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Recovery Pattern of Behavioral Responses in Female Zebrafish to Short-term Amitriptyline Hydrochloride Exposure

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Amitriptyline (AMI) is a tricyclic antidepressant (TCA) that has been widely used to treat depression. The widespread use has caused AMI to enter the environmental waters with a maximum concentration of $\mu\text{g/L}$. However, knowledge of its persistent toxic effects on fish is still limited. Therefore, this study exposed female zebrafish to $10\mu\text{g/L}$ of AMI for seven days, followed by a 21-day recovery in AMI-free water. The results showed that AMI could induce persistent or delayed hypoactivity and abnormal social behavior in zebrafish. AMI exposure also significantly altered the brain levels of 5-HT, 5-HIAA, and ACH. Correlation analysis revealed that 5-HT and ACH were negatively correlated with locomotor activity, and 5-HT was also positively correlated with social interaction. Our finding demonstrated that AMI could cause persistent abnormal behavior in zebrafish and alter the brain neurotransmitter levels in zebrafish, suggesting that those long-term impact should not be ignored.

Key words: Amitriptyline; Zebrafish; Neurotransmitter levels; Behavior; Recovery

INTRODUCTION

Amitriptyline (AMI) is a kind of tricyclic antidepressant (TCAs) that has been widely used to treat depression (Kaur and Malik 2013). The TCAs can achieve antidepressant effects by inhibiting serotonin and norepinephrine reuptake, and topical application of AMI also can relieve neuropathic pain (Abdollahi and Mostafalou 2014; Thompson and Brooks 2015; Chang *et al.*, 2021). The widespread use has caused TCAs to enter the environmental waters through human excretion, disposal of unused medicines, medical effluent discharges, and others (Li *et al.*, 2013; Choi *et al.*, 2018; Chang *et al.*, 2021). As a result, the TCAs residues in water may lead to continuous exposure of aquatic organisms to the active ingredients of such drugs, which pose potential risks to aquatic systems (Lajeunesse *et al.*, 2011).

Antidepressants can affect brain neurotransmitter levels in fish, thereby influencing a series of behaviors even at the environmentally relevant concentration (David *et al.*, 2018; Qiu *et al.*, 2022a). Previous studies have reported that exposure to antidepressants could affect the activity, socialization, survival, growth, predator, learning, and social interactions of aquatic organisms (Yang *et al.*, 2014; Eisenreich and Szalda-Petree 2015; Qiu *et al.*, 2022a). In mammals, discontinuation of antidepressants could induce various withdrawn syndromes (Cosci and Chouinard 2020). Similarly, exposure to fluoxetine for 6 days resulted in a decreased serotonin

level and impaired hunting ability of bass, and the serotonin level did not return to the control levels during the 6-day recovery period (Gaworecki and Klaine 2008). Exposure to venlafaxine (250 and $500\mu\text{g/L}$) caused an increase in predatory behavior and a significant decrease in brain serotonin levels in bass during exposure, with the serotonin levels returning to normal during the 6-days recovery period (Bisesi *et al.*, 2014). Short-term exposure to antidepressants could have long-term adverse effects on fish.

Zebrafish is a model organism and has been widely used in ecotoxicological studies (Grabicova *et al.*, 2014; Arnnok *et al.*, 2017; Melvin 2017). This model organism has previously been used to study behavioral changes caused by various neurotoxic substances (Bisesi *et al.*, 2014; Demin *et al.*, 2017; Chen *et al.*, 2021). In a previous study, we reported that short-term AMI exposure at $40\mu\text{g/L}$ induced long-term hypoactivity and abnormal schooling behavior and found that the upregulated brain 5-HT may serve as the central modulator of the above persistent behavioral impacts (Qiu *et al.*, 2022a). Considering that AMI residues can be up to $6.7\mu\text{g/L}$ in wastewaters (Kasprzyk-Hordern *et al.*, 2009; Richmond *et al.*, 2018), fish may temporarily be exposed to AMI at relatively high concentrations due to periodic emissions from wastewater treatment plants. Thus, more insights into the recovery pattern of behavioral responses to short-term AMI exposure are critical for comprehensively assessing their risks.

Therefore, in the present study, we exposed zebrafish to AMI at $10\mu\text{g/L}$ (near the environmentally relevant concentration) for 7 days and then transferred zebrafish to AMI-free water to recover for 21 days. Locomotor activity and social behavior of zebrafish were examined at the end of the exposure and on days 7 and 21 of the recovery period. The brain neurotransmitter was also measured to evaluate their relationship to behavior. This

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study aimed to investigate the recovery pattern of female zebrafish to short-term AMI exposure and the role of brain neurotransmitters in those processes.

MATERIALS AND METHODS

Test organism

Female zebrafish (AB-strain, six months after hatching) were maintained in two 16-L circular glass aquariums (40 fish per tank) containing 12 L of dechlorinated tap water (conductivity at 0.50–0.53 mS/cm; $27\pm 1^\circ\text{C}$; light: dark cycle=14 L:10 D h). The fish were fed with *Artemia nauplii* (<24 h after hatching) twice per day, and half of the water volume was changed every 24 h.

Chemicals

Amitriptyline hydrochloride (CAS No. 439–14–5) was purchased from the Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). Other reagents at analytical grade were purchased from the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Exposure and recovery

The test solution of AMI ($10\ \mu\text{g/L}$) was prepared by pipetting calculated amounts of the amitriptyline hydrochloride stock solution into dechlorinated tap water. In the control group, only dechlorinated tap water was used. For the exposure experiment, 10 healthy individuals were introduced into 3-L glass cylindrical tanks that contained 2.5 L of a test solution, with three replicates ($n=3$). Thus, a total of 60 female zebrafish were used in this study. The exposure was conducted for 7 days (not feeding), and half of the test solutions were renewed every 24 h. After the 7-day exposure, half of the zebrafish (i.e., 5 fish of each replicate) were transferred into dechlorinated tap water to recover for 21 days. The recovery test was conducted under the same conditions mentioned in section 2.1, and fish were fed with *A. nauplii* (<24 h after hatching) during the recovery period.

Behavioral assay

The behavioral assay was conducted on day 0 (R–0, i.e., immediately after the 7-day exposure), 7 (R–7), and 21 (R–21) of the recovery period. For this assay, five zebrafish in each tank (as one group) and transferred to an aquarium ($20\times 9\times 10\ \text{cm}$) containing 1000 mL of dechlorinated tap water. After a 10-min acclimation, fish behavioral traits behavior was tracked for 10 min using the DanioVision system (Noldus, Wageningen, Netherlands) and then analyzed using the EthoVision XT software (Vison 11.5; Noldus). The fish locomotor activity was judged based on the threshold values reported previously (Chen *et al.*, 2021), and the shoaling behavior is defined as all five individuals staying together in an area with a diameter of 7 cm (i.e., the distance between an individual and the other individuals is $\leq 3.5\ \text{cm}$) (Miller and Gerlai 2012).

Brain neurotransmitter levels

At R–0 (i.e., the end of the 7-day exposure period) and R–21 (i.e., the end of the 21-day recovery period), five fish in each tank were placed into an ice water bath ($0\text{--}4^\circ\text{C}$) for euthanasia (Wallace *et al.*, 2018). Subsequently, the whole brain was extracted and stored at -80°C . For assaying brain neurotransmitter levels, 4 individuals were pooled as one sample, and 3 replicates were used for each treatment group. The homogenate and supernatant for those assays were prepared following the method described by Qiu *et al.* (2022b). Briefly, each sample was homogenized with 9 vol (*w/v*) phosphate buffer (10 mM, pH 7.2–7.4). Then, the homogenate was centrifuged at $6000\times g$ for 5 min, and the supernatant was collected. The concentrations of serotonin (5-HT), 5-Hydroxyindoleacetic acid (5-HIAA), acetylcholine (ACH), and acetylcholinesterase (ACHE) in each sample were conducted using the corresponding ELISA Kit (Bomei Biotechnology Co., Ltd., Hefei, Anhui, China). The units of each parameter were normalized by the total protein amount ($\mu\text{g}\text{-protein}$).

Statistical Analysis

A generalized linear model (GzLM) was used to analyze the combined effects of AMI and sampling time on the behavioral traits and the neurotransmitter levels. Following the GzLM analysis, a simple effects analysis was conducted to examine the difference between exposure and control within either time point. For the correlation analysis, data were first normalized to percentages respective to the control, and the correlations of neurotransmitter levels with those of behavioral traits were tested for significance using Pearson correlation analysis. All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL).

RESULTS

Impacts on the behavior related to locomotor activity

No mortality was observed in all the experimental groups during the four-day exposure. As shown in Fig. 1, significantly altered behavioral traits related to locomotor activity were usually observed on R–7 and R–21. The GzLM model evaluated that AMI exhibited a significant main effect on the duration of low mobility (DLM), while the interaction between AMI and sampling time (ST) exhibited significant effects on the DLM (Table 1). Although no significant difference in the average swimming velocity (ASV) (Fig. 1A) and frequency of low mobility (FLM) (Fig. 1C) was observed at any time point, the maximum swimming velocity (MSV) of zebrafish in the exposure group significantly increased on R–7 (Fig. 1B, $p<0.01$). The DLM of zebrafish in the exposure group did not significantly change on R–0, but significantly increased to 244% ($p<0.01$) and 230% ($p<0.01$) of that in the control group on R–7 and R–21, respectively (Fig. 1D). Thus, short-term exposure to AMI could induce delayed hypoactivity in zebrafish.

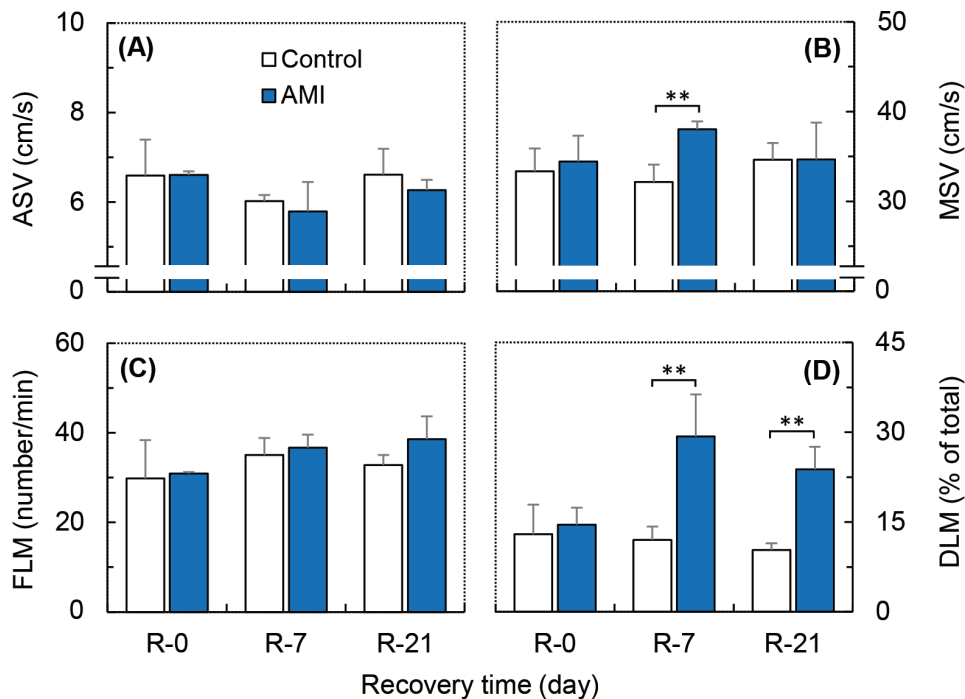


Fig. 1. Variations in the average swimming velocity (ASV, A), maximum swimming velocity (MSV, B), frequency of low mobility (FLM, C), and duration of low mobility (DLM, D) of zebrafish (*Danio rerio*) exposed to amitriptyline at $10 \mu\text{g/L}$. Data are mean \pm SE ($n=3$), and asterisks indicate significant differences (** $p<0.01$).

Table 1. Summary of generalized linear model testing the statistical significance for the effect of AMI, sampling time (ST), and their interaction (AMI \times ST) on the behavioral traits of zebrafish (*Danio rerio*)

| | Locomotor activity | | | | Social interaction | | | |
|----------------------------|--------------------|------|------|--------|--------------------|--------|------|--------|
| | ASV | MSV | FLM | DLM | ADI | FBC | DSH | DSO |
| AMI ($df=1$) | 0.50 | 2.78 | 1.30 | 23.1** | 12.3** | 3.20 | 0.19 | 12.0** |
| ST ($df=2$) | 4.76 | 0.67 | 3.11 | 5.07 | 32.6** | 10.4** | 0.37 | 19.8** |
| AMI \times ST ($df=2$) | 0.28 | 4.77 | 0.88 | 8.65* | 15.9** | 8.0* | 5.26 | 19.5** |

* Wald Chi-Square is listed; df : degree of freedom; asterisks indicate statistical significance (* $p<0.05$; ** $p<0.01$).

Impacts on the behavior related to social interaction

As shown in Fig. 2, significant alteration in the behavioral traits related to social interaction could be observed on R-0, R-7, and R-21. The GzLM model evaluated that AMI exhibited significant main effects on the average distance between individuals (ADI) and duration of solitary (DSO); the ST exhibited significant main effects on the ADI, FBC, and DSO; and their interaction significantly affected the ADI, frequency of body contact (FBC), and DSO (Table 1). The ADI of zebrafish in the exposure group did not significantly change on R-0, but significantly decreased on R-7 ($p<0.01$) and R-21 ($p<0.01$), respectively (Fig. 2A). The FBC of zebrafish in the exposure group significantly decreased on R-0 ($p<0.01$) but did not significantly change on R-7 and R-21 (Fig. 2B). The duration of shoaling (DSH) of zebrafish in the exposure group significantly decreased on R-0 ($p<0.05$) but significantly increased on R-7 ($p<0.05$) (Fig. 2C). The DSO of zebrafish in the expo-

sure group did not significantly change on R-0, but significantly decreased on R-7 ($p<0.01$) and R-21 ($p<0.01$), respectively (Fig. 2D).

Impacts on the brain neurotransmitter levels

As shown in Fig. 3, significantly altered brain neurotransmitter levels could be observed on both R-0 and R-21. Serotonin (5-HT) in the exposure group did not significantly change on R-0, but significantly increased on R-21 (Fig. 3A, $p<0.01$). 5-Hydroxyindoleacetic acid (5-HIAA) in the exposure group significantly decreased on R-0 (Fig. 3B, $p<0.01$) but not on R-21. Acetylcholine (ACH) in the exposure group significantly increased on both R-0 and R-21 (Fig. 3C, $p<0.01$). Acetylcholinesterase (ACHE) in the exposure group did not significantly change on R-0 and R-21.

Correlations analysis

As shown in Table 2, the 5-HT level was significantly correlated with DLM (positive, $p<0.05$), ADI (negative,

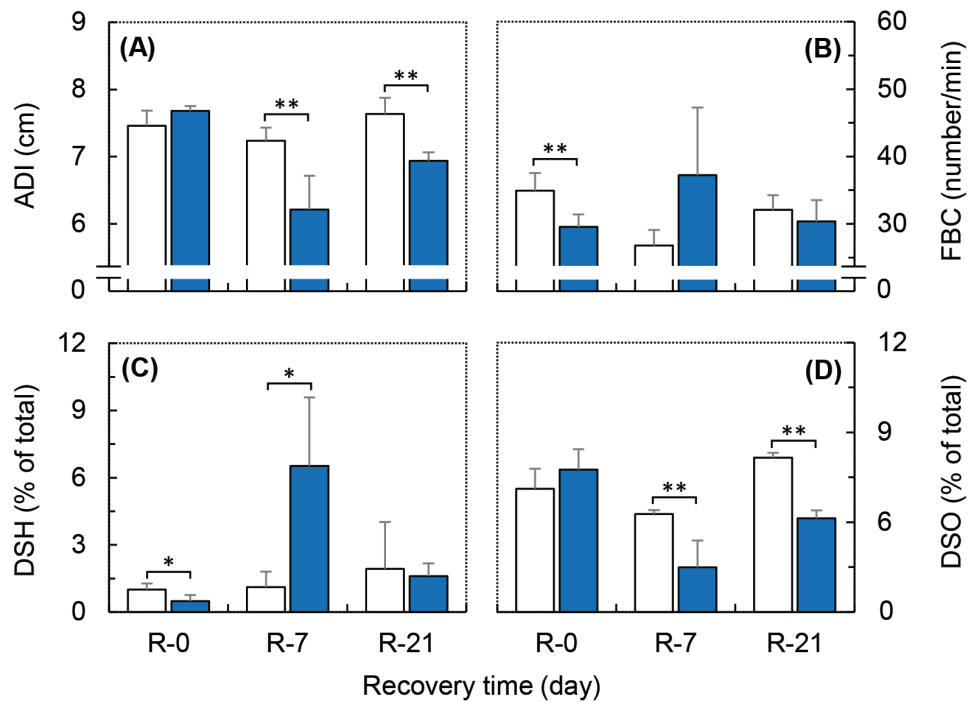


Fig. 2. Variations in the average distance between individuals (ADI, A), frequency of body contact (FBC, B), duration of shoaling (DSH, C), and duration of solitary (DSO, D) of zebrafish (*Danio rerio*) exposed to amitriptyline at 10 $\mu\text{g/L}$. Data are mean \pm SE ($n=3$), and asterisks indicate significant differences (* $p<0.05$; ** $p<0.01$).

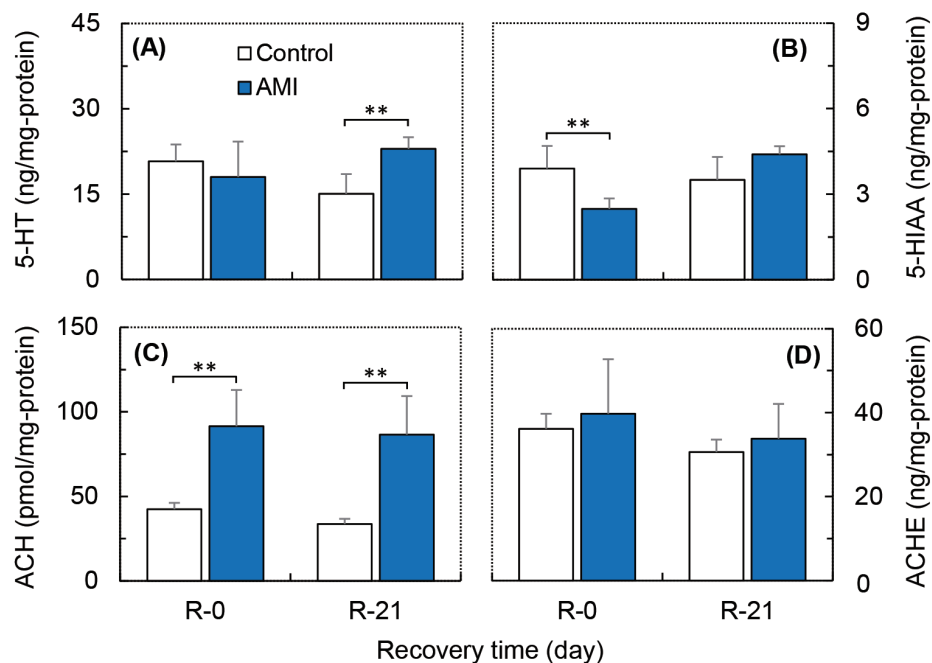


Fig. 3. Variations in the brain neurotransmitter levels of juvenile and adult zebrafish (*Danio rerio*) exposed to amitriptyline at 0 (control) and 10 $\mu\text{g/L}$ for seven days. (A) Serotonin (5-HT); (B) 5-Hydroxyindoleacetic acid (5-HIAA); (C) Acetylcholine (ACH); (D) Acetylcholinesterase (ACHE). Data are mean \pm SE ($n=3$), and asterisks indicate significant differences (** $p<0.01$).

$p<0.05$), and DSO (negative, $p<0.01$), and the ACH level was significantly and positively correlated with DLM ($p<0.05$). However, no significant correlation was observed between the 5-HAA (or ACHE) and the behavioral parameters.

DISCUSSION

Our results demonstrated that 7-days exposure to AMI induced delayed hypoactivity and increased social interaction in zebrafish. Our previous study showed that

Table 2. Pearson correlation between the brain neurotransmitter levels and behavioral parameters of zebrafish (*Danio rerio*)

| | Locomotor activity | | | | Social interaction | | | |
|--------|--------------------|--------|-------|--------|--------------------|--------|--------|----------|
| | ASV | MSV | FLM | DLM | ADI | FBC | DSH | DSO |
| 5-HT | 0.016 | 0.267 | 0.101 | 0.648* | -0.547* | 0.132 | -0.164 | -0.666** |
| 5-HIAA | 0.109 | 0.064 | 0.018 | 0.439 | -0.416 | 0.243 | -0.161 | -0.492 |
| ACH | -0.146 | -0.092 | 0.357 | 0.601* | -0.388 | -0.139 | 0.023 | -0.220 |
| ACHE | -0.054 | -0.031 | 0.017 | 0.203 | -0.128 | -0.386 | -0.034 | 0.115 |

Asterisks indicate significant correlation (* $p < 0.05$; ** $p < 0.01$).

exposure of zebrafish to AMI (at $40 \mu\text{g/L}$) reduced locomotor activity and increased schooling behavior, both of which were reduced after 21 days recovery period (Qiu *et al.*, 2022a). Similar to our findings, Sehonova *et al.* (2019) demonstrated that exposure to AMI (at $300 \mu\text{g/L}$) could reduce the swimming distance of zebrafish in the dark, and Bisesi *et al.* (2014) found that venlafaxine exposure could increase the time for the bass to feed, while the time spent by the bass feeding gradually decreased with the recovery period. Thus, insights into the recovery pattern of behavioral responses to short-term AMI exposure are critical for comprehensively assessing their risks.

The locomotor activity integrates physiological abilities that enable the fish to generate and coordinate the energy needed for basic functions such as migrating or avoiding predators (Gaworecki and Klaine 2008; Eisenreich and Szalda-Petree 2015). Previous studies have demonstrated that antidepressants can reduce feeding rates of juvenile Eurasian perch (*Perca fluviatilis*) at both low and high prey densities, and such effects may alter the stability of the predator-prey system and the community structure (Hedgespeth *et al.*, 2014). On the other hand, shoaling behavior is an aggregation behavior, and the groups among multiple individuals can reduce the risk of predation (Buske and Gerlai 2012; Gerlai 2014). These behavioral changes may further affect fish populations by influencing foraging efficiency and predator avoidance (Demin *et al.*, 2017; Meshalkina *et al.*, 2018). Therefore, the effects of AMI on zebrafish causing delayed hypoactivity and abnormal schooling behavior should not be ignored.

To evaluate the possible mechanisms involved in the behavioral change of zebrafish to AMI, we assessed the levels of 5-HT, 5-HIAA, ACH, and ACHE in the brain of zebrafish. Our results showed that altered DLM was significantly associated with increased 5-HT in the ACH, and altered social behavior was significantly associated with 5-HT.

The increased brain 5-HT level in zebrafish is consistent with the mode of action of AMI, which exhibits an inhibitory effect on 5-HT reuptake transporters, resulting in the increased 5-HT levels (Abdollahi and Mostafalou 2014). As a monoamine neurotransmitter, 5-HT is a major modulator of other excitatory and inhibitory neurotransmitter systems in the central nervous system, which has been associated with stress response, feeding, motor activity, social interaction, and migration

in fish (Bisesi *et al.*, 2016; Horzmann and Freeman 2016). For example, Mennigen *et al.* (2011) reported that antidepressants could increase serotonin concentrations and alter 5-HT receptor function through chronic exposure. On the other hand, 5-HIAA is a product of 5-HT, and the level of 5-HIAA is often used to measure 5-HT neuronal activity (Horzmann and Freeman 2016; McDonald 2017). Previously, Meshalkina *et al.* (2018) found that zebrafish exposed to AMI ($50 \mu\text{g/L}$) for a fortnight showed reduced serotonin turnover (5-HIAA/5-HT ratio), which led to significantly reduced locomotor activity. Consistent with our results, acute fluoxetine (an antidepressant) exposure also reduced 5-HIAA concentrations in the zebrafish brain, which may have contributed to their decreased social interaction (Winberg and Thörnqvist 2016). Thus, our findings supported that the serotonergic system plays an important role in modulating fish behavioral responses to antidepressants.

We also found that AMI affected the locomotor activity of zebrafish and was significantly associated with changes in the level of ACH. Acetylcholine (ACH) is the predominant neurotransmitter in the parasympathetic nervous system, and its neurotransmission is widespread in the central nervous system, helps regulate the release of other neurotransmitters, and is associated with learning and memory (Horzmann and Freeman 2016). Furthermore, The significantly elevated brain ACH levels may be explained by negative feedback to the AMI, as all TCAs can block the action of ACH (Abdollahi and Mostafalou 2014). Previous studies have reported that abnormal ACH levels led to reduced locomotor activity in zebrafish (Mennigen *et al.*, 2011; Sarasamma *et al.*, 2018). Chinese minnow exposed to psychotropic drugs showed a significant increase in anxiety-type behavior, accompanied by a significant increase in ACH level (Gao and Yang 2022). Considering that ACH is a typical target of TCAs (Abdollahi and Mostafalou 2014), its mechanisms in mediating behavioral responses to those antidepressants should be addressed in future studies.

AUTHOR CONTRIBUTIONS

L. LI, K. DONG and C. CHEN performed the exposure test, analyzed the data, and wrote the paper. K. CHEN, Y. SHI, and C. CHEN performed the behavioral experiments and participated in the data analysis. X. QIU designed the study, supervised the work, wrote the paper, and provided facilities and resources. Y.

SHIMASAKI and Y. OSHIMA designed the study, wrote the paper, and provided resources. All authors assisted in editing the manuscript and approved the final version.

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