

**Effects of parental exposure to amitriptyline on the survival, development,
behavior, and gene expression in zebrafish offspring**

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Abstract

In mammals, parental exposure to amitriptyline (AMI) has been proven to contribute to congenital disabilities in their offspring. However, no studies have paid attention to the adverse effects of parental exposure to amitriptyline on fish offspring. In this study, we exposed adult zebrafish (F0) to AMI (0.8 µg/L) for 21 days. Subsequently, these zebrafish (F0) were allowed to mate, and their offspring (F1) were collected to culture in clean water for 5 days. The mortality rate, average hatching time, and heart rate at 48 hours post-fertilization (hpf) of F1 were investigated. Our results showed that parental exposure to AMI induced tachycardia and increased mortality in F1 zebrafish. Under a light/dark transition test, F1 larvae born from AMI-exposed parents exhibited lower locomotor activity in the dark period and decreased thigmotaxis in the light period. The transcriptome analysis showed that parental AMI exposure dysregulated some key pathways in their offspring. Through the prediction of key driver analysis, six differentially expressed genes (DEGs) were revealed as key driver genes involved in protein processing in endoplasmic reticulum (*hspa5*, *hsp70.1*, *hsp90a*), ribosome (*rps27a*) and PPAR signaling pathway (*pparab* and *fabp2*). Considering that the concentration of AMI residual components in natural water bodies may be over our test concentration (0.8 µg/L), our findings suggested that toxicity of parental exposure to the offspring of fish should receive greater attention.

Keywords: antidepressant; behavior responses; key driver analysis; parental exposure; zebrafish

1 Introduction

Tricyclic antidepressants (TCAs), such as amitriptyline (AMI), desipramine, and nortriptyline, have been widely used for the treatment of depression, anxiety, migraine, and other chronic pain for decades (Abdollahi and Mostafalou, 2014). The TCAs can enter the environment through human excretion, sewage effluent, and expired drug disposal (Sehonova et al., 2018; Wilkinson et al., 2022). With their extensive use globally, TCAs have become the most frequently detected pharmaceutical pollutants in aquatic ecosystems (Wilkinson et al., 2022; Wu et al., 2015). For AMI, its residual concentrations in urban waters, including wastewater treatment plant (WWTP) influents and effluents, river water, and drinking water, are usually in the range of ng/L to µg/L, with a maximum concentration of 6.7 µg/L detected in WWTP influent (David et al., 2018; Kasprzyk-Hordern et al., 2009; Wu et al., 2015). In particular, Lhotský et al. (2017) found that the maximum concentration of AMI could reach 7.46 µg/L in groundwater contaminated by the pharmaceutical industry in the Czech Republic.

Increasing studies have proven that AMI could induce various adverse effects on aquatic organisms, generally dependent on the exposure concentrations (Meshalkina et al., 2018; Qiu et al., 2022a; Shi et al., 2023; Shi et al., 2022). Even at environmentally relevant concentrations, AMI could significantly affect the growth, development, behavior, and gene expression of fish at early life stages (David et al., 2018; Sehonova et al., 2019; Wu et al., 2017; Yang et al., 2014). For example, Yang et al. (2014) reported that AMI (from 10 ng/L to 1 mg/L) could significantly affect zebrafish embryos in a concentration-dependent manner, and exposure to AMI at 0.01-1 µg/L was sufficient to induce growth inhibition and oxidative stress. Using the high-throughput RNA sequencing (RNA-Seq) method, Wu et al. (2017) found that AMI at 0.1 µg/L could significantly disturb gene expression involved in multiple biological processes and

signaling pathways in zebrafish larvae. Therefore, the adverse effects of AMI exposure on aquatic species and their potential ecological risk have received significant attention.

Recent studies also demonstrated that exposure to antidepressants could disturb the reproductive potential and population dynamics of aquatic species. For example, Venkatachalam et al. (2023) reported that zebrafish exposed to 10 µg/L sertraline for two weeks showed a significant decrease in reproductive ability (i.e., decreases in egg numbers and fertilization rate), Hong et al. (2022) found that whole life cycle exposure to citalopram at 1 µg/L decreased the offspring number of zebrafish. It has also been found that citalopram exposure could affect the population dynamics of crustaceans (Fong and Ford, 2014; Sehonova et al., 2018). Furthermore, Vera-Chang et al. (2018) reported that a 6-day fluoxetine exposure during early zebrafish development could induce transgenerational hypocortisolism and behavioral disruption, and Al Shuraiqi et al. (2021) found that parental exposure to fluoxetine could alter the swimming activity of zebrafish offspring in a non-monotonic manner. Considering the widespread pollution of TCAs in aquatic ecosystems, details about the impacts of parental exposure to those pollutants (including AMI) on fish offspring are essential for assessing their potential risks. However, no studies focused on the potential impacts of parental exposure to AMI on the offspring of fish, not to mention the underlying mechanisms.

In the present study, we hypothesized that parental exposure to AMI may induce adverse effects on the survival, development, behavior, and gene expression of fish offspring. To test this hypothesis, we exposed female and male zebrafish to AMI at a low concentration (0.8 µg/L) for 21 d, mated them, and investigated variations in the survival, development, behavioral responses, and transcriptional gene expression in their offspring within 5 days post-fertilization (dpf). Our study aimed to assess the potential adverse effects and related mechanisms of parental exposure to AMI on

zebrafish offspring.

2 Materials and methods

2.1 Test organism

Four-month-old female and male zebrafish (AB-strain) were obtained from Nanjing Ezerinka Biotechnology Co., LTD. (Nanjing, China). The zebrafish were separately cultured in glass aquariums (DIA = 28 cm; H = 30 cm) with 16 L dechlorinated tap water (0.50-0.53 mS/cm). The water temperature was 27 ± 1 °C, and the light: dark cycle was 14:10 h. The fish were fed *Artemia* nauplii (<24 h after hatching) twice per day, and half of the water was changed every two days. Under these conditions, zebrafish were acclimated in our laboratory for two weeks before experiments. All the maintenance and experiments followed the Guide for the Care and Use of Laboratory Animals (8th edition) by the National Research Council. All procedures of experiments were approved by the Jiangsu University Animal Care and Use Committee, Zhenjiang, China (2018-0053).

2.2 Chemicals

Amitriptyline hydrochloride (>98%, analytical standard) was purchased from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). Other experimental reagents (sodium chloride: $\geq 99.5\%$, analytical reagent; ethanol: 99.5%, analytical reagent) were purchased from Sinopharm Chemical Reagent Co., LTD. (Shanghai, China).

2.3 Parental exposure experiment

The parental exposure concentration of AMI was 0.8 µg/L, which is equivalent to 1/10000 of its 96 h-LC₅₀ (i.e., 8 mg/L) to zebrafish larvae (Ali et al., 2011). Previous studies reported that AMI concentrations could reach 0.2 µg/L in river water (Pivetta et al., 2020), 0.42 µg/L in WWTP effluent (David et al., 2018), 6.7 µg/L in WWTP influent

(Kasprzyk-Hordern et al., 2009), and 7.46 µg/L in polluted groundwater (Lhotský et al., 2017). Thus, the exposure concentration of AMI used in this study is within the range of environmentally relevant concentrations. Moreover, it has been reported that waterborne exposure to AMI at this concentration level (0.1-10 µg/L) was sufficient to disturb the growth, development, and gene expression in zebrafish larvae (Wu et al., 2017; Yang et al., 2014).

A total of 60 healthy adult zebrafish (30 females and 30 males; F0 generation) were used for parental exposure. For the AMI exposure group, 15 individuals of the same sex were introduced into a glass beaker containing 3 L of test solution, and females and males were exposed separately in 2 different beakers. The same procedure was performed for the control group, except that the dechlorinated tap water was used as the test solution. The exposure was conducted for 21 days under the same conditions described in section 2.1, except that the test solution was changed every two days to ensure optimal water quality. The fish were fed *Artemia* nauplii (<24 h after hatching) twice per day. In addition, water samples (20 ml in each tank) were collected before renewal and used to confirm the realistic AMI concentrations in exposure systems.

2.4 Collection and maintenance of F1 embryos

Because male and female zebrafish (F0) were maintained in separate beakers during parental exposure, they could only lay eggs after being placed together for mating. To reduce unnecessary stress, the breeding and embryo collection were conducted only once at the end of parental exposure. To collect embryos, F0 females and males were randomly selected and placed into spawning boxes (containing 2 L of AMI-free dechlorinated tap water) with a ratio of 3:3, resulting in 5 groups for the control and AMI exposure group. A transparent baffle was placed in each spawning box to separate females and males, and all spawning boxes were put in an incubator (14L:10D, 28 °C).

The following day, after removing the baffle, the male and female fish were allowed to mate to lay eggs. About 20 minutes later, the fertilized embryos (F1 generation) were collected and transferred to a clean Petri dish (120 mm in diameter) containing an appropriate E3 medium (Zou et al., 2017).

For both groups, 150 embryos were randomly assigned to 90 mm diameter Petri dishes (i.e., 50 per Petri dish, $n = 3$), which were used for the physiological and behavioral assay. All Petri dishes were put in an incubator (14L:10D, 28 °C), and the mortality, hatching time, and development condition were observed and recorded at 2, 4, 8, 24, 48, 72, 96, and 120 hours post-fertilization (hpf). During this time, all dead individuals were removed immediately upon noticing. At 48 hpf, 5 embryos in each Petri dish were selected and used for detecting heart rate under a stereomicroscope (SZX16, Olympus, Japan). In addition, the remaining F1 embryos were cultured under the same condition and used for transcriptome analysis.

2.5 Behavioral test of F1 larvae

At 120 hpf, 8 larvae per Petri dish were randomly selected for the light-dark locomotion test (24 larvae in the control and AMI exposure group, respectively). These F1 larvae were assigned to 24-well plates, following Qiu et al. (2022b). The behaviors of zebrafish larvae were recorded using a DanioVision System (Noldus, Wageningen, Netherlands) and analyzed using EthoVision XT Vision 11.5 (Noldus). The behavioral parameters (ASV: average swimming velocity; DTT: duration of thigmotaxis; DHM: duration of high mobility) are described in detail in Table S2.

2.6 Transcriptome Analysis of F1 Larvae

For transcriptome analysis, 50 individuals were randomly selected and pooled in a Freezer Storage Tube (as one sample), with 3 replicates for each treatment group. The samples were frozen in liquid nitrogen (10 min) and then stored at -80 °C until RNA

extraction. The TRIzol reagent (Invitrogen, Carlsbad, CA) was used to extract and purify total RNA. Subsequently, the constructions of the cDNA library and the RNA sequencing followed our previous study (Qiu et al., 2022a). After quantification of gene expression using RSEM (v1.2.12), the differentially expressed genes (DEGs) testing was completed by using DESeq2 (Love et al., 2014), with the condition of Q-value $\leq$ 0.05 and fold change (FC) cutoff of ≥ 1.5 . Subsequently, functional enrichment analysis (GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes) was conducted. Moreover, gene set enrichment analysis (GSEA) was conducted using the GSEA software (Subramanian et al., 2005), and the significant enrichment was identified based on the normalized enrichment score (NES) and FDR q-value ($|NES| \geq 1.5$, $FDR-q \leq 0.25$). The protein-protein interaction (PPI) framework of the key driver gene was constructed using the Cytohubba plugin in Cytoscape software (v3.9.1, UAS). Details about the transcriptome analysis and statistical evaluation methods are shown in the supporting information.

2.7 Statistical analysis

A generalized linear model was applied to determine significant differences in physiological parameters (i.e., survival, hatching time and heart rate) and behavioral traits between control and AMI-exposed offspring ($n = 3$; each Petri dish was considered as one replicate), with SPSS 16.0 (Chicago, IL).

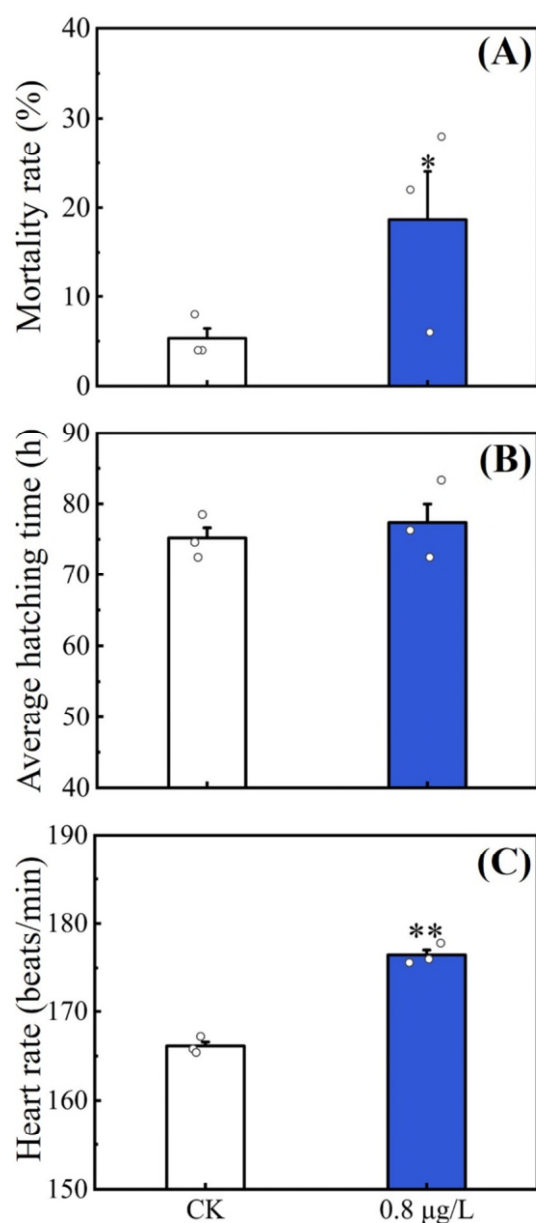
3 Results

The AMI was not detected in the dechlorinated tap water collected from the control group, and its concentrations in test solutions collected from the AMI exposure group were 82.5–104% of the nominal concentration (i.e., 0.8 $\mu\text{g/L}$; Table S1). All the F0 zebrafish survived during the 21-day exposure. After pairwise breeding (3 pairs in a

spawning box), the average number of embryos per spawning box in control and AMI-exposure groups were 537 ± 111 and 694 ± 227 , respectively, without significant difference.

3.1 Mortality and hatching of F1 generation

The F1 generation parentally exposed to AMI exhibited a significantly higher mortality rate compared with the control group ($p < 0.05$, Fig. 1A). However, parentally exposure to AMI did not noticeably affect their average hatching time (Fig. 1B). At 48 hpf, there was a significant increasing in the heart rate of F1 embryos born from AMI-exposed parents ($p < 0.01$, Fig. 1C).



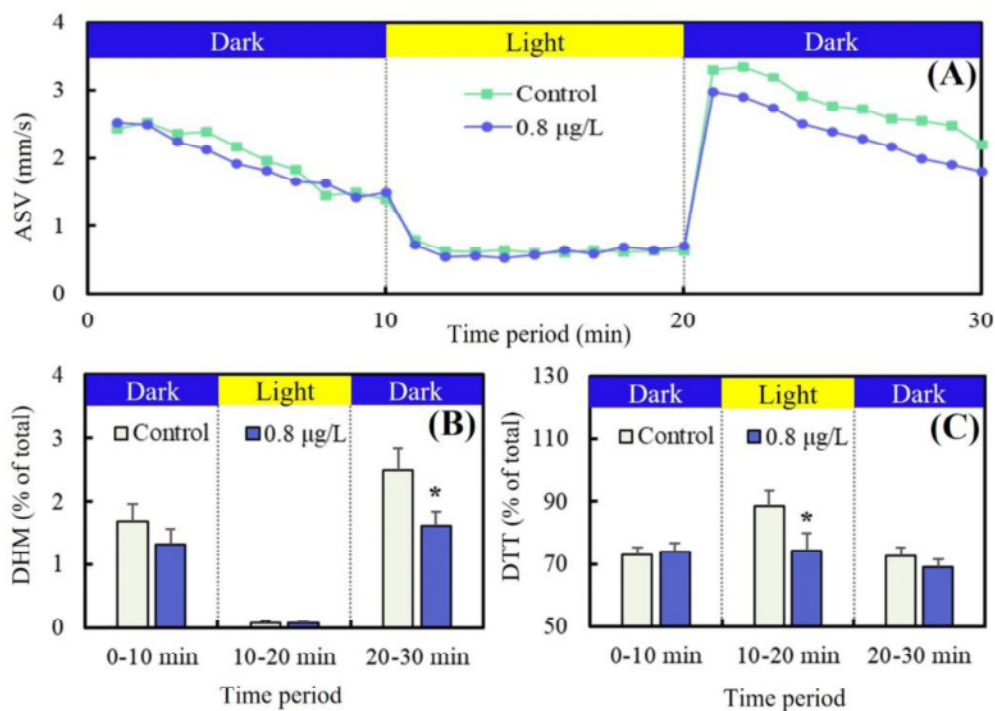
197

198 **Fig. 1.** Impacts of parental amitriptyline exposure on the survival, hatching and heart
 199 rate of zebrafish offspring. (A) The mortality rate at 120 hpf; (B) Average hatching time;
 200 (C) Heart rate at 48 hpf. Data are shown as means ± SE ($n = 3$, each Petri dish was
 201 considered as one replicate), and asterisks indicate significant differences (* $p < 0.05$,
 202 ** $p < 0.01$). CK: F1 offspring born from parents in the control group; AMI: F1
 203 offspring born from parents exposed to amitriptyline at 0.8 µg/L.

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3.2 Responses in behaviors of F1 larvae during Light-Dark test

Regardless of whether the parents were exposed to AMI, their offspring (i.e., F1 larvae) displayed an active state during dark periods and a resting state during light periods (Fig. 2A, more details in Fig. S2). As shown in Fig. 2B, the F1 larvae parentally exposed to AMI exhibited a significantly lower duration of high mobility (DHM) in the second dark period, indicating reduced locomotor activity. As shown in Fig. 2C, the F1 larvae parentally exposed to AMI also exhibited a significantly lower duration of thigmotaxis (DTT) during the light period. As thigmotaxis is a well-validated index of anxiety in animals and humans (Schnörr et al., 2011), the decreased DTT reflected that the offspring born from parents exposed to AMI exhibited a lower anxiety-like state.



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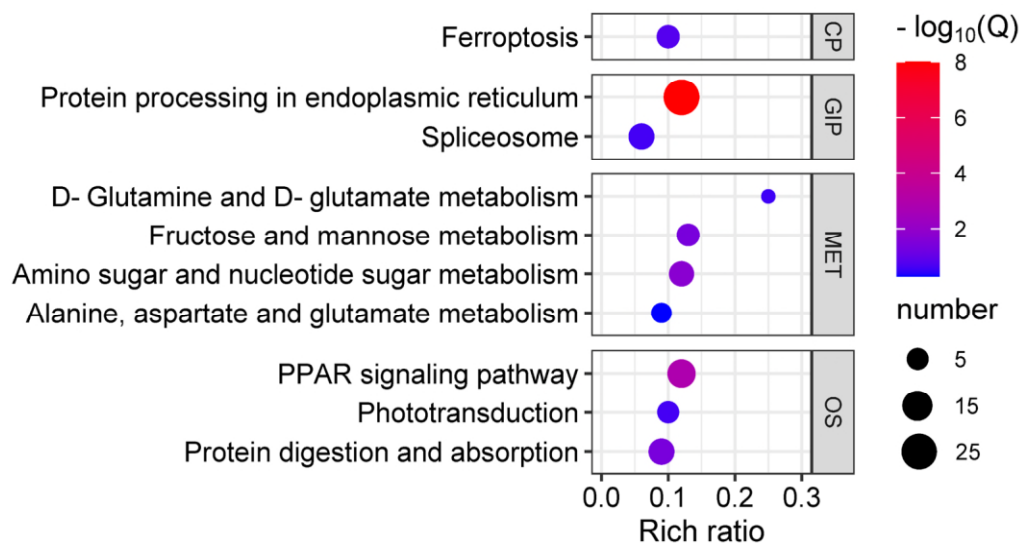
218 **Fig. 2.** Effects of parental amitriptyline exposure on the behavioral responses of
219 offspring to light-dark transition. The navy blue and yellow above the charts separately
220 present the dark and light conditions. (A) ASV: average swimming velocity (mm/s; data
221 are shown as the mean value in each minute interval). (B) DHM: duration of high
222 mobility. (C) DTT: duration of thigmotaxis. A detailed explanation of the abbreviations
223 is presented in Table S2. The unit (% of the total) of DHM and DTT indicates the
224 percentage of accumulated time for the high mobility or thigmotaxis in zebrafish larvae
225 to the total observation time of each dark or light period (i.e., 10 min). Data are shown
226 as means $\pm$ SE ($n = 3$, each Petri dish was considered as one replicate), and asterisks
227 indicate significant differences ($*p < 0.05$).

3.3 Transcriptional responses in F1 larvae

This study detected 587 differentially expressed genes (DEGs) in the F1 larvae parentally exposed to AMI, including 231 up-regulated genes and 356 down-regulated genes. According to our results of KEGG pathway analysis, those DEGs were enriched in some crucial pathways that play important roles in organisms (Fig. 3, more details in Table S4). Among these pathways, protein processing in endoplasmic reticulum, amino sugar and nucleotide, phototransduction, protein digestion and absorption were also identified as significantly enriched pathways in a GSEA analysis (Table S5).

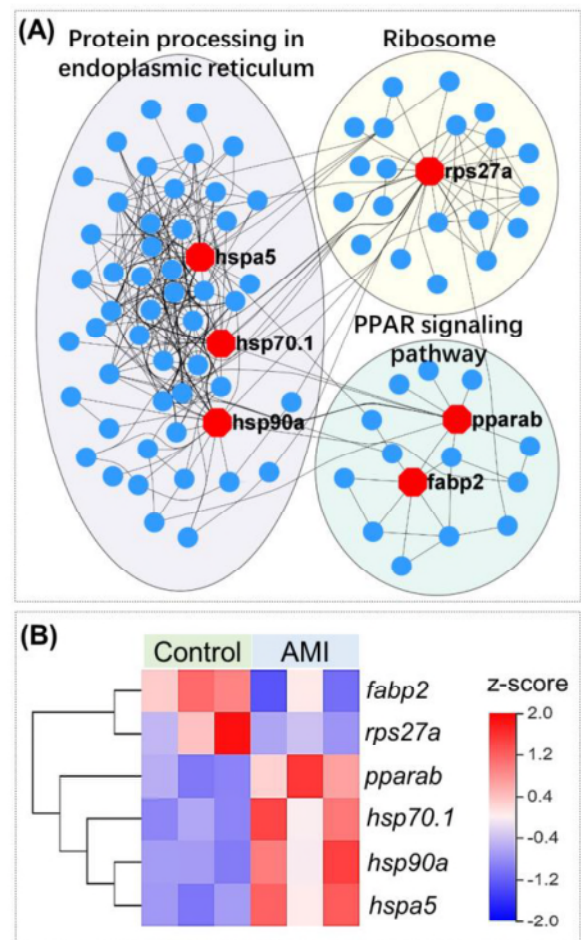
According to the PPI network (Fig. 4A), six DEGs (i.e., *hspa5*, *hsp70.1*, *hsp90a*, *rps27a*, *pparab* and *fabp2*) were revealed as key driver genes involved in protein processing in endoplasmic reticulum (*hspa5*, *hsp70.1*, *hsp90a*), ribosome (*rps27a*) and PPAR signaling pathway (*pparab* and *fabp2*). The expression of *fabp2* and *rps27a* was down-regulated, but *pparab*, *hsp70.1*, *hsp90a* and *hspa5* were up-regulated (Fig. 4B).

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243

244 **Fig. 3.** Enrichment bubble chart depicting the top ten enrichment KEGG pathways
245 analyzed and calculated from the mRNA-Seq analysis of the F1 larvae samples from
246 the AMI-exposed F0 zebrafish. The text in the gray box on the right shows the names
247 of the first-level KEGG category (CP: cellular processes; GIP: genetic information
248 processing; MET: metabolism; OS: organismal systems). Abbreviations: Q: the *p*-value
249 after the multiple hypothesis test correction; number: the number of differentially
250 expressed genes (DEGs) in a pathway; Rich ratio: the proportion of the number of
251 DEGs in a pathway to the number of all genes annotated to that pathway.



253

254 **Fig. 4.** Key driver analysis (KDA) for the differentially expressed genes (DEGs) in the
255 F1 offspring. (A) Protein-protein interaction (PPI) network showing key driver genes.
256 (B) Heatmap indicating the differential expressions of six key driver genes in the F1
257 larvae samples born from the control and AMI-exposed parents. The expression levels
258 of all samples (columns) are normalized using the Z-scores method.

259

4 Discussion

Our results demonstrated that parental exposure to AMI could disturb the survival, development, behaviors, and gene expression of zebrafish offspring, which supports our hypothesis. Previously, Vera-Chang et al. (2018) reported that fluoxetine exposure could induce hypocortisolism and behavioral disruption in zebrafish offspring, and Al Shuraiqi et al. (2021) found that parental exposure to fluoxetine could alter the swimming activity of zebrafish offspring in a non-monotonic manner. Considering the importance of early life survival and behaviors for fish population dynamics (Brodin et al., 2014; Qiu et al., 2023; Sehonova et al., 2018), these adverse effects of parental exposure to antidepressants (including AMI) may induce ecological consequences in natural habitats.

4.1 Abnormal development of offspring induced by AMI parental exposure

Previous studies have demonstrated the implications of antidepressants on fish reproduction. For example, Hong et al. (2022) reported that the whole life cycle exposure to citalopram (at 1, 10, and 100 µg/L) and venlafaxine (at 10 and 100 µg/L) could induce reproductive inhibition in zebrafish, based on the mean number of egg production obtained from 3 batches of reproductive tests. In addition, Lister et al. (2009) also found that exposure to fluoxetine (32 µg/L) significantly reduced the reproductive capacity of zebrafish, based on the decreased cumulative egg production over a 7-day exposure period. In the present study, the reproductive test (i.e., breeding and embryo collection) was conducted only at the end of parental exposure, which may result in insufficient data for assessing fish reproductive capacity. Therefore, although we did not detect a significant difference in the average number of embryos per spawning box in the control and AMI-exposure groups, it should not be lightly assumed that chronic AMI exposure did not affect fish reproductive performance.

Furthermore, our results showed that parental exposure to AMI was associated with significantly higher mortality and tachycardia in zebrafish offspring (Fig. 1). Heart rate is commonly used to evaluate cardiotoxicity and developmental toxicity of chemical substances in zebrafish embryos (Craig et al., 2006). Previous studies have reported that waterborne exposure to AMI could increase the mortality of zebrafish embryos (Yang et al., 2014) and disrupt the heart development of common carp (*Cyprinus carpio*) in their early life stages (Sehonova et al., 2017). On the other hand, cardiovascular toxicity of TCAs has also been well demonstrated in mammals, and it has been reported that prenatal exposure to antidepressants could increase the risk of congenital cardiac rhythm in newborns (Gentile, 2014; Thanacoody and Thomas, 2005). Moreover, Sehonova et al. (2017) also reported that cardiotoxicity was the main cause of the increased mortality in common carp (*C. carpio*) exposed to TCAs (amitriptyline, nortriptyline, and clomipramine).

4.2 Abnormal behavior of offspring induced by AMI parental exposure

The light/dark transition test suggested that parental exposure to AMI could induce abnormal behavioral responses in F1 zebrafish larvae, as evidenced by the hypoactivity during the second dark period and decreased thigmotaxis during the light period (Fig. 2). Hypoactivity and reduced anxiety are typical responses of animals exposed to antidepressants. For instance, Sprowles et al. (2016) found that perinatal exposure to citalopram (a selective serotonin reuptake inhibitor) could decrease motion activity in rat offspring, and Suryanto et al. (2021) found that exposure to antidepressants (e.g., AMI, amoxapine, and sertraline) could induce hypoactivity of zebrafish during the dark period. Hypoactivity induced by pollutants may result in fish being more easily caught by predators and reduced predation efficiency (Brodin et al., 2014; Qiu et al., 2017).

During the light period, zebrafish larvae parentally exposed to AMI exhibited less

thigmotaxis, a well-validated index that reflects their anxiety (Schnörr et al., 2011). In other words, parental exposure to AMI could reduce anxiety and increase the exploratory behavior of zebrafish larvae. Previously, Martin et al. (2020) revealed that exposure to fluoxetine caused a non-monotonic reduction of anxiety behavior in female mosquitofish (*Gambusia holbrooki*). However, natural anxiety behavior is essential for the escape strategy of aquatic species in the face of danger; the modulation of decision-making under an anxiety-like state increases survival from predation (Brodin et al., 2014; Perrot-Minnot et al., 2017). Thus, we inferred that the reduced locomotor activity and thigmotaxis may result in organisms being unable to escape in time when faced with danger, thereby reducing their survival.

4.3 Overview of enrichment analysis

Comparative analysis of the transcriptional profiles resulted in 587 DEGs (231 up-regulated and 356 down-regulated), suggesting a global change in gene expression in zebrafish larvae parentally exposed to AMI. Enrichment analysis suggested that the transcriptional disruption of several pathways could be associated with the observed abnormal development and behavioral responses in zebrafish larvae parentally exposed to AMI. For example, the GSEA analysis indicated that the cardiac muscle contraction pathway in zebrafish offspring was up-regulated after parental exposure to AMI (see Table S5), which may be a factor in the observed tachycardia (Kagemoto et al., 2015). Previously, Wu et al. (2017) reported that AMI may cause changes in the heart rate of zebrafish larvae by inducing the expression of the gene *act1a*, which is involved in the regulation of muscle contraction in cardiomyocytes, and Sehonova et al. (2019) also found significantly up-regulated expression of *nkx 2.5* gene (encoding a transcription factor that regulates heart development) in *Xenopus tropicalis* embryos exposed to 30 µg/L AMI for 2 days.

Moreover, dysregulated phototransduction in zebrafish larvae parentally exposed to AMI was identified by KEGG (Fig. 3) and GSEA analysis (Table S5). Phototransduction is the sensory transduction of the visual systems, which involves converting light into electrical signals in photoreceptor cells (Shimmura et al., 2017). Previously, Qian et al. (2021) found that abnormal behavioral traits in the light/dark transition test may be due to the dysregulation of the phototransduction pathway in zebrafish larvae exposed to boscalid. Furthermore, Sun et al. (2023) also reported that the disruption of the phototransduction pathway may be a reason for the hypoactivity of zebrafish larvae. Taken together, we inferred that those abnormal behaviors of zebrafish offspring may be associated with the disruption of the phototransduction pathway. However, it should be noted that our transcriptomic data were derived from whole larval samples, making it difficult to correlate disruptions in most of the enriched pathways with behavioral alterations specifically. Further cell- or tissue-specific studies will help to better establish the specific associations between transcriptomic data and behavioral changes.

4.4 Dysregulation of pathways related to endoplasmic reticulum stress

Dysregulated protein processing in the endoplasmic reticulum in zebrafish larvae parentally exposed to AMI was identified by KEGG analysis (Fig. 3). Proteins are generally folded in the endoplasmic reticulum (ER). However, when unfolded proteins accumulate in the lumen, the ER stress would be induced to affect cell homeostasis (Scriven et al., 2007). The ER has been revealed as a novel target of antidepressants in mammals (Yang et al., 2018), and Ma et al. (2016) found that exposure to fluoxetine (an antidepressant) contributed to apoptosis in glioma cells through CHOP-dependent ER stress-related pathways. In addition, ER stress was associated with depress-like behavior in rats (Timberlake and Dwivedi, 2015). In zebrafish, dysregulation of ER

function has been associated with reduced thigmotaxis, anxiety, and movement (Chavali et al., 2023; Wang et al., 2023). Thus, we speculated that the decrease in anxiety may be related to the ER stress in zebrafish offspring induced by parental AMI exposure.

In the present study, three up-regulated DEGs (*hspa5*, *hsp70.1* and *hsp90a*) were predicted as the key driver genes that may be involved in the modulation of the protein processing in the endoplasmic reticulum of zebrafish larvae parentally exposed to AMI (Fig. 4). As members of the heat shock protein 70 (HSP70) family, binding immunoglobulin protein (GRP78/BiP, encoded by *hspa5*) serves a primary role in regulating ER homeostasis and anti-apoptotic mechanisms (Walter and Ron, 2011), and the heat shock protein 70.1 (HSP70.1, encoded by *hsp70.1*) plays an important role in protecting the lysosomal membrane integrity and also served protective roles against ischemia or oxidative injury (Stetler et al., 2010). The up-regulated expression of these genes implies that parental exposure to AMI may induce ER stress in zebrafish offspring. As a member of the HSP90 family, heat shock protein 90 (HSP90, encoded by *hsp90*) serves important roles in the folding of nascent proteins, refolding of misfolded proteins, and removing misfolded proteins (Han et al., 2020). It can be inferred that the abnormal expression of HSPs in the F1 larvae could lead to the dysregulation of pathways related to ER stress, and further affect the folding and normal functions of proteins. These impacts may contribute to cell and organ damage and then further affect the survival and behaviors of zebrafish offspring.

4.5 Dysregulation of the ribosome pathway

As the site of protein synthesis, the ribosome is an intercellular structure made of both RNA and proteins. It serves crucial roles in the growth, development and survival of organisms (Moss et al., 2007). The results of GSEA (Table S5) and KDA showed a

significant upregulation in the ribosome pathway, with *rps27a* as a key driver gene (Fig. 4). Dysregulation of ribosome biogenesis may result in potential aberrance of cell proliferation, ribosomal stress, and some metabolic disorders in organisms (Wang et al., 2015). Ribosome proteins are important for embryonic development, and the deficiency of ribosomal protein S7 (RPS7) has been proven to induce abnormal brain development and impaired hematopoiesis in zebrafish embryos (Duan et al., 2011). In our study, the key driver gene involved in the ribosome, *rps27a* (UBCEP80), was down-regulated in zebrafish offspring parentally exposed to AMI (Fig. 4B). The RPS27a (encoded by *rps27a*) participates in the ribosome biogenesis and the production of ubiquitin in organisms (Luo et al., 2023). Previously, *rps27a* was defined as a hub gene in the MAPK signaling pathway of zebrafish exposed to bisphenol S (Yang et al., 2021). Moreover, the overexpression of protein RPS27a is an indicator of ER stress (Yilmaz et al., 2021). Based on these previous findings, we inferred that the abnormal expression of *rps27a* could lead to ER stress disorders and even behavioral changes in zebrafish offspring after parental exposure to AMI.

4.6 Dysregulation of the PPAR signaling pathway

Significant enrichment of the PPAR signaling pathway, which is essential to regulate the lipid metabolism of organisms (Den Broeder et al., 2015), was identified in zebrafish offspring parentally exposed to AMI (Fig. 3). Dysregulation of the PPAR signaling pathway in fish induced by antidepressants has been reported by many previous studies. For example, Colville et al. (2022) revealed that exposure to fluoxetine at 0.05 µg/L for 96 h caused abnormal regulation of the PPAR signaling pathway in fathead minnows (*Pimephales promelas*), and Rodrigues et al. (2020) found that venlafaxine at 400 ng/L dysregulated the PPAR receptors and induced the dysfunction of lipid metabolism in zebrafish. Moreover, Meade et al. (2023) reported

that antidepressants and other chemical mixtures detected in the water body disrupt the expressions of genes involved in the PPAR signaling pathway, phototransduction, and ribosome pathways in zebrafish larvae.

Our KDA suggested that two key driver genes, *pparab* and *fabp2*, were involved in the PPAR signaling pathway. PPAR- α , encoded by *pparab*, is essential to regulate the release of energy in the lipid breakdown process (Den Broeder et al., 2015). Furthermore, the PPAR- α is also a vital nervous system regulator, and its deficiency would inhibit the proliferation of neuronal and glial precursors in zebrafish embryos (Hsieh et al., 2018). On the other hand, the fatty acid-binding protein 2 (FABP2), encoded by *fabp2*, is essential to help maintain the steady of fatty acids (Shao et al., 2022). It has been reported that *fabp2* is a key factor in regulating fatty acid response genes (e.g., *ppar* superfamily), and its overexpression may induce fatty acid accumulation and other lipid metabolism disorders in fish (Zhao et al., 2020). Moreover, Wu et al. (2022) found that *fabp2* played an important role in affecting the behaviors of zebrafish by using the gene knockout method. Based on the findings of these previous studies, we inferred that parental AMI exposure may disturb the neuronal development and lipid metabolism of their offspring through dysregulating the PPAR signaling pathway, and further cause developmental and behavioral abnormalities of zebrafish embryos and larvae.

5. Conclusion

Parental exposure to AMI adversely affected the survival, development, behavior, and gene expression of zebrafish offspring, including higher mortality, abnormal heart rates, and decreased swimming activity and anxiety. Enrichment analysis suggested that the transcriptional disruption of several pathways could be associated with the observed

abnormal development and behavioral responses in zebrafish larvae parentally exposed to AMI. For example, dysregulated phototransduction may be a crucial factor in the abnormal behavioral response to the light/dark transition, and the up-regulated cardiac muscle contraction pathway may contribute to the observed tachycardia. Furthermore, parental exposure to AMI also induced the disturbance of pathways related to endoplasmic reticulum stress and PPAR signaling. Six genes were predicted as key driver genes involved in those pathways, and their differential expressions may notably disrupt the function of the related proteins regarding parental AMI exposure. However, since our study only included two generations (i.e., F0 and F1) and lacked information on AMI concentration in F1 embryos, it is impossible to distinguish the detailed mechanisms underlying the observed impacts of parental exposure on F1 zebrafish. The possible mechanisms may include the maternal transfer of AMI (*in-ovo* exposure), poor sperm quality due to paternal AMI exposure, or even transgenerational epigenetic effects. Therefore, further assessments across multiple generations are needed to determine the full magnitude of adverse effects of parental AMI exposure on fish.

Acknowledgments

This work was supported by the following funds: Natural Science Foundation of China (Grant No. 32071623), the Natural Science Foundation of Jiangsu Province of China (Grant No. BK20191433), and the Jiangsu Provincial Double-Innovation Program (Grant No. 1711370013). Thanks for supporting the Student Scientific Research Project from Jiangsu University (Grant No. 22A102).

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Liu, Anqi; Chen, Chen; Chen, Kun; Shi, Yanhong; Grabowski, Robert C. ...

2024-02-20

Elsevier

<https://doi.org/10.1016/j.scitotenv.2023.169173>

Liu A, Chen C, Chen K, et al., (2024) Effects of parental exposure to amitriptyline on the survival, development, behavior, and gene expression in zebrafish offspring. Science of the Total Environment, Volume 912, February 2024 Article number 169173