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Movement, technology and discovery in the zebrafish

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Abstract

Zebrafish provide unique opportunities for optogenetic studies of behavior. Here, we review the most recent work using optogenetic and imaging approaches to study the neuronal circuits controlling movements in the transparent zebrafish. Specifically, we focus on what we have learned from zebrafish about neuronal migration, network formation and behavioral control, and what the future may hold.

Introduction

Optogenetics is an emerging field that utilizes light and genes to interrogate the nervous system [1–3]. It is difficult to imagine a vertebrate animal better suited to optogenetic technology than the zebrafish (*Danio rerio*). A see-through, behaving animal provides at least two unique opportunities to understand how the vertebrate nervous system generates behavior. First, one can perform time-lapse imaging in developing animals to determine how neurons take shape and coalesce into functional networks. Second, one can monitor and manipulate neuronal activity in behaving animals to study the functional organization of these networks once they're assembled.

The advantages of zebrafish for studies of neural circuits and behavior are often touted and are the subject of recent reviews [4–6]. Here, we will cover work over the past couple years that has used optogenetic and imaging strategies to study the development and operation of neuronal networks that control movements in zebrafish. Our goal is to highlight what we have learned by studying zebrafish motor networks and to what degree this reflects information of consequence beyond the zebrafish model.

Formation of neurons, nuclei and layers

The vertebrate nervous system is populated by a wide variety of neurons organized into nuclei, columns and layers. Our understanding of neuron formation, migration and organization is largely based on a series of static images collected at different time points. Several recent studies have focused their attention on these issues using more dynamic *in vivo* time-lapse approaches in zebrafish.

Neurons are spun out at different time periods, a process that contributes to neuronal diversity. To sustain neuronal proliferation, asymmetric cell division often occurs, where

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one cell becomes a neuron while the other remains a progenitor that can give rise to additional neurons. Until recently, it was thought that during asymmetric cell division, the more apical daughter cell (the closest to the progenitor pool) remained a progenitor, while the more basal daughter cell (located further away from the progenitor pool) became the neuron. A very recent study has overturned this idea by visualizing dividing progenitor cells in the caudal hindbrain and rostral spinal cord of embryonic zebrafish [7]. These observations reveal that during asymmetric cell division, the cell that maintains contact with the basal membrane of the neural tube becomes the progenitor, while neurons derive from the more apical daughter cell. This evidence contrasts with expectations based on prior work and reveals a previously unappreciated level of complexity to the replenishment of progenitor pools.

Another form of cell division that contributes to neuronal diversity is one in which a progenitor generates neurons with two different identities. In the spinal cord, earlier work showed that cells from one dorsoventral domain, the p2 progenitor domain, generate two distinct neuronal subtypes: excitatory V2a cells and inhibitory V2b cells. Both classes of neuron have axons that project on the same side of the body. What was not known, however, was how these cells were generated. A recent study used time-lapse imaging to investigate this issue and revealed that the final division of pair-producing progenitors in the zebrafish spinal cord produces one excitatory V2a neuron and one inhibitory V2b neuron [8]. Delta-Notch interactions between the two daughter cells play a critical role in the proportional generation of the two classes of spinal neurons [8,9]. This finding has interesting implications regarding the assembly of spinal motor networks, where excitatory and inhibitory interneurons with similar morphologies are generated in equal numbers. This might help to provide a rough balance between excitation and inhibition that seems functionally important in many networks.

Once neurons are generated, they very often migrate to their final destination where they form either nuclei or laminae. This process plays an important role in the organization of neuronal circuitry. Migration of hindbrain motor nuclei has been the source of recent interest in zebrafish [10–12]. For example, facial branchiomotor neurons (FBMNs) undergo a posterior migration from their site of birth in a more rostral region of hindbrain (rhombomere 4 or r4) into more caudal regions (rhombomere 6–7 or r6–7). Once there, FBMNs migrate laterally to their ultimate destination. Despite their more caudal location, axons from FBMNs still exit at r4 where they innervate muscles of the 2nd brachial arch (corresponding to the muscles that control facial expression, middle ear and upper neck in mammals). The advantage of migration for circuit function is unknown, but it might act to minimize the cost of wiring up networks [13]. The effects of perturbing migration on the wiring and subsequent function of motor nuclei should offer some insight into the role of migration, which occurs widely in nervous systems.

In mammals, the cerebellum is a striking laminated structure whose role in motor coordination is intensely studied. The development of the cerebellum in zebrafish, including the migration and the genetic specification of identified cerebellar network components, has now become a focus of study [14–19]. Remarkably, even in 4–5 day old larval zebrafish, the equivalents of granule cells, Purkinje cells, and eurydendroid cells, in addition to climbing, parallel and mossy fiber pathways, are already present [14–18]. The several recent studies describe the neuronal types in larvae and adults [16], identify the patterns of proneural gene expression associated with the generation of these cell types, whose production persists into adulthood [17], and reveal that the continued proliferation into adulthood is correlated with the absence of the external germinal layer seen in amniotic vertebrates [18]. The new work also identifies mutants that alter granule and Purkinje cell numbers [16] and reveals the differentiation of granule neuron subtypes [14] and a role for cadherins in their migration

[15]. While these developmental studies are fundamental in their own right, a simple, accessible, functional and intact cerebellum should also provide a wonderful opportunity to study the essentials of cerebellar network function with the array of tools available in zebrafish.

Identifying motor networks

To study neuronal networks, it helps if you can reproducibly identify them. The expression of reporters driven by promoters for specific genes, like transcription factors, has been a successful way to identify motor networks in zebrafish in the past [20–22]. Our own work has used transgenic lines of fish to investigate the contribution of different classes of spinal neuron to locomotion. Studies of excitatory spinal neurons have revealed that switches in interneuron class occur during changes in swimming speed [23]. So, it is likely that more than one spinal premotor network is responsible for producing the full range of locomotor movements in zebrafish, as also appears to be the case in mammals [24]. In addition, we recently found that there are inhibitory interneurons that are shared between different motor behaviors, while others are dedicated to particular movements [25]. Dedicated neurons playing roles in escape have segmental, especially powerful commissural inhibitory connections that block motor output on the side opposite and escape bend. Another dedicated class is a large ascending inhibitory neuron only activated during struggling that may shape the pattern of activity in the spinal cord to give rise to the bends that propagate from tail to head during struggling, opposite the direction found during swimming. The picture that is emerging is that a core network of shared spinal neurons may be shaped by more specialized interneurons to produce an assortment of motor behaviors in vertebrates [26].

As we mentioned above, neurons develop at different times, which plays a crucial role in their ultimate identity. We, like others, have used optogenetic birth-dating strategies to identify neurons and networks according to when they differentiated [20,27,28]. Work in the spinal cord suggests that networks differentiate in a very logical progression; those that ultimately control the fastest/strongest movements develop first, with those controlling increasingly slower/weaker movements added with time [20,28]. The sequential addition of neurons according to speed/force generates a topographic map of recruitment in the larval spinal cord [29]. This work is beginning to forge links between patterns of development and later function and is likely to impact studies of nervous system organization more broadly.

Another way to selectively light up neuronal networks in zebrafish is to use an enhancer/trap strategy [30–33]. Recently, a zebrafish enhancer/trap line was used to elucidate the contribution of a specific class of spinal interneuron to locomotion [34]. Enhancer/trap lines often have mosaic, random expression, however in this particular line of fish the only neurons labeled in the spinal cord were an identified class of commissural local (CoLo) glycinergic interneuron. Using a combination of electrophysiology, imaging, ablation and behavioral approaches, the authors determined the context in which CoLo neurons are active and their connectivity, and were able to selectively remove one population, and only that population, and test the behavioral consequences. This study represents a very compelling test of the contribution of a spinal neuron to a vertebrate behavior. The work demonstrated unambiguously that interneurons in spinal cord can play a critical role in filtering out conflicting commands from the brain.

Monitoring and manipulating motor networks

One way to begin to understand how identified networks contribute to behavior is to determine when they are active. Imaging neuronal activity in behaving animals has been possible in zebrafish for quite some time, but one drawback to even the most recent

approaches is the requirement for the fish to be at least partially restrained [35–37]. While this is far better than some of the more invasive procedures required for electrophysiology, ideally you want to monitor neuronal activity in freely swimming fish when they can exercise their full behavioral repertoire.

A recent study demonstrated the feasibility of monitoring neuronal activity in freely swimming fish by using the Gal4-UAS system to drive the expression of a bioluminescent fusion protein GFP-Aequorin in the nervous system of larval zebrafish, [38]. The approach involved targeting GFP-Aequorin to a small set of cells and then detecting photons emitted from the whole animal, which must have, by inference, arisen from the labeled neurons it contained. The authors went on to show that a ‘neuroluminescent’ signal could be detected from a small group of hypocretin-positive neurons in the hypothalamus, which correlated well with periods of increased locomotor activity, as would be predicted from their presumed role during sleep-like states in larval fish [39,40]. While the approach is dependent on very specific targeting of small subsets of neurons to be confident about the source of the emitted photons, nonetheless it offers a powerful way of correlating activity in identified neurons with natural behavior.

Once the neurons active during a behavior have been identified, a critical next step to revealing their role in behavior is to manipulate their activity to reveal how the behavior changes. Zebrafish embryos and larvae have often been used as a proving ground for constructs that manipulate neuronal activity with light. In most cases, movements are the functional read-out. One of the earliest *in vivo* demonstrations of channelrhodopsin-2 (ChR2) was an experiment performed in zebrafish [41]. Flashes of blue light caused spikes in somatosensory trigeminal and Rohon-Beard neurons expressing ChR2, which, as you’d expect, caused zebrafish embryos to twitch on cue. An *in vivo* demonstration of light-activated glutamate receptors (LiGluRs) was also provided by expression in groups of neurons in zebrafish larvae, which included somatosensory neurons [42]. Surprisingly, in this study activation of LiGluRs in sensory neurons with light did not elicit any motor activity, but rather prevented the normal response to tactile stimulation. This finding is difficult to interpret without some indication of what the activation of LiGluRs was doing to the zebrafish neurons.

More recently, methods to silence neuronal activity *in vivo* have been demonstrated in zebrafish. By expressing halorhodopsin (NpHR) throughout the larval nervous system, a recent study showed that activation of NpHR can suppress firing in as yet unidentified, but spontaneously active hindbrain neurons [43]. By systematically silencing different regions of the hindbrain, the authors revealed a region in which NpHR-mediated neuronal silencing elicited a curious ‘rebound’ activation of swimming. It is unclear why silencing neurons should lead to a delay in swimming. Future work examining the identity of networks in this region, their activation patterns and their connectivity, could provide some explanation. The same group also recently demonstrated the application of optical tools to examine an identified motor network in larval zebrafish. Pan-neuronal expression of both ChR2 and NpHR was used to optically activate and silence, respectively, different regions of the brain to probe the premotor circuitry responsible for the optokinetic response [44]. The conclusion reached was that the saccade-generating circuit in zebrafish was in many ways similar to its mammalian counterpart. In addition to NpHR, another recently proven way to silence neuronal activity in living zebrafish is via a potassium-selective ionotropic glutamate receptor, called HyLighter, which when expressed in motoneurons can reduce the probability of touch-evoked movements [45].

One recent optogenetic perturbation led to a rather unexpected result [46]. Kolmer-Agduhr (KA) neurons are GABAergic neurons in the spinal cord that contact the central canal, but

whose function was unresolved. Using a series of Gal4-UAS driver lines, optical stimulation with LiGluRs, and genetic silencing with tetanus toxin light chain (TeTxLC), the authors investigated the role of KA cells during swimming behavior. A common result from the application of neuromodulatory chemicals to larval zebrafish is an effect on the occurrence of swim bouts, with no obvious effect on the patterning of the rhythm within bouts [47–49]. Consistent with this observation, the authors demonstrate that perturbing KA cells can modulate the occurrence of swim bouts in larval fish. However, in contrast to the expected inhibitory role of GABA, KA cells instead appear to play an excitatory role because their activation enhances the production of swimming, while their silencing reduces it. GABA can play excitatory roles early in embryonic development, but these studies of zebrafish larvae are well past these early embryonic stages, so how this excitatory effect on swimming comes about is a mystery. Future work determining the context in which these neurons are active, as well as the nature and patterns of their connectivity, should resolve this mystery.

Outlook

The usefulness of any model system is its ability to establish principles that apply broadly, well beyond the model itself. For studies of neuronal network differentiation, assembly and function, the zebrafish is an extremely valuable model. To make strong links to other vertebrate systems, regardless of level of analysis, the key is to use genetic and/or anatomical homologies that unite vertebrates. Conserved genetic programs underlie much of the construction of the vertebrate nervous system, from spinal cord to cortex. In addition, zebrafish have simpler, yet homologous brain structures to other vertebrates, including mammals [50–52]. Studies in the future using zebrafish will hopefully throw some light on the neural mechanisms underlying more complex behaviors. If we are to do so, however, one key lesson from studies of zebrafish motor networks is that simply perturbing neurons, without being able to place perturbations in the context of the activity patterns and connectivity of neurons, can be more puzzling than informative. A rigorous approach to circuit/behavior busting requires detailed information about activity and connectivity to complement the elegant optogenetic approaches that now allow us to perturb discrete, identifiable neuronal classes with such precision.

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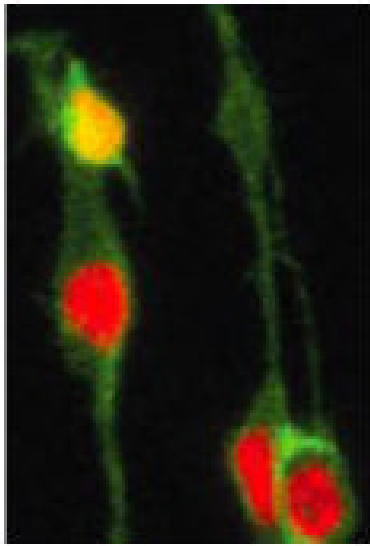
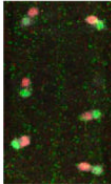

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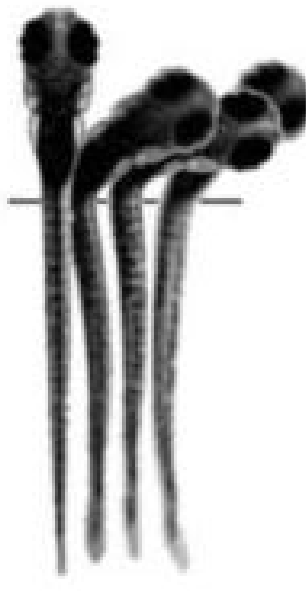
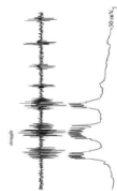
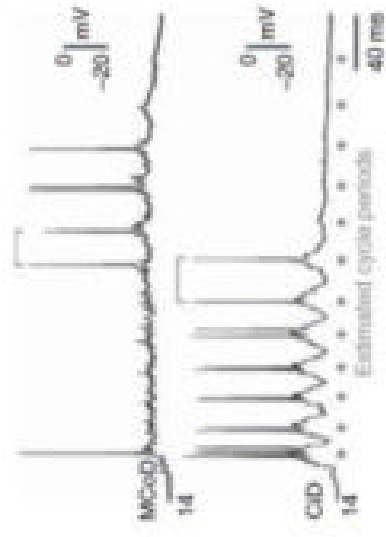
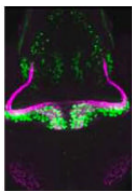
- using transgenics to visualize raphe-specific pet1 expression. *J Comp Neurol* 2009;512(2):158–182. [PubMed: 19003874]
52. Mueller T, Wullmann MF, Guo S. Early teleostean basal ganglia development visualized by zebrafish *dlx2a*, *lhx6*, *lhx7*, *tbr2* (*eomesa*), and *gad67* gene expression. *J Comp Neurol* 2008;507(2):1245–1257. [PubMed: 18181142]

Table 1

Selected recent results from the zebrafish model. Column at the left shows images from the relevant papers. **Row 1:** Live Images of neurons in the neural tube during cell division from Fig. 1 in ref. ⁷. Yellow cell has adopted a neuronal fate. **Row 2:** Horizontal view of pairs of neurons that arose from the same neuronal precursors, immunolabeled for the markers Chx10 (red) and Scl (green). From Fig. 2 in ref. ⁸. **Row 3:** Horizontal view of the brainstem motor pools (green) from a perturbation of Hox expression to explore the migration of motor pools. Red is 3A10 staining of neurons including the Mauthner cell (asterisk). From Fig. 3 in ref. ¹⁰. **Row 4:** Visualization of cerebellar structure in 5 day old zebrafish with antibodies to Parvalbumin7 and VGlut1. From Fig. 13 in ref. ¹⁶. **Row 5:** Recordings from two spinal interneurons in larval fish showing switches in activity from one to the other as swimming slows. From Fig. 7 in ref. ²³. **Row 6:** Recordings form a spinal nerve (top) and an inhibitory interneuron showing the interneuron is activated during a struggling motor pattern. From Fig. 2 in ref. ²⁶. **Row 7:** Missing escape tail bend after selective elimination of one class of spinal neuron. From Fig. 7 in ref. ³⁴. **Row 8:** Left, Image of hypocretin neurons in a horizontal view of the brain (rostral at the top) along with the distribution of photons captured from the neurons, which are expressing GFP-Aequorin. From Fig. 5 in ref. ³⁸.

Discovery	Approach	Ref
 <p>Neurons derive from the apical rather than the basal daughter cell in asymmetric cell divisions</p>	Direct imaging of cell division in the intact nervous system	7
 <p>Pair producing progenitors can give rise to one excitatory and one inhibitory neuron, possibly achieving an initial excitatory and inhibitory balance in the CNS</p>	Direct imaging of the generation of cell types in the intact spinal cord	8
 <p>Facial motoneurons migrate caudally, so the somata end up far away from the exit site of their motor axons. This puzzling movement may have implications for ideas of wiring minimization.</p>	Imaging of migration of neurons in vivo	10_12

Discovery	Approach	Ref
<p>Even very young 4–5 day old zebrafish have the major cerebellar neuronal classes, offering a simple model used to examine the development and, perhaps eventually, the function of these networks.</p>	<p>Developmental studies of cerebellum</p>	<p>14, 19</p>
<p>Increases in the speed of locomotion are accompanied by shifts in the neuronal networks, so that there is not one network for all speeds. Spinal neurons differentiate from those driving the fastest movements to those driving the slowest ones.</p>	<p>Functional imaging and physiology of spinal cord</p>	<p>23, 28</p>
<p>Some spinal inhibitory neurons are shared by different motor behaviors while others are involved in just one, suggesting that the output of a core network is shaped by specialized neurons to produce different behaviors</p>	<p>Electrophysiology of spinal cord</p>	<p>26</p>
<p>The functional role of a spinal interneuron in generating behavior was conclusively demonstrated.</p>	<p>Enhancer trapping, imaging, physiology, and behavior</p>	<p>34</p>



Discovery	Approach	Ref
<p>Two important approaches were demonstrated in zebrafish - one to monitor neuronal activity in freely swimming fish by using GFP-Aequorin and the other to excite and inhibit neurons with light-activated tools.</p>	<p>Using light to visualize and perturb neuronal activity.</p>	<p>38,41,46</p>

