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Sound shock response in larval zebrafish: A convenient and high-throughput assessment of auditory function

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ABSTRACT

Given that hearing ability can be challenged in diverse ways, it is necessary to develop an easily conducted, high-throughput method for assessing potential auditory risks. Measuring the acoustic startle response (ASR) has become a critical behavioral method in hearing research using zebrafish (*Danio rerio*). In this study, changes in the activity of zebrafish larvae (10 days post fertilization (dpf)) due to exposure to a sudden easily-generated broad-band noise were automatically and objectively recorded and analyzed without building sophisticated equipments. A significant increase in activity was induced by the noise stimulation and the alterations were impaired by gentamicin. In addition, a clear dose-response trend was observed between gentamicin exposure and the impaired activity, and a similar phenomenon was observed between gentamicin exposure and damage to hair cells. Our results suggested that alterations in the activity induced by a broad-band noise can potentially be used as an efficient assay for assessing hearing ability.

1. Introduction

Hearing disability is commonly due to gene mutations or extrinsic challenges, such as excessive noise exposure and certain therapeutic drugs. The rapid development of the fields of personal genomics and bioinformatics has accelerated the identification of genes and mutations that are potentially associated with deafness. In addition, several kinds of therapeutic drugs have been thoroughly demonstrated to be related to hearing disability, such as aminoglycoside antibiotics and chemotherapeutic drugs (Berg et al., 1999; Qu et al., 2015; Fuchs et al., 2016), implying that other therapeutic drugs may harm hearing. Verifying whether candidate mutations or candidate therapeutic drugs are ototoxic or otoprotective is highly beneficial for the diagnosis and prevention of hearing disability, but it requires high-throughput screening of hearing disabled animals.

Although the zebrafish auditory system is not as developed as that of humans, the two systems share many similarities. Zebrafish have otolithic vestibular organs, such as the saccule and utricle, that are similar to mammalian vestibular organs (Popper and Fay, 1973; Yao et al., 2016), and the mechanosensory hair cells of the inner ear transduce mechanical stimuli into electrical impulses, which are centrally conveyed (Hudspeth, 2005). Furthermore, hair cell death is a common denominator of irrecoverable sensory input loss in many forms of hearing impairment (Gillespie and Muller, 2009), and zebrafish also have hair cells in the lateral line that are organized into neuromasts and remarkably similar to that of inner ear (Williams and Holder, 2000; Owens et al., 2007). These hair cells can be directly manipulated and observed.

In addition to cellular detection, electrophysiological techniques, such as recording the auditory evoked potential and microphonic potential, have been useful for assessing the auditory capabilities of zebrafish (Nicolson et al., 1998; Lu and Tomchik, 2002; Ladich and Fay, 2013; Yao et al., 2016). While these methods are rapid and precise, the fish must be physically restrained, and recording potentials involves invasive surgeries that are difficult to perform.

Therefore, noninvasive and non-restraining methods of behavioral analysis are becoming increasingly ideal for the evaluation of hearing ability. As early as the beginning of the 19th century, conditioning methods involving pulse trains, similar to Pavlovian or respondent conditioning, were used to assess the hearing ability of fish (Parker, 1902, 1903), but it was time-consuming to train fish in response to sound. Then, in 1970s, larval zebrafish at 5 days post fertilization (dpf) were found to perform a “C-start” startle response in reaction to vibration from a spring-generated click (Kimmel et al., 1974). Since then,

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various kinds of acoustic startle responses (ASR) have been applied to assess the hearing ability of fish, such as the rapid escape reflex (Bang et al., 2002; Gibb et al., 2006; Colwill and Creton, 2011) and prepulse inhibition (Bhandiwad et al., 2013). Meanwhile, most of the assays mentioned above depend on a high-speed camera, such as 1000 frames per second (fps). With the rapid development of automated methods to measure and analyze animal movements, objective determinations of responses can now be achieved with an image analysis program that compares images taken with a normal CCD video camera with a speed at 25 fps before and after the presentation of a stimulus.

In this work, zebrafish larvae were stimulated with a broad-band noise, and changes in activity, i.e., the variation in ASR, were quantified to assess hearing ability. The activity of larvae were automatically and objectively measured before and after sound stimuli and then analyzed with commercial equipment. Gentamicin, a well-known ototoxic drug, was then used to validate this method.

2. Materials

2.1. Animal care

Zebrafish eggs were obtained by random mating between sexually mature individuals (AB strain) and then treated with pronase to remove the external membrane. Beginning at 5.5 h post fertilization (hpf), the naked eggs were randomly grouped into different exposure conditions (described below in Section 2.2) and raised in 90-mm dishes coated with agarose at 28.5 °C under a 14:10-h light:dark cycle (lights on at 08:00 AM). Then, at 7 dpf, the larvae that were developing normally were selected and transferred to 3 L tanks and raised with *Paramecium caudatum* until behavioral testing at 10 dpf. All procedures complied with local and international regulations for animal experimentation, and the protocols were approved by the National Institutes of Health guide for the care and use of Laboratory animals (http://oacu.od.nih.gov/UsefulResources/APD_Zebrafish.pdf) and the institutional animal care committee of the Children's Hospital of Fuda University.

2.2. Chemical exposure

Gentamicin sulfate (A506614-0005, Sangon Biotech, China) was mixed with water from the zebrafish system to appropriate concentrations before the experiments, and the larvae were exposed to gentamicin at either 2.6 μM or 14 μM from 5.5 hpf to 72 hpf; the drug was refreshed every day. Eggs or larvae from each group were inspected daily under a dissection microscope during exposure, and eggs with arrested development or obvious malformations were excluded. At the end of the exposure period, the zebrafish were carefully rinsed with fresh water from the experimental system three times before being transferred back to the normal rearing conditions.

2.3. Acoustic startle response

2.3.1. Noise exposure

Two intermittent noise stimulations were used in the experiments. Larvae in 24-well plates were exposed to a broad-band noise at 96 dB in a ZebraBox (ViewPoint Life Sciences); the background/baseline sound was 62 dB. The broad-band noise was computer generated and played through two commercial loudspeakers in air, which were placed in the chamber, but not physically connected to the chamber (Fig. 1), and the sound intensity within the box was measured with a noise detector (AS804, Smart Sensor). The noise exposure procedure is described in detail in Fig. 2.

2.3.2. Behavioral assessment

Behavioral tests were carried out with 10 dpf zebrafish larvae in 24-well plates. All experiments were performed 2 h after the beginning of the light cycle and 2 h before the beginning of the dark cycle.

The zebrafish larvae were carefully transferred to a 24-well plate; a single larva and 1 mL of system water was placed in each well. The plate was then placed into a ZebraBox (ViewPoint Life Sciences) which is equipped with infrared illumination for imaging in the dark.

To eliminate the influence of visual stimulation, the entire experiment was conducted in a completely dark environment. After a 30 min acclimatization period, zebrafish were monitored under ambient sound for 5 min to capture the background/baseline larvae activity counts. This protocol and that of the experimental procedure are shown in Fig. 2.

Zebrafish activity was quantified using the quantization mode of ZebraLab software as previously described (Liu et al., 2014). The videos were taken at 25 fps and pooled into 1 s time bins to assess the ASR and into 1 min time bins to assess the visual motor response (VMR).

2.4. Dye labeling and imaging

After the behavioral tests, the larvae were labeled with FM1-43 (F-35355, Invitrogen) to assess the functional status of the hair cells in the lateral lines. Before the experiments, FM1-43 was dissolved in sterilized water to a concentration of 200 ng/μL and kept at −20 °C in the dark. The working FM1-43 solution was freshly diluted from concentrated stock to 4 ng/μL with PBS before labeling. The larvae after behavioral tests were immersed in FM1-43 for 90 s followed by 3 washes with PBS for 90 s each.

After labeling, stained larvae were anesthetized with 200 mg/L tricaine methanesulfonate (A5040-25G, Fluka), and the number of the neuromasts in the zebrafish lateral line was observed randomly under an epifluorescence microscope (M205 FA, Leica) and photographed.

2.5. Data presentation and statistical analysis

Data are presented as the mean ± SEM. Statistical analyses were performed and graphs were created using GraphPad Prism software (version 5.0).

One-way ANOVA followed by Dunnett's multiple comparison post hoc tests was performed to compare the gentamicin-treated groups with the controls to assess the effects of gentamicin on the ASR, and a probability level of 5% was used as the minimal criterion of significance.

Student's *t*-tests (two-tailed) were performed to analyze the behavioral changes in response to noise and light stimulation within each gentamicin concentration group. The minimum criterion for significance was 5%.

3. Results

3.1. Exposure to a sudden broad-band noise induced a significant increase in the activity of zebrafish larvae

An innate response was induced by a broad-band noise and evaluated according to the changes in activity counts. As shown in Fig. 3A, when experiencing a sudden broad-band noise at 96 dB for the first time, zebrafish in the non-drug treated control groups exhibited significantly higher activity than before the noise exposure (see Movie S1); the larvae activity counts fluctuated between 20 and 40 before noise exposure and then climbed to 90 after exposure. After recovering for 5 min, a second exposure to the sudden noise stimulus also resulted in a significant and robust increase in the larvae activity counts that were similar to that during the first noise exposure (see Movie S2). The results indicated that a significant increase in the activity counts of zebrafish larvae could be elicited by a sudden broad-band noise.