Embryonic exposure to fentanyl induces behavioral changes and neurotoxicity in zebrafish larvae

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The use of fentanyl during pregnancy, whether by prescription or illicit use, may result in high blood levels that pose an early risk to fetal development. However, little is known regarding the neurotoxicity that might arise from excessive fentanyl exposure in growing organisms, particularly drug-related withdrawal symptoms. In this study, zebrafish embryos were exposed to fentanyl solutions (0.1, 1, and 5 mg/L) for 5 days post fertilization (dpf), followed by a 5-day recovery period, and then the larvae were evaluated for photomotor response, anxiety behavior, shoaling behavior, aggression, social preference, and sensitization behavior. Fentanyl solutions at 1 and 5 mg/L induced elevated anxiety, decreased social preference and aggressiveness, and behavioral sensitization in zebrafish larvae. The expression of genes revealed that embryonic exposure to fentanyl caused substantial alterations in neural activity (*bdnf*, *c-fos*) and neuronal development and plasticity (*npas4a*, *egr1*, *btg2*, *ier2a*, *vgf*). These results suggest that fentanyl exposure during embryonic development is neurotoxic, highlighting the importance of zebrafish as an aquatic species in research on the neurobehavioral effects of opioids in vertebrates.

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16 ## Abstract

17 The use of fentanyl during pregnancy, whether by prescription or illicit use, may result in high
18 blood levels that pose an early risk to fetal development. However, little is known regarding the
19 neurotoxicity that might arise from excessive fentanyl exposure in growing organisms,
20 particularly drug-related withdrawal symptoms. In this study, zebrafish embryos were exposed to
21 fentanyl solutions (0.1, 1, and 5 mg/L) for 5 days post fertilization (dpf), followed by a 5-day
22 recovery period, and then the larvae were evaluated for photomotor response, anxiety behavior,
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26 exposure to fentanyl caused substantial alterations in neural activity (*bdnf*, *c-fos*) and neuronal
27 development and plasticity (*npas4a*, *egr1*, *btg2*, *ier2a*, *vgf*). These results suggest that fentanyl
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29 as an aquatic species in research on the neurobehavioral effects of opioids in vertebrates.

30 ## Introduction

31 Opioids, a group of alkaloids and derivatives isolated from the opium poppy plant, are frequently
32 used to treat cancer pain, post-operative pain from surgery, and other types of chronic pain. In
33 the past two decades, drug dependence and addiction due to the nonmedical use of opioids have
34 been identified as a significant public health problem (Coyle et al. 2018).
35  Chronic accumulation of toxic opioid metabolites in adults can result in opioid-induced
36 neurotoxicity, which includes hallucinations, delirium, confusion, seizures, and nociceptive
37 hypersensitivity (van den Bosch et al. 2017; Herlinger & Lingford-Hughes 2022). Additionally,
38 the exposure of women to excessive doses of opioids, including from illicit sources, raises the
39 risk to fetal development, which is extremely concerning. From 2000 to 2007, a survey of 1.1

40 million Medicaid-enrolled women in 46 states and the District of Columbia in the United States
41 revealed that one in five of these women obtained an opioid prescription during pregnancy
42 (Desai et al. 2014). Certain birth abnormalities, including congenital heart disease, spinal
43 deformities, hydrocephalus, and glaucoma, have been associated with the use of opioid
44 analgesics in early pregnancy (Broussard et al. 2011; Martins et al. 2019). Self-reported opioid
45 usage among pregnant women rose 14-fold between 2000 and 2012, contributing to a 400% rise
46 in newborn morbidity (Winkelman et al. 2018; Honein et al. 2019). Prenatal exposure to opioids
47 also caused behavioral changes in infants, including psychomotor developmental impairments,
48 cognitive delays, and cognitive function deficits (Patrick et al. 2012; Conradt et al. 2019).
49 Minnes et al. proposed a significant correlation between anxiety, aggression, feelings of
50 rejection, disruptive or inattentive behavior in newborns and prenatal opioid exposure (Minnes et
51 al. 2011). However, the neurotoxic implications of early developmental exposure to opioids are
52 still unclear.
53   The zebrafish (Danio rerio) has been widely employed in genetic and developmental research
54 in recent years (Bailey et al. 2013). With the varying chemical structures of new psychoactive
55 substances, such as synthetic cannabinoids (Garcia-Gonzalez et al. 2021), synthetic opioids
56 (Kolesnikova et al. 2021), and hallucinogenic drugs (Demin et al. 2022), the zebrafish model
57 provides an efficient and rapid method to study the toxicity assessment and mechanisms of these
58 compounds. Zebrafish have homologous opioidergic genes to humans and a diverse behavioral
59 profile that can be used to validate the neurotoxicity generated by opioids (Stevens 2009; Demin
60 et al. 2018). For instance, chronic morphine treatment at 1.5 mg/L induced anxiety-like effects in
61 adult zebrafish (Cachat et al. 2011), resulting in a significant increase in time spent near the
62 water surface and a decrease in systemic cortisol levels. Diacetylmorphine (heroin) exposure
63 induced psychostimulant-like hypermobility in adult zebrafish, but did not alter anxiety-like
64 behavior (Stewart et al. 2015). The zebrafish conditioned positional preference test is an
65 important behavioral experiment to assess drug reward effects. Adult zebrafish can develop
66 addictive behavior when given substances like fentanyl, oxycodone, bupivacaine, phencyclidine,
67 and chlorpheniramine (Brock et al. 2017). Since the opioid system is active during zebrafish
68 embryonic development (Sarmah et al. 2020), the zebrafish larvae can be used to examine the
69 toxicity of prenatal opioid exposure. Zebrafish absorb drugs through the embryonic chorion or
70 skin during early stages of embryonic development (Martins et al. 2018), whereas adult humans
71 absorb fentanyl through the skin, intravenously, or orally. Despite differences in the mode of
72 ingestion of anesthetics, the teratogenicity of fentanyl has been demonstrated in studies with
73 zebrafish embryos tested. For example, zebrafish embryos have revealed that developmental
74 fentanyl exposure produced cardiotoxicity and respiratory depression, along with reduced
75 swimming ability in larvae (Kirla et al. 2021; Zaig et al. 2021; Cooman et al. 2022). However,
76 these studies have been limited to tests on larvae that have developed to day 5, and only superior
77 behavioral testing on more neurodevelopmental zebrafish larvae can provide a comprehensive
78 evaluation of the neurobehavioral toxicity induced by fentanyl exposure.

79 Fentanyl and its analogs are ultrashort-acting opioids with selective μ-receptor affinity and
80 gamma aminobutyric acid (GABA) agonist actions, which results in a complex neurotoxic
81 mechanism (Kim et al. 2017). Remifentanil, which is a synthetic opioid analgesic drug, binds to
82 μ-opioid receptors and indirectly enhances glutamate toxicity in patients, resulting in neuronal
83 degeneration and epilepsy (Angst et al. 2003). Fentanyl binding to G-protein-coupled receptors
84 also induced excessive dopamine release, which caused oxidative stress and neuronal damage
85 (Cunha-Oliveira et al. 2008). As with other kinds of nerve damage, disturbance of
86 neurotransmitter homeostasis appears to be involved in the etiology of opioid neurotoxicity
87 (Kofke et al. 2000). More research is needed to find out if fentanyl is neurotoxic to developing
88 organisms, especially to see how it affects behavior in animal models.
89 Fentanyl has a maximum plasma concentration of ug/L levels when used as an analgesic or
90 anesthetic. For example, when fentanyl is used as an anesthetic for corrective surgery for
91 congenital heart defects, the maximum plasma concentration of fentanyl in the patient is 30 ± 8
92 μg/L (Newland et al. 1989). We tested fentanyl-induced neurotoxicity in zebrafish larvae at
93 concentrations of 0.1, 1 and 5 mg/L, a range that correlates with blood concentrations of fentanyl
94 following illicit use of fentanyl analogs in humans (0.00013-2.1 mg/L) (Dwyer et al. 2017). To
95 determine if exposure to fentanyl during the first five days post fertilization causes behavioral
96 changes and neurotoxicity in zebrafish larvae, we evaluated the larvae's performance on days 10-
97 14 post fertilization in terms of photomotor response, anxiety levels, shoaling behavior,
98 aggressive behavior, and behavioral sensitization. The expression of neurological damage marker
99 genes was further examined to provide evidence of neurotoxicity.
100

Materials & Methods

Chemicals

103 Fentanyl hydrochloride powder (> 99.8% purity, CAS 1443-54-5) was purchased from Shanghai
104 Yuansi Standard Science and Technology Co., Ltd. All other reagents and chemicals were
105 purchased from Sigma-Aldrich (analytical grade, Shanghai). Fentanyl hydrochloride powder was
106 dissolved in distilled water and then diluted to 0.1, 1, 5 mg/L in E3 medium (5 mM NaCl, 0.17
107 KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$). The Trizol reagent was acquired from Jiancheng
108 (Nanjing, China). Reverse transcriptase kits and SYBR green reagents were purchased from
109 Takara (Dalian, China).
110

Zebrafish maintenance and reproduction

112 In this study, 4-6-month-old zebrafish of the wild type were obtained from the Chinese Zebrafish
113 Resource Center in Wuhan. Prior to the experiment, zebrafish were kept in a 40-liter tank for 15
114 days at a density of 10 per liter and fed twice daily with brine shrimp. The water in the aquarium
115 system was constantly filtered and oxygenated (pH 6.8-7.2, 28 ± 1°C, conductivity at 500–600
116 μS), and the room was lit for 14 hours and dark for 10 hours (lights on at 8:00 am). Fertilized
117 eggs were collected by generating spawning activity in adult zebrafish. The night before the
118 experiment, male and female zebrafish were positioned in a tank in a 2:1 ratio, separated by a

119   transparent acrylic panel. The next morning at 8:00 am, the divider was removed to trigger
120   natural spawning under the stimulation of light, and eggs were collected within 1 h. Normally
121   developed embryos were chosen for tests following standard techniques (Howe et al. 2013). The
122   larvae were kept in an incubator at 28°C and checked daily for mortality or deformities.
123   Zebrafish larvae do not require additional feeding for 5 days after fertilization. From 5 to 10 days
124   after fertilization, zebrafish larvae were subjected to a daily straw worm feeding in the morning.
125   From 10 to 14 days after fertilization, zebrafish larvae were fed once daily with fungus shrimp
126   larvae in the morning. At the end of all experiments, zebrafish larvae were anesthetized by ice
127   water and euthanized with 1% sodium hypochlorite. All procedures were approved by the
128   Zhejiang University Experimental Animal Welfare and Ethical Review Committee (Ethical
129   approval number: ZJU20220147).
130
131   **Zebrafish exposure to fentanyl**
132   Randomly selected zebrafish embryos were transferred to fentanyl solutions or E3 medium (5
133   mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$) and placed in standard 90 mm
134   Petri dishes within 2 hours of fertilization (50 embryos per Petri dish, 146 embryos were usedper
135   concentration). Petri dishes were kept in the incubator at a 14-hour light: 10-hour dark cycle,
136   with 15 mL of exposure solution replaced daily (i.e. half of the exposure solution was renewed).
137   After the 2 hpf to 5 days post-fertilization (dpf) exposure test, all zebrafish larvae in the fentanyl
138   and control groups were transferred to E3 solution to begin a five-day recovery period and fed
139   once daily until 14 dpf. Larvae that completed the behavioral tests (Fig. 1) were returned to the
140   petri dishes in the incubator. Different zebrafish were used for different behavioral tests. The
141   mortality rate was less than 5% during the experiment. The absence of significant malformations
142   during zebrafish embryonic development, such as pericardial edema, spinal curvature and
143   delayed yolk sac uptake, was confirmed by stereomicroscopy (Olympus, SZX2).. Gene
144   expression associated with neuronal damage was examined in zebrafish larvae at 5 dpf after
145   exposure to fentanyl (Fig.1).
146
147   **Photomotor response**
148   Following methods previously reported (Tao et al. 2020), we examined the photomotor response
149   of zebrafish larvae at the end of fentanyl exposure (5 dpf) and at the end of the recovery period
150   (10 dpf). Briefly, 24 larvae per concentration were transferred to 24-well plates containing 1.5
151   mL of E3 medium per well. The larvae's behavior was monitored utilizing the DanioVision™
152   observation system (Noldus). Once the zebrafish larvae had acclimated to the dark for 10
153   minutes, their responses to three successive 10-minute light at an intensity of 100 lux and 10-
154   minute dark periods were studied. The EthoVision XT 10 software (Noldus) was used to analyze
155   the video data. Anomalous data due to video tracking failures were removed. The swimming
156   distances of larvae during light (visible light) and dark (infrared light) periods were examined.
157
158   **Thigmotaxis behavior**

159 Zebrafish prefer to swim to the edge, which is associated with anxiety (Yang et al. 2017). At 10
160 dpf, a total of 24 randomly selected larvae per concentration were transferred to 24-well plates
161 according to the previous report (Yang et al. 2017). After an acclimation period of 30 min, the
162 behavioral characteristics of zebrafish were then recorded under 10 min of light (intensity of 100
163 lux) and 10 min of darkness for a total of 20 min. In the 24-well plate, a circle was created in the
164 middle of each well, with the inner circle's area equal to half of each well's total area. The
165 EthoVision XT 10 software was used to measure and analyze the time in the central zone and
166 entries into central zone as two thigmotaxis parameters.

167 **Light/dark preference test**
168 24 larvae were taken at random from each concentration group at 10 dpf and placed into the
169 wells of the 24-well plate (1 mL of E3 medium in each well). The bottom of the 24-well plate
170 was covered with a black and white acrylic plate, which divided the circular wells into two large
171 equal areas of light and dark zones (Chen et al. 2018). The DanioVision^{TM} system was used to
172 collect data on zebrafish behavior under light conditions every 60 seconds for 6 minutes. The
173 amount of time zebrafish larvae spent in the light zone and the number of times they moved from
174 the light zone to the dark zone were used to examine zebrafish preference for light and dark.

175 **Shoaling behavior**
176 Following methods previously reported (Chen et al. 2018), we examined the shallow behavior of
177 zebrafish larvae at 11 dpf. In brief, 20 larvae were picked at random from each concentration
178 group and placed in 90-mm glass Petri dishes (10 larvae and 30 mL of E3 medium per Petri
179 dish). The movements of zebrafish were tracked for 8 minutes, with data being collected every
180 15 seconds. The first 2 minutes were used to get the fish used to visible light, and the last 6
181 minutes were used to study how the fish behaved when they were in groups. Zebralab software
182 was used to calculate the inter-individual distance (IID) and the nearest neighbor distance (NND)
183 as indicators of shoaling behavior.

184 **Mirror-attack test**
185 At 12 dpf, 12 larvae were taken at random from each fentanyl concentration group and
186 transferred to rectangular 6-chamber polypropylene dishes (North Star Plastics in Jinan District,
187 China) for mirror-attack studies. A mirror paper (3.5 × 1.5 cm) was placed to one side of each
188 chamber (3.5 × 3.5 × 1.5 cm) of the dish (Chen et al. 2021). Zebrafish in separate chambers were
189 unable to see one another. The behavior of zebrafish was monitored using a ZebraLab behavioral
190 monitoring station for 8 minutes under light conditions, with data collected every 60 seconds.
191 The attack zone was established at 1 cm from the mirror paper using EthoVision XT 10 software.
192 The number of times the fish entered the attack zone and the amount of time zebrafish spent in
193 the attack zone were determined.

194 **Sensitization behavior**
195 At 14 dpf, 12 zebrafish larvae from each concentration group were randomly moved to a 0.01
196 mg/L fentanyl solution with 10 minutes and quickly transferred to 96-well plates (300 μL of 0.01
197 mg/L fentanyl solution per well). The behavior of the larvae was monitored. Once the zebrafish
198 larvae had acclimated to the dark for 10 minutes, their responses to three successive 10-minute

199   light (a light intensity of 100 lux) and 10-minute dark periods were studied. The swimming
200   distances of larvae during light (visible light) and dark (infrared light) periods were examined to
201   determine the behavioral effects of repeated exposure of larvae to the fentanyl.
202   **Quantitative real-time polymerase chain reaction (qPCR)**
203   Changes in transcript levels regulating neural activity (*bdnf, c-fos*) as well as neuronal
204   development and plasticity (*npas4a, egr1, btg2, ier2a, vgf*) were examined in zebrafish larvae at
205   5 dpf after fentanyl exposure. Thirty larvae were randomly selected from each concentration
206   group and total RNA was extracted using Trizol reagent (Takara, Japan) after hypothermia
207   anesthesia.RNA was quantified by measuring the absorbance at 260 nm and diluted to 200 ng/L.
208   Takara's cDNA first strand synthesis kit (Dalian, China) was then used to reverse-transcribe 1 μg
209   of total RNA. Following the manufacturer's protocol, quantitative analysis by qPCR was
210   performed using the CFX-96™ Real-Time PCR Detection System (BioRad, USA). The cycling
211   conditions were as follows: 95°C for 3 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30
212   s, and 72 °C for 30 s. *β-actin* expression levels were used to normalize the relative expression
213   ratios of neural-related genes, which were then evaluated using the $2^{-(\Delta\Delta Ct)}$ method (Livak &
214   Schmittgen 2001). The primer sequences are provided in Supplementary Table S1.
215   **Statistical analysis**
216   Normal data were described by mean and standard error of the mean, while non-parametric data
217   were described by median and interquartile range. One-way analysis of variance (ANOVA) was
218   used to investigate differences between the treatment and control groups. The normality of the
219   behavioral data was tested using Kolmogorov-Smirnov, and the homogeneity of variance was
220   tested using Bartlett's test. Welch's ANOVA was used when the data did not meet the assumption
221   of homogeneity of variance. Each treatment was compared to the control group using the
222   appropriate post hoc test (Dunnett's T3 multiple comparison test) when differences were found to
223   be statistically significant. Prism 9 (GraphPad, USA) was utilized to analyze significant
224   differences, and statistical significance thresholds for all experiments were set at $*p < 0.05$, $**p$
225   < 0.01, $***p < 0.001$ and $****p < 0.0001$.
226   # Results
227   **Fentanyl exposure resulted in decreased behavioral capacity**
228   The behavioral capacity of zebrafish larvae at 5 dpf was evaluated by the distance traveled under
229   light and dark stimuli. The findings indicated that zebrafish larvae moved farther during the dark
230   period than they did during the light period (Fig. 2A). Fentanyl exposure led to a concentration-
231   dependent decrease in the average total moving distance of zebrafish larvae under both light and
232   dark conditions (Figs. 2B and 2C). When compared to the control group, zebrafish larvae
233   exposed to 0.1, 1, or 5 mg/L of fentanyl experienced significant reductions in the amount of
234   distance they traveled during the light phase (Fig. 2B) (Welch's ANOVA test, $F (3.000, 45.06) =$
235    $15.61, p < 0.0001$; Dunnett's T3 post hoc test, all $p < 0.0001$) . Only the group that was given 0.1
236   mg/L of fentanyl did not experience a significant reduction in travel distance during the dark
237   phase in comparison to the control larvae (Fig. 2C) (one-way ANOVA, $F (3, 87) = 17.58, p <$

238 0.0001; Dunnett's T3 post hoc test, $p = 0.2158$ for 0.1 mg/L, $p = 0.0024$ for 1 mg/L, $p < 0.0001$
239 for 5 mg/L).
240 Fentanyl continued to impair the behavioral abilities of zebrafish larvae after a 5-day recovery
241 interval (Fig. 2D). Although there were no significant differences in the travel distance in the
242 control and fentanyl-exposed groups during light periods (Fig. 2E) (one-way ANOVA, F (3, 81)
243 = 1.666, $p = 0.1808$), zebrafish that had been exposed to 1 or 5 mg/L fentanyl up to 5 dpf and
244 then recovered for 5 days, traveled significantly smaller distances than control fish in the dark
245 conditions (Fig. 2F) (one-way ANOVA, F (3, 85) = 3.096, $p = 0.0311$;Dunnett's T3 post hoc test,
246 $p = 0.0429$ for 1 mg/L, $p = 0.0199$ for 5 mg/L).
247 **Fentanyl exposure resulted in increased anxious behavior**
248 When exposed to fentanyl for 5 dpf and then recovered for 5 days in the clean medium, zebrafish
249 larvae showed a decrease in their preference for the central region (thigmotaxis), which indicated
250 increased anxiety. Compared to control fish, 10 dpf larvae exposed to fentanyl spent significantly
251 smaller time in the central region (Fig. 3A) (Welch's ANOVA test, F (3.000, 42.71) = 5.937, $p =$
252 0.0018; Dunnett's T3 post hoc test, $p = 0.0135$ for 0.1 mg/L, p = 0.0069 for 1 mg/L, $p = 0.0004$
253 for 5 mg/L) and made significantly fewer entrances into the central region (Fig. 3B) (one-way
254 ANOVA, F (3, 85) = 11.66, $p <0.0001$; Dunnett's T3 post hoc test, $p <0.0001$ for 0.1 mg/L, $p =$
255 0.0003 for 1 mg/L, $p = 0.0011$ for 5 mg/L). Another important indicator of high levels of anxiety
256 in zebrafish larvae is the increased preference for light (Bai et al. 2016). Here, the time zebrafish
257 spent in the light region was positively correlated with fentanyl concentration (Fig. 3C), and all
258 concentrations were significantly increased compared to the control (Welch's ANOVA test, F
259 (3.000, 42.79) = 35.42, $p < 0.0001$; Dunnett's T3 post hoc test, $p = 0.0347$ for 0.1 mg/L, $p <$
260 0.0001 for 1 mg/L, $p <0.0001$ for 5 mg/L). Also, compared to the control group, the larvae in the
261 1 mg/L and 5 mg/L fentanyl groups moved between the light and dark areas significantly more
262 often (Fig. 3D) (one-way ANOVA, F (3, 89) = 4.537, $p = 0.0052$; Dunnett's T3 post hoc test, $p =$
263 0.0057 for 1 mg/L, $p = 0.0064$ for 5 mg/L).
264 **Fentanyl exposure produced deficient social behavior**
265 For shoaling at 11 dpf, zebrafish exposed to 1 or 5 mg/L fentanyl showed significantly increased
266 inter-individual distance (IID) (one-way ANOVA, F (3, 188) = 8.934, $p < 0.0001$; Dunnett's T3
267 post hoc test, $p = 0.0003$ for 1 mg/L, $p = 0.0011$ for 5 mg/L) and increased nearest neighbor
268 distance (NND) (Welch's ANOVA test, F (3.000, 65.59) = 5.363, $p = 0.0023$; Dunnett's T3 post
269 hoc test, $p = 0.0038$ for 1 mg/L, p = 0.0023 for 5 mg/L) compared to control fish (Figs. 4A and
270 4B). Zebrafish larvae exposed to fentanyl at 1 or 5 mg/L entered the mirror zone significantly
271 less frequently than control fish (Fig. 4C) (one-way ANOVA, F (3, 44) = 6.621, $p = 0.0009$;
272 Dunnett's T3 post hoc test, $p = 0.0182$ for 1 mg/L, $p = 0.0004$ for 5 mg/L). Although the
273 fentanyl-exposed fish spent more time in the mirror region than control fish, the differences were
274 not significant (Fig.4D) (one-way ANOVA, F (3, 44) = 0.8351, $p = 0.4818$).
275 **Behavioral sensitization to a fentanyl challenge at 0.01 mg/L**
276 Behavioral sensitization refers to the enhanced motor stimulus response that occurs during
277 repetitive, intermittent exposure to a specific drug, and in the present study we evaluated this by

278   the motor ability of zebrafish in photomotor response. The locomotor response to a repeated
279   fentanyl stimulus at 14 dpf was studied in zebrafish that had been exposed to fentanyl at 0.1, 1 or
280   5 mg/L during the first 5 dpf, then recovered in clean E3 medium up to 14 dpf. Zebrafish larvae
281   exposed to fentanyl during development experienced behavioral changes after re-exposure to
282   fentanyl (Fig. 5A). After the 0.01 mg/L fentanyl challenge, zebrafish larvae in the 5 mg/L
283   fentanyl group showed significant increases in swimming distance during light (Fig. 5B) (one-
284   way ANOVA, F (3, 37) = 3.473, p = 0.0255; Dunnett's T3 post hoc test, p = 0.0297) and dark
285   periods (Fig. 5C) (one-way ANOVA, F (3, 37) = 3.864, p = 0.0168; Dunnett's T3 post hoc test, p
286   = 0.0080).
287   **Fentanyl exposure alters the expression of genes related to neuro-development**
288   We further examined how different concentrations of fentanyl affected the transcriptional
289   regulation of genes regulating neural activity (*bdnf*, *c-fos*) and neuronal development and
290   plasticity (*npas4a*, *egr1*, *btg2*, *ier2a*, *vgf*). At 5 dpf, fentanyl exposure at a concentration of 5
291   mg/L significantly increased the relative expression of *bdnf* (one-way ANOVA, F (3, 8) = 9.878,
292   p = 0.0046; Dunnett's T3 post hoc test, p = 0.0181), *c-fos* (one-way ANOVA, F (3, 8) = 5.327, p
293   = 0.0261; Dunnett's T3 post hoc test, p = 0.0172), *napas4a* (one-way ANOVA, F (3, 8) = 23.49,
294   p = 0.0003; Dunnett's T3 post hoc test, p = 0.0003), *egr1* (one-way ANOVA, F (3, 8) = 12.199, p
295   = 0.0024; Dunnett's T3 post hoc test, p = 0.0090) in comparison to control larvae (Figs. 6A-D).
296   At concentrations 1 and 5 mg/L, fentanyl exposure significantly upregulated *btg2* (one-way
297   ANOVA, F (3, 8) = 76.72, p < 0.0001; Dunnett's T3 post hoc test, p = 0.000 for 1 mg/L, p
298   <0.0001 for 5 mg/L) and *ier2a* (one-way ANOVA, F (3, 8) = 11.51, p =0.0028; Dunnett's T3
299   post hoc test, p = 0.0116 for 1 mg/L, p = 0.0013 for 5 mg/L) genes relative to controls (Figs. 6E
300   and 6F). For *vgf*, all the fentanyl groups showed significant increase relative to control group
301   (Fig. 6G) (one-way ANOVA, F (3, 8) = 152.4, p < 0.0001; Dunnett's T3 post hoc test, p =
302   0.0184 for 0.1 mg/L, p = 0.000 for 1 mg/L, p < 0.0001 for 5 mg/L)
303
Discussion
305   In recent years, zebrafish have been widely utilized as a model aquatic vertebrate for
306   toxicological research. Thus far, research on the toxicological effects of fentanyl on zebrafish has
307   focused on cardiac development, respiratory depression, and behavioral inhibition. For example,
308   in the zebrafish embryotoxicity (zFET) test, the lethal concentration of fentanyl was 83.17 μM
309   (28.40 mg/L) and the sublethal value (endpoint including pericardial edema, irregular heartbeat,
310   yolk sac edema, and head cord deformities) was 16.15 μM (5.52 mg/L) in 5 dpf zebrafish larvae
311   (Kirla et al. 2021; Cooman et al. 2022). The range of fentanyl concentrations (0.1-5 mg/L) used
312   in the current study exhibited no teratogenic effects on zebrafish development, but caused
313   behavioral alterations in numerous motor and social behavioral tests, demonstrating that low
314   concentrations of fentanyl could cause neurobehavioral toxicity.
315   Neurodevelopment in zebrafish embryos is a complex process and if, at any stage, the process
316   is altered, interrupted or inhibited, the consequences could be permanent. We chose to expose
317   zebrafish embryos to fentanyl at 4 hpf to 5 dpf, this was in consideration of the fact that the

318   major organs of the zebrafish would have completed their developmental maturation during this
319   period. A growing number of studies have used various behavioral endpoints in zebrafish to
320   assess the effects of chemicals and contaminants (Rosa et al. 2022; Zhang et al. 2021). As soon
321   as possible after undergoing the recovery period, we tested for complex behaviors, which are
322   usually not reliably measured at 5 dpf. Complex behaviors (for example, social behavior,
323   memory, and learning) can reveal more about toxicological effects than simple behavioral
324   analyses of model animals (Hong & Zha 2019). Our findings demonstrate that exposure to
325   fentanyl during embryo development has a concentration-dependent effect on anxiety, shoaling,
326   aggression, social preference, and sensitization in zebrafish. Zebrafish larvae show a stable
327   motor response to continuous alternating light and dark stimuli at 6 dpf, i.e. light inhibits
328   behavior and dark promotes behavior, which needs supraspinal input from the brain and is a
329   crucial sign for determining neurodevelopment (Hong & Zha 2019). In our investigation,
330   fentanyl administration reduced the distance traveled of 5 dpf larvae during cycles of light and
331   dark. Other opioids revealed similar toxicity in zebrafish larvae. For example, zebrafish
332   displayed decreased swimming speed after exposure to different fentanyl analogues at 1 μM
333   (0.34 mg/L for fentanly and 0.35 mg/L for butyrfentanyl) (Pesavento et al. 2022). The high
334   sensitivity of fentanyl for mammalian μ-type opioid receptors and the similarities between
335   zebrafish and mammalian opioid systems (Steenbergen et al. 2011) make zebrafish useful for
336   studying fentanyl toxicity in animals and even humans. *Oprm1* (Opioid receptor mu1)
337   expression is detectable in numerous nerve centers at 24 hours post fertilization in zebrafish
338   embryos (Sarmah et al. 2020). The motor impairment caused by fentanyl in zebrafish may be a
339   result of the analgesic and sedative effects provided by the binding of opioid receptors (Gampfer
340   et al. 2020; Zaig et al. 2021). In fact, after the recovery period, we observed no significant
341   difference in the behavioral capacity of zebrafish larvae exposed to all concentrations of fentanyl
342   under bright conditions compared to the control group, indicating that behavioral capacity under
343   light conditions was recovered. However, the behavioral abilities of zebrafish larvae exposed to
344   1.0 mg/L and 5.0 mg/L concentrations of fentanyl under dark conditions remained significantly
345   lower than those of the control group, which may be due to the anxiety-induced effects on larval
346   behavior under non-preferred conditions (darkness). (Gampfer et al. 2020)
347   During the later phases of zebrafish larval development after the recovery period, anxiety,
348   sociability, and other behaviors were assessed further. Commonly, zebrafish thigmotaxis tests
349   and light/dark preference tests have been employed to evaluate the anxiety-inducing effects of
350   substances (Peng et al. 2016; Yang et al. 2017; Chen et al. 2018). Zebrafish larvae like to swim
351   near the edge and have a strong dark avoidance behavior (Steenbergen et al. 2011). In our
352   investigation, fentanyl dramatically decreased the proportion of time spent in light areas while
353   increasing the propensity to explore intermediate locations, indicating that fentanyl
354   administration enhanced zebrafish anxiety. Opioid-like anxiety behaviors have been found in rats
355   on long-term morphine use and have been demonstrated to include functional abnormalities in
356   the limbic dopaminergic system (Raghav et al. 2021; Deji et al. 2022).

357   Zebrafish are highly social animals that prefer swimming in groups (Mansur Bde et al. 2014).
358   The increase of IID and NND in the shoaling test indicated that zebrafish have decreased social
359   cohesion (Chen et al. 2018). Typically, zebrafish do not begin to shoal until a few days after
360   hatching, and this propensity steadily develops during development. Zebrafish undergo
361   alterations in their dopaminergic and serotonergic systems as their shoal behavior matures.
362   Disruption of early neurodevelopment (Chen et al. 2021; Fu et al. 2021; Wang et al. 2022) and
363   neurotransmitter systems (Santos et al. 2021) in the zebrafish brain may be responsible for
364   reduced shoal behavior. Another well-established tool for researching zebrafish sociality is the
365   mirror attack test, which is used to assess zebrafish aggression (Qiu et al. 2021). We found that
366   the exposure to fentanyl enhanced the frequency of mirror attacks and the proportion of time
367   spent in the attack area. Even though there haven't been many behavioral studies of opioid
368   withdrawal in fish, chronic aggression has been observed in mouse models after opioid
369   withdrawal (Piccin & Contarino 2020), and morphine made both male and female mice less
370   social (Piccin et al. 2022).
371   Sensitization to addictive drugs plays a significant role in the onset and recurrence of relapse
372   and drug-seeking behavior. In this study, embryonic fentanyl exposure in zebrafish resulted in an
373   enhanced behavioral response to the fentanyl challenge. Zebrafish exhibit stronger behavioral
374   responses after repeated exposure to cocaine, nicotine, and ethanol, which is consistent with our
375   findings (Pisera-Fuster et al. 2019). In adult zebrafish, behavioral sensitization generated by a
376   single morphine exposure resulted in significant behavioral sensitization after repeated and
377   intermittent morphine (1.25-10 μM, 0.36-2.85 mg/L) treatment (Bian et al. 2022). The dosages
378   employed to elicit behavioral sensitization in zebrafish are typically low, owing to the fact that
379   excessive concentrations produce a decrease in locomotor activity. The behavioral sensitization
380   we found with fentanyl in zebrafish may be due to a negative reinforcement mechanism, i.e., a
381   heightened behavioral response generated by ceasing or avoiding an aversive stimulus such as a
382   withdrawal medication (Koob 2013), which should be further validated on other animal models.
383   The genes addressed in this article that were related to essential neuronal activities may be
384   early markers of potentially harmful impacts on neural development in zebrafish embryos, which
385   can result in altered organismal behavior, memory, and even energy balance. Brain-derived
386   neurotrophic factors (*bdnf*) play critical roles in neuronal cell proliferation, differentiation, and
387   maintenance, as well as synaptic plasticity, learning, and long-term memory. The neurotrophic
388   factor bdnf has been identified as a possible downstream target of c-fos and *npas4a* (Lin et al.
389   2008; Lu 2011). In zebrafish, a reduction in aggressive behavior was observed to be mediated by
390   an increase in *bdnf* expression in the brains (Theodoridi et al. 2017). The *c-fos* is an excellent
391   biomarker for identifying synaptic activation (Torres-Hernandez et al. 2016), and the relative
392   expression of *c-fos* was more than twice as high in zebrafish exposed to high fentanyl as it was in
393   the control group. Experiments on mice revealed a correlation between *c-fos* expression in a
394   specific subset of brain areas and opioid hyperalgesia and physical withdrawal symptoms
395   (Alvarez-Bagnarol et al. 2022). In addition, the expression level of *c-fos* mRNA in the zebrafish

396 brain increased considerably following nicotine abstinence in the zebrafish nicotine withdrawal
397 experiment (Ponzoni et al. 2021).
398   The *npas4a* homolog is a brain-specific activity dependent gene involved in forebrain
399 development. For example, *npas4a* gene deletion during embryonic development resulted in
400 forebrain defects and reduced expression of specific forebrain markers (Lin et al. 2008). As part
401 of the control of excitation-inhibition equilibrium in brain circuits, *npas4a* has also been shown
402 to be upregulated in proportion to the severity of seizures (Bloodgood et al. 2013). Animals'
403 learning and behavior in regards to fear have recently been linked to changed amounts of *npas4a*
404 expression in zebrafish (Torres-Hernandez et al. 2016; Baker & Wong 2021). The *egr1* is an
405 inducible transcription factor in the brain that regulates synaptic plasticity, memory, as well as
406 neuronal cell development and differentiation (Boyer et al. 2013). It has been proposed that b-
407 cell translocation gene 2 (*btg2*) and *ier2a* are key regulators of vertebrate neural development
408 (Moriya et al. 2016). Previous investigations associating *btg2* deletion in zebrafish and mice with
409 aberrant aggression raise the possibility that both genes are crucial for brain processes related to
410 aggression (Malki et al. 2016; Liu et al. 2020). The *vgf* (VGF nerve growth factor-inducible), a
411 neuropeptide involved in crucial neural processes such as synaptic plasticity, neurogenesis, and
412 brain neurite growth, is produced by brain-derived neurotrophic and nerve growth factors
413 (Mizoguchi et al. 2017; Takeuchi et al. 2018) and has been shown to have neuroprotective
414 effects on retinal ganglion cells (Takeuchi et al. 2018). In addition, *vgf* knockout led to increased
415 sadness and antisocial behavior in mice, as well as decreased brain weight and memory
416 impairment (Mizoguchi et al. 2017). Depression, schizophrenia, and bipolar disorder are related
417 to aberrant levels of *vgf* in the human brain, indicating that this gene is involved in the
418 pathophysiology of mental diseases (Lin et al. 2015).
419
420

Conclusions
421
422 In conclusion, we reveal for the first time that exposure of zebrafish embryos to fentanyl resulted
423 in elevated anxiety, decreased social preference and aggressiveness, and induced behavioral
424 sensitization. The expression of genes showed that embryonic exposure to fentanyl at
425 concentrations of 5 mg/L caused substantial alterations in neural activity (*bdnf*, *c-fos*) and
426 neuronal development and plasticity (*npas4a*, *egr1*, *btg2*, *ier2a*, *vgf*). The results demonstrated
427 the neurotoxicity of fentanyl at nonteratogenic concentrations to developing organisms,
428 providing evidence for the risk of fentanyl use during pregnancy.
429 .
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Acknowledgements
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Competing Interests
433
434 The authors declare there are no competing interests.

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Data Availability

437 The following information was supplied regarding data availability:

438 Photomotor response, thigmotaxis behavior, light/dark preference, shoaling behavior, mirror-

439 attack, sensitization behavior were examined for statistical analyses to compare significant

440 differences in behavioural changes in   zebrafish larvae at different concentrations and

442 are available in the Supplemental File.

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Figure 1

Figure 1. A schematic diagram showing zebrafish embryos exposed to 0, 0.1, 1, 5 mg/L
fentanyl and sampled at different time points for behavior tests.
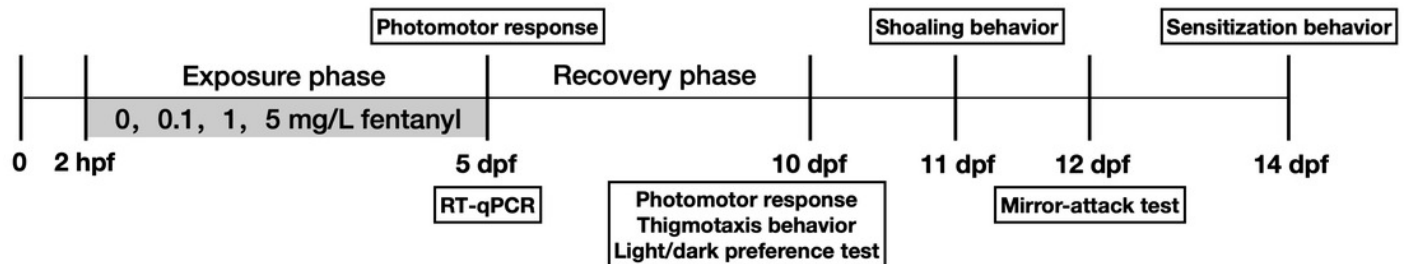
Figure 2

Figure 2. Effect of fentanyl exposure on the locomotor activity of zebrafish larvae at 5 dpf and 10 dpf.

Behavioral tests were performed on zebrafish larvae after they were exposed to 0.1, 1, and 5 mg/L fentanyl in E3 medium from 2 hours post fertilization to 5 dpf and 10 dpf (n = 3). (A) Distance traveled following fentanyl exposure at 5 dpf. (B) Total distance travelled during the light period at 5 dpf. (C) Total distance travelled during the dark period at 5 dpf. (D) Distance traveled after the recovery period at 10 dpf. (E) Total distance travelled during the light period at 10 dpf. (F) Total distance travelled during the dark period at 10 dpf. Values are plotted as mean ± SEM. Significance was defined as * $p < 0.01$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.
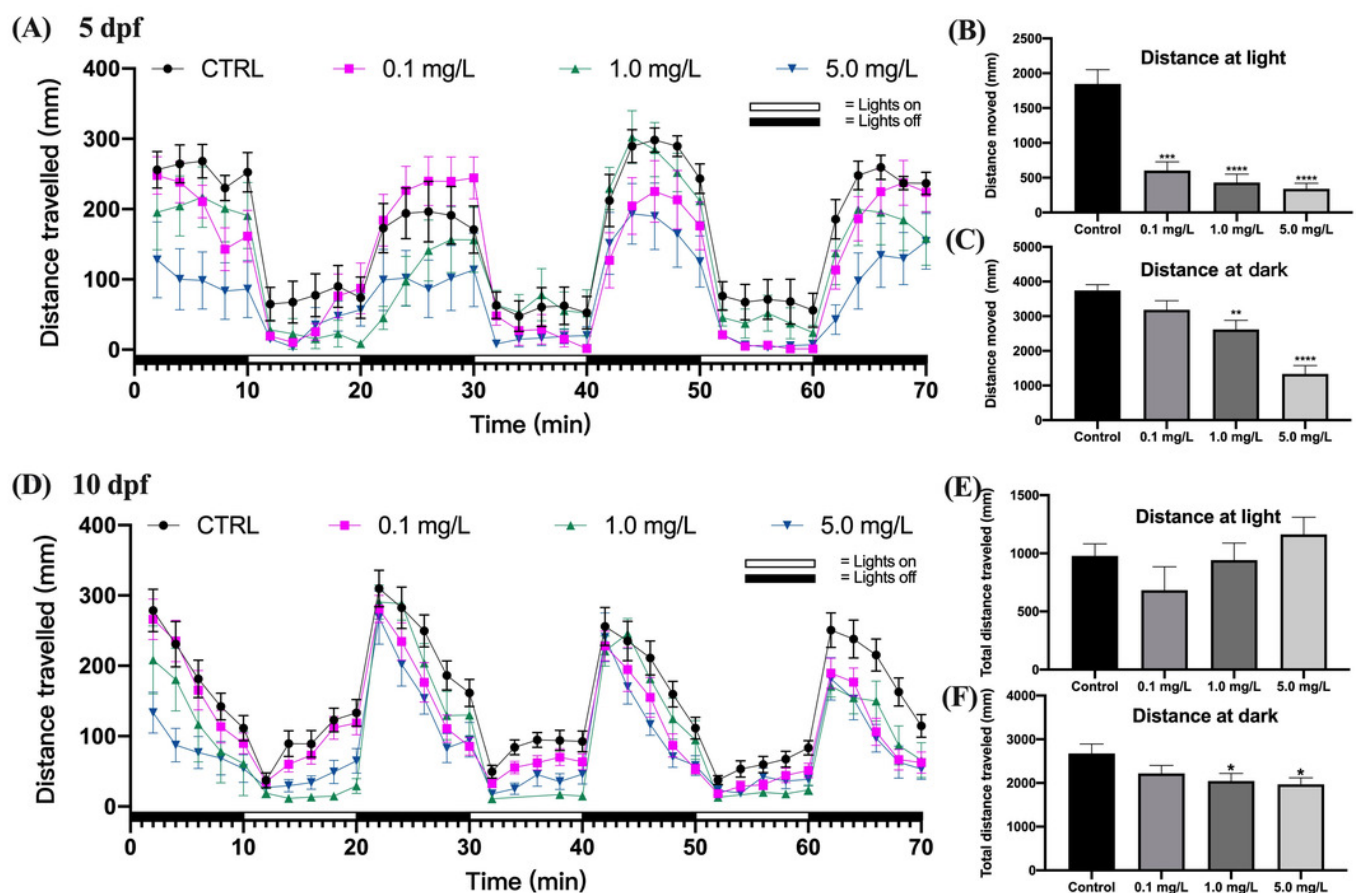
Figure 3

Figure 3. The effect of fentanyl exposure on larval thigmotaxis behavior and light/dark preference.

Zebrafish embryos were exposed to 0.1, 1, and 5 mg/L fentanyl in E3 medium from 2 hours post fertilization to 5 dpf, followed by a 5-day recovery interval, and behavioral tests were performed at 10-11 dpf. (A-B) Light/dark background preference tests were performed on larvae at 10 dpf (n = 3). (C-D) Thigmotaxis behavior was assessed on larvae at 11 dpf (n = 3). Values are plotted as mean ± SEM . Significance was defined as * p < 0.05, ** p < 0.01, *** p < 0.001,**** p < 0.0001.
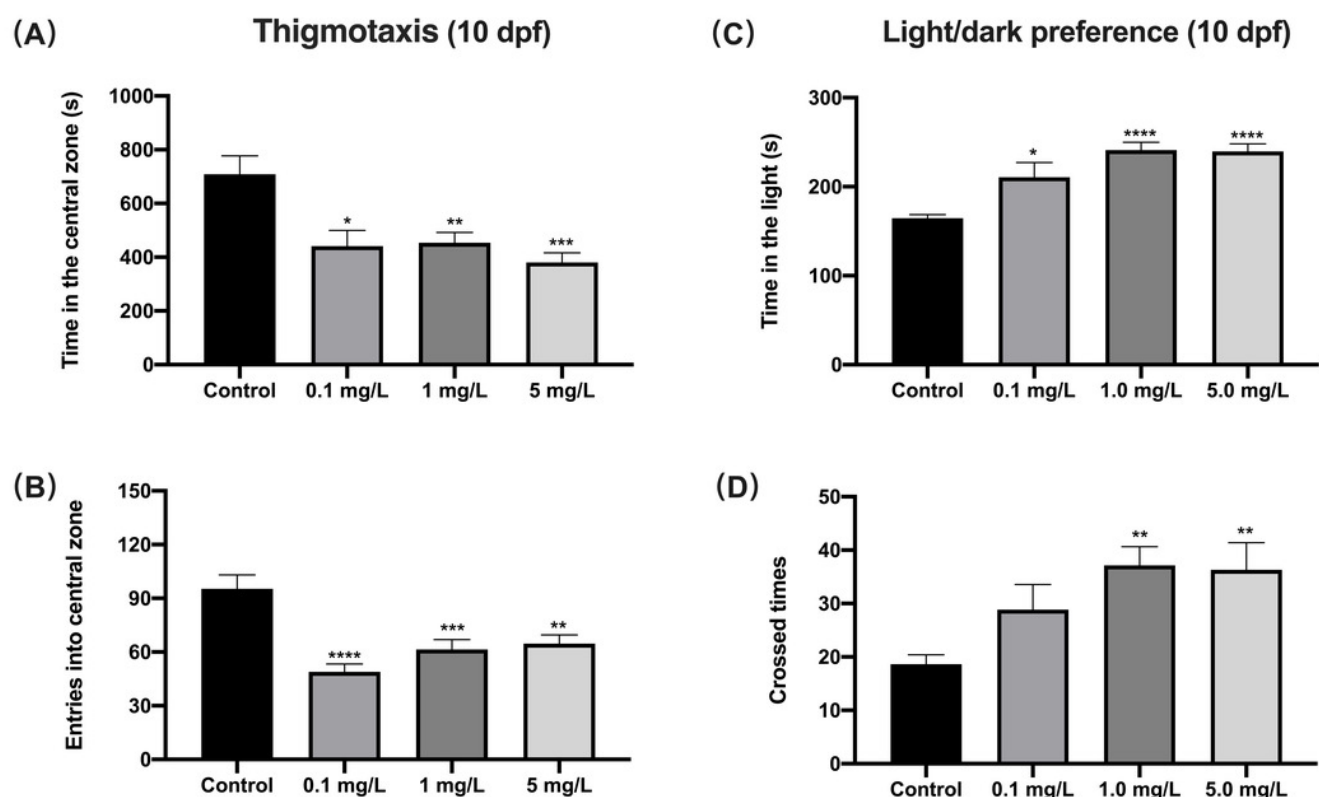
Figure 4

Figure 4. The effect of fentanyl exposure on shoaling and mirror attack behavior of larvae.

Zebrafish embryos were exposed to 0.1, 1, and 5 mg/L fentanyl in E3 medium from 2 hours post fertilization to 5 dpf, followed by a 5-day recovery interval, and behavioral tests were performed 11-12 dpf. (A-B) Shoaling behavior evaluated using larvae at 11 dpf (n = 3). Inter-individual distance (IID) and nearest neighbor distance (NND). (C-D) Mirror attack behavior evaluated using larvae at 12 dpf (n = 3). Values are plotted as mean ± SEM. Significance was defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
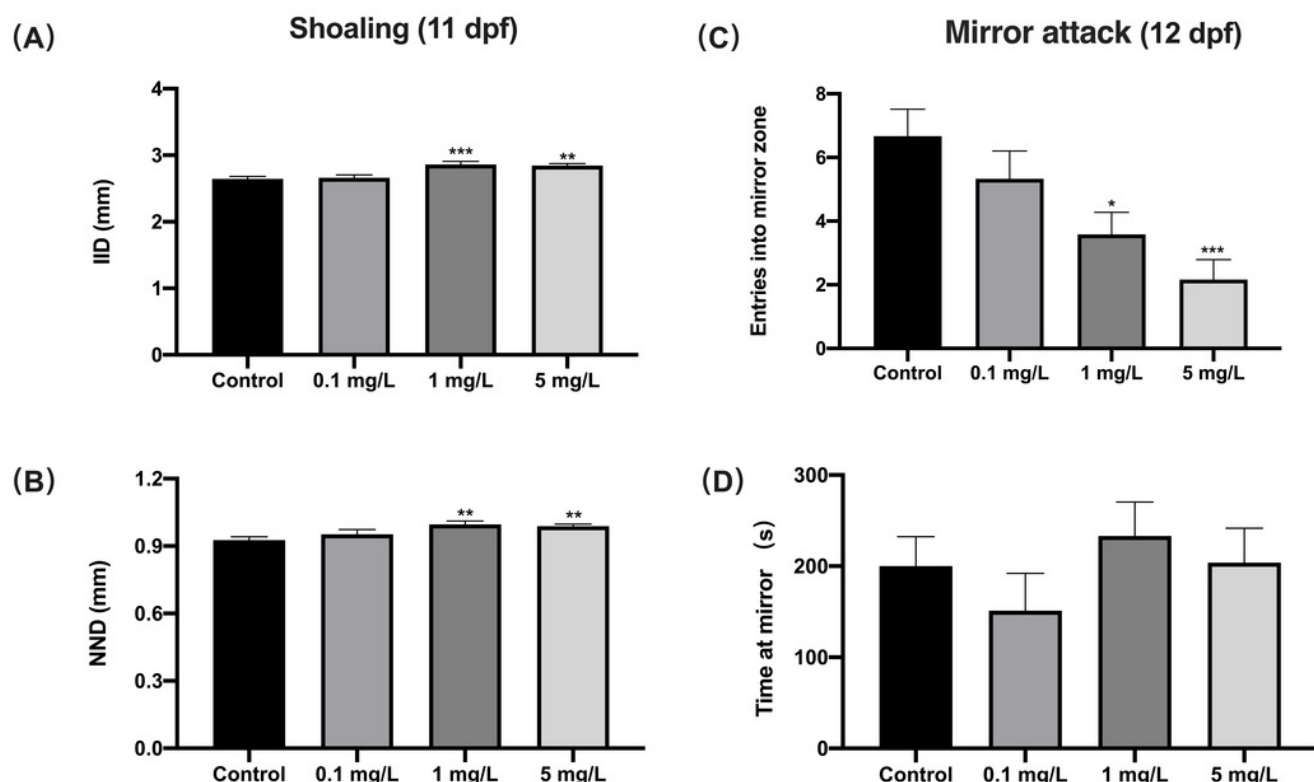
Figure 5

Figure 5. Behavioral sensitization after re-exposure to 0.01 mg/L fentanyl at 14 dpf.

Zebrafish embryos were exposed to 0.1, 1, and 5 mg/L fentanyl in E3 medium from 2 hours post fertilization to 5 dpf, followed by a 5-day recovery interval, and behavioral tests were performed at 14 dpf (n = 3). (A) Distance travelled every two minutes after the fentanyl challenge. (B) Total distance travelled during the light period. (C) Total distance travelled during the dark period. Values are plotted as mean ± SEM. Significance was defined as * p < 0.05, ** p < 0.01.
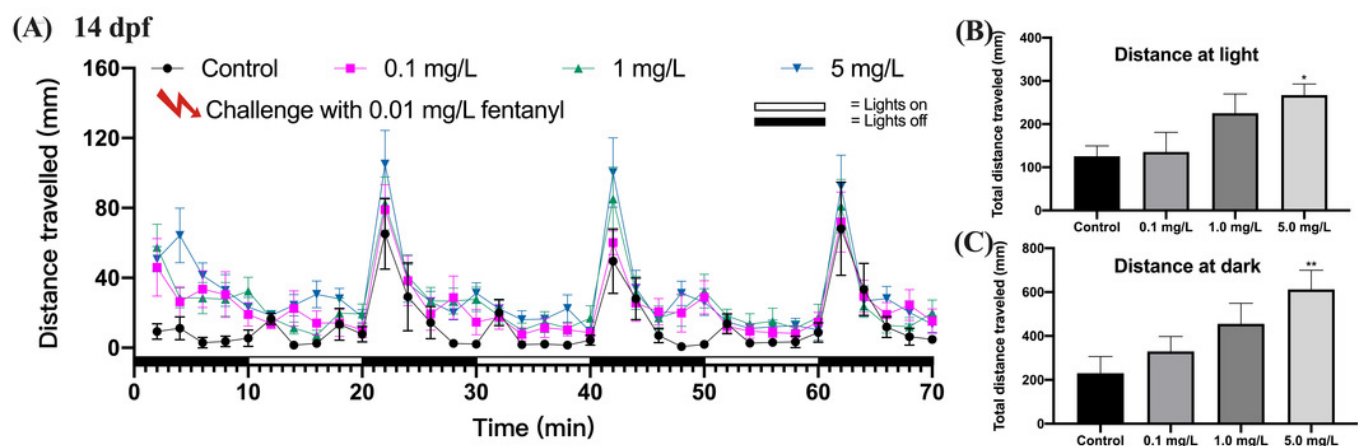
Figure 6

Figure 6. Relative mRNA expression levels of neurodevelopmental marker genes in zebrafish at 5 dpf after fentanyl exposure.

(A-B) Genes regulating neural activity (*bdnf*, *c-fos*). (C-G) Genes regulating neuronal development and plasticity (*egr1*, *napas4a*, *vgf*, *btg2* and *ier2a*). Data are expressed as mean ± SEM. Significance was defined as *p < 0.05, **p < 0.01 and ***p < 0.001.