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Authors

Wang, Zejun

Dai, Zhuoya

Han, Xiaowen

et al.

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A potential mechanism of triclosan-induced neurotoxicity in zebrafish at environmental Concentrations: Splicing disruption of *tecra* mediates the circRNA266/miR-214/dapk2-gad2 axis

Zejun Wang^{a,*,1}, Zhuoya Dai^{a,1}, Xiaowen Han^a, Xincong Su^a, Xiao Yang^a, Xuedong Wang^a, Jin Yan^a, Qiuhui Qian^a, Huili Wang^{b,**}, Randy A. Dahlgren^c

^a National and Local Joint Engineering Laboratory of Municipal Sewage Resource Utilization Technology, School of Environmental Science and Engineering, Suzhou University of Science and Technology, Suzhou, 215009, China

^b School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou, 325035, China

^c Department of Land, Air and Water Resources, University of California-Davis, CA, 95616, USA

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ABSTRACT

Many environmental pollutants are known to exhibit neurotoxic effects; however, the initiating molecular events and their associated adverse outcome pathways (AOPs) remain largely unclear. This study investigated the role of circRNA266 and its target miRNA in Triclosan (TCS)-induced neurodevelopmental toxicity in zebrafish (*Danio rerio*) based on candidates circRNAs identified via high-throughput sequencing. Toxicological effects were characterized at multiple levels, including phenotype alterations, motor behavior, neural damage, and the expression of marker genes. TCS exposure was found to upregulate circRNA266, thereby reducing the availability of free miR-214. As a crucial neuroprotective factor, miR-214 downregulation was associated with increased expression of *dapk2* and *gad2*, resulting in elevated inhibitory neurotransmitter levels and neuronal apoptosis. Furthermore, molecular docking analysis suggests that the upregulation of circRNA266 by TCS may occur via its interaction with U2AF1, thereby disrupting the splicing of the *tecra* gene. While high concentrations of TCS caused marked neurotoxic phenotypes and behavioral impairments, environmentally relevant concentrations did not elicit overt symptoms. Nonetheless, the activation of key molecular events within the AOP framework suggests a potential neurotoxic risk even at low exposure levels. This study is the first to confirm that TCS induces neurotoxicity in zebrafish by interfering with the splicing of the *tecra* gene and mediating the regulatory role of the circRNA266/miR-214/*dapk2-gad2* signaling axis. These findings provide novel insights into the molecular mechanisms of TCS-induced neurotoxicity and propose a potential new AOP model for environmental risk assessment.

1. Introduction

Triclosan (TCS), a broad-spectrum antibacterial agent, is commonly used in daily products such as hand sanitizers, soaps, various medical devices, and disinfectants (Li et al., 2019a). Recommended concentrations typically range from 0.05 % to 0.3 % (0.5–3 g/L) in aqueous solutions such as mouthwash and hand sanitizer (Yueh and Tukey, 2016). In agriculture, TCS is also applied in irrigation water disinfection (Mendez et al., 2016), with recommended dosages of 2–5 g/m³ to inactivate pathogens such as bacteria and parasite eggs. It enters aquatic

environments primarily through domestic wastewater and effluent from wastewater treatment plants, where it is only partially removed during treatment processes (Magro et al., 2020). Consequently, TCS has been widely detected in various environmental matrices, including surface water, sediments, and even drinking water sources (Liang et al., 2022; Jagini et al., 2019; Diao et al., 2022; Milanovic et al., 2023; Huang et al., 2025; Ogutverici et al., 2016), with concentrations typically ranging from ng/L to µg/L levels (Yueh and Tukey, 2016; Wang et al., 2025). The highest reported aquatic concentration reached 86.2 µg/L in riverine systems (Jagini et al., 2019). Moreover, TCS is lipophilic and resistant to

* Corresponding author.

** Corresponding author.

E-mail addresses: wangzj9135@163.com (Z. Wang), whuili@163.com (H. Wang).

¹ Co-first author: Zejun Wang and Zhuoya Dai are the co-first authors.

biodegradation, making it prone to bioaccumulation in aquatic organisms. It has also been detected in human matrices, including urine, plasma, and breast milk (Zhou et al., 2021; Lee-Sarwar et al., 2018; Kim et al., 2020), indicating widespread exposure and systemic absorption. Given its ubiquitous environmental distribution and chronic low-level exposure, assessing the hazard and mechanistic toxicity of TCS—especially at environmentally relevant concentrations—has become increasingly important for ecological risk assessment and regulatory policymaking.

Numerous studies have demonstrated that TCS exposure induces a range of toxic effects, including neurotoxicity (Diao et al., 2022; Zhao et al., 2022a, 2025; Chen et al., 2025; Liu et al., 2019a; Ling et al., 2020; Hao et al., 2022a), cardiotoxicity (Saley et al., 2016; Diao et al., 2023; Wan et al., 1987), abnormal lipid metabolism (Liu et al., 2019b, 2024; Huang et al., 2020; Wang et al., 2020; Tenkov et al., 2022; Li et al., 2023a), and reproductive toxicity (Qiao et al., 2022; Li et al., 2024; Wang et al., 2024a, 2024b; Duan et al., 2022; Hao et al., 2022b). Among these, neurotoxicity has garnered particular attention. TCS has been shown to interfere with the function of several key enzymes and regulatory proteins, such as the RNA-binding protein CELF2 (Wang et al., 2021a), the demethylase ALKBH5 (Wang et al., 2023), and proteins involved in the nuclear factor kappa B (NF- κ B) signaling pathway (Liu et al., 2021), thereby contributing to neurotoxic outcomes. In recent years, increasing attention has been paid to the regulatory role of TCS in modulating aberrant microRNA (miRNA) expression—small non-coding RNAs that serve as post-transcriptional regulators of gene expression. For instance, TCS exposure has been shown to alter miRNA expression profiles in zebrafish (*Danio rerio*) and other model organisms (Wang et al., 2019). A 2019 study reported that the upregulation of miR-137 mediates TCS-induced neurotoxicity (Liu et al., 2019a). Similarly, miR-219 (Ling et al., 2020) and miR-144 (Diao et al., 2022) have been implicated in TCS-induced disruptions in oligodendrocyte differentiation and activation of PKC/MAPK signaling, respectively, leading to neural impairments. In parallel with this, the competing endogenous RNA (ceRNA) network has emerged as a novel post-transcriptional regulatory mechanism (Asadi et al., 2023; Thomson and Dinger, 2016). In contrast to conventional miRNA-mediated regulation, the ceRNA network functions via a more intricate and tightly regulated mechanism, wherein messenger RNAs (mRNAs) and other transcripts containing miRNA response elements (MREs) compete for miRNA binding, thus influencing the expression of downstream genes (Asadi et al., 2023). Further exploration of this model may yield novel insights into the mechanisms of TCS-induced neurotoxicity and enhance our understanding of miRNA-mediated toxic responses.

Circular RNA (circRNA) is a class of non-coding RNA molecules characterized by covalently closed circular structures, exhibiting high stability and pronounced tissue specificity (Memczak et al., 2013). They are abundantly expressed in the cytoplasm of eukaryotic cells and exhibit pronounced tissue- and developmental-stage-specific expression, especially in the brain (Mehta et al., 2021; Özdemir and Arslan, 2022). Emerging evidence suggests that circRNAs play vital roles in neurodevelopment, neuronal plasticity, and the pathogenesis of neurological disorders (Li et al., 2021; Galli et al., 2025). Recent studies have revealed that circRNAs regulate gene expression through various mechanisms, notably by acting as microRNA response elements (MREs), or "miRNA sponges," thereby modulating the bioavailability of miRNAs and influencing downstream gene networks (Memczak et al., 2013; Zhao et al., 2022b; Barrett and Salzman, 2016). Seminal work by Sebastian et al. first demonstrated the miRNA sponge function of circRNAs, showing that specific circRNAs could bind to miR-7 in neuronal tissues, effectively suppressing its activity (Memczak et al., 2013). Notably, several circRNAs have been identified as key regulators of neurodevelopment. For instance, circEdis was found to regulate neuronal differentiation and innate immunity via the *Edis*–*Relish*–*castor* axis in *Drosophila* (Xiong et al., 2022). Studies in mammalian models have shown that circRNAs are dynamically expressed during neural stem cell

differentiation (Yang et al., 2018), and differential N6-methyladenosine (m6A) methylation patterns of circRNAs have been observed in the brains of Alzheimer's disease models (Zhang et al., 2023), suggesting epitranscriptomic regulation of circRNA function and associated with neurogenetic disorders.

In addition to their roles in neurodevelopment, circRNAs have gained attention in the field of neurotoxicology. Environmental and chemical exposures can disrupt circRNA expression profiles in the brain, potentially contributing to neurotoxicity through ceRNA network dysregulation. For example, toxicogenomic studies revealed that circRNA_13845 interacts with miR-361-3p and miR-702-3p to regulate *traf2* and the NF- κ B pathway, while exposure to the flame retardant DCIPP alters circRNA expression in zebrafish (Li et al., 2020). Other compounds such as cypermethrin, chlorpyrifos, and glyphosate have also been shown to modulate circRNA levels in the brain, affecting MAPK signaling and neuronal apoptosis (Özdemir and Arslan, 2022; Yu et al., 2018; He et al., 2021). Moreover, circRNAs have been implicated in the neurotoxicity induced by classical toxicants such as paraquat, aluminum, and manganese. For instance, manganese exposure triggers ferroptosis-mediated cognitive impairment through circHmbox1(3,4) (Wei et al., 2024), while paraquat-induced oxidative stress alters circRNA m6A modification, influencing neuronal survival (Chen et al., 2021). In a study on aluminum-induced hippocampal toxicity, ceRNA network analysis linked specific circRNAs to cognitive dysfunction and synaptic signaling disruption (He et al., 2021). Beyond environmental toxicants, circRNAs also modulate drug-induced neurotoxicity. CircRNA_001372 was shown to mitigate inflammation and apoptosis in propofol-induced neuroinflammation by regulating the PI3K/Akt/NF- κ B pathway through miR-148b-3p (Wang et al., 2021b). In methamphetamine-treated neurons, novel circRNAs have been identified that may underlie addiction-related neuroadaptations (Li et al., 2019b). Collectively, these studies underscore the emerging significance of circRNAs as key regulators of neurodevelopment, neurodegeneration, and neurotoxicity (Shao and Jiang, 2021). As research progresses, circRNA–miRNA–mRNA interaction networks (ceRNA networks) are being increasingly recognized not only as mechanistic pathways in neuronal health and disease but also as promising biomarkers for chemical-induced neurotoxicity (Lu et al., 2019). However, while computational predictions and expression profiling have advanced rapidly, experimental validation of circRNA functions in toxicological contexts remains limited, warranting further investigation.

In our previous study, we performed a preliminary screening of 29 key circRNAs that were differentially expressed following TCS exposure (Wang et al., 2024b). Building upon these findings, we further explored the potential of the ceRNA network as a key mechanism underlying chemical-induced toxicity, with the aim of uncovering novel mechanisms of TCS-induced neurotoxicity. We begin by evaluating the toxicological effects of TCS using a gradient exposure strategy, focusing on phenotypic alterations, locomotor behavior, structural changes in the nervous system, and the expression of neural marker genes. Subsequently, we assess the functional role of circRNAs in mediating TCS-induced neurotoxicity by performing loss- and gain-of-function assays for selected candidates. To further elucidate the regulatory interplay between circRNAs and their downstream miRNA targets, we construct a circRNA/miRNA interaction network within the ceRNA framework. Based on integrated phenotypic and molecular data, we propose a mechanistic adverse outcome pathway (AOP) model that links circRNA–miRNA interactions to TCS-induced neurotoxicity. Finally, we explore whether environmentally relevant concentrations of TCS are sufficient to trigger similar neurotoxic responses, providing insight into the ecological and human health risks posed by chronic low-dose exposure.

2. Materials and methods

2.1. Chemical reagents and test organisms

TCS (CAS No. 3380-34-5, 99.9 % purity) was purchased from Sigma Aldrich (St. Louis, USA), and the chemical solvent, acetone (99.9 % purity), was purchased from Zhejiang Zhongxing Chemical Reagent Co.

According to previous research (Yan et al., 2024), the zebrafish were cultured and allowed to spawn naturally. When the embryos developed for six hpf, high-quality embryos were screened, collected, and transferred into Petri dishes for exposure experiments.

2.2. Verification of circRNA266 circular structure

Prior to conducting toxicity experiments, the circular structure of circRNA266 was verified using Sanger sequencing and RNase R digestion. Specific primers were then designed as templates for reverse transcription PCR or real-time fluorescence quantitative PCR after obtaining cDNA by reverse transcription of RNase R-treated RNA from 120 hpf zebrafish, and the PCR details are shown in section 2.11. PCR products were purified and sequenced to verify the sequence at the circular junction.

2.3. Screening of miRNAs targeted by circRNA266

In this study, miRanda was used to predict the miRNAs that can potentially bind to circRNA266, and DIANA (<http://snf-515788.vm.okeanos.grnet.gr/>) was employed to analyze their functions. Target genes were predicted through Targetscan (https://www.targetscan.org/fish_62/) and subjected to KEGG analysis. The expression levels of corresponding miRNAs under different TCS concentrations was then detected.

2.4. Dual-luciferase reporter assay

A mutant of the circRNA266 binding site was synthesized using the targeted gene mutation kit (Takara, Japan). The wild-type (circRNA266-WT) and mutant (circRNA266-MT) sequences were amplified, ligated into the pmirGLO vector using T4 DNA ligase, and transformed to obtain WT-pmirGLO and MT-pmirGLO plasmids. These plasmids were co-injected with miRNA mimics into zebrafish embryos. After 48 hpf, embryos were homogenized using a sterile stick, and the supernatant was collected into a 96-well plate. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). PCR amplification details and sequences of circRNA266-WT and circRNA266-MT are shown in Table S1.

2.5. Sample preparation and exposure experiments

Taking into account environmental concentrations, median lethal concentration, and previously reported LC₅₀ values for TCS (Wang et al., 2021a), three gradient exposure concentrations (62.5, 125, and 250 µg/L) and three environmentally relevant concentrations (2, 10, and 30 µg/L) were selected. High-quality embryos were screened and transferred to Petri dishes for exposure. Each dish contained 80 zebrafish embryos exposed to different TCS concentrations up to 120 hpf, with exposure solutions renewed every 24 h.

As circRNA266 expression was positively correlated with TCS concentration, knockdown experiments were conducted to validate its functional role. Given the synergistic relationship within the circRNA/miRNA network, simultaneous regulation of both components was necessary to evaluate their contribution to TCS-induced neurotoxicity. circRNA266 knockdown was performed via injection of small interfering RNA (siRNA), while target miRNAs were overexpressed or suppressed using microinjection of mimics and inhibitors. These reagents were synthesized by Shanghai Gemma Gene Pharmaceuticals (Shanghai,

China), with sequences listed in Table S2.

2.6. Phenotypic observation

Due to the transparency of zebrafish and the ease of visual monitoring, developmental phenotypes serve as a clear indicator of toxicity. Twenty-four embryos were randomly selected from each treatment group at various developmental stages and photographed under a stereomicroscope (SZX16; Olympus, Japan) following light anesthesia with 80 mg/L MS-222. Subsequently, hatching rate, heart rate, body length, pigmentation level, and the percentage of larvae with uninflated swim bladders were quantified using ImageView software (version 9.0.13657). Neuronal development was assessed using the transgenic zebrafish line Tg(*elavl3*:EGFP), in which neurons are labeled with enhanced green fluorescent protein (EGFP) under the *elavl3* promoter.

2.7. Behavior of zebrafish larvae

Motor behavior, an important indicator of central nervous system function, was assessed by evaluating spontaneous and stimulus-induced movement responses. At 120 hpf, zebrafish larvae from each group were transferred to 96-well plates and placed into the DanioVision observation chamber. For spontaneous movement testing, following a 5-min acclimation, locomotor trajectories were recorded for 5 min in a dark environment using a high-resolution camera. Movement distance and speed were analyzed using EthoVision XT software to evaluate autonomous motor activity. For the acoustic startle response test, an eight-level sound stimulus protocol was applied. After acclimation, movement was recorded for 5 min, and the average swimming speed was used to assess auditory sensitivity and stress responsiveness. For light-dark cycle analysis, alternating light and dark periods of 10 min each were applied for two full cycles (light:dark = 10 min:10 min). After a 5-min acclimation, larval movement was recorded, and speed data were analyzed to evaluate activity changes in response to light-dark transitions.

2.8. Lateral line neural mound observation

The lateral line system is a key sensory organ in zebrafish originating from the epidermis. The lateral line neural mound contains neuromast hair cells, and their loss may result in hearing impairment or vestibular dysfunction. To assess development of this structure, scanning electron microscopy (SEM) was performed. Zebrafish larvae exposed for five days post-fertilization (5 dpf) were collected, 20 larvae per group, and transferred into centrifuge tubes. After repeated rinsing, dehydrated and dried zebrafish samples were mounted onto SEM stubs, and neuromast hair cells of the lateral line were observed under a scanning electron microscope.

2.9. ROS staining

A ROS detection kit (developed in Nanjing) was used to assess reactive oxygen species (ROS) levels in 72 hpf larvae from both control and treated groups. Twenty-three larvae from each group were transferred into separate 1.5 mL centrifuge tubes and incubated with 10 µM of the fluorescent ROS probe DCFH-DA in the dark for 30 min. Following incubation, larvae were washed three times with PBS, anesthetized using 0.03 % MS-222, and observed under an IX73 fluorescence microscope, where images were captured.

2.10. AO apoptosis staining

After exposure until 3 days post-fertilization (dpf), zebrafish larvae were collected and grouped in 1.5 mL centrifuge tubes. Larvae were rinsed twice with 1 mL PBS at low speed. After removing excess PBS, AO apoptosis staining solution was added in the dark. Following 15 min of staining, the solution was removed, and larvae were washed three times

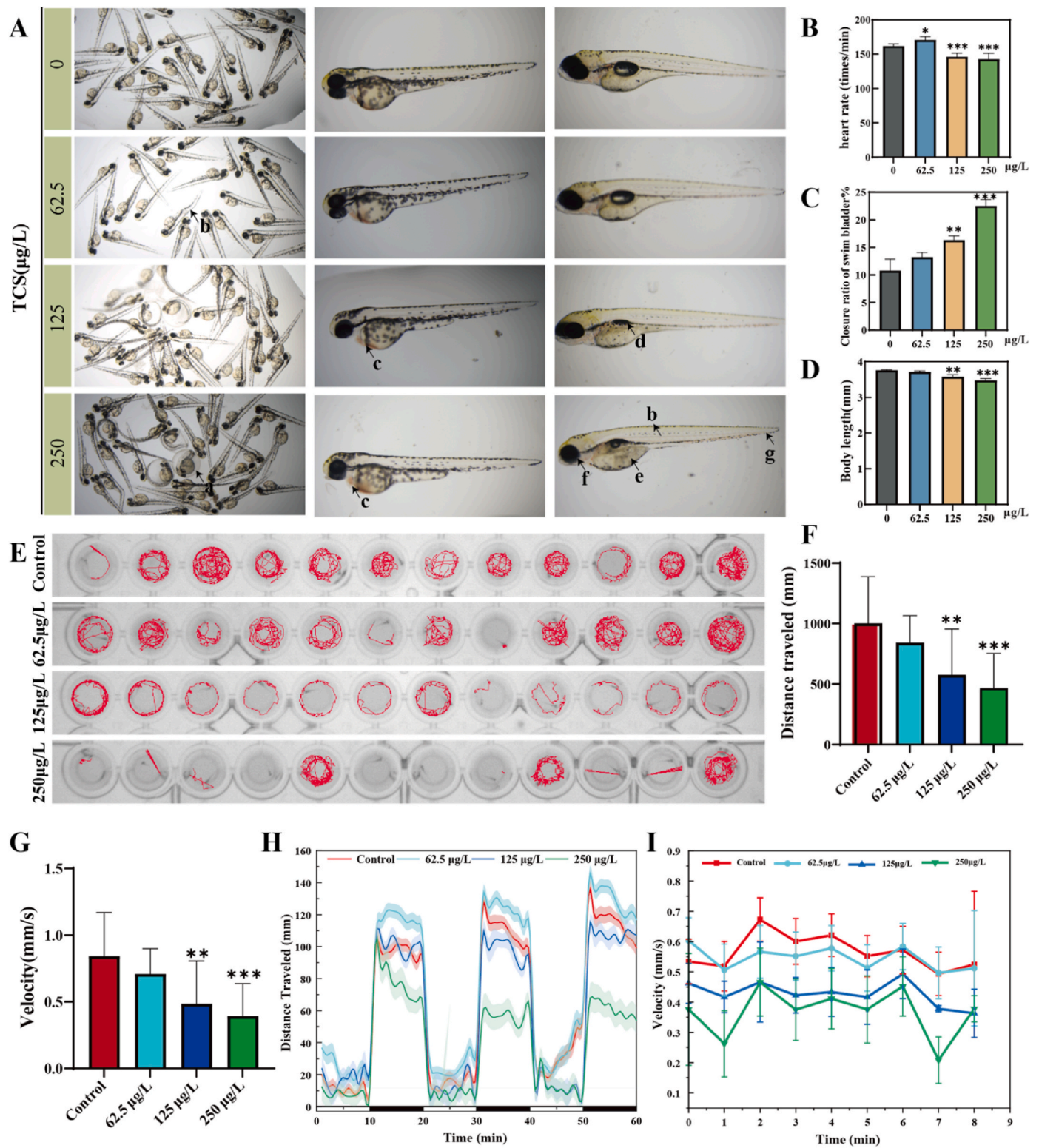


Fig. 1. Developmental status and locomotor behavior of zebrafish under gradient TCS exposure. (A) effects of TCS on the growth and development of zebrafish larvae (a: delayed hatching; b: spinal curvature; c: cardiac bleeding; d: closure of the pouch; e: melanin deposition; f: microphthalmia; g: shortening of body length). (B–D) Three quantitative developmental indices: heart rate, bladder closure ratio, and body length. (E) Autonomous motion trajectory map of zebrafish. (F) The distance of autonomous movement within 20 min. (G) The speed of autonomous movement within 20 min. (H) Response of zebrafish treated with TCS to changes in light and dark; (I) Response of zebrafish treated with TCS to acoustic stimulation. Statistical significance $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), Mean $\pm$ SD.

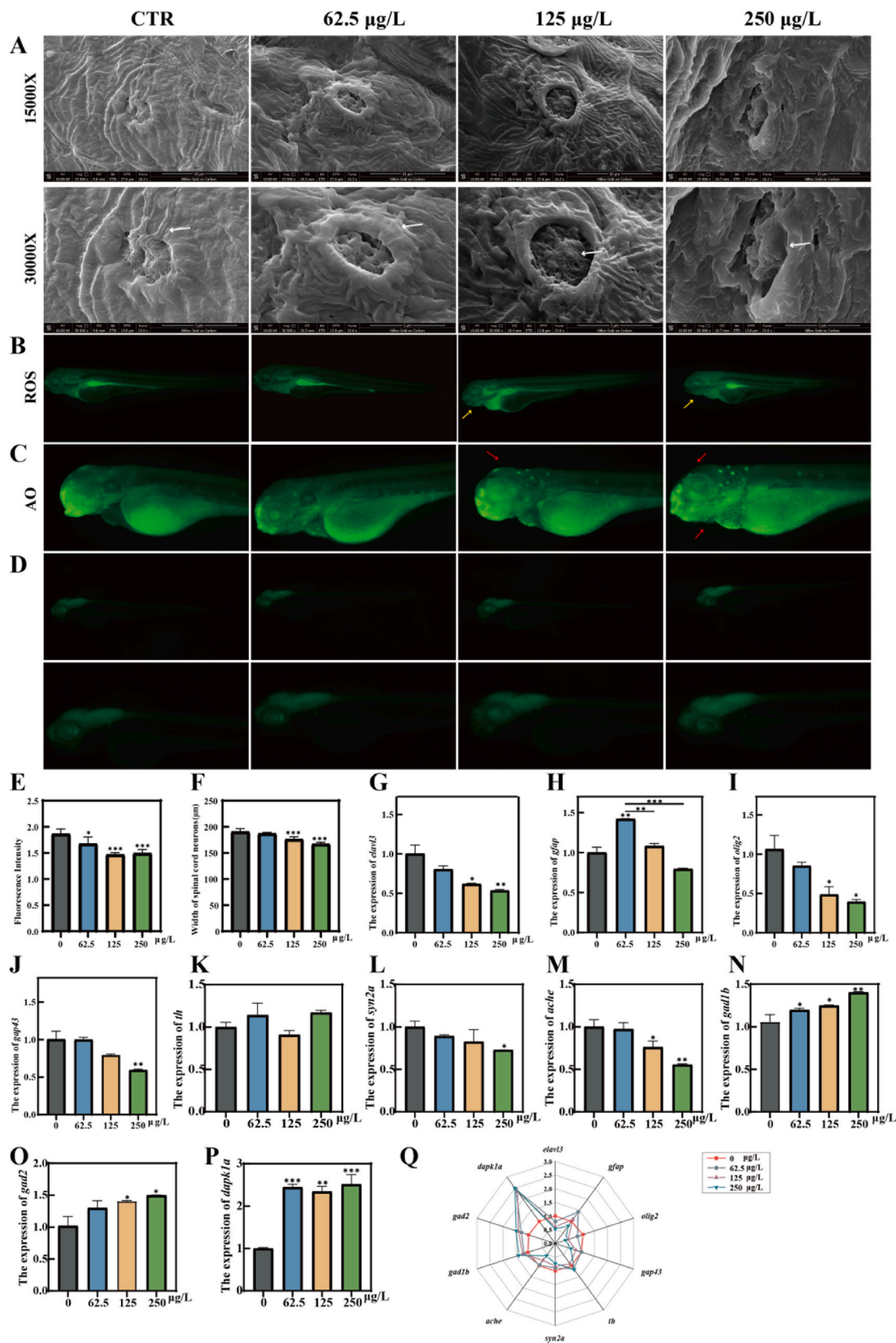


Fig. 2. The neural damage and neuronal development abnormalities in zebrafish under gradient TCS exposure. (A–D) the effect to the neural mound structure, the accumulation of reactive oxygen species, the area of cell apoptosis and Tg (*elavl3*: EGFP) zebrafish neurons. (E) the neuron fluorescence intensity of Tg (*elavl3*: EGFP) zebrafish. (F) the spinal cord neuron width of Tg (*elavl3*: EGFP) zebrafish. (G–J) expression levels of neuronal development marker genes *elavl3*, *gap43*, *gfp*, and *olig2*. (K–P) expression of genes related to neuronal signal transmission including *syn2a*, *ache*, *th*, *dap1a*, *gad2*, and *gad1b*. (Q) radar charts for all genes. Statistical significance $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), Mean $\pm$ SD.

in the dark with PBS. Stained larvae were transferred to glass slides, anesthetized with tricaine (MS-222), and imaged under a stereo fluorescence microscope.

2.11. RT-qPCR

Total RNA from each experimental group was extracted using ET101 TransZol reagent (Transgen, China). RNA was quantified and reverse-transcribed using the RR047A PrimeScript™ RT reagent kit with gDNA Eraser (Takara, China). Specific primers were used for RT-qPCR on a Bio-Rad CFX96 instrument with AQ141 TransStart Tip Green qPCR Supermix (Shanghai, China). All qPCR reactions were conducted in triplicate, with 20 larvae per replicate. The PCR reaction system is detailed in Table S3. Target genes included four neural development markers (*elavl3*, *gfap*, *olig2*, and *gap43*), neurotransmitter-related genes (*th*, *syn2a*, *ache*, *gad1b*, *gad2*), and death-associated protein kinases (*dapk1a*, *dapk2*).

2.12. Molecular docking

circRNAs are produced from pre-mRNA via alternative splicing and are highly conserved (Liu et al., 2019c). across species. Components of the spliceosome play a critical role in maintaining the balance between circular and linear RNA isoforms (Liang et al., 2017). To explore possible initiating events, 44 splicing-related proteins were selected for molecular docking analysis. These included spliceosomal complexes (U1, U2, U4, U5, U6 snRNPs), the U2 snRNP auxiliary factor (U2AF), serine/arginine-rich (SR) proteins, additional SR proteins, and proteins involved in splice site selection. The 3D structures of the candidate target proteins were predicted using AlphaFold (<https://alphafold.ebi.ac.uk/>). AutoDock software was employed to simulate the binding patterns of TCS with each target protein. From the top 10 docking conformations, the optimal binding pose was selected, and PyMOL 1.7.6 (www.PyMOL.org) was used for molecular visualization.

2.13. Statistical analysis

For each treatment group, three biological and technical replicates were conducted. Data are expressed as mean $\pm$ standard deviation (SD). Biological and technical replicates and specific sample sizes for each test are detailed in Table S4. One-way analysis of variance (ANOVA) was used for statistical evaluation. Significance levels were defined as $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

3. Results and discussion

3.1. Acute exposure to TCS induces neurotoxicity in zebrafish

3.1.1. Developmental abnormalities induced by TCS concentration gradient

This study first investigated the neurotoxic effects of TCS on zebrafish embryonic development. Different TCS concentrations elicited varying toxic effects on hatching rates and phenotypic abnormalities (Fig. 1A). At 62.5 $\mu\text{g/L}$, zebrafish exhibited a slight decrease in hatching rate and pigmentation. Higher concentrations (125 $\mu\text{g/L}$ and 250 $\mu\text{g/L}$) significantly reduced hatching rates after 72 h, accompanied by pigment loss, cardiac hemorrhage, spinal curvature, and delayed swim bladder development.

Further quantitative analyses assessed heart rate, swim bladder inflation (closure ratio), and body length at 120 hpf (Fig. 1B–D). At 62.5 $\mu\text{g/L}$, heart rate increased significantly ($P < 0.05$), while closure ratio and body length showed slight but non-significant reductions. At 125 $\mu\text{g/L}$ and 250 $\mu\text{g/L}$, both heart rate and body length were significantly decreased. These findings suggest that TCS impairs zebrafish development in a concentration-dependent manner.

3.1.2. Behavioral abnormalities induced by TCS concentration gradient

To further evaluate the neurotoxic effects of TCS, behavioral analyses were conducted. As shown in Fig. 1E, zebrafish larvae exposed to 62.5 $\mu\text{g/L}$ TCS exhibited movement patterns similar to controls. However, higher concentrations (125 $\mu\text{g/L}$ and 250 $\mu\text{g/L}$) led to irregular movement trajectories, reduced turning behavior, and diminished exploratory activity. Quantitative analysis confirmed that TCS exposure caused a concentration-dependent reduction in movement distance and speed (Fig. 1F and G). While 62.5 $\mu\text{g/L}$ showed no significant change, 125 $\mu\text{g/L}$ and 250 $\mu\text{g/L}$ resulted in significant reductions ($P < 0.01$).

In the light–dark transition assay (Fig. 1H), larvae exposed to 62.5 $\mu\text{g/L}$ showed heightened sensitivity, characterized by increased speed and elevated stress responses, possibly due to low-dose neural stimulation. In contrast, high-concentration exposure diminished sensitivity. A similar trend was observed in the acoustic stimulation test (Fig. 1I), where larvae at 62.5 $\mu\text{g/L}$ showed increased maximum velocity in response to sound, while those at 125 $\mu\text{g/L}$ and 250 $\mu\text{g/L}$ exhibited impaired responses. These findings indicate that TCS suppresses locomotor activity and reduces sensory responsiveness, likely due to disruption of nervous system function.

3.1.3. Nervous system damage caused by TCS concentration gradient

As shown in previous results, TCS impairs motor and sensory function in zebrafish. The lateral line system—a crucial sensory structure—detects water flow, pressure, and temperature and converts mechanical stimuli into neural signals. The lateral colliculus contains neuromast hair cells essential for auditory and vestibular function, and their loss can lead to sensory deficits. Scanning electron microscopy revealed well-formed neuromasts in control and low-dose (62.5 $\mu\text{g/L}$) groups, with dense hair cell clusters and intact structures (Fig. 2A). However, higher concentrations caused visible deformation and hair cell loss, suggesting TCS-induced sensory impairment.

Oxidative stress is a key factor in neurodegenerative processes (Shefa et al., 2019). ROS and AO staining were used to evaluate oxidative stress and apoptosis in TCS-exposed embryos. TCS significantly increased ROS accumulation in the head, trunk, and heart in a concentration-dependent manner (Fig. 2B). Similarly, AO staining showed limited apoptosis in control and low-dose groups but extensive apoptosis in the head and heart at 125 $\mu\text{g/L}$ and 250 $\mu\text{g/L}$, indicated by dense green fluorescence (Fig. 2C). These findings confirm that TCS induces oxidative stress and apoptosis, contributing to neural damage.

Transgenic zebrafish Tg(*elavl3*:EGFP) were used to assess neuronal development. In the control group, strong fluorescence was observed in the brain with clear diencephalon–hindbrain boundaries (Fig. 2D). In contrast, high-concentration groups exhibited disrupted neuronal structures and diminished fluorescence. Quantitative analysis (Fig. 2E and F) showed significantly reduced fluorescence intensity and spinal cord width ($P < 0.001$), suggesting inhibited neurogenesis. These results demonstrate that TCS impairs nervous system development in a dose-dependent manner.

3.1.4. Neural marker genes altered by TCS concentration gradient

To investigate the molecular mechanisms underlying TCS-induced neurotoxicity, we analyzed the expression of neural marker genes. Genes associated with neurons (*elavl3*), oligodendrocytes (*olig2*), astrocytes (*gfap*), and axon growth (*gap43*) were assessed (Fig. 2G–J, Q). Expression of *elavl3*, *olig2*, and *gap43* decreased with increasing TCS concentrations, indicating suppression of neuronal differentiation, axonal growth, and oligodendrocyte development. *gfap* exhibited a biphasic (hormetic) response—altered at low concentrations but downregulated overall at higher doses—suggesting concentration-specific effects on astrocyte regulation.

To further explore neurotransmission and cell survival, we examined neurotransmitter-related genes (*th*, *syn2a*, *ache*, *gad1b*, *gad2*) and cell death-associated kinases (*dapk1a*, *dapk2*) (Fig. 2K–P, Q). *syn2a* and *ache* were significantly downregulated, indicating impaired neurotransmitter

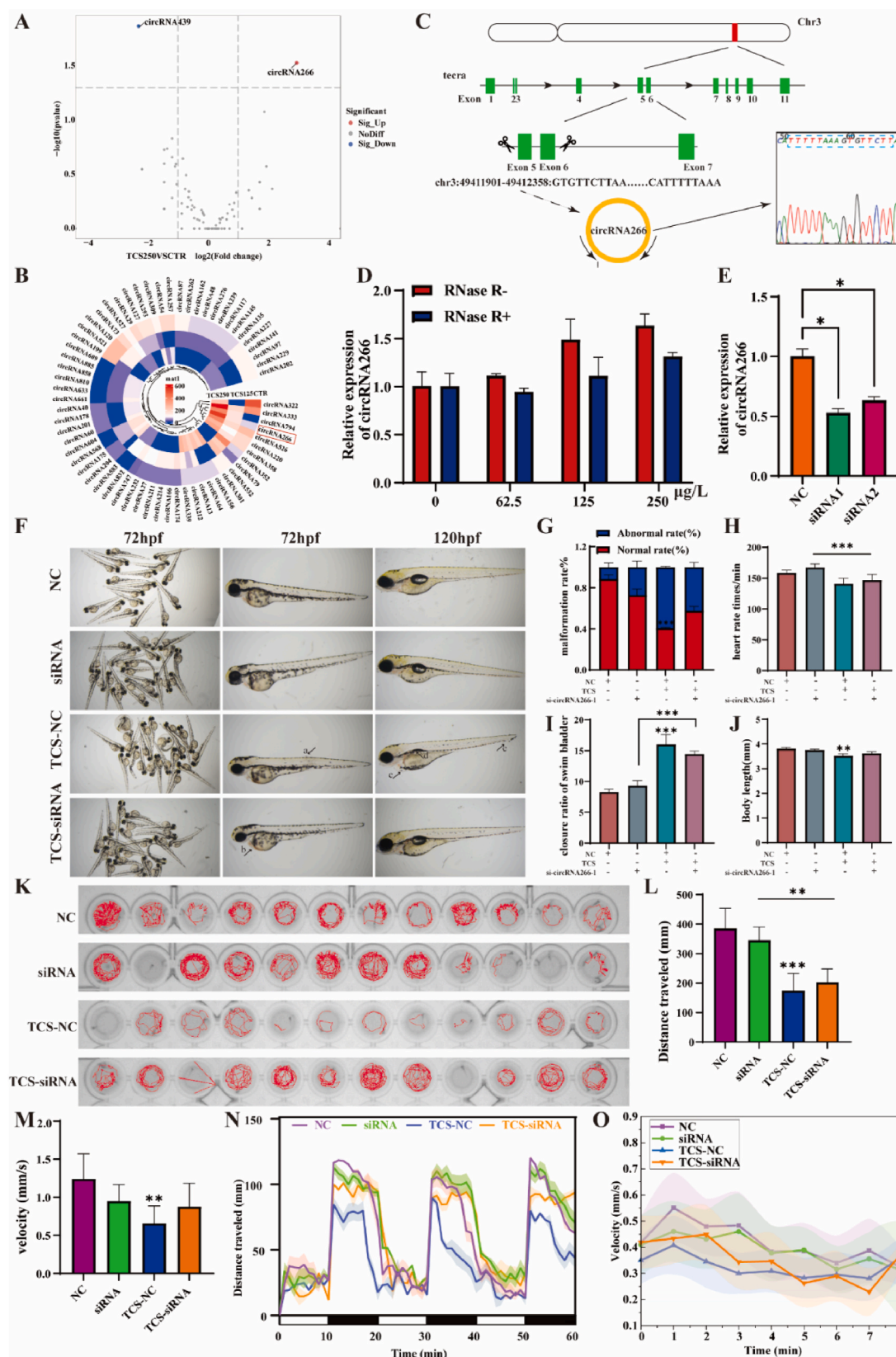


Fig. 3. TCS induces neurotoxicity toxicity and behavioral abnormalities in zebrafish by upregulating circRNA266. Notes: (A) Volcanic map of DE circRNAs. (B) Heat map of DE circRNAs. (C) The formation mechanism of circRNA266. (D) Cyclic structures verification of circRNA266. (E) Knockdown efficiency of siRNAs on circRNA266. (F) Observation on the phenotype of embryos. (G–J) Four quantitative developmental indices: malformation rate, heart rate times, closure ratio of swim bladder and body length. (K) Autonomous locomotor trajectories of zebrafish. (L–M) The quantification of moving distance and speed. (N) Distance traveled in light-dark rhythmic. (O) Sensitivity of Zebrafish to Sound Stimulation. Statistical significance: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***), Mean $\pm$ SEM.

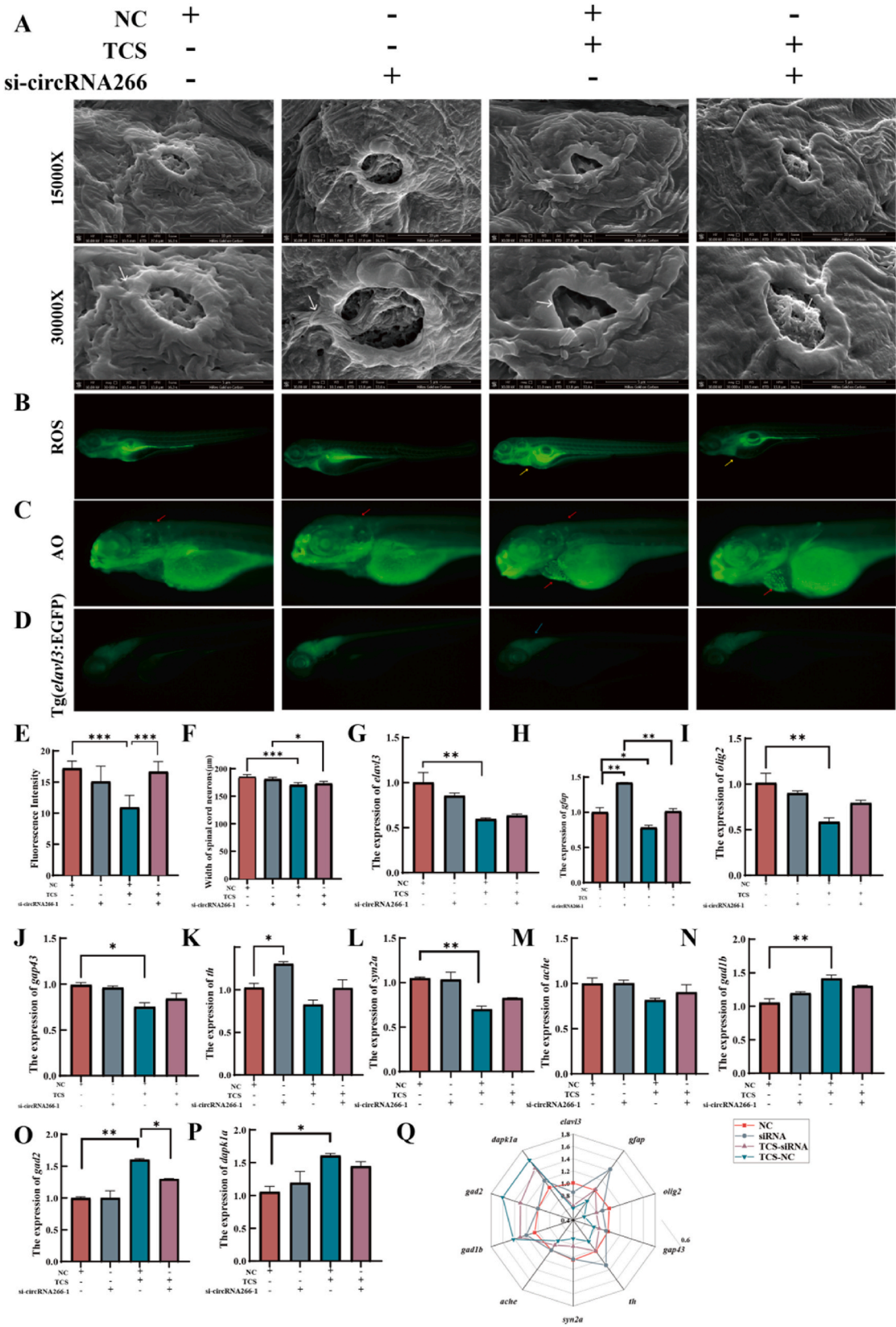


Fig. 4. TCS induces nervous system damage and alters relative expression of neural marker genes in zebrafish by upregulating circRNA266. Notes: (A–D) The Observation on neural mounds, ROS, apoptosis, and Tg(*elavl3*:EGFP). (E) The quantitative fluorescence intensity of positively expressed *elavl3*. (F) The quantitative width of spinal cord neurons. (G–J) Relative expression of fourbiomarkers related to nerve cell development. (K–M) Relative expression of three biomarkers related to neurotransmitter-related genes. (N–O) Relative expression of two biomarkers related to inhibitory neurotransmitters. (P) Gene biomarker related to death-associated protein kinases. (Q) Radar statistical chart of gene markers. Statistical significance: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***), Mean $\pm$ SEM.

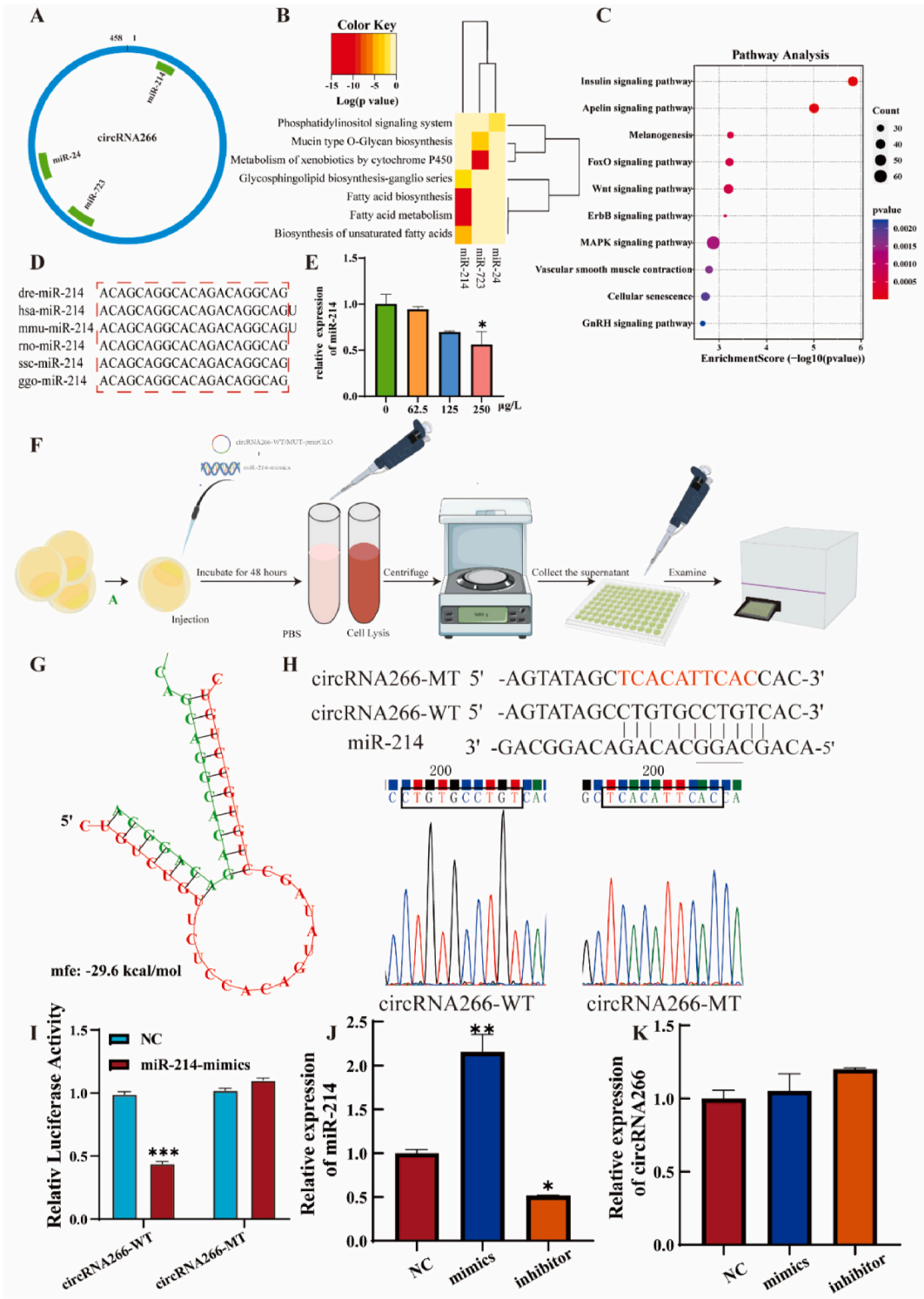


Fig. 5. Analysis of the regulatory relationship between circRNA and its target miRNA. Notes: (A) The binding sites of the three target miRNAs on circRNA266. (B) Cluster heatmaps of three target miRNAs. (C) KEGG analysis of miR-214 target genes. (D) Conservative comparison of miR-214 among different species. (E) Relative expression of miR-214 under different concentrations of TCS exposure. (F) The experimental process of dual luciferase experiment. (G) Schematic diagram of the binding of circRNA266 and miR-214. (H) Key sequences of circRNA266-WT and circRNA266-MT. (I) The result of the dual luciferase experiment. (J–K) Relative expression of miR-214 and circRNA266 after mimics and inhibitor injected. Statistical significance: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***), Mean $\pm$ SEM.

release and degradation. The expression remained relatively unchanged. *gad1b* and *gad2* were upregulated, suggesting increased synthesis of the inhibitory neurotransmitter GABA. In parallel, *dapk1a* and *dapk2* expression levels were significantly elevated, indicating enhanced apoptotic activity consistent with AO staining results. Collectively, these

findings demonstrate that TCS disrupts neural gene expression, neurotransmission, and neuronal viability in a concentration-dependent manner.

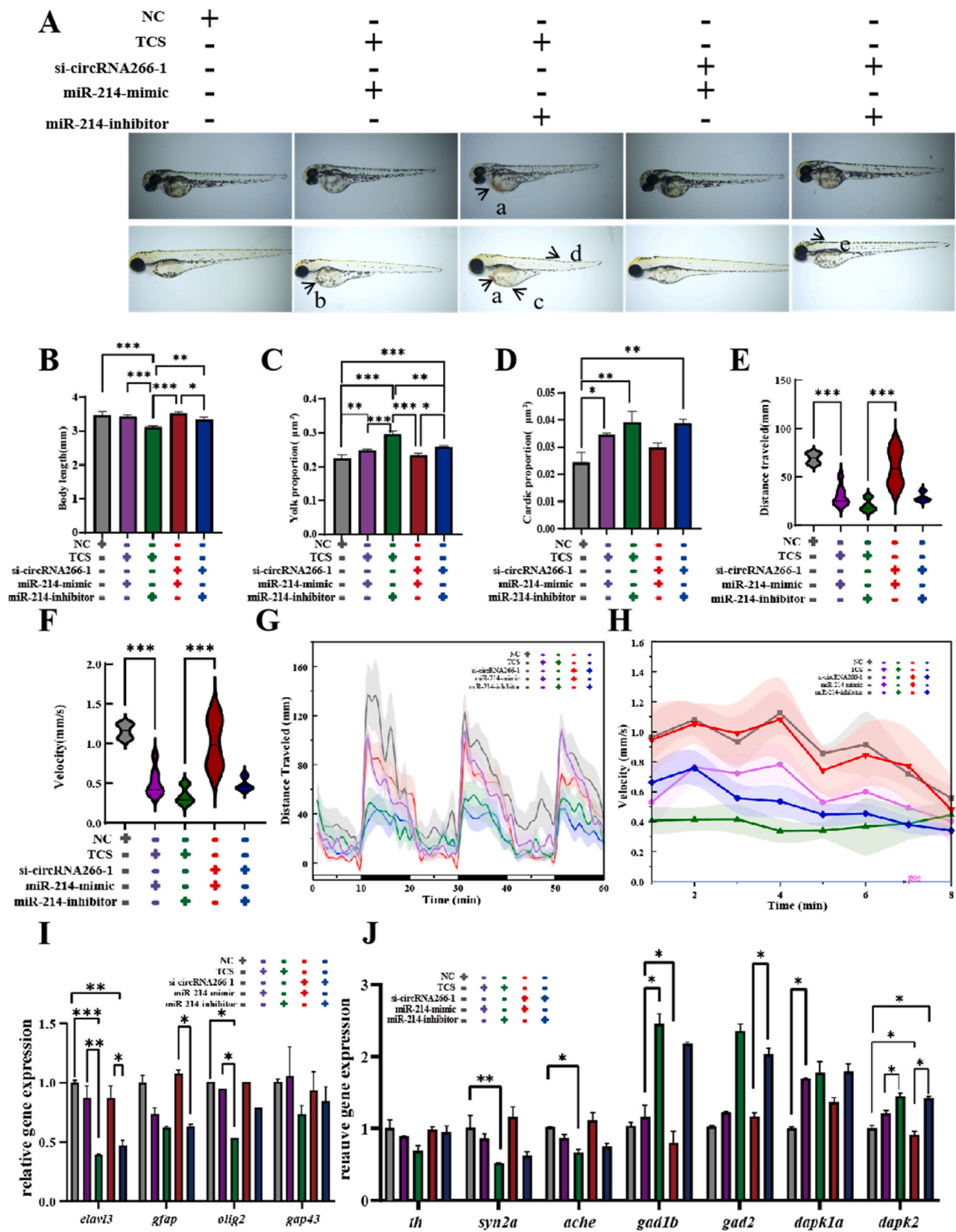


Fig. 6. TCS induces neurotoxicity in zebrafish by the circRNA266/miR-214 regulatory network. (A) Observation on the phenotype of embryos(a: cardiac bleeding; b: pericardial edema; c: yolk cyst; d: body length shorten; e: abnormal pigmentation). (B–D) Three quantitative developmental indices: body length, yolk proportion, and heart area. (E–F) The quantification of moving distance and speed under autonomous locomotor state. (G) Distance traveled in light-dark rhythmic. (H) The velocity in acoustic stimulation. (I–J) The gene biomarkers related to nerves. Statistical significance: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***), Mean $\pm$ SEM.

3.2. TCS induces neurotoxicity in zebrafish through the circRNA266/miR-214 network

3.2.1. CircRNA266 upregulation is a key event for TCS-induced neurotoxicity in zebrafish

This study demonstrates that TCS disrupts neuronal development and induces neurobehavioral abnormalities in zebrafish. Our previous research showed that TCS alters circRNA expression, potentially contributing to neurotoxicity, cardiotoxicity, and lipid metabolism disorders via miRNA adsorption (Wang et al., 2024b). High-throughput sequencing identified circRNA266 as significantly upregulated and circRNA439 as downregulated at 250 µg/L TCS (Fig. 3A). Among 29 key circRNAs, only circRNA266 exhibited a dose-dependent increase (Fig. 3B), indicating its potential role in TCS-induced neurotoxicity.

CircRNA266 is derived from exons 5 and 6 of the *tecra* gene (chr3:49411901–49412358, 458 bp) and is generated through reverse splicing (Fig. 3C). Sanger sequencing confirmed the back-splice junction within its sequence. RNase R digestion experiments (Fig. 3D) showed that circRNA266 resisted degradation, indicating a stable circular structure across different TCS exposure levels. These results highlight circRNA266 as a key molecular event in TCS-induced neurotoxicity.

3.2.2. TCS causes neurotoxicity in zebrafish by inducing upregulation of circRNA266 expression

Epigenetic assessments (Fig. 3F) showed normal development in the NC and siRNA groups, while zebrafish exposed to TCS (TCS + NC) displayed severe abnormalities, including delayed hatching, pericardial edema, uninflated swim bladder, reduced eye size, and shortened body length. Knockdown of circRNA266 (TCS + siRNA) alleviated these defects, improving growth and reducing deformities such as spinal curvature and swim bladder malformation. Quantitative analysis (Fig. 3G–J) confirmed that circRNA266 knockdown significantly improved developmental indices.

Thermographic tracking of locomotor behavior (Fig. 3K) revealed that larvae in the NC and siRNA groups maintained dense, regular swimming patterns, whereas TCS exposure resulted in sparse, irregular movements. CircRNA266 knockdown restored swimming activity, increasing both distance and speed (Fig. 3L and M). In the light–dark cycle (Fig. 3N) and acoustic stimulation experiments (Fig. 3O), TCS exposure diminished responsiveness, while circRNA266 knockdown reinstated normal behavioral responses, including increased movement in darkness and enhanced reaction speed after auditory stimuli. These findings suggest that circRNA266 modulates TCS-induced sensory impairments, particularly in auditory and perceptual processing.

Scanning electron microscopy was used to evaluate the development of zebrafish neural mounds (Fig. 4A). In the NC and siRNA groups, neural structures were intact, with abundant, organized hair cell bundles. In contrast, TCS exposure (TCS + NC) led to severe damage, reducing hair cells and narrowing neural mound openings. CircRNA266 knockdown under TCS exposure (TCS + siRNA) partially restored these structures. AO and ROS staining (Fig. 4B and C) showed that TCS-induced apoptosis and oxidative stress in brain, heart, and eye tissues were significantly alleviated by circRNA266 knockdown. In Tg(*elavl3*:EGFP) zebrafish, TCS reduced neuronal density and disrupted midbrain–hindbrain boundaries (Fig. 4D), while circRNA266 knockdown restored neuronal integrity, increasing *elavl3* expression and spinal cord width (Fig. 4E and F). These findings identify circRNA266 as a critical regulator of TCS-induced neurodevelopmental impairment.

Gene expression analysis revealed that circRNA266 knockdown slightly decreased *elavl3*, *olig2*, and *gap43* levels while significantly increasing *gfap* expression under normal conditions (Fig. 4G–J). Under TCS exposure, neurodevelopmental genes were downregulated, but circRNA266 knockdown partially reversed this effect, restoring *gfap* to NC levels. Additionally, circRNA266 knockdown upregulated neurotransmitter-related genes (*ache*, *th*, *syn2a*) and downregulated apoptosis- and GABA-related genes (*dapk1a*, *gad2*, *gad1b*) (Fig. 4K–Q),

suggesting its role in modulating neuronal signaling and survival. Collectively, these data support the involvement of circRNA266 in mediating TCS-induced neurotoxicity.

3.2.3. Screening and bioinformatic analysis of miRNAs targeted by circRNA266

Bioinformatic predictions identified miR-214, miR-24, and miR-723 as top candidate miRNAs interacting with circRNA266 (Fig. 5A). Enrichment analysis suggested involvement in lipid metabolism, cytochrome P450 pathways, mucin-type O-glycan biosynthesis, and sphingolipid metabolism. miR-214 showed the highest enrichment in the sphingolipid pathway, implicating it in circRNA266-mediated neurotoxicity (Fig. 5B). KEGG pathway analysis (Fig. 5C) revealed that miR-214 participates in Apelin, Wnt, FoxO, MAPK, and melanogenesis signaling, with MAPK being the most enriched. miR-214 is highly expressed in the heart, cerebellum, and midbrain, is evolutionarily conserved, and plays key roles in muscle coordination, neurodevelopment, and cell cycle regulation (Fig. 5D).

RT-qPCR analysis (Fig. 5E) demonstrated that miR-214 expression decreased with increasing TCS concentrations, inversely correlated with circRNA266 expression. Dual-luciferase reporter assays (Fig. 5F–I) confirmed direct binding between circRNA266 and miR-214. Further knockdown and overexpression experiments (Fig. 5J and K) revealed that circRNA266 unidirectionally regulates miR-214, supporting its role as a downstream target.

3.2.4. Effects of the circRNA266/miR-214 network on TCS-induced neurotoxicity

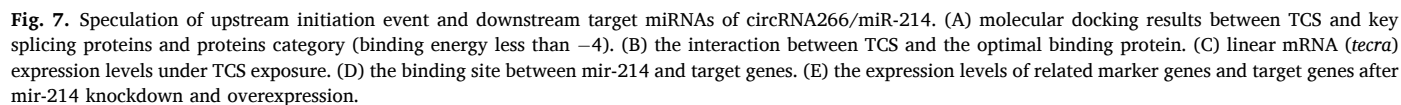
Simultaneous upregulation of circRNA266 and downregulation of miR-214 resulted in the most severe developmental deformities, while the reverse—circRNA266 knockdown with miR-214 overexpression—led to the least morphological defects (Fig. 6A). Intermediate malformations were observed in the other two groups. This trend was confirmed by quantitative measurements of body length (Fig. 6B), yolk sac area (Fig. 6C), and heart area (Fig. 6D), where the most severe impairments were observed in the circRNA266-up/miR-214-down group.

Behavioral data showed that circRNA266 upregulation and miR-214 downregulation had the strongest negative effects on swimming distance and speed (Fig. 6E and F). In contrast, circRNA266 knockdown and miR-214 overexpression restored movement. Similar improvements were observed in light–dark (Fig. 6G) and sound stimulation assays (Fig. 6H), reinforcing the role of the circRNA266/miR-214 axis in neurobehavioral regulation. Additional experiments on neural mound development (Fig. S1) showed that the circRNA266-up/miR-214-down group exhibited the most structural abnormalities, including narrow openings, protrusions, and underdeveloped cilia. These features aligned with AO and ROS staining results, confirming increased apoptosis and oxidative stress in this group.

Expression analysis of neural marker genes (Fig. 6I and J) showed that the circRNA266-up/miR-214-down group exhibited the greatest suppression of genes involved in neuronal development (*elavl3*, *gfap*, *gap43*, *olig2*) and neurotransmission (*ache*, *syn2a*, *th*), along with the highest expression of GABA-related (*gad1b*, *gad2*) and apoptotic markers (*dapk1a*, *dapk2*). In contrast, circRNA266 knockdown and miR-214 overexpression restored expression levels toward those of controls. These results demonstrate that TCS disrupts zebrafish neurodevelopment via circRNA266 upregulation and miR-214 downregulation, highlighting the mechanistic significance of this regulatory axis in neurotoxicity.

3.3. A new mechanism of TCS-induced neurotoxicity: circRNA266/miR-214-mediated AOP network

Aberrant pre-mRNA splicing has been implicated in the pathogenesis of various human diseases, including hematological malignancies and



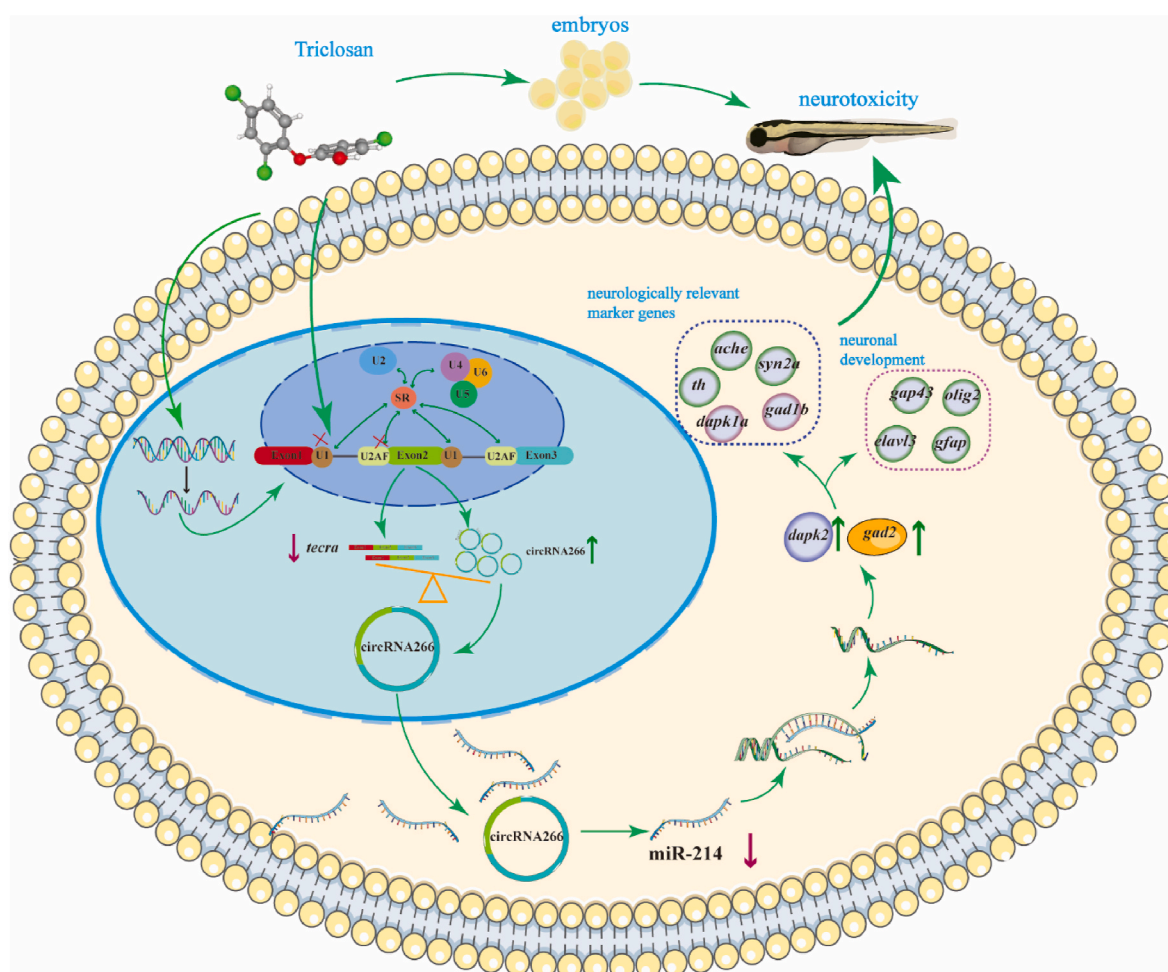


Fig. 8. The mechanism of TCS-induced neurotoxicity in zebrafish through a ceRNA regulation network based on circRNA266.

neurological disorders (Hsu et al., 2015). Pharmacological inhibition or depletion of spliceosomal components has been shown to increase circular RNA (circRNA) levels while decreasing the expression of corresponding linear mRNAs (Liang et al., 2017). The observed increase in circRNA266 is closely associated with the splicing of the *tecra* gene, where spliceosome components play a key regulatory role. Molecular docking analysis identified the U2 complex—particularly U2AF1—as the most susceptible target for TCS binding (Fig. 7A). The U2 complex, composed of U2 snRNA and associated proteins, recognizes the branchpoint sequence of pre-mRNA, triggering conformational changes that facilitate intron excision and exon ligation during spliceosome assembly. U2AF1 specifically binds the 3' splice site and is essential for constitutive and enhancer-dependent splicing. Fig. 7B shows stable hydrophobic interactions between U2AF1 and TCS. U2AF1 is known to regulate splicing patterns in both in vivo and in vitro systems, with particular relevance in hematological disorders such as myelodysplastic syndrome (Li et al., 2019c). Moreover, the increase in circRNA266 was accompanied by a decrease in linear transcript levels of the same locus (Fig. 7C), indicating that TCS alters *tecra* gene splicing through enhanced U2AF1 activity, thereby increasing circRNA266 and reducing its linear isoforms.

While TECRA has been linked to evolutionary processes and lipid metabolism (Gaudet et al., 2011), the functions of circRNA266 remain poorly understood. This study reveals a novel neuromodulatory role for circRNA266, potentially via its function as a MRE that sequesters miR-214. Following TCS exposure, circRNA266 is upregulated and miR-214 is concurrently downregulated. Notably, miR-214 is enriched in pathways related to lipid synthesis and glycosphingolipid

biosynthesis (Fig. 5B and C)—both of which are vital for neural development. As a lipid-rich organ, the brain relies on tightly regulated lipid metabolism, and disruptions are implicated in neurodegenerative diseases such as Parkinson's and Alzheimer's (Luo et al., 2024). Abnormal glycosphingolipid metabolism also impairs neuronal development (Wang et al., 2024c; Russo et al., 2018). miR-214 has also been implicated in several cancers, including melanoma, glioma, and hepatocellular carcinoma (Wang et al., 2022; Sun et al., 2019; Sharma et al., 2015) and has demonstrated neuroprotective effects in mouse and human neuroblastoma cell lines (Wang et al., 2015; Wu et al., 2019; Liu et al., 2022; Zhang et al., 2024). While its roles in zebrafish have largely focused on cartilage, muscle, and pigmentation (Roberto et al., 2018; Samman et al., 2025), our findings extend its relevance to neural protection.

Target prediction and validation indicated that miR-214 regulates key genes within the MAPK and Wnt signaling pathways. MAPK signaling, one of the most conserved cascades, regulates over 100 biological processes, including neuronal injury repair and axonal regeneration (Shiraki and Shimizu, 2023; Wang and Wang, 2020). Similarly, Wnt signaling governs cell proliferation, migration, and differentiation, and is essential for embryonic and neural crest development (Ding and Wang, 2017). Notably, *gad2* and *dapk2* were identified as direct targets of miR-214. Functional assays confirmed that miR-214 overexpression suppresses *gad2* and *dapk2* expression (Fig. 7D and E), thereby modulating neuronal apoptosis, proliferation, and neurotransmission. The *dapk2* encodes a kinase involved in apoptotic regulation (Chang et al., 2023), while *gad2* marks GABAergic neurons (Mu et al., 2023). These results indicate that TCS modulates *gad2* and *dapk2* via the

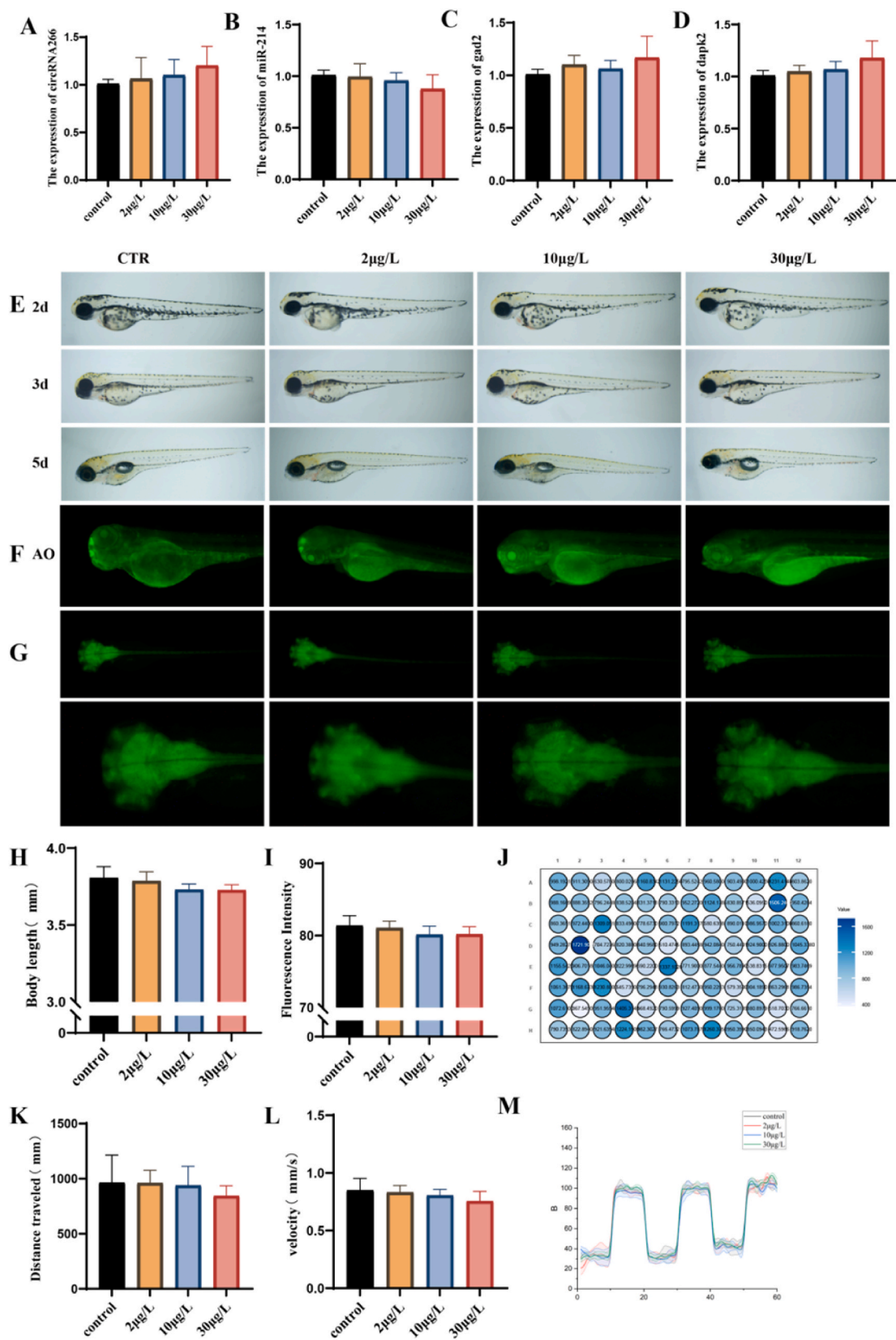


Fig. 9. Developmental status and motility behavior of zebrafish exposed to environmental concentration TCS. (A). Expression level of *circRNA266* under TCS exposure. (B). Expression level of *miR-214* under TCS exposure. (C). Expression level of *gad2* under TCS exposure. (D). Expression level of *dapk2* under TCS exposure. (E). Effects of TCS Exposure on the Growth and Development of Juvenile Zebrafish. (F). Phenotype of head apoptosis in juvenile fish exposed to TCS. (G). Representative image of 72 hpf TG (*elavl3*: EGFP) transgenic zebrafish exposed to TCS. (H). Quantitative results of zebrafish body length under TCS exposure on the fifth day. (I). Quantitative results of fluorescence intensity in the brain of TG (*elavl3*: EGFP) transgenic zebrafish. (J). Spontaneous swimming distance heatmap of 120 hpf

zebrafish fry exposed to TCS. (K–L): 20 min autonomous movement distance and movement speed. (M). The response of zebrafish to light dark changes under TCS exposure.

circRNA266/miR-214 axis, disrupting neurogenesis, apoptosis, and synaptic function in the zebrafish lateral nervous system. GABA, an inhibitory neurotransmitter, dampens neuronal excitability by binding specific receptors and blocking synaptic transmission (Li et al., 2023b). The combined effects of GABAergic dysregulation and increased apoptosis likely underlie reduced responsiveness to sensory stimuli, including light-dark transitions and acoustic cues.

Based on these findings, we propose a mechanistic model for TCS-induced neurotoxicity (Fig. 8) and present a corresponding AOP network (Fig. S2). TCS modulates circRNA266/miR-214 signaling, leading to disrupted neuronal proliferation, apoptosis, and synaptic transmission, ultimately impairing lateral line system function. As a sensory structure vital for auditory and vestibular perception, the zebrafish lateral line relies on neural hair cells. Dysfunction in this system, coupled with GABAergic signaling imbalance, may account for the observed reductions in responsiveness to environmental stimuli.

3.4. Environmental concentrations of TCS have the potential to induce neurotoxicity via circRNA266/miR-214-mediated AOP network

The above findings describe a novel mechanism of TCS-induced neurotoxicity in zebrafish at high concentrations. Building on this, we investigated whether environmentally relevant concentrations of TCS could similarly activate this neurotoxic mechanism.

We first examined the expression levels of circRNA266 and miR-214 following exposure to environmentally relevant concentrations of TCS (Fig. 9A and B). As TCS concentration increased, circRNA266 expression was upregulated, while miR-214 levels declined. Notably, the expression of *gad2* (associated with inhibitory neurotransmission) and *dapk2* (involved in apoptosis)—both validated downstream targets of miR-214—was negatively correlated with miR-214 expression (Fig. 9C and D). These results indicate that even at low, environmentally realistic concentrations, TCS activates the circRNA266/miR-214 axis and modulates the expression of its downstream targets.

To assess toxicological effects at the organismal level, multiple phenotypic and behavioral endpoints were evaluated. Morphological analysis revealed no significant differences in body length between TCS-exposed and control groups (Fig. 9E), although a non-significant downward trend was observed (Fig. 9H). Similarly, AO staining and neuronal morphology (Tg(*elavl3*:EGFP)) showed no significant structural changes (Fig. 9F and G). Quantification of brain fluorescence intensity revealed a slight, non-significant decline with increasing TCS concentration (Fig. 9I). Behavioral analyses—including spontaneous locomotion and responses to light–dark transitions—also showed no statistically significant alterations (Fig. 9J–M). Although swimming distance and velocity were reduced in TCS-exposed groups, these decreases were not statistically significant (Fig. 9K and L).

In contrast to the pronounced neurotoxicity observed at high TCS concentrations, environmentally relevant exposure did not induce observable neurotoxic effects at phenotypic or behavioral levels. However, activation of key molecular initiating events within the adverse outcome pathway (AOP) framework suggests a latent neurotoxic risk at environmental concentrations.

Importantly, phenolic environmental contaminants structurally similar to TCS—such as bisphenol A (BPA), its analogs, and parabens (Sakhi et al., 2018)—are ubiquitously present in aquatic environments. Given the high binding affinity observed between TCS and the splicing factor U2AF1 in molecular docking studies, it is plausible that other structurally related phenolics may also target U2AF1 or homologous splicing regulators. Moreover, accumulating evidence indicates that bisphenol-type pollutants, including BPA, disrupt miRNA expression profiles (Ramírez et al., 2021). A key question remains: does miRNA

dysregulation result from phenolic contaminant-induced splicing interference that alters circRNA expression? If this regulatory axis is confirmed as a common mechanism among phenolic compounds, it would provide a molecular rationale for developing circRNA/miRNA-based biomarkers for early detection of neurotoxicity and support the refinement of environmental risk assessment models for these contaminants.

CRedit authorship contribution statement

Zejun Wang: Writing – review & editing, Methodology, Conceptualization. **Zhuoya Dai:** Writing – original draft, Data curation. **Xiaowen Han:** Visualization, Software, Formal analysis. **Xincong Su:** Data curation. **Xiao Yang:** Methodology, Formal analysis. **Xuedong Wang:** Visualization, Supervision, Investigation. **Jin Yan:** Visualization, Supervision, Investigation. **Qiuhui Qian:** Project administration, Data curation. **Huili Wang:** Visualization, Supervision, Investigation. **Randy A. Dahlgren:** Writing – review & editing.

Ethics approval

This study was approved by the Ethics Committee of Suzhou University of Science and Technology, Suzhou, China.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2025.122398>.

Data availability

Data will be made available on request.

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