Visual Prey Capture in Larval Zebrafish Is Controlled by Identified Reticulospinal Neurons Downstream of the Tectum

Ethan Gahtan,1,2,3 Paul Tanger,1 and Herwig Baier3
1Department of Psychology, University of Massachusetts, Amherst, Amherst, Massachusetts 01003, 2Department of Psychology, Humboldt State University, Arcata, California 95521, and 3Department of Physiology, Program in Neuroscience, University of California, San Francisco, San Francisco, California 94158-2722

Many vertebrates are efficient hunters and recognize their prey by innate neural mechanisms. During prey capture, the internal representation of the prey’s location must be constantly updated and made available to premotor neurons that convey the information to spinal motor circuits. We studied the neural substrate of this specialized visuomotor system using high-speed video recordings of larval zebrafish and laser ablations of candidate brain structures. Seven-day-old zebrafish oriented toward, chased, and consumed paramecia with high accuracy. Lesions of the retinotectal neuropil primarily abolished orienting movements toward the prey. Wild-type fish tested in darkness, as well as blind mutants, were impaired similarly to tectum-ablated animals, suggesting that prey capture is mainly visually mediated. To trace the pathway further, we examined the role of two pairs of identified reticulospinal neurons, MeLc and MeLr, located in the nucleus of the medial longitudinal fasciculus of the tegmentum. These two neurons extend dendrites into the ipsilateral tectum and project axons into the spinal cord. Ablating MeLc and MeLr bilaterally impaired prey capture but spared several other behaviors. Ablating different sets of reticulospinal neurons did not impair prey capture, suggesting a selective function of MeLr and MeLc in this behavior. Ablating MeLc and MeLr neurons unilaterally in conjunction with the contralateral tectum also mostly abolished prey capture, but ablating them together with the ipsilateral tectum had a much smaller effect. These results suggest that MeLc and MeLr function in series with the tectum, as part of a circuit that coordinates prey capture movements.

Key words: prey capture; tectum; reticulospinal; laser ablation; zebrafish; visuomotor

Introduction

Visuomotor behaviors depend on multisynaptic pathways that link the photoreceptors in the retina to muscle activity in the periphery. To elicit a particular behavior, stimulus features have to be recombined and channeled into separate neural pathways. For example, recognition and approach of prey are elicited by a distinct combination of stimulus cues, which have to be extracted rapidly and faithfully from a complex visual scene. The neuronal activity pattern signifying prey is conveyed from visual circuits to premotor circuits, which in turn send commands to motor neurons in the spinal cord. The ensemble of premotor neurons encoding identity and location of an external object and triggering an innate response to that object has been referred to in the ethological literature as a “command releasing system” (Ewert, 1987). Such a system may be exclusively dedicated to a particular behavior, although it is debated whether this is a general feature of CNS organization (Metzner and Juranek, 1997; Edwards et al., 1999; Kupfermann and Weiss, 2001; Sowards and Sowards, 2002).

The larval zebrafish brain is well suited for an investigation of visuomotor coding (Gahtan and Baier, 2004). Zebrafish display several stereotyped responses to visual stimuli, and the neural substrate underlying these behaviors is composed of a small number of neuronal elements. Retinal ganglion cells (RGCs) project to 10 distinct areas in the brain (Burrill and Easter, 1994), consistent with functional segregation of behavioral pathways. Laser ablation of the optic tectum, the largest retinorecipient structure, leaves optomotor responses (OMRs) and optokinetic responses (OKRs) unaffected, two visuomotor reflexes that compensate for self-motion (Reoer and Baier, 2003). This result suggests that the individual visual pathways in these larval fish are already dedicated to processing behavior-specific information.

In zebrafish, motor commands generated in the brain are conveyed to the spinal cord through a small number of reticulospinal (RS) neurons. There are ~150 RS neurons, the cell bodies of which are arranged in a bilaterally symmetric array in midbrain and hindbrain. Few attempts have been made to link identifiable premotor systems to certain behaviors. Best known are the Mauthner neuron (or M cell) and its segmental homologs MiD2 and MiD3, which play a critical role in fast-start escapes (Zottoli et al., 1987; Faber et al., 1989; Eaton et al., 1991; Svoboda and Fetcho,
Animals. Zebrafish of the Tübingen Long and Long-Finned Gold strains were kept and bred according to standard procedures. Larvae were raised in 9 cm Petri dishes at a density of <40 per dish. A Bm3cmGFP transgenic line was used for laser ablations and behavioral experiments. In this line, membrane-bound green fluorescent protein (GFP) is expressed in approximately one-half of all RGCs, including those projecting to the stratum fibrosum et griseum superficiale of the tectum, and in mechano-sensitive hair cells in the skin and the inner ear (Xiao et al., 2005). Furthermore, a Shc:GFP transgenic line (Neumann and Nüsslein-Volhard, 2000) was used to visualize RGC axons simultaneously with backfilled RS neurons. Shh:GFP labels all RGC types, in addition to subpopulations of neurons in the ventral thalamus (Roeser and Baier, 2003).

Depending on the experiment, GFP was imaged either with a confocal laser-scanning microscope (MRC-1024; Bio-Rad, Hercules, CA), a compound fluorescence microscope (Axioskop II; Zeiss, Oberkochen, Germany), or an epifluorescence-equipped dissecting microscope (MZ FLIII; Leica, Nussloch, Germany).

Retrograde labeling of RS neurons. Zebrafish larvae, aged 4–5 dpf, were anesthetized by 0.03% 3-amino-benzoic acid ethyl ester methanesulfonate (MESAB; Sigma, St. Louis, MO) to anesthetize them before tracer injections. Anesthetized larvae were placed into an agar-coated Petri dish, and a glass injection pipette containing the tracer solution was inserted, under a dissecting light microscope, through the lateral muscle and into the ventral spinal cord. The injection was made at the level of the 20th myotome, approximately three-quarters down the length of the spinal cord. Care was taken to avoid penetrating intersegmental blood vessels. For ablations, a 0.75% solution (w/v) of Alexa Fluor dextran [molecular weight (MW), 10,000] in distilled H2O was injected, and, for confocal anatomy studies, a 0.5% solution of Texas Red dextran (MW, 10,000; Invitrogen) was used. Tracers were pressure ejected from the pipette tip using a PicoPump (World Precision Instruments, Sarasota, FL). These tracers enter RS neurons through severed axonal processes at the spinal injection site (Gahtan and O’Malley, 2003). At least 12 h were allowed for retrograde filling of hindbrain cell bodies and dendrites. Multiple descending neurons, on both sides of the hindbrain and midbrain, were labeled in most injected larvae. The present study focused on two pairs of individually identifiable descending neurons in the nucleus of the medial longitudinal fasciculus (nMLF), because their anatomy suggested that they may receive tectal input. As a control for bilateral ablation of the MeLr and MeLc neurons, an equal number of RS neurons from outside of the nMLF were ablated. These control ablations targeted any four neurons among a subset of 14 individually identifiable neurons within rhombomeres 4–6, including the Mauthner neuron and its two segmental homologs. Because dye injections did not always label these cells of interest, injected fish were first screened for suitable labeling patterns.

Laser ablations. GFP-expressing larvae were sorted at 2 or 3 dpf. At 6 or 7 dpf, larvae were first anesthetized in a 0.63% MESAB solution and then embedded in 1.2% agar, dorsal side down, against glass coverslip. The coverslip was then inverted onto a depression microscope slide so that the brain could be visualized through the dorsal surface of the head. Two methods of laser ablation were used, which gave similar results. Tectum ablations, and RS ablations done in conjunction with tectum ablation, were performed using a MicroPoint (Photon Instruments, Arlington Heights, IL) laser system (equipped with a VSL-337ND-S nitrogen laser; Laser Science, Franklin, MA) on a Zeiss Axioskop II compound microscope. The primary UV laser pumped a 488 nm coumarin laser, which was aimed via a 20× (for tectum ablations) or 40× (for RS ablations) microscope objective (Plan-Neofluar 20×, numerical aperture, 0.5; 40×, numerical aperture, 0.8; Zeiss). These ablations were performed under simultaneous visual control through GFP optics. Laser power was attenuated by at least 50% with neutral gray filters to minimize incidental tissue damage. The laser was used at a pulse rate of 2–10 Hz. During the ablation procedure, the fish were positioned to achieve the clearest view of the targeted neurons or axons. Under these conditions, ablation of one tectal lobe took between 3 and 10 min, and ablation of nMLF neurons took ~1 min (see Results). Tectum and nMLF ablations were done either bilaterally or unilaterally, as well as alone or in combination. In experiments comparing the effects of nMLF ablation and ablation of non-nMLF RS neurons, ablations were performed using a confocal laser microscope (Leica TCS SL, with a >30 mW laser intensity at the focal plane, using the 488 nm argon laser line; the confocal system was used in conjunction with a Leica DM LFS compound microscope equipped with a 40× water-immersion objective lens). In these ablations, a 488 nm laser was used in scanning mode and set at low intensity to identify and target individual RS neurons. The beam was then parked in a single position at the center of the targeted neuron and increased to maximum intensity. Under these conditions, it took ~90 s to ablate a single RS neuron.

Prey capture assay. Live paramecia cultures and protozoa food pellets were obtained from Carolina Biological Supply (Burlington, NC). Cultures were grown in 500 ml plastic flasks, at 28.5°C, to a density of ~100 paramecia per milliliter, and new cultures were started every 2–4 weeks.

The prey capture assay was run immediately after measurement of spontaneous activity (see below), and the groups of ablated and control larvae remained in the same 60 mm dishes for both procedures. Between 0.5 and 2.0 ml of paramecia culture was added to the larvae’s dishes to achieve a concentration of ~50 paramecia per fish. A similar volume was added to dishes with embryo medium, but no fish, to determine the effect of embryo medium on paramecia viability over the assay period. Each dish was placed on a glass plate and illuminated from below with a fiber optic cable, and video images of the dish were recorded with a high-speed digital camera positioned above the dish. Either a Motionscope PCI from Redlake Imaging (San Diego, CA) or a P-L A741 from PixeLINK (Ottawa, Ontario, Canada) was used. Two hundred video frames, captured at 60 Hz, were recorded for each dish at each time point, and dishes were recorded successively, maintaining their serial order throughout the experiment.

An off-line imaging-based counting method was used to determine the number of paramecia in each dish immediately after they were added (time 0) and again every hour for 5 h. Paramecia in healthy cultures remain in constant motion, allowing individual paramecia to be counted by tracking their path across the 200 video frames (encompassing 3.3 s). The video frames were first projected using the SD z-projection method in ImageJ, a java-based image-processing package developed and made freely available by the National Institutes of Health (for details on the projection method, see http://rsb.info.nih.gov/ij/). This procedure highlights changes in pixel values from one frame to the next and subtracts stable background pixels. This method allows the movement of paramecia and fish to be easily distinguished from each other and from background noise (see Fig. 1A). Paramecia were marked and counted manually from the projected image, which was saved, whereas the larger movie file was deleted.

Results were expressed as the average number of paramecia per fish (calibrated to be ~50) relative to the same number at the starting time point. A decline in the percentage of counted paramecia over time could be caused by predation by the larvae as well as "natural" paramecia death (natural being defined here as “not by predation”). We therefore always ran a control plate, containing paramecia but not fish, alongside each experiment. The number of paramecia counted in experimental wells was adjusted at each time point to correct for the measured natural
Prey capture performance in ablated and control groups was analyzed using a repeated-measures ANOVA. The number of paramecia was counted six times for each plate, at time 0, and once per hour for 5 h, and therefore sampling time was analyzed as a within-subjects repeated measure. There was a significant main effect of group ($F_{(3,240)} = 11.35; p < 0.01$), and a significant time by group interaction ($F_{(4,960)} = 4.20; p < 0.01$), indicating that the groups differed in the rate of decline in paramecia. One-tailed Bonferroni’s $t$ tests were used for subsequent pairwise comparisons of treatment groups at individual sampling points.

**Analysis of prey encounter behaviors.** To investigate how tectum and MeLr/MeLc ablation might disrupt prey capture performance, high-speed video recordings of individual prey encounter episodes were analyzed in separate groups of bilateral tectum ablated, bilateral MeLr/MeLc ablated, and injected control larvae (all were Brm3c:mGFP transgenics). Two larvae from each treatment condition were placed in 35 mm clear Petri dishes containing ~3 ml of embryo medium. The video recording method and apparatus were the same as that described for paramecia counting experiments, except that the camera was set to record for the period preceding a manual trigger. Between 15 and 30 paramecia were added to a dish, after which the dish was observed continuously for 1 h using a video preview monitor attached to the camera (the camera itself remained in record mode). Ten prey encounter episodes were recorded for later analysis. A prey encounter episode was defined as any time at which a paramecia moved within a semicircular area extending 5 mm around the head of the larva (with the straight edge of the semicircle running between the two eyes), followed by movement of the fish. These criteria were based on our previous observations of prey capture episodes and visual orienting responses in control larvae and probably represent a conservative estimation of the fish’s visual abilities. Analysis of prey encounters was according to Borla et al. (2002). The larvae’s head yaw (the angular displacement of the head during successive side-to-side bends of a swim bout) and the fish-to-paramecia distances were measured using automated image analysis procedures in ImageJ.

**Locomotor activity assay.** Because prey capture requires swimming, spontaneous activity was measured to determine whether laser ablations of the tectum or the nMLF disrupted locomotor performance. Spontaneous activity was tested in groups of between three and six individual larvae, aged 7–8 dpf. Each group was maintained in a 60 mm Petri dish containing ~6 ml of embryo medium. Each larva within a group received the same treatment: bilateral tectum ablation, bilateral nMLF ablation, or control larvae with intact eyes and sibling with fluorescent prelabeled of the targeted structure (tectum, nMLF, or both). Larvae in control groups were also imaged to ensure that they showed a fluorescent signal in the corresponding structure. In the case of tectum ablations, this meant that control larvae were all Brm3c:mGFP transgenics. In the case of nMLF ablations, control larvae had received spinal injection of fluorescent tracer and were confirmed to have labeled neurons in the nMLF. All control larvae were siblings of ablated larvae and received identical treatment with the exception of the ablation procedure.

Dishes of ablated and control larvae were placed on a glass plate positioned above a digital camcorder (TRV-9; Sony, Tokyo, Japan). Light-diffusing plastic above the dishes produced a uniform light background in recorded images. Images were captured directly to the computer at a rate of 0.5 Hz for 20 min, creating a 600 frame movie file. Images were recorded using Adobe Premiere software (Adobe Systems, San Jose, CA) and analyzed using ImageJ. For the analysis, pixel values from pairs of successive movie frames were subtracted such that only changes caused by fish movements or random noise remained. After this background subtraction, the software could be configured to locate and count all of the fish in a frame, based on their characteristic size and contrast (specified in pixel area and pixel intensity, respectively). For each pair of frames, only fish that moved were counted, because stationary pixels were subtracted out. Individual fish within a group could not be distinguished in this analysis; therefore, activity was measured for the group as a whole, specifically, results were expressed as the percentage of larvae within the group that moved from one frame to the next, averaged across the 20 min (600 frame) observation period.

**OKR assay.** The OKR was tested in larvae with unilateral nMLF lesions, using previously published procedures (Roese and Baier, 2003; Orger et al., 2004). Briefly, a computer-generated image of a rotating black and white grating was projected onto the inner walls of a white drum (height, 50 mm; inner diameter, 56 mm). Larvae were mounted in methyl cellulose in a 30 mm Petri dish, dorsal up and facing out toward the moving grating. Stimulus velocity for the sine wave grating was 20° per second. Stimuli were presented to one eye only by restricting the stimulus to 180° of the visual field, and each eye was tested on both temporal-to-nasal and nasal-to-temporal stimulus directions. Eye positions of individual fish were recorded on a computer at four frames per second. Eye movements of the stimulated eye only were plotted as angle (relative to the axis of the fish) over time, and the saccade rate was measured manually in representative parts of the OKR trace. Paired-samples $t$ tests were used to compare saccade rates between ablated and intact sides within fish. Fish tested in the OKR assay were not used in other behavioral tests.

**Results**

**Prey capture is a coordinated series of actions controlled by visual feedback.** We observed in high-speed video recordings that zebrafish larvae often detected a paramecium from a distance and then oriented toward it, bringing the prey object in front of their mouth. The fish then approached and, if necessary, pursued, and swallowed the paramecium (Fig. 1) (Budick and O’Malley, 2000). Paramecia of the variety used in this study swam steadily at a rate of ~3 mm/s. Zebrafish larvae occasionally chased a paramecium over distances of several body lengths, making a variable number of turns as needed, before they finally ingested it (Fig. 1 B; see supplemental movie of this particular episode, available at www.jneurosci.org as supplemental material). These observations indicate that the appetitive phases of this behavior are under continual control of sensory, presumably visual, feedback.

**A novel behavioral assay records prey capture performance by zebrafish larvae.** We devised a prey capture assay that allowed us to record the rate of predation by zebrafish larvae under different experimental conditions, as described in Materials and Methods. Briefly, we added ~50 paramecia per fish to dishes containing three to six fish larvae and counted their number every hour for 5 h (Figs. 1, 2). The decline in paramecia number, corrected for their natural disappearance when no fish was present, was directly related to the efficiency by which zebrafish consumed them. We found that paramecia were eaten at a steadily declining rate, after a negative exponential with a half time of ~2 h. The gradual drop in the rate of prey catching is probably better explained by the steady decrease in the density of paramecia. Satiation may also be a factor at later sampling points. The new assay provided a rapid, robust, and quantitative readout of prey capture by larval zebrafish.

**Prey capture primarily depends on an intact optic tectum and is not lateralized.** To begin to explore the neural substrates of prey capture behavior, we first tested whether the optic tectum was involved, as was reported for other vertebrates in various food-seeking behaviors. For this experiment, we laser ablated the retinotectal neuropil in transgenic zebrafish whose RGC axons were labeled with GFP (Fig. 2A). This method is able to remove retinotectal visual input, while leaving other retinofugal projections (e. g., to the pretectum, thalamus, and hypothalamus) intact (Roese and Baier, 2003) (the effectiveness of the ablations was demonstrated by...
postablution dye injections into the eye, which labeled pretectal retinofugal projections but not those in the tectum. GFP-transgenic sibling larvae served as controls. We found that fish with bilateral tectum lesions consumed substantially fewer paramecia than unablated animals (Fig. 2A, unablated controls, labeled GFP + Light). Post hoc comparisons showed that the bilateral ablation group had less overall decline in paramecia than the unablated control group (mean difference, 27.3; \( p < 0.01 \)), and this difference was significant at all sampling points except the start point.

When bilaterally tectum-ablated fish were present, paramecia numbers dropped at a rate of ~12% per hour, compared with 40% per hour in controls. The paramecia capture rate by unilaterally tectum-ablated fish was intermediate between the control and bilaterally ablated groups at each sampling point (~25% per hour) (Fig. 2B). This effect was symmetric with regard to the left or the right tectum (Fig. 2B, data shown for the 5 h sampling point only) (the overall mean difference in paramecia consumption between the left and right tectum ablation groups was 5.9%). The fact that ablation of either the left or the right tectum resulted in a similar impairment suggests that prey capture is not lateralized in larval zebrafish. The natural decline of paramecia in the absence of any fish was very rarely >5% per hour, which is substantially less than when bilateral tectum-ablated fish were present (12% per hour). This suggests that tectum-ablated animals were still able to consume prey. In summary, these lesion experiments demonstrated that paramecia capture depends, to a large extent, albeit not exclusively, on an intact optic tectum.

**Prey capture is primarily driven by vision**

From our high-speed video recordings, it appeared that zebrafish larvae use their visual sense to detect and to pursue paramecia. The results of tectum ablations are also consistent with a prominent role for vision; however, they do not exclude a major contribution by other sensory modalities, because the tectum is known to receive input from several other sensory systems. We therefore tested the role of vision in our prey capture assay using two independent methods. First, we measured paramecia consumption by normal zebrafish larvae held in constant darkness (Fig. 2B, the group labeled GFP+ Dark). Although the initial decline in paramecia was 40% per hour for the fish in the light, it was only ~10% for fish kept in darkness. The average for the dark group was significantly lower at each sample point (Fig. 2B). Interestingly, the bilaterally tectum-ablated group did not differ significantly from the dark-kept group (Fig. 2B), indicating that most if not all of the vision-driven prey capture depends on the tectum. Second, we tested prey capture performance in a completely blind mutant line, *lakritz*, which lacks RGCs but is otherwise normal and viable (Kay et al., 2001). *lakritz* mutants were significantly impaired relative to wild-type siblings tested in normal ambient light and were very similar to wild-type siblings kept in darkness (overall mean difference from sibling control, 33% \( p < 0.01 \) and 37% \( p < 0.01 \) for dark-kept siblings and mutants, respectively) (Fig. 2C). These results confirm that prey capture depends, to a large degree, on vision.

**MeLr and MeLc are selectively involved in visual prey capture**

We next asked whether we could delineate the prey-capture pathway further, beyond the tectum and into premotor areas of the brain. Tectal cells do not extend projections into the spinal cord, but they do form monosynaptic connections with RS cells that do so. Among them are the M cell, as well as neurons in the nMLF, located ventrally to the tectum. The M cell has been shown by others to be dispensable for prey capture (Borla et al., 2002). The nMLF, in contrast, had not been tested previously. We decided to focus on those neurons in the nMLF that are tectorecipient and that, at the same time, project axons to the spinal cord.

Neurons in the nMLF were backfilled from the spinal cord
taken at 2 GFP expression in RGCs terminating at the tectum in GFP-expressing larvae. The left panel is a projection of a confocal image stack showing nMLF, extended elaborate dendrites into the ipsilateral tectum form a bilaterally symmetric pair situated in the lateral part of the with Texas Red dextran (see Materials and Methods) and imaged. The right panels show an enlargement of the boxed section containing the tectum on both sides. These are single images acquired under epifluorescence, showing the same section before (top) and after (bottom) laser ablation of the left tectum. After 3 min of visually guided lasing, GFP expression is no longer visible in the left tectum but is unchanged in the right tectum. The effects of tectum ablation and ambient lighting on prey capture performance. Larvae tested in the dark (GFP + Dark) captured significantly fewer paramecia than untreated controls (GFP + Light). Bilateral tectum ablation (Bilat Tect Abl) impaired performance to a similar degree as performance between right- and left-tectum-ablated groups, shown for the 5 h sampling point only (R.Tec and L.Tec, respectively).

**Figure 2.** Prey capture requires the tectum and is vision dependent. A, Laser ablation of the tectum in GFP-expressing larvae. The left panel is a projection of a confocal image stack showing GFP expression in RGCs terminating at the tectum in Bsm3cmGFP transgenic larvae (20 images taken at 2 μm intervals). The optic chiasm and GFP-expressing hair cells are also visible. The right panels show an enlargement of the boxed section containing the tectum on both sides. These are single images acquired under epifluorescence, showing the same section before (top) and after (bottom) laser ablation of the left tectum. After 3 min of visually guided lasing, GFP expression is no longer visible in the left tectum but is unchanged in the right tectum. B, The effects of tectum ablation and ambient lighting on prey capture performance. Larvae tested in the dark (GFP + Dark) captured significantly fewer paramecia than untreated controls (GFP + Light). Bilateral tectum ablation (Bilat Tect Abl) impaired performance to a similar degree as testing in darkness, whereas unilateral tectum ablation (Unilat Tect Abl) had an effect intermediate between bilateral ablation and control conditions. There was no significant difference between right- and left-tectum-ablated groups, shown for the 5 h sampling point only (R.Tec and L.Tec, respectively). C, Prey capture performance of blind lakritz mutants (lak) and wild-type siblings tested in normal lighting or in darkness. The poor performance of blind mutants and larvae tested in darkness relative to controls supports the vision dependence of prey capture.

with Texas Red dextran (see Materials and Methods) and imaged. We found that two identified neurons, MeLr and MeLc, which form a bilaterally symmetric pair situated in the lateral part of the nMLF, extended elaborate dendrites into the ipsilateral tectum (Fig. 3). RGC axons terminate in four superficial layers of the zebrafish tectum (Xiao et al., 2005). The dendrites of MeLr and MeLc, in contrast, innervated a deeper layer and are concentrated in the medial and caudal boundary of the tectum. This position roughly corresponds to the expected position of neurons in the output layers of the tectum (Meek and Schellart, 1978; Nguyen et al., 1999; Xiao et al., 2005). Based on their morphology, MeLr and MeLc are thus prime candidates for involvement in tectum-dependent visuomotor behaviors.

MeLr and MeLc were laser-ablated under visual control after retrograde labeling from the spinal cord (Fig. 4A, B). Similar procedures have been shown to produce selective killing of targeted neurons (Liu and Fetcho, 1999; O’Malley et al., 2003). After bilateral lesion of MeLc and MeLr, prey capture was substantially decreased relative to injected but unablated controls (mean difference in paramecia decline was 19.16%; p = 0.05) (Fig. 4C). The effect of ablating an equal number of non-nMLF RS neurons was also tested to control for laser exposure and to determine whether RS control of prey capture was mediated by a large, distributed network or a smaller, more dedicated group. Control ablations targeted a random subset of four well labeled RS neurons among a group of 14 identified neurons within rhombomeres 4–6. This group includes the Mauthner neuron. The control ablated group performed similarly to unablated controls in the prey capture assay and significantly better than the bilateral MeLr and MeLc ablated group (mean difference in paramecia decline, 21.91%; p < 0.05) (Fig. 4D). This experiment provides initial evidence that MeLr and MeLc are required for prey capture.

**MeLr and MeLc function in a behavioral pathway downstream of the tectum**

Bilateral tectum and bilateral MeLr and MeLc ablation both impaired prey capture, and our anatomical data suggested that MeLr and MeLc are downstream of the tectum. Alternatively, MeLr and MeLc could have a tectum-independent role in prey capture. To test whether the tectum and MeLr/MeLc were linked in the same neural pathway, we performed dual ablations, taking advantage of the fact that MeLr and MeLc dendrites were located only in the ipsilateral tectum. We ablated the retinotectal neuropil on one side, leaving the tectum on the other side intact, and combined this operation with the ablation of either the contralateral or the ipsilateral MeLr/MeLc. If tectum and MeLr/MeLc are in the same pathway, we expect the contralateral ablation to reduce prey capture to a much greater extent than the ipsilateral ablation. The effect of contralateral ablations may in fact be as great as that after bilateral tectum ablation. If tectum and MeLr/MeLc are downstream of the tectum, although the difference is not statistically significant. One possible explanation for this trend is that the tectum relays relevant visual information to RS neurons other than MeLr and MeLc. In that case, bilateral MeLr and MeLc ablation would leave all additional tectoreticular connections intact, whereas the
combined contralateral ablation would eliminate all tectoreticular connections on one side of the brain, along with the connection to MeLr and MeL on the other. The combined contralateral ablation would therefore sever more tectoreticular connections than the bilateral MeLc and MeLr ablation. Although MeLr and MeLc may not be the only tectorecipient RS neurons important for prey capture, these experiments firmly place MeLr and MeLc in a tectoreticulospinal pathway for prey capture.

Ablations reduce orientation toward prey

Prey capture begins with prey recognition and localization, followed by orientation and swimming toward the prey, and culminates in a strike. We asked at what stage the behavior was disrupted by our ablations. To address this question, high-speed (125 frames per second) video recordings of individual prey encounters were analyzed in bilateral tectum-ablated, bilateral MeLr/MeLc-ablated, and control larvae. The first 10 prey encounters, defined as a paramecium moving at close distance (<5 mm) and within the visual range of the larva, were monitored in each group and analyzed using frame-by-frame playback of video recordings. Swimming responses during prey encounters (10 per experimental group) fell into two specific classes, one termed “orienting swims” and the other termed “routine swims,” which closely correspond to the “routine turns” described previously by Budick and O’Malley (2000) (Fig. 5). A distinguishing feature of these two behaviors is the head yaw angle (the angular displacement of the head during successive side-to-side bends of a swim bout): for orienting swims, this angle is much smaller than for routine swims (Fig. 5A–C, three swimming trajectories with the corresponding yaw). Across all three groups, head yaw angle showed a bimodal distribution peaking at ~5° for orienting swims (4.80 ± 0.31 for successful and 6.08 ± 0.67 for unsuccessful captures) and 16° for routine swims (15.67 ± 1.85), with a dip in the distribution at 10° (Fig. 5D). Almost one-half (6 of 14) of the orienting swims (<10°) culminated in prey capture, whereas none (0 of 16) of the routine swims (>10°) did (Fig. 5D). In addition, orienting swims (<10°) invariably led to an approach (i.e., shortening of the distance between fish and paramecium) at the end of the episode (14 of 14), whereas routine swims (>10°) almost always (14 of 16) resulted in an increase in fish-to-paramecium distance, as would be expected from the uncorrelated motion of two objects.

Control larvae oriented in 8 of 10 encounters toward the paramecium and successfully captured the paramecium four of those eight times. Tectum-ablated larvae showed only one orienting swim during the 10 encounters and had no successful capture during a 1 h observation period. Bilateral MeLr-ablated larvae showed five orienting swims, two of which were successful (Fig. 5E). The lack of orienting in the tectum ablation group suggests that these larvae were impaired in their ability to detect paramecium (yaw angle difference, p < 0.001; two-tailed t test, assuming unequal variance). Orienting responses by MeLr-ablated larvae were reduced mildly compared with controls (p = 0.05) but not significantly different from the tectum-ablated group (p = 0.75). The kinematics of routine and orienting swims were very similar between all three groups with respect to total distance traveled (pairwise comparisons, p = 0.47, 0.63, and 0.69, respectively) and other parameters. These data show that lesions of tectum and MeL have qualitatively similar effects on the orientation toward prey.

Locomotion and eye movements are unaffected by ablations of MeLr and MeLc

Could nMLF ablation impair prey capture through an unspecific effect on locomotor control? Zebrafish larvae exhibit characteristic, age-specific patterns of spontaneous swimming activity (Cahill et al., 1998; Drapeau et al., 2002). Therefore, a spontaneous activity assay was used as a measure of general locomotor functioning. Time-lapse videos of spontaneous swimming of groups of larvae were recorded and analyzed in terms of the average percentage of the group that remained in motion (i.e., fish that had changed position from one video frame to the next) across the 20 min recording period. Blind lakritz mutants showed normal levels of spontaneous swimming frequency and moved similar distances as wild-type sibling controls, suggesting that spontaneous activity level is not vision dependent (percentage of group in motion, 0.84 ± 0.10 and 0.94 ± 0.06 for lakritz mutants and wild-type controls, respectively). Bilateral tectum-ablated and bilateral MeLr/MeLc-ablated groups also showed normal levels of spontaneous swimming activity relative to their respec-
The effect of MeL ablation, and combined MeL and tectum ablation, on prey capture. The location of the targeted MeLc neuron and the neighboring MeLr neuron are marked with an asterisk for reference (M). The dendritic projection of the right MeLr neuron is clearly visible, innervating the ventromedial tectum. The M cell is oriented toward paramecia in their field of view. This result visually guided prey capture. Tectum-ablated animals almost never oriented toward paramecia in their field of view. During a chase, they hold their heads still (horizontal yaw angle is only $\sim 5^\circ$, as opposed to $16^\circ$ during routine swims) and steadily decrease the distance to the prey. To delineate the neural pathway underlying prey detection, we then performed laser ablations of candidate CNS structures and tested the lesioned animals using the new behavioral assay. The results of these various lesion studies allowed us to make a number of strong predictions about the neural pathway involved in prey capture, although they may play additional roles in other tectum-dependent behaviors.

**Discussion**

Zebrafish larvae, at the age of only 1 week after fertilization and with a body length of $<0.5$ cm, readily prey on live paramecia, by either chasing the prey or remaining stationary and sucking the prey into their mouths (Borla et al., 2002). Using a quantitative behavioral assay of prey capture performance, we investigated how zebrafish accomplish this feat. We first showed that that prey capture is predominantly visually mediated. Wild-type fish in the dark and blind mutants (lakritz) catch only one-fifth of the paramecia compared with control fish in the light. High-speed video recordings of individual capture episodes revealed that fish often make rapid orienting turns and pursuit swims toward paramecia that were presumably only detectable by vision. During a chase, they hold their heads still (horizontal yaw angle is only $\sim 5^\circ$, as opposed to $16^\circ$ during routine swims) and steadily decrease the distance to the prey. To delineate the neural pathway underlying prey detection, we then performed laser ablations of candidate CNS structures and tested the lesioned animals using the new assay. The results of these various lesion studies allowed us to make a number of strong predictions about the neural pathway underlying prey capture.

First, we found that integrity of the tectum is indispensable for visually guided prey capture. Tectum-ablated animals almost never oriented toward paramecia in their field of view. This result...
confirms previous electrophysiological and lesioning studies in other vertebrates (Ewert et al., 2001; Lomber et al., 2001; Doubell et al., 2003). For instance, in trout, activation of the tectum elicits orienting movements, the direction and amplitude of which depend on the site of stimulation (Herrero et al., 1998b). However, our study also showed that the tectum is not always necessary for prey consumption, because paramecia numbers still drop at measurement. We find that crossed tectum lesions severely impaired prey capture, similar to unilateral tectum ablations. If tectum and MeLr/MeLc on one side, reside in the lateral portion of the nMLF, the only midbrain structure with descending projections into the spinal cord. MeLr and MeLc extend elaborate dendrites into the deeper layers of the tectum. Here, the cell bodies of tectofugal projection neurons are situated (Meek and Schellart, 1978; Nguyen et al., 1999), which, in other teleosts, have been reported to send a predominantly ipsilateral and excitatory projection to the nMLF (Bosch and Paul, 1993; Herrero et al., 1998a; Niida et al., 1998; Zompa and Dubuc 1998a,b; Isa and Sasaki, 2002; Torres et al., 2002; Perez-Perez et al., 2003). MeLr and MeLc extend axons into the spinal cord, arborizing predominantly in its rostral segments, in which their axon terminals colocalize with motor neurons and interneurons (Gahtan and O’Malley, 2003). Axon branching decreases caudally, but stem axons still reach the most caudal spinal segments. Their morphology thus made MeLr and MeLc excellent candidates for relaying information from the tectum to spinal motor circuits. Bilateral ablation of MeLr and MeLc resulted in a severe prey capture deficit that almost equaled that of bilateral tectum ablations. MeLr- and MeLc-ablated fish showed fewer orienting swims than unablated controls, and, even when they did orient, prey capture was rarely successful. Control ablations of other identified RS neurons did not produce significant prey capture deficits. To our knowledge, this finding is one of very few that links a specific behavior to a small number of identified neurons in a vertebrate. We verified experimentally that MeLr and MeLc acted downstream of the tectum, taking advantage of their exclusively ipsilateral connection. We reasoned that if tectum and nMLF are in the same pathway, combined “crossed” ablations of the tectum on one side and MeLr/MeLc on the other would reduce prey capture to a degree comparable with bilateral ablations of either structure. Ipsilateral, “uncrossed,” ablations, in contrast (of tectum and MeLr/MeLc on the same side), should have the same, intermediate effect as unilateral tectum ablations. If tectum and MeLr/MeLc, however, are acting in different pathways we, expect both combinations to have about the same effect (the magnitude of which would be difficult to predict). We find that crossed ablations severely impaired prey capture, similar to unilateral tectum ablations, whereas uncrossed ablations resulted in an intermediate phenotype, similar to unilateral tectum ablations (Fig.
worsen prey capture performance. This finding strongly suggests that MeLr and MeLc relay signals from the ipsilateral tectum to spinal motor neurons.

Although prey consumption was substantially diminished in MeLr/MeLc-ablated larvae, the severity of this deficit was not as strong as that of bilaterally tectum-ablated larvae. This difference can be explained by additional connections from the tectum to the RS array, including the crossing dendritic projection of medial nMLF cells that innervates the contralateral tectum in zebrafish (Fig. 4A) (Gahtan and O’Malley, 2003) and a descending projection from the tectum to hindbrain RS neurons. We predict that these connections may also participate in the transmission of prey information. Ablation of the entire nMLF, although technically possible (it consists of ~20 cells on each side of the brain), would be difficult to verify, because not all RS cells are labeled by retrograde tracing in any given animal. Therefore, some cells may escape the operation, and their continued presence after laser treatment would remain undetected.

To show behavioral specificity of MeLr and MeLc, we tested spontaneous swimming, as well as OMR and OKR. All three behaviors were unaffected in ablated larvae. We paid particular attention to the OKR, because studies in fish (Torres et al., 1995), frogs (Kostyk and Grobstein, 1987), and mammals (Buttner-Ennever and Buttner, 1988; Blanks, 1990) have shown that the nMLF influences eye movements. Neurons within the rostral interstitial nMLF provide direct premotor input to the trochlear nerve, which innervates the superior oblique muscle, and other nMLF neurons influence eye movements through a cerebellar circuit (Blanks, 1990). However, our study suggests that the nMLF neurons controlling eye movements (if present in zebrafish) are not identical to MeLr or MeLc.

The nMLF resides in the tegmentum. In amphibians, a tegmental area called the large-celled column (LCC) has been implicated previously in prey recognition (Ewert, 1987; Ewert et al., 2001). It is likely that lesions of the LCC in previous studies encompassed the amphibian homologs of MeLr and MeLc and thus led to similar effects on prey capture to those seen here in fish larvae. What could the function of MeLr and MeLc be? From our high-speed video analysis of prey encounters, it appears that MeL- ablated fish are less able to orient toward prey, mimicking the behavioral deficit after tectum ablation. Thus, MeLc and MeLr may be part of a sparse ensemble of neurons that transmits tectal information on prey location to the motor periphery. Additional studies will be aimed at identifying the other elements of this visuomotor pathway. The fact that the RS neurons in larval zebrafish are individually identifiable, together with our ability to target single neurons for laser ablation, has allowed us to assign a behavioral function to only two identified neurons, rather than hundreds of indistinct cells. Our studies are therefore beginning to trace a dedicated neural pathway, at single-cell resolution, for a complex behavioral program in the vertebrate brain.

References
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