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Research report

Chronic fluoxetine treatment induces anxiolytic responses and altered social behaviors in medaka, *Oryzias latipes*

Satoshi Ansai[a,*], Hiroshi Hosokawa[b,**], Shingo Maegawa[b], Masato Kinoshita[a]

[a] Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan
[b] Department of Intelligence Science and Technology, Graduate School of Informatics, Kyoto University, Yoshida-Honmachi, Sakyo-ku, Kyoto 606-8501, Japan

HIGHLIGHTS

- Behavioral effects of fluoxetine on medaka were evaluated using five paradigms.
- Chronic fluoxetine treatment induced anxiolytic responses.
- Chronic fluoxetine treatment altered socially-evoked behaviors.
- Chronic fluoxetine treatment reduced horizontal but not vertical locomotor activity.

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ABSTRACT

Medaka (*Oryzias latipes*) is a small freshwater teleost that is an emerging model system for neurobehavioral research and toxicological testing. The selective serotonin reuptake inhibitor class of antidepressants such as fluoxetine is one of the widely prescribed drugs, but little is known about the effects of these drugs on medaka behaviors. To assess the behavioral effects of fluoxetine, we chronically administrated fluoxetine to medaka adult fish and analyzed the anxiety-related and social behaviors using five behavioral paradigms (diving, open-field, light–dark transition, mirror-biting, and social interaction) with an automated behavioral testing system. Fish chronically treated with fluoxetine exhibited anxiolytic responses such as an overall increased time spent in the top area in the diving test and an increased time spent in center area in the open-field test. Analysis of socially evoked behavior showed that chronic fluoxetine administration decreased the number of mirror biting times in the mirror-biting test and increased latency to first contact in the social interaction test. Additionally, chronic fluoxetine administration reduced the horizontal locomotor activity in the open-field test but not the vertical activity in the diving test. These investigations are mostly consistent with previous reports in the other teleost species and rodent models. These results indicate that behavioral assessment in medaka adult fish will become useful for screening of effects of pharmaceutical and toxicological compounds in animal behaviors.

1. Introduction

Medaka, *Oryzias latipes,* is a small freshwater teleost fish that originally inhabited East Asia and has been used as a vertebrate model in physiology, embryology, and genetics for the past 100 years [1]. Its biological advantages as a model system are mostly similar to those of zebrafish (*Danio rerio*): short generation time (2–3 months), transparency of embryos, and availability of genomic information [2,3]. There are several additional advantages in medaka over zebrafish such as smaller genome size (800 Mb), availability of highly polymorphic inbred strains, and viability over a wide temperature range (6–40 °C) [3]. These features contribute to the wide use of medaka as an aquatic (eco) toxicological model [4]. More recently, it has become clear that medaka exhibits complex social and/or visually-evoked behaviors, such as shoaling/schooling [5–7], aggressive behaviors [8], mating preference [9,10], mate-guarding behaviors [11], social learning [12], mirror approaching [13], predation [14], and startle response [15]. These behavioral features serve as an emerging model system for neurobehavioral research.

The selective serotonin reuptake inhibitor (SSRI) class of antidepressant such as fluoxetine is one of the most widely prescribed groups of pharmaceuticals, which block serotonin reuptake into

* Corresponding author. Fax: +81 75 753 6400.
** Corresponding author.
E-mail addresses: ansai@kais.kyoto-u.ac.jp (S. Ansai), hosokawa@i.kyoto-u.ac.jp (H. Hosokawa).

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the presynaptic cells by serotonin transporter and therefore effectively increases the extracellular serotonin levels in the synaptic clefts [16]. Various studies have demonstrated the presence of these pharmaceuticals in environmental waters and their diverse effects on aquatic organisms [17,18]. Previous reports using medaka have investigated the effects of fluoxetine on embryonic development, reproduction, and estradiol level [19,20] and the uptake and bioaccumulation [21,22]. However, there is few studies on the behavioral effects of fluoxetine in medaka except to a report about altered photomotor response in hatching larvae [23], while the effects on adult complex behaviors have been shown in some teleost species such as aggressive behavior in the bluehead wrasse (*Thalassoma bifasciatum*) [24], the Siamese fighting fish (*Betta splendens*) [25], and the Gulf toadfish (*Opsanus beta*) [26], locomotor behavior in Chinook salmon (*Oncorhynchus tshawtscha*) [27] and sheepshead minnow (*Cyprinodon variegates*) [28], and anxiety-related behavior in the zebrafish [29–31] and fathead minnow (*Pimephales promelas*) [32].

In this study, we aimed to characterize the behavioral alterations induced by chronic fluoxetine treatment in medaka adult fish. The behavioral effects, especially on the anxiety-related and social behaviors, were examined by five behavioral test paradigms including diving test, open-filed test, light–dark transition test, mirror approaching test, and social interaction test that are regarded as effective paradigms to evaluate locomotor activities, anxiety-related behaviors, and social behaviors in teleost [13,29,33–35]. The behavioral data of testing fish was acquired and analyzed by an automated behavioral testing system consisting of a video tracking software and test chambers for automated quantification of the medaka behavior.

2. Materials and methods

2.1. Fish and housing conditions

The d-rR strain medaka fish [36] is maintained as a closed colony with little polymorphisms among the individuals and is a parental strain of a inbred strain Hd-rR whose whole genome was sequenced [2], which is used for a standard laboratory strain in a wide range of scientific research. We used this strain in this study to evaluate the pharmacological effects with minimal effects of both genetic and environmental backgrounds.

Embryos were raised in embryo culture medium (0.1% NaCl, 0.003% KCl, 0.004% $CaCl_2{\cdot}2H_2O$, and $MgSO_4{\cdot}7H_2O$) on a 14/10 h light/dark cycle at 26 °C. Hatched fish were raised in groups of 8–10 fish per 2-L tank with recirculating filtered water at 27 °C. The fish tanks were illuminated by white fluorescent tube on a 14/10 h light/dark cycle. The fish were fed newly hatched *Artemia* nauplii and commercial powdered foods (for larval fish: Sweetfish Super Gold #0, Oriental Yeast, Tokyo, Japan; for juvenile and adult fish: Otohime B2, Marubeni Nisshin Feed, Tokyo, Japan, for juvenile and adult fish) once or twice a day. The 4 month-old fish were subjected to the following pharmacological manipulation and the behavioral testing. The standard length of the examined male or female fish was 20.03 ± 1.20 or 20.22 ± 1.69 mm, respectively (n = 24 each). After the experimentation, all tested fish were sacrificed by deep anesthesia using MS-222 (Sigma–Aldrich, St. Louis, MO). This work was conducted using the protocols approved by the Animal Experimentation Committee of Kyoto University (permission number: 26–71).

2.2. Pharmacological manipulations

Stock solutions of 1 mg/mL fluoxetine hydroxychloride (F132, Sigma–Aldrich) was prepared in distilled water and kept at 4 °C until use. The stock solution was diluted to 100 ng/mL with conditioned water (0.003% Red Sea Salt, Red Sea Aquatics, Houston, TX; 0.05% Tetra Vital, Tetra, Melle, Germany; 0.05% Tetra AquaSafe, Tetra; 0.025% Tetra ContraChlorine, Tetra). Two groups of 12 fish per tank (including both male and female) were exposed to 2 L of fluoxetine-containing water for 10 days with tank water changes every 2 days. Their respective controls were kept in conditioned water without fluoxetine but in otherwise identical conditions.

2.3. Behavioral testing

2.3.1. Apparatuses and software

All apparatuses for behavioral testing were set up in a wooden cube (36 cm height × 36 cm width × 38 cm depth) with a door to stabilize the testing condition. Video images of the test fish were recorded using a USB webcam (DC-NCR300UY, Hanwha, Seoul, Korea) from front of the testing tank (diving and light–dark transition test) or above the tank (open-field, mirror biting, and social interaction test). The testing tanks for the diving and light–dark transition test were illuminated using an LED panel light (300 lx) attached to the top panel, and the tanks for other paradigms were illuminated four light bars (550 lx) attached to each side panel at 75 mm height from the bottom panel.

Behavioral data were acquired and analyzed by custom-made software with OpenCV computer vision library. Images were captured every 100 ms. Captured images were subtracted background image and binarized. The calculated gravity points of binarized fish objects were used as fish position. All behavioral parameters used in this study including swimming distance and velocity were calculated by the time course of fish position.

2.3.2. Testing procedures

Behavioral testing including five paradigms (open-field, diving, light–dark transition, mirror biting, and social interaction) was performed over five days. Each fish was subjected to open-field and diving test at the first and second days, to light–dark transition and mirror biting test at the third and forth days, and to social interaction test at the fifth day.

Before introducing test fish into the test tank, they were individually introduced in a 1 L pre-treatment tank by a fishnet. The sex of each test fish was determined by anal fin shape, and then was transferred to the tank for the behavioral testing.

2.3.3. Diving test

Diving test is a well-established paradigm to quantify stress responses and anxiety-related behaviors in zebrafish [29]. The test was conducted using a rectangular tank (200 mm length × 50 mm width × 150 mm height) consisting of white acrylic panels except a transparent acrylic panel at the front face and filled with the conditioned water to a height of 120 mm (Fig. 1 A). The tank was divided into five virtual horizontal areas (24 mm height each) (Fig. 1B). Fish were individually introduced into the test tank, and their behaviors were recorded for 10 min to calculate distance traveled (mm), freezing duration (s), the time spent in the top area (s), latency to enter the top (s), the number of entries into the top, and the time spent in the bottom area (s).

2.3.4. Open-field test

Open field test is a behavioral paradigm to assess anxiety levels and locomotor activity in zebrafish [33]. The test was performed in a white acrylic tank (200 mm length × 200 mm width × 75 mm height) filled with the conditioned water to a height of 20 mm (Fig. 2A). The bottom of the tank was virtually divided into center (120 mm × 120 mm, 60% of length and width) and peripheral (the area within 80 mm from the walls) areas (Fig. 2B). Fish were individually introduced into the center of the tank, and their trajectories were recorded for 15 min. Distance traveled (mm), freezing