Imaging the Functional Organization of Zebrafish Hindbrain Segments during Escape Behaviors

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Summary

Although vertebrate hindbrains are segmented structures, the functional significance of the segmentation is unknown. In zebrafish, the hindbrain segments contain serially repeated classes of individually identifiable neurons. We took advantage of the transparency of larval zebrafish and used confocal calcium imaging in the intact fish to study the activity of one set of individually identified, serially homologous reticulospinal neurons (the Mauthner cell, MiD2cm, and MiD3cm) during behavior. Behavioral studies predicted that differential activity in this set of serially homologous neurons might serve to control the directionality of the escape behavior that fish use to avoid predators. We found that the serially homologous cells are indeed activated during escapes and that the combination of cells activated depends upon the location of the sensory stimulus used to elicit the escape. The patterns of activation we observed were exactly those predicted by behavioral studies. The data suggest that duplication of ancestral hindbrain segments, and subsequent functional diversification, resulted in sets of related neurons whose activity patterns create behavioral variability.

Introduction

Vertebrate brains are segmented structures (Lumsden and Keynes, 1989; Rubenstein et al., 1994; Guthrie, 1995). The segmentation is most obvious in the hindbrain where a series of repeated units can be defined at all levels from the gross anatomical to the molecular in both developing and adult brains (Metcalf et al., 1986; Fraser et al., 1990; Trewavas et al., 1990; Clarke and Lumsden, 1993). In animals as different as chicks and fish, these segments contain repeated, serially "homologous" neurons (Trewavas et al., 1990; Lee and Eaton, 1991; Clarke and Lumsden, 1993). Although the anatomical segmentation is evident even at the level of single, identifiable neurons, the functional significance of the segmentation is unknown. One obvious question is whether similar neurons in successive segments act together as a functional unit, or are functionally independent. Because segments are thought to have arisen by duplication of ancestral segments, subsequent divergence of serially repeated neurons might constitute one mechanism for the evolution of behavioral diversity. Here, we examine the functional organization of one set of serially repeated neurons in zebrafish larvae.

Zebrafish larvae are a particularly favorable preparation for study of hindbrain because many of their hindbrain neurons are individually identifiable from fish to fish (Kimmel et al., 1982; Metcalfe et al., 1986; Hanneman et al., 1988). We have focused on one particular set of hindbrain cells that includes the well-studied Mauthner cell in hindbrain segment 4 and two other neurons, MiD2cm and MiD3cm, in segments 5 and 6. All of these cells are morphologically similar, each having two major dendrites and an axon that crosses in the brain and descends along the length of the contralateral spinal cord. This set of homologs is of particular interest because one of the set, the Mauthner cell, is already known to play an important role in the escape behaviors used to avoid predators. There are, however, no physiological data from the other cells to provide insight into the functional organization of this serial set of neurons.

During escapes, a stimulus on one side of the fish leads to a very fast and forceful C-shaped bend to the opposite side (Foreman and Eaton, 1993). This is produced, at least in part, by the output of the Mauthner cell that fires a single action potential during the escape, thereby exciting motoneurons along the contralateral side of spinal cord (Fetcho and Faber, 1988; Faber et al., 1989). This is followed by a counter bend that propels the fish through the water. The extent of the initial C-bend and the subsequent direction of the escape vary in a systematic and, apparently, adaptive manner (Eaton and Emberley, 1991). Sensory stimuli from behind the fish lead to a weak C-bend and an escape movement directed forward, away from the location of the sensory stimulus. Stimuli at the head lead to the strongest C-bends, with the fish making up to a 180° turn directed away from the stimulus. Thus, the escape movement varies in a way that assures that the fish moves away from a potential threat.

Although there is strong evidence that the Mauthner cell initiates the escape behavior, several observations indicate that the Mauthner cell is not acting alone. The variability of the escape with the location of the triggering sensory stimulus suggests the involvement of additional neurons, because the Mauthner cell only fires a single spike and, by itself, produces a stereotyped output (Nissancov et al., 1990). Also, lesion studies show that after depletion of the Mauthner cell, goldfish can still produce robust escapes, suggesting that other neurons can mimic the function of the Mauthner cell (Eaton et al., 1982). These observations led to the hypothesis that there might be neurons other than the Mauthner cell involved in the escape. Anatomical work revealed the presence of MiD2cm and MiD3cm, and these are thought, based upon their morphological similarities to the Mauthner cell, to be the two most likely candidates to act in concert with it. Foreman and Eaton (1993) have proposed an explicit model of how the combined activity in the Mauthner cell and its serial homologs might contribute to the directional control of the escape behavior. They propose that rostral stimuli might activate the entire set of cells (Mauthner cell, MiD2cm, and MiD3cm) leading to a very powerful C-bend and a large direction change, whereas caudal stimuli would activate only the Mauthner cell producing a smaller C-bend and the associated weaker turn.
Thus, the prior work has generated two predictions about the activity patterns in MiD2cm and MiD3cm. First, if these are the neurons that can functionally substitute for the Mauthner cell, then they should also be activated during at least some escapes. Second, the Foreman and Eaton model predicts that escapes elicited by rostral stimuli should activate the entire set of homologs, whereas caudal stimuli should activate just the Mauthner cell. These predictions have not been tested previously because no one has been able to record the activity of MiD2cm and MiD3cm during escapes. We have recently developed methods to label neurons with a calcium indicator and observe their activity patterns during behavior by using confocal calcium imaging in the transparent, posthatching zebrafish larvae (Fetcho and O’Malley, 1995). Here, we show that this approach can be used to label and monitor the activity of hindbrain neurons in the live fish, which allowed us to evaluate these predictions.

We asked first whether the Mauthner cell and its homologs were activated together during escapes, and second, how their pattern of activation varied during escapes elicited by sensory stimuli at different locations. We examined both questions by directly observing which members of this set of homologs showed fluorescence increases during escapes elicited by a pair of piezoelectric tappers that were used to apply a sudden light touch to either the head or the tail. We found that the activity pattern is exactly that predicted by Foreman and Eaton (1993). The observations are consistent with the idea that this set of similar neurons in successive hindbrain segments forms a functional group and that the activity pattern within the group contributes to variability in the form of the behavior (Morton and Chiel, 1994). The organization into serial functional groups spanning hindbrain segments may extend to other serially repeated neurons present in the hindbrains of both zebrafish and other vertebrates.

Results

Imaging Identified Hindbrain Neurons In Vivo
Reticulospinal neurons were retrogradely labeled in larval fish by injecting calcium green dextran into the caudal spinal cord. Large injections labeled many reticulospinal neurons bilaterally, as illustrated in Figure 1A, which shows a projection from a stack of images acquired from the hindbrain of a living fish. This array contains several sets of serially homologous neurons. We were specifically interested in the set that includes the Mauthner cell and its segmental homologs (MiD2cm and MiD3cm). Small, unilateral injections of calcium green dextran into ventral spinal cord more selectively labeled this set on the contralateral side of the brain (Figure 1B). The identity of the cells was confirmed in three-dimensional reconstructions of confocal data sets obtained from the living animal; the characteristic lateral and ventral dendrites and decussating axons of these cells were easily distinguished in these reconstructions. The ability to label reproducibly this set by injections restricted to ventral, postanal spinal cord indicates that not only is their dendritic morphology similar, but the axons of the segmental homologs have similar trajectories in caudal spinal cord.

Calcium Responses in Mauthner Cells
Because nerve cells have voltage-gated calcium channels, we expected to observe fluorescence responses in calcium green dextran–labeled reticulospinal neurons when they fired action potentials during escapes. To confirm our ability to detect such increases, we initially studied the Mauthner cell because of the compelling evidence that it fires in conjunction with escapes (Zottoli, 1977; Eaton et al., 1982). Mauthner cells in unanesthetized fish (n = 86) showed robust fluorescence increases ranging from 12% to 110% in conjunction with escapes elicited by an abrupt, gentle tap to the ipsilateral side of the head (Figure 2A; escapes were monitored by visualizing the large, rapid movement of the fish). These fluorescence changes correspond to calcium increases...
Figure 2. Comparison of Calcium Green and Lucifer Yellow Dextran

(A) Response of a calcium green dextran labeled Mauthner cell during an escape elicited by a head tap. Panels show successive images (in pseudocolor) taken at 400 ms intervals. The color scale on the left represents fluorescence intensity (blue, lowest; red, highest) for both (A) and (B). The asterisk marks the frame in which the tap and escape occurred. A movement artifact from the escape is evident at the top of this frame. Since these images are acquired line by line at 2 ms/line and the movement artifact lasts only about 30 lines (60 ms), this response was the result of a brief movement, as occurs in escapes. The fluorescence of the cell increases quickly, but decays slowly over subsequent frames. The size of the increase was about 50%, as illustrated in the inset plot of fluorescence versus time. The starting fluorescence level was normalized to 100% in the plot.

(B) Fluorescence images of a Mauthner cell filled with the nonindicator, lucifer yellow dextran. An escape was elicited in the frame marked by the asterisk. The inset shows that the fluorescence did not change. This control experiment demonstrates that movement artifact cannot explain the fluorescence changes observed in cells labeled with calcium green dextran. The data point for the frame containing the escape was omitted from the plot because movement of the cell during the escape precludes measurement of the fluorescence of the cell. Scale bars represent 10 μm in (A) and (B).
ranging from 25 nM to 735 nM, assuming a resting calcium of 100 nM. In experiments in which both Mauthner cells were monitored, only the Mauthner cell ipsilateral to the head stimulus responded to stimuli at or just above threshold for escapes. Stimuli that were well above threshold sometimes produced responses in both Mauthner cells, possibly as a consequence of the generation of multiple escapes.

In these experiments, we purposely did not paralyze the fish so that we could visually monitor its behavior. One consequence of this was a movement artifact during the escape (e.g., frame marked by asterisk in Figure 2A). Although the agar returned the fish very close to its initial position, there remained the concern that the movement might induce some artifactual change in fluorescence by changing, for example, the path length of the emitted light. Since calcium responses have not previously been imaged in single neurons in the brain of an intact vertebrate, we were interested in both ruling out movement related artifacts and establishing the minimal fluorescence signal that could be reliably ascribed to calcium dynamics. To this end, we backfilled Mauthner cells with a calcium insensitive dye, lucifer yellow dextran, and monitored the fluorescence of these cells during escapes under conditions identical to those used in the calcium imaging experiments. In these fish (n = 5), head taps again produced robust escape behaviors, but only very minimal, if any, fluorescence changes, as illustrated in Figure 2B. Since lucifer yellow dextran does not respond to calcium increases, these changes provide a measure of the magnitude of movement related artifacts and indicate that after the escape attempt, the fish returns quite closely to its original position, presumably due to constraint by the agar. When fluorescence increases did occur in lucifer experiments, they were typically less than 1% or 2%, and the largest ever observed was 4%. These small increases might be due to the focal plane not being precisely set at the maximally bright focal plane, or perhaps to some slight compression of the fish during the escape. All of the increases seen with lucifer yellow dextran were considerably smaller than the calcium responses in zebrafish neurons labeled with calcium green dextran in which the fluorescence increases to sensory stimulation were usually much greater than 10%, well above the level that could be explained by shifts in the position of the fish.

Comparison of Antidromic and Sensory Responses
The fluorescence increases observed in calcium indicator-labeled Mauthner cells during escapes support a link between an action potential in the Mauthner cell and a rise in intracellular calcium. However, we wanted to confirm that we could detect the fluorescence increase associated with a single spike and to explore the relationship between spikes and the calcium rise. The ideal approach would be to record intracellularly from the cell and elicit one spike or a series of spikes. Unfortunately, all of the neurons in the larvae are small, with the 12–14 μM diameter Mauthner cell being among the largest. While this small size is favorable for imaging (the larger surface to volume ratio results in larger calcium signals), it makes intracellular recording more difficult. Instead, we placed an extracellular metal stimulating electrode next to the Mauthner cell axon in caudal spinal cord and stimulated it antidromically using an approach that is standard in Mauthner cell studies. The Mauthner axon is easily seen in bright field due to its large diameter, allowing the close apposition of the electrode to the axon. Antidromic stimulation of the cell (in an anesthetized fish) produced small fluorescence increases (12%–19%) in the Mauthner cell soma in response to a single stimulus, and larger increases in response to a rapid train of 2, 5, or 10 stimuli, as shown in Figure 3A. Figure 3B shows that the responses were similar when the same set of stimuli was repeated. The size of the calcium response to a single antidromic spike, as well as the accumulation and extrusion of calcium after trains of action potentials, were similar to that observed previously in zebrafish motoneurons (Fetcho and O’Malley, 1985) and in cultured bullfrog sympathetic neurons (O’Malley, 1994).
The small increase due to a single antidromic spike was comparable to the smallest increases observed in the Mauthner cell during escapes elicited by a tap. The small tap-evoked increases occurred in early trials in a series of escapes elicited at 2 min intervals. However, the Mauthner cell responses were often larger, ranging from 30% to 70% in most experiments. This was somewhat surprising because the evidence, including data from larval and adult fish, indicates that the Mauthner cell in teleost fishes fires only a single action potential each time it triggers an escape behavior (Zottoli, 1977; Featherstone et al., 1991). However, our data do not allow us to rule out the possibility that the larger calcium responses are a consequence of multiple action potentials.

Although the increase in the size of the responses in the Mauthner cell with repetitive escapes is an interesting phenomenon that may reflect plasticity in the size of the calcium change evoked by a single spike or an increase in the number of spikes fired (O’Malley and Fetcho, 1996, Soc. Neurosci., abstract), we were mainly concerned with whether or not the cell was responding during different sensory stimuli. Thus, the large size of the responses typically seen was helpful in determining whether or not the cell had been activated. There was no evidence that these larger calcium responses were a consequence of damage because they recovered rapidly and completely and could be observed reliably in conjunction with escapes for up to 132 trials spread over as long as 2 days. Thus, the cells remained healthy and could be monitored physiologically for an extended time, as long as the illumination was minimized.

**Calcium Responses at Threshold**

The experiments with antidromic stimulation indicate that the smallest fluorescence increases in the Mauthner cell during escapes could be explained by the calcium influx associated with a single action potential. They do not, however, rule out the possibility that some of the fluorescence responses are a result of subthreshold postsynaptic potentials. To determine whether we could detect fluorescence increases during large, subthreshold input to the Mauthner cell, we varied the voltage applied to the piezoelectric crystal driving the head tap and examined the fluorescence changes in the Mauthner cell at stimulus strengths near threshold for eliciting an escape (i.e., a strength where the Mauthner fires only 50% of the time). Figure 4 shows the responses of a Mauthner cell to a series of stimuli of different strength, including several near the threshold for escapes. The calcium response only occurred when an escape was produced. In cases where the fish showed no evidence of producing the escape, no calcium response resulted. This included trials in which the stimulus strength was the same for escape and nonescape trials. Thus, in the absence of escapes, there is little if any fluorescence change in the soma, even when one would expect there to be a large postsynaptic potential in the cell. This strengthens the link of the somatic calcium responses to both the behavioral response and to the firing of action potentials by the Mauthner cell. It also supports other evidence that the calcium increases in the soma seen with this approach are associated with the firing of action potentials rather than subthreshold postsynaptic potentials (O’Donovan et al., 1993; McClellan et al., 1994; Fetcho and O’Malley, 1995).

**Activation Pattern of Segmental Homologs**

After using the well-studied Mauthner cell to establish the reliability of the technique as an indicator of neuronal firing, we went on to study the responses of MiD2cm and MiD3cm, for which there were no prior physiological data.
Figure 5. Differential Activation of the Mauthner Cell and Its Homologs during Escapes

Each row shows a trial consisting of a sequence of images during which an escape was elicited by an ipsilateral touch to the head or tail. The color scale (applies to all figures) represents fluorescence intensity (blue, lowest; red, highest).

(A) Response of MID3cm during an escape elicited by a head tap. MID3cm is shown at relatively high magnification to illustrate clearly the fluorescence increase. The tap was applied during the third frame. The escape event transiently causes the cell to move out of the frame, which provides a convenient record of the behavioral event. This movement frame is omitted from subsequent rows.

(B) Simultaneous imaging of both the Mauthner cell (left) and MID3cm (far right) during a head stimulus. Both cells respond.

(C) Same field as in (B), but with a tail stimulus. The Mauthner cell responds but not MID3cm.

(D) Simultaneous imaging of the Mauthner cell (left) and MID2cm (right) during a head stimulus. Both cells respond.

(E) Same field as in (D), but with a tail stimulus. Only the Mauthner cell responds. Arrows mark the first frame after the escape in (B)–(E).

Images were acquired at 400 ms intervals. Scale bars: 10 μm in (A), 15 μm in (B)–(E). The size of the fluorescence increases in each trial were as follows: 41% (A); 62% in Mauthner cell, 21% in MID3cm (B); 53% in Mauthner cell, 2% in MID3cm (not significant, see Figure 2) (C); 21% in Mauthner cell, 22% in MID2cm (D); 67% in Mauthner cell, none in MID2cm (E).

data. We compared the responses of the Mauthner cell and its homologs during escapes elicited by one of the two tappers positioned to stimulate either the head or the tail. The Mauthner cell and its serial homologs (MID3cm and MID3cm) all responded to a touch on the ipsilateral side of the head at a strength above threshold for eliciting an escape (Figures 5A, 5B, and 5D). These responses consisted of fluorescence increases ranging from 9% to 41% in the homologs and 21% to 110% in the Mauthner cells. The fluorescence changes far outlasted the movement artifact; they returned nearly to baseline in 4–6 s, which is typical for calcium signals in neuronal somata (Regehr et al., 1989; Lev-Ram et al., 1992; Yuste et al., 1994). In marked contrast with the response of this group during head taps, only the Mauthner cell responded to ipsilateral tail stimuli even when the stimuli were well above the threshold for escapes (Figures 5C and 5E).

If the homologs are indeed playing a role in determining the magnitude of the escape response, then their
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Figure 6. Quantification of Escape Responses of the Mauthner Cell and Its Homologues over Successive Trials

Each row shows a series of successive trials in which we alternately elicited escapes by either head or tail stimuli. Each point represents the mean pixel intensity per frame in a rectangular box placed over the cell (400 ms between frames). The escape was elicited during the sixth frame in each trial. In some trials, the cell moved out of the field during the frame in which the escape occurred. Because the fluorescence of the cell could not be measured when it was not in the frame, there is no data point for those movement frames, leading to a discontinuity in the plot. A 2 min rest was allowed between each trial. Each row is from a different fish. The mean pixel intensity was normalized so that the starting fluorescence baseline is 100 in each trial. This normalization corrects for any slow changes in the baseline brightness of the cells (due to bleaching for example) and allows an easy comparison of successive trials. (Top) The Mauthner cell responds repeatedly during escapes elicited by either head or tail taps. MiD2cm (middle) and MiD3cm (bottom) cells both respond during escapes elicited by head taps, but not during escapes produced by tail taps.

trial-to-trial behavior should consistently show a differential response to head and tail stimulation. Addressing this issue requires not only that the in vivo recordings be relatively stable, but that successive trials be directly observed without signal averaging. High efficiency optics and the large fluorescence signals of calcium indicators were important for this because they allow the use of minimal excitation light, providing stable recording conditions. This allowed the recording of neuronal responses over a series of successive trials in which head and tail stimuli were alternately applied to elicit escapes (Figure 6). Throughout these trials, head stimulation activated all of the cells (Mauthner cell, MiD2cm, and MiD3cm), whereas tail stimulation activated only the Mauthner cell; such differences were observed in 17 fish, with no exceptions to the differential response of MiD2cm and MiD3cm to head and tail stimuli that elicited escapes.

Timing and Threshold of Homolog Responses

These results suggest that the homologs of the Mauthner cell are involved in the production of the more vigorous escapes produced by stimulation of the head. Because the escape behavior is a very fast event (the initial C-bend is completed 20–25 ms after the stimulus onset; Eaton and Farley, 1975), the segmental homologs must be activated close in time to the Mauthner cell if they are to contribute to the initial escape bend. To examine the time course of activation, the laser scanning microscope was used in a one-dimensional imaging mode, i.e., a single line across these cells was repetitively scanned. This provided a much faster acquisition rate (2 ms per line) than the 400 ms needed for two-dimensional images. These experiments showed that MiD3cm and the Mauthner cell are activated within 30 ms of one another (Figure 7A). While the movement artifact prevented a more exact determination of the response latency, both cells were synchronously activated over a series of trials, within the limits of the detection system (Figure 7B).

If MiD2cm and MiD3cm contribute to the escape along with the Mauthner cell, then their threshold should be similar to that of the Mauthner cell, which is known to fire an action potential only in conjunction with escapes (Zottoli, 1977). The relative thresholds for activating these cells were determined by varying the strength of the stimulus applied to the head or tail. When the head stimulus was near or at threshold (50% probability of response) for an escape, the Mauthner cell and its homologs responded together whenever an escape occurred (e.g., Figure 7). The Mauthner cell showed a very clear all or none response with large fluorescence increases during escapes and none in response to threshold stimuli that did not lead to an escape. In some animals, as in Figure 7, the homologs of the Mauthner cell also had a clear threshold, responding only in conjunction with escapes. In other fish, the homologs showed a weak response to head stimuli that did not elicit an escape, but even in these cases, they produced a larger increase in fluorescence in conjunction with escape events. MiD2cm and MiD3cm did not respond to tail stimuli at any strength, even when those stimuli were three times threshold for eliciting an escape.

Discussion

A role for the Mauthner cell homologs in escapes would help to explain both the normal adaptive variability of escapes as well as the production of escapes in fish without Mauthner cells, thus unifying a series of observations made over the past 15 years. Until now, it had not been possible to obtain functional data from the identified Mauthner cell homologs (Metcalfe et al., 1986; Foreman and Eaton, 1993). This is a consequence of the smaller size of MiD2cm and MiD3cm, which makes it difficult both to find them and to record their electrical
Figure 7. Responses of the Mauthner Cell and MiD3cm near the Threshold for Escapes

(A) The fluorescence of a line (black line in top panel) that passed through both the Mauthner cell (left) and MiD3cm (right) was monitored repeatedly as shown in the bottom panel. Consecutive lines were acquired at 2 ms intervals (vertical scale bar, 200 ms) and are displayed from top to bottom. An escape elicited by a tap to the ipsilateral side of the head is indicated by the arrows. The trace was briefly disrupted by the escape movement, but following the movement, the fluorescence of the vertical bands representing the two cells had increased, indicating that both cells had responded rapidly, within 30 ms of one another. (B) Quantification of responses from the cells in (A) over several trials in which the head was tapped at a strength near threshold for escape (escapes occurred in 67% of the trials). The points show the average intensity of successive sets of 15 lines (i.e. 30 ms per data point) plotted over time. Successive trials, 2 min apart are separated by dashed lines. The starting baseline in each trial was normalized to 100 as in Figure 3. Repeated stimuli at a constant stimulus strength near threshold led to escapes in trials 2, 4, 5 and 6, but not in 1 and 3. When an escape occurred, both the Mauthner cell and MiD3cm responded, but neither responded when there was no escape.

Horizontal scale bar, 20 μm. Size of increases in (A): 70% in Mauthner cell; 34% in MiD3cm.

activity. Our data indicate that the Mauthner cell and its serial homologs (MiD2cm and MiD3cm) in the hindbrain do indeed function together during escapes. They respond together during the large escape turns produced by lateral stimulation on the head, but in response to stimulation of the tail, only the Mauthner cell is activated. This is exactly the pattern of activation predicted by behavioral studies. It suggests a population code, with the extent of the bend related to the extent of activation of a population of serially homologous hindbrain cells (Eaton et al., 1991). We examined only the extremes of the escape behavior by using stimuli that elicit the weakest and strongest escapes. Intermediate forms of the escape might be produced not only by variations in which cells are activated, but also by changes in the degree of activation of individual cells.

The large size of the somatic calcium signals seen during sensory activation of MiD2cm and MiD3cm suggests that these cells are firing one or more action potentials. These responses are unlikely to result from subthreshold excitation. In the case of the Mauthner cell, there is usually no detectable calcium increase from stimuli that are just below threshold (Figures 4 and 7), even though such stimuli lead to a large synaptic input (Faber et al., 1991). This is not unexpected, as action potentials should produce considerably larger calcium signals than subthreshold inputs because action potentials will open high threshold calcium channels and NMDA-gated channels (if the NMDA receptors and glutamate are present). The somatic calcium signals observed in MiD2cm and MiD3cm after sensory stimulation (Figures 6 and 7) are typically larger than those produced either by single antidromic spikes in the Mauthner cell (Figure 3) or after direct firing of single action potentials in cultured bullfrog neurons (O’Malley, 1994). In addition, we are aware of no imaging studies reporting detectable somatic calcium signals due to subthreshold EPSPs. All of these observations support the conclusion that MiD2cm and MiD3cm are firing in response to head stimuli that elicit escapes.

The differential activation we observed among the Mauthner cell, MiD2cm, and MiD3cm most likely arises from differences in the sensory input to this set of hindbrain neurons. Based upon previous work, the most likely possibility is that rostral stimulation leads to activation of the auditory system, whereas caudal stimulation activates cutaneous and/or lateral line inputs (Eaton et al., 1984). All of these are known to excite the Mauthner cell in goldfish, consistent with its responses to both head and tail stimulation (Faber et al., 1989). Previously, there was no information about the inputs to MiD2cm and MiD3cm. Our work shows that they respond only to head stimulation, suggesting that they receive little or no input from the cutaneous or lateral line system. The similar thresholds and conjoint activation of MiD2cm and MiD3cm with the Mauthner cell in response to head stimuli are consistent with their sharing common rostral sensory inputs, most likely from the auditory system. Such differences in sensory input to escape neurons is not unprecedented, as they are well documented in crayfish. The crayfish system has obvious parallels with zebrafish in that rostral and caudal stimuli also elicit different forms of escape by differential activation of giant neurons (Wine and Krasne, 1972).

Our observations that MiD2cm and MiD3cm are coactivated with the Mauthner cell during escapes and
that they share a similar axonal trajectory are consistent with their playing a role in the generation of escapes. Although this evidence confirms previous predictions, it is still correlative, and nothing is known about the output connections of MiD2cm and MiD3cm. A more causal relationship could be established by lesioning the cells in the live fish and observing the effects on performance. Our data lead to the prediction that lesioning MiD2cm and MiD3cm should reduce the performance of escapes elicited by head taps, but not those from tail taps. The expectation is that such lesions should make the C-bend to a head stimulus more like that to a tail stimulus. We have preliminary data that neurons in intact zebrafish can be deleted by using optical methods, so it should eventually be possible to evaluate this prediction.

The escape system is particularly attractive for studies of how activity in neuronal populations determines vertebrate behaviors because the behavior is produced by a population of neurons, but the number of neurons and synapses in the circuit is manageably small (Faber et al., 1989). The ability to image the activity of any of the array of hindbrain neurons and to delete them and monitor behavioral changes offers the prospect of a more complete understanding of how a population of neurons in a vertebrate determines the form of a behavior. These approaches should prove useful for future studies of neural circuits not only in the normal animal, but also in the many behavioral mutants that have been generated by large scale mutagenesis of zebrafish (Mullins and Nusslein-Volhard, 1993; Driever et al., 1995).

Our work has several implications concerning the functional organization of hindbrain segments. Hindbrain segments are thought to have arisen from the duplication of an ancestral segment, with subsequent evolutionary divergence of the segments (Metcalf et al., 1986). This would lead to the similar structural organization in successive segments observed in vertebrates. Our observations indicate that another consequence of this duplication is the production of a series of functionally related neurons in successive segments. The serially homologous neurons studied retain a similar functional role in escapes, but they are not complete functional clones of one another. Instead, there is variability in the sensory inputs that drive the cells and this is associated with variability in the resulting escape behavior. The duplication of segments may have permitted an expansion of the behavioral repertoire of the animal by producing segmental groups of neurons involved in particular behaviors. A subsequent evolutionary divergence of the inputs and outputs of these cells would allow for behavioral diversification, much as gene duplication and divergence has led to diversification at the molecular level. This organization of the hindbrain into serial sets of functionally related neurons is likely to be a very general one because the hindbrain has changed relatively little during the evolution of vertebrates, as illustrated by the very similar organization of escape circuits in fish and startle circuits in mammals (Crue and Nieuwen, 1984; Lingenhohl and Friauf, 1994; Butler and Hodos, 1996).

Experimental Procedures

A 50% solution of calcium green dextran (10,000 MW) in 10% Hank's solution was pressure injected via a glass microelectrode into the caudal (postanal) spinal cord of posthatching larval zebrafish (Danio rerio, usually within the first 2 weeks after hatching) that were anesthetized with 0.02% 3-aminobenzoic acid ethyl ester (Fetcho and O'Malley, 1995). In most cases, this injection was into ventral cord to selectively label the Mauthner cell and its homologs without disrupting more dorsal sensory pathways. After injection, the fish were allowed to recover and were maintained in 10% Hank's solution. We only studied fish that exhibited no obvious disruptions of swimming or escape behaviors following the injection. Twelve or more hours later, the fish were briefly anesthetized, embedded in soft agar on a petri dish (Eaton et al., 1984), and then rinsed with 10% Hank's solution to allow recovery from the anesthetic. Confocal images were obtained by looking into the head of the intact fish using a Zeiss IM35 inverted microscope with a 50 × Leitz 1.0 NA objective and a Biorad MRC 600 laser-scanning confocal imaging system (Hernandez-Cruz et al., 1990; O'Malley, 1994; Fetcho and O'Malley, 1995). The transparency of the fish allowed us to not only see neurons inside the living animal but also allowed easy monitoring of the viability of the fish by observing the heart beat and blood flow. To confirm the identity of the cells studied physiologically, stacks of images showed the morphology of successive confocal sections were acquired. Signal averaging, usually seven frames, was used when acquiring this morphological data. Maximum projections were made from stacks of these sections. The image stacks were also reconstructed in three-dimensions using the VolVis program (Sobierajski et al., 1995), allowing us to examine the details of the dendritic morphology and axonal projections of each cell.

Escapes were elicited by an abrupt touch produced by the displacement of a small glass probe attached to a piezoelectric crystal. The voltage applied to the crystal could be varied to change the excursion of the glass probe. Two probes were used: one to stimulate the head and the other the tail. All stimuli were ipsilateral to the reticulospinal neurons, in the region of the otolith for the head stimulus and postanally for the tail stimulus. The tail stimulus was located rostral to the calcium green injection site so as to activate sensory neurons whose pathways to the brain were presumably not damaged by the injection. The responses of the Mauthner cell to tail stimuli indicated that ascending sensory pathways were indeed intact. The stimuli produced an escape movement during which the cell(s) moved briefly out of the plane of section, but then returned rapidly because the agar controlled the resting position of the fish. The escape attempt could also be observed visually (with protective goggles to block the laser light) through a dissecting scope mounted above the inverted microscope. Free swimming larval zebrafish generate a variety of movements subsequent to the escape (Eaton and Farley, 1975). Although the initial escape event is very obvious in the agar, we could not distinguish subtle differences in movement following escapes. However, the responses we observed were clearly linked to the initial escape movement and not subsequent behavior, because they occurred whether or not the fish continued to move after the initial escape event.

The fluorescence intensity of neurons during escapes was monitored either by collecting a sequence of images of a cell or group of cells (usually at 400 ms intervals) or by repeatedly scanning a single line through the cells at 2 ms intervals. To assure that an increase in the brightness of the cell was not the result of movement to a brighter plane, we collected a series of optical sections spanning the cell(s) (this was done for each block of trials). We then focused at the brightest focal plane immediately prior to each trial. In experiments with multiple cells in the field, we picked a focal plane where the cells of interest were at or near their maximal fluorescence. AThis organization of the hindbrain into serial sets of neurons is particularly attractive for studies of neural circuits not only in the normal animal, but also in the many behavioral mutants that have been generated by large scale mutagenesis of zebrafish (Mullins and Nusslein-Volhard, 1993; Driever et al., 1995).

Our work has several implications concerning the functional organization of hindbrain segments. Hindbrain segments are thought to have arisen from the duplication of an ancestral segment, with subsequent evolutionary divergence of the segments (Metcalf et al., 1986). This would lead to the similar structural organization in successive segments observed in vertebrates. Our observations indicate that another consequence of this duplication is the production of a series of functionally related neurons in successive segments. The serially homologous neurons studied retain a similar functional role in escapes, but they are not complete functional clones of one another. Instead, there is variability in the sensory inputs that drive the cells and this is associated with variability in the resulting escape behavior. The duplication of segments may have permitted an expansion of the behavioral repertoire of the animal by producing segmental groups of neurons involved in particular behaviors. A subsequent evolutionary divergence of the inputs and outputs of these cells would allow for behavioral diversification, much as gene duplication and divergence has led to diversification at the molecular level. This organization of the hindbrain into serial sets of functionally related neurons is likely to be a very general one because the hindbrain has changed relatively little during the evolution of vertebrates, as illustrated by the very similar organization of escape circuits in fish and startle circuits in mammals (Crue and Nieuwen, 1984; Lingenhohl and Friauf, 1994; Butler and Hodos, 1996).

Experimental Procedures

A 50% solution of calcium green dextran (10,000 MW) in 10% Hank's solution was pressure injected via a glass microelectrode into the
of free calcium by knowing the resting calcium level, the $K_D$ of the indicator, and the dynamic range. The latter two numbers were unknown for calcium green dextran in living neurons. We determined them by using cultured bullfrog sympathetic neurons whose accessibility and large size relative to zebrafish neurons allowed the stable whole cell patch recordings needed for such determinations. The cultured cells were patch clamped and filled with free calcium set at fixed levels, using 10 mM BAPTA. The cells were then flooded with calcium via repetitive voltage pulses under voltage clamp and the maximal fluorescence increase that could be obtained at the differing resting calcium levels determined. This allowed the intracellular $K_D$ of calcium green dextran to be determined. The average value obtained, 250 nM, was quite close to the in vitro value (252 nM; Molecular Probes, Eugene, Oregon). The maximum dynamic range obtainable (upon raising calcium from subnanomolar levels to indicator-saturating levels) was only 8.7-fold, considerably less than the range obtainable in intracellular solution in cuvettes (15-fold). Based on these numbers, and by assuming a resting calcium level (e.g., 100 nM), the fluorescence responses may be equated to changes in free calcium. Since the actual resting calcium levels are not known, the calcium responses in the paper are expressed in terms of relative fluorescence increases. An estimate of the sizes of the increases based upon the calibration data is provided in the Results.

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References


