Rhythmic Motor Activity Evoked by NMDA in the Spinal Zebrafish Larva

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McDearmid, Jonathan R. and Pierre Drapeau. Rhythmic motor activity evoked by NMDA in the spinal zebrafish larva. J Neurophysiol 95: 401–417, 2006. First published October 5, 2005; doi:10.1152/jn.00844.2005. We have examined the localization and activity of the neural circuitry that generates swimming behavior in developing zebrafish that were spinalized to isolate the spinal cord from descending brain inputs. We found that addition of the excitatory amino acid agonist N-methyl-d-aspartate (NMDA) to spinalized zebrafish at 3 days in development induced repeating episodes of rhythmic tail beating activity reminiscent of slow swimming behavior. The neural correlate of this activity, monitored by extracellular recording comprised repeating episodes of rhythmic, rostrocaudally progressing peripheral nerve discharges that alternated between the two sides of the body. Motoneuron recordings revealed an activity pattern comprising a slow oscillatory and a fast synaptic component that was consistent with fictive swimming behavior. Pharmacological and voltage-clamp analysis implicated glycine and glutamate in generation of motoneuron activity. Contralateral alternation of motor activity was disrupted with strychnine, indicating a role for glycine in coordinating left-right alternation during NMDA-induced locomotion. At embryonic stages, while rhythmic synaptic activity patterns could still be evoked in motoneurons, they were typically lower in frequency. Kinematic recordings revealed that prior to 3 days in development, NMDA was unable to reliably generate rhythmic tail beating behavior. We conclude that NMDA induces episodes of rhythmic motor activity in spinalized developing zebrafish that can be monitored physiologically in paralyzed preparations. Therefore as for other vertebrates, the zebrafish central pattern generator is intrinsic to the spinal cord and can operate in isolation provided a tonic source of excitation is given.

INTRODUCTION

Much of our understanding of how behavior is generated has arisen from the study of the neuronal circuits that drive motor output in the vertebrate spinal cord. While considerable insight has been obtained from the study of higher vertebrates such as the cat (Rossignol et al. 1998), the rat (Kiehn and Butt 2003) and the mouse (Whelan 2003), lower vertebrate models such as the lamprey (Grillner et al. 1998) and the frog tadpole (Roberts et al. 1998) have proven invaluable. The attraction of lower vertebrate motor systems lies in their anatomical simplicity, stereotyped motor behaviors and tractability to physiological techniques. Their study has enabled the most detailed descriptions of the assembly and function of neuronal circuitry currently available (Grillner et al. 1998; Roberts et al. 1998). In vertebrates, in the absence of inputs from higher centers in the brain, the spinal cord retains the capacity to generate coordinated locomotor activity patterns provided an exogenous source of excitation is given (Barry and O’Donovan 1987; Cazalets et al. 1990; Cohen and Wallen 1980; Dale and Roberts 1984; Douglas et al. 1993; Forssberg and Grillner 1973; Hernandez et al. 1991; Houssaini et al. 1993; Jankowska et al. 1967; Kjaerulf et al. 1994; Kudo and Yamada 1987; Poon 1980; Wallen and Williams 1984; Wheatley and Stein 1992). Therefore the circuitry responsible for generating locomotion [the central pattern generator (CPG)] is intrinsic to the spinal cord. Isolation of the spinal rhythm generating circuit by removing higher-order structures greatly simplifies the task of relating neural activity to behavior because it reduces the influence of extrinsic inputs on the core rhythm-generating circuit.

Recently the zebrafish embryo has arisen as a leading model system for the study of vertebrate development. Early behaviors and the motoneuron activity that drives them have already been examined in some detail (Buss and Drapeau 2001; Masino and Fetcho 2005; Saint-Amant and Drapeau 2000, 2001). The use of zebrafish as a genetic model is currently burgeoning with a number of mutations affecting motor behavior identified and in some cases characterized at molecular and cellular levels (Cui et al. 2004; Gleason et al. 2004; Granato et al. 1996; Hirata et al. 2004, 2005; Lorent et al. 2001; Luna et al. 2004; Masino and Fetcho 2005; Ono et al. 2001, 2002, 2004; Zeller and Granato 1999; Zhang and Granato 2000; Zhang et al. 2004). Understanding the physiological properties of spinal neurons is thus important for understanding the development of normal and mutant spinal circuits. These studies would be facilitated by the ability to isolate the CPG for locomotion from extrinsic inputs. However, it is not known whether the zebrafish CPG is intrinsic to the spinal cord or distributed throughout regions of the spinal cord and/or brain.

To study the ontogeny of the spinal swimming circuit, we have examined locomotor activity in spinalized zebrafish by transecting the rostral spinal cord, thereby isolating the spinal cord from higher CNS structures. We have studied the properties of N-methyl-d-aspartate (NMDA)-induced activity at different developmental stages using kinematic analyses of locomotion, peripheral nerve recordings of fictive motor activity, and single motoneuron current and voltage recording of NMDA-induced synaptic activity. We provide the first evidence in zebrafish that rhythmic activity can be generated when the spinal cord is isolated from the brain provided a tonic source of excitation (NMDA) is given.

METHODS

Zebrafish maintenance and experimental preparation

Zebrafish were raised from a colony maintained according to established procedures (Westerfield 1995), and all procedures were approved in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
carried out in compliance with the Canadian Council for Animal Care and McGill University.

Fish were prepared for recording as described previously (Drapeau et al. 1999). Briefly, larvae were anesthetized in 0.02% tricaine (MS-222, Sigma) dissolved in fish saline (containing, in mM, 134 NaCl, 2.9 KCl, 2.1 CaCl₂, 1.2 MgCl₂, 10 HEPES, and 10 glucose; osmolarity 280–290 mosM, pH 7.2; MgCl₂ was omitted in magnesium-free saline) and pinned to a silicone elastomer (Sylgard)-lined dish by placing two tungsten pins through rostral and caudal areas of the notochord. The skin was subsequently removed using fine forceps and the tricaine washed off and replaced with fish saline containing the neuromuscular blocker d-tubocurarine (15 μM, Sigma) to maintain muscle paralysis during the physiology experiments.

For patch-clamp recordings, collagenase was also added for 10 min to allow partial digestion of the musculature before being washed off in fish saline containing d-tubocurarine. Muscle fibers were then aspirated from a one- or two-segment region of the trunk to expose the underlying spinal cord.

Spinalization was performed by completely transecting the spinal cord using a glass (10 μm tip diameter) microelectrode at the desired region. After transection the caudal tip of the lesioned brain was clamped with forceps and pulled away from the preparation to ensure that total spinal lesion had been obtained. In some preparations, the brain was removed altogether. Comparable results were obtained using both methods. The number of somites rostral to the transection were counted and used to reference the rostrocaudal level at which transection occurred.

Kinematic studies

High-speed video was used to analyze behavior in spinalized zebrafish. For these experiments, larvae were embedded in 1% low-melting-point agarose (Gibco) and their tails subsequently freed so that they could move without obstruction. Fish were then spinalized and allowed to recover for 5–30 min during which time, the dish was washed repeatedly with fresh fish saline. Tail-beat activity was observed with a Zeiss (Germany) dissecting microscope and filmed at a frequency of 500 frames/s using a Photron Fastcam PCI high-speed video camera attached to the dissecting microscope. Data were captured to a memory buffer on the Fastcam acquisition card and then written to disc as either a series of JPEG images or as an AVI file. Because at a capture rate of 500 Hz, the frame buffer was only capable of capturing ca. 2 s of activity, experiments in which episode duration and frequency were analyzed were performed using a capture frequency of 60 Hz, permitting around 16 s of data acquisition. This enabled analysis of several episodes of activity per recording.

For experiments in which strychnine was used to disrupt NMDA-induced tail flexions, we found that this drug had no effect when added to spinalized fish already exposed to NMDA. This is presumably a result of the relatively poor penetration of drugs in minimally dissected zebrafish preparations (note the skin was not removed for kinematic experiments, and this likely impedes drug access dramatically). Therefore we bathed fish in 10 μM strychnine for ≤1 h to allow penetration of the drug before NMDA was added to the dish, thereby preventing rundown of the NMDA effect during the experiment.

Electrophysiology

Peripheral nerve recordings were performed according to established procedures (Masino and Fetcho 2005) with minor modifications. Briefly, large-bore (ca. 15 μm tip diameter) extracellular electrodes were filled with curare-free extracellular fish saline and placed at the dorsoventral midline of the intermyotomal cleft to be recorded. Extracellular voltage was monitored using Axopatch 200B amplifiers at a gain of 500 and a low-pass filter setting of 5 kHz. A digital acquisition rate of 40 kHz was used. Subsequently recordings were filtered off-line with a low- and high-frequency filter of 100 and 2,000 Hz, respectively.

For current-clamp experiments, a potassium-glucuronate-based solution comprising (in mM) 116 K-glucuronate, 16 KCl, 2 MgCl₂, 10 HEPES, 10 EGTA, and 4 Na₂ATP was used. For voltage-clamp recordings a Cs-glucuronate-based solution was used that contained (in mM) 116 Cs-glucuronate, 16 CsCl, 2 MgCl₂, 10 HEPES, 10 EGTA, and 4 Na₂ATP. Lidocaine N-ethyl bromide (QX-314) was added to the pipette solution to block voltage-gated sodium channels during voltage-clamp recordings. A pH of 7.2 and osmolarity of 280–290 mosM was maintained for all intracellular solutions. Sulfonfrodamine B (0.2%) was also added to all intracellular solutions, confirming identification and complete dialysis of motoneurons with fluorescent imaging. An Axopatch 1D amplifier (5-kHz low-pass filter setting) was used for all patch-clamp recordings captured at a digital acquisition rate of 20–40 kHz.

It has previously been demonstrated, using methods that do not interfere with chloride homeostasis, that at the resting membrane potential glycine is depolarizing in zebrafish neurons (Brustein et al. 2003b; Saint-Amant and Drapeau 2000). Therefore in whole cell patch-clamp experiments, patch pipette solutions contained a total of 20 mM chloride, setting the equilibrium potential for chloride at around −46 mV.

During NMDA-induced activity in 3-day-old larvae, 80- to 150-pA negative current was sometimes injected into motoneurons to bring the troughs of slow oscillations near the resting potential of −60 mV, thereby amplifying this component of the activity. The resting potential of recorded motoneurons ranged between −58 and −70 mV with an average resting potential of −63.9 ± 0.7 mV. Input resistances varied between 100 and 200 MΩ in 2- and 3-day-old fish and 1–1.5 GΩ in 1-day-old fish.

Drugs

All drugs were obtained from Sigma except for TTX, which was obtained from Tocris (UK). Concentrations of drugs used were as follows: NMDA, 80–850 μM; QX-314, 0.5 mM; TTX, 1 μM; CNQX, 10 μM; APV, 50 μM; serotonin, 1–50 μM; and strychnine, 1–50 μM. For physiology experiments, pharmacological effects could be observed 5–12 min after addition to the fish saline. However, during kinematic studies, reduced drug access (presumably because the skin was not removed for these experiments) meant that it was not possible to obtain effects of strychnine unless fish were incubated in the drug for ≤1 h.

Analysis

High-speed video analysis was performed with Photron Fastcam Viewer 2.1 software. Tail-beat frequency was measured by counting the number of times the caudal tip of the tail reached maximum deflection to either the left or right side of the body. Time to maximal tail deflection was determined from time-code information embedded in each frame of the captured video.

Physiological analysis was performed using Clampfit 8 (Axon Instruments). Measurements of peripheral nerve episode duration (ED) episode period (EP), episode frequency, burst duration (BD), rostrocaudal delay, cycle period (CP), and contralateral phase were performed using cursor measurements in Clampfit 8 software. Measurements of motoneuron oscillation period (OP), oscillation frequency, episode duration (ED), PSP and PSC frequency, tonic potential, and action potential threshold were also performed using cursor measurements in Clampfit. To obtain averages for peripheral nerve recordings, 200–250 cycles of activity were analyzed from excerpts of activity taken 1–6 min after induction of a stable rhythmic activity with NMDA.

Rhythmic PSC and PSP amplitudes and frequencies were determined from consecutive measurements made on excerpts of 50–100
excitatory postsynaptic currents (EPSCs) taken 1–3 min after induction of a stable rhythmic activity with NMDA. Values for action potential threshold were determined as the membrane potential at which EPSPs initiated action potential firing. For spectral analysis of whole cell recordings, power spectra were generated in Clampfit from 40-s excerpts of data. A spectral resolution of 2 kHz, the highest resolution afforded by the analysis software, was used. Results are presented as means ± SE throughout the text.

RESULTS

NMDA swimming behavior in spinal zebrafish larvae

In all other vertebrate preparations studied to date, the excitatory amino acid NMDA can reliably evoke patterns of rhythmic motor activity in the isolated spinal cord. To examine if this was also the case in zebrafish larvae, we began by filming trunk movements generated by NMDA in nonparalyzed fish that had been spinalized to remove inputs from the brain. Fish at 3 days were chosen for the study because at this stage, they have already hatched from their egg membrane and, in untransected preparations, generate a well-characterized pattern of swimming behavior (Buss and Drapeau 2001, 2002).

To analyze motor activity, we first embedded fish in agarose, their trunks freed so the tail could move unrestricted in the dish. Fish were then spinalized (i.e., the rostral spinal cord was completely severed) and subsequently filmed using a high-speed (500 Hz) camera to facilitate detection of the rapid undulations that occur during swimming behavior. The results obtained during these experiments are summarized in Table 1.

While untransected fish often swam after restraint in agarose, no spontaneous motor activity was observed after spinalization (n = 34). Subsequent addition of NMDA (80–500 μM) to the bathing media evoked random flexions of the tail that eventually organized into rhythmic undulations characteristic of swimming behavior (n = 34). This activity could even be observed in preparations where the entire head had been removed (see supplemental video 1). Undulations appeared to comprise waves of muscle contraction that propagated in a rostrocaudal direction and alternated rhythmically between the two sides of the body. Figure 1A depicts frames taken from a recording of a fish spinalized at the sixth somite, ~4 min after addition of 170 μM NMDA to the bathing media (the video is available on-line as supplemental video 2). Frames were selected to show side to side flexions of the trunk which repeat in a rhythmic fashion to generate, in this excerpt, a 21.5 ± 4.0-Hz beating of the tail. Examination of tail-beat frequencies in all fish revealed an average frequency of 18.2 ± 0.7 Hz (Fig. 1B).

The frequency is around half of that reported in untransected 3-day-old fish (Buss and Drapeau 2001) although it is within the range for low-frequency swimming.

It was apparent during these studies that the rhythmic tail-beating activity was broken into discreet, repeating episodes that were separated by quiescent periods where no rhythmic activity was observed (though occasional random flexions or twitches of the tail were often observed during these periods). To characterize the episodic nature of NMDA-induced activity in more detail, we recorded prolonged (20 s) periods of activity (capture frequency = 60 Hz) in five fish and found that discreet episodes of tail-beating activity occurred on average every 1.4 s (average frequency = 0.71 ± 0.05 Hz, Fig. 1C) and lasted around half a second in duration (average = 0.55 ± 0.04 s, Fig. 1D). Therefore rhythmic tail beating occupied around 39% of each episode cycle and inactivity occupied the remaining 61%.

Episodes would cycle repeatedly in this fashion for several minutes (maximum observed duration was 14 min) until the NMDA was washed off.

Peripheral nerve recordings of fictive swimming activity in spinal zebrafish larvae

Because NMDA was capable of eliciting swim-like undulations of the trunk in spinalized zebrafish, we sought to determine if this behavior was generated by a coordinated pattern of nervous system activity with the appropriate temporal characteristics for swimming. We began by using extracellular recording techniques (Masino and Fetcho 2005) to record NMDA-induced fictive motor patterns from peripheral nerves in paralyzed, spinalized zebrafish at 3 days in development. A summary of the results obtained during these experiments is presented in Table 2.

In normal fish saline that contained 15 μM d-tubocurarine to prevent muscle contraction, no peripheral nerve activity was detected in spinalized animals. However, addition of NMDA (300–600 μM) evoked a regular pattern of peripheral nerve activity. As can be seen in Fig. 2A, fictive activity was broken up into repeating episodes of rhythmic peripheral nerve discharges with each episode separated by a quiescent period where little to no activity was observed (n = 12 fish). Fictive episode periods (the time in seconds between the onset of each successive episode, determined from EP in Fig. 2B) were ~1.7 s in duration, giving an average fictive episode frequency of 0.58 ± 0.01 Hz (Fig. 2C). Each episode (determined from ED in Fig. 2B) lasted on average 0.74 ± 0.02 s (Fig. 2D). Therefore peripheral nerves generated fictive activity for around 43% of each episode cycle and were quiescent for the remaining 57%. Episodes typically repeated in this fashion for the duration of the experiment (28 min maximum). As described in the following text for NMDA-induced activity, and previously for intact larvae (Masino and Fetcho 2005), each episode presumably reflects a bout of fictive swimming and consists of multiple brief discharges that presumably generate individual myotomal muscle contractions during swimming.

If NMDA-induced activity recorded from peripheral nerves was the fictive correlate of swimming, each episode should share common temporal features with those seen during swimming in untransected aquatic vertebrates. In aquatic animals, episodes of swimming are generated by a rhythmic series of alternating (side-to-side) muscle contractions that progress rostrocaudally (with a brief intersegmental delay) down each side of the body (Cohen and Wallen 1980; Fetcho and Svoboda

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tail-beat episode duration, s</th>
<th>Tail-beat episode frequency, Hz</th>
<th>Tail-beat frequency, Hz</th>
</tr>
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<tr>
<td></td>
<td>0.55 ± 0.04 (5)</td>
<td>0.71 ± 0.05 (5)</td>
<td>18.2 ± 0.7 (34)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Values in parentheses are the number of fish examined to obtain the presented mean.
We therefore determined the frequency, duration, rostrocaudal progression, and contralateral phasing of fictive bursts within each episode of activity to determine if NMDA-induced peripheral nerve activity was indeed the neural correlate of swimming.

Figure 2E depicts a representative example of six cycles of fictive nerve activity taken from within an episode of NMDA-evoked activity in a spinalized fish at 3 days in development. Measurement of the duration of each fictive burst (BD, Fig. 2E) across all recordings (n = 12) revealed that they were relatively brief, ranging between 1.8 and 19.0 ms, with an average of 10.3 ± 0.1 ms (Fig. 2F). This was similar to fictive burst durations observed during spontaneous activity in untransected wild-type fish (mean BD = 13.0 ± 0.1 ms, n = 3, not shown) and to burst durations reported previously in older larval zebrafish (Masino and Fetcho 2005).

The frequency of fictive burst discharges were determined from cycle period (the time, in milliseconds, between onset of two successive peripheral nerve discharges, CP in Fig. 2E) measurements. Cycle periods ranged from 22.8 to 140.6 ms with an average of 48.7 ± 10.2 ms, giving an average fictive burst discharge frequency of 21.2 ± 0.1 Hz (n = 12 fish, Fig. 2G), closely matching the rhythmic 18.2 ± 0.7 Hz tail-beat frequency observed in unparalyzed spinalized fish exposed to NMDA (Fig. 1B).

In intact zebrafish larvae, a brief (<1.5 ms) rostrocaudal delay occurs between successive myotomes during fictive motor activity (Buss and Drapeau 2002; Masino and Fetcho 2005). To assess whether NMDA-evoked fictive activity in spinalized zebrafish also progressed in a rostrocaudal direction, we placed extracellular electrodes at two different intermyotomal clefts on the same side of the body (Fig. 3A, n = 4). The rostrocaudal delay (the time delay, in ms, between onset of fictive burst discharges at the recorded rostral and caudal clefts, Δt in Fig. 3A), was then measured. As expected, fictive burst discharges typically propagated rostrocaudally down the body of the fish such that the rostral peripheral nerve discharges

<table>
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<th>Parameter</th>
<th>Mean ± SE (n)</th>
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<tr>
<td>Fictive episode duration, s</td>
<td>0.74 ± 0.02 (12)</td>
</tr>
<tr>
<td>Fictive episode frequency, Hz</td>
<td>0.58 ± 0.01 (12)</td>
</tr>
<tr>
<td>Fictive burst discharge frequency, Hz</td>
<td>21.2 ± 0.1 (12)</td>
</tr>
<tr>
<td>Fictive burst duration, ms</td>
<td>10.3 ± 0.1 (12)</td>
</tr>
<tr>
<td>Delay per intermyotomal segment, ms</td>
<td>1.66 ± 0.04 (4)</td>
</tr>
<tr>
<td>Contralateral phase</td>
<td>0.520 ± 0.004 (5)</td>
</tr>
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Values are means ± SE. Values in parentheses are the number of fish examined to obtain the presented mean.
preceded the caudal peripheral nerve discharges on each cycle of activity. The delay per myotomal segment ranged from –3.9 to 6.7 ms with an average of 1.66 ± 0.04 ms (Fig. 3C). While some delays were negative (i.e., caudorostral in progression), these comprised only 2.4% (26 of 1,069) of all measured cycles. As such, the large majority of cycles progressed longitudinally down each side of the body in a head to tail direction, as seen during fictive swimming in untransected fish (Buss and Drapeau 2002; Masino and Fetcho 2005).

Finally, NMDA-induced activity should alternate rhythmically between the two sides of the body if it was to reflect swimming behavior. To determine if this was the case, we recorded simultaneously from peripheral nerves on contralat-
eral sides of the trunk \((n = 5, \text{Fig. } 3B)\). The degree of alternation was quantified by determining the point at which onset of the contralateral nerve discharged relative to the cycle period. The resulting contralateral phase ratio \((\Delta t/CP \text{ in Fig. } 3B)\) gives an indication of the degree of alternation between the two sides of the body, with 0.5 being precise alternation. The mean contralateral phase was 0.520 ± 0.004 (Fig. 3D), representative of an alternating activity pattern similar to that seen in intact larvae (Masino and Fetcho 2005).

**NMDA motoneuron activity patterns in spinalized zebrafish larvae**

Because peripheral nerve recordings revealed a pattern of fictive activity evoked by NMDA that was appropriate for swimming, we next sought to determine if motoneuron activity also shared common features with those observed during fictive locomotion in intact zebrafish larvae (Buss and Drapeau 2001). To do this, we used whole cell patch-clamp techniques to monitor motoneuron activity in 3-day-old spinalized zebrafish exposed to NMDA with the expectation that these patterns underlie the rhythmic motor activity observed in kinematic and peripheral nerve recordings. The results of measurements obtained during patch-clamp experiments are summarized in Table 3.

Spinalization resulted in silencing of network activity such that no patterned synaptic activity was observed in recorded motoneurons. Subsequent addition of NMDA \((100–650 \mu M)\) increased the occurrence of postsynaptic potentials (PSPs) and depolarized cells by 17.7 ± 1.7 mV \((n = 20, \text{Fig. } 4A)\). Similar large, steady depolarizations induced by NMDA have been reported in intact zebrafish (Cui et al. 2004). After this depolarization, we observed that a regular pattern of activity developed, which typically presented as a repeating series of slow depolarizing oscillations, during the peaks of which rapid PSPs

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**FIG. 3.** Rostrocaudal and contralateral phasing of NMDA-induced peripheral nerve activity in spinal 3-day-old zebrafish. A: ipsilateral peripheral nerve recordings taken from the 7th (L7) and 14th (L14) intermyotomal clefts on the left hand side of the body (see schematic) of a fish spinalized at the 5th somite and bathed in 300 μM NMDA. □, burst activity in the rostral (L7) peripheral nerve recording. △t indicates the rostrocaudal delay. B: contralateral peripheral nerve activity recorded from the 7th intermyotomal cleft on the left hand side of the body (L7) and the 9th intermyotomal cleft on the right hand side of the body (R9, see schematic) of a 3-day-old zebrafish spinalized at the 5th somite and bathed in 400 μM NMDA. □, burst activity recorded from the right hand side of the body (R9). C and D: histograms of the rostrocaudal delay per myotomal segment (measured in \(n = 4\) fish, C) and the contralateral phase (measured in \(n = 5\) fish, D).
capable of driving action potentials occurred. Figure 4B (an expanded section of the activity underlined in Fig. 4A) shows two subsequent slow oscillations with rapid PSPs that drive action potentials occurring at the peaks of these oscillations. Hyperpolarizing current was often injected into cells to make the slow oscillations larger in amplitude, thereby making it easier to quantify this aspect of the activity (Fig. 4C).

In intact zebrafish, synaptic activity in motoneurons presents as a tonic depolarization on which occur rapid PSPs that drive action potentials in a rhythmic fashion (Buss and Drapeau 2001). In spinalized zebrafish exposed to NMDA, there was also a tonic component (the slow oscillations) and a rapid PSP component. We examined each of these aspects of the NMDA-induced motoneuron activity in more detail, beginning with the slow oscillations. The oscillatory period (OP in Fig. 4C) was on average around 2 s such that oscillations occurred at an average frequency of 0.50 ± 0.01 Hz (Fig. 4D). The slow oscillations appeared to gate the rapid PSPs, which typically were clustered at the peaks of each slow oscillation (Fig. 4B).

As such, the rapid PSPs occurred in discrete episodes. Measuring the duration of each PSP episode (ED in Fig. 4D) gave an average of 0.90 ± 0.01 s (Fig. 4E). Therefore the rapid PSPs occupied ~45% of each oscillatory cycle, while the remaining 55% had little to no rapid PSP activity.

We next examined the rapid PSPs that occurred during the peaks of the slow oscillations. The PSPs were capable of crossing action potential threshold (mean = −38.0 ± 0.9 mV). While the rapid PSPs often appeared rhythmic (Fig. 4F, *), irregular PSPs interspersed between the rhythmic potentials at the peaks of the slow oscillations (Fig. 4F, →). Irregular PSPs similar to the ones we observed occur during fictive locomotion in intact zebrafish (Buss and Drapeau 2001) and are glycineergic in origin. It is likely the irregular PSPs observed here are also glycineergic since, as detailed in the following text, they were abolished by strychnine and because glycineergic currents isolated using voltage-clamp techniques were irregular in timing. Despite the presence of irregular PSPs, spectral analysis of motoneuron activity revealed that rhythmic potentials could still be detected. As shown in Fig. 4G, which depicts the power spectrum of the neuron shown in 4C, a distinct peak in power could be detected at ~20 Hz, reflecting the average measured PSPs frequency of 23.4 ± 1.6 Hz (n = 20 recorded cells).

**TABLE 3. Parameters of NMDA-induced motoneuron activity in spinal zebrafish**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value ± SE (n)</th>
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<tr>
<td>PSP episode duration, s</td>
<td>0.90 ± 0.01 (20)</td>
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<tr>
<td>Slow oscillation frequency, Hz</td>
<td>0.50 ± 0.01 (20)</td>
</tr>
<tr>
<td>PSP frequency, Hz</td>
<td>23.4 ± 1.6 (20)</td>
</tr>
<tr>
<td>Glutamate slow oscillation frequ.</td>
<td>0.6 ± 0.2 (6)</td>
</tr>
<tr>
<td>Glutamate PSP frequ. Hz</td>
<td>21.8 ± 2.4 (6)</td>
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Values are means ± SE. Values in parentheses are the number of fish examined to obtain the presented mean.

**Currents underlying the NMDA-evoked rhythm in spinal zebrafish larvae**

We used whole cell voltage-clamp techniques to attempt to identify currents underlying NMDA-induced activity in motoneurons. Only two types of synaptic activity, glycineergic and glutamatergic, have been shown to occur during rhythm generation in motoneurons of intact zebrafish larvae (Buss and Drapeau 2001). These currents can be isolated during rhythmic synaptic activity by voltage clamping at the reversal potential for chloride ions (to isolate the glutamatergic cation current) or cations (to isolate the glycineergic current). This method can be used to circumvent the use of pharmacological antagonists, which act on neurons of the entire spinal cord and may cause indirect changes to the rhythmic activity observed in motoneurons (Buss and Drapeau 2001). We first isolated the cationic component of the NMDA-induced rhythm in motoneurons of 3-day-old spinalized zebrafish (n = 6). The chloride ion reversal potential was determined experimentally in each motoneuron by identifying the reversal potential of spontaneous glycinergic synaptic currents (around −46 mV). The cation current had both a slow oscillatory component and a rapid postsynaptic current (PSC) component. The slow oscillations (Fig. 5Ai, top) occurred at an average frequency of 0.6 ± 0.2 Hz, whereas the rapid PSCs (Fig. 5Ai, bottom) occurred at the peaks of these oscillations with a mean frequency of 21.8 ± 2.4 Hz. The rapid PSCs presented as distinct peaks in power occurring at ~20 Hz in spectral analysis plots (e.g., Fig. 5Ai plots the power spectrum for activity shown in Fig. 5Ai).

Motoneurons were then voltage clamped at the cation reversal potential (around 0 mV, n = 6) to isolate the chloride current (Fig. 5Bi). Again the cation reversal potential was determined experimentally in each motoneuron by identifying the reversal potential of spontaneous glutamatergic synaptic currents. We found that the peaks of the chloride current occurred in a less regular fashion than the cation current. As such, while spectral analysis revealed a peak frequency at ~20 Hz, frequencies on the whole were far more variable (e.g., Fig. 5Bii plots the power spectrum for activity shown in Fig. 5Bi). The isolated chloride current was presumably glycineergic because it was abolished by strychnine (Fig. 5Bi).

We sought to confirm that the observed cation and chloride currents were generated by activation of glutamate and glycine receptors, respectively, by using pharmacological agents to block these receptors during voltage recording of motoneurons in spinalized zebrafish. We began by adding the pan-specific glycine receptor antagonist strychnine. In intact, spontaneously active preparations, strychnine abolishes the irregular PSPs that occur during rhythmic motoneuron activity (Buss and Drapeau 2001). We observed an analogous effect here whereby the irregular PSPs that occur during the peaks of the slow oscillations were abolished, leaving rhythmic PSPs (Fig. 5C, n = 5). Subsequent addition of the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) in the presence of strychnine (n = 4) completely abolished NMDA-induced activity after prolonged exposure (ca. 14 min). However, prior to cessation of activity, CNQX blocked the rapid PSPs that drive action potentials leaving summating PSPs that were presumably generated by NMDA receptor activation (Fig. 5D). Finally, addition of the NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV) also invariably abolished all network activity (Fig. 5E, n = 4).
NMDA slow oscillations in motoneurons require magnesium ions

In other locomotor networks, slow, self-sustaining NMDA-evoked membrane potential oscillations that depend on the presence of magnesium can be observed (e.g., Sigvardt et al. 1985; Sillar and Simmers 1994a,b; Wallen and Grillner 1985). To determine a possible requirement for magnesium ions in the generation of the slow NMDA-induced oscillations, 3-day-old spinalized zebrafish were bathed in magnesium-free saline containing NMDA. As can be seen from the panels in Fig. 6A, NMDA-induced slow oscillations (Fig. 6Ai) were abolished when magnesium-free fish saline was washed into the bath (Fig. 6Aii). The slow rhythm recovered on re-addition of magnesium ions to the bath (Fig. 6Aiii).
magnesium (not shown). Magnesium ions therefore appear to be required for the slow rhythmic component of NMDA-induced activity.

Previous studies in a range of vertebrates has shown that slow NMDA-induced membrane oscillations persist in the absence of action potentials (Hochman et al. 1994; MacLean et al. 1997; Scrymgeour-Wedderburn et al. 1997; Sigvardt et al. 1985; Sillar and Simmers 1994a,b; Tresch and Kiehn 2000; Wallen and Grillner 1985). To determine if this was also the case in zebrafish, the sodium channel blocker tetrodotoxin (TTX) was added to spinal zebrafish bathed in NMDA (\(n = 5\)). TTX blocked all network activity (Fig. 6\(Bi, cf. Bii\)). However, under these conditions, no membrane potential oscillations were observed (Fig. 6\(Bii\)). Because NMDA-induced oscillations are voltage dependent, we manipulated the membrane potential between \(-90\) and \(-10\) mV an attempt to unmask oscillations. However, we were unable to observe oscillations at any membrane potential.

There is precedence in frog tadpoles to suggest that TTX resistant oscillations are also conditional on the presence of serotonin (Scrymgeour-Wedderburn et al. 1997; Sillar and Simmers 1994a,b). We therefore added serotonin to the recording solution in the presence of NMDA (\(n = 5\)). However, we were still unable to unmask membrane potential oscillations (Fig. 6\(Biii\)). Hence, preliminary data suggest that, at least in larval motoneurons, the slow component of the NMDA-induced rhythm appears to be dependent on NMDA and magnesium and does not persist in the presence of TTX.
toneuron activity elicited by NMDA in two somite sections of the spinal cord \((n = 5)\) revealed that this activity comprised a rhythmic glutamatergic current (Fig. 7Bi) and a less rhythmic glycinergic current (Fig. 7Bii) reminiscent of that seen in whole spinal cord preparations (Fig. 5, A and B). Therefore the circuitry that generates rhythmic activity is likely local in nature.

**Strychnine disrupts contralateral alternation of fictive motor discharges in spinalized zebrafish larvae**

Glycinergic inhibition coordinates alternation of antagonistic muscle groups in all vertebrate motor systems studied to date (Arshavsky Yu et al. 1993; Butt et al. 2002; Grillner et al. 1995). Motoneuron recordings typically reveal strong, rhythmic glycinergic inhibitory potentials during the contralateral phase of motor activity, when motoneurons innervating the antagonistic muscle are active. However, no rhythmic hyperpolarizing glycinergic activity has been reported in zebrafish motoneurons. Instead, glycinergic inputs on motoneurons are irregularly timed, ranging in frequency from 20 to 500 Hz, a range that extends well above the normal frequency range for swimming (Buss and Drapeau 2001) (see also Fig. 5Bi). Furthermore, the equilibrium potential for chloride is depolarized relative to the resting potential in the developing zebrafish (Brustein et al. 2003b; Saint-Amant and Drapeau 2000). This therefore raises the question as to whether glycinergic transmission plays a role in coordination of contralateral alternation in zebrafish. To address this issue, we studied the effects of strychnine on fictive motor behavior in spinalized zebrafish bathed in NMDA. We began by performing kinematic analysis of the effects of strychnine. Here, intact larvae were bathed in 10 \(\mu\)M strychnine (lower concentrations did not have an effect in these minimally dissected preparations). When fish began to show seizure-like contractions (Hirata et al., 2005), the spinal cord was subsequently transected and NMDA (300–600 \(\mu\)M) added to elicit motor activity. Under these conditions, strychnine caused a severe disruption in NMDA-induced rhythmic swimming (Fig. 8A, \(n = 16\), see also supplemental video 3).

We next examined the effects of strychnine on peripheral nerve activity in spinalized zebrafish. In the presence of NMDA (300–600 \(\mu\)M), we found that strychnine had a much stronger effect, presumably owing to increased drug access afforded by removal of the skin overlying the trunk. As such, equivalent or higher concentrations (10–50 \(\mu\)M) to those used in kinematic studies abolished NMDA-induced motor activity \((n = 6\) fish, Fig. 8Bii cf. Bi). We therefore tested lower

![Fig. 6.](image1.png) **Fig. 6.** The NMDA-induced slow oscillations are magnesium dependent but not intrinsic to motoneurons of 3-day-old zebrafish. A: voltage recording of a 3-day zebrafish motoneuron bathed in 500 \(\mu\)M NMDA (i). Washing magnesium-free saline into the bath abolishes the slow oscillations (ii). B: voltage recording of a 3-day zebrafish motoneuron bathed in 650 \(\mu\)M NMDA (i). Washing TTX into the bath abolishes network activity (ii). No rhythm is observed after addition of 10 \(\mu\)M serotonin (iii). † † † in A and B = –60 mV.

![Fig. 7.](image2.png) **Fig. 7.** NMDA-evoked rhythmic activity in a two somite segment of the 3-day-old zebrafish spinal cord. Ai: voltage recording of a motoneuron within an isolated 2-segment section of a 3-day zebrafish spinal cord bathed in 850 \(\mu\)M NMDA · · · · · · · · 60 mV. ii: expanded trace taken from underlined section in A. Bi: 2-s excerpt of glutamatergic activity in a motoneuron voltage clamped at –42 mV from a 2 somite segment (somites 6 and 7) of the spinal cord in a 3-day zebrafish and bathed in 750 \(\mu\)M NMDA ii: 2-s excerpt of glycinergic activity in same cell voltage clamped at 0 mV.
concentrations. At concentrations of 1–8 μM strychnine, NMDA-induced motor activity persisted (Fig. 8Bi). Analysis of burst durations in fish exposed to these lower strychnine concentrations (n = 5 fish) revealed that bursts ranged from 4.4 to 109.6 ms with an average duration of 23.0 ± 0.6 ms (Fig. 8C), around twice that observed when strychnine was absent from the bathing media (10.3 ± 0.1 ms, Fig. 2F). Measurement of discharge frequency revealed a mean of 22.1 ± 0.4 Hz (Fig. 8D), close to that seen in preparations not treated with strychnine (21.2 ± 0.1 Hz). Finally, the contralateral phase was found to be disrupted in strychnine treated fish. As the histogram in Fig. 8E demonstrates, the contralateral phase had a
mean of 0.39 ± 0.01 with a peak around zero (i.e., synchronous motor discharge on the 2 sides of the body). Therefore the predominant effect of strychnine was to disrupt coordination across the two sides of the body such that discharges between contralateral peripheral nerves became synchronous on the majority of cycles.

We also examined the effects of strychnine on the frequency and duration of fictive motor episodes. As can be seen from the example in Fig. 8F, NMDA-induced activity was still broken into discreet episodes in the presence of strychnine (n = 5 fish). These episodes occurred at a mean frequency of 1.0 ± 0.1 Hz (Fig. 8G) and duration of 0.30 ± 0.01 s (Fig. 8H). Therefore there was roughly a doubling in episode frequency and a halving of episode duration compared with control fish (cf. Fig. 2, C and D).

**Ontogeny of motor activity in spinal zebrafish**

The effect of NMDA on spinalized zebrafish at earlier stages in development was also investigated to determine at what point the spinal cord acquires intrinsic rhythm-generating properties. In the intact zebrafish, swimming activity first emerges at ~27 h in development (Saint-Amant and Drapeau 1998). This early form of activity is characterized by slow (ca. 10 Hz) undulations of the trunk that alternate between opposite sides of the body. We therefore examined the effects of NMDA on spinalized zebrafish at around 30 h in development, shortly after the onset of swimming behavior. Addition of NMDA (500–850 μM), after depolarizing cells by 26.0 ± 8.0 mV, invariably evoked rapid PSPs occurring at an average frequency of 7.6 ± 0.7 Hz (n = 11, Fig. 9A). In a small proportion of cells (3 of 11), a slow (0.4 ± 0.2 Hz) oscillatory component

![Picture](https://example.com/image.png)

**Fig. 9.** NMDA-induced rhythmic activity in spinal 1-day-old zebrafish. A: 10-s excerpt of a motoneuron recording depicting rhythmic synaptic activity in a motoneuron of a spinalized (at the 4th somite) zebrafish at 31 h in development bathed in 700 μM NMDA. ii: expanded period of activity taken from region highlighted with bar in i depicting rhythmic PSPs (→) and intermittent glycineric potentials (*). Bi: 10-s excerpt of a voltage recording of NMDA-evoked motoneuron activity in a 32-h-old zebrafish spinalized at the 4th somite that had both fast and slow rhythmic components. ii: expanded period of activity taken from region highlighted with bar in i depicting rhythmic PSPs (→) and intermittent glycineric potentials (*). Ci, left: 2.5-s excerpt of glycineric activity in same cell voltage clamped at 0 mV. C: 20 pA. Right: expanded region underlined with bar. D: selected frames of kinematic analysis depicting coiling behavior in the trunk of a zebrafish spinalized at the 5th somite and bathed in 700 μM NMDA.
was also observed in coincidence with the fast component \((n = 3; \text{Fig. 9B})\). Voltage-clamp recordings from embryonic motoneurons \((n = 15)\) revealed the presence of a rhythmic glutamatergic current (Fig. 9Ci) and an intermittent glycinergic current (Fig. 9Cii). The glycinergic current in embryonic preparations did not summate as it did in 3-day-old fish (Fig. 9Cii). To determine if the synaptic drive in embryonic motoneurons was the neural correlate of swimming, the heads of these fish were embedded in agarose and their trunks were freed. The spinal cord was then transected and NMDA \((500–850 \text{ mM})\) was added to the bathing media. In only 2 of 41 preparations were alternating \((3 \pm 0.5 \text{ Hz})\) undulations of the trunk sufficient to propel the fish in a swim-like manner observed (data not shown). The remainder of preparations \((39 \text{ of 41})\) generated strong \(1.0 \pm 0.1-\text{Hz}\) coiling flexions of the trunk unrelated to swimming and characteristic of the earliest form of behavior previously described in the intact embryo (Fig. 9D) (Saint-Amant and Drapeau 1998).

At 2 days in development, when hatching occurs, zebrafish generate a pattern of swimming activity termed burst swimming that is characterized by infrequent, uninterrupted periods of motor activity at relatively high (ca. 50 Hz) frequency (Buss and Drapeau 2001). When spinalized zebrafish at this stage in development were bathed in NMDA \((400–800 \mu\text{M})\), after a \(23.5 \pm 0.9-\text{mV}\) depolarization of the resting potential, the majority of preparations \((n = 13 \text{ of 16})\) generated a pattern of synaptic activity that appeared to lack rhythmicity when compared with fish at 3 days in development (Fig. 10A). In only 3 of these 16 embryos did we observe a pattern of activity that had obvious rapid PSP and slow oscillatory components.

**FIG. 10.** NMDA-induced rhythmic activity in spinal 2-day-old zebrafish. A: voltage recording of synaptic activity in a motoneuron of a spinalized (at the 6th somite) zebrafish at 2 days in development bathed in 500 \(\mu\text{M}\) NMDA. ii: expanded period of activity taken from region highlighted with bar in i. B: voltage recording depicting the slow rhythmic component of NMDA evoked activity in a spinalized (at the 5th somite) zebrafish at 2 days in development. ii: expanded period of activity taken from region highlighted with bar in i. c: voltage-clamp recording of rhythmic glutamatergic (i) or nonrhythmic glycinergic (ii) motoneuron activity of a 30-h zebrafish spinalized at the 5th somite and bathed in 700 \(\mu\text{M}\) NMDA. Di: selected frames of taken from high-speed \((500 \text{ Hz})\) kinematic analysis depicting lack of rhythmic activity in the trunk of a 2-day zebrafish spinalized at the 5th somite and bathed in 700 \(\mu\text{M}\) NMDA.
In these cells, the frequency of the slow oscillations was 0.4 ± 0.1 Hz and was generally weaker than that seen at 3 days because it depolarized the membrane by only 4.3 ± 0.5 mV. The fast component occurred at a frequency of 9.1 ± 0.7 Hz, much lower than the frequency of PSPs during motor activity in the intact fish (52.0 ± 4.0 Hz) (Buss and Drapeau 2001).

However, when motoneurons of fish at 2 days in development were voltage clamped to isolate the glycineergic and glutamatergic components of NMDA-evoked activity, a rhythmic glutamate current (Fig. 10Ci) and an irregular glycinergic current (Fig. 10Ci) were clearly visible in all recorded cells (n = 5). To determine if the synaptic drive at these earlier stages was sufficient to generate swimming behavior, the heads of these fish were embedded in agarose and their trunks were freed. The spinal cord was then transected and NMDA (200–850 μM) was added to the bathing media. Swimming activity was never observed during these behavioral experiments (n = 36). Instead weak, rapid, nonrhythmic muscle contractions with intermittent strong body flexions were seen that did not generate undulations characteristic of swimming in the intact 2-day zebrafish (Fig. 10D).

**DISCUSSION**

In vertebrates, locomotor behavior is generated by rhythmic oscillating circuits that alternately activate pairs of antagonistic muscles such as flexor/extensor muscles in limbed vertebrates or axial swimming muscles in aquatic animals. These circuits are local to the spinal cord and capable of rhythm generation in isolation if a source of (unpatterned) excitatory activity is provided. For example in the lamprey, tonic stimulation of reticulospinal neuron regions elicits locomotion (McClellan and Grillner 1984). Because reticulospinal neurons are primarily glycineergic in nature (Buchanan et al. 1987; Ohta and Grillner 1989), it is likely that descending unpatterned glutamatergic activity is sufficient to induce locomotor behavior. Furthermore, excitatory amino acid agonists evoke rhythm generation in a range of vertebrates such as the chick (Barry and O’Donovan 1987), cat (Douglas et al. 1993), lamprey (Cohen and Wallen 1980; Poon 1980; Wallen and Williams 1984), mouse (Hernandez et al. 1991), mud puppy (Wheatley and Stein 1992), rabbit (Fenaux et al. 1991), rat (Cazalets et al. 1990; Houssaini et al. 1993; Kjaerulf et al. 1994; Kudo and Yamada 1987), and Xenopus tadpole (Dale and Roberts 1984). We show here that the 3-day-old zebrafish spinal cord can also generate rhythmic motor activity after spinalization and exposure to NMDA, demonstrating that the CPG for swimming is intrinsic to the spinal cord and does not require patterned input from the brain to produce rhythmic behavior.

**Similarity between NMDA-induced activity in spinal zebrafish and motor activity in intact zebrafish**

Several findings indicate that NMDA induces swimming in spinalized zebrafish. First kinematic studies revealed a clearly alternating undulation of the tail, occurring at a frequency of ca. 20 Hz that resembled low-frequency swimming behavior in intact, freely behaving fish. Analysis of peripheral nerve activity in paralyzed preparations revealed a fictive pattern of motor discharge that shared all the temporal characteristics of swimming behavior. Specifically, cycles of fictive burst discharge, occurring at a frequency of ca. 20 Hz, progressed rostrocaudally (with a brief delay between myotomal muscle blocks) down each side of the body in an alternating fashion. Therefore it is likely that peripheral nerve activity in spinalized zebrafish exposed to NMDA is the fictive correlate of swimming behavior previously described in intact zebrafish larvae (Buss and Drapeau 2001, 2002; Masino and Fetcho 2005). The phasing of synaptic components during NMDA-induced motoneuron activity also shared several common features with the activity thought to underlie swimming in intact zebrafish (Buss and Drapeau 2001). In spinalized zebrafish exposed to NMDA, rhythmic (ca. 20 Hz) PSPs capable of driving action potentials appeared to be glutamatergic and most likely determined the frequency of muscle contractions during locomotion. This activity was similar to the rhythmic glutamatergic activity that drives action potentials during fictive contractions in intact zebrafish larvae (Buss and Drapeau 2001). Furthermore, irregular PSPs were also observed during NMDA-induced synaptic activity in motoneurons of spinalized zebrafish. These PSPs appeared to be glycineergic in origin because they could be abolished by strychnine and because glycine currents recorded in voltage clamp were less rhythmic than glutamatergic currents. This mirrors the observations previously reported in the intact zebrafish (Buss and Drapeau 2001) where glycineergic potentials occur at variable frequencies and summate to generate a tonic conductance. The authors speculated that it was the tonic nature, rather than the irregular timing, of the glycine current that was important for rhythm generation in motoneurons of zebrafish, causing a decrease in the membrane input resistance and time constant that regulates action potential firing. Our data indicate that glycine functions in a similar manner during NMDA-induced activity in spinalized fish. Finally, during fictive locomotion in intact zebrafish, PSPs occur during a sustained tonic depolarizing potential. We also observed that NMDA-induced PSP activity occurred coincident with tonic depolarizing potentials. The principal difference was that these tonic potentials oscillated in a slow, repeating manner.

**Slow oscillations and gating of NMDA-induced episodes**

The slow depolarizing oscillations observed in the presence of NMDA appear to gate the rapid PSPs and irregular glycineergic PSPs such that little synaptic activity was observed during the troughs of the slow oscillations. These oscillations are too slow (0.5 Hz) to generate natural swimming activity in zebrafish (which occurs in the frequency range of ca. 20–100 Hz); however, they roughly match the duration and frequency of NMDA-induced episodes of peripheral nerve discharges. The inference is therefore that these oscillations pattern NMDA-induced peripheral nerve discharge activity into brief (0.5 s) swim episodes that occur at a frequency of ca. 0.5 Hz. In an analogous fashion, the intensity of peripheral nerve discharges oscillates at a similar frequency during NMDA-induced fictive motor activity in Xenopus (Reith and Sillar 1998), an effect though to arise from the voltage sensitivity of NMDA receptors (see following text).

The slow oscillations may play a role in development of more mature locomotor patterns in the developing zebrafish: swimming behavior transitions from episodes of uninterrupted...
“burst” swimming in 2-day-old fish to repeating episodes of intermittent “beat-and-glide” activity at 4 days whereby motoneurons continuously alternate between brief (ca. 200 ms) rhythmically active (beat) periods and longer (400 ms) quiescent (glide) periods (Buss and Drapeau 2001). The slow NMDA-evoked rhythm reported here closely matches the periodicity of natural beat-glide swimming. While it has previously been suggested that the duration of beat periods is determined by accommodation of action potentials in zebrafish (Buss et al. 2003), development of strong voltage-dependent properties of the NMDA receptor may also be required for ontogeny of beat-glide motor activity. Indeed, the sharp increase in occurrence of slow NMDA induced oscillations between 1 and 3 days in development during our experiments suggests that this may be the case.

In vertebrate motor systems such as the lamprey (Sigvardt et al. 1985; Wallen and Grillner 1985), the frog tadpole (Scrymgeour-Wedderburn et al. 1997; Sillar and Simmers 1994a,b; but see following text), and the rat (Hochman et al. 1994; MacLean et al. 1997) slow NMDA-evoked membrane potential oscillations persist in the presence of TTX. These oscillations depend on the presence of magnesium ions (Sigvardt et al. 1985; Sillar and Simmers 1994; Wallen and Grillner 1985), which block the NMDA receptor at negative membrane potentials (Mayer and Westbrook 1984) and impart bistability of membrane excitability in the presence of NMDA. In this study, we found that slow NMDA-induced network oscillations were conditional on the presence of magnesium but did not persist in TTX. In the developing tadpole nervous system, NMDA-induced membrane oscillations are not observed after application of TTX and NMDA alone (Scrymgeour-Wedderburn et al. 1997; Sillar and Simmers 1994). Co-application of serotonin, however, can unmask oscillations in a proportion of motoneurons (Scrymgeour-Wedderburn et al. 1997). The propensity to observe these oscillations is strongly regulated during development, with studies suggesting that very few embryonic neurons (12%) and the majority of larval neurons (70%) can generate TTX-resistant NMDA-induced oscillations after serotonin application. While we failed to observe any effect of serotonin in the present study, it should be noted that serotonin has no reported effect on zebrafish behavior until later stages in development, when it shortens quiescent periods between episodes of swimming (Brustein et al. 2003a). The absence of TTX resistant oscillations reported here may also reflect the relative immaturity of the developing larval zebrafish. It is very likely that only a proportion of zebrafish neurons (perhaps premotor neurons) are mature enough to produce NMDA-induced oscillations in NMDA at this stage of development, a phenomenon that may have precluded their observation owing to the relatively small pool of neurons examined during the current study.

Effects of strychnine on NMDA-induced motor activity

Glycinergic inhibitory transmission is the common mechanism that governs alternation of antagonistic muscle pairs in vertebrates (Arshavsky Yu et al. 1993; Butt et al. 2002; Grillner et al. 1995). In aquatic vertebrates, strychnine disrupts contralateral alternation of fictive motor activity, causing synchronous discharges across the two sides of the body (Alford and Williams 1989; Alford et al. 1990; Cohen and Harris-Warrick 1984; Hagevik and McClellan 1994; Roberts et al. 1985). This effect most probably arises because phasic mid-cycle inhibitory glycinergic potentials observed in these systems prevent coincident bilateral activation of spinal interneurons and motoneurons. The glycinergic synaptic drive observed in developing zebrafish motoneurons has been reported to be primarily tonic (Buss and Drapeau 2001). Furthermore, the equilibrium potential for glycine is depolarized from the resting membrane potential in zebrafish motoneurons (Brustein et al. 2003b; Brustein and Drapeau 2005; Saint-Amant and Drapeau 2000), a common feature of the developing vertebrate CNS (Aguayo et al. 2004). This therefore raises the question as to whether glycine is required for coordination of activity between opposite sides of the spinal cord. However, because we observed a dramatic disruption of alternation between contralateral sides of the body in the presence of strychnine, it appears that the function of glycinergic transmission is conserved in the zebrafish spinal pattern generator. It should also be noted that while the glycinergic synaptic drive did not share the highly regular nature of the glutamatergic drive, spectral analysis did reveal a peak frequency at around swimming frequency (see Fig. 5bii). The most parsimonious conclusion would be that glycine causes a shunting inhibition in motoneurons that, while irregular, is strongest mid-cycle, thereby causing reciprocal alternation of motoneuron activity between the two sides of the body. However, it remains possible that glycine coordinates alternation through an atypical mechanism.

Strychnine also affected the duration of peripheral nerve bursts during NMDA-induced fictive motor activity in spinalized zebrafish as has been shown to occur in frog tadpoles (Perrins and Soffe 1996; Roberts et al. 1985). Because it has previously been shown that a modest increase in motoneuron spiking occurs in zebrafish motoneurons after strychnine exposure (Buss and Drapeau 2001), this likely accounts for the increase in motor burst durations reported here. We found that the frequency of motor output was not affected by strychnine, in fitting with previous studies in intact zebrafish larvae (Buss and Drapeau 2001). However, this finding is contrary to the effects observed in the lamprey (Cohen and Harris-Warrick 1984; Grillner and Wallen 1980; McPherson et al. 1994) and Xenopus embryo (Dale 1995) where cycle periods decrease after strychnine exposure. Computer simulations of motor networks in these systems have implicated the strength and duration of mid-cycle inhibition as an important determinant of motor frequency (Dale 1995; Hellgren et al. 1992). It is therefore possible that the lack of a strong hyperpolarizing mid-cycle inhibition during fictive motoneuron activity accounts for the lack of effect of strychnine on fictive swimming frequency in zebrafish.

Development of NMDA-induced activity

While it is clear that intact fish swim in response to touch by ~30 h in development, we were not able to pharmacologically elicit swimming with NMDA until 3 days in development. At 2 days, the synaptic drive in motoneurons typically appeared arrhythmic and unpatterned after NMDA application. However, separation of currents under voltage clamp did reveal rhythmic glutamatergic and less patterned glycinergic currents. It would therefore appear that the rhythmic glutamatergic drive elicited by NMDA is too weak to generate swimming at this point in development. In zebrafish, it would therefore appear that the rhythmic glutamatergic drive elicited by NMDA is too weak to generate swimming at this point in development.
stage in development. This effect likely arises from the relatively low number of neurons at 2 days in development that may be insufficient to generate a strong, coordinated excitatory drive after spinal cord transection (which further diminishes the number of CPG neurons that can be recruited during NMDA-evoked motor activity). Conversely, it may be that there are insufficient numbers of NMDA receptors at spinal synapses to enable pharmacological activation of a stable motor pattern. Spinalized fish at around 30 h in development also typically did not swim after application of NMDA, and this was likely for the same reasons. Furthermore, extensive electrical coupling between muscle fibers (Buss and Drapeau 2000) and between neurons (Saint-Amant and Drapeau 2001) may also affect the ability of the embryonic network to generate swimming behavior in response to NMDA. Nevertheless, spinalized embryos were on occasion able to generate rhythmic trunk flexions in the presence of NMDA.

How can this finding be consolidated with the lack of swimming in isolated trunks of two day fish? Embryonic cells of the spinal network, though few in number have relatively high-input resistances. Further, embryonic neurons are extensively electrically coupled (Saint-Amant and Drapeau 2001), an effect that will presumably strengthen the amplitude and temporal coordination of the activity of a very small, immature neural network. This means that very little information is lost during synaptic transmission. This contrasts with fish at 2 days where neurons, though greater in number, have much lower input resistances making them comparatively “leaky.” Perhaps these factors help the transected embryonic spinal cord generate NMDA-induced rhythmic activity patterns even in the absence of large numbers of neurons participating in the CPG. Whatever the explanation, it appears that NMDA only reliably evokes swimming behavior in the isolated trunk at ∼3 days in development and may only be useful as a tool for studying behavior subsequent to this period. Prior to this stage, voltage-clamp experiments may nonetheless be useful to determine the properties of the spinal CPG in isolation.

In summary, we find that the CPG for swimming in zebrafish is intrinsic to the spinal cord. The CPG comprises glutamatergic and glycnergic inputs that generate a locomotor drive when an exogenous source of excitation is given. This activity is sufficient to drive swimming behavior by 3 days in development. The isolated zebrafish spinal cord preparation should prove a useful tool when trying to determine whether mutations or other manipulations affecting motor behavior arise due to deficits in the spinal CPG or deficits in the brain.

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REFERENCES


Brustein E and Drapeau P. Serotonergic modulation of chloride homeostasis during maturation of the locomotor network in zebrafish. J Neurosci In press.


Buss RR and Drapeau P. Activation of embryonic red and white muscle fibers during fictive swimming in the developing zebrafish. J Neurophysiol 87: 244–251, 2002.


