Activity of pectoral fin motoneurons during two swimming gaits in the larval zebrafish (Danio rerio) and localization of upstream circuit elements

Matthew H. Green¹ and Melina E. Hale¹,²
¹Committee on Computational Neuroscience, University of Chicago, Chicago, Illinois; and ²Department of Organismal Biology and Anatomy, University of Chicago, Chicago, Illinois

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Green MH, Hale ME. Activity of pectoral fin motoneurons during two swimming gaits in the larval zebrafish (Danio rerio) and localization of upstream circuit elements. J Neurophysiol 108: 3393–3402, 2012. First published October 3, 2012; doi:10.1152/jn.00623.2012.—In many animals, limb movements transition between gait patterns with increasing locomotor speed. While for tetrapod systems several well-developed models in diverse taxa (e.g., cat, mouse, salamander, turtle) have been used to study motor control of limbs and limb gaits, virtually nothing is known from fish species, including zebrafish, a well-studied model for axial motor control. Like tetrapods, fish have limb gait transitions, and the advantages of the zebrafish system make it a powerful complement to tetrapod models. Here we describe pectoral fin motoneuron activity in a fictive preparation with which we are able to elicit two locomotor gaits seen in behaving larval zebrafish: rhythmic slow axial and pectoral fin swimming and faster axis-only swimming. We found that at low swim frequencies (17–33 Hz), fin motoneurons fired spikes rhythmically and in coordination with axial motoneuron activity. Abductor motoneurons spiked out of phase with adductor motoneurons, with no significant coactivation. At higher frequencies, fin abductor motoneurons were generally nonspiking, whereas fin abductor motoneurons fired spikes reliably and nonrhythmically, suggesting that the gait transition from rhythmic fin beats to axis-only swimming is actively controlled. Using brain and spinal cord transections to localize underlying circuit components, we demonstrate that a limited region of caudal hindbrain and rostral spinal cord in the area of the fin motor pool is necessary to drive a limb rhythm while the full hindbrain, but not more rostral brain regions, is necessary to elicit the faster axis-only, fin-tucked swimming gait.

forelimb; gait transition; limb-axial coordination; midbrain locomotor region

Limb and axial locomotor gaits of vertebrates are driven by motoneurons of the spinal cord and caudal hindbrain and are shaped by mechanical properties of the body and interactions of the body with the physical environment. Motoneuron activity reflects the combined activity of local and distant neural circuits and directly controls the activity of skeletal muscle. Passive properties of muscles, tendons, and skeletal elements, as well as forces due to properties of the surrounding environment, translate and adapt neuronal activity into the movement kinematics we observe. Dissecting the roles of the nervous system and the peripheral body elements in limb and axial movement provides fundamental information about how locomotor gaits are organized, function, and are coordinated during behavior. More specifically, examining how regions of the nervous system contribute to locomotor gaits provides fundamental information to begin to address circuit structure and the evolution of brain and behavior more broadly.

In larval zebrafish, the pectoral fins (forelimbs) and axial morphology are actuated rhythmically during steady swimming and can be used independently or together. Similar to limbs of other aquatic vertebrates such as salamanders (Azizi and Horton 2004; Ijspeert et al. 2007), the pectoral fins are typically active at low frequencies, with or without axial movement. When coactive with the axis during slow swimming, the left and right pectoral fins alternate rhythmically and appear to be coordinated with axial undulations. The fin-axial movement pattern transitions to axial-only locomotion at high frequencies, during which the pectoral fins stop beating and are positioned along the side of the body (Thorsen et al. 2004). While diverse pectoral fin and axial movement behaviors have been studied in a number of species over a wide phylogenetic and developmental range, we know little about either the motor control of the pectoral fin system (but see Drucker and Jensen 1997; Lauder et al. 2006; Westneat and Walker 1997) or the neural basis of coordination of movement between the pectoral fins and the body axis.

Limb morphology, including muscles, skeleton, connective tissue, and skin, forms a controllable and deformable mechanical system. The morphology of the pectoral fin in larval zebrafish is relatively simple, with one abductor and one adductor muscle receiving input from four pectoral fin nerves to power fin movement (Ma et al. 2010; Thorsen and Hale 2007; Uemura et al. 2005). These muscles are located distal to the body wall on either side of an endoskeletal disk (Grandel and Shulte-Merker 1998), and the fin extends further, beyond these muscles, as a peripheral membrane. As the fin moves away from the body during abduction, the proximodistal axis of the fin bends posteriorly and is drawn rostrally along the body. On the return adduction stroke, the fin forms a relatively flat plane and is brought back toward the body (Green et al. 2011, Fig. 3).

Regionalization of function is a well-known property of the nervous system. In tetrapod taxa, regions of the nervous system that generate rhythmic limb movement have been localized with surgical lesions. In rats, rhythmic hindlimb networks are located in the lumbar and caudal thoracic segments of spinal cord (Kjaerulf and Kiehn 1996), whereas rhythmic forelimb circuitry is located in the cervical and rostral thoracic segments of cord (Ballion et al. 2001). Similarly, in salamanders the networks generating rhythmic forelimb movement are localized to cervical spinal cord (Cheng et al. 1998; Wheatley and Stein 1992). These foundational experiments isolated minimal or near-minimal regions of the nervous system that can gen-
erate rhythmic limb activity; however, additional circuitry is likely required for gait transitions between distinct patterns of limb activity. In support of this, a semi-intact preparation of the salamander including the midbrain locomotor region (MLR) was shown to produce limb gait transitions from rhythmic limb walking to nonrhythmic, limbs-tucked swimming during electrical stimulation of the MLR (Cabelguen et al. 2003).

The location of fin circuitry upstream of the pectoral fin motoneurons is unclear. Pectoral fin motoneurons in larval zebrafish and other ray-finned fishes are clustered in a pool that extends from caudal hindbrain to rostral spinal cord (Ma et al. 2010), indicating that the networks generating pectoral fin movement include both spinal and hindbrain components. Like tetrapods, fishes possess an MLR, which is likely involved in control of motor patterns during prey capture (Gahtan et al. 2005) and is a clear candidate for control of transitions between pectoral fin gaits.

In this work we used the larval zebrafish fictive preparation to examine the active control of the pectoral fin rhythmic movement and its coordination with the body axis. This work complements studies of fin morphology, movement, performance, and fluid dynamics to develop a broader understanding of the contributions of active control and passive system properties on swimming. We aimed to describe the pattern of adductor and abductor motoneuron activity and its coordination with axial nerve activity during fictive swimming. In the fictive swimming preparation we can elicit the same range of axial swim frequencies observed in swimming fishes, but, as the preparation is held immobile during recording, typical sensory feedback experienced during swimming, such as of fluid flow around the body and movement in the visual field, is lacking, allowing us to assess feedforward elements of motor control in isolation.

We examined both rhythmic swimming gaits: slow swimming, when the fins and body axis are both beating rhythmically, and fast swimming, when the axis alone is undulating. With these data we aimed to address several fundamental hypotheses about fin function and coordination with the axial system: 1) The gait transition between slow swimming, when fin and axial rhythms are coordinated, and fast, axial-only, swimming, involves actively controlled tucking of the fins against the body with arrhythmic adductor motoneuron activity. 2) The frequency at which larval zebrafish transition between swimming gaits based on pectoral fin activity corresponds to the frequency at which the transition between pre-motor drive is observed in the axial system and is not dependent on sensory feedback. 3) The pattern of fin bending during abduction and adduction, in which marked curvature is observed during abduction but not adduction, is reflected in fin motoneuron activity. Specifically, we hypothesized that coactivation of abductor and adductor stiffen the fin during adduction.

Additionally, we aimed to assess the general location of elements of the upstream circuits that drive motoneuron activity patterns in zebrafish, using brain and spinal cord transections. Such assessment informs comparison between the slow and fast swimming gaits and provides important information to further explore the pectoral fin circuit. On the basis of previous studies we hypothesize that 1) the slow swim rhythm can be generated with local circuits in the region of the fin motor pool but that 2) midbrain circuits are necessary to generate the fast axis-only swim gait.

METHODS

Animals. Embryos of wild-type zebrafish [Danio rerio (Hamilton 1822)] and transgenic zebrafish [Tg(cREST2-hsp70:GFP) and Islet-1:GFP] were obtained from a laboratory breeding population maintained at 28.2°C. Embryos and larvae were raised at 28.2°C in 10% Hanks’ solution on a 14:10-h light-dark cycle until experimental use at 4–5 days postfertilization (dpf). Experiments were approved by the Institutional Animal Care and Use Committee at the University of Chicago.

Electrophysiology. Electrophysiological experiments were performed as described previously (Liao and Fetcho 2008; Liu et al. 2011; McLean et al. 2007), with minor modifications to record from pectoral fin nerves and motoneurons. Extracellular and whole cell patch electrodes were constructed from borosilicate glass pipettes (Harvard Instruments; 0.86-mm inner diameter, 1.5-mm outer diameter). Patch pipettes were pulled to have 1- to 2-μm tip diameters (P-97 pipette puller; Sutter Instruments, Novato, CA) and resistances of 10–20 MΩ when filled with intracellular solution (in mmol/l: 125 K gluconate, 2.5 MgCl₂, 10 EGTA, 10 HEPES, 4 Na₃ATP; adjusted to pH 7.3 with KOH). Extracellular pipettes were constructed from patch pipettes by manually breaking the tip and fire-polishing with a microforge (model MF-830; Narishige, Tokyo, Japan) so that the final tip width was 10–20 μm. Patch and extracellular electrodes were positioned with motorized micromanipulators (PATCHStar, Scientifica) under visual guidance (BX-51WI upright fluorescence microscope, Olympus, with ×10 and ×40 water-immersion objectives). Electrode signals were amplified with a MultiClamp 700B amplifier and digitized at 40 kHz with a Digidata 1440A digitizer (Molecular Devices, Sunnyvale, CA). Signals were low-pass filtered with an analog Bessel filter (1-kHz cutoff for extracellular signals, 10-kHz cutoff for intracellular signals). Electrode resistance and capacitance compensation were used for whole cell recordings.

Fish were immersed in 0.1% a-bungarotoxin (Invitrogen, Grand Island, NY) dissolved in extracellular solution (in mmol/l: 134 NaCl, 2.9 KCl, 1.2 MgCl₂, 10 HEPES, 10 glucose, 2.1 CaCl₂) adjusted to pH 7.8 with NaOH until they stopped moving (5–10 min). After immobilization, fish were washed with distilled water and placed in extracellular solution until recording. To record from pectoral fin nerves, extracellular electrodes were placed on the central region of the proximal, muscular area of the fin and suction was applied to draw fin tissue into the electrode tip. An extracellular electrode was placed on the myotomal clef between muscle segments 13 and 16 to record axial nerve activity. To record from pectoral fin motoneurons, patch electrodes were advanced into the ventral half of the nervous system below muscle segments 2–4, where much of the fin motor pool is located (Myers 1985; Thorsen and Hale 2007; Uemura et al. 2005). A positive pipette tip pressure of 15–30 mmHg was maintained to prevent tip clogging (pressure transducer model DPM1B; Fluke, Everett, WA). When the electrode tip was in contact with a target cell, pressure was reduced to atmosphere and a holding potential of −65 mV was applied. This was usually sufficient to form a gigahm seal, but in some cases a gentle suction was applied to obtain a seal. After gigahm seal formation, whole cell access was achieved by application of gentle pulses of suction to rupture the cell membrane. Whole cell patch recordings were performed in current-clamp mode. Immediately after whole cell access was obtained, resting membrane potentials were close to −65 mV; however, over the duration of recordings the resting membrane potential gradually increased. Recordings were terminated if the resting membrane potential exceeded −50 mV. We did not correct for the liquid junction potential because we were primarily interested in spiking activity and relative changes in the membrane potential.
Morphological analysis of patched cells. Images of recorded cells were acquired with a CCD camera (ORCA II; Hamamatsu) and imaging software. Pectoral fin motoneurons were morphologically identified with two methods: 1) by injecting rhodamine-dextran dye (Texas Red, mol wt 10,000; Invitrogen) into the fin muscles 6–12 h prior to electrophysiological experiments, which retrogradely labeled fin motoneuron cell bodies (Thorsen and Hale 2007), or 2) by including red dye in the intracellular solution (1% sulforhodamine B; Sigma) and tracing the axon of a recorded cell to the pectoral fin. Abductor motoneurons could be distinguished from adductor motoneurons by performing patch recordings in transgenic fish (Tg;cCREST2-hsp70:GFP); Uemura et al. 2005), in which abductor motoneurons expressed green fluorescent protein (GFP) but adductor motoneurons did not express GFP. For 2 of the 21 recorded motoneurons, we were unable to anatomically verify that their axons entered the pectoral fin because of incomplete filling of the axon; however, these two cells were included in our analysis as adductor motoneurons based on the following reasoning: 1) The axons were sufficiently filled to confirm that they exited the nervous system, indicating that these two cells were motoneurons. 2) These two cells did not express GFP, indicating that they were not abductor motoneurons, so they were either adductor or axial motoneurons. 3) Spikes recorded from these cells occurred synchronously or at a small delay to bursts of activity recorded from an axial nerve located at midbody (caudal to the intracellular recording site). This timing of rhythmic spiking, relative to rhythmic bursting of a caudally located axial nerve, would not be expected for axial motoneurons, which have been shown to fire in a traveling wave from rostral to caudal, so that more rostrally located motoneurons spike before more caudally located motoneurons (Masino and Fetcho 2005). We therefore concluded that these two cells were adductor motoneurons.

Transections. Transections of the brain and spinal cord were performed with a tungsten surgical tool under a fluorescence dissection microscope (MZ6; Leica). Fish were anesthetized in MS222 before transections were performed. For posttransection electrophysiology, we used the same methods as described for the intact fictive preparation. To assess the location of transections we used the Islet-1:GFP transgenic line (Higashijima et al. 2000), which allowed morphological analysis of patched cells. Images of recorded cells were acquired with a CCD camera (ORCA II; Hamamatsu) and imaging software. Pectoral fin motoneurons were morphologically identified with two methods: 1) by injecting rhodamine-dextran dye (Texas Red, mol wt 10,000; Invitrogen) 6–12 h prior to transections according to methods described previously (McLean and Fetcho 2004).

Data analysis and statistics. Electrophysiological data were analyzed with code written in MATLAB (MathWorks, Natick, MA). Spikes in extracellular records were detected with a threshold set equal to three standard deviations of the baseline noise. For intracellular records, spikes were detected with a threshold set to −10 mV. Bursts of spikes were identified with a clustering algorithm (k-means; MATLAB Statistics Toolbox). k-Means clustering was applied to spike times recorded from each channel. k-Means requires an estimate of the number of clusters as an input, so we manually counted the number of bursts from the raw data prior to the use of k-means clustering. To mark the occurrence of a burst, we used the mean time of all spikes belonging to a burst.

Cycle duration, $T$, was measured as the time interval between two consecutive bursts in extracellular records from axonal nerves. Swimming frequency was computed as the inverse of the axial cycle period, $f = 1/T$. The phase, at time $t$, during a cycle beginning at time $t_0$, and of duration $T$ is $\phi(t) = (t - t_0)/T$. We measured the phases of the axial cycle at which spikes were fired by pectoral fin motoneurons. If a spike occurred at time $t_{\text{spike}}$, then the phase of this spike was defined by $\phi(t_{\text{spike}}) = (t_{\text{spike}} - t_0)/T$. Summary statistics and tests were computed with the MATLAB Statistics Toolbox and the Circular Statistics Toolbox (Berens 2009). Circular statistics were used to summarize phase results because phase is conveniently described as periodic quantity (cyclically ranging between 0 and 1).

RESULTS

Activity of pectoral fin motoneurons during high- and low-frequency swimming. We elicited a range of swim frequencies in our fictive preparation that is comparable to that recorded in behavioral experiments (Müller and van Leeuwen 2004; Thorsen et al. 2004) and in previous studies of axial motoneuron physiology (Masino and Fetcho 2005; McLean et al. 2007, 2008). We additionally observed spontaneous bouts of activity in the lower range of swim frequencies. Overall, we examined the activity of pectoral fin motoneurons and axial motoneurons over swim frequencies ranging from 16 Hz to 74 Hz. Pectoral fin motoneuron activity was recorded with either extracellular nerve recordings (Fig. 1A1), which allowed simultaneous monitoring of abductor and adductor activity, or intracellular, whole cell patch recordings (Fig. 1A2), which gave more precise measurements of the spiking of individual cells as well as subthreshold membrane potential.

After a brief electrical stimulus applied to the skin of the tail, the frequency of axial nerve bursting began high and decreased smoothly over the duration of the swim bout (Fig. 1B). Abductor nerves and individual abductor motoneurons showed little or no activity during higher axial cycle frequencies but became rhythmically active as the axial cycle frequency decreased (Fig. 1, B and C). In contrast, adductor nerves and individual adductor motoneurons showed consistent, nonrhythmic spiking activity during higher-frequency axial cycles and transitioned to rhythmic bursting activity during lower-frequency axial cycles (Fig. 1, B and D).

We first used extracellular nerve recordings to examine the transition of the fin-axial motor pattern. We analyzed 124 swim bouts recorded from 6 fish. In all swim bouts for which abductor and adductor nerve activity were recorded simultaneously (84 bouts from 4 fish), the onset of rhythmic abductor nerve activity and the onset of rhythmic adductor nerve activity occurred within a single axial cycle period. For the remaining two fish, a single electrode was placed on the fin to record from either the abductor or the adductor nerves. Before the onset of rhythmic abductor and adductor nerve activity, the average axial cycle frequency was 32.74 ± 6.81 Hz (mean ± SD) and ranged from 16.07 Hz to 62.53 Hz. While adductor nerves showed little or no activity during this period, adductor nerves were active. The pattern of adductor activity was nonrhythmic, consisting of an uninterrupted series of closely timed spikes. The mean duration of this initial period of nonrhythmic adductor nerve activity was long (110.4 ± 63.0 ms) compared with the shorter duration of subsequent rhythmic bursts (17.2 ± 12.5 ms; $P < 0.0001$, Welch’s $t$-test comparing duration of pretransition adductor nerve activity to duration of posttransition adductor nerve bursts). After the transition to rhythmic abductor and adductor nerve activity, the mean axial cycle frequency was 22.52 ± 2.74 Hz and ranged from 16.87 Hz to 31.84 Hz. These data demonstrate a decrease in the mean axial cycle frequency across the transition between nonrhythmic and rhythmic fin nerve activity ($P < 0.0001$, Welch’s $t$-test comparing pretransition and posttransition cycle frequencies) yet a substantial overlap in the ranges of cycle frequencies observed before and after this transition. This is demonstrated in the

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example shown in Fig. 1B, in which two axial cycles immediately before the onset of rhythmic fin nerve activity have frequencies similar to the axial cycles after the transition.

We next used intracellular, whole cell patch recordings to further quantify the relationship between pectoral fin motoneuron activity and axial cycle frequency. In contrast to nerve recordings, in which overlapping spike waveforms made precise spike counts difficult, patch recordings allowed measurement of the number of spikes fired by individual pectoral fin abductor motoneurons. Following a previous analysis of axial motoneuron and interneuron recruitment over a range of swimming frequencies (McLean et al. 2008), we counted the number of spikes fired by patched pectoral fin abductor motoneurons during each axial swimming cycle (Fig. 2A). We then plotted the number of spikes recorded from abductor motoneurons during each swimming cycle decreased with increasing axial cycle frequency (Fig. 2B1), ranging from a peak value of $2.0 \pm 1.5$ spikes for axial cycle frequencies between 20 and 25 Hz to a minimum value of $0.1 \pm 0.2$ spikes for axial cycle frequencies between 55 and 60 Hz. The percentage of axial cycles during which the abductor motoneurons fired at least one spike also decreased rapidly with increasing frequency (Fig. 2B2), ranging from 94% for axial cycle frequencies between 15 and 20 Hz to 7% for axial cycle frequencies between 55 and 60 Hz. In contrast, the mean number of spikes recorded from adductor motoneurons did not change substantially with increasing cycle frequency (Fig. 2C1), ranging from $0.8 \pm 0.8$ spikes to $1.2 \pm 1.0$ spikes. The percentage of axial cycles during which adductor motoneurons fired at least one spike ranged from 56% to 75%, with no clear trend of increase or decrease in percentage across axial cycle frequencies (Fig. 2C2). These data demonstrate that abductor motoneurons are largely nonspiking during high-frequency swimming, whereas adductor motoneurons are active during both low- and high-frequency swimming.

Soma sizes and input resistances of fin motoneurons. Given that abductor and adductor motoneurons showed distinct firing patterns during swimming, we tested whether there were differences in the intrinsic properties of these two cell classes. The soma sizes of patched pectoral fin motoneurons (e.g., Fig. 2D1) were comparable to previously reported measurements of
We used extracellular nerve recordings to examine the coordination of abductor, adductor, and axial motoneuron activity. At lower swimming frequencies, when fin motoneurons were rhythmically active, bursts of spikes fired by abductor nerves and adductor nerves occurred at nonoverlapping phase ranges with respect to the axial cycle (Fig. 3). The mean cycle phases of the first and last spikes of rhythmic abductor nerve bursts were 0.58 ± 0.12 and 0.81 ± 0.21, respectively (Fig. 3C). In contrast, the mean cycle phases of the first and last spikes of rhythmic adductor nerve bursts were 0.02 ± 0.13 and 0.28 ± 0.14, respectively (Fig. 3C). Phase data demonstrated that rhythmic bursts of spikes recorded from abductor nerves occurred nearly in alternation with rhythmic bursts recorded from adductor and axial nerves. There was no evidence of substantial coactivation of abductor and adductor nerves; however, in 75 of 940 abductor-adductor burst pairs analyzed we found that the first spike of an adductor burst occurred at a slightly earlier phase than the last spike of the corresponding abductor burst, indicating a small, variable overlap between abductor and adductor bursts at the transition from abduction to adduction. A caveat to these data is that we were not able to record from nerves innervating the dorsal and ventral margins of fin muscle, and so it is possible that we missed coactivation involving these regions.

Transsections to localize pectoral fin motor networks. To localize the neuronal networks driving rhythmic and nonrhythmic pectoral fin motor patterns, we performed surgical transections of the brain and spinal cord and evaluated the capacity of the remaining nervous system to produce pectoral fin motor patterns, using pairwise extracellular nerve recordings from adductor and axial nerves and from abductor and adductor...
nerves. Transections of the brain (Fig. 4A) were made at two positions: 1) at the caudal edge of the nuclei of the third and fourth cranial nerves, which lies immediately caudal to the MLR (Fig. 4B) and 2) at the rostral border of the nucleus of the tenth cranial nerve (Fig. 4C), which is located in the caudal hindbrain (Higashijima et al. 2000). In combination with this caudal hindbrain transection, an additional transection of the spinal cord, at the caudal edge of the swim bladder (7th myotomal segment), was made to determine whether caudal regions of spinal cord were necessary for generating pectoral fin motor patterns.

After the rostral transection, near the midbrain-hindbrain boundary, recordings from adductor and axial nerves demonstrated fictive swimming bouts during which nonrhythmic adductor nerve activity transitioned to rhythmic activity (we analyzed 61 bouts recorded from 5 fish; e.g., Fig. 5A). Swim bouts that included transitions from nonrhythmic to rhythmic adductor nerve activity were evoked with a single pulse of electrical stimulation applied to the otolith (amplitude 0.3–0.6 mA and duration 0.5 ms). We also recorded spontaneous bouts of lower-frequency rhythmic fin and axial nerve activity that were not analyzed. The mean duration of the initial, nonrhythmic bursts of spikes at distinct phases of the axial swim cycle during low-frequency swimming. A: example of rhythmic fin and axial nerve activity is highlighted by a box. The asterisk marks the stimulus. B: expanded view of the nerve recording data boxed in A. The first and last spikes of each burst are marked with open and closed symbols, respectively. Triangles denote abductor nerves, squares denote adductor nerves, and circles denote axial nerves. C: pooled data from 6 fish showing the mean phases of the first and last spikes of rhythmic fin and axial nerve bursts, which are indicated by the left and right sides of each box, respectively. Phase statistics for 2 full cycles are plotted. Error bars indicate SD and show variability of first and last spike phases across all recorded bursts. Phase is measured with respect to the axial nerve rhythm, so that the centers of each axial nerve burst defined phase zero. To plot the phase on a linear axis that is easy to compare to the raw data, we used negative phase values to indicate the previous axial cycle and phase values >1 to indicate the following axial cycle (i.e., phase −0.5 corresponds to phase 0.5 of the previous cycle, and phase 1.5 corresponds to phase 0.5 of the next cycle). Ab, abductor; Ad, adductor; Ax, axial.
The mean duration of adductor nerve activity was 89.5 ± 32 ms and was significantly longer than the mean duration (12.1 ± 1.0 ms) of subsequent rhythmic adductor nerve bursts (Welch’s t-test). During nonrhythmic adductor nerve activity, the mean frequency of rhythmic axial nerve bursts was 35.71 ± 9.52 Hz which is significantly higher than the mean axial cycle frequency (28.22 ± 3.31 Hz) during rhythmic adductor nerve activity (Welch’s t-test). Overall, swim frequencies ranged from 20.04 Hz to 65.88 Hz.

After the caudal transection of the hindbrain, at the rostral border of the nucleus of the tenth cranial nerve, bouts of rhythmic fin and axial nerve activity could be routinely evoked by a single pulse of electrical stimulation (amplitude 0.3–0.6 mA and duration 0.5 ms) applied to the midline of the transected edge of the brain. We analyzed 70 bouts recorded from 5 fish (e.g., Fig. 5B), among which 3 fish were also transected at the spinal cord near the caudal edge of the swim bladder. Swim bouts also occurred spontaneously. We were unable to evoke transitions from nonrhythmic fin nerve activity to rhythmic fin nerve activity in fish transected at the caudal hindbrain location, even when stimulation amplitude was increased to 1.5 mA. The mean axial cycle frequency for caudal hindbrain-transected fish with intact spinal cords was 22.58 Hz and ranged from 16.75 to 34.81 Hz. This was not significantly different from the mean frequency of rhythmic axial or abductor nerve activity recorded from intact fish (P = 0.11, 1-way ANOVA comparing abductor and axial frequencies from intact fish to axial frequencies from fish transected at the caudal hindbrain) but was significantly larger than the frequency of abductor nerve activity (18.83 ± 3.25 Hz) recorded from fish that were transected at both the caudal hindbrain and spinal cord levels (P < 0.0001, Student’s t-test).

To determine whether coordination of rhythmic fin and axial nerve activity was affected by transections, we measured the phases of the first and last spikes of rhythmic fin and axial nerve bursts with respect to the adductor rhythm and compared these measurements to those taken from intact fish (Fig. 5C). The basic pattern of nonoverlapped, alternating activity of abductor and adductor nerves was preserved in transected fish (Fig. 5C); however, we found significant differences in the mean phases of the first and last spikes of abductor bursts comparing fish with combined caudal hindbrain and spinal cord transections to intact fish (P < 0.0001, Student’s t-test). These differences in mean phase values of the first and last spikes of abductor nerve bursts were not large (first spike mean phase of 0.41 vs. 0.47 and last spike mean
phase of 0.65 vs. 0.73, for transected vs. intact fish, respectively). A similarly small, but significant, difference was found by comparing the phases of first spikes of bursts recorded from axial nerves of transected and intact fish (first spike mean phase of 0.79 vs. 0.73 in transected vs. intact fish; \( P < 0.0001 \), Watson-Williams test). This difference may be due to small changes in electrode placement on different fish, and so while it is suggestive of subtle changes in fin control, additional work is needed to substantiate these findings. For the broader patterns observed, these data show that a small region of hindbrain and spinal cord, extending from the rostral border of the nucleus of the tenth cranial nerve to the caudal edge of the swim bladder (at the 7th myotomal segment), can produce rhythmic pectoral fin nerve activity including abductor-adductor alternation, albeit at a lower frequency than was typically recorded from intact fish and with small differences in burst phases. Additionally, these data show that brain regions rostral to the midbrain-hindbrain border are not necessary for initiating high-frequency swimming and the transition between the nonrhythmic and rhythmic fin motor patterns.

**DISCUSSION**

In this study we show that a fictive preparation can produce rhythmic and nonrhythmic pectoral fin motor output within frequency ranges that are consistent with previous behavioral studies in which fish were allowed to move freely (Budick and O’Malley 2000; Green et al. 2011; Müller and van Leeuwen 2004; Thorsen et al. 2004). The consistency between free-swimming behavior and motoneuron activity in our fictive preparation allowed us to examine the neural control of a gait transition in fin movement.

We found that rhythmic abduction and adduction of the pectoral fin during low-speed swimming are driven by alternating activity of abductor and adductor motoneurons. We found that during high-speed swimming consistent adduction of the fins, which holds them tucked against the body, is actively driven by nonrhythmic activity of adductor motoneurons and a lack of activity in abductor motoneurons. Examining trials in which frequency of axial swimming varied, we found that the fin motor pattern switched between nonrhythmic activity and rhythmic activity with changes between high and low axial motor pattern frequencies. That the gait transition can be elicited in the fictive preparation in an appropriate frequency range indicates that this transition is not dependent on sensory feedback from the animal’s movement or interaction with the environment but is centrally coordinated with the circuits that control axial swim frequencies. Our transection experiments demonstrate that the fundamental control of the gait transition and coordination of the fins with the axis occurs in the hindbrain and spinal cord. We suggest that the pectoral fin system in larval zebrafish provides a valuable comparative model for understanding the neural correlates of a limb-based gait transition in a genetic model organism for which recordings from neurons can be routinely conducted in vivo.

**Transition of fin motoneuron activity.** Speed-associated gait transitions between limb motor patterns are common to aquatic and terrestrial vertebrates. Gait transitions during locomotion in terrestrial animals are triggered when musculoskeletal forces reach a critical level (Farley and Taylor 1991) or when switching between gaits results in a minimization of energy expenditure (Griffin et al. 2004). In fishes, a gait transition between fin-driven propulsion and body-driven propulsion with increasing speed allows for the recruitment of additional muscle mass (Kendall et al. 2007; Korsmeyer et al. 2002) or overcomes the mechanical limits of pectoral fin gaits used at slower swim speeds (Hale et al. 2006). Our recordings from immobilized larval zebrafish indicate that the pectoral fin gait transition can be driven by a feedforward motor program, since sensory feedback from water flow or from a changing visual scene is largely lacking in the immobilized fictive preparation.

During high-speed swimming larval zebrafish, and fishes generally, keep the fins tucked against the body. Two plausible mechanisms for fin tucking during high-speed swimming are that 1) tucking is passive, resulting from no motor drive to either the abductor or adductor muscles, or 2) tucking is actively driven by motoneuron activity. Our data indicate that in larval zebrafish the fins are tucked actively by sustained, nonrhythmic adductor motoneuron activity coupled with no, or low-level, abductor motoneuron activity. We hypothesize that sustained, nonrhythmic adductor motoneuron activity holds the fins in a tucked position in freely swimming fish, and that this may reduce hydrodynamic drag on fish during fast swimming. Abductor nerve spiking above an axial swimming frequency of 32 Hz was rare and often consisted of single, isolated spikes rather than rhythmic bursts. The swimming frequency above which we saw no consistent abductor motoneuron activity is in line with a previous study of freely swimming larval zebrafish, in which the pectoral fins were observed to be tucked against the body at swimming frequencies at or above 36 Hz (Thorsen et al. 2004).

As the limbs make a transition from beating rhythmically to being actively tucked, the body axis is continuously shifting to higher swim frequencies. We hypothesized that the swim frequency at which larval zebrafish transition between swimming gaits corresponds to the frequency at which the transition between premotor drives is observed in the axial system. Despite the continuous range of axial swim frequencies, a distinct change occurs in the population of active premotor interneurons driving this behavior. Multipolar commissural descending excitatory interneurons (MCoDs) in the axial network, which have axons that cross the midline and descend caudally in cord, show a steep drop in spiking activity with increasing swimming frequency, with no spikes occurring during cycle frequencies exceeding 50 Hz (McLean et al. 2008). The pattern of MCoD recruitment is strikingly similar to the recruitment pattern we observed in abductor motoneurons. This suggests that pectoral fin motoneurons and MCoDs are part of the same, slow swimming neuronal network; however, ablation of MCoDs does not impair rhythmic fin movements (McLean et al. 2007), and the cell bodies of MCoDs are located caudal to the pectoral fin motor pool (Hale et al. 2001; McLean et al. 2008), which suggests that fin motoneurons do not receive direct excitatory synaptic connections from MCoDs. We instead hypothesize that MCoDs and abductor motoneurons share a common source of synaptic input.

Further work is needed to understand the mechanisms driving the distinct firing patterns of abductor and adductor motoneurons during high-speed swimming. We found no evidence of differences in the size or input resistance of abductor and adductor motoneurons, which suggests that synaptic input, or more subtle intrinsic cellular properties such as cell type-specific ion channel expression, is playing a role in shaping the distinct firing proper-

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tially that the hindbrain was transected at a caudal location (further discussed below) provides some evidence that descending synaptic input, which possibly targets adductor motoneurons specifically, is important for driving adductor motoneurons to fire nonrhythmically during high-frequency swimming.

**Coordination of fin and axial motoneuron activity and adductor-adductor motoneuron activity.** Coordination of limb and axial movement occurs during walking in tetrapods and swimming in larval fishes. Our data suggest that, in larval zebrafish, coordinated movement of the pectoral fins and body axis during slow swimming is enforced by coordinated (i.e., phase locked) rhythmic spiking of pectoral fin and axial motoneurons.

Undulations of the body axis are driven by a rostrocaudal traveling wave of activity, with phase lags between activity of axial nerves innervating different myotomal segments (e.g., Masino and Fetcho 2005). Therefore, although rhythmic fin and axial activity is phase locked, the precise phase difference between spiking of fin and axial motoneurons is dependent on the location of axial nerve recording along the rostrocaudal axis. Since we chose to record from axial nerves near midbody (myotomal segments 13–16), we found a nearly alternating pattern of rhythmic activity between adductor motoneurons and axial motoneurons and a nearly in-phase pattern of rhythmic activity between adductor motoneurons and axial motoneurons. This is consistent with behavioral data showing that fin abduction occurs nearly synchronously with bending on the opposite side of the trunk or, equivalently, in alternation with bending on the ipsilateral side of the trunk (Thorsen et al. 2004). For both adductor and adductor motoneurons, the phase of spiking is likely governed by subthreshold oscillations of the membrane potential during low-frequency swimming, which appear to restrict the occurrence of spikes to the depolarized phase of the oscillation.

We had originally hypothesized that coactivation of abductor and adductor motoneurons during the abduction phase of the fin beat could explain an asymmetry in fin bending (Green et al. 2011), in which the fins remain rigid during addition but bend posteriorly during abduction; however, activity of abductor and adductor motoneurons occurred, by and large, at nonoverlapped phase ranges of the swim cycle, indicating an alternating drive to the abductor and adductor muscles during the fin beat. We sometimes observed a small overlap between abductor and adductor bursts at the transition from abduction to adduction; however, the majority of bursts showed no overlap. This suggests that asymmetric fin bending is a passive, mechanical phenomenon. As our recordings were limited to nerves and motoneurons innervating the middle and ventral regions of the pectoral fin muscles, it is possible that we missed a more complex pattern of coactivation in more rostral motoneurons innervating the dorsal regions of the pectoral fin muscles; however, it seems unlikely that this would have a large effect on midstroke bending of the fin. Further study of the material properties and hydrodynamics of fin beats will elucidate the mechanisms and potential functions of asymmetric fin bending.

**Location of pectoral fin circuitry.** The capacity of small regions of the nervous system to produce rhythmic high activity is well known and has been demonstrated in both limb and axial motor systems (e.g., Ballion et al. 2001; Cheng et al. 1998; Cohen and Wallén 1980; Kjaerulf and Kiehn 1996; Wheatley and Stein 1992) as well as hindbrain regions producing respiratory rhythms in mammals (Smith et al. 1991). Our data indicate that networks of the caudal hindbrain and rostral spinal cord can generate the pectoral fin rhythm. Further examination of this region may yield insights into the organization of the central pattern generator driving rhythmic fin beats.

Descending pathways from the hindbrain to spinal cord are important for the initiation of distinct modes of locomotion, as is well demonstrated by the escape system in fishes (reviewed by Zottoli and Faber 2000). The importance of the hindbrain in the initiation of rhythmic swimming has recently been demonstrated by a hindbrain-spinal cord preparation of adult zebrafish, which initiates rhythmic swimming when the hindbrain or hindbrain-spinal cord boundary is stimulated (Kyriakatos et al. 2011). Previously, a semi-intact preparation of the salamander that included the MLR was shown to produce a gait transition between rhythmic limb activity and limbs-tucked, nonrhythmic activity as the level of stimulation to the MLR was increased (Cabelguen et al. 2003). We found that a single pulse of stimulation applied to the otolith was sufficient to evoke a higher-frequency, fins-tucked motor pattern and a transition to the lower-frequency, rhythmic fin motor pattern in larval zebrafish following the removal of brain regions rostral to the hindbrain. Our data suggest that an intact MLR is not needed to produce the pectoral fin gait transition. It is possible that stimulation of the MLR in salamanders activated hindbrain networks that generated the higher-frequency, limbs-tucked gait or that multiple descending pathways (from either the MLR or the hindbrain) can generate gait transitions. Alternatively, salamanders and larval zebrafish may have different pathways of descending control over the selection of locomotor gait or zebrafish may have the same MLR-related pathway but can generate comparable gait transitions with hindbrain circuits. In any case, our experiments demonstrate that networks within the hindbrain and rostral spinal cord are critical for the generation of nonrhythmic and rhythmic pectoral fin gaits in larval zebrafish.

**Comparison to other limb systems.** The transition we observed in the pectoral fin motor pattern of larval zebrafish is comparable with electromyographic recordings from salamander forelimb muscles, which show that distinct forelimb muscles switch either from silence to rhythmic activity or from tonic to rhythmic activity as the frequency of axial bending decreases across a swimming to walking gait transition (Delvolvé et al. 1997; Ijspeert et al. 2007). As discussed by Thorsen and colleagues (2004), the kinematics of the larval pectoral fins mirror the kinematics of the legs in salamanders during both the slow swimming/walking gait and the faster swimming, limbs-tucked gait. It is possible that this similarity in motor patterns arises from a shared, primitive limb-axial motor circuit in a common ancestor to fish and tetrapods. Alternatively, the limb-axial motor pattern could have evolved independently in these two organisms. The more basic pattern of alternating extensor-flexor motor drive within the limb appears to be a strong commonality between pectoral fin motor systems and limb motor systems in other vertebrates such as mammals (reviewed in Kiehn 2006).

Other aspects of the fin-axial motor pattern in larval zebrafish differed from the motor pattern measured in salamanders. First, the frequency range of walking does not overlap with the frequency range of limbs-tucked swimming in salamanders (Delvolvé et al. 1997; Ijspeert et al. 2007), whereas we often observed lower-frequency swimming during nonrhythmic fin activity, prior to the switch to the rhythmic pectoral fin gait, demonstrating that there is some overlap in the swimming frequency ranges across the nonrhythmic to rhythmic fin gaits. Second, the axial motor
pattern during walking in salamanders is a standing wave, in contrast to the traveling wave seen in fishes (e.g., Cohen and Wallén 1980; Masino and Fetcho 2005). These broad differences between salamander and larval zebrafish motor patterns during a kinematically similar gait transition may be due to the distinct morphologies of these animals or to the distinct physical environments for walking versus swimming and the demands that such differences place on the organization of motor circuits that control the movement of the limbs and body. Further studies of limb motor patterns from a broader range of aquatic and terrestrial vertebrates may shed light on species-specific innovations that may have modified the function of a primitive limb motor circuit.

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