Research report

Early life exposure to chronic unpredictable stress induces anxiety-like behaviors and increases the excitability of cerebellar neurons in zebrafish

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ABSTRACT

Anxiety is a common emotional disorder in children. To understand its underlying mechanisms, chronic unpredictable stress (CUS) has been established as a stress model in zebrafish. By using the tall tank test, the stress response reliability could be improved in adult fish which has not been confirmed in larvae. In addition, the increasing evidences have shown that cerebellum plays important roles in anxiety. Whether CUS will affect cerebellar neuronal activity remains unknown. We found that CUS exposure to larvae (from 10 to 17 days post fertilization) induced anxiety-like behaviors and social cohesion impairments within 1–2 d after CUS, including a prolonged freezing time, an increased time spent at the bottom of tank, an increased thigmotaxis index, and an increased interindividual distance. Our results showed that the four behavioral tests were homogeneous, especially the tall tank test either anxiety-like behaviors or the basal locomotion. Furthermore, we found that CUS enhanced the excitability of cerebellar neurons, as the amplitude, frequency, time to peak and half-width of spontaneous firing significantly decreased, as well as the amplitude of excitatory post-synaptic current when compared with the control group. CUS also activated hyperpolarization-activated cyclic nucleotide-gated and potassium channels of cerebellar neurons. Multiple linear regression analysis showed that the total distance in bottom (tall tank test) was correlated positively with outward Na+ -K+ currents (r = 0.848, P = 0.016), and the thigmotaxis index (open field test) correlated with action potential amplitude (r = 0.854, P = 0.030). Altogether, early life CUS transiently induced an anxiety-like behavior which could be more accurately assessed by combining the tall tank test with other behavior tests in young zebrafish. CUS increased the excitability of cerebellar neurons might provide new targets to treat emotional diseases such as anxiety.

1. Introduction

Stress response, organisms responding to stressors and trying to maintain homeostasis [1], is primarily through the sympathetic nervous system and hypothalamic-pituitary-adrenal (HPA) axis [2–4]. Although an acute response of organisms is essential for its adaption to a changing environment, the repeated and long-term stress response will not only imbalance its stress response ability, but also lead to a variety of adverse health problems, such as growth impairments, emotional disorders, immune dysfunction and so on [5–7]. In order to understand neuro-pathological mechanisms under such conditions, several animal models have been introduced in recent years. Among them, chronic unpredictable stress (CUS) is one of the recognized clinically applicable stress models in animal, which simulates the neuropsychiatric results of patients [8,9]. In this model, the animals were chronically subjected to unpredictable stressors inducing a variety of neuropsychiatric phenotypes.

Zebrafish (Danio rerio) is becoming an essential model organism to
be used to study the neurobehavioral alterations resulted from stress. The molecular, cellular and neurobehavioral adaption to stressors found in humans is exhibited in zebrafish, such as cortisol release, augmented oxidative stress, HPA-axis activation, center avoidance and so on[10-13]. Commonly, the time spent around the wall during the behavior testing indicates the anxiety-like behavior of fish [14,15]. There are several CUS models have been established in zebrafish[9,16,17]. However, there was a discrepancy about CUS-induced explorative response when testing their behaviors such as social cohesion [9,16,17]. Usually, stressed fish showed a reduced exploration of a novel environment[18,19], and the novel tank test is widely used to characterize adult and young fish anxiety phenotypes[16,20,21]. It had also been demonstrated that by using the tall tank test could lead to greater differences in behavioral responses between individuals therefore improved its response reliability in adult fish [22], which has not been confirmed in young zebrafish.

It is proposed that zebrafish mainly respond to stress exposure through the neuroendocrine regulation mechanism [11,23,24]. This CUS-induced explorative and scototaxis neurobehavioral response was also associated with reduced neurogenesis and mitochondrial dysfunction [9,16,19,23]. So far, we know little about the changes of brain sensitive regions and neural circuits after CUS [25]. Interestingly, increasing evidences have shown that cerebellum might play more function than previously thought [26]. Recent results suggest that the involvement of structural and functional deficits in cortico-striato-thalamo-cerebellar circuitry in social anxiety disorder [27]. Therefore, to accurately assess the altered neurobehavioral response to early life CUS exposure in zebrafish larvae by using the tall tank test combining with other commonly used tests, and to understand the underlying electrophysiological mechanisms of CUS-induced behavioral disorders with special reference to cerebellum is the prime objective of the present study.

2. Material and methods

2.1. Zebrafish care and husbandry

All experiments and animal handling were performed according to the Guide for the Care and Use of Laboratory Zebrafish by the China Zebrafish Resource Center and were approved by the Animal Care and Use Committee at Zhejiang University School of Medicine (16779). Wild-type (WT) zebrafish AB strain was housed in a modular zebrafish system (Haisheng, China), and all fish were kept in a 10-h dark / 14-h light cycle, and 28 ± 0.5 °C filtered and UV sterilized water. We collected fertilized embryos within 1 h after mating. After the collection, the fertilized embryos (≤50) were put into Petri dishes (90 mm in diameter) containing embryonic media (E3) (pH = 7.0), and then move to an incubator (28 °C). Change the water in the morning and evening every day. At 5 days post fertilization (dpf), take them out of the incubator and put into a breeding tank (780 ml, n ≤ 50). Feed Paramecium at 5–7 dpf, Paramecium + #100 at 7–15 dpf, three times a day, and change water 20 min after each feeding. After 15 dpf, put them into the circulation system, feed #150 + shrimp at 15–30 dpf, and feed adult fish regular food + shrimp after 30 dpf. The fish tank is cleaned once a week. The pH value of circulating water is 7.0–8.0; the conductivity is generally about 500–800 µS/cm.

2.2. Experimental design and CUS

CUS exposure was done from 10 to 17 dpf in the stressed group (see Fig. 1). Control group was simultaneously raised without CUS exposure. Fish of both groups were tested by using tall tank, shoaling behavior and novel tank tests at early stage after CUS (18 dpf). Another set of fish were tested by the novel tank test at 18 dpf followed by the open field test at intermediate stages after CUS (19 dpf). Fish of additional set were left undisturbed after the end of CUS tested with tall tank and novel tank tests at late stages after CUS (25 dpf) to assess the persistence of anxiety-

Fig. 1. Experimental design and CUS. CUS exposure was done from 10 to 17 dpf in the stressed group. Control group was simultaneously raised without CUS exposure. Fish of both groups were tested by using tall tank (control: n = 98; stressed: n = 112), shoaling behavior (control: n = 80; stressed: n = 88) and novel tank tests (control: n = 66; stressed: n = 54) at early stage after CUS (18 dpf). Another set of fish were tested by novel tank tests at 18 dpf followed by open field tests at intermediate stages after CUS (19 dpf) (control: n = 62; stressed: n = 50). Fish of additional set were left undisturbed after the end of CUS tested with tall tank (n = 118; stressed: n = 81) and novel tank (n = 126; stressed: n = 90) tests at late stages after CUS (25 dpf) to assess the persistence of anxiety-like behaviors. For CUS, five stressors were used. Briefly, from 10 to 17 dpf, the fish in the stressed group were exposed to two stressors (randomly assigned) per day, applied at random times between 8 am and 8 pm to maintain unpredictability. Fish in the control group remained undisturbed except routine feeding and tank cleaning.
like behaviors. For CUS, five stressors were used according to the previously reported (Fig. 1): (i) Chasing: put the fish into a 250 ml tank with circulating water (n = 30), and rotate it clockwise and uniformly along the tank wall with a plastic dropper for 5 min (ii) Turbulences: put the fish into a 250 ml tank (n = 30) with two bubble stones respectively located at the left and right bottom. After the power supply of the air pump is turned on, an increased air bubbling lasts for 3 min followed by resting for 1 min, and repeats three times. (iii) Hyperosmotic shock: put the fish into a 250 ml tank (n = 30) with 100 mM NaCl for 10 min, and then wash with circulating water for 3 min / time, three times in total. (iv) pH drop: put the fish into a 250 ml tank (n = 30) with pH = 4 solution for 3 min and then wash with circulating water for 3 min / time, a total of 3 times. (v) Light flashes exposure: the light flashes were produced by a custom-made panel of white LEDs controlled by a microcontroller board (Arduino Uno). Put the fish into a 70 ml dish (n = 30) with circulating water, first adapt to the dark conditions for 3 min, and then start flashing the lamp for 10 min (6 MW / cm² light flashes and 5 Hz). Briefly, at 10 dpf, fish were randomly assigned to control or stressed groups and housed in the nursery tank. From 10–17 dpf, the fish in the stressed group were transferred to the holding tank to expose to two stressors (randomly assigned) per day, applied at random times between 8 am and 8 pm to maintain unpredictability. Fishes in the control group remained undisturbed except routine feeding and tank cleaning.

2.3. Behavioral analysis

Fish were tested during the light cycle in a room with a HD digital camera, and were transferred to the testing area at least one hour before the initiation. Tracking of fish behavior was done by using the ImageJ with wrMTrck plugin [28]. Detailed information about the test tools is provided in Supplementary file 1.

2.4. Tall tank test

It had been demonstrated that the tall tank test led to greater differences in behavioral responses between individuals therefore improved its response reliability in adult fish [22]. There has no report about anxiety-like behavior in zebrafish larvae assessed by using the tall tank test. Here, we scaled the tall tank size to be suitable for larvae (Fig. 3A) [22]. Video tracking for 15 min immediately after placed in the tank and the first 5 min was used for analysis [29]. We also divided the tall tank into top, middle and bottom zones for analysis as previously described [22,30].

2.5. Open field test

Open field test utilizes the innate avoidance of novel open space to measure anxiety-like behaviors. By using an individual petridish (diameter = 3.5 cm, depth = 1.8 cm) (Fig. 3H), a single fish was introduced into the central area and video-tracked for 10 min. The time spent in the peripheral zone was evaluated. To calculate the thigmotaxis index, the petridish were divided into three concentric circles of equal distance, the ratios of time the fish spent in the outermost-circle zone vs total test time represents the anxiety-like phenotype probability [31,32].

2.6. Novel tank test

The experiment was carried out in a small water tank (6.3 cm height X 12.8 cm length X 1.2 cm width), as shown in Fig. 4A. The tank is divided into three equal virtual horizontal sections marked by dividing lines on the outer wall. The behavior was recorded for 10 min (6 fishes per test) with the last 8 min were included in the analysis. The positions of fishes were detected by ImageJ ROI manager plugin. The vertical positions were calculated by averaging the normalized vertical positions of all fish (bottom = 0, surface = 1 ; every 0.3 s). The ratios of fish in the bottom third (every 30 s) and interindividual distances (every 0.3 s) were measured of each group.

2.7. Shoaling behavior test

The shoaling behavior test was estimated with/without acclimation [16]. Briefly, 8–10 fish per group were released into the center of a transparent disk, and their behaviors were immediately recorded for 10 min (without acclimation) and for another 20 min (with acclimation). The camera was fixed at the top of the swimming tanks. Interindividual distance is the average of all distances between individuals in a...
2.8. In vivo electrophysiological recording

In vivo electrophysiological recordings were prepared as previously described [33]. Larvae were transferred to recording chamber with 50 μL tricaine (E10521, Sigma) to anesthetize for 5 min. By using ~200 μL low melting point agarose (1%), the larvae were quickly adjusted to dorsal up position using tweezers under an upright stereoscopic microscope and then were immersed in 3–4 ml external solution. Removing the agarose from the top of the target brain area, and then we used a plastic pipette to suck out excess agarose and exposed the surface of the brain tissue by using forceps to tear the skin along the midline of two tectal hemispheres. After removing the larvae to recording setup, we checked whether the surgery was good enough for recording at a high magnification objective. The chamber perfused with external solution at a rate of ~2 ml/min (23–25 °C) continuously. The extracellular solution contained the following (in mM): 134 NaCl, 2.9 KCl, 2.1 CaCl\(_2\), 1.2 MgCl\(_2\), 10 HEPES, and 10 glucose (pH = 7.8, adjusted with NaOH, 290 mOsmol/l).

Whole-cell voltage/current-clamp recordings from cerebellar neurons in zebrafish were obtained [33,34]. In brief, recording electrodes
were pulled from borosilicate glass (1.5 mm OD, 0.84 mm ID; World Precision Instruments, USA) with a micropipette puller (PC-100; NARISHIGE, Japan). The patch pipette filled with intracellular solution (3–7 MΩ) containing the following (in mM): 110 KCl, 6 NaCl, 2 CaCl$_2$, 2 MgCl$_2$, 10 HEPES, and 10 EGTA (pH = 7.4, adjusted with KOH, 270 mOsmol/l). Recording signals were amplified with an Axopatch 700B amplifier and pCLAMP software (Molecular Devices, USA). The holding potential for recording excitatory post-synaptic currents (EPSCs) was 70 mV. Whole-cell recordings were sampled at 10 kHz and low pass filtered at 8-pole Bessel. Recording can be accepted if the series resistance is between 10 and 30 MΩ and varied < 20% throughout the recording. Recordings were discarded if the rest membrane potential (RMP) > -40 mV. All data were off-line analyzed by pCLAMP. The MiniAnalysis Program (Synaptosoft Inc., USA) was used to analyze inter-event interval and amplitude of EPSCs. As hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are widely distributed in the nervous system, we also measured the inward or outward Na$^+$-K$^+$ currents ($I_h$ or $I_A$). Once the membrane is hyperpolarized, the HCN channel generates a $I_h$. The $I_A$ was obtained by applying voltage steps starting from – 84 mV to + 66 mV.

2.9. Statistical analysis

We used Image J software to analyze the behavior tracking data. Statistical analysis was performed by GraphPad Prism 8.0 (GraphPad Software, USA). The normal distribution of data was tested by the

Fig. 4. CUS led to reduced exploration behavior and impaired social cohesion. A Novel tank test was used to assess anxiety-like behavior. B Typical images showing the position of control (left) and stressed (right) fish at the beginning of the novel tank test. The novel tank is placed vertically. The solid line at the top represents the water surface (surface = 1) and the bottom third of the tank indicates by a dotted line. The arrows represent the fish. C The vertical positions of all fish in the novel tank test. D Proportion of fish in the bottom third of the tank. E The mean distance between individuals for all fish. F Shoaling was tested for 20 min in a horizontal arena. G Typical images display the positions of stressed and control fish. The arrows show the fish’s positions. H The average distances between inter-individuals for all fish. I-J Total swimming distances and average velocities of both groups for 10 min. Data are presented as mean ± SEM. * P < 0.05; ** P < 0.01; **** P < 0.0001.
Kolmogorov-Smirnov test. The comparison was made with Student’s two-tailed t test or non-parametric Mann-Whitney U test. Multiple linear regression analysis (Enter method) was used to analyze the correlation between the significantly different electrophysiological indexes and the behaviors and stressors (SPSS 23.0, IBM, Armonk, NY, USA). Data were presented as mean ± standard error of the mean (SEM). A P-value < 0.05 was considered significant.

3. Results

3.1. The survival of zebrafish was significantly decreased during CUS

During CUS from 10 to 17 dpf (corresponding to 0–7 d post-CUS), the survival in the control group decreased from 100% to 75%, while the survival in the stressed group decreased rapidly to 55% (Fig. 2, the stressed group vs the control group: \( P = 0.016 \)). After CUS, there was no significant difference in survival between the stressed and control group.

3.2. CUS induced anxiety-like behaviors in zebrafish

Our study implemented a custom-designed tall tank to evaluate anxiety-like behaviors one day post-CUS (Fig. 3 AB). Zebrafish in the control group had an average swimming trajectory in the tall tank test; while, stressed group spent more time in the bottom zone \( (P < 0.01, \text{degree of freedom (DF)} = 208, t = 5.008, \text{Fig. 3 CD}) \) and showed less entries into the top zone \( (P < 0.01, \text{DF} = 208, t = 2.743, \text{Fig. 3 E}) \). Zebrafish freezing time of stressed group was significantly higher than that of control group \( (P < 0.01, \text{DF} = 208, t = 2.763, \text{Fig. 3 F}) \). In

Fig. 5. Anxiety-like behaviors and impaired social cohesion recovered to the normal level 8 d post-CUS. A Typical swimming trajectory of stressed and control fish during the tall tank. B Total distances travelled by the fish in top, middle and bottom zone. C The spent time of fish at top, middle and bottom of the tank. D The number of entries into the top and bottom zone. E Fish freezing times of both groups, with less than 2.5 mm locomotion defined as a ‘freezing’ event. The probability of freezing events in both groups. G Typical images display the positions of both groups 8 d post-CUS in novel tank. The arrows show fish’s positions. H The vertical positions of all fish in the novel tank test. I Proportions of fish in the bottom third of the tank. J The mean distances between individuals for all fish. Data are presented as mean ± SEM. * \( P < 0.05 \).
addition, zebrafish in the stressed group had a 60% probability of freezing event, significantly higher than that 20% in control group ($P < 0.05$, Fig. 3G). Next, we used open field test to determine whether CUS caused anxiety-like behaviors two days post-CUS by analyzing the thigmotaxis index of freely swimming fish in a circular petri dish (Fig. 3I). The thigmotaxis index of fish in the stressed group was significantly higher than that in the control group ($P < 0.05$, $DF = 110$, $t = 2.002$, Fig. 3J). There was no significant difference between the stressed and control group from total distance to average velocity during the tall tank and open field tests ($P > 0.05$, $DF = 110$, $t = 0.946$, Fig. 3KL).

3.3. CUS led to reduced exploration behavior and impaired social cohesion

In the novel tank test, zebrafish are usually less exploratory, the longer they stay in the lower half zone of the tank and the less they enter the upper half zone, suggesting that they are experiencing anxiety from a changing environment (Fig. 4B) [35]. The vertical position of all fish in the stressed group was significantly lower than that in the control group ($P < 0.05$, $DF = 112$, $t = 2.534$, Fig. 4C), and had a higher proportion of fish in the bottom third of the tank ($P < 0.05$, $DF = 19$, $t = 2.293$, Fig. 4D). We also evaluated the distances between individuals indicating their social interaction behavior among conspecifics. As shown in Fig. 4E, the stressed group had a larger mean distance between individuals than control group ($P < 0.05$, $DF = 270$, $t = 2.113$). We further analyzed the social cohesion in horizontal direction (Fig. 4G), and found that zebrafish in the stressed group were more inclined to swim alone and lack social interaction as the average distance between inter-individuals was significantly higher than that in the control group ($P < 0.01$, $DF = 576$, $t = 2.923$, Fig. 4H). The total swimming distance and average velocity in stressed group was also significantly lower than those in control group during the novel tank test ($P < 0.0001$, $DF = 155$, $t = 8.941$, Fig. 4IJ).

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**Fig. 6.** Effects of CUS on electrophysiological properties of cerebellar neurons. A. Infrared DIC images of the cerebellar region in zebrafish at low and high magnification. B–D. Bar plots show the RMP, $C_m$, and $R_{in}$ of cerebellar neurons in the control ($n = 13$, $N = 6$) and stressed fish ($n = 7$, $N = 5$). E. Typical recording displays spontaneous spiking activities of a cerebellar neuron in the control and stressed fish. F–I. Bar plots illustrate the amplitude, frequency, time to peak and half-width of AP of cerebellar neurons in the control ($n = 8$, $N = 4$) and stressed fish ($n = 6$, $N = 4$). Data are presented as mean ± SEM. * $P < 0.05$. ** $P < 0.01$. 

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3.4. Anxiety-like behaviors and impaired social ability recovered to the normal level 8 d post-CUS

We carried out tall tank (Fig. 5A) and novel tank tests (Fig. 5G) 8 d post-CUS to explore possible persistent effects of CUS on anxiety-like behaviors. We found that in the tall tank test, except a higher freezing time in the stressed group ($P < 0.05$; $DF = 197$, $t = 2.363$, Fig. 5 E), there was no significant difference between the stressed and control group of the duration spent in each part of tank ($P > 0.05$; $DF = 197$, $t = 0.614$ (top); $DF = 197$, $t = 0.280$ (middle); $DF = 197$, $t = 2.568$ (bottom); Fig. 5 BC), the amounts of entries into upper and lower parts of the tank ($P > 0.05$; $DF = 197$, $t = 0.279$ (top); $DF = 197$, $t = 0.675$ (middle); $DF = 197$, $t = 0.647$ (bottom); Fig. 5 D), and the probability of freezing events ($P > 0.05$, Fig. SF). In the novel tank test (Fig. 5G), there was no statistically significant difference between the stressed and control group in various indicators including vertical positions of all fish (Fig. 5 H, $DF = 214$, $t = 1.158$), ratios of fish in bottom third (Fig. 5 I, $DF = 36$, $t = 0.360$), and interindividual distances (Fig. 5 J, $DF = 515$, $t = 1.151$) ($P > 0.05$ respectively). Our results indicated that neither the anxiety-like/exploration behavior nor the social ability was disturbed one week after CUS.

3.5. Effects of CUS on electrophysiological properties of cerebellar neurons

To investigate the effects of CUS on electrophysiological properties of cerebellar neurons, a series of in vivo whole cell recordings were performed in the stressed zebrafish 1 d post-CUS (Fig. 6A). It was interesting that CUS significantly decreased the RMP ($P < 0.01$; $DF = 19$, $t = 3.145$) when compared to the control group. Other membrane properties, such as membrane capacitance ($Cm$) and Rin (input resistance) were not significantly affected ($P > 0.05$; $DF = 19$, $t = 1.192$ ($Cm$); $DF = 19$, $t = 0.867$ (Rin); Fig. 6 B-D). Furthermore, for the spontaneous spikes (Fig. 6 E), CUS resulted in lower amplitude ($P < 0.05$, $DF = 12$, $t = 2.698$), frequency ($P > 0.05$, $DF = 12$, $t = 2.698$), time to peak ($P < 0.01$, $DF = 12$, $t = 4.296$), and half-width of action potential (AP) ($P < 0.01$, $DF = 12$, $t = 4.584$) respectively compared to the control group (Fig. 6 F-I).

CUS induced a dramatic decrease in EPSC amplitude of cerebellar neurons ($P < 0.01$, $DF = 14$, $t = 1.002$), but not for the frequency ($P > 0.05$, $DF = 14$, $t = 0.212$) (Fig. 7 AB). Cumulative distribution analysis demonstrated that CUS shortened the amplitude of EPSCs (Fig. 7 B). Finally, we found that the $I_h$ and $I_A$ was significantly affected by CUS ($P < 0.05$; $DF = 19$, $t = 2.398$ ($I_h$); $DF = 19$, $t = 1.695$ (Rin); Fig. 7 D), indicating that CUS activated HCN channels and blocked potassium channels in cerebellar neurons (Fig. 7 E).

We further analyzed the correlation between the significantly different electrophysiological indexes (Figs. 6 and 7) and the behaviors and stressors in the stressed group. Multiple linear regression analysis showed that the total distance at the bottom (tall tank test) was correlated positively with $I_h$ ($P = 0.028$, $DF = 6$), that is, the greater the amplitude of $I_h$, the greater the total distance in bottom; the thigmotaxis index (open field test) correlated with AP amplitude ($P = 0.03$, $DF = 5$), that is, the larger the AP amplitude is, the larger the trend index is. There

Fig. 7. CUS induced a dramatic decrease of EPSC’s amplitudes in cerebellar neurons. A Representative EPSCs recordings of cerebellar neurons in control and stressed fish. B CUS induced a significant shift (leftward) of the distribution of amplitudes but not for inter-event intervals of EPSCs. C Summarized data showing that CUS rapidly decreased the amplitude of EPSCs but did not affect inter-event intervals (control: $n = 9$, $N = 4$; stressed: $n = 7$, $N = 3$). D Representative $I_h$ and $I_A$ traces in response to voltage stimulations. E CUS activated HCN channels and blocked potassium channels in cerebellar neurons (control: $n = 14$, $N = 6$; stressed: $n = 7$, $N = 4$). Data are presented as mean ± SEM. * $P < 0.05$; ** $P < 0.01$. 

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4. Discussion

Although several CUS models have been established in zebrafish inducing an explorative and scototaxis (dark/light preference) neuro-behavioral response [9,16,17], i.e., an anxiety-like behavior, so far there has been no reported using the tall tank test to evaluate anxiety-like behaviors for CUS-larvae. Here, we established a CUS model, demonstrated through the tall tank test combined with novel tank, open field and shoaling behavior tests and demonstrated that these zebrafish exhibited anxiety-like behaviors and social impairments.

In this study, we found that zebrafish with an 8-ν-CUS exposure (10–17 dpf) induced a transient anxiety-like behavior which not manifested 5 d post-CUS, which is in line with many other reports of chronically stressed fish [16,19,36,37]. So far, there have been a large number of CUS models established in zebrafish using multiple stressors (conditions) or treating with a variety of duration [9,16,17]. Basing on previous reports, the fish was usually subjected to 2–9 CUS conditions for 7–21 d, and was exposed to two stressors (randomly assigned) per day, applied randomly between 8 am and 8 pm to maintain unpredictability [9,16,17,36]. In this study, we applied a relatively long CUS duration (8 d) and the more stressors (5 types) [16], but whether this early-life CUS would affect their long-term outcomes needing further experiments. Generally, early-life stress can lead to two different behavioral responses: (i) increased susceptibility to psychiatric disorders or (ii) resilience. Barbara, et al. created a CUS protocol to assess the effects of early experiences in adult zebrafish and found that zebrafish exposed to 7 d of CUS showed a decreased anxiety-like phenotype in adult; while fishes exposed to 14 d of CUS showed an opposite anxious phenotype compared to 3 and 7 d of CUS in adult [38]. The outcome of early life stress is related to the intensity and duration of the stressor, and whether it changes the neural circuit of anxiety. Although the current findings did not find any anxiety-like behavior at 25 dpf (8 d after CUS), it might take some time for the brain to form an anxiety neural circuit, and then the anxious phenotype appears in adulthood.

Stressed fish also showed a reduced exploration of a novel environment in the present study, as supported by the literature [18,19,36]. It has been demonstrated that larvae prefer deep water, and this behavior could already emerge in the first week of life [39]. The novel tank test is widely used to characterize adult and young fish anxiety phenotypes [20,21], that exploits the fish’s natural diving response when placed in a new environment [39]. We also observed a robust diving response in larvae at 18 – 20 dpf (data not shown) and was consistently enhanced by the stress (Fig. 4C and D), which is in line with the previous reports [16]. Usually, the time spent around the wall indicates the anxiety-like behavior of fish [14,15]. It has been demonstrated that by using the tall tank test could lead to greater differences in behavioral responses between individuals therefore improved its response reliability in adult fish [22]. In this study, we modified the model size for <21 dpf zebrafish and found that CUS induced an anxiety-like behavior as these fishes spend more time at the bottom, reluctantly entered into the upper zone (Fig. 3C - G). This was quite consistent with those anxiety-like behaviors observed in novel tank and open field tests [19,37,40–42]. When comparing the duration and total distance in the bottom zone in tall tank test with those in the novel tank test, we found that both were significantly higher than that in the novel tank test of the stressed fishes (Supplementary file 2 Fig. S1AB). We observed a robust diving response in larvae enhanced by stress in tall tank test (Fig. 3CD and Supplementary file 2 Fig. S1AB). Our results indicated that the tall tank test is a more suitable assay for measuring stress-induced anxious states in young zebrafish. Shoaling behavior test is also an important indicator for anxiety states. Previous study found that CUS had no effect on shoaling for larvae [16]. Not similar to this, we found that CUS resulted in a weakened shoaling for larvae (Fig. 4H), which might be due to CUS induced a reduced social communication [40,43,44]. In our novel tank test, the increased inter-individual distances in stressed group also proved this social impairment in CUS-fish (Fig. 4E). There was a discrepancy about CUS duration on this social cohesion: 7 ν-CUS increased but 14 ν-CUS reduced social cohesion [17], while 15 ν-CUS had no effect on social cohesion [45]. This may be due to the heterogeneity of social and anxiety-related behavior among zebrafish strains [43], or different experimental conditions. Altogether, our results not only established that an 8 ν-CUS caused anxiety-like behaviors and social impairments in zebrafish, but also revealed that these kinds of behaviors varied in different strains and ages of zebrafish as well as in different tests being used.

Another important factor to consider when assessing anxiety-like behavior is basal locomotion. Stressors, such as dark-light transition test may affect the basal locomotion in larvae. This change in reactive basal locomotion reflects an adaptive ability of fish to optimize

**Fig. 8.** Correlations of the significantly different electrophysiological indexes and the behaviors and stressors in the stressed group. A Multiple linear regression analysis showed that the total distance in bottom (tall tank test) was correlated positively with $I_c (P = 0.028, DF = 6)$. B The thigmotaxis index (open field test) correlated with AP amplitude ($P = 0.03$, DF = 5). Pearson correlation analysis between the total distance in bottom and $I_c$ in tall tank test (C) and in open field test (D); between the thigmotaxis index and AP amplitude in tall tank test (E) and in open field test (F).
showed that the basal locomotion of CUS group had significantly decreased compared to the control group during the shoaling behavior test but not for tall tank, novel tank and open field tests i.e., this reduced basal locomotion might indicate CUS-fish had developed a new adapting pattern or might due to the order of exposed-stressors.

In addition, we know little about the changes of brain sensitive regions and neural circuits after the CUS. There are increasing evidences showing that cerebellum might play more roles in brain function such as cognition, memory and emotion regulation [26,49], than previously thought [25,49]. To explore underlying mechanisms of CUS-induced anxiety-like behavior, in vivo whole-cell recordings were made on cerebellar neurons in zebrafish. We found that CUS increased the excitability of cerebellar neurons. It has been demonstrated that early postnatal tobacco smoke exposure triggers anxiety-like behavior and reduces synaptic protein expressions in the cerebellum [50], which might affect synaptic transmission and firing activity of neurons, results in a decreased amplitude of EPSCs and spontaneous firing of cerebellar neurons as we found after the CUS. Rodent models have demonstrated that CUS can lead to reduced synaptic plasticity and dendritic spines, reduced dendrite length and complexity, and impaired neurogenesis in multiple brain regions including hippocampus, amygdala and prefrontal cortex [51]. We found that the total distance at the bottom (tall tank test) was correlated positively with $I_{T, I}$, that is, the greater the amplitude of $I_{T, I}$, the greater the total distance at the bottom; the thigmotaxis index (open field test) correlated with AP amplitude, that is, the larger the AP amplitude is, the larger the trend index is. These novel results have not been reported previously. Whether modulating these properties of cerebellar neurons can alleviate the anxious phenotype; how the brain to form an anxiety neural circuit involving the cerebellum after CUS, needs further study, which might provide new targets to treat emotional diseases.

The main limitation of this study is that we did not know the long-term outcomes (both behavioral and pathological) of these fishes with early life CUS. Also, underlying mechanisms of alterations in electrophysiological properties of cerebellar neurons caused by the CUS, such as molecular and morphologic regulations remain uncertain.

In the present study, early life CUS transiently induced an anxiety-like behavior which could be more accurately assessed by combining the tall tank test with other behavior tests. CUS increased the excitability of cerebellar neurons might providing new targets for treating emotional diseases such as anxiety.

Ethics approval
The study was approved by the Animal Care and Use Committee at Zhejiang University School of Medicine (16779). All experiments using zebrafish were performed in accordance with the Guide for the Care and Use of Laboratory Zebrafish by the China Zebrafish Resource Center.

Consent for publication
Not applicable.

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Authors’ contributions
W-J and J-KW designed the experiments; W-J and Y-BH performed the main experiments; Y-BH, Z-PL, B-MY and S-J performed the behavioral test analysis with the help of W-D; W-J performed the electrophysiological data analysis; W-J, W-JP, L-ZX and J-KW wrote and revised the manuscript. All authors approved the final version of the manuscript and agreed to be accountable for all aspects qualify for authorship.

Competing interests
The authors declare that they have no competing interests.

Data Availability
Data will be made available on request.

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