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Using Imaging and Genetics in Zebrafish to Study Developing Spinal Circuits *In Vivo*

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Abstract

Imaging and molecular approaches are perfectly suited to young, transparent zebrafish (*Danio rerio*), where they have allowed novel functional studies of neural circuits and their links to behavior. Here, we review cutting-edge optical and genetic techniques used to dissect neural circuits *in vivo* and discuss their application to future studies of developing spinal circuits using living zebrafish. We anticipate that these experiments will reveal general principles governing the assembly of neural circuits that control movements.

Keywords

optical; molecular; neural networks; spinal cord; locomotion; *Danio rerio*

INTRODUCTION

Technological advances fuel scientific discovery. The recent confluence of optical and genetic approaches heralds a new era in neuroscience (reviewed in: Fetcho and Bhatt, 2004; Miesenbock, 2004; Deisseroth et al., 2006; Miller, 2006; Herlitze and Landmesser, 2007), where networks of neurons will be monitored and manipulated simultaneously in intact behaving animals, rather than a few cells at a time in dissected ones. One model system immediately capable of taking advantage of new “optogenetic” tools is the zebrafish.

Biologists have historically used zebrafish to study basic mechanisms of development because their eggs are fertilized externally, their embryos are optically transparent and they develop quickly. For example, only 4–5 days after egg fertilization, zebrafish are freely swimming with a behavioral repertoire that allows them to pursue prey and avoid predators (Kimmel et al., 1995). The use of zebrafish to examine mechanisms of development has provided a range of molecular tools for physiologists, from mutagenesis to targeted gene expression (reviewed in: Rasooly et al., 2003; Sprague et al., 2006). Thus, the transparency of zebrafish at these early stages, their rapid locomotor development, and their genetic accessibility make them ideal subjects for studying the assembly of motor networks using optogenetic tools.

While zebrafish are established models for development (reviewed in: Granato and Nusslein-Volhard, 1996; Grunwald and Eisen, 2002) and have been used to image and record from motor circuits *in vivo* (reviewed in: Fetcho and Liu, 1998; Drapeau et al., 2002; O’Malley et al., 2003), they have only recently become the focus of studies combining genetic and optical approaches to examine neural circuits and their links to behavior

(Gleason et al., 2003; Higashijima et al., 2003; Hale et al., 2004; Li et al., 2005; Kimura et al., 2006; Smear et al., 2007). Here, we review recent optogenetic techniques and outline future experiments using these approaches to study spinal circuit assembly in zebrafish.

Specifically, we are interested in using the power of this model to address the following questions: How do you define components of a functional circuit in developing spinal cord? How does the structure of identified spinal circuits change during development and what impact does this have on function? What processes orchestrate structural and functional changes to produce mature locomotion? Are there any principles of organization in motor systems that might be revealed more easily early on in life? Providing answers to these developmental questions will undoubtedly yield greater insight into the operation of neural networks in adults. Watching a circuit build itself can help us understand how it fundamentally works.

In considering these issues, it is important to keep in mind the principal advantage of optogenetic approaches, namely, the targeted and minimally invasive delivery of agents designed to image or perturb the structure and function of neural circuits *in vivo*. So how is this accomplished in zebrafish?

TRANSGENESIS AND IMAGING

Zebrafish formally arrived as a genetic model system with the generation of clones almost 3 decades ago (Streisinger et al., 1981). Since then, the number of approaches designed to perturb or insert genes in developing zebrafish has increased substantially (reviewed in: Kuwada, 1995; Lekven et al., 2000; Westerfield, 2000; Corey and Abrams, 2001; Amsterdam and Hopkins, 2006). Arguably, the most useful for the dissection of neural circuits is the use of transgenesis to label specific cell types. In the past, the generation of DNA constructs with the appropriate promoter and reporter elements required a significant level of expertise. However, recent technological advances, including homologous recombination into bacterial artificial chromosomes (BACs), have obviated the normally labor-intensive cloning approaches used to generate these constructs. More detailed descriptions of BAC-based homologous recombination can be found elsewhere (Jessen et al., 1998; Lee et al., 2001; Thermes et al., 2002; reviewed in: Yang et al., 2006). Suffice to say this approach, as well as transposon-mediated methods (reviewed in: Kawakami, 2005), have greatly simplified the generation of transgenic zebrafish, putting molecular tools in the hands of novices.

The first stable transgenic lines of zebrafish with tissue-specific expression of reporter constructs were created using constitutively active promoters (Higashijima et al., 1997; Long et al., 1997; Higashijima et al., 2000). However, more precise temporal control of expression can be achieved using promoters that rely on heat (Halloran et al., 2000), hormones (de Graaf et al., 1998), or light (Yu et al., 2007) to activate them.

Of course, the selection of the promoter depends entirely on the question you hope to address, as does the selection of the reporter. For *in vivo* optical studies, a veritable rainbow of fluorescent proteins is available (reviewed in: Miyawaki, 2005; Shaner et al., 2005). Many are man-made derivatives of green fluorescent protein (GFP) originally discovered in jellyfish (Shimomura et al., 1962), while others have been found in corals (Matz et al., 1999). There are also proteins, whose fluorescence can be switched on and off by light, like Dronpa (Habuchi et al., 2005) or converted from green to red, like Kaede (Ando et al., 2002), which adds yet another level of control (reviewed in: Lukyanov et al., 2005).

Genetically encoded fluorescent indicators of neuronal activity and transmitter release are also available (reviewed in: Fetcho and Bhatt, 2004; Miyawaki, 2005; Knopfel et al., 2006),

further extending the capability of *in vivo* imaging to probe neural circuits. Genetic approaches can also be used to silence or activate neurons, as well as to map out circuit connectivity (reviewed in: Kiehn and Kullander, 2004; Callaway, 2005; Miyoshi and Fishell, 2006; Herlitze and Landmesser, 2007). Together, these technologies open up the exciting prospect of identifying, monitoring, and controlling neural circuits in developing zebrafish with light.

Imaging of labeled specimens is typically accomplished in three principal ways, wide-field epifluorescence, confocal microscopy, and two-photon microscopy. Without going into too much detail (see: Yuste and Konnerth, 2005), the salient differences can be summarized according to the modes of excitation and detection. Wide-field epifluorescence uses a light source to excite fluorophores broadly within the specimen, and the fluorescence of a large region is then detected. Confocal microscopy uses lasers and mirrors to scan a spot of light through the specimen and narrow optical sections are extracted at the detection end using an optically conjugate pinhole. For two-photon microscopy, specialized lasers and the nonlinear optical properties of fluorophores provide very localized excitation, so any detected fluorescence must be from the focal point. Nothing so far can match the localized excitation provided by two-photon imaging, which minimizes out-of-focal-plane photobleaching and phototoxicity, and can provide a means to precisely trigger photoactivatable agents (reviewed in: Callaway and Yuste, 2002). For most studies in zebrafish, however, confocal microscopy is more readily available and more versatile than multiphoton imaging.

We have chosen to use these optical and genetic methods to study spinal circuits, because they operate within a very well-defined functional context, movement. This provides a simple setting to ask questions about how neural circuits are designed to produce particular behaviors. So, what have we learned about spinal circuits in zebrafish?

DEFINING DEVELOPING SPINAL NETWORKS

Spinal neurons are traditionally defined using a combination of qualities, including morphology, firing properties, and transmitter phenotype (reviewed in: Jankowska, 2001). Similarly, spinal circuits and networks are assessed by patterns of connectivity (reviewed in: Edgley, 2001). Unfortunately, all these are features that can change during development (reviewed in: Landis, 1990; Bate, 1999), making the reliable identification of functional circuits at different ages more difficult. One notable exception is a local reflex inhibitory circuit in spinal cord that can be identified at different developmental stages using a stimulation paradigm (Xu et al., 2005; Mentis et al., 2006). So-called Renshaw cells are a source of feedback inhibition to motor neurons and are reliably identified by antidromic stimulation of the ventral roots. Nonetheless, it is extremely difficult to assess the contribution of identified neural circuits to developing motor patterns. Ideally, readily identifiable classes of spinal neuron would be labeled and monitored *in vivo*, as the animal develops and the networks are brought on-line. This is exactly the sort of unprecedented opportunity provided by optogenetics in zebrafish.

By the time zebrafish have passed 4 days of development [Fig. 1(A)], they are freely swimming and have a diverse array of anatomically distinct spinal neuron classes. For simplicity, the current list has been summarized schematically in Figure 1. There are two classes of motor neurons, primary and secondary [Fig. 1(Bi)], so named due to their respective emergence during development (Myers et al., 1986). Spinal sensory inputs include mechanoreceptive Rohon-Beard cells and the afferents of dorsal root ganglia [Fig. 1(Bii)]. Interestingly from a developmental standpoint, Rohon-Beard cells disappear soon after the fifth day of development, and their function is taken over by the dorsal root ganglia

(Bernhardt et al., 1990). From an evolutionary standpoint, this replacement process also occurs in amphibians (Hughes, 1957), but well before changes in spinal network activity associated with metamorphosis (Combes et al., 2004). It is still unclear why this occurs. Finally, there are also a number of glutamatergic, glycinergic, and GABAergic spinal interneurons, in addition to one class of spinal serotonergic interneuron [Fig. 1(C)].

While a majority of these classes were originally discovered and described at earlier stages of development, there are some classes of spinal interneuron that were not (e.g., MCoDs, UCoDs, CoLAs, VeMes; see Fig. 1 legend for definitions). It is unclear whether this is merely an artifact of differences in the labeling method or a consequence of their later arrival. Similarly, there are classes of embryonic interneuron, whose presence has not been observed in larvae (e.g., commissural bifurcating or CoB, ipsilateral caudal or IC, ventral longitudinal descending or VeLD). Again, it is unclear whether they disappear, or are simply unrecognizable in their more-differentiated states.

This raises one problem with normal “fix and stain” methods, which stitch together a life history from different snap-shots in time. These methods can lead to mistakes, because they depend on a correct matching between the same neurons at different stages of development. For example, in zebrafish, VeLD interneurons in embryos and MCoD interneurons in larvae have somata located in similar positions, which originally led to the incorrect proposal that VeLDs and MCoDs were one and the same (Bernhardt et al., 1990). However, VeLDs are GABAergic and ipsilaterally projecting (Bernhardt et al., 1992), while MCoDs are glutamatergic and contralaterally projecting (Higashijima et al., 2004). So, unless VeLDs undergo a dramatic change in both axonal trajectory and transmitter phenotype, it is unlikely that they are MCoDs, which raises the question, what happens to VeLDs? This could have important consequences for developing motor patterns, because the function of GABA changes dramatically, as chloride gradients shift during development (reviewed in: Ben-Ari, 2002). Specifically, GABA becomes hyperpolarizing in adults. Therefore, the role inhibitory circuits play in embryonic motor patterns, could differ from their role in more mature ones (Chub and O'Donovan, 2001; Jean-Xavier et al., 2007). Fortunately, the fate of GABAergic VeLD interneurons should be an easy issue to resolve in zebrafish. Labeling VeLDs with vital dyes early and tracking their development *in vivo* will directly assess whether they are present and unchanged in larvae or transform into MCoDs, or even disappear like Rohon-Beard cells.

Although there are some developmental issues to resolve in the nomenclature, it is nevertheless clear that the classes of spinal neuron present in larvae represent a functioning network. After 4 days of development, zebrafish larvae are fully functioning animals that navigate through their environment using swimming movements that are virtually indistinguishable from adults (McHenry and Lauder, 2006). Furthermore, spinal motor neurons and interneurons studied in adult zebrafish or adult goldfish with identical morphology perform the same function (Westerfield et al., 1986; Fetcho and Faber, 1988; Liu and Westerfield, 1988; Fetcho, 1990). So, how do we get to the larval network from an embryonic one? As the VeLD example illustrates, it would be advantageous to use definitions other than morphology and transmitter phenotype, which could then be used to track circuits through development.

One recent successful strategy is the use of transcription factors. As their name suggests, transcription factors regulate DNA transcription and are thus well placed to control the ultimate identity of neurons. Landmark studies describing the discrete top-down patterning of transcription factor domains have revolutionized studies of spinal circuits (reviewed in: Jessell and Sanes, 2000; Goulding et al., 2002). In zebrafish, genes for the transcription factors *Engrailed-1* (*En1*) and *alx* (a zebrafish homolog of *Chx10*) define two discrete

classes of spinal interneuron [Fig. 1(C)]. CiAs are labeled by *En1* and provide a source of ipsilateral inhibition that gates sensory inputs during locomotor activity (Higashijima et al., 2004). CiDs are labeled by *alx* and provide a source of ipsilateral excitation that drives locomotor activity (Kimura et al., 2006). The hope is that distinct transcription factors will pick out distinct classes of interneuron, whose function could be inferred from their structural features. This function could then be tested using physiological recording and perturbation methods.

More important perhaps than consistent markers during development in the same species, is the ability to use these same markers to compare circuits between species. Both *En1* and *alx* (*Chx10*) have been shown to label neurons with similar morphology and transmitter phenotype in mammals (Saueressig et al., 1999; Thaler et al., 1999), but in mammals they are heterogeneous in other respects, indicating functional subdivisions within the populations.

In addition to transcription factors, other markers could be used to selectively label networks of spinal neurons in transgenic animals. Because firing properties and synaptic inputs play an important role in defining neurons and circuits, ion channels (Diez-Garcia et al., 2005), calcium buffers (Meyer et al., 2002), or receptors (Hantman and Perl, 2005; Sato et al., 2005; Prober et al., 2006) could be used as defining features. It is even conceivable that a combination of two or more factors could be used as markers. Fluorescent proteins can be split in two and will only yield fluorescence when reunited in the same cells (Zhang et al., 2004). This means that two different promoters (e.g., a transcription factor and an ion channel) could be used to drive reporter expression, and only neurons where both reporters were expressed, would become fluorescent. Similarly, combinatorial gene expression strategies, like Cre-mediated site-specific recombination (Thummel et al., 2005), could be used to selectively label neural circuits *in vivo*.

The key for developmental studies is to use markers that will reliably mark different classes of spinal neuron, so that their anatomy and connectivity can be assessed repeatedly at many consecutive time points [Fig. 2(A)]. This will then set the stage for an examination of the changes in identified spinal circuits during development and how these drive changes in motor behavior.

DEVELOPMENT AND SPINAL NETWORK STRUCTURE/FUNCTION IN ZEBRAFISH

Zebrafish undergo a very stereotyped sequence of locomotor development (Saint-Amant and Drapeau, 1998), as do all animals, including humans (Thelen, 1979; Piek and Carman, 1994; D'Elia et al., 2001). The first movements (>17-hours-old) are spontaneous, powerful muscle contractions that involve coiling of the whole body [Fig. 3(A)]. Next (>27-hours-old) are more coordinated swimming movements with less powerful muscle contractions, which are typically only elicited when the zebrafish is startled [Fig. 3(B)]. Finally (>96-hours-old), spontaneous, goal-directed swimming emerges, with even weaker muscle contractions that involve mostly the tail and the newly functional pectoral fins [Fig. 3(C)]. This sequence of development results in zebrafish larvae that can move smoothly through a range of movements, from fast escapes to slow swimming.

Since spinal motor patterns change both during development and later in life to produce a range of movements, is it possible that these phenomena are linked? For example, are the bending movements produced by embryos and the escape bends produced by larvae generated by the same network? Does the network that produces swimming movements emerge as a refinement of this early network? This is not inconceivable, given that escape

and swimming circuits share the need to drive muscle contractions on the same side of the body, while inhibiting them on the other (Fetcho and Faber, 1988). How do we test this? Questions about circuit assembly can be addressed on many levels of organization, from the synapse to the network.

Synaptic Dynamics

Neurons communicate through synapses, which govern the level of excitability and the patterning of activity within a circuit. The process of tagging synaptic markers with fluorescent proteins provides a means of monitoring dynamic wiring processes *in vivo*. This does not appear to adversely affect function. For example, GFP has been fused to postsynaptic receptors (David-Watine et al., 1999) and ion channels (Marshall et al., 1995; Grabner et al., 1998), with no measurable effect on activity.

Presynaptic and postsynaptic components have been used with great success *in vivo* to track developing synapses. Synaptophysin is a protein associated with synaptic vesicle recycling (Valtorta et al., 2004). In zebrafish, the ingrowing axons of retinal ganglion cell (RGC) axons have been labeled with a synaptophysin: GFP reporter and monitored as they innervate the optic tectum (Meyer and Smith, 2006). A similar approach using tagged synaptophysin was also performed *in vivo* in *Xenopus* to label developing motor neuron axons (Javaherian and Cline, 2005) and developing RGC axons (Ruthazer et al., 2006). These studies clearly demonstrated a direct link between synaptogenesis and axonal elaboration, whereby new axon branches emerge from newly formed synapses. The same process also occurs in dendrites (Niell et al., 2004). In this case, PSD-95, a scaffolding protein associated with the postsynaptic density of excitatory synapses (Sheng, 2001), was fused to GFP and used to label dendritic synapses of tectal neurons in zebrafish.

With respect to presynaptic and postsynaptic communication, *in vivo* studies of the developing neuromuscular junction in zebrafish suggest that axons are not required for the formation of postsynaptic specializations, but are necessary for their stabilization (Flanagan-Steet et al., 2005). In addition, once these stable postsynaptic specializations are formed, axons tend to compete over the same sites (Walsh and Lichtman, 2003). This arrangement, where functional synapses act as points from which new axonal or dendritic arbors grow, provides an elegant means to construct functional circuits.

Selectively labeling spinal networks with presynaptic and postsynaptic markers will allow tests of the relevance of these mechanisms in central motor networks. It will be interesting to see if spinal interneuron axons similarly compete over dendritic space and how motor neuron (or interneuron) dendrites react to this competition. For instance, when hunting for postsynaptic connections, do emerging premotor axonal branches focus on a single motor neuron, or a homonymous pool of them? Will dominant interneurons emerge or will synaptic connections be distributed evenly within the class? How does the relative distribution of synapses change during development? To what degree is connectivity reflected in motor function?

Cellular Dynamics

The morphology of a neuron has long been suggestive of its function (Ramon y Cajal, 1904). The fact that neuronal form changes so dynamically during development provides an opportunity to study exactly how form contributes to function in the nervous system. Axonal trajectory and dendritic arborizations are major anatomical-defining features of neurons. There is a great deal of work examining the molecular mechanisms underpinning axon guidance and synaptogenesis (reviewed in: Tessier-Lavigne and Goodman, 1996), a field in which zebrafish are beginning to contribute *in vivo* information (reviewed in: Hutson and

Chien, 2002). However, a recent report using optogenetic approaches in zebrafish has revealed a previously underappreciated role for dendritic targeting in shaping neuronal connectivity in vertebrates (Mumm et al., 2006).

In the retina, the innerplexiform layer is comprised of laminar synaptic connections between RGCs and their presynaptic partners, bipolar and amacrine cells (reviewed in: Mumm et al., 2005). In zebrafish, RGCs were individually labeled with GFP and their dendritic arbors tracked during development. Remarkably, they found that dendrites from RGCs selectively grew toward their future synaptic laminae, where axons from amacrine cells were already present. A similar role for dendritic structure dictating the pattern of circuit connectivity (and thus function) has been described in the olfactory (Jefferis et al., 2004) and motor (Landgraf et al., 2003) systems of flies. At least with respect to the olfactory system, it is clear that transcription factors also play a key role in the patterning of dendrites (Komiya and Luo, 2007), further reinforcing their importance in the formation of neural circuits. Genetic programs that can shape both axonal and dendritic patterning provide some clues as to how the developing nervous system can generate functional networks within such a dynamic environment.

Similar dendritic targeting strategies may be used in central motor circuits. A study in zebrafish embryos imaging the interaction of Mauthner cell axons with primary motor neurons in spinal cord demonstrated that primary motor neurons have highly dynamic dendritic filopodia (Jontes et al., 2000). Like the Mauthner cell, zebrafish spinal interneurons also have a very distinctive morphology, which could also predict their function (Bartelmez, 1915). By individually labeling neurons with either expression of GFP, or the selective activation of Dronpa (Aramaki and Hatta, 2006), driven by the appropriate promoter, it will be possible to track the dendritic and axonal changes in spinal circuits that are associated with changes in motor behavior. What is the relative contribution of axonal versus dendritic targeting to the formation of spinal circuits? Do all spinal circuits follow the same rules and how this might reflect the diversity of cell types? What does this tell us about the limits of circuit design and the function of spinal networks?

Network Dynamics and Activity

Identifying the cells, circuits, and networks that underlie behavior, is a fundamental pursuit of neurobiology. Sensory systems have proven fruitful ground for optogenetics to investigate how the activity of neuronal networks instructs behavior (Wang et al., 2003; Suh et al., 2004; Marella et al., 2006; Verhagen et al., 2007). This is because controlled stimuli can be delivered that elicit known behavioral responses, and the resulting responses in sensory networks monitored. Two areas, where the activity of networks has been imaged during development, are the olfactory and visual systems of zebrafish.

In zebrafish, odors are communicated by the activity patterns of olfactory glomeruli in the olfactory bulb (Friedrich and Korsching, 1997), as they are in the olfactory bulb of mice (Rubin and Katz, 1999) and the antennal lobe of flies (Wang et al., 2003). Using a genetically encoded calcium indicator, inverse pericam, the response of glomeruli to amino acids and bile acids was recently imaged during development (Li et al., 2005). This study found acid-evoked responses in the olfactory bulb that coincided with the emergence of behavioral responses to these odors. Critically, however, a gross pattern of functional topography was established early [Fig. 4(A)]. This pattern was later refined, suggesting that the adult neuronal circuits were a product of local refinements and elaborations, as opposed to large scale reorganizations of the olfactory bulb. This is certainly a parsimonious solution to producing the complexity observed in adults, which would not compromise the function of the network. In other words, mature neural circuits emerge from elaborations of an embryonic ground state, allowing them to continue to function as they develop.

A study of the visual system of larval zebrafish imaging the emergence of activity patterns also points in a similar direction (Niell and Smith, 2005). As for odors in the olfactory bulb, a topographic map representing the visual field is established early in the optic tectum (the fish correlate of the superior colliculus), at stages when they first begin to respond to visual stimuli. However, while the gross pattern of organization is laid down from the beginning, visual acuity improves substantially as larvae get older. It is likely that this improvement is due to a fine-tuning of the retinotopic map based on experience, since visual acuity did not improve in larvae raised in complete darkness. Like the olfactory bulb, visual circuits are unlikely to be the product of random wiring, followed by the pruning of inefficient synapses, but rather an immediate construction of a functional circuit, upon which refinements and elaborations generate maturity.

The same imaging approaches used in the olfactory bulb and optic tectum could be used to determine the relative contribution of spinal circuits to different locomotor behaviors during development. In addition, to test their time of differentiation, the photoconvertible protein Kaede could be used as a method of *in vivo* time stamping. Kaede turns from green to red in the presence of UV light (Ando et al., 2002), and has been expressed in the nervous system of zebrafish using neuron specific promoters (Kimura et al., 2006; Sato et al., 2006). Kaede protein that continues to be generated after conversion is green, so the presence of red Kaede protein is an indication that the cells differentiated before the conversion time point [Fig. 5(A,B)]. These approaches would conclusively address whether mature behaviors are also a product of network refinement or the emergence of new circuits, or a mixture of the two.

To test function, it is important to move beyond anatomical description. The beauty of studying motor networks to address issues of network assembly is that there is a clear functional context, from which predictions about perturbation experiments can be made. Specifically, optogenetic perturbations can test with unmatched precision how activity and/or external inputs shape the architecture of nascent networks and their behavioral output.

PERTURBING SPINAL NETWORKS AND LOCOMOTOR DEVELOPMENT

The reason why olfactory and visual centers are such popular subjects for network assembly is their topographic organization. Such regularity provides a powerful means to study how dendrites and axons interact during synaptogenesis to construct functional networks. You can predict where a dendrite should arborize and what axons should contact it. It now appears that the spinal cord may also be topographically organized. It is textbook knowledge that the spinal cord can be divided into 10 laminae, based on broad cytological and functional features. However, it was not clear how interneurons within or between these laminae might be organized to produce various strengths and speeds of movement.

Using a combination of electrophysiological, imaging, genetic, and perturbation approaches in larval zebrafish, we recently demonstrated that the location of a neuron in spinal cord could predict when it was recruited during swimming (McLean et al., 2007). The most ventral motor neurons and excitatory interneurons would fire rhythmically at the slowest swimming frequencies, with progressively more dorsal ones recruited as swimming speed increased [Fig. 6(Ai)]. For inhibitory interneurons, the opposite pattern was apparent; dorsal interneurons would fire at the slowest swimming speeds, with progressively more ventral ones recruited as swimming speed increased [Fig. 6(Ai)]. This topography of recruitment could at least be partly explained by matching gradients in neuronal excitability. The most ventral excitatory and dorsal inhibitory interneurons had the highest input resistances and were therefore the most excitable [Fig. 6(Aii)]. At present, it is unclear what mechanisms in the brain could contribute to the recruitment pattern we observe. We do not know whether or not there are distinct descending inputs that selectively activate slower versus faster spinal

circuits. However, given the pattern of excitability, there is not necessarily a need for selective drive, since the proper circuits could be selected based on a general level of drive to spinal cord.

There is also evidence that this topography may be related to age. We already know that primary motor neurons, which are born first, participate in the fastest swimming behaviors. However, a recent study using Kaede driven by the *alx*-promoter has shown that the oldest, excitatory CiD interneurons are also the dorsal-most, and contribute to the most powerful motor behaviors in hatchling zebrafish embryos [Kimura et al., 2006; see also, Fig. 5(B)]. It is not known whether the same holds true for larvae, but given the pattern of recruitment we observed, it is certainly plausible. Thus, we would predict that the dorsal-most excitatory interneurons and the ventral-most inhibitory interneurons are the oldest [Fig. 6(Aiii)]. This type of organization likely enables larval zebrafish to move efficiently through their speed range [Fig. 6(B)]. The fact that these patterns of recruitment may reflect time of neuronal differentiation raises the possibility that spinal networks emerge in accord with the strength of the movement to which they contribute. The strongest movements and the neurons driving them emerge first during development with weaker movements and neurons added later. In retrospect, such gradients in excitability, recruitment, and age make sense given how important gradients are for setting up spinal patterning (Briscoe et al., 1999).

How do we test the contribution of activity or external inputs to the topographic organization of spinal circuits? Pioneering investigations of developing spinal networks in the past relied on pharmacological perturbations to alter network activity and chemical or physical lesions to remove external inputs (reviewed in: Sillar et al., 1998; O'Donovan, 1999; Nishimaru and Kudo, 2000; Clarac et al., 2004). The arrival of more sophisticated optical and genetic approaches, particularly suited to the zebrafish model, now mean that these perturbations can be extremely precise. For the purposes of this review, we have organized different approaches according to their ability to chronically or acutely perturb network function.

Chronic Perturbations

In mutagenesis, genes are randomly disrupted using either UV light or chemical agents, and their contributions to development are reflected in the phenotype. The genes are then typically identified using a combination of candidate gene and positional cloning approaches. The large-scale Tübingen screen generated a number of zebrafish mutants with severe locomotor deficits (Granato et al., 1996). Unfortunately, from the point of view of central motor circuit development, a majority of the mutant phenotypes studied so far are a consequence of defects in muscle-specific genes (Ono et al., 2001; Ono et al., 2002; Downes and Granato, 2004; Gleason et al., 2004; Hirata et al., 2004; Zhang et al., 2004; Schneider and Granato, 2006). A few, however, have turned out to have central deficits.

For example, zebrafish *bandoneon* mutants develop normal spontaneous coiling movements, but fail to develop early embryonic swimming movements due to a mutation in a glycine receptor β subunit gene (Hirata et al., 2005). Zebrafish shocked mutants exhibit altered early coiling movements due to a mutation in the glycine transporter-1 gene (Cui et al., 2005), but do eventually develop swimming movements. These mutants could be extremely useful for dissecting the relative contribution of glycinergic signaling during different stages of locomotor development.

The usefulness of a mutation approach relies on the ability to rapidly screen and recognize deficits that would hint at changes in the circuit in which you are interested. This can be tricky with locomotion, because the movement deficits may not be a result of central defects, as illustrated above. One exquisite example of high throughput screening to identify

circuit malfunction is the recent identification of a sensorimotor gating mutant, *Ophelia* (Burgess and Granato, 2007). Using a combination of high-speed imaging and automated movement analysis, the authors isolated mutant fish that failed to attenuate escape responses elicited shortly after a weaker stimulus as normal fish do. Because this attenuation is the zebrafish form of prepulse inhibition (PPI), and impaired PPI is symptomatic of many neuropsychiatric disorders, *Ophelia* mutants are anticipated to shed light on the genes and circuitry underlying this phenomenon (Burgess and Granato, 2007). Indeed, zebrafish hold great promise for uncovering the molecular and cellular basis of human diseases reviewed in (Amsterdam and Hopkins, 2006; Lieschke and Currie, 2007).

It is important to consider downstream effects that may result from the generation of mutations. The nervous system is, of course, very plastic. This is clearly illustrated by the shocked mutation, where a secondary consequence of disrupted neural activity was a persistence of an embryonic state of electrical coupling in the muscle fibers, which exacerbated the phenotype (compare: Luna et al., 2004; Cui et al., 2005). Sometimes these homeostatic phenomena can also be exploited to investigate neural network plasticity. A mutation in the *des/notch1a* gene that results in extra Mauthner cells (Gray et al., 2001), was used to test how a functional neural circuit would cope with duplications (Liu et al., 2003). Zebrafish larvae with extra Mauthner cells are not hyperactive, but rather exhibit normal escape movements [Fig. 7(A)]. The escape circuit reconfigures itself, such that each extra Mauthner cell covers a smaller, nonoverlapping territory, dividing the outputs along spinal cord [Fig. 7(B)]. This demonstration of network homeostasis during formation of the escape circuit helps explain how startle circuits in mammals with much larger numbers of neurons might have evolved.

There are also a number of other chronic methods for perturbing developing circuits *in vivo*. Morpholino oligonucleotides can be injected into single-cell zebrafish embryos and will bind to messenger RNA to prevent protein synthesis, providing a useful means to “knockdown” gene function (Nasevicius and Ekker, 2000). Morpholinos have been used to reveal the contribution of identified currents to develop spinal circuit excitability and behavior *in vivo* (Pineda et al., 2005), as well as the role glycinergic signaling plays during spinal interneuron differentiation (McDearmid et al., 2006). Conversely, constructs can be injected into single-cell embryos to chronically enhance gene function. For example, injection of *hoxb1b* mRNA produces a segmentation duplication in the hindbrain, which results in two rhombomeres that contain Mauthner cells (Hale et al., 2004). This approach provides another elegant means to investigate how ectopic cells integrate into a functional circuit. A similar strategy could be used to probe the consequences of ectopic components within spinal circuits, given the correct promoter.

Cell-specific promoter driven strategies can also be used to functionally silence networks. By expressing tetanus toxin light chain to prevent transmitter release (Sweeney et al., 1995) or an inward rectifying potassium channel (Kir2.1) to hyperpolarize cells (Johns et al., 1999), a relatively recent study demonstrated the importance of spontaneous release and firing activity on the establishment and maintenance of topographic connections in the olfactory bulb (Yu et al., 2004). Kir2.1-mediated silencing has also been used to demonstrate the importance of activity in the formation of RGC axonal contacts in developing zebrafish optic tectum (Hua et al., 2005). Another study in zebrafish has demonstrated that cell-specific expression of tethered peptide toxins can modulate ion channels and receptors *in vivo* (Ibanez-Tallon et al., 2004). Ideally, however, it is better to have precise spatial and temporal control over a perturbing agent, which is exactly what optogenetic strategies can provide.

Acute Perturbations

Lasers provide a powerful means to target cells and to selectively remove them from the circuit quickly, minimizing any accommodative measures that may take place during development. The earliest studies using lasers *in vivo* to test the contribution of interneurons to behavior include perturbations of visual orientation in flies (Geiger and Nassel, 1981) and chemotaxis in worms (Bargmann et al., 1993). In zebrafish, laser ablations have been used *in vivo* to selectively perturb the neural circuits underlying escape behavior (Liu and Fetcho, 1999), visuomotor responses (Roeser and Baier, 2003), prey capture (Gahtan et al., 2005), and swimming (McLean et al., 2007). Lasers have also been used during zebrafish development to assess the contribution of neuroepithelial cells to axon guidance (Kuwada, 1986) and the dynamics of myelination by oligodendrocytes (Kirby et al., 2006). In flies, laser ablation experiments have examined competitive mechanisms underlying the formation of dendritic territories in the peripheral nervous system (Gao et al., 2000). Interestingly, this study found that competitive interactions between neighboring neurons were apparent at larval, but not embryonic stages of development, supporting age-dependent differences in the wiring dynamics.

Laser ablation approaches are limited by the fact that only a handful of cells can be removed at a time. So, while they may be perfect for testing the contribution of relatively small nuclei to development, they are less useful for removing large populations. Here, phototoxic agents could prove more useful. One recent example of a genetically encoded means to ablate cells is called KillerRed (Bulina et al., 2006). KillerRed is a dimeric red fluorescent protein developed from a homolog of GFP that generates reactive oxygen species when illuminated with green light. This is sufficient to kill cells within minutes, but only the ones illuminated. This means a single cell, a subset, or a whole population can be ablated depending on the expression pattern and the degree and localization of illumination.

Clearly, killing cells by an irreversible process is not ideal. Fortunately, there are now acute ways to reversibly activate and silence neurons. A recent example of this uses a combination of channelrhodopsin-2 (ChR2) and *Natronomonas pharaonis* halorhodopsin (NpHR) to rapidly activate and silence neurons, respectively, using light (Zhang et al., 2007). These proteins have nonoverlapping spectra (meaning they can be used simultaneously) and utilize a nonselective cation channel (ChR2) and a chloride-ion pump (NpHR) to alter neuronal excitability. In embryonic chick spinal cord, ChR2 and a different inhibitor of neuronal activity, vertebrate rat rhodopsin 4 (RO4), have been used to manipulate spontaneous activity (Li et al., 2005). More targeted studies using these approaches will provide the ability to assess the contribution of spontaneous activity within identified classes of interneuron to network assembly. They also open up the exciting possibility of artificially coupling or uncoupling sets of developing neurons to test the effects of coincident activity on wiring.

Light is not the only way to control perturbations. A ligand-based approach was recently used to reversibly perturb spinal rhythmogenesis in developing mammals (Gosgnach et al., 2006). Interneurons arising from a domain called V1 in spinal cord consist of at least two classes; Renshaw cells, which mediate recurrent inhibition to motor neurons and Ia inhibitory interneurons, which mediate feed-forward inhibition to antagonistic motor pools. Both of these classes are labeled by the transcription factor *En1*, which as mentioned above, labels a single class of spinal interneurons in zebrafish larvae, CiAs. V1 interneurons in mice were selectively silenced by conditionally expressing the *Drosophila* allatostatin G-protein-coupled receptor and then bath-applying allatostatin. The allatostatin receptor couples to endogenous mammalian inward rectifying potassium channels, which decrease input resistance and thus neuronal excitability (Lechner et al., 2002). The perturbation of the locomotor rhythm that resulted was reversed by simply washing off the allatostatin.

Allatostatin produced a slowing of the motor rhythm that corroborated the slow locomotor rhythms observed in mice where V1 interneurons were chronically removed in the same study. The contribution of V1 interneurons to faster locomotor outputs is supported by detailed physiological studies of *En1*-positive neurons in frog embryos, which are more reliably active during faster swimming movements (Li et al., 2004). *En1* labels ascending inhibitory interneurons in frog embryos identical to zebrafish CiAs (Higashijima et al., 2004). Other more recent examples of acute perturbation strategies utilize light-gated glutamate receptors to excite neurons (Szobota et al., 2007) or ligand-gated chloride channels to inhibit them (Lerchner et al., 2007).

Of course, perturbations need to be assessed by some means. *In vivo* imaging of dendritic fields and axonal arbors will certainly report changes in the location of inputs and outputs, but this is only an inference of network connectivity. It would be useful to have a more direct measure of the degree to which perturbations affect network connectivity. A recent study reports the fabrication of a genetically targetable trans-synaptic tracer that labels only monosynaptically connected partners (Wickersham et al., 2007). This technique improves upon previous ones, where multiple neurons tended to be labeled at different rates, making it difficult to separate direct from indirect connections. It could be imagined that single identified spinal motor neurons or interneurons labeled in such a way would provide *in vivo* maps of changes in connectivity, not only during development, but also after selective perturbations.

Another means to assess connectivity is through activity. For instance, individual interneurons could be labeled and activated using ChR2 and then responses in likely postsynaptic partners imaged, using either global expression of a genetically encoded calcium indicator or bulk loading with membrane permeable, acetoxymethyl dyes. A similar strategy has been used to assess connectivity in the visual cortex (Peterlin et al., 2000). This would rapidly assess functional outputs. Conversely, the activity of a postsynaptic cell could be recorded using conventional electrophysiology and individual activation of a group of presynaptic neurons labeled with ChR2 could rapidly assess functional inputs (Petreanu et al., 2007). Collectively, these techniques would provide unequal access to the processes governing spinal network assembly and function.

Perturbation experiments are best when driven by predictions. Thanks to the array of anatomically distinct spinal neurons and the topography we have discovered in zebrafish, we would predict that dendritic arborizations and axonal trajectories also follow topographic profiles according to speed of movement. Thus, the location of dendrites in spinal cord could also reflect a functional field, in the same way the dendritic location of RGCs do. These patterns could easily be perturbed in a predictable way by optogenetically altering network excitability or by removing inputs. The effect on locomotor rhythms could then be assessed.

As a model system, it is important that zebrafish studies reveal principles of organization that allow predictions about similar functional circuits in humans and other animals. Axial circuits likely reflect the primitive condition, from which limb circuits are derived. It is encouraging that shared markers, like the transcription factor *En1*, reveal morphological and functional similar neurons from species as disparate as fish (Higashijima et al., 2004), frogs (Li et al., 2004), and mice (Gosgnach et al., 2006), because the findings from developing zebrafish may reveal features of organization that apply broadly among vertebrates. It is unclear how the recruitment topography we have described in zebrafish relates to organization of mammalian spinal cord. However, we would predict that if interneurons could be birth-dated in mammals, then the older ones would participate in the more powerful locomotor maneuvers. We would also predict that *Chx10*-labeled V2 interneurons in

mammals, which in zebrafish represent the relatively early born *alx*-labeled CiDs (Kimura et al., 2006), contribute to faster speeds of mammalian locomotion, since they are specialized for faster swimming speeds in zebrafish (Bhatt et al., 2007; McLean et al., 2007). The power of concrete predictions from a simple model system is that you can test the role of interneurons in the correct context. For instance, the contribution of *Chx10*-labeled V2 interneurons to spinal rhythmogenesis may not be fully appreciated if perturbation experiments are conducted and the animals are only tested at lower speeds, when these neurons might not be as important.

The bottom line is that zebrafish can reveal principles of organization during development that should apply broadly among vertebrates. These principles need to provide clear predictions to allow for rigorous testing of the breadth of their explanatory power, including their applicability to the motor systems of mammalian species.

CONCLUDING REMARKS

To understand motor systems, it is important to look through development at a tractable behavior within a model system where the circuits can be identified, monitored, and perturbed through time. At present, zebrafish are the best vertebrate system to accomplish this. The sequential emergence of distinct motor behaviors that zebrafish perform later in life generates a number of questions regarding how neural circuits are organized to produce movement. It also provides the opportunity to investigate the strategies used by the nervous system to maintain function while still under construction. All of the optical and genetic tools are now in place to search for general principles of neural circuit development and organization using the zebrafish model.

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A Larval zebrafish (4-5 days post-fertilization) **Bi Larval spinal motor neurons ii Sensory** **C Larval spinal interneurons** **Figure 1.**

Identified classes of spinal neuron in larval zebrafish. (A) Photograph of a larval zebrafish taken from the side. The head is to the left, in this and subsequent images. (B) Schematic depictions of identified classes of (i) motor neurons and (ii) sensory neurons. Rostral primary (RoP), middle primary (MiP), and caudal primary (CaP) motor neurons are distinctive based on their location with respect to one another and their nonoverlapping regions of axial muscle innervation (m, middle; d, dorsal; v, ventral). Secondary motor neurons can be divided into classes that innervate either the pectoral fins or axial musculature. Rohon-Beard (RB) sensory neurons and dorsal root ganglion (DRG) cells provide the principle source of mechanosensory input to spinal cord. (C) Schematic

depictions of identified classes of spinal interneurons, organized according to transmitter phenotype. Abbreviations: CiD, circumferential descending; MCoD, multipolar commissural descending; UCoD, unipolar commissural descending; VeMe, ventral medial; CoSA, commissural secondary ascending; CoPA, commissural primary ascending; CiA, circumferential ascending; CoBL, commissural bifurcating longitudinal; CoLo, commissural local; CoLA, commissural longitudinal ascending; DoLA, dorsal longitudinal ascending; KA, Kolmer-Adhur; VeSe, ventral serotonergic. List compiled from Myers (1985); Bernhardt et al. (1990); Hale et al. (2001); Higashijima et al. (2004); McLean and Fetcho (2004); Thorsen and Hale (2007). See main text for more details.

Figure 2.

Tracking the development of identified classes of spinal interneuron *in vivo*. (A) Confocal time lapse imaging of two inhibitory circumferential ascending (CiA) cells stochastically labeled with GFP using *En1* as a promoter. CiAs are so named due to their primary ascending axon, however, they also have a secondary descending one. Successive images at different time points (hours postfertilization noted in respective images) illustrate the growth of the descending axon (at thin and thick white arrows), as well as the elaboration of the dendrites. Scale bar is 50 μm . Image reproduced from Higashijima et al. (2004) with permission (Copyright 2004 by the Society for Neuroscience).

A ~24 hours old (coiling)

B ~48 hours old (swimming)

C ~96-120 hours old (swimming)

Figure 3.

Stereotyped emergence of locomotor behavior in zebrafish. (A) Photograph of 1-day-old zebrafish embryo taken from the side, below which is a sequence of images captured at a rate of 1000 frames per second. The sequence illustrates spontaneous, embryonic coiling behavior. Each image is 20 ms apart. (B) Photograph of a 2-day-old zebrafish embryo taken from the side, below which is an image sequence captured at a rate of 1000 frames per second. The sequence illustrates swimming behavior elicited by a tactile stimulus to the tail. Each image is 2-ms apart. (C) Photograph of a 4- to 5-day-old zebrafish larva taken from the side, below which is an image sequence captured at a rate of 1000 frames per second. The sequence illustrates spontaneous swimming behavior, shortly followed by swimming evoked

by a tactile stimulus to the tail. Each image is 2-ms apart. See Saint-Amant and Drapeau (1998); Budick and O'Malley (2000); Buss and Drapeau (2001); Thorsen et al. (2004); and the main text for more details.

A Developing olfactory responses to scents

Figure 4.

Genetically encoded calcium imaging of olfactory bulb activity during development. (A) Inverse pericam expression driven by the neuronal promoter *HuC* labels cells in the olfactory bulb (left). Color-coded response patterns from the area outlined in green captured using wide-field epifluorescence and a cooled CCD camera are to the right and below. The responses to 14 different odor stimuli at 3 and 5 days post fertilization (dpf) from the same larva are illustrated. While signals are certainly stronger in older larvae, the broad spatial organization of odor responses is already apparent early on. Image reproduced from Li et al. (2005) with permission (Copyright 2005 by the Society for Neuroscience). Odorant abbreviations can be found in the original citation.

A Kaede conversion process

Figure 5.

Tracking spinal network differentiation *in vivo*. (A) Schematic illustrating the photoconversion of Kaede protein using ultraviolet (UV) light. In this example, Kaede is restricted to the nervous system. Before conversion Kaede is green, and after conversion it is red. Any neurons containing red Kaede must have been present before conversion. Neurons with no red Kaede differentiated after conversion. (B) An example of the Kaede approach to time-stamp a specific class of spinal excitatory interneuron, circumferential descending (CiD) cells. Kaede expression was driven selectively into CiDs using an *alx* promoter. The top panel shows a merge of the red and green channels imaged using a confocal microscope, which are individually displayed below. The arrows indicate cells with both red and green

fluorescence (yellow), while arrowheads indicate cells with red fluorescence, but marginal levels of green fluorescence. The time of conversion and imaging is as detailed in (A). Scale bar is 20 μm . Image in (B) is reproduced from Kimura et al. (2006) with permission (Copyright 2006 by the Society for Neuroscience).

A Spinal interneuron topography

i. Swimming speed

ii. Input resistance

iii. Age?

B Grading swimming speed

Figure 6.

Gradients of recruitment in spinal cord and the relation to swimming speed. (A) Schematic illustrations of the dorso-ventral gradients of inhibitory and excitatory interneurons with respect to (i) swimming speed, (ii) input resistance, and, more speculatively, (iii) age. (B) Cartoon illustrating a larval zebrafish responding to a tactile stimulus with an escape bend and a bout of swimming. As the speed of the swimming bout decreases, so too does the shade. This is meant to reflect the relative participation of spinal networks driving slow versus faster movements according to the topographic arrangement illustrated in (A). The image sequence was captured at a rate of 1000 Hz and each image is 4-ms apart.

A Normal escape response in *des* mutant

B Axons from supernumerary Mauthner cells

Figure 7.

Deadly seven/notch1a (des) mutants display homeostatic plasticity in the escape circuit. (A) An escape in response to a tactile stimulus to the head appears normal in this *des* mutant. The larva turns quickly from the stimulus and begins to swim away. Images were captured at 1000 Hz, and every third frame is shown. The time in milliseconds is marked on the frames. Scale bar is 550 μ m. (B) A mutation in the *des/notch1a* gene results in multiple Mauthner cells, two of which have been electroporated here with different colored fluorescent dyes and imaged using a confocal microscope. Arrowheads denote axon collaterals, which do not form in the same location as the merged image at the bottom

demonstrates. Scale bar is 10 μm . Images reproduced from Liu et al. (2003) with permission (Copyright 2003 by the Society for Neuroscience).