zooplanktivores in the surface waters of the southern sea of Korea. Arch. Environ. Contam. Toxicol. 69, 340–351.

Lei, L., Liu, M., Song, Y., Lu, S., Hu, J., Cao, C., Xie, B., Shi, H., He, D., 2018. Polystyrene (nano) microplastics cause size-dependent neurotoxicity, oxidative damage and other adverse effects in *Caenorhabditis elegans*. Environ. Sci. Nano 5, 2009–2020.

Limonta, G., Mancia, A., Benkhalqui, A., Bertolucci, C., Abelli, L., Fossi, M.C., Panti, C., 2019. Microplastics induce transcriptional changes, immune response and behavioral alterations in adult zebrafish. Sci. Rep. 9, 15775.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408.

Lusher, A.L., Tirelli, V., O'Connor, I., Officer, R., 2015. Microplastics in Arctic polar waters: the first reported values of particles in surface and sub-surface samples. Sci. Rep. 5, 14947-14947.

MacPhail, R.C., Brooks, J., Hunter, D.L., Padnos, B., Irons, T.D., Padilla, S., 2009. Locomotion in larval zebrafish: influence of time of day, lighting and ethanol. Neurotoxicology 30, 52–58.

Maximino, C., Silva, R., da Silva, S. d N., Rodrigues, L. d S., Barbosa, H., de Carvalho, T., Leão, L.K., Lima, M., Oliveira, K.R., Herculano, A., 2015. Non-mammalian models in behavioral neuroscience: consequences for biological psychiatry. Front. Behav. Neurosci. 9, 233-233.

Ma, H., Pu, S., Liu, S., Bai, Y., Mandal, S., Xing, B., 2020. Microplastics in aquatic environments: toxicity to trigger ecological consequences. Environ. Pollut. 261, 114089.

Menco, B.P., Birrell, G.B., Fuller, C.M., Ezeh, P.I., Keeton, D.A., Benos, D.J., 1998. Ultrastructural localization of amiloride-sensitive sodium channels and Na^+, K^+-ATPase in the rat's olfactory epithelial surface. Chem. Senses 23, 137–149.

Munday, P.L., Dixson, D.L., Donelson, J.M., Jones, G.P., Pratchett, M.S., Devitsina, G.V., Døving, K.B., 2009. Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. Proc. Natl. Acad. Sci. USA 106, 1848.

Naidoo, T., Glassom, D., 2019. Decreased growth and survival in small juvenile fish, after chronic exposure to environmentally relevant concentrations of microplastic. Mar. Pollut. Bull. 145, 254–259.

Newman, J.C., Verdin, E., 2017. β-Hydroxybutyrate: a signaling metabolite. Annu. Rev. Nutr. 37, 51–76.

Pannetier, P., Morin, B., Le Bihanic, F., Dubreil, L., Clérandeau, C., Chouvellon, F., Van Arkel, K., Danion, M., Cachot, J., 2020. Environmental samples of microplastics induce significant toxic effects in fish larvae. Environ. Int. 134, 105047.

Prokić, M.D., Radovanović, T.B., Gavrić, J.P., Faggio, C., 2019. Ecotoxicological effects of microplastics: examination of biomarkers, current state and future perspectives. TrAC Trend Anal. Chem. 111, 37–46.

Rochman, C.M., 2018. Microplastics research-from sink to source. Science 360, 28–29.

Rong, J., Su, W., Guan, X., Shi, W., Zha, S., He, M., Wang, H., Liu, G., 2018. Ocean acidification impairs foraging behavior by interfering with olfactory neural signal transduction in black sea bream, *Acanthopagrus schlegelii*. Front. Physiol. 9, 1592.

Schild, D., Restrepo, D., 1998. Transduction mechanisms in vertebrate olfactory receptor cells. Physiol. Rev. 78, 429–466.

Van Sebille, E., Wilcox, C., Lebreton, L., Maximenko, N., Hardesty, B.D., Van Franeker, J. A., Eriksen, M., Siegel, D., Galgani, F., Law, K.L., 2015. A global inventory of small floating plastic debris. Environ. Res. Lett. 10, 124006.

Shamchuk, A.L., Blunt, B.J., Lyons, D.D., Wang, M.Q., Gasheva, A., Lewis, C.R., Tomlin, K., Hazard, E.S., Hardiman, G., Tierney, K.B., 2018. Nucleobase-containing compounds evoke behavioural, olfactory, and transcriptional responses in model fishes. FACETS 3, 79–102.

Shi, W., Han, Y., Guan, X., Rong, J., Su, W., Zha, S., Tang, Y., Du, X., Liu, G., 2019. Fluoxetine suppresses the immune responses of blood clams by reducing haemocyte viability, disturbing signal transduction and imposing physiological stress. Sci. Total Environ. 683, 681–689.

Sorensen, P.W., Hara, T.J., Stacey, N.E., 1987. Extreme olfactory sensitivity of mature and gonadally-regressed goldfish to a potent steroidal pheromone, 17α,20β-dihydroxy-4-pregnen-3-one. J. Comp. Physiol. A 160, 305–313.

Tang, Y., Rong, J., Guan, X., Zha, S., Shi, W., Han, Y., Du, X., Wu, F., Huang, W., Liu, G., 2020a. Immunotoxicity of microplastics and two persistent organic pollutants alone or in combination to a bivalve species. Environ. Pollut. 258, 113845.

Tang, Y., Zhou, W., Sun, S., Du, X., Han, Y., Shi, W., Liu, G., 2020b. Immunotoxicity and neurotoxicity of bisphenol A and microplastics alone or in combination to a bivalve species, *Tegillarca granosa*. Environ. Pollut. 265, 115115.

Tatti, R., Bhaukaurally, K., Gschwend, O., Seal, R.P., Edwards, R.H., Rodriguez, I., Carleton, A., 2014. A population of glomerular glutamatergic neurons controls sensory information transfer in the mouse olfactory bulb. Nat. Commun. 5, 3791-3791.

Tierney, K.B., Baldwin, D.H., Hara, T.J., Ross, P.S., Scholz, N.L., Kennedy, C.J., 2010. Olfactory toxicity in fishes. Aquat. Toxicol. 96, 2–26.

Wang, J., Li, Y., Lu, L., Zheng, M., Zhang, X., Tian, H., Wang, W., Ru, S., 2019. Polystyrene microplastics cause tissue damages, sex-specific reproductive disruption and transgenerational effects in marine medaka (*Oryzias melastigma*). Environ. Pollut. 254, 113024.

Williams, C.R., Gallagher, E.P., 2013. Effects of cadmium on olfactory mediated behaviors and molecular biomarkers in coho salmon (*Oncorhynchus kisutch*). Aquat. Toxicol. 140–141, 295–302.

Wyeth, R.C., Braubach, O.R., Fine, A., Croll, R.P., 2011. Videograms: a method for repeatable unbiased quantitative behavioral analysis without scoring or tracking. In: Kalueff, A.V., Cachat, J.M. (Eds.), Zebrafish Neurobehavioral Protocols. Springer, pp. 15–33.

Yin, L., Liu, H., Cui, H., Chen, B., Li, L., Wu, F., 2019. Impacts of polystyrene microplastics on the behavior and metabolism in a marine demersal teleost, black rockfish (*Sebastes schlegelii*). J. Hazard. Mater. 380, 120861.

Zhou, W., Han, Y., Tang, Y., Shi, W., Du, X., Sun, S., Liu, G., 2020. Microplastics aggravate the bioaccumulation of two waterborne veterinary antibiotics in an edible bivalve species: potential mechanisms and implications for human health. Environ. Sci. Technol. 54, 8115–8122.