Optical and Genetic Approaches Toward Understanding Neuronal Circuits in Zebrafish

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SYNOPSIS. Optical and genetic tools are beginning to revolutionize the studies of neuronal circuits. Neurons can now be labeled with conventional or genetically encoded indicators that allow their activity to be monitored during behavior in intact animals. Laser ablations and genetic inactivation offer ways to perturb activity of specific cells to test their contributions to behavior. These approaches promise to speed progress in understanding vertebrate networks in genetic models such as zebrafish. Here we review some of the progress in applying these tools, with an emphasis on our work to develop and apply these approaches in the zebrafish model.

INTRODUCTION

Like many neurobiologists, those of us who study motor control aim to understand how the nervous system produces behavior. In our case, the behavior is some sort of movement. Understanding the neuronal basis of behavior is notoriously difficult. Much of the difficulty arises because behavior emerges from the interactions at many levels of organization in the nervous system and in the body as a whole. At a cellular level, understanding how neuronal circuits produce behavior requires information about the neurons involved in the behavior, their active and passive membrane properties which determine how they will respond to inputs, the wiring diagram of the neuronal network, and the synaptic properties of the connections in it. Much of the focus of work on motor circuits, particularly among vertebrates, has been at these cellular levels of organization. This is largely a consequence of the availability of tools such as sharp and patch microelectrode recording, which allow one to monitor relatively easily the activity of neurons and to explore their cellular properties and connectivity. These cellular methods have led, over many years, to important, remarkably detailed accounts of the neurons and the wiring for various motor behaviors of both invertebrates and vertebrates (Roberts et al., 1998; Selverston et al., 1998; Grillner and Wallen, 2002).

While this information is essential in any study of motor circuits, other levels of analysis are equally important for a complete understanding of how the nervous system generates the behavior. Among vertebrates, individual neurons typically contribute little to the behavior, which is produced by patterned activity in populations of cells. Cellular analysis leads to a wiring diagram, but it is not easy with intracellular microelectrodes to monitor the activity of more than a couple of neurons at a time. Consequently, the links between activity in populations of neurons and the behavioral output are usually missing. Since it is likely that changes in behavioral output are produced by changes in population activity (something well established for motoneurons in vertebrates), monitoring activity in populations of interneurons during behavior is an essential part of explaining the behavior.

Simply knowing the activity patterns of the cells and a wiring diagram for a circuit does not causally link the neurons to a behavior. Very often a cellular analysis leads to a plausible wiring diagram for a circuit, which offers some idea of what individual cell types might be contributing to the production of the motor pattern and the behavior. These data form a foundation for model building to explore if the known circuit could generate the studied motor pattern. Such modeling shows whether the known neurons could, in principle, generate the motor pattern, but it does not provide a real test of the contributions of the neurons to behavior. It might be, for example, that other, unidentified neurons are in fact more important contributors to the motor pattern than those identified by electrophysiological recording. Electrophysiological techniques do not typically provide an unbiased sample of the large numbers of neurons in the vertebrate nervous system, so important classes of neurons might be missed.

Linking neurons explicitly to the behavior requires some sort of perturbation of the cells to explore how that perturbation alters the motor pattern and the behavior. These experiments are difficult and, for the most part, have not been accomplished even in the best-studied motor circuits of vertebrates. The difficulties lie in the inability to selectively remove or alter the activity of a specific cell type. In some important motor areas such as hindbrain and spinal cord, cells of a particular type are not grouped into nuclei that could be targeted with either drugs for inactivation or with microelectrodes for electrolytic lesions. Even when cells are in discrete nuclei, the nuclei are not homogeneous and drug application or lesions inevitably perturb more than one cell type. The challenge is to perturb the system in a cell specific way to examine the behavioral consequences. This would allow a test of predictions from models of the network function and provide a better view of what particular classes of neurons actually contribute to behavior. This has been

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done in invertebrate systems where cell number is smaller, but is much more problematic among vertebrates because of the large number and heterogeneity of their neurons (Selverston and Miller, 1980; Bargmann et al., 1993).

Optical and genetic approaches offer a solution to the problems of both monitoring activity in populations of cells and perturbing that activity. The activity of cells can be monitored with fluorescent indicators that monitor calcium levels or membrane potential. If the cells can be filled in intact animals and imaged in vivo, the activity of populations of identified cells can be monitored during behavior. Activity can also be perturbed with optical methods by using lasers to target particular labeled cells for ablation. These optical methods are even more powerful if they can be combined with genetics. Genetic tools offer the prospect of targeting expression of proteins to particular cell types. The increasing number of genetically encoded indicators provides elegant tools for monitoring activity. Activity of neurons can also be perturbed genetically by targeted expression of proteins that alter the firing of neurons.

Our recent work has been directed toward the development and application of optical and genetic methods in studies of motor circuits in zebrafish. In the following sections, we review our progress in developing some of the tools and place it in the context of some important recent work in other model systems.

**Why Zebrafish?**

Our early studies of motor circuits focused on goldfish. We switched to studies of zebrafish because it is the most favorable current vertebrate model for combining optical and genetic methods. Zebrafish have the additional advantage of being closely related to goldfish (Meyer et al., 1993), so we could make use of previous data from electrophysiological studies in goldfish to direct our studies of zebrafish.

Zebrafish have been a significant model in developmental biology for over a decade. Their initial choice as a prospective model by George Streisinger in the late 1960s was motivated by the desire for a vertebrate model in which one could discover mutations that affect development (Grunwald and Eisen, 2002). Zebrafish offered an animal in which there were large numbers of accessible offspring that developed externally and were transparent throughout their early development. Mutations that affect development are obviously easier to find if one can simply watch the developing embryo.

Zebrafish develop rapidly. Hatching begins at about two days after fertilization, continuing through day 3 and sometimes later (Kimmel et al., 1995). They are free swimming at about 5 days of age, at which time they must move around to feed and avoid predators in order to survive. Throughout this time they are nearly transparent, except for scattered pigment cells (Fig. 1). This allows one to observe development, and, for our purposes, to image cells throughout the brain and the spinal cord. They thus offered the prospect of using fluorescent indicators to image activity of cells in an intact animal. The transparency alone is motivation enough to choose zebrafish, since there are few groups of vertebrates (only some larval fishes and amphibians) in which one can easily image neurons throughout the nervous system of the intact living animal.

Zebrafish, however, have the additional advantage of an increasingly broad range of genetic tools, including both the ability to make transgenic fish as well as hundreds of mutant lines with various developmental and functional deficits (Haffter et al., 1996; Higashijima et al., 1997; Meng et al., 1997; Higashijima et al., 2000; Lin 2000). It is this unique combination of transparency and genetic tools that was our primary motivation for switching from goldfish to zebrafish. We initially set out to image activity of neurons during behavior with fluorescent calcium indicators.

**Optical Monitoring of Activity—Non-Genetic Methods**

Fluorescent calcium indicators have been used widely to study the important roles of calcium in cellular function. They can also serve as an indirect measure of neuronal activity because all neurons have voltage-gated calcium channels that allow entry of calcium during neuronal activity. Application of calcium indicators to studies of activity patterns in neuronal networks expanded when it was discovered that neurons in intact circuits could easily be backfilled with these indicators (O’Donovan et al., 1993). This allowed for the selective labeling of neuronal populations based upon their projection patterns. Their activity could then be studied under different circumstances by simply imaging the fluorescence changes associated with the neuronal activity.

We initially combined the advantages of the calcium indicators with the relative ease of imaging inside live larval zebrafish. We first tested fluorescent indicators by injecting them into muscle to backfill motoneurons in spinal cord (Fetcho and O’Malley, 1995). The indicator we found most useful was calcium green coupled to a 10,000 molecular weight dextran. This indicator was favorable because it filled the cells well and had reasonably strong fluorescence even at resting calcium levels, so we could identify the cells prior to their activation. We imaged the cells with confocal microscopy, which allowed us to collect high quality morphological data from the intact backfilled neurons, so we could identify the imaged cells and relate structure to function. Confocal imaging is not, however, necessary for imaging calcium changes in the cells in spinal cord. If one is willing to sacrifice some image quality, it is possible to image the activity of spinal neurons with an intensified CCD camera.

Our initial studies of motoneurons indicated that the calcium green produced robust fluorescence signals during activity in the motoneurons. The calcium green labeling and signals persisted for weeks after labeling. The persistence is likely a consequence of the attached
dextran, which may prevent the cell from easily removing the molecule from the cytosol. Studies in which we filled cells and then electrically stimulated to antidromically activate their axons showed that there was roughly a 10% increase in fluorescence for a brief (0.2 msec) stimulus that should lead to firing of a single action potential. This is important, because if the indicator is to monitor effectively the activity of cells, it must be able to report even single action potentials. Otherwise, the data might lead to the false conclusion that a weakly active cell (firing one or few spikes) is not active during a particular behavior.

We have not yet imaged calcium changes of neurons in vivo in zebrafish while simultaneously monitoring the neuronal electrical activity, to directly relate fluorescence changes to activity. There is good direct evidence from other systems such as brain slices, however, that the calcium green can detect single action potentials (Smetters et al., 1999). The correlation of electrical activity and calcium has become more feasible (though still difficult) in zebrafish now that patch recording from zebrafish neurons has become routine (Drapeau et al., 1999) Such simultaneous imaging and recording would serve to document better the relation between activity and fluorescence of calcium indicators, but would not totally resolve the problem of interpreting negative responses of neurons imaged in vivo, where the optical conditions and intensity of labeling can vary from preparation to preparation. Both of these can affect the ability to detect weaker fluo-
rescence changes in neurons and increase the likelihood of false negative conclusions regarding activity.

Since it is not possible to record electrophysiologically from each of the neurons in a particular preparation to confirm that we can detect single spike responses in each cell (if that were easy, then there would be no need for the imaging), we have used less direct methods. These include, most importantly, showing the we can detect fluorescence responses in cells to single antidromic stimuli, along with evidence that a cell can in fact produce a detectable response under some behavioral conditions. Still, conclusions about the lack of activity in a neuron based on calcium indicators are the weakest; the safer conclusion is that a cell showing a fluorescence increase is active.

We have imaged the fluorescence responses in the neuronal soma, where the evidence indicates that we can detect the firing of action potentials. Indirect evidence indicates that we cannot detect subthreshold changes in calcium levels when we image the fluorescence of the soma as a whole. In particular, we do not observe increases in fluorescence in conditions when a cell should be receiving strong, but sub-threshold synaptic input. For example, the giant Mauthner cells in fish produce a large flip of the tail (C-start) that is used to escape from predators (Zottoli and Faber, 2000). If a fish is stimulated to produce such an escape by a tap on the body that is just at threshold for eliciting the behavior, the Mauthner cell only lights up when an escape is produced. It shows no somatic fluorescence increase in those trials in which an escape does not occur, even though the stimulus is at threshold for the firing of the cell, so there should be a large synaptic input to the neuron (O’Malley et al., 1996; Fetcho et al., 1998). It might be that we could detect subthreshold changes if we imaged the smaller dendrites in the living fish, but we have not systematically attempted to do so.

The large signals from calcium indicators provide important data about which cells are active and reveal the pattern of activity in groups of cells in vivo. The indicators, however, are relatively slow, with a time course of decay of the fluorescence in the soma that is seconds long. Under ideal conditions (e.g., well filled neurons in slices, where movement is absent) it is possible to see the fluorescence changes from successive action potentials visible as fluorescence increases superimposed on the decay of fluorescence from previous responses (Smetters et al., 1999). It is also possible to obtain higher temporal resolution by using the confocal microscope to monitor the intensity of a line through the cells of interest, which can be done quickly (2msec/line) (Fetcho and O’Malley, 1995). The situation, however, is complicated in vivo where preparations can move and the exact timing of the increase may be difficult or impossible to detect because of movement artifacts. Consequently, we have chosen to use the indicators primarily to observe which cells are active, without attempting to explore the timing of the activity on a millisecond time scale.

Higher temporal resolution might be possible in paralyzed preparations, but we have not invested effort along these lines because there was initially much to learn simply based on information about which cells were active. Indeed, our focus has been to image the activity of the cells in animals that can actually produce as much of the normal motor behavior as possible. The movement of these preparations precludes high temporal resolution.

We have used the backfilling approach to address several questions concerning the function of spinal and hindbrain neurons in zebrafish. Initially we examined the activation of motoneurons during escapes and found that when we imaged groups of motoneurons and elicited escapes by a tap on the opposite side of the body, all of the motoneurons were activated (Fetcho and O’Malley, 1995). This included both the largest, primary motoneurons as well as smaller so-called secondary ones. Escapes are the most powerful movements produced by axial musculature, so a maximal recruitment of the motoneurons might be expected. Nonetheless, this work showed that one could image the activation of groups of neurons during behavior in an intact vertebrate.

We moved from studies of motoneurons, where we had a good idea of what to expect, to studies of other neurons where less was known. This included studies of neurons in hindbrain and in spinal cord (O’Malley et al., 1996; Ritter et al., 2001; Gahtan et al., 2002). Our focus on hindbrain was motivated by the observation that the hindbrains of all vertebrates are segmentally organized. Work in zebrafish showed that the hindbrain segments contained morphologically similar reticulospinal neurons in successive segments. The idea that the segments arose via duplication events in evolution led to the prediction that the serially repeated neurons would be functionally related to one another. Testing this idea was difficult with conventional approaches, because many of the hindbrain neurons are not organized into discrete nuclei as in other brain regions, so it is difficult to monitor the activity of identified cells electrophysiologically. The optical approach offered a means to approach this question.

Our experiments focused on one serial set of hindbrain neurons including the Mauthner cell in hindbrain segment 4 and two other cells (MiD2cm and MiD3cm) similar to it in hindbrain segments 5 and 6 (O’Malley et al., 1996). Previous work had shown that these cells were morphologically similar, each having two major dendrites and a commissural axon that ran down the opposite side of spinal cord (Metcalfe et al., 1986; Lee and Eaton, 1991). The presence of the Mauthner-like cells was known for some time, but functional studies of the cells were not accomplished because there were only two cells in each segment and they were not easily identified physiologically. The presence of the cells and the segmental organization of the hindbrain led to the idea that the cells might, like the Mauthner cell, play a functional role in escape behavior (Foreman and Eaton, 1993).
Foreman and Eaton (Foreman and Eaton, 1993) proposed that the presence of a serial set of Mauthner-like neurons in the hindbrain might help to explain the diversity in the escape behavior. The form of the escape behavior fish produce in response to a threat varies depending upon the location of the stimulus that produces the escape. Stimuli from the front produce the largest and fastest turns, with the fish turning about 180 degrees to swim away from the potential threat. Stimuli from behind lead to a slower, weaker turn, with the fish escaping forward away from the threat. Foreman and Eaton published a simple model in which the hindbrain neurons were differentially activated in the different forms of the escape. In particular, they proposed that in the largest, fastest escapes to rostral stimuli, the Mauthner was activated along with both of the Mauthner-like neurons, whereas in the weaker escapes the Mauthner cell was the only one of the three activated.

We tested this model by backfilling the cells with calcium green dextran and imaging the calcium responses of the neurons when escapes were elicited by stimulating the head or the tail (O’Malley et al., 1996). We found that a tap on the head activated all three hindbrain neurons, whereas tail stimuli activated just the Mauthner cell. This pattern of activation was consistent with the predictions of the Forman and Eaton model, and suggested that the form of the escape might be determined in part by which of the serially repeated hindbrain neurons were activated. Although the stimulus in our case was a touch and in the Foreman and Eaton studies of goldfish was vibrational, the directionality of the behavioral responses in each case is similar. We infer that the patterns of activity of the cells are as well. Our data, like all of the calcium indicator data, provide some idea of which cells might participate in the different forms of the behavior. They do not, however, reveal what the cells contribute to the behavior. The perturbation experiments we used to address this are dealt with later in this paper.

We also used the ability to image the active cells with calcium green dextran and imaging the calcium responses of the neurons when escapes were elicited by stimulating the head or the tail (O’Malley et al., 1996). We found that a tap on the head activated all three hindbrain neurons, whereas tail stimuli activated just the Mauthner cell. This pattern of activation was consistent with the predictions of the Forman and Eaton model, and suggested that the form of the escape might be determined in part by which of the serially repeated hindbrain neurons were activated. Although the stimulus in our case was a touch and in the Foreman and Eaton studies of goldfish was vibrational, the directionality of the behavioral responses in each case is similar. We infer that the patterns of activity of the cells are as well. Our data, like all of the calcium indicator data, provide some idea of which cells might participate in the different forms of the behavior. They do not, however, reveal what the cells contribute to the behavior. The perturbation experiments we used to address this are dealt with later in this paper.

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When we imaged the largest CiD cells in zebrafish that were backfilled with calcium green dextran, we found that the cells were consistently activated in escapes. In contrast, they were not activated in swimming (Fig. 2). This indicated that there was some distinction between the interneurons activated in the two behaviors. Such a distinction was also supported by our imaging of another commissural cell type (called MCoD [Hale et al., 2001]), with an axon that crosses spinal cord and descends ventrally in cord for many segments. These cells are most likely excitatory interneurons based on staining for glutamatergic markers. We found with calcium imaging that the MCoDs were activated in swimming, but not in escapes, just the reverse of the situation for the CiD cells. Thus, there were differences in the activation of the interneurons in the two behaviors.

Our initial notion that CiD cells might be shared by escape and swimming was thus refuted in its simplest form. One caveat, however, is that in our agar preparations, the range of swimming frequencies was reduced relative those seen in freely swimming fish. We could not elicit the highest swimming frequencies in the agar preparation, so we cannot rule out the possibility that CiDs are indeed activated in swimming, but only at higher frequencies. Nonetheless, the imaging revealed differences in the activation of different clas-
ses of interneurons during swimming and escape. These differences may in part underlie the differences in the kinematics of the two behaviors.

The calcium indicators provide a means for monitoring the active cells, but would seem less applicable to measuring inhibition within the central nervous system. They have, however, been used to study the time course of inhibition on the Mauthner cell in larval zebrafish. In a very clever series of experiments, Takahashi et al. (Takahashi et al., 2002) took advantage of the fact that the calcium influx in a Mauthner cell during an action potential depends upon the amplitude of the action potential. When an action potential occurs together with inhibition, the size of the action potential and the associated calcium signal are both reduced. By pairing antidromic activation of a Mauthner cell back-filled with calcium green dextran with stimulation of other pathways that produce inhibition of the cell, they could monitor the level of inhibition produced by the other pathways by measuring the reduction in the calcium signal produced by the antidromic stimulus. This reduction reflected the shunting and reduced amplitude of the antidromic action potential by the inhibition. They were able to show a match between the time course of the reduction of the calcium signals and the time course of the electrophysiologically measured inhibition. This optical approach allowed them to demonstrate that the major inhibitory pathways onto the Mauthner cell were present at as early as four days of life in the fish. The ability to image the levels of inhibition in a non-invasive way sets the stage for studies of changes in inhibition that are thought to underlie plasticity in the escape networks (Charpier et al., 1995; Oda et al., 1995).

**GENETICALLY ENCODED INDICATORS**

One concern about using backfilling with calcium indicators is that in order to fill the cells, the evidence indicates that the processes of the neurons must be damaged. Given this, there is the possibility that such damage would disrupt the circuits of interest. In our backfilling experiments, we studied neurons with long axons and filled them far from the soma (from caudal spinal cord to fill hindbrain neurons for example). When the backfilling is carefully done, the behaviors we studied are not altered in any obvious way based on filming the movements of backfilled animals. Nonetheless, the damage of backfilling precludes the study of large numbers of local interneurons with short axons, because labeling many would require many injections and the local circuits would likely be disrupted. It would be better if there were ways to label the cells that would not damage them at all. We have used two approaches to accomplish this.

In the first, we injected calcium green into one of the blastomeres in the developing fish (Cox and Fetcho, 1996). The indicator was then parceled out to the progeny neurons as the injected cell underwent the cell divisions as the animal developed. Injections at, for example, the four-cell stage produced a larval fish with roughly 1/4 of its cells containing a calcium indicator. The calcium indicator was responsive and the ap-
living animal. More recently, genetically encoded indicators have been developed based on derivatives of GFP (Miyawaki et al., 1997, 1999), and there are now a variety of such indicators. (Nagai et al., 2001a, b; Yu et al., 2003). The first of these was the ratiometric indicator cameleon, which is a fusion protein containing CFP and YFP along with calmodulin and a binding domain for calmodulin. (Miyawaki et al., 1997, 1999).

Rises in calcium levels lead to conformation changes that bring the CFP and YFP closer together resulting in changes in the extent of fluorescence resonance energy transfer (FRET) between the two. The FRET changes are monitored as the ratio of the fluorescence emission from YFP to that from CFP. More recently a series of other single wavelength indicators have been developed with differences in their calcium sensitivity and in the magnitude (dynamic range) of the signal they produce. (Nagai et al., 2001 a, b; Yu et al., 2003).

The genetic indicators were first used in vivo to monitor calcium changes in muscle and in neurons of nematodes. (Kerr et al., 2000). In the last year or so, a series of studies have been published applying these to studies of neurons in the central nervous system of fruit flies. (Diegelmann et al., 2002; Fiala et al., 2002; Reiff et al., 2002; Wang et al., 2003). These studies are very important as some of the first to apply the genetic indicators to address key neurobiological questions. Flies have been notoriously intractable to conventional physiological techniques. The genetic indicators offer a much easier way to monitor the physiology of the neurons. Their use in studies of mutant and transgenic lines will allow more easy study of the functional disruptions produced by particular mutations, so we can expect the application of the indicators will become routine.

We have tested some of these indicators in zebrafish and found that both yellow cameleon (2.1) and G-CaMP produce detectable calcium signals in zebrafish. (Higashijima et al., 2003). Most of our work has focused on developing the use of cameleon, because its ratiometric nature obviates many of the problems associated with movement artifacts that we encounter in our preparations in which the fish can partially move. When using single wavelength indicators, a change in brightness might be a consequence of either a change in calcium levels or a movement of the preparation that shifts the cell to a different focal plane. Not all parts of the cell have the same amount of indicator so a shift in focal plane could lead to a change in brightness from a single wavelength indicator without any calcium change. The ratiometric indicators solve this problem because the ratio is independent of indicator concentration and, for a given calcium level, is the same in different focal planes through a cell. We have generated stable lines of fish with cameleon and inverted pericam under control of a general neuronal promoter (Huc) (Higashijima et al., 2002, 2003). The expression of the calcium indicator in all neurons does not kill the fish, even though the indicators act to buffer calcium. We have worked mostly with the cameleon

Fig. 3. Transgenic labeling of neurons in living zebrafish. A: A projection from a stack of confocal images of a lateral view through the caudal head and rostral spinal cord of the GFP transgenic line produced by Higashijima et al., (Higashijima et al., 2000). Rostral is to the left. A V marks the vagal motor nucleus. Cell bodies of spinal motoneurons form a band caudal the vagal motoneurons. The axons of the motoneurons are visible exiting in the ventral roots (arrow). B: A lateral view of a single, well filled caudal primary axons of the motoneurons labeled by transient expression of GFP and viewed in a projection from a stack of confocal images through the fish. The axon of the motoneuron leaves cord and extends ventrally where it branches extensively in the muscle. Scale bars = 20 μm.

approach accomplished the goal of producing an animal in which neurons were filled with calcium indicator without disrupting their processes. The drawback, however, was that the filled neurons were much less well filled than by backfilling and, consequently, it was much more difficult to identify the labeled cells. In some cases, such as the olfactory epithelium, the labeled cells were identifiable. In other cases, such as spinal cord, the round somata of the neurons were evident, but it was hard to identify many of the cell types because dendrites and axons were not clearly filled.

A more powerful approach to accomplish the same goal is to take advantage of the possibility of using transgenic methods in zebrafish. The production of transgenic zebrafish has become routine (Higashijima et al., 1997, 2000; Long et al., 1997; Lin, 2000). The first transgenic lines with labeled neurons were lines expressing green fluorescent protein (GFP) in motoneurons (Fig. 3). These have proven a powerful tool for marking cells for studying motoneuron structure and development, which can easily be visualized in the

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Fig. 4. Lesions of motoneurons and hindbrain neurons. A,B: Images of spinal motoneurons before (A) and after (B) laser ablation of the motoneuron marked by an asterisk in A. C,D: Images of the Mauthner and the two more caudal hindbrain neurons (MiD2cm and MiD3cm) before (C) and the day after (D) laser killing the three hindbrain neurons. The axon stump of the ablated Mauthner cell is marked by and arrow in D. Scale bars = 10 μm.

One of the strengths and also the challenges of using genetic indicators is the targeting of the indicator to particular subsets of neurons. The ability to drive expression in many or all of a particular class of neurons would move us much closer to the goal of using optical methods to monitor the pattern of activity in populations of neurons during behavior. The challenge is to generate lines in which the indicators are expressed in particular subsets of neurons. There are many Gal4 lines of flies based on enhancer trap screens in which it is possible to drive expression of proteins in different patterns throughout the nervous system. (Brand and Perrimon, 1993; Brand and Dormand, 1995) This allows easier targeting of subsets of neurons in flies than in other species. Targeting among vertebrates requires promoter sequences that drive expression of proteins in particular subsets of neurons. The ability to label unique sets of cells depends on having a gene whose expression is restricted to a particular cell type. Developmental studies of vertebrates are defining transcription factors that direct the differentiation of particular classes of neurons (Jessell 2000; Moran-Rivard et al., 2001; Pierani et al., 2001; Goulding et al., 2002). The promoters of these transcription factors provide tools for targeting subsets of cells. Although they label restricted subsets of neurons, the extent to which they define only one class of neuron is still largely unknown (Wenner et al., 2000). Pilot work in zebrafish indicates that such transcription factors as well as other genetic markers of neuronal phenotypes, such as the transporters for neurotransmitters, offer viable tools for driving expression of genetically encoded indicators in subsets of neurons (Higashijima et al., 2001, 2002).

Perturbation Experiments—Optical Lesions

Information about the activity patterns of neurons obtained with conventional or genetic indicators along with cellular data about transmitter phenotypes and connectivity allow for the formation of hypotheses about what particular neurons may contribute to be-
behavior. Testing these hypotheses requires some sort of perturbation of the activity of the neurons. If their contributions to behavior are understood then one should be able to predictably alter the behavior by perturbing the activity of the neurons.

Such perturbations are hard among vertebrates because there are not good ways to alter activity in a particular cell type. Instead, these experiments typically involve gross lesions, or various approaches to inactivation such as cooling or application of anesthetics or inhibitory neurotransmitters. These affect large regions of the nervous system and invariably alter activity in many cell types.

Cell specific lesions offer a more powerful assessment of the contribution of neurons to behavior. Indeed, studies of invertebrates have led the way in this regard because methods to photoablate cells were pioneered in invertebrate systems (Selverston and Millner, 1980; Bargmann et al., 1993). Invertebrates offer the additional advantage that often there are few neurons in a particular class (sometimes one!) and this means that one needs to kill or inactivate only a few cells to test their contributions to behavior. The challenges among vertebrates are to achieve cell specific ablation or inactivation and then be able to accomplish this for a whole class of cells that may number in the hundreds or thousands.

Zebrafish offer several advantages in this regard. First, the optical properties of the animal that facilitate imaging in vivo also allow one to focus laser beams onto individual cells in the intact fish to kill the cells. Second, there are relatively few cells in each class, so the numbers to be killed are small enough that they can be removed on a cell-by-cell basis. Finally genetics of fish offer the possibility to alter activity by expressing proteins that interfere with activity or with transmitter release.

Our early efforts to perturb activity in zebrafish were directed toward ablations of neurons in vivo. We showed that one could use the phototoxicity of the calcium indicators to kill cells. (Liu and Fetcho, 1999). Normally, the cells are illuminated minimally to image calcium levels while maintaining the integrity of the cells. If, however, the intensity of illumination by the laser used for imaging is raised and focused on an individual cell, that cell can be killed selectively without affecting neighboring cells. These phototoxic effects are thought to be mediated by damage produced by free radicals arising via excitation of indicator molecules. In addition to using the phototoxicity of indicators, it is possible to use a higher energy laser (such as a pulsed nitrogen laser) to kill cells even when they are unlabeled by essentially heating a submicron spot in the nucleus of the cell. Cells visible in Nomarski optics can easily be targeted in zebrafish, much like cells in nematodes have been ablated for years. This is quicker (seconds) than using phototoxicity (which can sometimes take minutes in the brain) and is more appropriate for killing larger numbers of cells.

We used dye phototoxicity to ablate the neurons in the hindbrain Mauthner cell array to test hypotheses about their contribution to escapes that were formulated based upon behavioral work and our previous calcium imaging of the cells. (Liu and Fetcho, 1999). The calcium imaging indicated that the Mauthner cell was activated together with the other Mauthner-like neurons (MiD2cm and MiD3cm) in escapes elicited by a touch on the head, whereas the Mauthner cell was activated without the other two in response to a stimulus on the tail (O'Malley et al., 1996). This difference was correlated with the faster and large escape turns elicited by stimulating the head versus the tail. Prior studies lesioning the Mauthner cell in goldfish electrically led to very little change in the escape behavior experiments (Eaton et al., 1982; DiDomenico et al., 1988; Zottoli et al., 1999). Only a small increase in latency was observed. This small change in the behavior was thought to be a consequence of the ability of the other Mauthner-like hindbrain neurons to substitute for the Mauthner cell.

Our imaging data suggested a role for the other cells, but only in escapes to stimuli to the head. We predicted that killing all three cells would lead to fish that escaped poorly to both head and tail stimuli, whereas killing just Mauthner would have a larger effect on tail stimuli because the Mauthner cell was activated without the other two cells in response to tail stimuli. We found that after killing all three cells there was a dramatic increase in the latency of the response (illustrated in Fig. 5), a significant decrease in the peak angular velocity of the initial escape turn, as well as an increase in the time it took to make the turn. The extent of the turn did not change much if at all. These observations were true for escapes elicited by either head or tail stimuli. If only the Mauthner cell was lesioned, the performance (latency, velocity and duration) was altered in escape to tail stimuli, but not to head (Fig. 5). These observations were consistent with the imaging data showing the Mauthner cell acting without the other cells to tail stimuli.

The ablation data helped to explain the apparent absence of large effects following Mauthner lesions in previous goldfish experiments (Eaton et al., 1982; DiDomenico et al., 1988; Zottoli et al., 1999). In those experiments, the directionality of the stimulus was not controlled, so it was not possible to know if the stimuli producing the escape were coming from the front or the back of the fish. Thus, the responses to rostral and caudal stimuli were pooled. If the changes in goldfish were similar to those we found after Mauthner lesions in zebrafish, with unchanged rostral responses and changed caudal ones, then pooling the two would lead to a masking of the changes in response to caudal (tail) stimuli by unchanged rostral responses. As a result, one might be able to detect only the largest changes, which happen to be in latency. Latency was the only measure for which changes (small ones) were observed in the goldfish work. Thus, in the case of zebrafish, knowing something about the pattern of activation of the cells and then separating the source of
the stimulus for the escape based on that pattern revealed the more important contribution of the Mauthner cell to caudal stimuli. This result also reveals how critical it is in lesion studies to test the performance of the animal in the most relevant behavioral context.

One puzzling aspect of the lesions was the absence of effects of the Mauthner lesion on escapes to head stimuli, even though it is normally activated by those stimuli. Our suspicion is that we might see effects in a different behavioral context. We tested the fish in escapes from rest. These are the least demanding escapes. It may be harder to do a high performance escape when the escape occurs in the midst of swimming and the drive to the muscles from the hindbrain neurons must be powerful enough to overcome ongoing conflicting muscle activity. In this case, a missing Mauthner cell might lead to a decrease in performance to a head stimulus. This might not be evident from rest because the other two cells might provide enough drive to produce a powerful turn in a situation where there is not conflicting muscle activity.

An important role of behavioral context in escape performance has also been suggested by Zottoli et al. (Zottoli et al., 1999) who showed that bilateral lesions of the Mauthner cell in goldfish reduce the frequency of occurrence of escapes. They concluded that this reflects the interactions of the Mauthner cell with the other hindbrain homologues. Their proposal is that a key role of the Mauthner cell is to bring to threshold other hindbrain neurons that are “primed” to be activated in a particular behavioral context. Sensory inputs from the environment that reveal obstacles to avoid or desirable hiding places could, for example, bias the trajectory of the escape initiated by the Mauthner cells by altering the excitability of hindbrain neurons driven by the Mauthner cell. Behavioral context may thus

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**Fig. 5.** Hindbrain lesions change the latency of the escape behavior. The top and bottom panels show the effect on the latency to escape of lesions of all three hindbrain neurons (array lesions; top) versus just the Mauthner cell (bottom). Each panel shows, on the left, histograms of the latency of fish to respond to a stimulus on the two sides of the head and on the two sides of the tail. On the right are shown the similar histograms to stimuli on head or tail after the lesions. Killing all three neurons in the array (top) dramatically increases the latency of the fish to respond to both head and tail stimuli on the lesioned side (in black), but not on the intact side. Killing just the Mauthner cell results in a large change in the latency to respond to tail stimuli on the lesioned side, but does not affect the responses to head stimuli.
matter both with respect to ongoing, potentially conflicting motor patterns as well as with respect to the sensory (environmental) situation in which the escape will occur.

In sum, the lesion studies have provided more causal links between the hindbrain neurons and behavior. They support the idea that the segmentally repeated hindbrain cells are functionally related to one another. Such segmentally repeated functional groups may be present throughout vertebrates since all vertebrate brains are segmented. In these studies relatively few neurons were killed. The challenge is to develop tools that will allow easy perturbation of large numbers of cells. We have made some progress along this line in spinal cord by using a pulsed nitrogen laser, but it still remains a difficult task.

PERTURBATIONS IN MUTANTS

One of the strengths of zebrafish is the ability to do mutagenesis and identify fish with single gene mutations (Haffter et al., 1996). This has proven powerful for identifying molecules in key developmental pathways. The mutants also offer the potential for insights into circuits and behavior as well. A number of mutants with movement deficits have been identified, (Granato et al., 1996), but in most cases it is not clear what genetic deficit leads to the movement problem (Ono et al., 2001, 2002).

Mutants with perturbations of central circuitry may offer unique alterations of circuits that provide another avenue to test hypotheses about the links between neurons and behavior. For example, a mutant line with extra Mauthner cells offers the possibility of examining the consequences of building the circuit with several cells as opposed to the normal single cell (Liu et al., 2003). It is difficult to add cells in other ways, so genetics provides a perturbation not otherwise easily accomplished.

Mutations that affect central circuits are nonetheless problematic. Since the genes in mutant lines might affect cells at different places in the nervous system and are defective from the beginning of life, there might be both multiple deficits as well as substantial secondary reorganizations of the circuits through development. As a result, it may be very difficult to identify the locus of the circuit or cellular change that is responsible for a behavioral change in the mutant. Interpreting the defect then depends upon a strong prior knowledge of the normal circuitry and its possible functional roles.

One case in which analysis of a mutant line has revealed changes that have implications for the neuronal control of movement is the analysis of the mutant space cadet (Lorent et al., 2001). This line produces uncontrolled rapid bending movements of the tail that seem, at least in part, a consequence of spurious activation of the Mauthner cell. An examination of the line revealed defects in a commissural pathway of neurons that may help to control the excitability of the Mauthner cell. Severing the pathway in normal fish mimicked the mutant phenotype, suggesting that the missing commissural pathway might explain the phenotype. Like most mutants though, the line has multiple deficits in central pathways. In this case, some knowledge of the Mauthner related circuits allowed a focus on defective pathways that were likely candidates to explain the phenotype. While such analysis of mutants provides complimentary evidence to imaging studies and ablations, the lingering possibility of multiple unknown deficits and developmental changes requires a careful approach to analyzing the mutants.

GENETIC PERTURBATION

The future of experiments to test the behavioral role of neurons in vertebrates lies in using reverse genetic tools to perturb function. This approach is now being realized in invertebrate systems including flies and worms (Moffat et al., 1992; Sweeney et al., 1995; Osterwalder et al., 2001; White et al., 2001a, b), and, to some extent, in mice (Watanabe et al., 1998). The strategy is to introduce a gene for a protein that will either kill the cells or alter the activity or the synaptic function of specific neurons. The tools applied to alter activity in flies use expression of potassium channels or altered potassium channels that hold the cell at resting potential and thus reduce activity or silence neurons (White et al., 2001b). Interfering with synaptic transmission in flies has been accomplished by expression of toxins such as tetanus toxin that cleave synaptic proteins and block transmission (Sweeney et al., 1995; Martin et al., 2002). Another permutation of this approach is the use of a temperature sensitive mutation of dynamin (called Shibire) which interferes with vesicle recycling at the synapse and indirectly blocks release (Poodry et al., 1973; Poodry and Edgar, 1979; Poodry, 1990; Chen et al., 1991).

The powerful application of tools such as this to study circuits and behavior requires the ability to express the perturbing protein in only a specific subset of neurons and then turn on the protein at a particular point in time. It does little good to express the protein from the beginning of development, as that would likely lead to plastic changes in the nervous system and secondary effects—essentially the same issue that arises in using mutant lines to study behavior.

The spatial and temporal control of proteins to inactivate cells has been accomplished in flies by using a system for conditional expression of proteins based upon an inducible Gal4 (Osterwalder et al., 2001). This involves activating a gene and a consequent delay while the expression of the perturbing protein ramps up. In contrast, the Shibire mutant offers a relatively rapid temperature dependent change in transmission, as it does not require induction of the gene, but rather depends on a temperature dependent change in the existing mutant dynamin protein that alters its function and blocks transmitter release (Poodry and Edgar, 1979; Chen et al., 1991). This sort of rapid effect on neuron function is critical to reduce potential compensatory changes in the nervous system. Recent work...
indicates that there may be powerful mechanisms that can compensate for over expression of some channels and thus maintain normal activity in the face of attempts to disrupt it by over expression experiments (MacLean et al., 2003).

The tools for silencing neurons are beginning to be applied to vertebrates. Spatial and temporal control of expression of proteins has been accomplished in mice, so such perturbations are feasible once good proteins for silencing activity in vertebrates have been identified. A recent novel approach using viral infection of an invertebrate receptor offers the possibility to silence neurons in many mammalian species, including primates (Lechner et al., 2002). The challenge among vertebrates is to develop the methods for targeting specific subsets of cells and for inducing expression with good temporal control. These approaches are not yet available for zebrafish, although there are efforts to develop the tools. Once they are available, the ability to combine them with the imaging of activity possible in zebrafish will make the model an even more important one for studies of links between neurons and behavior. Indeed, such studies will likely begin to focus even more on the vertebrate genetic model systems—zebrafish and mice—because of the power of the genetic tools for teasing apart the behavioral roles of different classes of neurons.

**Monitoring Synaptic Level Events and Plasticity**

Once the essential circuitry for a behavior is understood, the challenge moves to understanding how that circuit is altered through life in conjunction with the processes of learning and memory. The ability to image non-invasively *in vivo* offers the possibility of monitoring changes in the patterns of activity in cells over time as an animal learns. A good deal of plasticity, however, seems to occur at the synaptic level. The ability to image synaptic level events and possible sites of plasticity *in vivo* might prove powerful. In collaboration with the laboratory of Gail Mandel, we have recently used a GFP tagged calcium calmodulin dependent protein kinase II (CamKII) to image synaptic level events *in vivo* (Gleason et al., 2003). Prior work in culture showed that CamKII moves to postsynaptic densities on dendrites of hippocampal neurons when NMDA receptors on those dendrites are activated (Shen and Meyer, 1999; Meyer and Shen, 2003). We found that when we expressed CamKII-GFP in interneurons in zebrafish spinal cord, activation of glutamatergic inputs onto the interneurons led to the accumulation of CamKII-GFP at the postsynaptic densities, which consequently were brightly labeled after the stimulus. CamKII is strongly implicated in synaptic plasticity (Fink and Meyer, 2002). The ability to image its movements *in vivo* offers the possibility of mapping the distribution of synapses that might be undergoing plastic changes in different behavioral paradigms. This raises the possibility of imaging both normal circuit function as well as plastic changes in an otherwise intact vertebrate.

**Conclusions**

Most vertebrate motor behaviors are produced by activity in many neurons and in a variety of cell types. One of the important challenges in studies of vertebrate circuits and the links between neurons and behavior lies in the ability to non-invasively monitor the activity in populations of neurons to correlate that activity with behavior. Zebrafish offer an especially powerful model for accomplishing this because it is possible to label the neurons with fluorescent indicators by backfilling or by genetics and then image the activity of the neurons in the intact animal during behavior. In addition, the transparency of zebrafish offers the ability to kill specific neurons by laser ablation to test their contributions to behavior. Genetic approaches to more elegantly accomplish cell specific perturbations of activity are now being applied in invertebrate genetic models and soon we will be extended to mice and zebrafish. The combination of optical and genetic approaches with more conventional electrophysiological ones should accelerate progress in understanding the links between neurons and behavior in vertebrate genetic models such as mice and zebrafish.

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