Direct activation of the Mauthner cell by electric field pulses drives ultrarapid escape responses


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TELEOST FISH have among the fastest reaction times for escape responses in the animal kingdom, rapidly accelerating away from the strike path of predators within tens of milliseconds. Response latency is a critical factor in predator evasion, enabling prey to escape the strike zone and freely maneuver while avoiding detection by predators. In fish species that possess well-coordinated escape responses when exposed to electric field pulses (EFPs), sensory cells activate motoneurons and facilitate predator detection by integrating acoustic, mechanosensory, and visual stimuli. In addition, larval fish show well-coordinated escape responses when exposed to electric field pulses (EFPs). Sensitization of the Mauthner cell by genetic overexpression of the voltage-gated sodium channel SCN5 increased EFP responsiveness, whereas Mauthner ablation with an engineered variant of nitroreductase with increased activity (epNTR) eliminated the response. The reaction time to EFPs is extremely short, with many responses initiated within 2 ms of the EFP. Large neurons, such as Mauthner cells, show heightened sensitivity to extracellular voltage gradients. We therefore tested whether the rapid response to EFPs was due to direct activation of the Mauthner cells, bypassing delays imposed by stimulus detection and transmission by sensory cells. Consistent with this, calcium imaging indicated that EFPs robustly activated the Mauthner cell but only rarely fired other reticulospinal neurons. Further supporting this idea, pharmacological blockade of synaptic transmission in zebrafish did not affect Mauthner cell activity in response to EFPs. Moreover, Mauthner cells transgenically expressing a tetrodotoxin (TTX)-resistant voltage-gated sodium channel retained responses to EFPs despite TTX suppression of action potentials in the rest of the brain. We propose that EFPs directly activate Mauthner cells because of their large size, thereby driving ultrarapid escape responses in fish.

escape response; Mauthner cell; electric pulse; nitroreductase; SCN5

Low survival rates among fish larvae necessitate that escape responses emerge very early in development, and indeed by 5 days postfertilization (dpf) zebrafish larvae execute rapid escape swims when threatened by acoustic, tactile, or looming visual cues (reviewed in Fero et al. 2011). Natural predators of zebrafish larvae include adult fish and dragonfly nymphs (Engeszer et al. 2007), which use a hydraulic mechanism to achieve high-velocity labial strikes (Tanaka and Hisada 1980). Accordingly, behavioral response latencies in larvae are short—3 to 10 ms for acoustic and tactile stimuli (Kohashi and Oda 2008; Liu and Fetcho 1999) and ~400 ms for looming visual stimuli (Facchin et al. 2009). However, the fastest reaction time for escape responses, frequently <2 ms, is seen in response to an electric field pulse (EFP). Many fish species initiate well-coordinated escape swims in response to EFPs (Dunlop et al. 2006; Webb 1976, 1980; Yokogawa et al. 2012). Apart from their use in analyzing escape responses, EFPs are widely used in behavioral assays in fish, especially in paradigms for learning and memory where EFPs act as unconditioned aversive stimuli (Aoki et al. 2013; Bitterman 1964; Portavella et al. 2002; Rawashdeh et al. 2007; Shcherbakov et al. 2005; Valente et al. 2012). In addition, EFPs have been used to assess arousal states (Yokogawa et al. 2007), study nociception (Dunlop et al. 2006), and analyze autonomic and behavioral responses to stressors (Agetsuma et al. 2010; Lee et al. 2010; Mann et al. 2010).

Despite this extensive use in research, the sensory modality through which EFPs trigger behavioral responses in fish has not been identified, although it has been assumed that, as in mammals, EFPs act on cutaneous nociceptors (Dunlop et al. 2006). However, we observed that in zebrafish larvae the first trunk movements in response to an EFP are detectable within 2 ms of the stimulus onset. The extreme short latency of EFP responses suggested that they are mediated by an unusual neuronal mechanism. Using genetic and physiological techniques we investigated the neuronal circuitry by which EFPs elicit ultrarapid escape responses.
MATERIALS AND METHODS

Fish husbandry. Zebrafish husbandry and tol1 transgenesis have been described previously (Yokogawa et al. 2012). EnSCP1:Gal4ffy264 (y264), EnREx2-cfos:Gal4ffy269 (y269), and EnREx2-cfos:Gal4ffy270 (y270) were isolated in an enhancer trap screen (Bergeron et al. 2012). y264 shows strong expression of Gal4 in the Mauthner cell and has additional stochastic expression in other reticulospinal neurons, the anterior lateral line ganglia, and sparse cells in the spinal cord, hindbrain, cerebellum and forebrain (see Fig. 3B). For Tg(UAS:Elb:Bgi-epNTR-TagRFPT-oPre)y268 (UAS:epNTR-RFP), zebrafish codon optimized nfsb was synthesized (Genscript), fused to TagRFPT, and cloned into pT1UMP (Yokogawa et al. 2012). Mutations T41L, N71S, and F124W (Jaberipour et al. 2010; LinWu et al. 2012) were introduced by PCR mutagenesis to generate an “enhanced-potency” nitroreductase (epNTR). Recently, a similar set of mutations was independently tested in zebrafish and also found to enhance-potency nitroreductase (epNTR). Recently, a similar set of mutations was independently tested in zebrafish and also found to enhance-potency nitroreductase (epNTR). Recently, a similar set of mutations was independently tested in zebrafish and also found to enhance-potency nitroreductase (epNTR). Recently, a similar set of mutations was independently tested in zebrafish and also found to enhance-potency nitroreductase (epNTR). Recently, a similar set of mutations was independently tested in zebrafish and also found to enhance-potency nitroreductase (epNTR). Recently, a similar set of mutations was independently tested in zebrafish and also found to enhance-potency nitroreductase (epNTR).

Behavioral analysis. Individual and group testing were performed in a 4.2-cm² arena above a translucent diffuser illuminated with an LED array. Individual testing of acoustic responses was performed in a grid of 1-cm² chambers. Responses were recorded with a high-speed camera (DVS Lightening RDT/1; DEL Imaging) at 1,000 frames/s and analyzed with Flote software (Burgess and Granato 2007). Acoustic startle responses, elicited as previously described, occur in two waves: short-latency C-starts (SLC) and long-latency C-starts (Burgess and Granato 2007). Except for Fig. 1C, only data from SLCs were analyzed. As larvae were most responsive to EFPs when oriented toward the anode, except for Fig. 2, A and B, results are for larvae oriented within 22.5° of this direction. Stimuli were generated with a digital-to-analog card (PCI-6221; National Instruments). EFPs were square DC pulses (2-ms duration, 1- to 9-V amplitude except as otherwise noted) or sinusoidal AC pulses (0.5- to 2-ms period, 2-ms duration, 9-V amplitude) across stainless steel wire mesh electrodes 1 or 4.2 cm apart for free-swimming larvae or 2.3 cm apart for head-embedded larvae.

Calcium imaging. Calcium fluorescence was monitored with either y264;UAS:GCaMP6s or larvae or Mauthner cells retrogradely labeled by injecting Calcium Green-1 dextran (C3713; Invitrogen) into the spinal cord. Larvae were head-embedded in 2% agarose with tail movement unrestricted. Imaging was performed with an Axio Imager compound microscope (Carl Zeiss), and time courses were recorded with μEye camera (IDS Imaging). Calcium fluorescence intensities were extracted with custom software. The microscope stage was affixed with a speaker, and a collar with electrodes was positioned inside the dish.

Pharmacology. ni,D-2-Amino-5-phosphonopentanoic acid (APV, 100 μM; A5282, Sigma), carbenoxolone (CBX, 1 μM; C4790, Sigma), NBQX (20 μM; N183, Sigma), strychnine (50 μM; S8753, Sigma) and tetrodotoxin (TTX, 1 μM; T-500, Alomone labs) were dissolved in E3 medium. Picrotoxin (100 μM; P1675, Sigma) was dissolved in DMSO. Larvae were embedded in agarose, and drugs were added to the bath solution and injected in the brain ventricle (2–4 nl) with a picospritzer (PV820, World Precision Instruments). Fish were tested before and 10 (TTX) or 30 (all others) min after drug delivery. For behavior testing y264;UAS:SCN5 larvae, RFP expression was visually confirmed at 4 dpf after anesthetization with 0.03% tricaine (MS-222, Sigma). Larvae were allowed to recover 40 h in E3 before testing.

Ablations. y264;UAS:epNTR-RFP, y269;UAS:epNTR-RFP, and y269;UAS:nfsb-mCherry larvae and RFP-negative siblings (controls) were treated with 10 mM metronidazole in E3 from 3 to 5 dpf. To detect cell death during genetic ablation, larvae were immersed for 1 h in 8 μM PhlPhiLux G122 (Oncolimmun) and washed three times before live imaging as previously described (Yokogawa et al. 2012). Laser ablations were performed in Gf(T2KSAG)y1229a(H1129) larvae, which express GFP in the Mauthner cell (Burgess et al. 2009). Mauthner cells, or RoV3 neurons for controls, were ablated with a Leica TCS-S5P II 2-photon confocal microscope with a ×20 0.95 NA objective and an 800-nm laser. Immunolabeling against GFP confirmed successful ablation of 70% of targeted cells. Only data from confirmed ablations were analyzed.

Statistical analysis. Analyses were performed with Gnumeric (http://projects.gnome.org/gnumeric/). Data in figures and text are means and SE.

RESULTS

EFPs induce well-coordinated swimming movements in larval zebrafish that strongly resemble C-start escape responses triggered by acoustic stimuli (Fig. 1, A and B, Supplemental Movie S1). Larvae show two types of responses to acoustic stimuli distinguished by latency and kinematics (Burgess and Granato 2007). In contrast, the vast majority of EFP responses in both AC and DC EFPs have been used in behavioral assays in fish, we tested the behavioral responsiveness to both stimulus types. Responsiveness to EFPs was greater for square (DC) field pulses compared with sinusoidal (AC) pulses of the same duration and amplitude (Fig. 1D). Using long-duration field pulses (10 ms), we observed that most EFP responses were evoked by the onset of the positive field potential, not the pulse offset (Fig. 1E), and increasing the duration of the square pulse did not increase responsiveness (Fig. 1F). Surprisingly, larvae showed a faster reaction time to EFPs than to acoustic stimuli (Fig. 1C, inset; electric 2.7 ± 0.06 ms, acoustic 5.1 ± 0.07 ms, P < 0.001) and we frequently noted EFP responses beginning within 2 ms of the stimulus onset, indicating a very short path for sensory-motor transmission. Ultrafast EFP responses are not a unique specialization of early-stage larval teleosts, because we observed the EFP response in 4–14 dpf larvae, 1-mo-old juveniles, and 2-mo-old adults (Fig. 1G). Moreover, these responses are not a unique specialization of zebrafish, because we recorded similarly rapid responses in larvae of three other phylogenetically well-separated lineages of teleost species (Fig. 1H; Supplemental Movies S2–S4). In each species, the EFP response was a well-coordinated swimming movement, initiated with a C-bend to one side, followed by a counterbend and swim. Similar to larval zebrafish, medaka, fathead minnows, and cavefish larvae all had shorter EFP responses.

1 Supplemental Material for this article is available online at the Journal website.
response latencies than acoustic response latencies (although the difference was not statistically significant for medaka). The remarkably short reaction time of larvae to EFPs led us to analyze the neural circuitry responsible for this behavior.

Fish that sense weak electric fields show orientation selectivity of responsiveness (Yager and Hopkins 1993). Likewise, larvae showed an orientation selectivity of responsiveness in the electric field, with maximal responsiveness to fields oriented toward the anode (Fig. 2, A–C). Larvae were more responsive when oriented toward the anode than toward the cathode (Fig. 2, A and B). At voltage intensities up to 2 V/cm, the initial C-bend of the escape response was highly stereotyped and little affected by voltage intensity, indicating that the EFP response is an all-or-nothing event (Fig. 2D). At intensities of 6–9 V/cm, larvae responded with C-starts; however, we observed greater variability in the duration and angular velocity of the initial bend (data not shown). SLC responses to acoustic stimuli are also all-or-nothing events, initiated by the Mauthner cells (Burgess and Granato 2007). Direct comparison of EFP responses to SLC responses in fish monitored in response to EFPs and acoustic stimuli presented on alternating trials. Each stimulus was calibrated to produce a behavioral (tail movement) response on 75% of trials. EFPs triggered large increases in calcium-dependent fluorescence in Mauthner cells similar to changes induced by acoustic stimuli (Fig. 3, C and D). Only small changes in fluorescence (ΔF/F: 0.023 ± 0.004, n = 9 cells; paired t-test, P < 0.001), possibly
reflecting subthreshold EPSPs, were seen in acoustic trials with no behavioral response, confirming that large signals report Mauthner cell firing (Fig. 3D). For the 15 Mauthner cells tested with 8 trials each, we detected superthreshold changes in fluorescence indicating neuronal activity in 44 acoustic trials and 65 EFP trials (Fig. 3E). All superthreshold changes in fluorescence were accompanied by a behavioral response for both types of stimulus. This demonstrates that EFPs activate the Mauthner cell at a level similar to acoustic stimuli and that Mauthner cell firing strongly correlates with behavioral responses to EFPs.

Do EFPs broadly activate neurons or selectively activate the Mauthner cell? To address this, we imaged calcium responses in additional reticulospinal neurons. As expected (Gahtan et al. 2002), other reticulospinal neurons also responded during acoustic trials; for the 16 reticulospinal neurons, we observed 26 responses to acoustic stimuli (A) showing angle, duration, and maximum angular velocity. n = 14 groups of 30 fish. *Paired t-test, P < 0.01.
to increase Mauthner cell membrane excitability. In contrast to methods such as channelrhodopsin stimulation that require exogenous stimuli and acutely drive neuronal activity irrespective of behavioral context, this strategy allows us to assess the contribution of a given neuron to a behavioral response under normal assay conditions. For these experiments, we used the voltage-gated sodium channel α-subunit from human myocyte (SCN5a), which activates and shows 50% conductance at −15 mV more negative potentials than α-subunits from brain or skeletal muscle and is slower to inactivate (Deschenes et al. 2001; Mantegazza et al. 2001). We generated a UAS:SCN5 transgenic line and, as proof of principle for SCN5a exogenous expression increasing neuronal excitability, drove expression in motoneurons using Gal4 enhancer trap line E(R2x:cfos:Gal4ff)y270 (Fig. 4A). In zebrafish larvae, stimuli that trigger large-angle escape turns recruit a larger fraction of motoneurons into the active pool, and these neurons fire more actively (Bhatt et al. 2007). Consistent with increased motoneuron excitability, y270;UAS:SCN5 larvae showed larger angle C-bends during escape responses. The larger angle of long-latency C-starts was due to an increase in the maximal angular velocity of the bend, as would be expected if a larger fraction of motoneurons were recruited (Fig. 4B). SLC responses are normally initiated with extremely high angular velocity, and thus the relatively small increase in bend angle after SCN5 expression may be due to a ceiling effect. We next drove expression of SCN5a in the Mauthner cell using the UAS:SCN5 transgenic line and enhancer trap line y264. To confirm SCN5 expression we used immunohistochemistry and saw expression in the Mauthner cell in y264;UAS:SCN5 larvae (Fig. 4C). y264;UAS:SCN5 larvae showed increased responsiveness to an acoustic stimulus, confirming that overexpression sensitized the Mauthner cell (Fig. 4D). The latency and kinematics of the acoustic short- and long-latency responses were similar between larvae expressing SCN5 in their Mauthner cells and clutchmates without the transgene (data not shown). y264;UAS:SCN5 larvae also showed greater responsiveness to an EFP but no change in response kinematics (Fig. 4E). This experiment correlates increased excitability of the Mauthner cell with enhanced responsiveness to the EFP implying that the Mauthner cell initiates this behavior. As an independent line of evidence, we examined the behavior of larvae presented with simultaneous subthreshold EFP and acoustic stimuli. Because acoustic stimuli are known to depolarize the Mauthner cell, we reasoned that if the EFP acts through a non-Mauthner response circuit simultaneous presentation with the acoustic stimulus would have an additive effect on responsiveness, whereas if both stimuli activate the Mauthner cell the combined presentation would have a supra-additive effect on responsiveness. We observed supra-additive responsiveness to simultaneous subthreshold acoustic and EFP stimuli, as expected if both stimuli stochastically trigger the Mauthner cell to fire (Fig. 4F).

To determine whether the Mauthner cells are required for EFP responses, we used the nitroreductase nfsB, which metabolizes metronidazole into a cell-impermeant cytotoxin, allowing cell-specific ablation (Pisharath et al. 2007). To improve ablation efficacy, we used a modified nfsB with increased activity through codon optimization and introduction of three amino acid changes, T41L, N71S, and F124W (Jaberipour et al. 2010; LinWu et al. 2012) (enhanced-potency nitroreductase, “epNTR”). Improved ablation efficiency by epNTR was initially shown by injection of mRNA into embryos (Fig. 5, A and B). After generating a stable UAS:epNTR-RFP line, we confirmed this by comparing the extent of cell loss after ablation to an unmodified UAS:nfsB transgenic line (Fig. 5C). We drove expression of epNTR in the Mauthner cells using y264;UAS:epNTR-RFP larvae. After 12 h of metronidazole treatment, we detected apoptosis in RFP-expressing Mauthner cells of y264;UAS:epNTR-RFP larvae with PhiPhiLux G1D2, a live fluorescent reporter of caspase-3-like activity (Fig. 6A) (Packard and Komoriya 2008; Yokogawa et al. 2012). After ablation of both Mauthner cells in y264;UAS:epNTR-RFP larvae, the Mauthner cell-independent long-latency response to acoustic stimuli was executed with normal response rates and kinematics (Fig. 6B) but the EFP response was completely lost (Fig. 6B). To
confirm that the loss of the EFP response was due to ablation of the Mauthner cell and not due to ablation of other NTR-expressing cells in \textit{y264;UAS:epNTR-RFP} larvae, we performed single-cell ablations of the Mauthner cells. Using J1229 larvae that express GFP in the Mauthner cells and other reticulospinal neurons (Burgess et al. 2009), we targeted and laser ablated Mauthner cells, or RoV3 neurons in controls. Similar to genetic ablations, EFP responses were lost after bilateral laser ablation of the Mauthner cells (Fig. 6, D and E), while after unilateral ablation all responses were initiated in a direction contralateral to the remaining Mauthner cell (Fig. 6, D and F). Together, these experiments show that the Mauthner cell is required for EFP responses.

The extreme short latency of EFP responses prompted us to ask how stimulus detection and transmission to the Mauthner cell could be accomplished so quickly. We detected responses within 2 ms of the onset of the EFP, remarkable given that the latency from Mauthner spike to trunk muscle contraction is \(~2\) ms (Eaton et al. 1977a). We hypothesized that EFPs directly activate the Mauthner cell, bypassing sensory structures. To evaluate this, we silenced neuronal inputs to Mauthner cells by injecting larvae with a cocktail of antagonists (APV, NBQX, picrotoxin, strychnine, CBX; see Materials and methods) designed to block glutamatergic, GABAergic, and glycinergic receptors and gap junctions (Fig. 7A). Injection of blockers drastically suppressed tail movements but was not lethal to larvae, which recovered and resumed spontaneous swimming after 3 h. For each larva, calcium responses to EFP and acoustic stimuli were recorded before and 30 min after drug injection, allowing us to examine the effect of synaptic block on individual Mauthner cells. Calcium imaging confirmed that after drug injection Mauthner cell responses to acoustic stimuli were completely lost (Fig. 7, B and C). In contrast, Mauthner cells in the same larvae retained responsiveness to EFPs, indicating that EFPs do not require synaptic transmission to drive Mauthner cell activity. These experiments are thus consistent with the notion that EFPs directly act on the Mauthner cell. However, although the cocktail of antagonists was designed to block the types of neurotransmission known to act on the Mauthner cell (Faber and Korn 1978) and did in fact effectively block the acoustic response, we cannot exclude the possibility that an uncharacterized neurotransmitter type relays EFP signaling to the Mauthner cell.

To address the possibility that other inputs to the Mauthner cell drive the EFP response, we isolated the Mauthner cell from other brain activity by taking advantage of the TTX resistance of SCN5a (Satin et al. 1992). We hypothesized that after action potentials were blocked throughout the brain by TTX injection into \textit{y264;UAS:SCN5} larvae, the expression of TTX-resistant SCN5a in the Mauthner cell should enable it to generate action potentials in response to an EFP. As with the cocktail of blockers, TTX injection paralyzed larvae but was not lethal to larvae, which recovered and resumed spontaneous swimming after 3 h. For each larva, calcium responses to EFP and acoustic stimuli were recorded before and 30 min after drug injection, allowing us to examine the effect of synaptic block on individual Mauthner cells. Calcium imaging confirmed that after drug injection Mauthner cell responses to acoustic stimuli were completely lost (Fig. 7, B and C). In contrast, Mauthner cells in the same larvae retained responsiveness to EFPs, indicating that EFPs do not require synaptic transmission to drive Mauthner cell activity. These experiments are thus consistent with the notion that EFPs directly act on the Mauthner cell. However, although the cocktail of antagonists was designed to block the types of neurotransmission known to act on the Mauthner cell (Faber and Korn 1978) and did in fact effectively block the acoustic response, we cannot exclude the possibility that an uncharacterized neurotransmitter type relays EFP signaling to the Mauthner cell.
Escape responses activated by EFP stimuli were not identical to those triggered by acoustic stimuli. C-start responses triggered by antidromically stimulating the Mauthner cell axon with electrodes chronically implanted in the spinal cord have a 12% smaller initial C-bend angle and a greatly reduced counterbend angle compared with acoustically evoked C-starts (Nissanov et al. 1990). This was interpreted to suggest that additional reticulospinal neurons participate in the escape network activated by acoustic stimuli. Our experiments largely support this model, as we also observed a small decrease in initial C-bend angle and a strong change in the duration and angle of the counterbend for EFP responses compared with acoustically evoked C-starts. However, an alternative explanation for the kinematic differences in the initial C-bend arises from how the Mauthner cell is activated by each stimulus. Action potentials in the Mauthner axon triggered by antidromic stimuli are smaller in amplitude than those elicited by orthodromic stimuli (Furshpan and Furukawa 1962), potentially leading to reduced synaptic drive to motoneurons. If correct, this could explain the reduced angular velocity and magnitude of the initial C-bend of the escape response evoked by electrical stimuli.

How do external electrodes selectively trigger escape responses? Action potentials generated by the Mauthner cell in larvae can be detected by external bath electrodes, demonstrating that the integument offers relatively low electrical resistance (Featherstone 1991; Prugh et al. 1982). We used EFPs of a magnitude comparable to those in previous studies with zebrafish (for example, Pradel et al. 1999; Valente et al. 2012). At these levels, calcium imaging revealed selective activation of the Mauthner cell and a specific short-latency behavioral response. Larger EFPs might activate other reticulospinal neurons, producing distinct behavioral responses, or broadly activate neurons in the brain, producing poorly coordinated movements. Two special characteristics of the Mauthner cell likely cause larvae to perform an escape response to the EFPs used here. First, unlike most other neurons, a single action potential in one Mauthner cell is sufficient to drive a coordinated C-start response (Nissanov et al. 1990), leading to the notion that the Mauthner cell is a “command-like” neuron (Eaton et al. 2001). Second, the large size of the Mauthner axons increases susceptibility to depolarization by electric fields. Large neuronal structures show greater sensitivity to electric stimulation because membrane resistance decreases as surface area increases (in proportion to length squared) while cytoplasmic resistance decreases more because of the larger increase in volume (in proportion to length cubed). Indeed, stimulating electrodes in the spinal cord selectively activate the Mauthner cells among other reticulospinal neurons (Eaton and Farley 1975; Nissanov et al. 1990). This was interpreted to suggest that EFPs directly activate the Mauthner cell and thus strongly suggesting that EFPs directly activate the Mauthner cell.
et al. 1990), and we rarely observed responses in non-Mauthner reticulospinal neurons using an EFP that activated Mauthner cells on 50% of trials. The EFP likely stimulates the Mauthner axons rather than soma, as larvae showed maximal responsiveness when the axons were longitudinally aligned with the field. This is the orientation most susceptible to a voltage gradient between stimulating electrodes (Ranck 1975).

Our findings raise the question as to the adaptive value of the large size of the Mauthner cells. It has been proposed that the large diameter of Mauthner axons facilitates rapid propagation of action potentials for short response latencies (Bennett 1984), 2) synchronous muscle activation during vigorous escape movements (Eaton et al. 1995), and 3) suppressing slower competing motor commands enabling reliable escape behaviors (Eaton et al. 1995). However, there are arguments against each of these ideas. The increase in conduction velocity provided by the larger diameter of the Mauthner axon makes only a small contribution to response latency because of the short distance to motoneurons in the spinal cord (DiDomenico et al. 1988; Eaton et al. 1995). To estimate this contribution in 6 dpf larvae we used the coefficient of conduction velocity to axon diameter determined for Mauthner cells (Funch et al. 1981). The difference in diameter between the Mauthner axon and other reticulospinal axons (4.7 and 1.1 μm) should increase conduction velocity by 4.5 m/s, allowing signals to reach the distal end of the spinal cord 0.6 ms more quickly.

Contrary to the second notion that the large diameter of Mauthner axons is necessary for synchronous muscle activation during vigorous escape movements, escape responses triggered by reticulospinal neurons with smaller axon diameters are similar in angle to or larger than those triggered by the Mauthner cells (Liu and Fetcho 1999). The reliability hypothesis as yet lacks empirical support. When both Mauthner cells fire near-simultaneously, the first to fire activates interneurons that suppress contralateral motoneurons enabling normal es-

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Fig. 6. EFP responses are Mauthner cell dependent. A: selective apoptosis in neurons expressing epNTR during metronidazole treatment. In a y264;UAS:epNTR-RFP larva with stochastic epNTR-RFP expression in the left Mauthner cell (arrow, top), PhiPhiLux G1D2 fluorescence is observed only in the same cell (arrow, bottom). B: acoustic LLC responsiveness and kinetics in y264;UAS:epNTR-RFP ablated larvae (gray) and nonablated sibling controls (black) (n = 4 plates of 5 fish for each group). C: EFP responsiveness in y264;UAS:epNTR-RFP ablated larvae (gray, n = 15 fish) and nonablated sibling controls (black, n = 17 fish). *t-Test, P < 0.01. D: immunolabeling of GFP in J1229 larvae after laser ablation of the left (top) or both (bottom) Mauthner cells. E: EFP responsiveness (E) and response direction (F, % of responses directed rightward) after unilateral or bilateral Mauthner cell laser ablations. Remaining Mauthner cell(s) are depicted. n is indicated in parentheses. *t-Test, P < 0.01.
cape behavior (Satou et al. 2009). However, non-Mauthner escape responses are initiated 3–30 ms after Mauthner-mediated escapes (Burgess and Granato 2007; Kohashi and Oda 2008; Liu and Fetcho 1999) and could therefore be suppressed even if the Mauthner axon was similar in size to other reticulospinal neurons.

An intriguing hypothesis is that the large size of the Mauthner cell enhances its susceptibility to ephaptic coupling. We observed that subthreshold field potentials (72 mV/mm) sensitize the Mauthner cell to concurrent auditory stimuli. The Mauthner axon’s sensitivity to electric fields may make it susceptible to ephaptic coupling from neighboring axons and trunk muscles. Large-magnitude extracellular potentials (~20 mV) at the axon hillock are known to inhibit action potential generation in Mauthner cells (Furshpan and Furukawa 1962). In mammals, even smaller-magnitude endogenous positive extracellular potentials (~0.2 mV) inhibit Purkinje cell firing (Blot and Barbour 2014) and application of negative extracellular potentials (~0.2 mV) near the axon initial segment enhances Purkinje cell firing (Blot and Barbour 2014). Whether ephaptic coupling enhances transmission along the Mauthner axon has yet to be determined. A potential source of ephaptic signaling is the extracellular muscle potential generated by the synchronous contraction of tail muscles during turns and swimming. Single fast-twitch muscle fibers can repetitively depolarize ~100 mV during fictive swimming (Horstick et al. 2013). Large biphasic muscle potentials (~0.8-mV amplitude) evoked during escape responses of larvae have been recorded with bath electrodes (Issa et al. 2011). Recordings from chronically implanted electrodes (Eaton et al. 1981) showed large extracellular potentials adjacent to the Mauthner cell during escape responses. These are likely to be generated by action potentials in trunk muscle (Issa et al. 2011). Muscle potentials generated during swimming bouts may sensitize the Mauthner cell to its other neuronal inputs, possibly contributing to the heightened startle responsiveness exhibited by moving, compared with stationary, larvae (Burgess and Granato 2007). The possible ephaptic coupling of these muscle potentials to the Mauthner axon is an intriguing avenue for future research.

Is the large size of the Mauthner cell a feature that enables fish to rapidly escape from naturally occurring electric fields? Because we found EFP responses in all four species of fish larvae tested here, a natural electric field source that triggers EFP responses should be common. Strongly electric fish that stun prey with electric organ discharges of tens or hundreds of volts are of restricted geographical distribution (Bennett 1971). Weakly electric fish that use electric pulses for communication...
and navigation are numerous, but these produce discharges of <1 V/cm (Scheich and Bullock 1974). As most other bioelectric sources underwater are at least two orders of magnitude weaker than the field strength required to elicit the EFP response (Patullo and Macmillan 2010), EFP responses may therefore not be triggered frequently under natural conditions. However, the stereotyped nature of escape responses is exploited by some predators to control the behavior of prey fish (Catania 2009). For example, during nocturnal predation, the catfish Malapterurus electricus deploys “prevolleys” of electric organ discharges thought to startle immobile prey into revealing themselves (Belbenoit et al. 1979). An intriguing possibility is that prevolleys take advantage of the hardwired EFP response described here to directly evoke Mauthner-dependent responses. Thus in some circumstances the large size of the Mauthner cells may carry an ethological cost.

In mammals, transcutaneous electric stimuli primarily activate C and Aδ peripheral sensory fibers (Koga et al. 2005). EFPs of magnitudes similar to those used here are commonly used in fish research and have been assumed to elicit reflex responses through a similar mechanism (Dunlop et al. 2006). Our findings strongly suggest that, at least for larval fish, such EFPs act directly on central neurons that trigger escape responses. Signaling to the Mauthner cell via peripheral sensory or other neurons is not required for an EFP response. Experiments using EFPs as sensory stimuli should therefore be interpreted with this in mind, as electric stimuli may not act as acute aversive stimuli.

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AUTHOR CONTRIBUTIONS


REFERENCES


