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Time for zebrafish

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A key challenge for identifying cellular bases of cognitive functions is to distinguish the contributions of different cell subtypes within complex circuits in a brain. Functional magnetic resonance imaging (fMRI) can reflect what brain areas are activated and electrophysiological recording can record neural activities adjacent to the electrodes, but neither can specify which and how neural subtypes *in vivo* contribute to a cognitive function, nor can they identify glial cells that support cognition (Pereira and Furlan, 2010). In the field of interval timing, considerable work has been done using the above two methods in organisms such as humans, primates, and rodents, yielding information on specific areas of the brain involved (Coull et al., 2011). However, a systems-level understanding from genetics to behaviors in the same organism is lacking. One way in which this can be achieved is to use a genetic system where specific neuronal ensembles or cell populations can be imaged and manipulated, while the resulting behaviors can be observed accordingly. One such system is the zebrafish (*Danio rerio*). The importance of zebrafish as a vertebrate model organism in neuroscience has been steadily increasing as more neural subtype-specific genetic markers and experimental tools become available. Although the rich behavioral repertoire of zebrafish has been characterized recently (Levin and Cerutti, 2009; Fero et al., 2010), its higher cognitive abilities, such as interval timing and time-based decision making, are relatively unknown compared with other species (Buhusi and Meck, 2005; Cheng et al., 2006; Penney et al., 2008). Here, we review some of the latest progress in optical imaging, zebrafish brain development, and timing behaviors to support the claim that the use of zebrafish model will extend our understanding of how different types of neurons work together in a vertebrate brain to generate the sense of time and determine complex time-based behaviors.

A major advantage of using zebrafish in neuroscience research is its transparent skull and brain during the larval stage of development, which enables *in vivo* fluorescence imaging of activity in large populations of cells. This can be done either by injecting calcium-sensitive dyes into the target brain areas, or by generating transgenic lines that express genetically encoded calcium indicators (GECIs, see Wilms and Hausser, 2009) in specific subsets of neurons. These fluorescent indicators report intracellular calcium concentration change triggered by action potentials, thus reflecting neural activity (Tian et al., 2009). From the calcium signals, neural firing rate information can be derived by de-convolution (Yaksi and Friedrich, 2006). With the neural populations identified and visible under the microscope, one can study how these defined neurons react to external stimuli (Dreosti et al., 2009, 2011), to internal stimuli (e.g., the sense of time) and during associative learning (Aizenberg and Schuman, 2011) by measuring the calcium signal change. Under the microscope, it is also possible to reconstruct a 3D functional map of the defined neurons after scanning multiple depths of the target area. This non-invasive imaging technique allows monitoring of neural activities across multiple sessions, which is critical for studying learning and memory (Aizenberg and Schuman, 2011). Interestingly, in addition to capturing images, two-photon microscopy allows ablations at single-cell resolution. This optical lesion technique enables examination of the functional consequences of loss of highly specific neural populations compared with surgical lesions. Besides, due to abundant neurogenesis persisting into adulthood in zebrafish (reviewed in Kizil, et al., 2011), functional recovery naturally occurs after brain lesions in the same fish. This provides excellent reversible brain lesions at single-cell level, which is difficult to conduct in mammalian models. In sum, it is now possible to effectively examine neural substrates underlying interval timing in zebrafish at single-cell resolution and in 3D.

Sumbre et al. (2008) recently demonstrated that larval zebrafish (between 5 and 14 days post fertilization, or dpf) could follow temporal patterns of rhythmic stimuli both at the neuronal level in the optic tectum (a visual area), as well as at the behavioral level in its tail flips. The temporal rhythm was established by repeatedly presenting a 200 ms visual (light flash) conditioned stimulus (CS) at a fixed inter-stimulus interval (ISI) of 4, 6, or 10 s. It was found that the tectal neurons entrained to the CS, such that the calcium signals increased transiently and, more importantly, in synchrony with the ISI. Moreover, this synchronous neural activity pattern continued for several cycles even after the flashing CS terminated. The same study also revealed that the tectal neurons in a fish that is as young as 4 days (one out of seven tested fish), but not 3 days old, was able to keep repeating a temporal pattern established by the ISI. From a developmental perspective, it is intriguing that a 4–5 days old zebrafish brain is sophisticated enough to react to a visual stimulus and to follow its temporal patterns. In general, zebrafish embryos start hatching after 2–3 dpf (henceforth called larvae) and the larvae need to hunt for food after their yolk is depleted at 5 dpf. Therefore, it is reasonable to assume that the developing zebrafish brain is mature enough and ready to function after 5 dpf, with timing external stimuli a part of its abilities. During development, dopamine (DA) neurons in the zebrafish brain mature at different time points ranging from 1 dpf in the diencephalon to 3 dpf in the telencephalon (Mahler et al., 2010). The zebrafish telencephalon is composed of the pallium and the subpallium, which is teleost analog of cortico-basal-ganglia circuits in mammals (Rink and Wullimann, 2002). The DA neurotransmitter systems, especially the ones in cortico-striatal

circuits, play a critical role in interval timing both in animals and humans (reviewed in Meck et al., 2008). Studying the potential timing functions of the DA neurons in the telencephalon may be a good place to start in zebrafish. A Gal4 driver that is active in DA neurons will allow us to label DA neurons in larval zebrafish for optical imaging, or to manipulate it, before, during, and after training on a timing task.

The behavior (rhythmic tail flips) shown by Sumbre et al. (2008) is driven by a flashing CS with the temporal patterns established by the ISI. It will be important to determine next whether larval zebrafish can associate the CS with biologically significant stimuli, such as food or danger (as an unconditioned stimulus, US), by guiding behaviors based on their sense of time between the CS and the US. This is a more sophisticated form of sensorimotor coordination than a simple reflex and is critical for associative learning (Balsam et al., 2010) and for survival. It was recently reported that larval zebrafish (20–35 dpf) could be conditioned to a 5-s light CS with a mild electrical shock as the US (Lee et al., 2010). In the study, after 10 CS–US conditioning trials that required the fish to swim to the non-CS side of a chamber to evade the full impact of the US, the fish showed a significant increase in swim speed at the 5th second of the 5-s CS in the probe trial (i.e., no US presented). This suggests that the fish adjusted their swimming behavior according to their expected time of the US delivery, which is consistent with previous findings in adult goldfish (Drew et al., 2005). To further explore interval timing in larval zebrafish, one can implement a subjective timing component in the task requirement, such as a trace interval between the CS offset and the US onset. Trace conditioning requires the animal to subjectively "bridge" the two stimuli by its own sense of time, because there is no external stimulus (i.e., CS free) to follow during the trace interval. An intact dorsal pallium is found to be critical in learning trace conditioning in adult goldfish (Vargas et al., 2009). In addition, the caudate nucleus (Flores and Disterhoft, 2009) and the hippocampus (Cheng et al., 2008) are also critically involved in trace conditioning in mammals. The analog of both regions can be found in the fish telencephalon (the caudate nucleus in the fish subpallium and the hippocampus in the fish lateral pallium; see Portavella and Vargas, 2005).

In conclusion, the highly conserved neurotransmitter systems and brain anatomy in the vertebrate brain allow us to investigate neural mechanisms of interval timing in zebrafish. We propose two critical experiments here. First, neural substrates of interval timing can be obtained by observing calcium signal change in specific neural populations in the zebrafish telencephalon as a function of the trace interval in a trace conditioning paradigm. Second, it is also crucial to observe how interval timing is affected when a particular set of neurons is disrupted, either by femtosecond laser ablation or optogenetics. Once the fundamentals of interval timing are established, the zebrafish will open a new window for research on interval timing and time-based decision making, especially as it can also be used for genetic or chemical screens (Lieschke and Currie, 2007).

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