The Journal of Neuroscience, November 15, 2001, *21*(22):8956–8965

In Vivo Imaging of Zebrafish Reveals Differences in the Spinal Networks for Escape and Swimming Movements

Dale A. Ritter,[1,2] Dimple H. Bhatt,[1] and Joseph R. Fetcho[1]

[1]*Department of Neurobiology and Behavior, State University of New York at Stony Brook, Stony Brook, New York 11794-5230, and* [2]*Heidelberg College, Tiffin, Ohio 44883*

Most studies of spinal interneurons in vertebrate motor circuits have focused on the activity of interneurons in a single motor behavior. As a result, relatively little is known about the extent to which particular classes of spinal interneurons participate in different behaviors. Similarities between the morphology and connections of interneurons activated in swimming and escape movements in different fish and amphibians led to the hypothesis that spinal interneurons might be shared by these behaviors. To test this hypothesis, we took advantage of the optical transparency of zebrafish larvae and developed a new preparation in which we could use confocal calcium imaging to monitor the activity of individual identified interneurons noninvasively, while we simultaneously filmed the movements of the fish with a high-speed digital camera. With this approach, we could directly examine the involvement of individual interneurons in different motor behaviors. Our work revealed unexpected differences in the interneurons activated in swimming and escape behaviors. The observations lead to predictions of different behavioral roles for particular classes of spinal interneurons that can eventually be tested directly in zebrafish by using laser ablations or mutant lines with interneuronal deficits.

Key words: interneurons; calcium imaging; zebrafish; spinal cord; escape; swimming

Spinal circuits can generate a variety of different movements, from swimming, struggling, and escape movements in fish and amphibians to walking, scratching, and galloping in limbed animals (Edgley et al., 1988; Roberts, 1990; McCrea, 1992; Giszter et al., 1993; Berkowitz and Stein, 1994; Bizzi et al., 1995; Fetz et al., 1996; Maier et al., 1998; Parker and Grillner, 2000). Although there are many studies of the spinal circuitry for movements, most have focused on the activity patterns and circuits underlying one behavior, usually studied in a paralyzed preparation producing a fictive motor pattern (Grillner et al., 1986; Roberts, 1990). Less is known about how spinal circuits generate different motor behaviors (but see Soffe, 1993).

The generation of different movements must, in part, be a consequence of differences in the activity of premotor interneurons in spinal cord. Whether these differences are in the cell types, firing patterns, or numbers of the interneurons involved has important implications for the functional organization of the spinal cord. It might be that different interneurons generate different motor patterns, as in some invertebrate systems (Heitler, 1985; Ramirez and Pearson, 1988). However, there is support from a variety of systems for idea that interneuronal circuitry is shared, with the same interneurons active in multiple behaviors (Berkinblit et al., 1978; Gelfand et al., 1988; Lockery and Kristan, 1990; Weimann et al., 1991; Giszter et al., 1993; Soffe, 1993; Bizzi et al., 1995). An understanding of which cell types are shared by different behaviors and which are not is fundamental to our understanding of spinal cord.

This information has been difficult to obtain for spinal circuits because it is hard to elicit different behaviors while recording the activity of identified interneurons. We set out to address this problem by developing a preparation in which we could examine the activity of identified spinal interneurons in a behaving animal. Our strategy was to take advantage of calcium indicators to image the activation of neurons in transparent larval zebrafish (Fetcho and O'Malley, 1995; O'Malley et al., 1996). This allowed us to image the active spinal neurons in an intact, partially retrained fish, while we simultaneously monitored the movements of the animal with high-speed video. We could then directly compare the activation of spinal neurons in different motor behaviors.

Here we report our initial studies in which we use this new preparation to image activity with single-cell resolution in partially moving fish. Our work was designed to determine whether particular classes of spinal interneurons are involved in descending escape pathways, in swimming circuits, or in both. We found robust differences in the activation of particular classes of interneurons during swimming and escape movements. The differences are surprising, because both swimming and escape involve bending movements that might be expected to share some spinal circuits. The differences we observed point to differing behavioral roles for at least some classes of spinal interneurons. These roles can eventually be tested directly in zebrafish by using laser ablations of neurons or mutant or transgenic lines of fish with interneuronal deficits (Granato et al., 1996; Liu and Fetcho, 1999; Higashijima et al., 2000).

MATERIALS AND METHODS

Experiments were performed on 5- to 10-d-old larval zebrafish (*Danio rerio*). At these times, the fish are freely swimming and feeding. The larval fish are nearly transparent, which allows imaging of neurons in the spinal cord or brain of the intact animal. Fish were raised at 28.5°C and

Received July 2, 2001; revised Aug. 30, 2001; accepted Sept. 6, 2001.

This work was supported by National Institutes of Health Grant NS26539. We thank N. Cambronero, E. Forbell, and S. Ross for help with pilot studies of CiDs. Melina Hale kindly provided the image in Figure 3*D*.

D.A.R. and D.H.B. contributed equally to this study.

Correspondence should be addressed to Dr. Joseph R. Fetcho, Department of Neurobiology and Behavior, SUNY Stony Brook, Stony Brook, NY 11794-5230. E-mail: jfetcho@notes.cc.sunysb.edu.

Copyright © 2001 Society for Neuroscience 0270-6474/01/218956-10$15.00/0

were then gradually acclimated to room temperature (25°C) over the course of a day or more before the experiments. Larval fish were fed powdered food (TetraMin baby fish food for egg layers; Tetra, Melle, Germany) or *Paramecium*.

Spinal neurons were labeled by backfilling with a 50% solution of calcium green dextran (molecular weight of 10,000) that was pressure-injected through a glass microelectrode into the spinal cords of fish anesthetized in 0.02% 3-aminobenzoic acid ethyl ester (Fetcho and O'Malley, 1995; O'Malley et al., 1996). Injections were into post-anal spinal cord, with different classes of interneurons labeled by injections into different dorsoventral positions in the cord. Imaging was performed ≥1 d after injections to allow time for the neurons to take up the indicator and for the fish to recover from the injection. Previous studies have shown that after careful injections the fish recover well, with little to no disruption of escape and swimming movements (Liu and Fetcho, 1999).

Fish were embedded in soft agar (1.2%), with the head and more rostral portions of the fish held in place by the agar and the caudal portion of the fish free to move. This allowed us to collect confocal images of the interneurons in the restrained portions of spinal cord while observing the movements of the adjacent, free portion of the tail. In early experiments, we used visual observations of the fish on the confocal stage to determine the type of movement. In these experiments, to confirm that we could reliably distinguish escapes from swimming, we visually examined the movements of the fish in response to various stimuli while at the same time filming the fish at high speed (1000 frames/sec) with a digital video camera. Our visual assessment of the movement was in good agreement with the high-speed video.

Although we were reasonably confident that we could distinguish swimming and escape movements visually, it was not easy, because these movements are very fast. Consequently, in later experiments we modified the experimental approach by mounting a high-speed digital camera above the stage of the confocal microscope so that we could directly capture images of the movements of the tail at the same time that we collected confocal images of the interneurons labeled with the calcium indicator. This allowed us to relate unambiguously the movements to the responses of neurons monitored by calcium imaging. We repeated all of the previous experiments with this approach. The conclusions of both methods were the same.

To image the fish with the high-speed camera, the stage of the inverted microscope used for the confocal imaging needed to be illuminated in a way that did not interfere with the confocal imaging. This was accomplished by filtering the light so that wavelengths of the illumination were similar to those of the 488 laser used for confocal imaging and would therefore be prevented from reaching the confocal detectors by the dichroic mirror and the emission filters.

We wanted to compare the escape or swimming movements in agar with those in freely moving fish to determine whether they were similar. From previous work, we had a large database of escapes (hundreds of trials) in freely moving fish in response to taps or squirts of water directed at the head or tail, so we were able to compare the escapes in agar with these (Liu and Fetcho, 1999). In addition, we collected a new series of high-speed (1000 frames/sec) movies of swimming fish to compare the movements and bending frequencies of freely swimming fish with those of restrained fish. Here our aim was to examine sequences over as much of the range of natural swimming speeds as possible for comparison with the movements in agar; therefore, we selected 36 bouts of swimming representing speeds from the slowest to the highest observed.

The calcium imaging was done with a Zeiss LSM 510 confocal system (Zeiss, Thornwood, NY) on an inverted microscope. The inverted scope allowed easy access from above, both for filming movements and for applying stimuli to elicit the movements. One drawback, however, is that the optics are best when there is minimal tissue between the imaged neurons and the objective lens, which comes from below. Thus, the best orientations for imaging were with the fish on its back or on its side, so that the spinal cord was close to the objective lens. Much of our imaging was done this way, and the fish performed swimming and escape movements in these orientations. To make sure that the orientation did not influence our conclusions, we repeated observations with the fish in an upright orientation. The results from this upright imaging, although limited because they were harder to obtain, were the same as when the fish was mounted on its back or its side.

The responses of neurons were imaged by using the argon laser 488 line. Low levels of illumination were used initially to identify a neuron or a group of neurons based on location and morphology. A series of optical sections through the cells were collected to determine the brightest intensity of any optical section before eliciting the behavior. We then acquired a series of images of the cells with minimal laser intensity (typically attenuations of 0.075 to 0.375% of maximum laser power), a maximally open confocal aperture, and a maximal photomultiplier gain to minimize the possibility of photodamage to the cells. In the midst of collection of the images, we elicited the behavior of interest. Escapes were elicited by an abrupt touch on the head with a glass probe attached to a piezoelectric crystal. Swimming was elicited by a sudden illumination of the head through a fiber optic strand directed at the head between the eyes. The stimulus and movements of the fish were visible in the high-speed movies of the fish. The time of the movements was also evident in the confocal images because of movement artifacts in the images. Although the fish is in agar, the portions in the agar can still move slightly. This leads to a brief movement artifact, after which the agar returns the embedded part of the fish very well to its original position. After eliciting a behavior, ≥2 min were allowed before the next trial to avoid habituation to the stimuli.

Confocal images were collected before, during, and after the movement to assess whether a particular cell increased in intensity because of a calcium influx associated with activity. We usually collected 50–100 successive images of the cells at intervals of 260–300 msec. In some cases we performed line scans through the cells, sampling the intensity in a single line through a cell every 1.9 msec. This allowed for higher temporal resolution, but the temporal resolution is limited by movement artifacts.

To rule out movement-related artifactual increases in intensity, a cell was considered to have responded only if its intensity after the stimulus was greater than that of the brightest optical section through the cell before stimulation. The fluorescence responses last seconds, whereas the movements are over in usually ≤100 msec, so it is possible to quantify intensity in stable images after the movement is complete. Fluorescence intensity was measured with the Zeiss LSM software as the mean intensity of pixels in the cell soma in each frame. After collecting calcium responses, we then increased the laser intensity and closed the confocal aperture so that we could collect better morphological data to confirm the identity of the neurons imaged.

Previous work in this system indicates that increases in fluorescence detected somatically are an indication that a cell has fired one or more action potentials (Fetcho and O'Malley, 1995; O'Malley et al., 1996). To test our ability to resolve responses in interneurons when they were minimally active, we imaged the cells while applying brief (0.2 msec) electrical shocks through a metal microelectrode placed near the axon of the labeled neuron in anesthetized fish (0.02% 3-aminobenzoic acid ethyl ester in 10% HBSS).

Cells might be unresponsive as a result of damage from the injection or from illumination and possible phototoxic effects. To minimize false-negative results attributable to damage, we only included cells that were capable of responding based on fluorescence increases in one of the behaviors we studied.

RESULTS

Behavior in agar

A key aspect of the work was the development of a preparation in which both escape and swimming movements could be elicited in the partially restrained fish. Escapes are easily elicited by a touch from a piezoelectric tapper on the head of the fish. The fish responds with a very rapid, large bend away from the side of the stimulus, followed by a return bend. An example of an escape bend in agar is shown in Figure 1.

We compared the movements of the tail in partially embedded fish with movements observed in freely moving animals to confirm that our stimuli elicited a behavior in embedded fish with the characteristic features of escape. The time from the start of the initial bend to maximal bending took 10–13 msec in the preparations we studied, consistent with the timing of the initial escape bend observed in freely moving fish in response to the same tapper stimulus, which also ranged from 10 to 13 msec in duration. The speed and large amplitude of these movements support the conclusion that they are escape behaviors. In addition, previ-

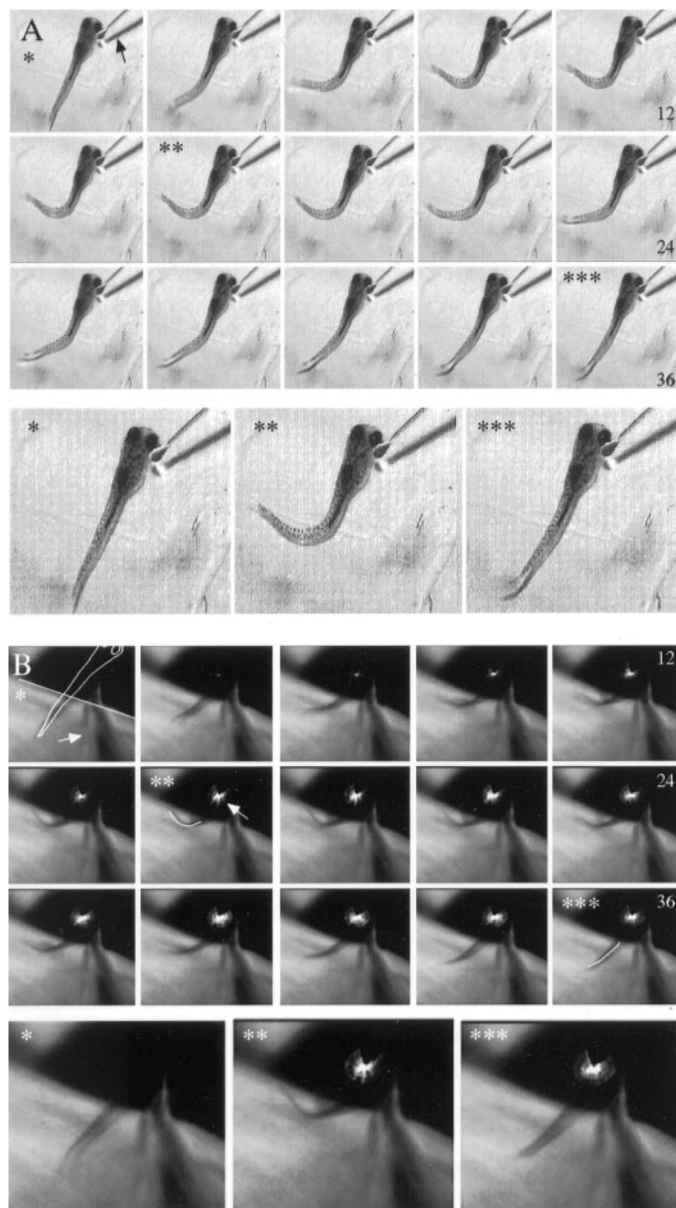

Figure 1. Escape behavior of partially restrained fish. *A* and *B* show high-speed camera images of escapes elicited by a glass tapper. We show every third frame from images collected at 1000 frames/sec, with enlarged frames marked by *asterisks*. Numbers indicate the time in milliseconds. In *A*, images were taken under full-light conditions, to show more clearly an escape with the fish partially embedded in agar. The glass tapper used to elicit the response is marked with an *arrow*. *B* shows frames captured during calcium imaging, for which the light intensity is lower and the fish is partially obscured because it is laying over the dark eye of the objective lens. White Teflon tape beneath the fish highlights its tail. The border of the tape is marked by the *line* in *frame 1* of *B*, in which the silhouette of the fish is outlined as well. The tapper (*B*, *arrow* in *frame 1*) and the scanning laser light (*B*, *arrow* in *frame 7*) are also evident. *Lines* on the small frames marked by *two* and *three asterisks* indicate the position of the midline of the tail. These frames are shown at larger magnification below to more clearly show the tail. Notice the large, fast bend away from the tapper in both *A* and *B*, indicative of an escape. Physiological data taken from the trial shown in *B* are shown in Figure 4*C* and quantified in Figure 5*B*, CiD1. The length of the fish is ~3.5 mm.

ous calcium-imaging data from embedded fish show that these tapper stimuli lead to activation of the Mauthner cell, a reticulospinal neuron whose activation is invariably associated with the production of an escape (O'Malley et al., 1996). The movement pattern and the Mauthner cell involvement make us confident that these are indeed escapes.

Swimming movements were more difficult to elicit in agar. To correlate neuronal activity with swimming as opposed to escape, it was important to obtain swimming movements that were not accompanied by an escape. This is difficult, because many stimuli with an abrupt onset lead to an escape bend. The most effective stimulus to elicit swimming without escapes was a light from a fiber optic strand directed at the center of the head. In good preparations, the onset of the light was followed by rhythmic alternating movements of the tail, with bends propagating from rostral to caudal. These are the characteristic features of the swimming movements observed in a freely moving fish. An example of these rhythmic swimming movements in an agar-embedded fish is shown in Figure 2. To elicit these movements reliably, the embedding must be done very carefully to avoid trauma to the small and delicate fish. The health of the fish can be monitored by watching the blood flow, which can be observed easily because of the transparency. Usually those fish with a robust blood flow in the brain and spinal cord after embedding also would reliably swim in the agar in response to a light stimulus or spontaneously.

The frequency of swimming movements in the preparations used for imaging ranged from 13 to 29 Hz, which overlapped the low end of the range observed in freely swimming fish (from 18 to 71 Hz in our preparations; see also Budick and O'Malley, 2000). As a result, our swimming data are from the lower half of the natural frequency range.

We studied the activity of two classes of interneurons during swimming and escape: circumferential descending interneurons (CiDs) and multipolar commissural descending interneurons (MCoDs) (Bernhardt et al., 1990; Hale et al., 2001). These cells were chosen for two reasons. First, they both have long descending axons in spinal cord, so they could be filled from caudal injections of the calcium indicator. This would lead to minimal disruption of spinal circuits, especially the inputs to the labeled cells, whose cell bodies are far from the labeling site. The presence of normal movement patterns after the labeling is consistent with a minimal disruption of the circuits by these caudal injections. The second reason for choosing these cells was that previous descriptions of their morphology suggested that they were likely to correspond to neurons activated in escape or swimming circuits in other species. The following sections describe the results for each cell type.

CiD morphology

The first class of neuron we studied was CiDs. These cells were backfilled by injections of calcium green dextran into dorsal spinal cord, which labeled a series of CiDs spread over 10–13 segments rostral to the injection. Over the course of our recent studies of zebrafish spinal neurons, we have collected images of well over 100 fluorescently labeled CiDs from intact fish. These confocal images of the cells in living fish, such as those in Figure 3*A–D*, show that the CiDs have a dorsally located, tear-drop-shaped soma with sparse dendrites. Their axon arises from the ventral side of the soma and extends ventrally and caudally to the vicinity of the Mauthner axon, which runs ventrally along the entire length of spinal cord. After approaching the ipsilateral Mauthner axon, the CiD axon turns dorsally and continues caudally and dorsally to join an axon bundle in dorsal cord, where it continues to run longitudinally in ipsilateral spinal cord for >10 spinal segments. There are several CiDs on each side in each body

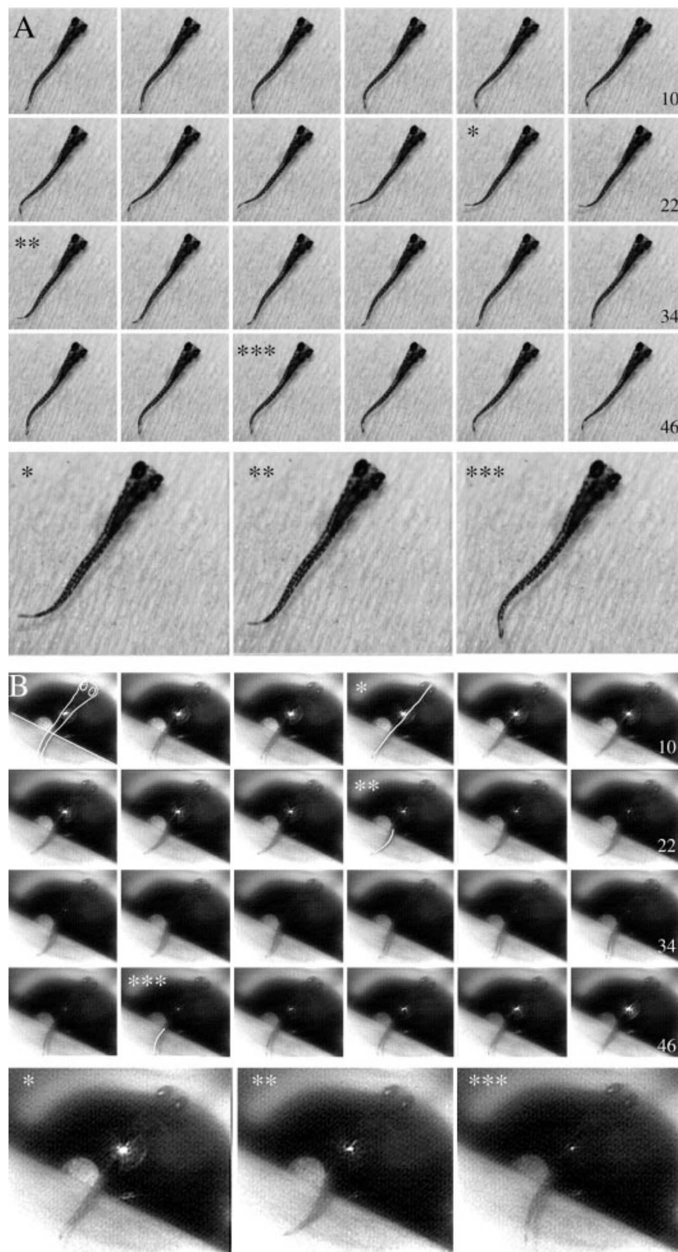

Figure 2. Swimming behavior of partially restrained fish. Swimming is shown here in both fully illuminated (*A*) and experimental (*B*) conditions. Frames are shown every other millisecond, and each set shows one full cycle of swimming, with *asterisked frames* enlarged to show key features of the movement. Tail movements during swimming are more subtle than those during an escape, with the tail moving from side to side as smaller bends propagate from front to back along the fish. The layout in *B* is similar to that in Figure 1*B*. The outline of the fish is marked in the first frame. *Asterisked frames* have the midline of the fish marked to indicate the direction of bending of the tail, which is also visible in the enlarged images of these frames at the *bottom* of the figure. Physiological data obtained from this trial are shown in Figure 6*B* and plotted in Figure 7*A*, MCoD1. The length of the fish is ~3.5 mm.

segment, including one relatively large CiD and approximately three smaller ones.

Our focus here is on the large CiDs, because their morphology is nearly identical to an interneuron in the escape circuit of goldfish whose connectivity has been studied by intracellular methods (Fetcho and Faber, 1988; Fetcho, 1992). In preparations in which both the large CiDs and the Mauthner axon were filled, the ventral process of the CiDs was observed to approach the output collaterals of the Mauthner axon, as in Figure 3*D*. This morphology is similar to that in goldfish, in which the Mauthner cell monosynaptically excites CiD-like interneurons at the contacts formed by the Mauthner axon collaterals. The morphological observations of the big CiDs supported the idea that they corresponded to the ipsilateral descending interneurons in the escape circuit of goldfish. This led us to predict that they would be activated in escapes and allowed us to evaluate the hypothesis from the previous studies of goldfish that these neurons might be shared by swimming and escape circuits (Svoboda and Fetcho, 1996).

Calcium imaging from CiDs

We imaged the responses of 60 large CiDs from 45 different fish in a total of 203 trials in which we elicited either escapes or swimming. In 20 of these cells, we were able to obtain both swimming ($n = 67$) and escape ($n = 77$) trials from the same fish for direct comparison of the responses in the two behaviors. The other 40 cells were studied in escapes only. The pattern of fluorescence changes in the two behaviors was the same for all of the large CiDs we studied, so we illustrate only representative examples here in Figures 4 and 5.

In goldfish, activation of the Mauthner neuron on one side leads to excitation of CiD-like cells on the opposite side of the body because the axon of the Mauthner cell crosses in the brain to monosynaptically excite contralateral motoneurons and interneurons (Fetcho and Faber, 1988). We imaged the responses of CiDs in zebrafish during escapes elicited by a tap on the contralateral side of the head to determine whether the CiD might participate in the large initial bend of the escape, as does the similar cell type in goldfish. The large CiDs in zebrafish showed robust increases in fluorescence in conjunction with escapes elicited by a tap on the head, as illustrated by the pseudocolor images of the cell shown in Figure 4. Quantification of the fluorescence changes in the cells over time (Fig. 5*B*) showed that these increases were rapid, peaking quickly in the first frame in which the cell was visible after the stimulus and then returning gradually to baseline over the course of 6–8 sec, as is typical for this calcium indicator. The percentage of increase in fluorescence ($\Delta F/F$) ranged from 11 to 67%, with most of the increases being $\geq$20%. Individual large CiDs responded consistently in successive trials. We saw no evidence that an individual large CiD would respond to head stimuli in some escapes and not others.

Although imaging successive frames suggested a rapid response of the cells, the frame imaging is slow compared with the movement. To obtain better temporal resolution, we used line scans to scan a line through a CiD at 1.9 msec intervals. These line scans revealed that the cells showed an elevated fluorescence in close association with the behavior, as in the example in Figure 4*D*. The movement artifact and the very short latency from neural activity to behavior prevent us from determining the exact time of onset of the increase in fluorescence in the cells; however, the fluorescence of the CiDs was elevated as early as we could observe them after the movement artifact.

Although the responses of CiDs in escapes were reproducible and substantial, we saw no increases above baseline in the fluorescence of the cells during swimming movements. Even CiDs with the largest increases in fluorescence in escapes, such as the one in Figures 4*C* and 5*B* (CiD1), showed no increase in swimming. This was true both when the swimming occurred in sepa-

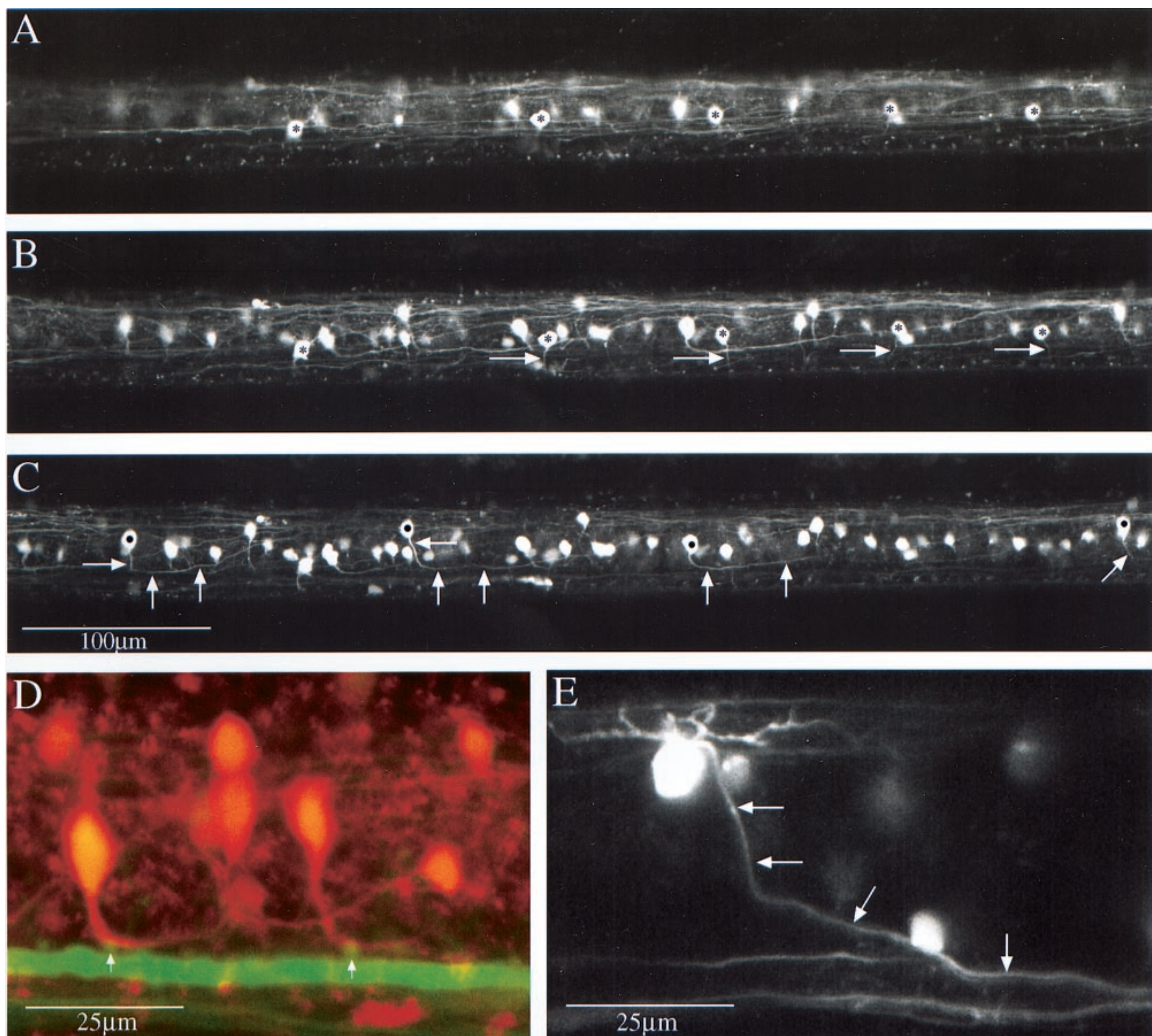

Figure 3. CiD and MCoD morphology in intact, living fish. *A–C* show a longitudinal array of spinal interneurons labeled by backfilling with calcium green dextran and viewed in a series of optical sections taken from most lateral in *A* to most medial in *C*. *Left* is rostral and *up* is dorsal. *Asterisked cells* are MCoDs, and an array of these cells can be seen in *A* and *B*. In B, MCoD axons are indicated by *arrows* and show a ventral course. The more medial CiDs are marked with *dots* in *C*. CiD axons, marked by *arrows* in *C*, show an initial ventral, circumferential course followed by a dorsal turn and descent in the dorsal longitudinal fasciculus. *D* is an image from a double labeling showing the relationship of CiDs (*red*) to the Mauthner axon (*green*). The *arrows* in *D* show the short presynaptic output collaterals of the Mauthner axon, which are apposed to the ventral process from CiDs. *E* is a dorsal view of a three-dimensional reconstruction of an MCoD to show the commissural process. The left and right sides of the cord are at the *top* and *bottom*, respectively. Rostral is to the *left*. The dendritic processes of the MCoD are evident at the *top*, as is the commissural axon (*arrows*), which descends in the medial longitudinal fasciculus (*rightmost arrow*) after crossing the cord.

rate trials from escapes, as in most of our experiments, as well as when a swim bout occurred with a short delay after an escape in a single trial. In swimming after an escape, the fluorescence from the escape peaked and declined, with no evidence for an additional increase during the swimming episode. Line scans of CiDs during swimming also showed no evidence of increases in fluorescence, which was expected, based on frame-scanning data. The decay of calcium responses measured with calcium green takes seconds, making frame scanning sufficient for detecting whether or not a cell has responded. The line scans are more useful for determining the onset of the response, which is much more rapid than the decay. In sum, the CiDs showed very robust increases in fluorescence when the fish escaped and no increase associated with swimming.

We tested our ability to resolve minimal activation of CiD neurons by applying brief electrical shocks (0.2 msec duration; 10–30 μA) through a metal microelectrode placed in the vicinity of their fluorescently labeled axon. We were able to detect responses to a single, brief shock, which produced increases in fluorescence of 6–13%. In previous intracellular recordings from the larger goldfish neurons, such single antidromic stimuli lead to a single action potential. Repetitive electrical stimuli near the CiD axons in zebrafish led to larger fluorescence increases, as shown previously in motoneurons (Fetcho and O'Malley, 1995).

J. Neurosci., November 15, 2001, *21*(22):8956–8965

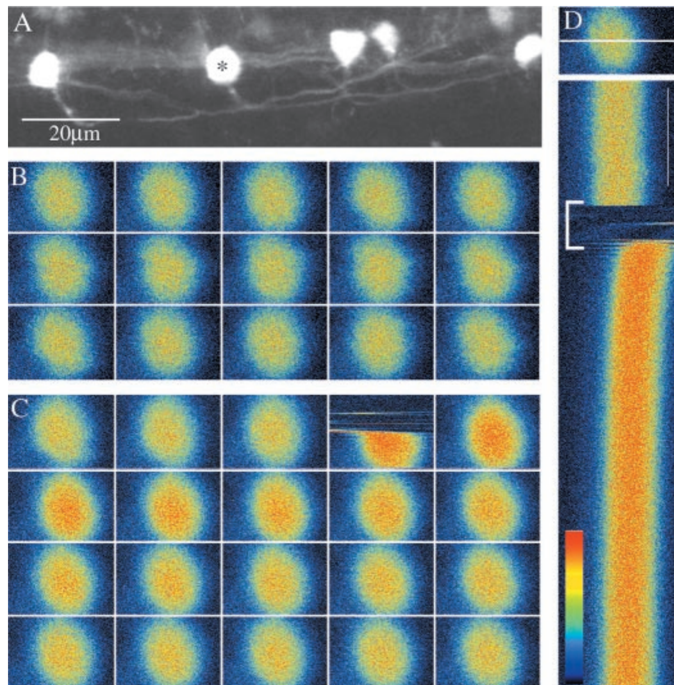

Figure 4. Calcium imaging of CiD responses. Calcium imaging was used to monitor the activity of the cell marked with an *asterisk* in *A* during swimming (*B*) and during escapes (*C, D*). The cell is shown in pseudocolor, with *red* representing the brightest intensity. Images were taken every 300 msec. In *B*, there is no increase in the fluorescence of the cell during swimming, which occurs in the first frame of the *second row*. In *C*, there is a marked increase in fluorescence of the same cell during an escape ($\Delta F/F$ = 50%; see the plot for this cell in Fig. 5*B*, CiD1). The movement for this trial was shown in Figure 1*B*. *D* shows a line scan of the same CiD during an escape. The *line* through the cell at the top was scanned every 1.9 msec. The *scanned lines* are *stacked below*, with time increasing as you move down (vertical scale bar at right, 100 msec). The movement artifact associated with the escape is *bracketed*. The *line* through the CiD showed a large increase in intensity ($\Delta F/F$ of 67%), with the increase evident as soon as the cell returned to the field after the escape. A *color bar* representing the pseudocolor map for this and Figure 6 is shown in *D*. The *bar* represents raw intensity values from 0 (*black*) to 255 (*red*).

These observations support our ability to detect even weak activity, produced by antidromic stimuli that can be expected to elicit one action potential.

MCoD morphology

MCoDs are large cells located ventral and lateral to the CiD neurons (Fig. 3*A*–*C*). We initially chose to study the MCoDs because of a previous report that they had long ipsilateral descending axons, which made them candidates for an excitatory cell type described in the swimming rhythm-generating circuits of lampreys and frog tadpoles (Bernhardt et al., 1990). However, our initial confocal observations of MCoDs, filled by injections into ventral spinal cord, revealed that their axons were commissural, as shown in Figure 3*E* (Hale et al., 2001). These axons arose from a multipolar cell body located in ventral lateral cord. The axon crossed the spinal cord ventrally at the level of the cell soma and then descended in contralateral ventral spinal cord for as many as 13 segments. A single injection into caudal spinal cord could fill many MCoD neurons rostral to the injection, because there is more than one MCoD per segment. All of the larval MCoDs we observed had the somatic morphology described by Bernhardt et al. (1990) for a cell type which they indicated had an ipsilateral axon and which they called ventrolateral descending interneurons, or VeLD cells. The observations of these cells in 27 fish for this study, as well as many more in a previous morphological study (Hale et al., 2001), indicate that these neurons are in fact commissural. We renamed these larval cells MCoDs based on these new morphological observations to distinguish them from a different embryonic cell type that does have an ipsilateral axon and is more appropriately called a VeLD cell (Hale et al., 2001).

Calcium imaging from MCoDs

We imaged the responses of 31 MCoD neurons in 27 fish, with a total of 117 trials of swimming or escape. In cells from 12 of these fish, we obtained both swimming (n = 56) and escape (n = 34) trials from the same cell for direct comparison of responses in swimming versus escape. The other 19 cells were studied only during swimming. MCoD neurons showed robust increases in fluorescence when the fish was swimming, as shown in Figures 6 and 7. These fluorescence increases ranged from 16 to >60%. A brief bout of swimming led to a fluorescence increase that returned gradually to baseline 5–8 sec after the peak of fluorescence (Fig. 7, MCoD2). Zebrafish typically swim in short bouts of three to six tail beat cycles. However, they sometimes produce multiple bouts after a light stimulus. Individual MCoDs, such as MCoD3 in Figure 7*A*, showed increases associated with each of a series of bouts of swimming. These repeated bouts of swimming could sometimes lead to a sustained elevation of fluorescence, with the fluorescence returning to baseline only after the swimming movements ceased for several seconds (Fig. 6*D*). Fluorescence increases from successive bouts could summate to give a greater increase than that seen in a single bout. Unlike escapes, which could only be elicited in response to a stimulus, fish in agar sometimes initiate swimming spontaneously during the confocal imaging. The MCoD neurons increased their fluorescence during this spontaneous swimming just as they did during swimming elicited by a light stimulus.

To achieve better temporal resolution, we performed line scans through MCoD neurons, one of which is shown in Figure 6*D*. We hoped to be able to observe successive rises in the fluorescence occurring at the same frequency as swimming. These would be expected if the neuron was rhythmically active at the same frequency as the swimming. However, we anticipated that these fluctuations might be difficult to discern because of the relatively slow time course of the indicator relative to the frequency of the swimming and because of movement artifacts. We saw some weak evidence of such repeated rises within a bout of swimming, but the movement of the fish made it difficult to determine conclusively whether they were present. What was clear from this higher temporal-resolution imaging was that the fluorescence increases occurred during the movements (which lasted only ~100–300 msec) and were sustained after them. One might expect that the fluorescence would increase before the movements if the neuron fired action potentials before movements began. However, for us to detect this, the cell would have to fire well before (20 msec or so) the movements began, because small increases are difficult to detect in only a few lines of a line scan at 1.9 msec/line. The fish are sufficiently fast enough that the delay between cell activity and movement is likely to be shorter than the temporal resolution of our imaging. The best we can confidently conclude at present is that the increase occurs very close in time to the movements and overlaps them.

Unlike the clear responses of MCoDs in swimming, we saw no increases in the fluorescence of MCoDs in response to taps that

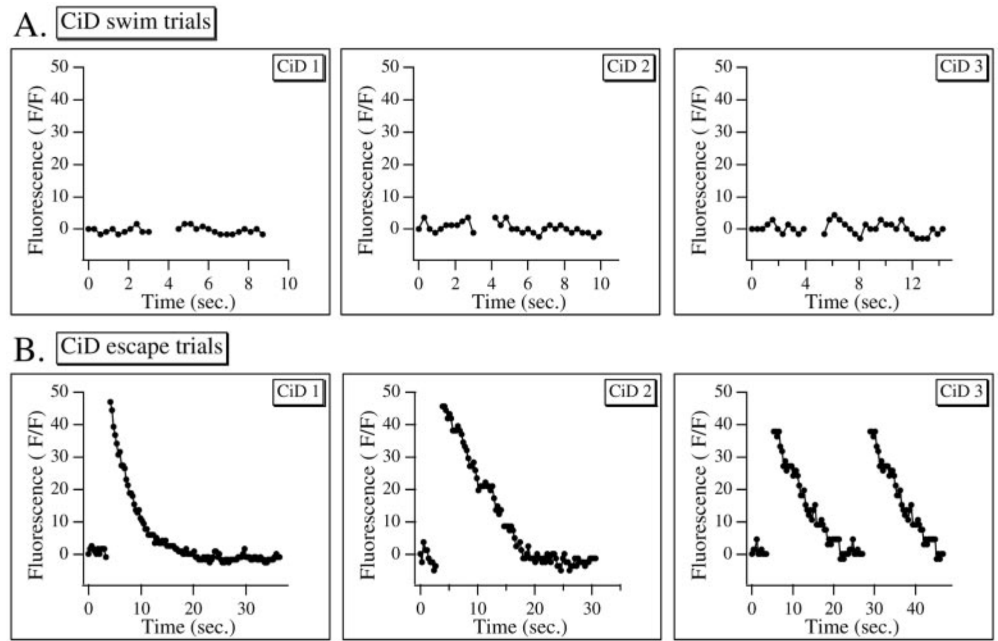

Figure 5. Quantifying representative CiD responses. These plots quantify the fluorescence changes ($\Delta F/F$) versus time for three different CiDs from three different fish. *A* shows fluorescence changes associated with swimming and *B* shows the intensity changes during escapes. The CiDs always showed no change in fluorescence compared with baseline levels during swimming bouts. In contrast, intensity changes during escapes were robust and consistent. The escape movements for CiD1 are shown in Figure 1*B* and the images of the same cell in Figure 4. The cell labeled CiD3 in *B* shows a double response that was a consequence of two successive escapes.

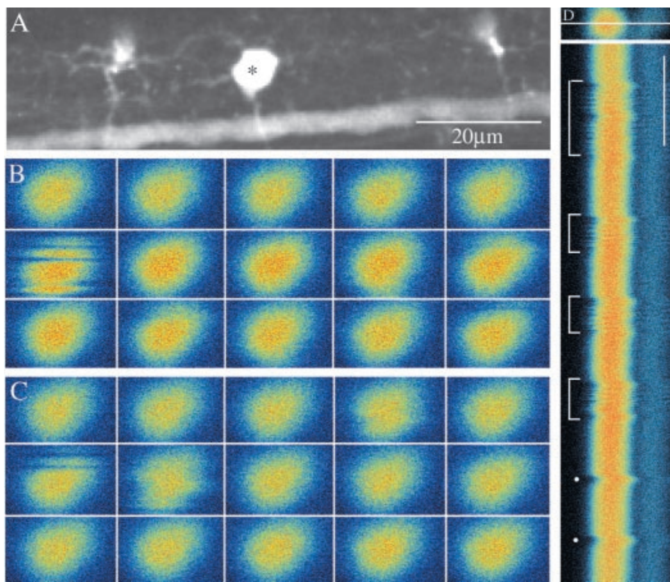

Figure 6. Calcium-imaging MCoD responses. Confocal calcium imaging of the MCoD marked by an *asterisk* in *A* is shown during swimming (*B, D*) and during an escape (*C*). Images were taken every 250 msec. During swimming (*B*), there is a clear increase in fluorescence ($\Delta F/F = 35\%$) (see the plot for this trial in Fig. 7*A*, MCoD1). The swimming movements for this trial are shown in Figure 2*B*. There is no increase in fluorescence during an escape (*C*, *frame 6*), (see the plot in Fig. 7*B*, MCoD1). Movement artifacts from swimming and escape are evident in the first frame in the *second row* of *B* and *C*, respectively. The line scan in *D*, taken from a different MCoD, shows the activity of an MCoD during multiple bouts of swimming with a layout similar to that in Figure 4*D*. The movement artifacts during each bout of swimming are *bracketed*, and two nonswimming movements are marked by *dots*. Note the increases in intensity after individual bouts and the summing effects from one bout to the next. *Time bar* in *D*, 100 msec.

led to an escape bend toward the side containing the neuron. Even repeated taps, which produce robust, summated increases in the fluorescence of the CiDs that respond in escapes, led to no detectable increase in the fluorescence of the MCoDs in both frame scans and line scans. As with the CiDs, we were able to detect the responses of MCoDs to a single antidromic stimulus, indicating that we could resolve even weak activation of the cell.

DISCUSSION

The extent to which individual identified spinal interneurons contribute to different motor behaviors has rarely been studied among vertebrates (Alstermark et al., 1990; McCrea, 1992; Soffe, 1993). This is because few preparations combine the ability to both elicit the motor behavior of interest and record the activity of identified cells. The preparation we describe allows us to image active spinal interneurons in an intact zebrafish that is partially free to move. Because we can image the neural activity and simultaneously film the movements, we can correlate active cells with the movements. The activity is monitored with optical methods, so the morphology of the recorded cell is clear in confocal optical sections of the living cell without subsequent tissue processing and staining. The physiological and morphological relationships are immediately available.

Our approach and preparation differ considerably from those typically used to study spinal circuitry. Most previous work, including our own, involved intracellular or extracellular recording from paralyzed preparations or from isolated spinal cord (Grillner et al., 1986; Fetcho 1990; Roberts, 1990; Soffe, 1993; Svoboda and Fetcho, 1996). In our work, the zebrafish is alert and partially moving, so the descending and sensory pathways are intact. This makes it easier to elicit a more complete set of motor behaviors and may also lead to activity patterns closer to the natural ones. One can easily monitor different cells and even groups of cells in the same preparation by simply changing the region of the spinal cord imaged.

There are disadvantages to the optical approach. We only indirectly measure activity by calcium levels. Electrophysiology is better for establishing the subthreshold input to cells and the exact number of action potentials they fire, but must be done in paralyzed preparations (Fetcho and Faber, 1988; Fetcho, 1990, 1992; Drapeau et al., 1999). Although pairwise recordings have not yet been done in larval zebrafish, such techniques can poten-

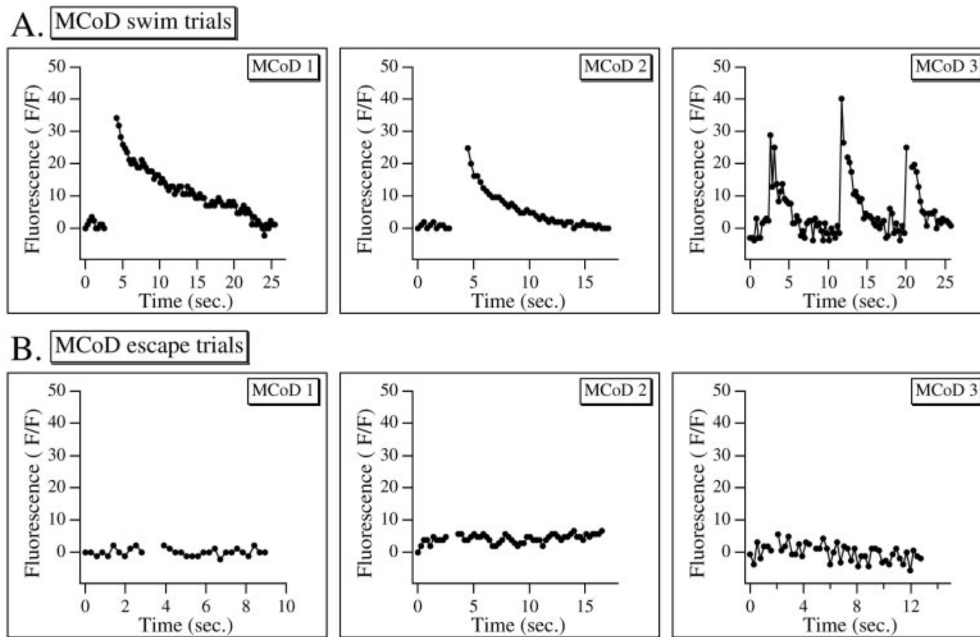

Figure 7. Quantifying representative MCoD responses. These plots show the fluorescence changes ($\Delta F/F$) versus time for three different MCoDs from three different fish. *A* shows fluorescence intensity changes associated with swimming and *B* shows the intensity changes during escapes. The swimming movements for MCoD1 are shown in Figure 2*B* and the images for the same trial are shown in Figure 6. MCoDs showed consistent increases during swimming but no increases in escapes. MCoD3 in *A* shows intensity increases during three successive bouts of swimming.

tially address questions of connectivity that current optical methods cannot. Thus, electrophysiological approaches can provide essential information not available with optical methods and vice versa. We have been using the two in a complimentary way through a combination of our imaging studies of behaving zebrafish with our electrophysiological observations from paralyzed preparations of the closely related goldfish, in which conventional physiology is easier.

Although there are few studies of the activity of identified spinal neurons in different behaviors, some strong evidence that interneurons can participate in the production of different axial motor patterns comes from a study of the interneurons activated during fictive struggling and swimming motor patterns in *Xenopus* tadpoles (Soffe, 1993). This work indicated that individual interneurons are active in both fictive swimming and struggling, pointing to substantial sharing of interneurons in different behaviors.

Our own previous electrophysiological work describing the spinal circuit of the Mauthner cell, which initiates the escape, led us to think that there might also be considerable overlap between interneurons involved in escape and swimming circuits, because the interneurons in the escape circuits of goldfish had patterns of connections that were similar to those in swimming networks in other species (Fetcho and Faber, 1988; Fetcho, 1990, 1992). The Mauthner cell appeared to tap into spinal circuits similar to those used for swimming. Therefore, we were surprised to find that the two classes of interneurons we studied in zebrafish showed no evidence of being shared by circuits for escape and swimming movements. One of the classes, the CiD, corresponds morphologically to a cell type studied previously with intracellular recordings in goldfish (Fetcho, 1992). These descending interneurons are excited monosynaptically by the contralateral Mauthner cell and in turn monosynaptically excite spinal motoneurons. Their connections make it likely that the cell contributes to the massive excitation of the motoneurons in the escape. Our imaging of the CiDs shows that they are active in escapes in zebrafish as well. However, we saw no evidence that they were active in swimming, although both swimming and escape involve bending movements of the body.

One concern about imaging activity is that nonresponses might have a very weak, undetected signal. This is difficult to rule out completely, but several lines of evidence support the conclusion that the CiDs are not activated as part of the spinal swimming circuit at the swimming frequencies in our experiments. We could detect responses in CiDs to a single brief antidromic electrical shock that should produce a single action potential (Fetcho and Faber, 1988). This, along with evidence from previous work, indicates that the calcium influx from a single spike is detectable with our imaging techniques (Fetcho and O'Malley, 1995; O'Malley et al., 1996). We studied enough cells and trials here that even if CiDs were active with only one spike we would have seen responses in at least some cells during swimming; however, we saw none. Finally, if the CiDs were involved in swimming, it is also likely that they would fire not just one spike, but several, with one or more spikes on each cycle of swimming, as in other swimming preparations, such as *Xenopus* tadpoles (Sillar and Roberts, 1993). This would lead to signals larger than that from a single spike, making it more likely that we would detect them. Thus, the evidence supports the conclusion that the CiDs are active in escapes but not in swimming at the speeds we studied.

We chose to study MCoDs (formerly VeLD cells) because they were thought to have ipsilateral descending axons that made them candidates for the excitatory interneurons described in swimming circuits in other species. However, the MCoDs are commissural, with long axons descending in ventral spinal cord (Hale et al., 2001). In contrast to CiDs, these MCoDs are active in swimming but not in escapes. Even repeated escape stimuli do not lead to any fluorescence increases in the cells, which makes us confident that they are not activated in escapes. The MCoDs are clearly active in swimming.

Recent data from *in situ* staining for the glutamate and glycine transporters cloned from zebrafish indicate that the MCoDs are glutamatergic and not glycinergic (Higashijima et al., 2001). Therefore, they are likely to be excitatory. Although long commissural excitatory interneurons with rhythmic inputs during swimming and connections with motoneurons have been identi-

fied in other species such as lampreys, their roles in swimming have not been emphasized (Buchanan, 1982; Grillner and Matsushima, 1991). Our data indicate that commissural excitatory cells are likely to be involved in swimming circuits in zebrafish and therefore warrant more attention in studies of swimming networks. These cells may drive the excitation of motoneurons and help to maintain the proper intersegmental phase relationships between the opposite sides of the body needed to produce the several bends that propagate along the body during swimming.

The differential activation of the CiDs and MCoDs in swimming and escape refutes the simple notion that spinal circuits are completely shared by the two behaviors. Although both behaviors involve axial bending movements, it appears that they recruit at least some interneuron populations in different ways. One key difference between the two behaviors is the speed of the bending. The interneurons optimal for faster bending movements might differ from those in slower movements, as suggested previously for commissural interneurons in escape circuits of goldfish (Fetcho, 1990). It may be, for example, that the large CiDs, which powerfully excite motoneurons in goldfish, are dedicated to the very large, fast bending movements of the escape. If they are involved in swimming, they might only be recruited during very fast and forceful swimming movements, above the range that could be elicited independent of escapes in our preparation. This is a possibility given the evidence from other systems that more interneurons are recruited at higher frequencies of swimming (Sillar and Roberts, 1993).

An alternative explanation of the differential activity in the interneurons follows from the observation that some of the key differences between axial bending movements such as escape, swimming, and struggling are the patterns of intersegmental coordination. It might be that long intersegmental interneurons such as the ones we studied are responsible for generating the different coordination patterns between segments, which would explain why different sets of intersegmental interneurons are active in different behaviors. This would be consistent with a similar role for propriospinal interneurons in cats, which seem to be important in the coordination of muscles across different joints during reaching movements (Tantisira et al., 1996). Perhaps more local interneurons would be shared by different behaviors, because the local patterns of coordination, such as reciprocal inhibition within segments, are similar in all of these behaviors. This might explain why the evidence supports sharing of interneurons in some studies and separation of the behavioral contributions of interneurons in others.

Whatever the explanation, there are differences in the activation of spinal interneurons in escape and swimming in zebrafish that indicate a functional subdivision of spinal interneurons used in different bending movements. Zebrafish offer two avenues for additional tests of the functional contributions of these cells. First, one might expect to find mutant lines of fish with perturbations in swimming movements but not escape, and vice versa (Granato et al., 1996; Lorent et al., 2001). Second, laser ablations could be used to selectively remove a class of cells with the prediction that one might find a deficit specific to swimming or to escape (Liu and Fetcho, 1999). The development of the zebrafish preparation and the identification of interneurons active in the different behaviors provide a foundation for future studies of mutant lines and for ablation experiments.

REFERENCES

Alstermark B, Kummel H, Pinter MJ, Tantisira B (1990) Integration in descending motor pathways controlling the forelimb in the cat. 17. Axonal projection and termination of C3–C4 propriospinal neurones in the C6-Th1 segments. Exp Brain Res 81:447–461.

Berkinblit MB, Deliagina TG, Feldman AG, Gelfand IM, Orlovsky GN (1978) Generation of scratching. II. Nonregular regimes of generation. J Neurophysiol 41:1058–1069.

Berkowitz A, Stein PS (1994) Activity of descending propriospinal axons in the turtle hindlimb enlargement during two forms of fictive scratching: phase analyses. J Neurosci 14:5105–5119.

Bernhardt RR, Chitnis AB, Lindamer L, Kuwada JY (1990) Identification of spinal neurons in the embryonic and larval zebrafish. J Comp Neurol 302:603–616.

Bizzi E, Giszter SF, Loeb E, Mussa-Ivaldi FA, Saltiel P (1995) Modular organization of motor behavior in the frog's spinal cord. Trends Neurosci 18:442–446.

Buchanan JT (1982) Identification of interneurons with contralateral, caudal axons in the lamprey spinal cord: synaptic interactions and morphology. J Neurophysiol 47:961–975.

Budick SA, O'Malley DM (2000) Locomotor repertoire of the larval zebrafish: swimming, turning, and prey capture. J Exp Biol 203:2565–2579.

Drapeau P, Ali DW, Buss RR, Saint-Amant L (1999) In vivo recording from identifiable neurons of the locomotor network in the developing zebrafish. J Neurosci Methods 88:1–13.

Edgley SA, Jankowska E, Shefchyk S (1988) Evidence that mid-lumbar neurones in reflex pathways from group II afferents are involved in locomotion in the cat. J Physiol (Lond) 403:57–71.

Fetcho JR (1990) Morphological variability, segmental relationships, and functional role of a class of commissural interneurons in the spinal cord of goldfish. J Comp Neurol 299:283–298.

Fetcho JR (1992) Excitation of motoneurons by the Mauthner axon in goldfish: complexities in a "simple" reticulospinal pathway. J Neurophysiol 67:1574–1586.

Fetcho JR, Faber DS (1988) Identification of motoneurons and interneurons in the spinal network for escapes initiated by the Mauthner cell in goldfish. J Neurosci 8:4192–4213.

Fetcho JR, O'Malley DM (1995) Visualization of active neural circuitry in the spinal cord of intact zebrafish. J Neurophysiol 73:399–406.

Fetz EE, Perlmutter SI, Maier MA, Flament D, Fortier PA (1996) Response patterns and postspike effects of premotor neurons in cervical spinal cord of behaving monkeys. Can J Physiol Pharmacol 74:531–546.

Gelfand IM, Orlovsky GN, Shik ML (1988) Locomotion and scratching in tetrapods. In: Neural control of rhythmic movements (Cohen AH, Rossignol S, Grillner S, eds), pp 167–199. New York: Wiley.

Giszter SF, Mussa-Ivaldi FA, Bizzi E (1993) Convergent force fields organized in the frog's spinal cord. J Neurosci 13:467–491.

Granato M, van Eeden FJ, Schach U, Trowe T, Brand M, Furutani-Seiki M, Haffter P, Hammerschmidt M, Heisenberg CP, Jiang YJ, Kane DA, Kelsh RN, Mullins MC, Odenthal J, Nusslein-Volhard C (1996) Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. Development 123:399–413.

Grillner S, Matsushima T (1991) The neural network underlying locomotion in lamprey: synaptic and cellular mechanisms. Neuron 7:1–15.

Grillner S, Brodin L, Sigvardt K, Dale N (1986) On the spinal network generating locomotion in the lamprey: transmitters, membrane properties, and circuitry. In: Neurobiology of vertebrate locomotion (Grillner S, Stein PSG, Forssberg H, Herman RM, eds), pp 335–352. London: Macmillian.

Hale ME, Ritter DA, Fetcho JR (2001) A confocal study of spinal interneurons in living larval zebrafish. J Comp Neurol 437:1–16.

Heitler WJ (1985) Motor programme switching in the crayfish swimmeret system. J Exp Biol 114:521–550.

Higashijima S, Hotta Y, Okamoto H (2000) Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the islet-1 promoter/enhancer. J Neurosci 20:206–218.

Higashijima S, Bhatt DH, Mandel G, Fetcho JR (2001) Neurotransmitter properties of spinal interneurons in embryonic/larval zebrafish. Soc Neurosci Abstr 27:830.4.

Liu KS, Fetcho JR (1999) Laser ablations reveal functional relationships of segmental hindbrain neurons in zebrafish. Neuron 23:325–335.

Lockery SR, Kristan WB (1990) Distributed processing of sensory information in the leech. II. Identification of interneurons contributing to the local bending reflex. J Neurosci 10:1816–1829.

Lorent K, Liu KS, Fetcho JR, Granato M (2001) The zebrafish *space cadet* gene controls axonal pathfinding of neurons that modulate fast turning movements. Development 128:2131–2142.

Maier MA, Perlmutter SI, Fetz EE (1998) Response patterns and force relations of monkey spinal interneurons during active wrist movement. J Neurophysiol 80:2495–2513.

McCrea DA (1992) Can sense be made of spinal interneuron circuits? Behav Brain Sci 15:633–643.
O'Malley DM, Kao Y-H, Fetcho JR (1996) Imaging the functional organization of zebrafish hindbrain segments during escape behaviors. Neuron 17:1145–1155.
Parker D, Grillner S (2000) The activity-dependent plasticity of segmental and intersegmental synaptic connections in the lamprey spinal cord. Eur J Neurosci 12:2135–2146.
Ramirez JM, Pearson KG (1988) Generation of motor patterns for walking and flight in motoneurons supplying bifunctional muscles in the locust. J Neurobiol 19:257–282.
Roberts A (1990) How does a nervous system produce behaviour? A case study in neurobiology. Sci Prog 74:31–51.
Sillar KT, Roberts A (1993) Control of frequency during swimming in *Xenopus* embryos: a study on interneuronal recruitment in a spinal rhythm generator. J Physiol (Lond) 472:557–572.
Soffe SR (1993) Two distinct rhythmic motor patterns are driven by common premotor and motor neurons in a simple vertebrate spinal cord. J Neurosci 13:4456–4469.
Svoboda KR, Fetcho JR (1996) Interactions between the neural networks for escape and swimming in goldfish. J Neurosci 16:843–852.
Tantisira B, Alstermark B, Isa T, Kummel H, Pinter M (1996) Motoneuronal projection pattern of single C3–C4 propriospinal neurones. Can J Physiol Pharmacol 74:518–530.
Weimann JM, Meyrand P, Marder E (1991) Neurons that form multiple pattern generators: identification and multiple activity patterns of gastric/pyloric neurons in the crab stomatogastric system. J Neurophysiol 65:111–122.