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Startle response memory and hippocampal changes in adult zebrafish pharmacologically-induced to exhibit anxiety/depression-like behaviors

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HIGHLIGHTS

- Assessment of startle responses and its habituation in adult zebrafish
- Ethanol and fluoxetine impair memory performance in this maze.
- Significant upregulation of hippocampal serotoninergic neurons

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ABSTRACT

Zebrafish (*Danio rerio*) are rapidly becoming a popular animal model for neurobehavioral and psychopharmacological research. While startle testing is a well-established assay to investigate anxiety-like behaviors in different species, screening of the startle response and its habituation in zebrafish is a new direction of translational biomedical research. This study focuses on a novel behavioral protocol to assess a tapping-induced startle response and its habituation in adult zebrafish that have been pharmacologically-induced to exhibit anxiety/depression-like behaviors. We demonstrated that zebrafish exhibit robust learning performance in a task adapted from the mammalian literature, a modified plus maze, and showed that ethanol and fluoxetine impair memory performance in this maze when administered after training at a dose that does not impair motor function, however, leads to significant upregulation of hippocampal serotoninergic neurons. These results suggest that the maze associative learning paradigm has face and construct validity and that zebrafish may become a translationally relevant study species for the analysis of the mechanisms of learning and memory changes associated with psychopharmacological treatment of anxiety/depression.

1. Introduction

There is convincing evidence that fish have significant cognitive ability [3]. Furthermore, environmental complexity has long been known to affect cognitive ability [15] through increased neuron density in the hippocampus or analogous brain structures [18,31,41]. Zebrafish (*Danio rerio*) are one of the most important vertebrate model organisms in genetics, developmental biology, neurophysiology, and the most widely used species of laboratory fish [34]. Zebrafish are also increasingly utilized in behavioral studies, such as investigations into the effects of drug exposure on learning and memory ([20]). In the current study, we used pharmacologically-induced anxious/depressed zebrafish to examine the effects on the behavioral complexity of learning and memory.

Ethanol withdrawal results in some behavioral abnormalities that parallel symptoms observed in human anxiety/depression and some antidepressants can reverse these symptoms. Indeed, most drugs of abuse, including ethanol, generate diverse behavioral and neurochemical effects. However, one feature common to many such drugs is the phenomenon of a "withdrawal syndrome" that results from termination of drug administration. Early drug withdrawal is characterized by adverse psychological and/or somatic symptoms. Withdrawal from ethanol precipitates a transient psychological condition that bears remarkable similarity to the symptoms of major depressive disorder in humans. Associated behavioral deficits in laboratory animals can be reversed by antidepressant treatment, suggesting that ethanol withdrawal may provide a basis for an animal model of depression [21,27].

Among the numerous neurotransmitter systems implicated in the pharmacological effects of ethanol, the serotonergic system has received particular attention. The serotonergic system has been shown to play an important role in the regulation of ethanol intake, preference, and dependence via central mechanisms. A decrease in serotonergic activity is also associated with depression. Selective serotonin re-uptake inhibitors (SSRIs) and some post-synaptic receptor agonists, which increase serotonergic activity in the synaptic space, are effectively used to reverse the symptoms of depression.

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The number of behavioral studies that have been conducted with zebrafish is orders of magnitude fewer compared to those of mice or rats [35,36]. Recently, however, several manuscripts focusing on zebrafish learning and memory have been published. For instance, zebrafish have been found to perform well in an avoidance learning paradigm [2], olfactory conditioning, appetitive choice discrimination, visual discrimination learning [6], place conditioning [8], automated learning paradigm [16], shuttle box active appetitive conditioning [26], active avoidance conditioning [42], and spatial alternation based memory tasks [39].

The startle response is a reflex evoked by exposure to unexpected, most often aversive, stimuli [32,38]. It exemplifies an evolutionarily conserved instinctive behavior which is observed in many species, including humans [12,13,19,29] and enables an organism to quickly react to perceived threats, avoid harm, and initiate adaptive fight-or-flight responses [11]. Startle assays are commonly used in neurobehavioral and psychopharmacological research. While anxiolytic drugs generally reduce startle responses, anxiogenic agents typically increase startle responses [12,14,17]. Furthermore, the startle response is based on cognitive processing of sensory information, which is highly relevant to modeling cognitive deficits, resembling those associated with anxiety/depression-like pathogenesis [14].

Startle responses have been examined in adult ([4,5]) and larval zebrafish [7,22]. In zebrafish, startling stimuli evoke a stereotyped behavior consisting of a rapid turn and swimming with high velocity away from the stimulus [25]. Habituation of the startle response is another important neural phenomenon manifested in the reduction of behavioral responses to startling stimuli over time [24,30,38,40]. Like any other type of habituation, startle habituation reflects cognitive functions and can be easily evaluated in zebrafish.

We have designed an associative learning task, adapted from the mammalian (rodent) literature, which was constructed to somewhat resemble classical plus-shaped maze paradigms. In this task, zebrafish are required to swim in a plus-shaped maze and to locate a chamber, which is paired with a visual cue (aquarium plant) or particular location (bottom right chamber) where the startle response will stop. We investigated whether zebrafish could learn the association conditioned stimulus, the startle, with a particular chamber(s). We also investigated whether ethanol, fluoxetine, or ethanol withdrawal affects temporal processes of learning and memory. We employed a dosing procedure of ethanol and fluoxetine that we have shown not to alter performance characteristics important in our learning task, i.e., motor function [27]. We explored whether this learning task has construct validity, i.e. whether disruption of a molecular mechanism known to be involved in learning and memory processes in mammals, would impair performance of zebrafish in this task. Our goal with the current investigation was; one, to show that zebrafish can perform well in an associative auditory discrimination learning paradigm similar to those employed with rodents; and two, to demonstrate that the paradigm has translational relevance, i.e., can detect learning and memory impairing effects both behaviorally and neurochemically.

2. Materials and methods

2.1. Animals/housing

Adult zebrafish (>90 days; 50/50 male/female) were acquired from a local commercial distributer (Pets Paradise 4 U, Enterprise, AL, USA). The fish were housed in 1 L tanks (Thoren Aquatic Systems, Inc. Hazleton, Pennsylvania, USA; recirculating high-density rack system) in groups of 10 fish per tank. The tanks were filled with de-ionized water treated with a Prime Freshwater® concentrated conditioner. The tank water was filtered using mechanical (sponge), chemical (activated carbon), and biological filtration units. The water temperature was maintained at 25–27 °C. Fish were kept on a 10/14 light–dark cycle (lights on 9 AM, off at 7 PM). Fish were fed a mixture of ground flake food (Tetramin Tropical Flakes; Tetra USA, Blacksburg, VA). All fish were maintained and procedures were performed in accordance with the Institutional Animal Care and Use Committee of Troy University, Troy, AL, USA.

2.2. Treatments

All of the fish were naive and given at least 7 days to adapt to the laboratory environment. Fish were randomly sorted into treatment groups (n = 10) and controls (n = 10) so that there was no confounding of breeding or holding conditions with respect to pharmacological treatment. Instead of continuous exposure, we adopted a regimen of intermittent ethanol exposure, as this allows better control of the dose. A dose-escalation study was conducted to ensure the optimal dose for all of the below treatments (Pittman et al., 2013; [28]). In addition, these dosages were based on previous work by Egan et al. [9] and Marthur et al. [23]. Behavioral testing followed chronic intermittent exposure to dissolved 3% ethanol (Fisher Scientific, USA) in a 1 L beaker filled with 900 mL tank water. Fish were gently transferred by net from their home tanks into the beaker for 1 h. After 2 weeks of daily exposure, the fish underwent abstinence/withdrawal from ethanol in their home tanks. Another group of fish (n = 10) was administered fluoxetine (100 μg/L) (Sigma-Aldrich, USA), beginning the day ethanol was discontinued, for 2 weeks. The observer was blind with respect to treatment.

2.3. Apparatus

Learning and memory were tested in a four-chambered maze (Fig. 1). Prior to testing, and between periods of testing, fish were housed in the above mentioned Thoren Aquatic Systems® home tanks. In this experimental setup, the tank (20″ × 10″ × 12″) faced the video-analysis web-camera/observer and was filled with home tank treated water. The testing apparatus was essentially a modification of a plus maze that was first employed in zebrafish by Al-Imari & Gerlai [1] and subsequently by Sison & Gerlai [35]. One important modification was that in the new maze 2 stimulus boxes (each measuring 2″ × 2″ × 2″) were placed inside the aquarium with additional adjacent non-stimulus compartments (2) at the end of each “arm”/compartment.

2.4. Experimental protocol

The maze was fitted with a “start box” in its central compartment (Fig. 1), thus the experimental fish could be released without disturbance. The learning task was preceded by an extensive habituation procedure. As with most learning tasks, our paradigm also required testing single subjects. Zebrafish are a highly social species and being placed in a novel setting alone may be stressful for them [10]. To minimize this negative aspect of the task and to facilitate active exploration of the maze all experimental fish were subjected to a habituation session every day for five consecutive days. For training, the experimental subject was netted singly into the release box positioned in the central compartment of the maze and was released into the maze. The fish were allowed to explore the maze for 5 min. Each fish had 5 consecutive trials with an interval of 5 min.

Due to the broad nature of a startle response, various procedures can elicit a startle response in zebrafish; including electrical, visual, or tapping-induced stimuli. An easy, inexpensive and practical stimulus to use is tapping, such as tapping on the side (left side) of the tank, as was performed in the current study. All behavioral testing sessions were video-recorded and manually recorded and quantified using an event recorder software application developed in our lab [27]. The percent of time experimental fish spent in different areas of the maze and the number of entries to these areas were quantified throughout training.