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Single stimulus learning in zebrafish larvae

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ABSTRACT

Learning about a moving visual stimulus was examined in zebrafish larvae using an automated imaging system and a t1–t2 design. In three experiments, zebrafish larvae were exposed to one of two inputs at t1 (either a gray bouncing disk or an identical but stationary disk) followed by a common test at t2 (the gray bouncing disk). Using 7 days post-fertilization (dpf) larvae and 12 stimulus exposures, Experiment 1 established that these different treatments produced differential responding to the moving disk during testing. Larvae familiar with the moving test stimulus were significantly less likely to be still in its presence than larvae that had been exposed to the identical but stationary stimulus. Experiment 2 confirmed this result in 7 dpf larvae and extended the finding to 5 and 6 dpf larvae. Experiment 3 found differential responding to the moving test stimulus with 4 or 8 stimulus exposures but not with just one exposure in 7 dpf larvae. These results provide evidence for learning in very young zebrafish larvae. The merits and challenges of the t1–t2 framework to study learning are discussed.

1. Introduction

The developing zebrafish (*Danio rerio*) has emerged as an important model system in biology, environmental toxicology, and neuroscience. Its small size, rapid development of sensory and motor functions, and its behavioral diversity combine to make the larval zebrafish an ideal candidate for high throughput automated analyses of behavior essential for rapid mutagenesis screening and detection of the effects of pharmaceutical and chemical agents. By 7 days post-fertilization (dpf), the zebrafish larva is approximately 5 mm long. It has a complex swimming repertoire consisting of several topographically distinct components that help co-ordinate its movements in three dimensional space at a variety of speeds (Wolman & Granato, 2012). This finely tuned motor system coupled with a highly developed tetrachromatic visual system enables the 7 dpf zebrafish larva to track and capture small prey, to avoid predators including adult zebrafish, and to maneuver without collision in typically densely populated larval colonies. Automated medium and high throughput assays have already been established to study spontaneous and stimulus-induced locomotor activity, social proximity, and visually-induced avoidance behavior in zebrafish larvae (Chen, Huang, Zheng, Simonich, Bai, Tanguay & Dong, 2011; Colwill & Creton, 2011a; Colwill & Creton, 2011b; Creton, 2009; Kokel & Peterson, 2011; Pelkowski, Kapoor, Richendrfer, Wang, Colwill & Creton, 2011; Powers, Wrench, Ryde, Smith, Seidler & Slotkin, 2010; Selderslaghs, Hooyberghs, De Coen & Witters, 2010). Here, we report three experiments using a semi-automated behavioral assay to study the effects of prior experience (learning) in zebrafish larvae.

Arguably the simplest procedure for studying learning is the repeated presentation of a stimulus. Demonstrations of habituation, sensitization, imprinting, and the mere exposure effect as well as studies of perceptual learning, latent inhibition, expectancy violation, priming and recognition memory all employ this basic procedure. However, proper assessment of the learning produced by this procedure is a more complicated matter. For example, many studies using this procedure to show habituation typically rely on the demonstration of a significant decline in responding to the stimulus, usually between the first and last presentations. Such an approach is inadequate for several reasons (Davis, 1970; Davis & Wagner, 1968; Davis & Wagner, 1969; Rescorla, 1988; Rescorla & Holland, 1976). Foremost, this design confounds the conditions for learning (input) with the assessment of learning (test) which can lead to serious misunderstandings about the nature of that learning. For example, Best, Berghmans, Hunt, Clarke, Fleming, Goldsmith, and Roach (2008) recently examined how the interstimulus interval (ISI) affected the rate of response decrement to an iterative auditory stimulus. Using a between-subjects design, different groups of 7 dpf WIK larvae were exposed to repeated stimulus presentations at 1, 5 or 20 s intervals, and the distance moved in the second immediately following each stimulus presentation was measured. The decline in the distance moved between the initial and terminal stimulus presentations was most pronounced in the larvae with the 1 s ISI. For the larvae with the

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20 s ISI, there was no significant change in distance moved. On the basis of these results, the authors concluded that habituation was slower with longer ISIs.

The opposite view that habituation is, in fact, superior with spaced stimulus presentations has been reached in studies that do not confound variations in the conditions for learning with concomitant variations in the testing conditions. For example, Wolman, Jain, Liss, and Granato (2011) exposed 6 dpf larvae in groups of 20 to a two hour habituation procedure consisting of a series of 480 1-s dark flashes with an ISI of 15 s. For one group, the series was split into 4 blocks of 120 flashes punctuated by 10 min intervals; for the other group, the stimuli were presented in a single block (massed). All larvae were then tested with a series of 10 1-s dark flashes with an ISI of 60 s. Larvae that had received a spaced pattern of stimulation showed a longer-lasting response decrement (dark flash failed to elicit an O-bend response) than those that had received a massed pattern of stimulation.

To guard against the potential pitfalls that can arise from confounding the conditions for learning with the assessment of that learning, Rescorla and Holland (1976) formulated an important general framework for all studies of elementary learning processes. They proposed the t1–t2 design, in which the input phase of a learning procedure is varied within or between animals at one point in time (t1) while the test phase is kept constant within or between animals and occurs at a subsequent point in time (t2). Intrinsic to this t1–t2 framework is the recognition that an appropriate control for determining learning about a stimulus is to compare behavior to that stimulus in a common test between animals that did or did not receive prior exposure to that stimulus. A significant difference in behavior during that common test would provide evidence for learning about the stimulus during the input phase.

We implemented the t1–t2 framework in a between-subjects design to study learning about a moving visual stimulus. Using a modified version of our automated visual avoidance assay (Creton, 2009; Pelkowski et al., 2011; Richendrfer, Pelkowski, Colwill, & Creton, 2012), larvae in Group B were exposed to 5 min periods of a plain white background alternating with a gray bouncing disk superimposed on the white background. Control larvae in Group S received the same exposure treatment except that the disk did not move. In previous work, we have demonstrated that larvae will swim away from a bouncing but not a stationary visual stimulus. The input phase was immediately followed by identical testing for both groups. Testing began with a presentation of the plain white background for 5 min immediately followed by presentation of the gray bouncing disk for 5 min. Using 7 dpf larvae, Experiment 1 established that 12 exposures to either a bouncing or stationary disk at t1 produced differential responding to the bouncing disk during testing at t2. Experiment 2 replicated the procedure of Experiment 1 with 5, 6, and 7 dpf larvae. Experiment 3 used the same procedure as Experiment 1 with 7 dpf larvae but with fewer stimulus exposures (1, 4 or 8) in the input phase.

2. Materials and methods

2.1. Embryo collection and rearing

Embryos were collected from a breeding population of adult male and female wild type zebrafish originally obtained from Carolina Biological Supply Co. (Burlington, NC) and maintained at Brown University as a genetically diverse outbred strain. The fish were housed in 20 gallon tanks at 28 °C and maintained on a 14 h light/10 h dark cycle. They were fed a combination of frozen or fresh brine shrimp and flake fish food once or twice per day. Embryos were collected for 2 h following light onset using shallow trays placed in the bottom of the tanks.

Embryos were grown at a density of approximately 250 embryos per liter of egg water (60 mg/L sea salt, Instant Ocean, in deionized water and 0.25 mg/L methylene blue as a mold inhibitor) in 2L plastic breeding tanks (Aquatic Habitats) and incubated at 28 °C. Unfertilized eggs were removed at 1 dpf. Dead larvae and other particles were removed on a daily basis and egg water in each tank was partially changed every other day to maintain water quality. Food supplements were not provided during this period because the larvae absorb nutrition from their yolk sac through 7 dpf (Jardine & Litvak, 2003).

2.2. Image collection

Details of the imaging system have been described previously (Pelkowski et al. 2011). Flat-bottom 6-well plates (Corning Costar No. 3506) were optimized for larval imaging by filling each well with 5 ml of agarose (0.5% w/v in deionized water). Agarose was allowed to set and then a center portion was punched out to create a 27 mm diameter × 5 mm deep swimming area surrounded by an agarose ring (Creton, 2009). Four 6-well plates with one larva per well were placed on the LCD screen (1366 × 768 pixel resolution and a brightness of 220 cd/m^2) of an inverted laptop on the bottom shelf of a tall cabinet (Fig. 1A–C). A plastic diffuser (Pendaflex 52345) was placed between the multiwell plates and the screen to avoid moiré patterns. Larvae were imaged from above by a 15 megapixel Canon EOS Rebel T1i digital camera with an EF-S 55–250 mm f/4.0–5.6 IS zoom lens using Canon's remote capture software. The following camera settings were used: image quality = 4752 × 3168 pixels, high-quality JPEG compression (0.6 MB/image), lamp = outdoor, iso-speed = 100, F = 5.0, and exposure time (Tv) = 1/20 s. In Experiment 3, we also used an 18 megapixel Canon EOS Rebel T2i digital camera with an EF-S 55–250 mm f/4.0–5.6 IS zoom lens with image quality = 5184 × 3456 pixels. All other camera settings remained the same.

2.3. Behavioral testing

In all three experiments, larvae were first exposed to alternating 5 min periods of a white background (RGB values were 255, 255, 255) with no visual stimuli (Fig. 1D) and 5 min periods of either a gray moving disk (1.35 cm in diameter) or an identical gray but stationary disk (RGB values were 120, 120, 120) on a white background (Fig. 1E). Exposure (t1) was immediately followed by testing (t2). For testing, all larvae were exposed to a 5 min period of the plain white background (Fig. 1F) followed by a 5 min presentation of the gray moving disk (Fig. 1G). Images were captured at the fastest rate allowed by the camera (6 s), a setting that generated 49 or 50 images in the 5 min recording periods. The visual stimuli were created in Microsoft PowerPoint. Using the custom animation feature, the moving gray disk was programmed to bounce back and forth in a straight line across the upper half of each well at a rate of 1.65 cm/s. The gray stationary disk was also presented in the upper half of each well but remained fixed in the center of the path traveled by the bouncing disk. Whether the stationary or bouncing disks appeared in the 12 wells on the right half or on the left half of the PowerPoint display was counterbalanced across subjects.

2.3.1. Experiment 1: basic demonstration of single stimulus learning

The input phase consisted of 12 5-min presentations of a white background (Fig. 1D) alternating with 12 5-min presentations of either the gray bouncing disk or the gray stationary disk (Fig. 1E) for 120 min. The test phase was the same for all larvae and consisted of a 5-min presentation of the white background (Fig. 1F) followed by a 5-min presentation of the gray bouncing disk