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Research report

Associative learning in zebrafish (*Danio rerio*) in the plus maze

Margarette Sison, Robert Gerlai*

Department of Psychology, University of Toronto, Mississauga, Canada

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ABSTRACT

Zebrafish has been gaining increasing amount of interest in behavioral neuroscience as this species may represent a good compromise between system complexity and practical simplicity. Particularly successful have been those studies that utilized zebrafish as a screening tool. Given the complexity of the mechanisms of learning, for example, forward genetic screens with zebrafish could potentially reveal previously unknown genes and molecular pathways that subserve this function. These screens, however, require appropriate phenotypical (e.g. behavioral) paradigms. A step in this direction is the characterization of learning abilities of zebrafish. Here we employ two classical learning tasks in a plus maze. In the first, zebrafish are required to associate a visible cue with food reward irrespective of the location of this pairing. In the second, zebrafish are required to associate the spatial location of food reward irrespective of intra-maze cues. Our results demonstrate that zebrafish perform well in both tasks and show significant acquisition of the association between cue and reward as well as between location and reward. We conclude that zebrafish, similar to classical laboratory rodents, may have utility in the biological analysis of simple as well as complex forms of associative learning.

1. Introduction

For the past three decades the zebrafish has been one of the favorite species of developmental biologists [34] due to the small size and prolific nature of this fish and to the fact that its development is fast (takes about 5 days from fertilization to get a free swimming little fish) and its embryo is mainly transparent throughout its development. As a result of these features numerous genetic tools have been developed and a substantial amount of genetic information has been amassed for zebrafish. By now the zebrafish is perhaps the third most genetically well characterized animal species after the mouse and the fruit fly [19]. Due to this accumulated knowledge on, and the available tool set for the zebrafish, disciplines other than developmental genetics have also taken notice of this fish. For example, behavior and behavioral neuroscience related publications on the zebrafish appear at an exponential rate [30]. Nevertheless, compared to the mouse, the main biomedical research species in the laboratory, the number of zebrafish behavioral tests is orders of magnitude fewer. For example, a PubMed (Medline) search with keywords "learning" and "mouse" returns 13520 publications whereas a similar search but with the keywords "learning" and "zebrafish" gives 82 publications, only less than one fourth of which is actually about some form, or mechanism, of learning in zebrafish.

Learning and memory are highly complex brain processes and despite decades of successful research of these phenomena, the number of molecular mechanisms discovered every year does not seem to plateau out. According to some estimates, about 30–40% of our genes are expressed in the brain and many of these genes are expected to be involved in some form of neuronal plasticity subserving different forms, types and/or phases of learning and memory [32]. Calculating with the conservative estimate of about 30 thousand mammalian genes (for mouse and human, for example), one may expect as many as 9–12 thousand genes potentially involved in learning and memory, a staggering number compared to the known few hundred genes so far associated with these phenomena. How can one investigate such mechanistically complex functions at the molecular, i.e. genetic, level? Some have argued that forward genetics, i.e. large-scale mutagenesis screens may have utility [7]. These screens have the potential to cover the entire genome and identify numerous mutations leading to the localization and identification of the genes and molecular pathways involved. The cornerstone of such screens is the phenotypical screening and characterization tools [30,13]. The zebrafish is particularly suitable for forward genetics [1] given its small size (4 cm when adult), ease of maintenance, prolific nature (2–300 eggs per spawning per female every other day), and the fact that numerous genetic markers have been developed for this species [21]. Also important is the translational relevance of zebrafish, i.e. the high nucleotide sequence homology and functional similarities between

* Corresponding author at: Department of Psychology, University of Toronto, 3359 Mississauga Road North, Rm 3035, Mississauga, Ontario L5L 1C6, Canada. Tel.: +1 905 569 4255(O)/4257(Lab); fax: +1 905 569 4326.
E-mail address: robert_gerlai@yahoo.com (R. Gerlai).

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mammalian and zebrafish genes (for review see [25]). However, there is a serious bottleneck in this research: the phenotypical testing tools that would be required for the screening are often rudimentary or not available at all [30]. The current paper is a step towards addressing this need.

An important step in this research is the characterization of learning abilities of zebrafish. For example, can zebrafish perform well in simple and complex associative learning tasks? Here we analyze associative learning in zebrafish in two tasks using a maze design principally similar to the classical radial arm maze employed for the analysis of spatial and non-spatial associative learning of rats and mice before [10]. In one task the fish are required to associate a visible cue with food reward while other intra-maze as well as extra-maze cues are made irrelevant, a single cue-based, simple, associative learning task. In the other task, the fish are required to find the location of the food reward irrespective of intra-maze cues. The latter task is often regarded as "spatial learning" [10,22], a form of complex associative learning termed relational learning [9]. Relational (and spatial) learning has been attributed to the function of the mammalian hippocampus [11]. However, the typical hippocampal anatomy, including the trisynaptic (dentate gyrus–CA3–CA1) circuitry is missing from the teleost brain, including the zebrafish brain [27]. Nevertheless, spatial learning has been demonstrated in a cyprinid species, the carp [28], closely related to zebrafish. Thus it appears that spatial (and thus relational) learning is not the distinct property of the mammalian hippocampal circuitry, but rather perhaps depends, at least partly, on mechanisms lower in the organization levels of the brain: for example, molecular pathways and/or synaptic function. Briefly, zebrafish represents a reductionist tool that does not possess the complex hippocampal circuitry of a mammal (nor does it have the cortex, the place where relational memories are believed to be stored [27,33,8]). Despite this simplicity, zebrafish have been found to perform well in learning tasks including a one trial avoidance learning paradigm [4], olfactory conditioning [6], shuttle box active appetitive conditioning [24], place conditioning [12], appetitive choice discrimination [3], active avoidance conditioning [36], alternation memory task, and even an automated learning paradigm [20] to mention but the most recent examples.

Here we propose that analysis of the zebrafish may allow one to identify the molecular and synaptic mechanisms fundamental to forms of associative learning including simple two-cue association and more complex relational associations at an evolutionary stage that long preceded the mammals. The goal of this paper is to demonstrate that zebrafish are capable of acquiring such associations and show therefore that it may have utility as a tool for the analysis of the mechanisms of these higher brain functions.

2. Methods

2.1. Animals and housing

Adult zebrafish (*Danio rerio*) were purchased at their age of three months from a local pet supplier (Big Al's Aquarium Warehouse Inc., Mississauga, Ontario, Canada). All fish were housed in 143 l glass tanks (91 cm length × 30 cm width × 50 cm height) at the University of Toronto Mississauga Vivarium (20 fish per tank). The holding tanks were filled with aged (biologically active) tap water (which was filtered in a system water cylinder for at least one week before its use in the holding tanks). The holding tanks were filtered by canister filters containing a biological (high surface area bacterial), chemical (activated carbon) and physical (porous sponge) filter media (EHEIM Classic Filter, Model 2213, Deizisau, Germany). The water temperature was kept constant at 27 °C. The holding tanks were illuminated by 13 W overhead fluorescent light placed directly above the tanks, and a 13 h/11 h light/dark cycle was maintained with lights on at 7 a.m. Fish were fed a 50–50% mix of ground freeze-dried krill (Aquatic Ecosystems, Florida) and TetraMin flakes (Melle, Germany) twice a day. All fish were allowed to acclimatize for one month in their holding tanks. After this period the fish were transferred to 10 l tanks (31 cm length × 16 cm width × 21 cm height, 1 fish per tank) that were filtered using Tetra-Whisper Power Filters (Model#1295520, VA, USA). The tanks were placed adjacent to each so that experimental zebrafish were not visually isolated from each other. In the 10 l holding tanks experimental zebrafish were fed Gelly Belly (Aquatic Eco-Systems Inc., Dade City, Florida, USA), a gelatinous mixture of krill, kelp and fish meal. Using this food substance allowed us to precisely determine the exact amount of food delivered to the fish during learning trials and we could also control the exact location of the food reward. Habituation to this novel food substance took one month, after which the experimental zebrafish entered the learning trials.

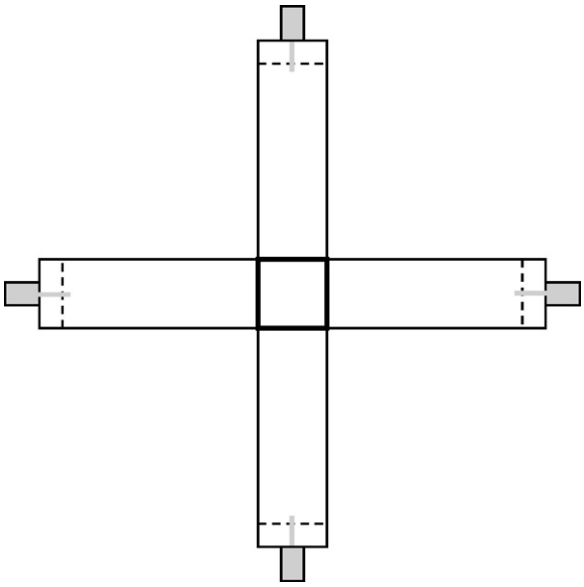

Fig. 1. The plus maze (4 arm radial maze). Note that each arm is equipped with a food dispensing syringe (grey rectangles) attached to Teflon tubing. The end of the Teflon tube may be positioned just behind or just in front of perforated plastic sheets (broken line). If the end of the tube is in front of the perforated sheet, the fish have access to food. Also note the center square piece (thick black line), which allowed us to place the fish in the maze and which could be lifted remotely using a pulley and nylon string system thereby allowing the fish to start the exploration of the maze with minimal experimenter interference.

2.2. Apparatus and procedure

The test apparatus (Fig. 1) was a four-arm, plus-shaped transparent Plexiglas maze similar to that employed by others [26,28]. Each arm of the maze was 35 cm long, 11 cm wide, and 20 cm high. The arms were connected to each other by an 11 × 11 cm center square into which a start box could be lowered and could be made accessible by lifting the start box. The maze was placed inside a large transparent Plexiglas tank (86 × 86 × 20 cm, width × length × depth) filled with water with temperature maintained at 27 °C by four symmetrically positioned 50-W thermostat controlled aquarium heaters (EHEIM JAGER Model 7357890, Deizisau, Germany). This arrangement allowed us to maintain constant water temperature inside the plus maze without having to put any objects (heaters and air-stones) that would disturb the fish or obscure them from the overhead digital camera (Optura 30, Canon, Japan) that monitored their behavior. The entire apparatus was placed on a rotating circular platform that allowed us to position the maze as required by the trials with ease.

2.2.1. Habituation trials

To acclimatize fish to the maze, they were administered four 2 h long habituation trials (one trial per day on consecutive days). On the first day 20 fish were placed in the maze at a time, on the second day 10 fish, on the third day 5, and on the fourth day 1 fish was placed in the maze at a time. For the 2 h long habituation trials all arms of the maze were baited, i.e. access to the food reward was allowed.

2.2.2. Shaping

In order to facilitate successful acquisition of the association between a visual cue (conditioned stimulus) and the food reward (unconditioned stimulus) a shaping procedure was conducted. The 2 h long habituation trials were followed by 12 short (5-min long) habituation trials (4 trials per day on 3 consecutive days) during which only one fish explored the maze at a time. On the first day of the short habituation trials all arms of the maze was baited with food and next to the food a red plastic cue card was also placed. This stimulus was chosen as it is expected to be clearly distinguishable for the tetrachromatic zebrafish and has been successfully utilized in the past [35]. On the following day three of the four arms were baited and marked by the visual cue and on the third day two of the arms were baited and marked with the visual cue. The gradual decrease of shoal size in the maze and the multiple exposures to the maze environment and cues was designed to minimize the possible