Review Article

Developmental Neurobiology
DOI 10.1002/dneu.20872

Behavioral genetics in larval zebrafish-learning from the young

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Running title: Zebrafish behavioral genetics

Key words: zebrafish, startle response, habituation, swimming, genetic screen, behavior

Received: Oct 20, 2010; Accepted: Dec 31, 2010

Abstract

Deciphering the genetic code that determines how the vertebrate nervous system assembles into neural circuits that ultimately control behavior is a fascinating and challenging question in modern neurobiology. Because of the complexity of this problem, successful strategies require a simple yet focused experimental approach without limiting the scope of the discovery. Unbiased, large-scale forward genetic screens in invertebrate organisms have yielded great insight into the genetic regulation of neural circuit assembly and function. For many reasons, this highly successful approach has been difficult to recapitulate in the behavioral neuroscience field's classic vertebrate model organisms – rodents. Here, we discuss how larval zebrafish provide a promising model system to which we can apply the design of invertebrate behavior based screens to reveal the genetic mechanisms critical for neural circuit assembly and function in vertebrates.

Introduction

Understanding how neural circuits form and then function to allow for organisms to interpret their surroundings and behave appropriately is a daunting task. Notwithstanding, dissecting the genetic program that dictates how neural circuits modulate behavior through sensory perception, cognitive processing, and motor output is one of neuroscience's most studied yet least understood questions. To begin to unravel the mechanisms critical for neural circuit assembly and function, it is critical to design a simplified and focused experimental approach without limiting the scope of the discovery. Mutagenesis screens using agents that randomly generate mutations in genes to disrupt a biological process of interest have been a highly successful approach. The strength of this approach is the ability to identify genes in an unbiased manner without prior assumption of the underlying molecular mechanisms involved. Indeed, invertebrate forward genetic analyses have provided great insight into how an organism's genetic makeup orchestrates the formation and function of its nervous system (Jorgensen and Mango, 2002; Margulies et al., 2005). The success of these invertebrate screens is tightly linked to their design – identifying deviations in simple, robust behaviors with characterized, accessible underlying circuits in an organism that comes with a well-stocked genetic toolbox. The dissimilar anatomical plan of invertebrate and vertebrate nervous systems and their inherent genetic divergence warrants further behavioral genetic analysis in a vertebrate model system. Nonetheless, these invertebrate studies provide a blueprint for an unbiased strategy to investigating the genetic basis of behavior in the most widely studied vertebrate organisms for behavioral neuroscience: mice and rats. Although ENU mutagenesis is widely utilized in the mouse (Godinho and Nolan, 2006;

Acevedo-Arozena et al., 2008), large-scale genetic screens for recessive behavioral mutations in rodents remain relatively impractical due to high costs, the inherent complexity and variability of adult rodent behaviors, and mutant viability into adulthood. Here, we propose the use of an alternative vertebrate model system, larval zebrafish, to which this powerful approach can be readily applied.

Zebrafish are a small diploid vertebrate that are amenable to forward genetic screens. Due to many of its features, it provides a promising model system to which we can apply the design of invertebrate behavior based screens to reveal the genetic mechanisms that dictate how neural circuits regulate behavior in vertebrates. Adults are small in size, prolific in generating offspring, and easy to maintain, which allows for a large number of lines to be maintained in a relatively small space, a necessity for large-scale screening. Embryos and larvae are transparent and develop rapidly: in a mere 5 days fertilized zygotes have become free swimming and self feeding larvae with a rich repertoire of stereotyped motor behaviors that operate on a simple blueprint of a vertebrate nervous system. Zebrafish also come with a well stocked genetic toolkit, including mutagenesis and chemical screening techniques, transgenesis, a variety of gene misexpression and multigenic approaches, cell transplantation, optogenetic circuit analysis, live imaging approaches, along with extensive genomic resources critical for mapping and cloning mutations. Taken together, zebrafish are an attractive vertebrate model for behavioral based forward genetic screening, and for the molecular genetic analysis of neural circuit formation and function.

The capable zebrafish larvae

Performing a genetic screen, isolating a mutation causing a specific behavioral phenotype, identifying the affected gene, and mapping its function to the underlying circuit are the landmark stages of a thorough behavioral genetic analysis. Similar to choosing an appropriate behavioral assay to study behavior, deciding on an appropriate animal age at which to perform the assay is equally critical. Recent studies have demonstrated the suitability of adult zebrafish to model aspects of complex behavior, such as reward, learning and memory, aggression, anxiety, shoaling and sleep (Spence et al., 2008; Mathur and Guo, 2010; Norton and Bally-Cuif, 2010; Sison and Gerlai, 2010), and genetic screens for drug addiction and visual behavior have been successfully executed in adults (Li and Dowling, 1997; Darland and Dowling, 2001; Webb et al., 2009). The growing characterization of adult zebrafish behavior and the expanding repertoire of adult behavioral assays represent an exciting opportunity to model complex, higher-level behaviors and neuropsychiatric disorders. However, using adult zebrafish for behavior based mutagenesis screens introduces many of the same problems that plague screens on adult rodents: behavioral complexity and experience based variability, mutant viability to adulthood, and less accessible and more complicated underlying circuitry.

Alternatively, utilizing zebrafish larvae, only 5-7 days old, to study behavior offers a more streamlined approach to dissecting and characterizing the neural substrates of behavior through forward genetic screening (Burgess and Granato, 2008). Many of the early, stereotyped behaviors reflect the “hard wiring” of the nervous system and provide

an opportunity to understand genetically specified behavior, while minimizing the influence of experience based remodeling and increased behavioral variability at adult stages. Performing a forward genetic analysis of larval behaviors also requires significantly less maintenance than using adult behaviors since embryos/larvae can be maintained in relatively high densities (60 larvae per 9 cm petri dish), without feeding for up to a week. Moreover, a prolific brood (often 100-200 embryos per cross) enables the comparison between large numbers of mutants and wild type siblings, controlling for genetic background effects and accounting for any intrinsic behavioral variability. Mutant viability through week one of development is quite reasonable, enabling a more complete analysis of the genome compared to adult behavior-based studies where mutant viability is significantly lower and precludes testing. Importantly, one-week old larvae are still transparent, allowing for the visualization and increased accessibility of a simple, yet functional nervous system in a live and free-swimming organism, critical for mapping a gene's function within the circuit driving the behavior of interest. Ideally, behavioral phenotypes identified in larval mutants should persist into adulthood to examine experience-based modulation of behavioral phenotypes, and hence gene function. Thus, zebrafish larvae offer a unique opportunity to execute behavior based large-scale genetic screens in a vertebrate model.

Larval Behaviors

By the end of their first week of life, larval zebrafish already possess a significant repertoire of stereotyped motor behaviors that allow them to navigate their environment. Larvae engage in slow ('scoot') and fast ('burst') swimming bouts, and a variety of

unique turning behaviors with specific kinematic properties that distinguishes each maneuver (Table 1/Budick and O'Malley, 2000; Muller and van Leeuwen, 2004; Gahtan et al., 2005; McElligott and O'Malley D, 2005; Burgess and Granato, 2007a, b). Moreover, larvae execute sensory directed locomotion by moving their bodies, fins, eyes and mouths in a coordinated manner in response to acoustic, tactile, olfactory, and visual stimuli. Capturing larval locomotor behavior using high speed video cameras at 1,000 frames per second, reveals that larvae execute relatively simple 'one behavior' sensorimotor responses, for example the optokinetic eye saccade (Clarke, 1981; Neuhauss, 2003), the acoustic startle C-bend turn (Kimmel et al., 1974; Eaton et al., 1977; Burgess and Granato, 2007b), or the dark flash induced O-bend turning behavior (Burgess and Granato, 2007a), as well as more complex behaviors, such as optomotor behavior, phototaxis, and prey capture (Table 1 and (Clarke, 1981; Brockerhoff et al., 1995; Orger and Baier, 2005; Burgess et al., 2010). These more complex larval behaviors are comprised of a sequence of individual, stereotyped behavioral routines or episodes that can only be distinguished with high temporal resolution imaging (~1,000 frames per second). For example, prey capture of paramecium involves eye movements to visualize the prey, subsequently executing a series of J-bend or routine turns to align the prey with the longitudinal axis of the larvae, and then initiating a forward swim culminating with an oral capture of prey (Borla et al., 2002; Gahtan et al., 2005; McElligott and O'Malley D, 2005). Since genetic analysis of behavior includes assignment of genetic function within the underlying circuit, it is critical to analyze behaviors with identified circuitry on an individual basis rather than complex behaviors on the whole.

This need to 'compartmentalize' complex behaviors was originally recognized by Nobel laureate Nico Tinbergen, best known for his groundbreaking studies on prey capture in wasps and on the mating behaviors in stickle backs (Tinbergen, 1951). By careful observation and analysis, Tinbergen divided the complex mating ritual of the three spined stickle back into multiple, 'simpler' episodes, each of which being triggered by a specific stimulus. Eventually, Tinbergen was able to substitute the natural stimuli with artificial stimuli to induce specific behavior episodes (Tinbergen and van Iersel, 1947). This led him to formulate the concept of 'fixed action patterns', in which complex behaviors are composed of string of individual behavioral episodes, each of them triggered by specific stimuli (Tinbergen, 1951). The universal nature of Tinbergen's concept has recently been manifested at the molecular-genetic level, most elegantly in studies on *Drosophila* courtship behavior (Stockinger et al., 2005; Dickson, 2008), and on zebrafish phototaxis (Burgess et al., 2009). Thus, the idea that complex behaviors are built from an organisms' repertoire of simpler behavioral 'modules' requires us to first identify and then describe these modules with great temporal resolution. Recently, great advances have been made in applying high-speed imaging and developing software to track larval movements at millisecond resolution (Burgess and Granato, 2007a, b; Fontaine et al., 2008; Burgess et al., 2010). As a result, a multitude of larval behaviors can now be classified based on defined kinematic properties, which has made possible accurate, high throughput screening for deficits in either simple or 'complex' behaviors in an experimenter-independent manner.

Genetic Screens for Larval Behavioral Genes

In the 1970s, geneticist George Streisinger began establishing zebrafish as a genetic model system for various aspects of development, particularly the nervous system. Understandably attracted by the relatively large eyes of zebrafish larvae, Streisinger, with the help of his colleagues after his early and untimely death in 1984, pioneered the genetic analysis of visually guided behavior in a small-scale screen that used gamma-irradiation to create mutants with visual deficits (Clarke, 1981; Chakrabarti et al., 1983; Walker and Streisinger, 1983). A subsequent large-scale genetic screen, performed in the 1990s by the Nusslein-Volhard and Boenhoffer groups in Tubingen, isolated several hundred mutations affecting the initiation and execution of visual and touch - evoked sensorimotor behaviors in zebrafish larvae (Granato et al., 1996; Neuhauss et al., 1999). The key to the success of these screens was using stimulus evoked, highly robust behaviors, with known and accessible underlying circuitry. Since these screens focused on isolating mutants that failed to initiate and execute simple sensorimotor responses, the majority of mutants showed defects in the formation of the underlying circuitry. For example, *belladonna* mutant larvae possess achiasmatic retinal ganglion cell axons and consequently, mutant larvae execute a reversed optokinetic response by shifting their eyes in the opposite direction of a moving visual stimulus (Karlstrom et al., 1996; Neuhauss et al., 1999; Rick et al., 2000). Mutations in *twitch twice/robo3* and *space cadet* result in improper execution of Mauthner cell dependent startle responses to acoustic or tactile stimuli (Granato et al., 1996; Burgess et al., 2009). Rather than initiating a single C-bend away from the stimulus, followed by a smaller counterbend and subsequent forward swimming, *twitch twice* and *space cadet* mutants initiate successive,

unilateral C-bends. The behavioral defects can be traced back to very specific wiring defects of the Mauthner neuron and its spiral fiber neuron inputs, respectively (Lorent et al., 2001; Burgess et al., 2009).

Lastly, a large group of 'accordion' mutants, characterized by the bilateral rather than unilateral contraction of body muscle results in a shortening of the larvae along the body axis (Granato et al., 1996). Each geneticist's dream that the seven accordion group mutants are caused by mutations in genes acting within one genetic pathway has in part become true. Cloning of several accordion group genes reveals that they encode components of the neural network to generate and mediate contralateral inhibition, a key circuit in generating alternating muscle contractions (Downes and Granato, 2004; Hirata et al., 2004; Lefebvre et al., 2004; Hirata et al., 2005; Wang et al., 2008; Hirata et al., 2009; Olson et al., 2010). Maybe not surprisingly, several of these 'accordion' group genes have human counterparts, which when mutated result in devastating movement disorders, including hyperekplexia and congenital myasthenic syndrome (Harvey et al., 2008; Engel et al., 2010). Taken together, screening for deficits in the initiation and execution of these simple sensorimotor behaviors (excluding mutants with obvious defects in muscle fiber development) isolated over 100 mutations, defining at least 30 genes (Granato et al., 1996; Neuhauss et al., 1999). Many have been characterized and molecularly cloned, and now serve as valuable models for a variety of human neurological disorders, ranging from congenital myasthenic syndrome to horizontal gaze palsy with progressive scoliosis (Lefebvre et al., 2004; Wang et al., 2008; Burgess et al., 2009).

Following the success of the Tubingen screens, the next logical step was to model higher level processing, such as sensory gating or learning and memory. This can be achieved by designing assays that measure the ability of larvae to modulate simple larval sensorimotor behaviors, and to perform genetic screens for mutants that properly *perform* the simple behavior, but which show deficits in *modulating* that behavior. Simple sensorimotor behaviors, such as reflexes, are not simply invariant reactions to stimuli, rather they are highly modifiable and provide paradigms for identifying the neural substrates underlying higher level processing. For example, the acoustic startle response, a conserved vertebrate behavior which involves a robust, whole body reaction to adverse stimuli can be modulated by environmental cues and experience.

The homology between the zebrafish and mammalian acoustic startle circuits and the well established capability of the mammalian acoustic startle circuit to modulate behavioral output based on prior experience suggests that the larval acoustic startle response is a suitable behavior for modeling higher level processing (Furshpan, 1964; Faber et al., 1989; Liu and Fetcho, 1999; Weber et al., 2002; Nakayama and Oda, 2004; Pilz et al., 2004; Szabo et al., 2006; Burgess and Granato, 2007b). Indeed, modulation of the startle response can be tested in various assays, which provide paradigms for identifying neural mechanisms underlying sensory information processing, learning, and cognitive dysfunction. These paradigms measure the nervous system's ability to modulate its sensitivity to incoming sensory stimuli, a process called sensory gating. Sensory gating allows the nervous system to minimize or exclude irrelevant stimuli. For

example, presentation of a weak, non-startling acoustic stimulus followed shortly by a robust stimulus suppresses startle responsiveness, a form of sensory gating known as prepulse inhibition (Geyer and Braff, 1982; Braff and Geyer, 1990; Freedman et al., 1991; Swerdlow et al., 2001; Burgess and Granato, 2007b). Using this paradigm, Burgess and Granato isolated mutants with reduced pre-pulse inhibition of the acoustic startle response, indicating that the underlying circuit is mature enough for sensory gating as early as 5 days post fertilization (Burgess and Granato, 2007b). In addition to pre-pulse inhibition, repeated presentation of identical, robust acoustic startle stimuli causes a rapid decrease in startle responsiveness, which represents a simple form of non-associative learning, called habituation (Burgess and Granato, 2007b; Best et al., 2008/ Wolman and Granato, unpublished). Taking advantage of these acoustic startle response paradigms to screen for mutations altering startle sensitivity, sensorimotor gating, and non-associative learning provides exciting opportunities to understand the neurogenetic substrates of higher level processing and may provide insight into psychiatric disorders such as schizophrenia, ADHD, addiction, and other cognitive disorders marked by sensory gating deficits.

Larval zebrafish: a promising future for behavioral neuroscience

Clearly, applying classic invertebrate strategies for executing behavior based forward genetic screens to larval zebrafish provides a powerful opportunity to dissect the genetic program guiding neural circuit assembly and function in a vertebrate system. By breaking down complex behaviors into series of simple behavioral modules, zebrafish researchers can easily unravel the mechanisms critical for assembly of neural circuits

required for the execution of specific behaviors and understand how intact circuits modulate these simple behaviors in freely swimming fish. Combining the fruits of forward genetics (mutants) with the ease of conducting large scale chemical screens (Kokel et al., 2010; Rihel et al., 2010), optogenetic and live circuit tracing techniques (Baier and Scott, 2009; Wyart et al., 2009), and temporal/spatial gene misexpression approaches (Halloran et al., 2000; Nasevicius and Ekker, 2000; Scott, 2009) provides exciting opportunities to understand how the nervous system allows organisms to interpret their surroundings and behave appropriately.

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Table 1. Simple Behavioral Modules and Complex Larval Behaviors

Simple Behavioral Modules	Description
Scoot Swim (Budick and O'Malley, 2000; Burgess et al., 2010)	Slow forward swim with low bend angle where maximal bend angle is at caudal portion of larva
Burst Swim (Budick and O'Malley, 2000; Gahtan et al., 2005)	Fast forward swim with larger bend angle, maximal bend angle is at mid-body of larva; swim speed is ~10x of scoot swim
Routine Turn (Budick and O'Malley, 2000; Burgess et al., 2010)	~60°, slow angular velocity turn that occurs spontaneously
J-bend Turn (McElligott and O'Malley D, 2005)	~30°, slow angular velocity turn initiated by a slight 'tail flip' of caudal portion of larva to one side; occurs spontaneously or to re-orient fish in-line with prey
C-bend Turn (Kimmel et al., 1974; Eaton et al., 1977; Burgess and Granato, 2007b)	120-180°, high angular velocity turn that initiates escape response to tactile or acoustic stimuli; occurs with very short latency (<10 msec) to stimulation
O-bend Turn (Burgess and Granato, 2007a)	~180°, lower angular velocity turn (vs C-bend) in response to sudden removal of light ('dark flash'); occurs within 100-500 msec of dark flash
Complex Behaviors	
Optokinetic Response (Clarke, 1981; Neuhauss et al., 1999; Neuhauss, 2003)	Lateral eye movements are used to track moving object, followed by a fast saccade to reset the eyes once the object has left the visual field
Optomotor Response (Clarke, 1981; Neuhauss, 2003)	Forward swims to follow moving visual stimuli (moving bars)
Prey Tracking (Borla et al., 2002; Gahtan et al., 2005; McElligott and O'Malley D, 2005)	To capture paramecia, larva will re-orient position relative to prey with a series of small, routine or J-bend turns, then swim forward to capture prey
Phototaxis (Brockerhoff et al., 1995; Orger and Baier, 2005; Burgess et al., 2010)	Positive – larvae initiate a turn toward weak light target, followed by a scoot or burst swim toward target Negative – larvae initiate a turn away from intense light targets
Escape Response (Kimmel et al., 1974; Eaton et al., 1977; Burgess and Granato, 2007b) **many others**	To acoustic or tactile stimulation, larva initiate a high speed C-bend away from stimulus direction, followed by a smaller counter bend, and burst, forward swimming
Sensorimotor Gating/Pre-pulse Inhibition (Burgess and Granato, 2007b)	Weak, 'sub-threshold' acoustic stimuli given 300 msec prior to delivery of strong, 'above-threshold' acoustic stimuli suppresses initiation of C-bend startle response behavior
Non-Associative Learning (Burgess and Granato, 2007b; Best et al., 2008)	Short interstimulus intervals between acoustic startle stimuli reduce C-bend startle responsiveness