Episodic Swimming in the Larval Zebrafish is Generated by a Spatially Distributed Spinal Network with Modular Functional Organization

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Despite the diverse methods vertebrates use for locomotion, there is evidence that components of the locomotor central pattern generator (CPG) are conserved across species. When zebrafish begin swimming early in development they perform short episodes of activity separated by periods of inactivity. Within these episodes, the trunk flexes with side-to-side alternation and the traveling body wave progresses rostrocaudally. In order to characterize the distribution of the swimming CPG along the rostrocaudal axis, we performed transections of the larval zebrafish spinal cord and induced fictive swimming using N-Methyl D-Aspartate (NMDA). In both intact and spinalized larvae, bursting is found throughout the rostrocaudal extent of the spinal cord and the properties of fictive swimming observed were dependent on the concentration of NMDA. We isolated series of contiguous spinal segments by performing multiple spinal transections on the same larvae. While series from all regions of the spinal cord have the capacity to produce bursts, the capacity to produce organized episodes of fictive swimming has a rostral bias: in the rostral spinal cord only 12 contiguous body segments are necessary, whereas 23 contiguous body segments are necessary in the caudal spinal cord. Shorter series of segments were often active, but produced either continuous rhythmic bursting or sporadic, non-rhythmic bursting. Both episodic and continuous bursting alternated between the left and right sides of the body and showed rostrocaudal progression, demonstrating the functional dissociation of the circuits responsible for episodic structure and fine burst timing. These findings parallel results in mammalian locomotion, and we propose a hierarchical model of the larval zebrafish swimming CPG.
KEYWORDS: Central pattern generator, transection, NMDA, locomotion
INTRODUCTION

Central pattern generators (CPGs) are neural circuits capable of producing a patterned motor output even in the absence of a patterned input (Marder and Calabrese, 1996). The spinal cord contains a CPG or CPGs that are important to the production of locomotion (Grillner, 2006). Despite the diverse methods vertebrates use for locomotion, there is evidence that components of the CPG are conserved across species (Grillner and Jessell, 2009; Kiehn, 2011). Larval zebrafish are an attractive species for studying motor systems (Fetch and Liu, 1998; Fetch, 2007; McLean and Fetch, 2011). While the neuronal circuits for some motor behaviors such as escape (O’Malley et al., 1996; Satou et al., 2009; Koyama et al., 2011) and early touch response (Downes and Granato, 2006; Pietri et al., 2009) have been characterized, the CPG circuits that control swimming in larval zebrafish are less well characterized (Kimura et al., 2006; McLean et al., 2008; Eklöf-Ljunggren et al., 2012).

Zebrafish begin swimming at 3 days post fertilization (dpf) (Fuiman and Webb, 1988; Buss and Drapeau, 2001; Muller and Von Leeuwen, 2004) and perform infrequent episodes of locomotion that last several seconds (Buss and Drapeau, 2001). Later, at 4 dpf, larvae transition to perform more frequent but shorter episodes of locomotor activity, sometimes described as a “beat and glide” pattern (Buss and Drapeau, 2001). The muscle contractions within these swimming episodes, in both swimming behaviors, are timed to alternate side-to-side and have rostrocaudal progression (Batty et al., 1984; Borla et al., 2002), which we collectively refer to as “coordination.” Similarly, paralyzed preparations of larval zebrafish produce episodes of peripheral nerve activity indicative
of motor neuron firing (Masino and Fetcho, 2005). Within episodes, peripheral nerve activity is organized into discrete bursts that have the same coordinated pattern as trunk flexions in free swimming zebrafish larvae (Masino and Fetcho, 2005), similar to the correspondence between fictive and real behavior in several other swimming vertebrates (Grillner, 1974; Cohen and Wallen, 1980; Kahn and Roberts, 1982; Fetcho and Svoboda, 1993). Normal swimming in larval zebrafish at 4 dpf and later, both real and fictive, is defined by episodic organization and coordination.

When mapping a CPG, it is helpful to know the distribution of its neuronal components. One approach to characterizing the distribution of these CPG components is through the use of lesions (Ho and O'Donovan, 1993; Kjaerulff and Kiehn, 1996). There are two benefits to this approach. First, locomotor functions produced by spatially distinct neuronal circuits can be dissociated. Sagittal sections of the spinal cord have been used in several species to demonstrate that the locomotor rhythm does not depend upon connections that cross the midline (Kahn and Roberts, 1982; Kjaerulff and Kiehn, 1996; Cangiano and Grillner, 2003). Second, lesions can be used to simplify the system being studied. Horizontal transections of the spinal cord have localized the CPG to the ventral spinal cord (Kjaerulff and Kiehn, 1996). Similarly, in a CPG composed of segmentally reiterated oscillators (Matsushima and Grillner, 1990), transections may allow investigation of simpler isolated oscillators.

Transections of the spinal cord produce “series” of contiguous spinal segments. Short series of spinal segments from dogfish and lamprey are sufficient to produce fictive
locomotion regardless of rostrocaudal position in the spinal cord (Grillner, 1974; Cohen and Wallen, 1980). Among limbed vertebrates, the bulk of the locomotor circuits are located in the spinal limb enlargements. In some limbed vertebrate model systems, such as embryonic chick, there is not a strong effect of rostrocaudal position within the limb enlargement on the capacity of segments to produce the locomotor rhythm (Ho and O’Donovan, 1993). Other model systems show a strong effect of rostrocaudal position on rhythm generating capacity (Kjaerulff and Kiehn, 1996), including extreme examples where a CPG is located in one or two spinal segments (Deliagina et al., 1983; Wheatley et al., 1994). A previous report in larval zebrafish showed episodic motor neuron spiking in an isolated series of two body segments (McDearmid and Drapeau, 2006), however, the effect the of rostrocaudal position of the segments and the coordination of the pattern produced was not described. Given the differences in rostrocaudal distribution of CPG elements between species, it was our objective to characterize the rostrocaudal distribution of the swimming CPG in larval zebrafish.

We hypothesized several possible architectures for the larval zebrafish swimming CPG: 1) The CPG is composed of segmentally reiterated oscillators, 2) There is a single CPG distributed throughout the entire spinal cord, or 3) There is a single CPG located in a small region of the spinal cord. We evaluated the episodic structure and coordination of the fictive swim pattern produced by series of spinal segments of different lengths in response to N-Methyl D-Aspartate (NMDA), an activator of the larval zebrafish swimming CPG (McDearmid and Drapeau, 2006). We found that only longer series (≥12 of 33 total spinal segments) produced episodic fictive swimming, and that shorter
series often produced continuous rhythmic bursting or sporadic, non-rhythmic bursting.

Fewer rostral spinal segments (≥12) than caudal segments (≥23) were necessary to produce episodic swimming. We also found that the degree of coordination, that is, side-to-side alternation and rostrocaudal progression, was not different between preparations that produced episodic fictive swimming and those that produced tonic bursting. This result prompted us to propose a model of the larval zebrafish swimming CPG that separates the circuits for episode generation and coordination.
MATERIALS AND METHODS

Animals and solutions

All procedures were approved by the Animal Care and Use Committee of the University of Minnesota Twin Cities. Wild type adult zebrafish (*Danio rerio*, Segrest Farms, Gibsonton, FL) were maintained in a zebrafish core facility. Adult zebrafish were set up to produce daily clutches of embryos with timed fertilization between 8:45 - 9:00am. Embryos and larval zebrafish were maintained in petri dishes filled with embryo water (60 µg/ml Instant Ocean® salt mix, Cincinnati, OH) in a 28.5°C incubator with a 14:10 light:dark cycle. Larval zebrafish 4 to 6 dpf were used in these studies. Chemicals and drugs were obtained from Sigma-Aldrich Chemical (St. Louis, MO), unless otherwise noted. Zebrafish Ringer’s solution was composed of (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).

Procedure

The following procedure was adapted from Masino and Fetcho (2005). Zebrafish larvae were anesthetized for 5 minutes with 0.02% Tricaine-S (Western Chemical, Ferndale, WA) in Ringer’s solution. For unilateral recordings, larvae were transferred to Sylgard®-lined dissecting dishes and pinned on their sides through the notochord using short pieces of fine tungsten wire (0.001” diameter). Typically, a rostral pin was placed between body segment (S) 5 and S8 and a caudal pin between S20 and S25 (Fig. 1A). In order to access the peripheral motor nerves and provide better perfusion access, the skin between the tungsten pins was removed using a sharp tungsten probe and fine
forceps (Fine Science Tools, Foster City, CA). For bilateral recordings, skin was removed from both sides of the larvae and the larvae were repositioned dorsal side up using additional pins to hold them in position. In order to prevent muscle contractions during recording, larvae were paralyzed using 5 µl of 0.1 mM α-bungarotoxin (Tocris, Ellisville, MO) added to the small amount (~15 µL) of Ringer’s solution in the dissection dish. The larvae were kept in α-bungarotoxin for 10 minutes before superfusion with Ringer’s solution began. Larvae were either left intact, spinalized (spinal cord transected at S3 in order to separate brain from spinal cord), or transected at multiple points along the body using a razor blade shard (FA-10 Feather S, Ted Pella, Redding, CA) clamped by a blade holder (Fine Science Tools, Foster City, CA). In the intact preparation, fictive swimming occurred spontaneously or was evoked by shining light on the fish. In the spinalized preparation, swimming was induced with 50 - 200 µM NMDA in Ringer’s solution.

Electrophysiology

After transferring the larva to the stage of an Olympus BX51 WI microscope, (Center Valley, PA), superfusion with Ringer’s solution at room temperature (21 - 25 °C) was started. Glass suction electrodes were fashioned from 1.5 mm o.d. x 1.12 mm i.d. glass tubes (A-M Systems, Sequim, WA) and pulled on a P-97 electrode puller (Sutter Instruments, Novato, CA). Electrodes were filled with Ringer’s solution. Tip sizes ranged from 9 to 15 µm. The electrodes were placed in electrode holders that were inserted into headstages (50 MΩ) attached to an Axon Multiclamp 700B amplifier (Molecular Devices, Union City, CA). The electrodes were positioned over the
peripheral nerves using micromanipulators (Siskiyou, Grants Pass, OR). Current clamp signals were digitized by an Axon Instruments Digidata 1440A (Molecular Devices, Union City, CA). Data was recorded using pClamp 10 software (Molecular Devices, Union City, CA). Signals were sampled at 10 kHz and band pass filtered to 100 - 1000 Hz.

**Data Analysis**

Organized fictive swimming in larval zebrafish consists of motor neuron bursts clustered into episodes (Fig. 1B,C). A program written in Matlab (Mathworks, Natick, MA) was used to analyze episodically organized extracellular peripheral nerve voltage recordings. The program detected the presence or absence of activity at each voltage sample \(v(n)\). For each \(v(n)\), the algorithm determined a voltage autocorrelation \(c_n(k)\) over a small window (3 ms) centered at \(v(n)\). These “windowed” autocorrelations were computed as \(c_n(k)=\sum_{i=-N_0}^{i=N_0} v(n-i)v(n-i-k)\) \((Eq. 1)\), where 3 ms windowing was implemented in \(Eq. 1\) by setting \(N_0 = (3 \text{ ms} \times f_{sam})/2\), where \(f_{sam}\) is the sampling frequency, and by setting \(v(j) = 0\) for \(j\) outside the interval \([n-N_0, n+N_0]\).

A subset of the autocorrelation values (lags) from \(Eq. 1\) were used to compute a test-statistic for each \(v(n)\) with the same lags \(\{k_0, k_2, \ldots, k_m\}\) used for all voltage samples. Building on \(Eq. 1\), for each \(v(n)\), a test-statistic \(c_n\) was computed as

\[c_n=\sum_{k=k_0}^{k=k_m} \sum_{i=-N_0}^{i=N_0} v(n-i)v(n-i-k),\] \((Eq. 2)\), where \(Eq. 2\) is the sum of the \(c_n(k)\) from \(Eq. 1\) specified by \(k_0\). \(k\) was set at \(k = [1,2]\) because we found that these values effectively...
separate the distributions of the test statistics \{c_n\} for samples of noise and samples of activity across a broad range of recording quality.

Finally, activity was considered present at \(v(n)\) only when \(c_n\) was greater than a detection threshold \(T\). \(T\) was set as the maximum of a set of \(\{c_n\}\) corresponding to the \{\(v(n)\)\} in one contiguous second of the voltage recording where activity was confirmed to be absent (typically the first second of the recording), and was set this way for each individual voltage recording to account for differences in baseline noise levels. Fictive locomotor bursts were detected, grouped into episodes and the burst and episode properties were determined as follows: Episode duration is the time from the onset of the first burst of an episode to the offset of the final burst in the same episode (Fig. 1C). Burst duration is the time from the onset to the offset of each burst, as defined by \(c_n\) and described above (Fig. 1D). Burst frequency is the inverse of the inter-burst period (IBP), which is defined for each pair of bursts within episodes as the time from the onset of the first burst to the onset of the second burst (Fig. 1D). IBPs between episodes (Fig. 1C) are excluded from burst frequency analysis. Rostrocaudal delay per body segment is the time between the onset of each burst in the more rostral recording and the onset of the corresponding burst in the more caudal recording divided by the number of intervening body segments. Contralateral phase is measured on a cycle by cycle basis as the time between the onset of each burst on the left and each corresponding burst on the right divided by the IBP of the left.
Because non-episodic swimming could not be processed using our Matlab program, the rostrocaudal delay and contralateral phase of non-episodically organized swimming was quantified using auto- and cross-correlation (Fig. 7). Signals were processed using python and the scipy signal library (http://www.scipy.org/) to rectify and low pass filter the signal to 90 Hz. Rostrocaudal delay was defined as the time of peak cross-correlation between the rostral and caudal signals (Fig. 7E). Rostrocaudal delay was normalized to the number of body segments separating the recording locations. Cross-correlation derived IBP was defined as the time of peak auto-correlation from 10 - 200 ms, and was converted to frequency by taking the reciprocal (Fig. 7C). Contralateral offset was defined as the average of the time of highest cross-correlation greater than zero and the absolute value of the time of the highest peak less than zero (Fig. 7G). Contralateral phase was defined as the contralateral offset divided by the IBP and expressed as a percentage.

**Episodic Organization**

In order to evaluate the degree to which the episodic structure of normal zebrafish locomotion was perturbed in these experiments, we developed a tool to quantify the degree of episodic organization (EO) of bursting. Because our Matlab program could not reliably identify bursts in non-episodically organized recordings, Clampfit (Molecular Devices) was used to detect bursts using a voltage threshold. Because many bursts crossed the threshold multiple times, crossings with an inter-event period of less than 15 ms were grouped into a single burst. The 15 ms threshold was chosen based on our observation that in these recordings most crossings within a burst occur within 12 ms.
and most inter-burst intervals are greater than 30ms. In normal swimming, the inter-
episode interval is much longer than the intra-episode burst period. In order to separate
“long” IBPs (inter-episode like) from “short” IBPs (intra-episode like), we defined a
critical value for separating the IBPs into short and long as the mean of all IBPs plus two
standard deviations (Fig. 1E). The means of the short and long IBPs were then
calculated. EO is defined as the log_{10} ratio of the mean long IBP to the mean short IBP.
To enhance readability, the EOs of spinalized and transected larvae are reported as a
percentage of the EO of fictive swimming in spinalized larvae at the same NMDA
concentration.

Statistical Analysis

For the comparisons between intact and NMDA induced fictive swimming along the
rostrocaudal axis, data was analyzed using a 2 (intact, spinalized NMDA) x 3 (rostral,
midbody, caudal) independent groups factorial design. All other tests were single
factor, independent group designs. Tests for significance were carried out using one
and two way ANOVAs and subsequent protected t-tests, or two tailed t-tests using
SYSTAT software (Sigma Plot, San Jose, CA). The Pearson’s correlation coefficient
was calculated using R (http://www.r-project.org/). Data with a p < 0.05 were accepted
as statistically significant. Data are expressed as mean with SD.
RESULTS

The Larval Zebrafish Spinal Cord Produces Fictive Swimming Throughout its Rostrocaudal Extent

In order to determine the baseline bursting activity at points along the rostrocaudal axis of the spinal cord, we measured motor neuron bursts during fictive swimming in intact and spinalized larval zebrafish. Spontaneous fictive swimming episodes were recorded from intact larvae (n = 21 peripheral nerves, 3 larvae), and NMDA (50 μM) induced fictive swimming was recorded in spinalized larvae, that is, larvae with a spinal transection at body segment 3 (n = 37 peripheral nerves, 17 larvae; Fig. 2A). Series of contiguous spinal segments will be referred to by the body segment of the rostral and caudal boundaries; e.g., a spinalized zebrafish is referred to as S3-33.

Both spontaneous (intact) and chemically-evoked (spinalized) swimming consists of episodes (Fig. 1B) that are composed of high frequency bursts (Fig. 1C,D). In both intact and spinalized larvae, peripheral nerves along the rostrocaudal extent of the spinal cord produced episodes of fictive swimming. In order to compare the motor output produced by different regions of the spinal cord, we grouped the data into three anatomical divisions: rostral (S1 to S10), midbody (S11 to S20) and caudal (S21 to 33). There were statistically significant differences in episode duration, burst duration and burst frequency between spontaneous (intact) and chemically-evoked (spinalized) fictive swimming (all F > 6.8, all p < 0.01; Fig. 2B-D). In intact larvae, there were no significant differences in the episode duration (Table 1, all t < 0.01, all p > 0.99), burst duration (Table 1, all t < 0.88, p > 0.38), or burst frequency (Table 1, all t < 0.71, all p > 0.57).
along the rostrocaudal axis (Fig. 2B-D, left). In spinalized larvae, there were no
significant differences in the episode duration (Table 1, all t < 0.39, all p > 0.7), or burst
frequency (Table 1, all t < 0.07, all p > 0.95) along the rostrocaudal axis (Fig. 2 B,D,
right), but the burst duration was significantly longer in the rostral region than in the
midbody or caudal regions (Table 1, t > 3.2, p < 0.002; Fig. 2C, right).

Fictive Swimming Characteristics Vary with NMDA Concentration

To determine the effect of NMDA concentration on the properties of fictive swimming,
we recorded activity from peripheral nerves in spinalized larvae (n = 6) at three
concentrations of NMDA: 50, 100 & 200 μM (Fig. 3). Because the burst duration of
NMDA-induced fictive swimming differs along the rostrocaudal axis (Fig. 2) and to
simplify our analysis, all recordings were performed in the midbody region due to its
relative accessibility. Episodic fictive swimming was observed at all three NMDA
concentrations (Fig. 3A). Increasing NMDA concentration significantly decreased
episode duration (Table 2, F = 20.3, p < 0.001; Fig. 3B). Increasing NMDA
concentration also significantly increased the number of episodes produced per minute
(Table 2, F = 28.8, p < 0.001; Fig. 3C). NMDA concentration did not have a significant
effect on burst duration (Table 2, F = 2.3, p = 0.13; Fig. 3D), but increased burst
frequency at 200 μM NMDA (Table 2, all t > 4.0, all p < 0.006; Fig. 3E). To determine
the independence of burst frequency and episode duration, we tested their correlation.
There was a significant negative correlation between episode duration and burst
frequency across NMDA concentrations (r = -0.63, t = -3.2, p = 0.006), but there was no
correlation between episode duration and burst frequency across preparations within NMDA concentrations \((r = 0.05, t = 0.2, p = 0.84)\).

Series of Contiguous Spinal Segments Retain the Ability to Produce Fictive Swimming Following Spinal Transection

To characterize the rostrocaudal distribution of the spinal locomotor circuit in spinalized (S3 transection) larval zebrafish, we performed a series of spinal transections at S8, S10, S15 or S20, dividing the spinal cord into series of spinal segments of different lengths. In all spinalized larvae with an additional spinal transection, episodically organized bursting was no longer observed at 50 \(\mu\text{M} \) NMDA \((n = 24; \text{Fig } 4\text{B-D})\). When the NMDA concentration was increased to 200 \(\mu\text{M} \), some of the series of contiguous spinal segments produced episodically organized bursting (Fig.4B-D). In spinalized larvae transected at S10 \((n = 5; \text{Fig. 4B})\), the caudal portion of the spinal cord (S10-33) produced normal, episodically organized bursting, while the rostral portion of the spinal cord (S3-10) produced regular, non-episodic bursting. In spinalized larvae transected at S15 \((n = 5; \text{Fig. 4C})\), the rostral portion of the spinal cord (S3-15) produced episodically organized bursting, while the caudal portion (S15-33) produced low frequency, sporadic bursting. In larvae transected at S8 and S20 \((n = 6; \text{Fig. 4D})\), the midbody series (S8-20) in most \((4 \text{ of } 6)\) larvae produced episodic bursting. The fictive swimming produced showed no significant differences in episode duration (Table 2, \(F = 2.3, p = 0.12; \text{Fig. 4E})\). There was a non-significant trend toward shorter burst duration in shorter series of spinal segments compared to spinalized larvae (Table 2, \(F = 3.0, p = 0.07; \text{Fig. 4E})\). There were no significant differences in burst frequency (Table 2, \(F = 1.0, p = 0.41\)
among the spinalized larvae and shorter series of spinal segments at 200μM NMDA (Fig. 4E).

Rostral Spinal Segments Have Greater Capacity for Producing Episodic Fictive Swimming

To map the distribution of episode organizing capacity along the rostrocaudal extent of the spinal cord, we compared the degree of episodic organization (EO) produced by series of contiguous spinal segments by their length and location along the spinal cord. EO is a tool for quantifying the degree to which bursts are organized into episodes (see Methods; Fig. 5A). To provide more closely matched lengths of spinal segments between rostral and caudal series, we measured bursting produced by spinalized larvae additionally transected at S20 (n = 4) as well as S10 and S15 (Rostral: 7, 12 & 17 segment series; Caudal: 13, 18 & 23 segment series). Because high NMDA concentrations were more likely to produce episodic fictive swimming in all series of spinal segments, 200 μM NMDA was used for this analysis. There was a positive correlation between the number of contiguous spinal segments and EO both for the rostral series (S3-10, S3-15 and S3-20) and for the caudal series (S10-33, S15-33 and S20-33) (r > 0.99 for both; Fig. 5B). Rostral series of spinal segments produced greater EO per contiguous spinal segment than caudal series (rostral = 5.3% (SD 0.6), caudal = 2.9% (SD 0.1), t = 7.3, p = 0.018). When comparing series of contiguous spinal segments that are roughly the same length (12-13 spinal segments) but from different regions of the spinal cord, there was a trend toward the rostral spinal segments producing fictive swimming with a higher EO than the caudal spinal segments, but this
trend was not statistically significant (S3-15 = 71% (SD 18), S8-20 = 66% (SD 16), S20-33 = 46% (SD 31), F = 1.7, p = 0.22; Fig. 5C).

Coordination of Episodically Organized Bursting in Transected Spinal Cord

To determine if bursting produced by series of contiguous spinal segments are coordinated (that is, produce side-to-side alternation and rostrocaudal progression), we performed two point recordings on spinalized and transected larvae. Quantification of delay and phase was performed using the first burst of each episode as a phase marker. Spinalized larvae produced fictive swimming with side-to-side alternation (n = 3) and rostrocaudal progression (n = 3) in the presence of 200 μM NMDA (Fig. 6A).

The S10-33 series, the shortest caudal series of contiguous spinal segments that produced episodically organized fictive swimming (EO = 67% (SD 13)), produced bursting with side-to-side alternation (n = 3) and rostrocaudal delay (n = 3; Fig. 6B).

The S3-15 series, the shortest rostral series of contiguous spinal segments that produces episodic fictive swimming (EO = 61% (SD 15)), also produced bursting with side-to-side alternation (n = 3) and rostrocaudal delay (n = 3) (Fig. 6C). Neither the rostrocaudal delay per body segment (S3-33 = 1.3ms (SD 0.75), S10-33 = 2.5ms (SD 1.2), S3-15 = 1.3ms (SD 0.38), F = 2.5, p = 0.15) nor the contralateral phase (S3-33 = 45% (SD 3.9), S10-33 = 49% (SD 5.0), S3-15 = 47% (SD 3.6), F = 0.36, p = 0.71) were significantly different between spinalized larvae and shorter series of spinal segments (Fig 6D, E).

Coordination of Non-Episodic Bursting in Transected Spinal Cord
Among the transection conditions that do not produce episodically organized bursting, some (e.g. S3-15, 100 μM NMDA; Fig. 4C) produced highly regular bursting (burst frequency = 14Hz (SD 0.82), coefficient of variation = 0.48Hz (SD 0.21)). Given the regularity of these bursts, we hypothesized that the putative episodic organization circuit was either inactive or functioned in an abnormal fashion that did not lead to episode termination, but that other swimming-related circuits were active. The S3-15 series transitioned from producing tonic bursting (EO = 26% (SD 2.1)) to episodic bursting (EO = 61% (SD 1.5)) when the NMDA concentration was increased from 100 μM to 200 μM. Therefore, to determine the necessity of episodic organization for coordination, we compared the coordination of bursts produced by this series of segments in 100 μM NMDA to the coordination of bursts produced in 200 μM (n = 3 per group). Because the bursting in 100 μM NMDA was non-episodic, we could not use the first burst of each episode as a phase marker (as in Fig. 6). Instead, we used cross- and auto-correlation to measure the inter-burst period (IBP) and burst frequency of individual recordings and the relative timing of bursts between recording sites. In order to facilitate correlation of biphasic bursts (Fig. 7A), recordings were rectified and low-pass filtered at 90Hz (Fig. 7B), and auto- and cross-correlations of the processed signal were performed (Fig. 7C,E). The burst frequency of the S3-15 series was significantly slower in 100μM than in 200μM NMDA (100μM = 14Hz (SD 1.6), 200μM = 19Hz (SD 1.7), t = 3.4, p = 0.043, Fig. 7D). The non-episodic bursting produced by the S3-15 series in 100 μM NMDA had a rostrocaudal delay per body segment significantly greater than zero (1.48ms (SD 0.18), t =14.6, p = 0.005). Rostrocaudal delay per body segment was significantly longer in 100 μM than 200 μM NMDA (200μM = 1.00ms (SD 0.07), t = 4.4, p = 0.02, Fig. 7D).
Contralateral phase was found by dividing the contralateral delay by the IBP (Fig. 7F). There were no significant differences between the contralateral phase of the S3-15 series in 100 μM and 200 μM NMDA (100μM = 52% (SD 0.02), 200μM = 50% (SD 0.03), t = 0.8, p = 0.49; Fig. 7H).
DISCUSSION

We have shown that fictive locomotion is produced along the rostrocaudal extent of the spinal cord in larval zebrafish (Fig. 2) and that this locomotor pattern can be generated from reduced series of spinal segments (Fig. 4). In the transected spinal cord, rostral spinal segments have greater potential for generating episodically organized fictive swimming (Fig. 5). The episodic fictive swimming produced by these series is coordinated normally (Fig. 6). Based on these findings, we return to our initial three hypotheses regarding the spatial distribution of the swimming CPG: 1) The CPG is composed of segmentally reiterated oscillators, 2) There is a single CPG distributed throughout the entire spinal cord, or 3) There is a single CPG located in a small region of the spinal cord. Our results are inconsistent with the third hypothesis. Neither the rostral (S3 - S10) nor caudal (S15 - S33) regions are necessary for organized locomotion (Fig. 5). While those transections leave open the possibility that the midbody segments S10 - S15 contain a critical population of neurons, the S8-20 series contains the largest contiguous midbody region but does not produce the most organized swimming (Fig. 5). Based on these results, we conclude that there are no critical segments or series of segments in the larval zebrafish swimming CPG. Our finding that there is a linear trend of EO against number of contiguous segments (Fig. 5) is suggestive of either a robust, distributed CPG or of segmentally reiterated oscillators that are too weak to drive fictive swimming independently, but we cannot distinguish between these possibilities on the basis of this data. We also found that normal functioning of the circuit responsible for generation of episodic organization is not necessary for coordination of motor bursts (Fig. 7). Our results support the hypothesis
that the zebrafish swimming CPG is composed of functionally separable circuits, one of which organizes episodes and another that coordinates bursting side-to side and rostrocaudally.

The Entire Spinal Cord Produces the Same Pattern of Fictive Swimming

Based on our observations of free-swimming larvae, we predicted that there might be different patterns of motor neuron bursting in the midbody and tail of the larvae. For example, one type of free-swimming behavior, slow start swimming, has a relatively tight lateral undulation in the midbody region that rapidly increases in amplitude in the far caudal region (Muller and Von Leeuwen, 2004). We proposed that this motion could be due to the whip-like snapping of a passive tail. To determine the spatial distribution of motor activity, we measured peripheral nerve activity at points along the rostrocaudal axis of the larvae. Our prediction of a passive tail was not observed in the range of swim frequencies or spinal segments recorded in our experiments (Fig. 2). Instead, the entire spinal cord produced the same pattern of motor neuron bursting. We concluded that the motor neuron output is distributed throughout the cord, though the interneurons that generate the motor pattern may not be.

Fictive Swimming Characteristics are Dependent on NMDA Concentration

Concentrations of NMDA between 50 and 200μM reliably evoked fictive swimming in spinalized larval zebrafish (Fig. 3). High concentrations of NMDA produce short duration episodes of high frequency bursting, while low concentrations produce long duration episodes of lower frequency bursting. The correlation we observed between
burst frequency and episode duration across NMDA concentrations is not observed across preparations following application of the same concentration of NMDA. This suggests that the effect of NMDA concentration accounts for the covariance of these variables and that episode duration and burst frequency are independent of one another.

Series of Spinal Segments Produce Fictive Swimming Following Transection

We performed a series of spinal transections that divided the spinal cord into isolated series of contiguous segments (Fig. 4). Under some conditions, these isolated series of spinal segments produced episodically organized fictive locomotion that was not statistically different from the output produced by spinalized larvae. The effect of these transections was to raise the threshold for production of organized episodes from 50 uM to 200 uM NMDA, and for some transection conditions, to abolish the capacity for generating episodically organized fictive swimming. The necessity of higher NMDA concentration may be due to removing ascending and descending intra-spinal excitatory projections (e.g. Satou et al. (2012)) or due to an injury-induced decrease in neuronal excitability. We found that rostral series of segments shorter than 12 segments were not able to produce episodic swimming (Fig. 4-5), and that more than 20 body segments were necessary to produce episodic swimming in the caudal region. This finding differs from a previous report that two isolated body segments were sufficient to produce locomotor-like bursting (McDearmid and Drapeau, 2006). There are several potential explanations for this discrepancy, including effects of strain, larval stage, NMDA concentration, and transection technique. We believe the most parsimonious
explanation arises from the intrinsic properties of motor neurons. McDearmid and Drapeau (2006) performed whole cell recordings from individual motor neurons while we use peripheral nerve recordings. Zebrafish motor neurons have intrinsic bursting properties (Buss et al., 2003) that may be activated by high doses of NMDA, possibly giving the appearance of episodic fictive swimming when recording from an individual neuron. However, these oscillations would not likely be correlated between motor neurons, and therefore would not be observed with peripheral nerve recording, unless a CPG was driving their activity.

The Rostral Spinal Cord has Greater Episode Organizing Potential

In these experiments, we found that rostral body segments are more capable of producing episodically organized swimming than caudal body segments following transection (Fig. 5). This difference is most dramatically demonstrated by the difference in motor output between the rostral series S3-15 and the caudal series S15-33 (Fig. 4C). Despite the rostral bias for episode generation, rostral spinal segments are not necessary for the production of episodes (Fig. 4). This finding is inconsistent with the hypothesis that the swimming CPG is localized to a small region of the cord, and suggests a more distributed model. The strong linear trend we find between the number of body segments and the EO score of the swim pattern (Fig. 5) could be interpreted in two ways. On the one hand, it could be that the episode circuit is segmentally reiterated. Based on this structure, we would predict repeating interneuron populations with progressively weaker net synaptic drive onto their targets. On the other hand, it could be that the episode organizing circuit is composed of a non-
segmentally organized network of neurons spread throughout the spinal cord. Based on this structure, we would predict a gradient of synaptic output from the episode circuit and an interneuron distribution that does not align to segmental boundaries. Modeling studies in tadpole (Wolf et al., 2009) provide a quantitative framework for the distributed hypothesis, but determining which hypothesis is more likely will require additional characterization of neuronal distribution throughout the larval zebrafish spinal cord.

Coordination of Bursts is Independent of Episodic Organization

We found that by manipulating the concentration of NMDA, we activated the putative coordination circuit without observing discretely organized episodes (S3-15, 100 μM NMDA; Figs. 4C, 7). The functional dissociation between the episode organization circuit and the coordination circuit we have observed is similar to models of the leech swimming circuit (Kristan et al., 2005) and multi-level models of the mammalian CPG (McCrea and Rybak, 2008). Therefore, we propose the following preliminary model of the functional organization of the zebrafish spinal locomotor CPG:

The hindbrain acts as an activator (Mori et al., 1978; Noga et al., 1988; Li et al., 2006; Arrenberg et al., 2009; Soffe et al., 2009) and makes excitatory connections with the spinal episode circuit (Hägglund et al., 2010; Li et al., 2010). The episode circuit acts as a gating center and makes excitatory connections (Buss and Drapeau, 2001; Kyriakatos et al, 2011) with the spinal coordination circuit. The coordination circuit sculpts excitatory input from the episode circuit into a coordinated output and makes excitatory and inhibitory connections with motor neurons (McLean et al, 2008; Kyriakatos et al,
When the hindbrain initiates a locomotor episode, it sends an excitatory signal to the episode circuit, initiating an up-state of high activity. The episode circuit excites the coordination circuit, which then begins driving the motor neurons in a coordinated fashion. The episode generator up-state self-terminates, ending the excitation to the coordination circuit. In the absence of excitation, the coordination circuit is silenced and the motor neurons stop firing.

Conclusions

In summary, using transections of the larval zebrafish spinal cord, we demonstrate the spatial and functional organization of the episode and coordination circuits in the spinal CPG. We show that there is a strong effect of rostrocaudal position on the ability of series of spinal segments to produce episodic swimming. Further, we show that normal coordination of bursting is not dependent upon episodic organization. Future work is necessary to determine whether or not the episode organization and coordination circuits are segmentally organized and how the putative episode and coordination circuits interact with one another.
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AUTHOR CONTRIBUTIONS

REFERENCES


Li WC, Roberts A, Soffe SR. Specific brainstem neurons switch each other into pacemaker mode to drive movement by activating NMDA receptors. *J Neurosci.* 30: 16609-20, 2010.


**FIGURE LEGENDS**

**Figure 1. Quantification of Episodic Organization.** (A) A diagram of a typical unilateral fictive swimming preparation. Two tungsten pins (filled circles) were used to stabilize the larvae. The region between these pins was skinned. (B - D) Peripheral nerve recording from the midbody region of a spinalized zebrafish during bath application of 200 μM NMDA. (C) The episode duration and inter-episodic long IBP are indicated by horizontal lines. (D) The intra-episodic short IBP and burst duration are indicated by horizontal lines. The inverse of the IBP is the instantaneous burst frequency. (E) Histogram of IBP distribution from the representative recording in B - D. The values that were used to calculate Episodic Organization (critical value, mean short IBP and mean long IBP) are indicated by dashed vertical lines.

**Figure 2. Fictive swimming is produced throughout the rostrocaudal extent of the spinal cord.** (A) Diagrams of larval zebrafish illustrating the design of the experiment. Recordings were performed in intact larvae (left) and spinalized larvae (right). The body of the larvae is divided into three regions: rostral (dark grey), midbody (light grey) and caudal (white). Circles above the illustration indicate location and number of recordings. Representative traces of spontaneous (intact) and chemically-evoked (spinalized) fictive swimming recorded in the midbody region are below the respective diagrams. (B-D) Plots of episode duration (B), burst duration (C) and burst frequency (D) against recording location and preparation type: rostral, midbody and caudal; intact (left) and spinalized (right). * Statistically significant difference.
Figure 3. The characteristics of fictive swimming depend on NMDA concentration. (A) Representative traces of NMDA induced fictive swimming in a spinalized larval zebrafish at 50, 100 and 200 μM NMDA. (B-E) Plots of episode duration (B), episodes per minute (C), burst duration (D) and burst frequency (E) against NMDA concentration: 50, 100 and 200 μM. * Statistically significant difference.

Figure 4. The length and location of contiguous segments determines the episodic nature of NMDA induced fictive swimming. (A-D) Representative traces illustrating the typical fictive motor output from each contiguous spinal region. Chemically-induced fictive locomotor activity in is shown at 50, 100 and 200 μM NMDA. A black box indicates experimental conditions that produce episodically organized fictive swimming that is quantified in panel E. (A) Fictive swimming activity produced by a spinalized larva. (B) Activity from S3-10 (top trace) and S10-30 (bottom trace). (C) Activity from S3-15 (top trace) and S15-33 (bottom trace). (D) Activity from S8-20. (E) Plots of episode duration (left), burst duration (middle) and burst frequency (right) against the series of contiguous spinal segments producing fictive swimming.

Figure 5. Production of episodic fictive swimming is rostrally biased. (A) Representative traces illustrating the patterns of bursting that correspond to episodic, intermediate and non-episodic EO scores. Fictive swimming was evoked by 200 μM NMDA. (B) Plot of % EO against the length of the series of contiguous spinal segments. The length of rostral spinal regions (open circular markers) was measured from the transection at S3 to the location of a more caudal transection. The length of
caudal spinal regions (filled diamond markers) was measured from the site of the midbody transection to S33. The rostral series were: S3-10, S3-15 and S3-20. The caudal series were: S10-33, S15-33 and S20-33. (C) Plot of % EO against transection type: rostral (S3-15), midbody (S8-20) or caudal (S15-30). Data were normalized to the average EO of spinalized larvae in 200 μM NMDA.

Figure 6. Episodic fictive swimming is coordinated following spinal transection. (A-C) Representative traces from ipsilateral (left) and bilateral (right) two point recordings in spinalized larvae. Fictive swimming was induced by bath application of 200 μM NMDA. Rostrocaudal delay in each ipsilateral record is indicated by a gray line. The timing of bursts from the left channel of each contralateral record is indicated by a filled gray box. (A) Spinalized larvae. (B) Transected larvae recorded in the S10-33 series. (C) Transected larvae recorded in the S3-15 series. (D, E) Plot of ipsilateral delay (D) and contralateral phase (E) against transection conditions: spinalized (S3-33), S10-33 and S3-15.

Figure 7. Non-episodic bursting is coordinated following spinal transection. (A) Representative traces from ipsilateral (left) and contralateral (right) two point recordings of fictive swimming in the S3-15 series. Bursting was induced by bath application of 100 μM NMDA. Rostrocaudal delay in the ipsilateral record is indicated by a gray line. The timing of bursts from the left channel of each contralateral record is indicated by a filled gray box. (B) The traces from A following processing. (C) Auto-correlation of the left processed signal in B. The gray line shows the IBP. (D) Plot of the burst frequency
determined by autocorrelation against NMDA concentration: 100 μM NMDA and 200 μM NMDA. (E) Cross-correlation of the left processed signal in B. The grey line shows the rostrocaudal delay. (F) Plot of the rostrocaudal delay against NMDA concentration. (G) Cross-correlation of the right processed signal in B. The grey line shows the contralateral delay reflected across 0 ms lag. (H) Plot of the contralateral phase against NMDA concentration. * Statistically significant difference.
Episodic Organization (% of spinalized control)

Number of Contiguous Spinal Segments

Episodic
EO = 87%

Intermediate
EO = 59%

Non-Episodic
EO = 30%

B

Episodic Organization (% of spinalized control) vs. Number of Contiguous Spinal Segments

Rostral - Caudal

C

Episodic Organization (% of spinalized control)

Rostral (S3-15)  Midbody (S8-20)  Caudal (S20-33)
<table>
<thead>
<tr>
<th>Preparation</th>
<th>Recording Location</th>
<th>Episode Duration (ms)</th>
<th>Burst Duration (ms)</th>
<th>Burst Frequency (Hz)</th>
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<tbody>
<tr>
<td>Intact, Spontaneous</td>
<td>Rostral</td>
<td>257 (34)</td>
<td>10.9 (0.89)</td>
<td>30 (1.2)</td>
</tr>
<tr>
<td></td>
<td>Midbody</td>
<td>254 (30)</td>
<td>11.1 (2.0)</td>
<td>30 (1.8)</td>
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<tr>
<td></td>
<td>Caudal</td>
<td>250 (28)</td>
<td>12.7 (1.3)</td>
<td>29 (2.3)</td>
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<tr>
<td>Spinalized, 50 μM NMDA</td>
<td>Rostral</td>
<td>3,484 (1,694)</td>
<td>11.7 (0.58)</td>
<td>16.5 (1.0)</td>
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<tr>
<td></td>
<td>Midbody</td>
<td>3,707 (1,575)</td>
<td>8.0 (0.67)</td>
<td>16.5 (1.1)</td>
</tr>
<tr>
<td></td>
<td>Caudal</td>
<td>3,575 (2,554)</td>
<td>7.2 (1.3)</td>
<td>16.5 (0.6)</td>
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Values are means (SD)
Table 2 – Properties of Chemically Evoked Fictive Swimming in Reduced Preparations of Larval Zebrafish

<table>
<thead>
<tr>
<th>Preparation</th>
<th>NMDA Concentration (μM)</th>
<th>Episode Duration (ms)</th>
<th>Episodes per Minute</th>
<th>Burst Duration (ms)</th>
<th>Burst Frequency (Hz)</th>
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</thead>
<tbody>
<tr>
<td>S3-33</td>
<td>50</td>
<td>3,150 (952)</td>
<td>12 (4.6)</td>
<td>11.2 (1.2)</td>
<td>15 (1.4)</td>
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<tr>
<td></td>
<td>100</td>
<td>1,950 (459)</td>
<td>24 (4.2)</td>
<td>12.0 (1.9)</td>
<td>16 (1.2)</td>
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<tr>
<td></td>
<td>200</td>
<td>898 (87)</td>
<td>33 (5.1)</td>
<td>13.8 (3.0)</td>
<td>21 (2.7)</td>
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<tr>
<td>S10-33</td>
<td>200</td>
<td>1,130 (372)</td>
<td>n.d.</td>
<td>10.6 (1.1)</td>
<td>19.5 (5.8)</td>
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<tr>
<td>S3-15</td>
<td>200</td>
<td>1,425 (386)</td>
<td>n.d.</td>
<td>8.9 (1.4)</td>
<td>18.3 (4.0)</td>
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<tr>
<td>S8-20</td>
<td>200</td>
<td>1,087 (412)</td>
<td>n.d.</td>
<td>9.8 (4.8)</td>
<td>16.3 (4.0)</td>
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</tbody>
</table>

Values are means (SD)

n.d. = not determined