Fictive Swimming Motor Patterns in Wildtype and Mutant Larval Zebrafish

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Larval zebrafish provide a unique model for investigating the mechanisms involved in generating rhythmic patterns of behavior, such as swimming, due to the array of techniques available including genetics, optical imaging, and conventional electrophysiology. Because electrophysiological and imaging studies of rhythmic motor behaviors in paralyzed preparations depend on the ability to monitor the central motor pattern, we developed a fictive preparation in which the activity of axial motor neurons was monitored using extracellular recordings from peripheral nerves. We examined spontaneous and light induced fictive motor patterns in wildtype and mutant larval zebrafish (4-6 days post-fertilization) paralyzed with curare. All spontaneous and light induced preparations produced alternation of motor activity from side-to-side (mean contralateral phase = 50.7 ± 7.0%; mean burst frequency = 35.6 ± 4.7 Hz) and a progression of activity from head-to-tail (mean ipsilateral rostrocaudal delay = 0.8 ± 0.5 msec per segment), consistent with lateral undulation and forward propulsion during swimming, respectively. The basic properties of the motor pattern were similar in spontaneous and light induced swimming. This fictive preparation can be used in combination with conventional electrophysiological and imaging methods to investigate normal circuit function as well as to elucidate functional deficits in mutant lines. Toward this end, we show that two accordion class mutants, accordion (acc) and bandoneon (beo), have alternating activity on opposite sides of the body, contradicting the hypothesis that their deficit results
from the absence of the reciprocal glycineergic inhibition that is typically found in
the spinal cord of swimming vertebrates.

KEYWORDS

Spinal Cord
Central Pattern Generator
Rhythmic Motor Behavior
Reciprocal Inhibition
Mutant line
INTRODUCTION

Neural circuits in spinal cord play essential roles in vertebrate movements including the limb movements used for walking as well as axial movements involved in undulatory swimming. Central pattern generators located in spinal cord can produce coordinated rhythmic motor output in the absence of sensory feedback. Zebrafish larvae are a good model for examining the mechanisms underlying rhythmic motor patterns because of the genetic and optical accessibility of the preparation (Fetcho and Liu 1998; Fetcho and O’Malley 1995; Fetcho et al. 1998; Granato et al. 1996; Higashijima et al. 2003; Higashijima et al. 2004; O’Malley et al. 1996; Ritter et al. 2001). We have been establishing the foundation for a combined genetic, conventional electrophysiological, and optical analysis of spinal circuits involved in motor behavior. One significant gap has been the lack of a fictive preparation in which spinal motor patterns can be monitored in a paralyzed animal. Such preparations are necessary to record the motor patterns in normal fish as well as to examine the motor pattern disruption in mutant lines. Here we report a fictive preparation using larval zebrafish that shows the features of the motor pattern for swimming. Our analysis of the pattern provides a background for future studies of the underlying circuits. We also use the fictive preparation to test an earlier hypothesis about the functional deficit in two accordion class mutant lines.
MATERIALS AND METHODS

Electrophysiological recordings

Wildtype (local commercial supplier; Brian’s wildtype) or accordion class mutant (accordion (acc), bandoneon (beo)) zebrafish larvae (4 to 6 days post-fertilization (dpf)) were anaesthetized with 0.02% Tricaine-S (Western Chemical Inc.) in an extracellular recording solution which contained (in mM) 134 NaCl, 2.9 KCl, 1.2 MgCl₂, 2.1 CaCl₂, 10 HEPES buffer, 10 glucose, adjusted to pH 7.8 with NaOH (Legendre and Korn 1994; Drapeau et al. 1999). The preparations were paralyzed with 0.01 mM d-tubocurarine (Sigma) added to the recording solution, which significantly reduced or abolished postsynaptic muscle activity based on patch recordings from muscle fibers (data not shown). Peripheral nerve recordings were observed even at much higher curare concentrations (0.05 mM) which completely abolished synaptic activity in the muscle fibers. The extracellular solution was bubbled with ambient air and superfused continuously at 22-to-26°C.

Larvae were pinned on their side to a Sylgard lined glass bottom petri dish with short pieces (~1 mm) of fine tungsten wire (0.001 in) pushed through the notochord - one pin placed near the air bladder and another near the anus. The skin between the two pins was removed with a pair of fine forceps. For paired bilateral extracellular recordings, larvae were re-oriented into a dorsoventral posture and held in place using additional tungsten wires placed between the wires in the notochord and along the body wall (Fig. 1A). For whole cell patch recordings, collagenase (0.1%, Sigma) in recording solution was applied to the
preparation for 3-to-5 min to prepare enzymatically the muscle fibers for removal. The collagenase solution was washed off and a large bore (~15 μm in diameter) glass microelectrode attached to an extracellular suction electrode holder was used to aspirate individual muscle fibers overlying a small section (2-to-3 segments) of the spinal cord. All preparations were observed by using a water immersion objective (X40, 0.80 NA, Olympus) on an upright microscope (BX51WI, Olympus) fitted with differential interference contrast (DIC) optics.

Extracellular recording techniques were used to monitor the activity of peripheral nerves during fictive behavior. Activity occurred spontaneously but was also elicited by shining a light source (flashlight) directly at the preparation. Extracellular suction electrodes (~15 μm tip diameter) pulled on a Flaming/Brown micropipette puller (P-97, Sutter Instruments) from borosilicate glass (1.5 mm OD, 1.12 mm ID, A-M Systems Inc., Carlsborg, WA) were filled with curare-free extracellular recording solution and placed in a suction electrode holder (E series, Warner Instruments or HL-U, Axon Instruments). The tip of the suction electrode was positioned at the dorsoventral midline of a myotomal cleft where the skin had been removed and a light suction was applied to ensure a tight seal with the underlying muscle tissue and peripheral nerves. All recordings were restricted to between body segments 7 and 15. In early experiments, extracellular signals were monitored with a differential AC amplifier (model 1700, A-M Systems) at a gain of 10,000 with the low- and high-frequency cutoff set at 300 and 500 Hz, respectively. Noise was reduced with a 60-Hz notch filter. In most experiments, however, a MultiClamp 700A (Axon Instruments) amplifier
was used to monitor extracellular voltage in current clamp mode at a gain of
1,000 ($R_f = 50 \text{ MOhm}$) with the low- and high-frequency cutoff at 100 and 4,000
Hz, respectively. We found that the current clamp approach produced a better
signal-to-noise ratio than did either conventional differential AC voltage
recordings or current recordings in voltage clamp mode.

Standard whole-cell patch recording techniques, modified from Drapeau et
al. 1999, were used to monitor the activity of motor neurons in vivo. As
described above, the fish were mounted on their side and the skin and muscle
overlying a portion of the spinal cord was removed. Patch electrodes (~15
MOhms) pulled on a Flaming/Brown micropipette puller (P-97, Sutter
Instruments) from borosilicate glass (1.5 mm OD, 0.86 mm ID, Warner
Instrument Co.) were filled with patch solution (in mM) 125 K gluconate, 2 MgCl$_2$,
10 HEPES buffer, 10 EGTA, 4 Mg ATP, adjusted to pH 7.2 with KOH. We did
not correct for junction potentials as we were only concerned with the timing of
activity in the motor neurons relative to ventral root activity. Positive pressure
(30-to-50 mm Hg) was applied to the patch electrode as it approached the
exposed surface of the spinal cord. The tip of the electrode was carefully
lowered until it broke into the cord. Motor neurons were targeted for recording
based on their size, shape and position in the spinal cord. Once the tip of the
patch electrode was directly apposed to a motor neuron, release of positive
pressure allowed a gigaohm seal to form. Suction pulses were applied to break
the seal for whole-cell voltage recordings. Whole-cell voltage was monitored with
a MultiClamp 700A (Axon Instruments) amplifier at a gain of 100 ($R_f = 5 \text{ GOhm}$)
filtered at 30 kHz and digitized at 66 KHz. The recordings were accepted for
data analysis if the resting membrane potential was more negative than -45 mV.
Neurons were labeled with 0.1% Sulforhodamine B (Sigma) added to the patch
solution and fluorescent images were acquired with a CCD camera (C-72-CCD,
Dage MTI, Michigan City, IN), a frame grabber (LG3, Scion Corp., Frederick, MD)
and imaging software (Scion NIH Image, Scion Corp.) for morphological
identification.

Data acquisition and analysis

Extracellular and whole cell voltage recordings were digitized using a
digitizing board (DigiData series 1322A, Axon Instruments), acquired using
pClamp 8.2 software (Axon Instruments) and analyzed offline with a spike train
analysis program written in Matlab 5.3 (Mathworks, Natick, MA).

In the analysis program, spikes were detected with a discrimination
window. When voltage crossed a lower threshold value, but did not exceed an
upper threshold, a spike event was detected and was indicated by a raster point
above the spike (Fig. 2A). The upper threshold eliminated transient artifacts in
the recording. To prevent multiple detection of the same spike, a refractory
period (1 msec), during which spikes could not be recognized, was applied after
each detected event. To ensure that all spikes were detected, the refractory
period was considerably shorter than the shortest interspike interval (~2 msec).
Spikes were then grouped into bursts as follows. After an interburst interval (~10
msec) elapsed without any spikes detected, the next spike event was identified
as the first spike of a burst. Subsequent spikes with interspike intervals less than the interburst interval were grouped into that burst. To eliminate the effects of stray spikes, single spike events were not considered as bursts. The median spike in each burst was indicated by a diamond above the burst (Fig. 2A). An episode of fictive activity was composed of a group of sequential bursts with interburst intervals >8 msec.

The analysis program was used to determine episode duration (ED), cycle period (T), burst frequency (BF), burst duration (BD), and duty cycle (D) for each recorded peripheral nerve. In addition, either or both the phase between paired recordings on opposite sides of the same body segment (contralateral phase (\(\phi_C\))) or the phase between paired recordings at different segments on the same side of the body (ipsilateral rostrocaudal phase (\(\phi_I\))) were measured.

Episode duration was defined as the interval in msec from the first spike of the first burst to the last spike of the last burst for the phase marker peripheral nerve (see below) (Fig. 2A) and cycle period as the interval in msec from median spike to median spike of consecutive bursts (Fig. 2A). The mean cycle period (\(T_X\)) across an episode was determined for each nerve (X). Burst frequency (Hz) was defined as the inverse of the cycle period (1 / \(T_X\)). The mean burst frequency was determined for each episode. Burst duration (BD) was defined as the portion of the cycle period (T) occupied by spike activity. Duty cycle (D) was defined as the percentage of the cycle period occupied by the burst duration ((D = BD / T) X 100). The mean duty cycle across an episode for each peripheral nerve was then displayed as box plots (normalized burst duration) in the phase
diagrams (Fig. 2B; described below in section on phase diagrams). To convey information regarding the variation of consecutive bursts within an episode, some figures plotted the dependent variable against burst position in the episode (BPE). BPE was defined on a burst-by-burst basis as the median spike time of a burst (MST) divided by episode duration (ED) and expressed as a percentage of the ED \( \text{BPE} = \left( \frac{\text{MST}}{\text{ED}} \right) \times 100 \).

A multivariate analysis of variance (MANOVA) with repeated measures (SuperAnova, Abacus Concepts, Berkeley CA) was used to compare the effects of spontaneous versus light induced activity on episode duration, cycle period, and burst duration. For the comparisons, four episodes from both spontaneous and light induced activities were selected from each of the six preparations examined. We picked episodes at the onset and offset of the activity as well as at various time points in between (Fig. 1B; extracellular peripheral nerve recordings). Onset was defined as episodic activity that occurred following a significant period (> 5 sec) of inactivity. Offset, however, was more difficult to define. In some cases it was clear since a “final” episode of activity occurred followed by a period of inactivity. In others, the “final” episode in the electrophysiological record was used as the offset episode because the robust nature of the activity gave no clear indication that the episodic activity would terminate. Individual episodes were further divided into an “early” and “late” component. The “early” component consisted of bursts from the first-half of each episode, while the “late” component consisted of bursts from the second-half of each episode. The mean value for each component (“early” and “late”) was
determined and used in the analysis. Specific contrasts were used to reveal embedded relationships within the MANOVA (see Results). The P-values from these contrasts are presented in Tables 1 and 2. Differences were considered significant at a level of $P < 0.05$. A model II principal axis regression analysis (Sokal and Rohlf 1995) was used to examine the slope of the relationships between various dependent variables (contralateral phase (Fig. 5C), rostrocaudal delay (Fig. 6D), and rostrocaudal phase (Fig. 6G)) and cycle period (T). We used Pearson's product moment correlation to determine the intensity of the association between these same dependent variables and cycle period.

The phase of a given peripheral nerve was defined on a burst-by-burst basis as the time difference ($\Delta_t$) between a burst's median spike ($t_x$) and the median spike of the corresponding burst in the phase marker nerve ($t_P$) ($\Delta_t = t_x - t_P$). The time difference was then normalized to the cycle period of the phase marker nerve and expressed as a percentage: ($\phi = (\Delta_t / T) \times 100$). An anti-phasic relationship between paired contralateral peripheral nerve recordings from the same body segment was indicated by a $\sim 50\%$ phase difference. In paired ipsilateral peripheral nerve recordings, the rostral recording site was defined as the phase marker nerve. A positive phase difference indicated a phase lag with respect to the phase marker nerve (rostrocaudal progression of activity as seen in swimming), while a negative phase difference indicated a phase lead with respect to the phase marker nerve (caudorostral progression of activity as seen in struggling).
Phase diagrams were used to illustrate phase differences between peripheral nerves (Fig. 2B). The beginning and end of each box plot indicated the average time of the first and last spike, respectively, in a series of bursts from a single episode relative to the median spike time of the bursts in the phase marker nerve. Error bars indicated the standard deviation around the mean first and last spike in a burst. The average median spike time of the bursts in the phase marker nerve, indicated by a dashed vertical line that bisected the phase box near its midpoint, was positioned at 100/0% phase on the diagram. The mean median spike time for bursts in each nerve was plotted on the phase diagram with respect to the phase marker nerve. A shift of the average median spike to the right of the 100/0% position indicated a phase lag while a shift of the average median spike time to the left of the 100/0% position indicated a phase lead.

RESULTS

*Unrestrained swimming behavior reflected in the wildtype fictive motor pattern*

To compare the overall pattern of fictive motor activity with swimming behavior in unrestrained fish, we used standard extracellular recording techniques to simultaneously monitor motor output in peripheral nerves located at various body segments (Fig. 1A). Unrestrained larvae produce an episode of swimming followed by a period of inactivity. We observed discrete episodes of spontaneous and light induced fictive activity separated by intervals of inactivity. At the developmental stages examined (4 to 6 dpf), this pattern of fictive activity
(Fig. 1B) mimicked the overall pattern of periodic episodes of swimming in unrestrained larvae (Budick and O’Malley 2000; Ritter et al. 2001; Muller and van Leeuwen 2004).

Timing between peripheral nerve and ipsilateral motor neuron activity

To establish that peripheral nerve activity was synchronized with membrane potential changes in motor neurons, we examined the timing relationships between activity recorded from peripheral nerves (extracellular) and membrane potential recorded from a single ipsilateral primary motor neuron (whole-cell patch) within 1-to-3 segments more caudal (n = 4 recordings from four fish). The identity of motor neurons was confirmed after recording by imaging the dye-filled cells. During an episode of swimming, the membrane potential of motor neurons depolarized coincident with an episode of activity in the peripheral nerves and remained depolarized throughout the entire episode (Fig. 3A). Riding on the depolarization were rhythmic events, some of which reached threshold (Fig. 3B). In paired recordings, each single spike or subthreshold event in the motor neuron occurred during a burst of activity in the nearby ipsilateral peripheral nerve (Fig. 3B). The cycle period (~30 msec) and thus burst frequency (~33 Hz) of the rhythmic depolarization in the motor neurons matched those recorded from peripheral nerves. All of these observations support the conclusion that the peripheral recordings were from axons of motor neurons.
Comparison of spontaneous and light induced activity in wildtype fish

To determine whether differences in the properties of the motor pattern were present between spontaneous and light induced activity, we compared features of the motor patterns produced spontaneously or induced by light. A multivariate analysis of variance (MANOVA) with repeated measures (see Methods) revealed there was no general effect of stimulus type (spontaneous or light induced) on episode duration ($P = 0.7$), cycle period ($P = 1.0$), or burst duration ($P = 0.2$) (Table 1). Subsequently, contrasts were used to test the effects of stimulus type on cycle period and burst duration between bursts which occurred ‘early’ or ‘late’ in episodes (see Methods). Significant differences were found between ‘early’ and ‘late’ bursts for both cycle period ($T$) and burst duration ($BD$) in spontaneous ($P_T = 0.02$ and $P_{BD} = 0.0001$, respectively) and light elicited ($P_T = 0.03$ and $P_{BD} = 0.0001$, respectively) activity (Table 2). In addition, a significant difference was evident between spontaneous and light induced activity for burst duration of ‘late’ bursts within episodes ($P_{BD} = 0.004$) (Table 2).

However, given the general overall similarities in the properties of spontaneous and light induced activity, data from both types of activity were merged for all subsequent analyses.

Temporal characteristics of fictive activity

Swimming in unrestrained fish is characterized by the lateral undulation of the body wall, which is generated by the alternation of muscle contractions that originate at or near the head and progress caudally (Cohen et al. 1980; Fetcho
and Svoboda 1993; Grillner and Kashin 1976; Grillner and Matsushima 1991; Grillner et al. 1991; Roberts 1990). To assess whether the fictive motor activity replicated the characteristics of unrestrained swimming, we analyzed paired peripheral nerve recordings with the spike train analysis program (see Methods).

Episode duration varied among different episodes within individual preparations (Fig. 4A), as well as across episodes from different preparations (Fig. 4B). Fictive episode durations ranged from ~91 to 967 msec (mean ED = 303 ± 137.3 msec, n = 199 episodes from 17 preparations) (Fig. 4B, C) and were comparable to, though somewhat longer than swim episodes observed in unrestrained fish (Buss and Drapeau 2001 (mean episode duration = 180 ± 20 msec; n=12); Brustein 2003 (mean episode duration = ~200 msec)). The mean number of consecutive bursts within an episode was 10.1 ± 4.7 bursts (n = 199 episodes from 17 preparations, range = 3 to 30 bursts).

Burst durations were regular within an episode (Figs. 2A, 4D), but could vary slightly from burst-to-burst (Figs. 4D). Overall, there was not a consistent tendency for burst duration to increase or decrease as the episode proceeded (Fig. 4E). However, in approximately 21% (39 of 186 episodes from 11 preparations) of the episodes examined, the first burst of an episode was markedly longer in duration than the following bursts. Burst durations ranged from ~1.0 (in rare (1.7%) cases there were only two spikes per burst) to 44.7 msec (mean BD = 7.9 ± 4.4 msec, n = 2000 bursts in 199 episodes from 17 preparations) (Fig. 4F) and scaled with period to occupy a constant fraction of the
cycle period. Duty cycles were lower (mean DC = 27.6 ± 13.7%, n = 2000 bursts in 199 episodes from 17 preparations) than the expected ~50% (Fig. 2B).

Burst frequencies were regular within an episode (Figs. 2A, 4G), but also could vary slightly from burst-to-burst (Figs. 4G). There was no consistent tendency for burst frequency to increase or decrease as the episode proceeded (Fig. 4H). However, in some episodes (~17%; 31 of 189 from episodes) the burst frequency at the start of an episode was markedly different than the burst frequency at the end of the episode. The burst frequency increased as the episode proceeded in 11 episodes and decreased as the episode proceeded in 20 episodes. Burst frequency ranged from 20.3 to 63.1 Hz (mean BF = 35.6 ± 4.7 Hz, n = 2000 bursts in 199 episodes from 17 preparation) (Fig. 4I) and was comparable to the swim (tail-beat) frequencies observed in unrestrained animals (Budick and O’Malley 2000; Ritter et al. 2001, 15 to 70 Hz; Buss and Drapeau 2001, 25-63 Hz; Muller and van Leeuwen 2004, 30 to 100 Hz in 3 dpf fish). Accordingly, cycle period ranged between 15.8 to 49.3 msec (mean T = 28.6 ± 3.7 msec, n = 2000 bursts in 199 episodes from 17 preparations).

Phase relationships during fictive activity

To examine the side-to-side pattern of activity between bursts during fictive episodes, we used paired extracellular electrodes to record simultaneously from contralateral peripheral nerves within the same body segment (Fig. 1A, B). A robust alternation of activity was observed (Fig. 2A, 5A) in each preparation examined (n = 11). There was no indication of a preference for activity to initiate
on a particular side of the fish (data not shown). The contralateral phase
difference was regular from burst-to-burst within an episode (Fig. 5B), showed a
slight tendency to decrease as cycle period increased ($r = -0.09, P = 0.04$; Fig.
5C), and was normally distributed across preparations (Fig. 5D) (mean
contralateral phase difference = $50.7 \pm 7.0\%$, $n = 537$ bursts in 55 episodes from
11 preparations).

To examine the progression of activity along the rostrocaudal axis of the
fish during fictive episodes, we used paired extracellular electrodes to record
simultaneously from ipsilateral peripheral nerves located at different body
segments (Fig. 1A). In all wildtype preparations ($n = 11$), we observed a
progression of activity from head-to-tail (Figs. 2A,B, 6A), consistent with
swimming behavior. A reversal in the progression of activity (i.e. tail-to-head), as
seen in struggling, was not observed (0 of 11) during spontaneous or light elicited
activity. However, struggling was observed when other forms of stimuli were
applied, such as repetitive electrical stimulation (~0.1-1.0 msec pulse width; ~20
µA (see Soffe 1993)) to the yolk sac or slight pressure on the dorsomedial aspect
of the head (data not shown). The rostrocaudal delay observed during fictive
activity was regular from burst-to-burst within episodes that produced regular
cycle periods (Fig. 2A, 6B) and increased as the number of body segments
between recording electrodes increased (Fig. 6C).

To compare the head-to-tail delays from different fish and to account for
the different number of segments separating the recording electrodes in the
preparations, we normalized the absolute rostrocaudal delay by dividing it by the
number of segments separating the recording electrodes. A small positive correlation between the normalized rostrocaudal delay and cycle period was found among pooled data from all preparations \((r = 0.16, P < 0.001; n= 1744\) bouts in 178 episodes from 11 fish), indicating that rostrocaudal delay increased as cycle period increased. The mean slope \((0.03 \pm 0.02; n = 11)\) of the regression lines fitted to the plots of normalized rostrocaudal delay versus cycle period from individual preparations was determined using a model II principal axis regression. A representative example of such a fit is shown for a preparation that produced cycle periods over a broad range \((\sim 15 \text{ to } 45 \text{ msec})\) during the fictive activity (Fig. 6D). The 95% confidence interval lines included the origin in 8 of the 11 cases, suggesting that the actual regression line could pass through, or very near to the origin.

The mean rostrocaudal delay per segment \((0.8 \pm 0.5 \text{ msec}; n = 1744\) bursts in 178 episodes from 11 preparations) was on average 2.8% of the mean cycle period \((28.6 \pm 3.7 \text{ msec})\). Since zebrafish larvae have \(\sim 33\) body segments, a 2.8% delay translated into approximately 92% of a wave of activity along the body at any point in time. Our measured average slope of 0.03 msec per segment for the regression of normalized rostrocaudal delay versus cycle time would produce \(\sim 99\%\) of a wave of activity along the body at any point in time. Both estimates showed that the fictive swimming activity represented \(\sim 90\) to 99% of a wave of activity along the body at any point in time. These estimates are consistent with high-speed video analyses of swimming behavior in which normal, unrestrained zebrafish larvae at 3-5 dpf have approximately one wave of
bending along the body at any point in time (Budick and O’Malley 2000; Liu and Fetcho 1999).

In many systems that generate rhythmic swimming motor patterns, such as lamprey (Grillner 1974; Grillner and Kashin 1976; Wallen and Williams 1984; Grillner and Wallen 2002), crayfish swimmeret (Mulloney 1997; Jones et al. 2003), *Xenopus* tadpoles (Tunstall and Roberts 1991; Tunstall and Sillar 1993; Tunstall et al. 2002), leeches (Pearce and Friesen 1988; Cang and Friesen 2002) and fish (Wallen and Williams 1984; Buchanan 1992; Fetcho and Svoboda 1993; Sigvardt and Williams 1996), rostrocaudal delay scales proportionally with cycle period to generate a constant rostrocaudal phase difference between adjacent segments across a range of cycle frequencies. To address this relationship in zebrafish larvae, we asked whether the rostrocaudal phase difference (normalized to a single body segment) within single episodes and among different episodes varied with cycle period. The rostrocaudal phase difference observed was regular from burst-to-burst within individual episodes (Fig. 2A, B, 6E) and varied with the distance between recording sites (Fig. 6F). More importantly, rostrocaudal delay scaled proportionally with cycle period when normalized to a single body segment, leading to a constant rostrocaudal phase (Fig. 6G) (mean rostrocaudal phase difference per segment = 2.6 ± 1.7%; n = 1744 bursts in 178 episodes from 11 preparations). The Pearson product moment correlation did not indicate a significant relationship between normalized rostrocaudal phase and cycle period (r = -0.01; P = 0.6).
Examination of the fictive motor pattern in motor mutants

To determine whether the pattern of fictive activity in the accordion class of motor mutants suggested a central mechanism potentially responsible for the behavioral phenotype, we monitored fictive peripheral nerve activity in these mutants to determine if the general properties of the motor pattern were like wildtype. Since these mutants (accordion (acc) and bandoneon (beo)) exhibited bilateral contractions during unrestrained, free swimming, we asked whether there was a loss of the normal side-to-side alternation in the mutants that might reflect a disruption of left/right coordination in the network. In all mutants examined, we observed rhythmic bursting within episodes as well as an anti-phasic (~50%) relationship of paired contralateral peripheral nerve recordings within the same body segment (Fig. 7A, B; bottom sweeps). This indicated that at least there was not a wholesale disruption of left/right coordination. A head-to-tail progression of activity was observed in all acc mutant preparations examined with recording electrodes placed at different points along the rostrocaudal axis of the body (2 of 2) (data not shown). There was no evidence for a reversal (tail-to-head) in the progression of activity, as seen in struggling, during spontaneous or light elicited activity (0 of 2).

Mutants from the beo subclass showed the more severe behavioral phenotype (data not shown), which was reflected in the extracellular peripheral nerve recordings (Fig. 7B). The fictive pattern of activity in beo was more disorganized when compared to either wildtype or acc (compare Figs. 1A, 7A, 7B). Even so, left/right coordination was maintained as indicated by the rhythmic
alternation of activity between paired contralateral peripheral nerve recordings in beo (Fig. 7B; lower trace).

DISCUSSION

The accessibility of the larval zebrafish preparation to optical, genetic, and electrophysiological techniques provides a unique model for investigating the mechanisms which underlie motor control of rhythmic patterns of behavior, such as swimming. Previous studies of axial muscle activity in larval zebrafish presented recordings from muscle fibers because the peripheral nerves were thought to be too small and inaccessible for extracellular recording of fictive motor activity (Buss and Drapeau 2001). Here we describe such a fictive preparation, in which activity in axial motor neurons was monitored using extracellular recordings from peripheral nerves (Fig. 1) to provide a detailed description of the activity pattern observed during spontaneous and light elicited fictive swimming (Fig. 2). The work provides a necessary foundation both for future studies of the underlying circuits as well as for understanding motor pattern disruption in mutant lines. Some of the work was previously presented in an abstract (Masino and Fetcho 2003).

We are confident that the activity observed in our recordings was due to motor neuron activity based on several lines of evidence. First, curare works in a concentration dependent manner to reduce and ultimately eliminate all postsynaptic activity in muscle fibers (data not shown). We could record peripheral motor activity at concentrations of curare that completely eliminated
postsynaptic potentials in muscle fibers, indicating that the recordings were from nerves and not muscle fibers. Second, the activity in peripheral nerves coincides with activity in individual motor neurons during fictive swimming. Finally, if the activity in the peripheral nerve recordings was due to an afferent component, then not only would the sensory input need to be rhythmic in the absence of movement, but it would also have to be rhythmic at a very high frequency (~30 Hz and greater) in synchrony with the ipsilateral motor neuron activity, both of which seem unlikely. Taken together, these observations suggest that the activity observed in our peripheral nerve recordings is the result of active motor neurons.

Normal unrestrained larval zebrafish produce both spontaneous and light elicited swimming behavior. Using the extracellular recordings from peripheral nerves, we found that fictive preparations also produced spontaneous and light elicited motor activity. Our analyses did not show any dramatic differences between the basic properties (e.g. episode duration, burst frequency, burst duration) of the two forms of activity in fictive preparations (Tables 1 and 2). Consequently, we primarily used light to elicit rhythmic activity because it allowed control over the onset of the motor output.

The motor pattern showed several features of the periodic episodes of swimming produced by freely swimming larvae, which typically swim for a short time, pause and then swim again. The fictive motor pattern that occurred spontaneously or in response to light also consisted of discrete episodes of rhythmic bursts of activity in peripheral nerves (Figs. 1 and 2). The mean
episode duration (303 ± 137.7 msec; n = 199 episodes) generated by fictive preparations (Fig. 4A-C) was longer than the mean episode duration observed during swimming in unrestrained fish (Buss and Drapeau 2001, mean episode duration = 180 ± 20 msec, n=12; Brustein 2003, mean episode duration = ~200 msec); however, the range of episode durations overlapped with that of free-swimming fish. Additionally, the range of rhythmic burst frequencies (20 to 63 Hz) recorded from individual nerves during episodes in fictive preparations (Fig. 4G-I) was similar to the range of tail beat frequencies observed in free-swimming fish (Budick and O’Malley 2000; Ritter et al. 2001, 15 to 70 Hz; Buss and Drapeau 2001, 25-63 Hz; Muller and van Leeuwen 2004, 30 to 100 Hz in 3 dpf fish during slow start swimming). These data suggested that the fictive motor pattern was that for swimming.

Curare was used in our experiments as a paralytic agent to block acetylcholine (ACh) receptors at the neuromuscular junction. Curare has been shown to function as a GABA antagonist (Lebeda et al. 1982; Wotring and Yoon 1995; Caputi et al. 2003). Since GABA can control or regulate the speed of locomotor activity (Kroggaard-Larsen and Johnston 1975; Tegner et al. 1993; Cazalets et al. 1994; Cazalets et al. 1998), it is possible that curare could modify the fictive swimming motor pattern in zebrafish larvae. We cannot rule out subtle effects of curare on the fictive motor pattern, however, the overlap of the burst frequencies we observed with the tail-beat frequencies in freely swimming fish suggests that curare does not dramatically change the frequency of the normal rhythm.
To confirm that the pattern of motor activity observed in fictive preparations was indeed swimming, we compared the pattern of activity monitored in the peripheral nerves of fictive preparations with the features common to patterns of activity recorded in electromyograms (EMGs) from freely swimming fish. Fishes (agnathans, cartilaginous, and bony fishes) and swimming salamanders and frog tadpoles generate a swimming electromyographic motor pattern with several common features (Cohen and Wallen 1980; Cohen et al. 1982; Fetcho and Svoboda 1993; Grillner 1974; Grillner and Kashin 1976; Mos et al. 1990; Roberts 1981; Williams et al. 1989). A side-to-side alternation of activity generates the lateral undulation of the body, with the burst duration occupying nearly half of the cycle time between successive bursts of activity in a segment. A similar, robust alternation of activity with a ~50% contralateral phase difference was evident in all fictive zebrafish preparations examined (Fig. 5). This pattern of alternating activity is consistent with the alternating bending seen during swimming. In fishes and amphibians, a traveling wave of activity originates at or near the head and progresses along the body toward the tail during swimming. In all fictive preparations examined, activity initiated at a more rostral location and progressed caudally (Fig. 6), consistent with the pattern that usually produces the forward propulsion necessary for swimming.

In swimming fishes, as cycle period varies with swim frequency, the burst duration remains proportional to period to generate a constant duty cycle of approximately 50% (Wallen and Williams 1984; Grillner and Kashin 1976;
Williams 1986). The burst duration scales with cycle time in our fictive preparations as well (Fig. 2B), but the duty cycles (mean duty cycle = 27.6 ± 13.7%) were lower than the expected ~50%. This might result from an under-sampling of the overall activity during swimming. Because the segmental ventral roots project out of the ventral spinal cord well medial to the lateral edge of the body wall, our superficially located recording electrodes probably sampled only a fraction of the motor axons from a particular segment.

Finally, in swimming animals as the wave propagates from head to tail, the ipsilateral rostrocaudal delay scales proportionally with cycle period to generate a constant rostrocaudal phase lag between adjacent segments across a range of cycle frequencies (Wallen and Williams 1984; Fetcho and Svoboda 1993; Cang and Friesen 2000; Grillner and Wallen 2002; Tunstall et al. 2002; Jones et al. 2003). The fictive preparations we studied also showed a constant rostrocaudal phase lag at different swimming frequencies (Fig. 6G).

Estimates of the relationship between rostrocaudal delay and cycle period indicated that the fictive zebrafish preparation produced approximately one full wave of activity (90 to 99%) along the body at any point in time. This result is consistent with observations made during swimming in lampreys, crayfish and leeches. In lamprey, the rostrocaudal delay is ~1% of the cycle time (Grillner et al. 1991; Williams et al. 1989). Because lampreys have ~100 body segments, there is about one complete wave along the body at any point in time. In crayfish, the rostrocaudal phase lag between the movements of each of the 4 pairs of swimmerets is approximately 25%, thus generating a full wave of activity
along the body at any point in time (Mulloney et al. 1998; Skinner and Mulloney 1998). Leeches also generate nearly one full wave of activity at any given time during dorsoventral undulatory swimming (Kristan et al. 1974; Hill et al. 2003). In contrast, however, adult goldfish generate less than a complete wave of activity (63%) along the body at any point in time (Fetcho and Svoboda 1993). This reduction in the extent of the wave of activity along the body may be due to the fact that adult goldfish are much less flexible than any of the other preparations mentioned above and thus mechanically are limited in their ability to generate a complete wave during swimming.

The baseline data from fictive preparations can be used to assess potential central deficits in different motor mutant lines. For example, a motor mutation originally identified by Granato et al. (1996) shows simultaneous bilateral contractions during swimming which result in the fish compressing along the rostrocaudal axis, leading to the mutant name ‘accordion’ (acc). The authors suggested that the disruption in motor behavior was due to a loss of glycinergic reciprocal inhibition in the spinal network that produced swimming. Recordings from fictive acc preparations allowed a quick assay of the integrity of their pattern generating networks. Paired contralateral peripheral nerve recordings within the same body segment showed that these mutants retained the ability to generate rhythmic bursts within episodes as well as a robust anti-phasic (~50% contralateral phase difference) relationship (Fig. 7A). This refutes the hypothesis that a wholesale disruption of reciprocal inhibition is responsible for generating the acc mutant phenotype.
These data are consistent with recent reports of the cloning of the acc gene, which show that the phenotype is produced by a primary deficit in the periphery rather than in the CNS (Gleason et al. 2004; Hirata et al. 2004). The acc mutation is in a gene that encodes the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase 1 (SERCA1). The mutation leads to an impaired Ca\(^{2+}\) reuptake in the sarcoplasmic reticulum of muscle and a slowed relaxation time of the muscle. The resulting overlap of muscle contractions on the two sides of the body leads to the accordion phenotype.

Bandoneon (beo), another ‘accordion’ class mutant identified by Granato et al. (1996), shows a more severe behavioral phenotype and a less well organized pattern of central motor activity than acc mutants. Nonetheless, they retain some rhythmic bursting and a side-to-side alternation during fictive swimming (Fig. 7B). This suggests that they too do not have a wholesale disruption of left/right coordination. Similar relatively simple recordings might help to identify sub classes of mutants which show disruptions of pattern that might reflect true central pattern generating deficits.

In conclusion, our development of a fictive preparation of larval zebrafish will allow for a more thorough exploration of the neural circuits involved in swimming, as well as in other rhythmic behaviors such as struggling. The activity of individual cells monitored by whole-cell patch recordings can be linked to the fictive motor pattern recorded from peripheral nerves. This preparation also simplifies the assessment of motor deficits in mutant lines. It will be increasingly useful for relating patterns of activity imaged in cells (with calcium and/or voltage
indicator dyes) to the motor output as well as for examining how genetic or optical perturbations of neurons affect the motor patterns.

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GRANTS

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FIGURE LEGENDS

Fig. 1. Extracellular peripheral nerve recording setup and electrical activity in the fictive preparation. A: Schematic diagram of a larval zebrafish at 4-6 dpf. Short horizontal lines beginning at the hindbrain and ending in the tail indicate segmentation. Fine tungsten wires are used to secure the fish in a dorso-ventral posture. Lateral wires (solid thick black lines) are pushed through the notochord – one near the air bladder and one near the tail. An additional four wires (4 black circles represent the wires end-on) are positioned against the body wall and lateral wires and pushed into the Sylgard dish to produce tension along the body. Extracellular suction electrodes are placed at various points along the midbody axis to monitor activity in peripheral nerves from axial motor neurons. B: Central motor output from a fictive preparation at a slow time base. Multiple synchronized bouts of episodic activity are observed in each of the peripheral nerve recordings. Note the fictive activity mimicked the pattern of swimming in unrestrained larvae – several episodes of activity each of which is followed by an interval of inactivity. Segment (S) number is indicated in parentheses. The gray box outlines a single episode of activity shown in Figure 2A.

Fig. 2. Recording of a single episode of fictive activity. A: Example of the spike train analysis. A raster point placed above each detected spike indicates individual events. The median spike in each burst is indicated by a black diamond above the burst. Open squares and open diamonds indicate the first
and last spike in a burst, respectively. Episode duration (ED), cycle period (T), and the time difference between median spikes from phase-locked bursts ($\Delta t$) are measured. Phase ($\phi$) is calculated as $\phi = (\Delta t / T) \times 100$. B: Phase diagrams display the phase relationships and duty cycles of the peripheral nerves from A. The black dashed vertical line (100/0%) indicates the occurrence of the average median spike of bursts in the right peripheral nerve in segment 11 (Right (S11)) (see Methods). The phase relationships of bursts in the other peripheral nerves are plotted on the phase diagram relative to this “phase marker”. The gray dashed vertical lines indicate the occurrence of the average median spike of bursts in the peripheral nerves in segments 11 (Left (S11); ~50%) and 16 (Right (S16); ~12%). Occurrence of the average median spike to the right of the dashed line indicates the phase lag. Error bars indicate standard deviation of the first and last spikes across bursts.

Fig. 3. Synchronized activity between ipsilateral peripheral nerves and a motor neuron. A: Paired whole cell (top trace; Left (S11)) and extracellular (bottom trace; Left (S15)) recordings show that the motor neuron is active during bursts of activity in an ipsilateral peripheral nerve. B: Rhythmic bursting within an episode shows that motor neurons generate a single spike or subthreshold event (*) that occurs during a burst in an ipsilateral peripheral nerve.

Fig. 4. Analysis of the general properties of fictive episodic activity. A: Plot of episode duration of episodes from two fish (each represented by a different
symbol).  B: Histogram plot of episode durations measured for each wildtype preparation (n=17).  C: Frequency histogram showing the variability of episode duration among preparations.  D: Plot of burst duration against burst position in episode (BPE; see Methods) within a single episode of activity for two fish (each represented by a different symbol).  E: Plot of burst duration against BPE for all preparations.  F: Frequency histogram showing the variability of burst duration among preparations.  G: Plot of burst frequency against BPE within a single episode of activity for two different fish (each preparation represented by a different symbol).  H: Plot of burst frequency against BPE for all preparations.  I: Frequency histogram showing the variability of burst frequency among preparations.

Fig. 5. Alternating (side-to-side) pattern of activity during a fictive episode.  A: Extracellular recordings indicate a robust alternation of activity ($\phi_c \approx 50\%$) between paired contralateral peripheral nerves from the same body segment (S9).  Gray boxes overlay the rhythmic burst activities in the Right (S9) peripheral nerve to indicate the alternating pattern of activity.  B: Plot of contralateral phase against BPE within a single episode of activity.  C: Plot of contralateral phase against cycle period (T) for all preparations (Pearson product moment correlation $= -0.09; P = 0.04$).  D: Frequency histogram showing the variability of contralateral phase among preparations.
Fig. 6. Rostrocaudal progression of activity during a fictive episode. A: Extracellular recordings indicate a head-to-tail delay between bursts in paired ipsilateral peripheral nerve recordings from different rostrocaudal points along the body. Gray boxes overlay the rhythmic burst activities in the Right (S8) peripheral nerve to indicate the head-to-tail delay of activity. B: Plot of rostrocaudal delay against BPE within a single episode of activity. C: Plot of mean rostrocaudal delay against the number of body segments separating the recording electrodes for two fish (each represented by a different symbol). Error bars indicate the standard deviation. D: Plot of rostrocaudal delay per segment against cycle period (T) for a single representative preparation (slope = 0.02). E: Plot of rostrocaudal phase against BPE within a single episode of activity. F: Plot of mean rostrocaudal phase against the number of body segments separating the recording electrodes for two fish (same fish and symbols as in C). Error bars indicate standard deviation. G: Plot of rostrocaudal phase normalized per segment against cycle period (T) for all preparations (Pearson product moment correlation = -0.01; P = 0.6).

Fig. 7. The side-to-side alternation of activity observed during fictive swimming in wildtype larvae is retained in the accordion class of mutants. A: Accordion (acc) mutants show episodic activity, similar to wildtype larvae, during fictive swimming (upper pair of traces). At a faster sweep speed, there is a clear alternation of bursting activity between paired contralateral peripheral nerve recordings (lower pair of traces; Left (S13) and Right (S13)). Gray boxes overlay the rhythmic burst
activities in the Right (S13) peripheral nerve to indicate the alternating pattern of activity. B: In *bandoneon* (*beo*) mutants, episodic activity is less organized and episode durations tend to be longer than in both wildtype and *acc* mutant fish. An alternation of bursting activity between paired contralateral peripheral nerve recordings is present (lower pair of traces; Left (S9) and Right (S9)), however, it is not as clear as that observed in either wildtype or *acc* fish. Gray boxes overlay the rhythmic burst activities in the Left (S9) peripheral nerve to indicate the alternating pattern of activity.
TABLES

Table 1. Comparison of the general properties of fictive activity between spontaneous and light induced motor patterns (MANOVA with repeated measures).

<table>
<thead>
<tr>
<th>Stimulus Type</th>
<th>Mean Episode Duration (msec)</th>
<th>Mean Burst Duration (msec)</th>
<th>Mean Cycle Period (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous</td>
<td>271.0 ± 138.0</td>
<td>6.8 ± 4.4</td>
<td>28.3 ± 3.2</td>
</tr>
<tr>
<td>Light Induced</td>
<td>327.4 ± 133.3</td>
<td>8.4 ± 4.0</td>
<td>28.5 ± 3.8</td>
</tr>
<tr>
<td>P value</td>
<td>0.7</td>
<td>0.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Table 2. Comparison of fictive activity between spontaneous and light induced motor patterns at ‘early’ and ‘late’ times within episodes.

<table>
<thead>
<tr>
<th>Stimulus Type</th>
<th>Mean Cycle Period (msec)</th>
<th>P value</th>
<th>Mean Burst Duration (msec)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early</td>
<td>Late</td>
<td>Early</td>
<td>Late</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>32.0 ± 4.0</td>
<td>30.1 ± 3.3</td>
<td>0.02</td>
<td>11.0 ± 8.3</td>
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<tr>
<td>Light Induced</td>
<td>31.9 ± 3.5</td>
<td>30.2 ± 3.4</td>
<td>0.03</td>
<td>11.4 ± 7.8</td>
</tr>
<tr>
<td>P value</td>
<td>0.9</td>
<td>0.8</td>
<td>0.08</td>
<td>0.004</td>
</tr>
</tbody>
</table>
Figure 1
Masino and Fetcho
Figure 2
Masino and Fetcho

A

Episode Duration

Left (S11)

Right (S11)

Right (S16)

\[ \phi = (\Delta_t / T) \times 100 \]

50 msec

B

Left (S11)
Right (S11)
Right (S16)

0 25 50 75 100/0 25 50 75 100

Phase (%)
Figure 3
Masino and Fetcho

A
Motor Neuron
Left (S11)

Peripheral Nerve
Left (S15)

B

* * *

20 mV

1 sec

10 mV

20 msec
Figure 4
Masino and Fetcho

A

B

C

D

E

F

G

H

I
Figure 5
Masino and Fetcho

A

\[ \phi_c = (\Delta_t / T) \times 100 \]

Left (S9)

Right (S9)

20 msec

B

Contralateral Phase (\(\phi_c\))

Burst Position in Episode (%)

C

Contralateral Phase (\(\phi_c\))

Cycle Period (msec)

D

Contralateral Phase (\(\phi_c\))

Count

Mean \(\phi_c = 50.7 \pm 7\)
Figure 6
Masino and Fetcho

\[ \phi_i = \left( \frac{\Delta_t}{T} \right) \times 100 \]

A

Right (S8)

Right (S17)

20 msec

B

Rostral-Caudal Delay (msec)

C

Rostral-Caudal Delay (msec)

D

Rostral-Caudal Delay (msec / segment)

E

Rostral-Caudal Phase

F

Rostral-Caudal Phase

G

Rostral-Caudal Phase

Burst Position in Episode (%)

Number of Segments

Cycle Period (msec)