Synaptic Drive to Motoneurons During Fictive Swimming in the Developing Zebrafish

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Buss, Robert R. and Pierre Drapeau. Synaptic drive to motoneurons during fictive swimming in the developing zebrafish. J Neurophysiol 86c: 197–210, 2001. The development of swimming behavior and the correlated activity patterns recorded in motoneurons during fictive swimming in paralyzed zebrafish larvae were examined and compared. Larvae were studied from when they hatch (after 2 days) and are first capable of locomotion to when they are active swimmers capable of capturing prey (after 4 days). High-speed (500 Hz) video imaging was used to make a basic behavioral characterization of swimming. At hatching and up to day 3, the larvae swarm infrequently and in an undirected fashion. They displayed sustained bursts of contractions (‘burst swimming’) at an average frequency of 60–70 Hz that lasted from several seconds to a minute in duration. By day 4 the swimming had matured to a more frequent and less erratic “beat-and-glide” mode, with slower (~35 Hz) beats of contractions for -200 ms alternating with glides that were twice as long, lasting from just a few cycles to several minutes overall. In whole cell current-clamp recordings, motoneurons displayed similar excitatory synaptic activity and firing patterns, corresponding to either fictive burst swimming (day 2–3) or beat-and-glide swimming (day 4). The resting potentials were similar at all stages (about -70 mV) and the motoneurons were depolarized (to about -40 mV) with generally non-overshooting action potentials during fictive swimming. The frequency of sustained inputs during fictive burst swimming and of repetitive inputs during fictive beat-and-glide swimming corresponded to the behavioral contraction patterns. Fictive swimming activity patterns were eliminated by application of glutamate antagonists (kynurenic acid or 6-cyano-7-nitroquinoxalene-2,3-dione and DL-2-amino-5-phosphonovaleric acid) and were modified but maintained in the presence of the glycinergic antagonist strychnine. The corresponding synaptic currents underlying the fictive drive to motoneurons during fictive swimming could be isolated on voltage clamp and consisted of cationic [glutamatergic postsynaptic currents (PSCs)] and anionic inputs (glycinergic PSCs). Either sustained or interrupted patterns of PSCs were observed during fictive burst or beat-and-glide swimming, respectively. During beat-and-glide swimming, a tonic inward current and rhythmic glutamatergic PSCs (~35 Hz) were observed. In contrast, bursts of glycinergic PSCs occurred at a higher frequency, resulting in a more tonic pattern with little evidence for synchronized activity. We conclude that a rhythmic glutamatergic synaptic drive underlies swimming and that a tonic, shunting glycinergic input acts to more closely match the membrane time constant to the fast synaptic drive.

INTRODUCTION

The use of simple model systems, such as the lamprey (Grillner et al. 1991, 1998; Rovainen 1979) and frog embryo (Dale and Kuenzi 1997; Roberts et al. 1986, 1998), has greatly increased the understanding of cellular aspects of the neural control of vertebrate locomotion. Zebrafish embryos and larvae share with lampreys and frog embryos the experimental advantages of a simple motor and sensory system (Drapeau et al. 1999) and are a popular vertebrate model system for a wide range of scientific disciplines (Eisen 1996; Laale 1977; Vascotto et al. 1997). An advantage of zebrafish is that they hold promise for identifying the genes controlling locomotion during development of the nervous system (Granato et al. 1996). An understanding of the physiology of the zebrafish locomotor system is necessary for eventually assessing genetic mutations and manipulations in zebrafish, thus ultimately increasing our understanding of the mammalian nervous system.

Movement depends on the activation of locomotor muscles, which are under the control of spinal motoneurons. In contrast to the complex motor system of adult fish, with five types of muscle fibers (de Graff et al. 1990; van Raamsdonk et al. 1983) and four classes of motoneurons (van Raamsdonk et al. 1983), the organization of the developing zebrafish is far simpler. Embryos and larvae have only two embryonic forms of muscle that have similar physiological properties (Buss and Drapeau 2000a). The motoneuronal pool is similarly reduced in complexity, with only primary and secondary motoneurons present at the early larval stages (Myers 1985). Primary motoneurons form first in development, are the largest motoneurons, and eventually branch extensively, making contact with nearly all fibers within their arborization (de Graff et al. 1990; Myers et al. 1986; van Raamsdonk et al. 1983; Westerfield et al. 1986). Primary motoneurons are generally recruited during fast swimming and the startle response (Fetcho and O’Malley 1995; Liu and Westerfield 1988). Secondary motoneurons form later in development, are smaller than primary motoneurons, branch less extensively in the muscle, and contact fewer fibers (de Graff et al. 1990; Myers et al. 1986; van Raamsdonk et al. 1983; Westerfield et al. 1986). This simplified motor system, when combined with the transparency of the zebrafish, allows visualization of individual neurons for patch clamping (Drapeau et al. 1999), making the zebrafish an excellent model organism for studying the neural control of locomotion.

The purpose of this study was to examine the development

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of swimming in zebrafish larvae from when they hatch (after 2 days) and are first capable of locomotion to when they are active swimmers capable of capturing prey (after four days). High-speed video imaging was used to make a basic behavioral characterization of swimming that was then compared with electrophysiological recordings made from identified motoneurons during fictive swimming in paralyzed larvae. The synaptic pharmacology of the network of neurons producing the swimming pattern was investigated by bath-applying antagonists of the major spinal cord synaptic neurotransmitters and examining their effects on the membrane potential changes occurring during fictive swimming. Voltage clamping was then used to isolate the cationic and anionic synaptic currents underlying the synaptic drive to motoneurons during fictive swimming. The findings are discussed in relation to the motoneuronal activity patterns observed during fictive locomotion in fishes and mammals. This work has been presented previously in abstract form (Buss and Drapeau 2000b; Buss et al. 1999).

METHODS

Swimming behavior

High-speed video (Motionscope 500, Redlake Camera Opticon) was used to analyze the swimming pattern of larval zebrafish aged 2.4–2.8 (n = 11) and 4.9–5.2 (n = 12) days postfertilization (dpf), referred to as day 2 and day 4. Eighteen larvae were placed in a 6-cm plastic petri dish illuminated by overhead halogen lighting. Spontaneously occurring swimming was filmed at 500 frames/s, and frames were stored on VCR/120 tape with a Panasonic AG7300 VCR. Images were captured to computer using NIH/Scion Image software for further viewing and analysis. Average camera jitter was ~3% and was not corrected.

Two distinct forms of swimming were observed in larval zebrafish: continuous bursts of swimming at day 2 (burst swimming) and an intermittent style of swimming characterized by tail beating followed by gliding (beat-and-glide swimming) at day 4. The terminology is taken from Hunter (1972), who used it to describe the swimming patterns of the larval anchovy. The swimming parameters were related to those that were measurable in patch-clamped motoneurons during fictive swimming. The parameters included swim duration, number of tail beats, tail-beat frequency, distance covered, and duration of either fictive swimming. The parameters included swim duration, number of tail beats, tail-beat frequency, distance covered, and duration of either fictive swimming. The parameters included swim duration, number of tail beats, tail-beat frequency, distance covered, and duration of either fictive swimming.

Preparation for recording

Experiments were performed on zebrafish (Danio rerio) larvae of the Longfin strain raised at ~28.5°C and obtained from a breeding colony maintained according to Westerfield (1993). Physiological results are taken from recordings made in 66 morphologically identified (dyed-filled) motoneurons. Zebrafish were examined at three ages: after hatching (2.0–2.8 dpf; referred to as day 2), a day later in development (3.1–3.4 dpf; referred to as day 3) and after the onset of active swimming and feeding (4.1–4.5 dpf; referred to as day 4). All procedures were carried out in compliance with the guidelines stipulated by the Canadian Council for Animal Care and McGill University as described previously (Drapeau et al. 1999). Larvae were anesthetized in 0.02% tricaine (MS-222, Sigma) dissolved in fish saline, pinned through the notochord to a silicone elastomer (Sylgard)-lined dish, and the skin overlying the axial musculature removed with a glass pipette and fine forceps. Muscle fibers were removed from one or two myotomal segments in the anal region by aspiration with a broken patch pipette to expose the spinal cord. Experiments were performed at room temperature (~22°C). The fish saline resembled the plasma of freshwater fish (Evans 1998; Heisler 1984; Holmes and Donaldson 1969; McDonald and Milligan 1992) and contained (in mM) 134 NaCl, 2.9 KCl, 2.1 CaCl_2, 1.2 MgCl_2, 10 HEPES, and 10 glucose, osmolality 280 to 290 mOsm and pH 7.8. In most experiments (n = 42), the neuromuscular blocker 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX, 10 μM, Sigma) was added to the dish for 10–20 min and then replaced with a 0.1% collagenase (type XII, Sigma) fish saline solution for 8–10 min. The collagenase was removed, and the preparation was perfused with fish saline for the remainder of the experiment. When α-tubocurarine (15 μM, Sigma) was used as a neuromuscular blocker (n = 24), it was added directly to the fish saline. There was no noticeable difference in the fictive swimming activity of zebrafish paralyzed with 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX, 10 μM) from RBl
ZEBRAFISH FICTIVE SWIMMING

RESULTS

Behavioral characterization of larval swimming

From hatching (after day 2) through day 3, zebrafish are largely inactive, lying on their sides on the substrate. Spontaneous swimming is infrequent and occurs in sustained bouts lasting from a few seconds to less than a minute. Changes in light intensity often evoked episodes of swimming. Figure 1 shows images taken every 10 ms while recording images at 500 Hz. The swimming effectively propels the larvae but is erratic, with many turns and displacements of the head, and lacks a definite direction (Fig. 1A). Tail-beat frequencies are very rapid, approaching 100 Hz in some instances and averaging 67 ± 6 Hz (n = 11). This form of swimming will be referred to as “burst swimming.” The kinematics of swimming is similar in day 2 and day 4 larvae in that swimming movements are eel-like (anguilliform) and are characterized by a wave traveling in a rostral to caudal direction (Fig. 1, insets). However, at day 4, the larvae maintained a more constant (forward) orientation. Furthermore, the structure of the swimming changed from sustained burst swimming to an intermittent style of swimming (Fig. 1B) where there is a period of active tail-beating and propulsion (beat period) followed by a period of inactivity where the larvae rapidly glide to a stop (glide period). Episodes of beating and gliding could repeat for longer than a minute or could persist for only a few cycles. This form of swimming will be referred to as “beat-and-glide swimming.” Beat-and-glide swimming was much less erratic than the earlier burst swimming; larvae made frequent turns, but their movements were more directed, and they could maintain a suspended position in the water column even though their swim bladders were usually not yet functional at this stage of development. The average duration of the tail-beat periods was 180 ± 20 ms (n = 12), and these periods were followed by a period of gliding lasting on average 390 ± 30 ms (Table 1). The mean distance traveled during the tail-beat period was 4.7 ± 0.5 mm, while on average 37 ± 7% of this distance was covered during the glide period. Two to eight tail beats occurred during the beat period (mean = 4.7 ± 0.5), and tail-beat frequencies averaged 35 ± 2 Hz (range = 25–63 Hz).

Properties of fictive swimming

The preceding behavioral observations revealed that larval zebrafish spontaneously initiate swimming and that changes in light intensity could induce swimming. As ventral roots are too small and inaccessible for recording in the zebrafish larva, we resorted to whole cell patch-clamp recordings from motoneurons to characterize the cellular and synaptic activity patterns during swimming. Motoneuron activity patterns were examined in paralyzed larval zebrafish with the expectation that the nervous system would continue to produce rhythmic activity destined to activate the myotomal locomotor muscle in a way appropriate for swimming. Current-clamp recordings revealed stable resting membrane potentials ranging from −60 to −76 mV at all stages examined (Table 2; mean = −69 ± 1 mV) and small, intermittently occurring (possibly spontaneous) postsynaptic potentials, which were never large enough to elicit action potentials (Fig. 2). Periodically, or in response to changes in illumination, motoneurons depolarized and fired.

TABLE 1. Comparison of free and fictive beat-and-glide swimming

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Free Swimming</th>
<th>Fictive Swimming</th>
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<tbody>
<tr>
<td>Mean beat period, ms</td>
<td>180 ± 20</td>
<td>220 ± 30</td>
</tr>
<tr>
<td>Mean glide period, ms</td>
<td>390 ± 30*</td>
<td>910 ± 100*</td>
</tr>
<tr>
<td>Mean tail beats/bend period</td>
<td>4.7 ± 0.5*</td>
<td>6.9 ± 0.8*</td>
</tr>
<tr>
<td>Mean tail beat frequency, Hz</td>
<td>35 ± 2</td>
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Values shown are means ± SE. Mean beat periods were significantly different from mean glide periods during both free and fictive swimming (not indicated in the Table). The number of larvae or motoneurons examined to calculate the presented mean values in free swimming was 12 and in fictive swimming was 11. * Significant difference of means (Student’s t-test or Mann-Whitney rank sum test); comparisons were made horizontally in the table.

Analyses

Analyses were performed using pClamp 6 or Axograph 4.4 (Axon Instruments). Measurements of fictive swimming rhythmic excitatory postsynaptic potential and current (EPSP and EPSC) frequency or amplitude, tonic depolarization or current, and action potential amplitude or threshold were made by eye (cursor measurement). Rhythmic EPSCs were detected using the template function of Axograph 4.4. Detected events were divided into two size groups, averaged, and the decay phase (τ) of the averaged EPSC fit to a single exponential function.

Mean rhythmic EPSC amplitudes and frequencies were determined from consecutive measurements made on the first 30–50 EPSCs occurring in an episode of fictive swimming. EPSP measurements were made on ~10 consecutive EPSPs, which occurred during periods of fictive swimming, where the synaptic drive was sufficient to drive occasionally the membrane to action potential threshold. The action potential threshold values presented are the membrane potential at which rhythmic EPSPs initiated action potential firing. Results are presented as means ± SE throughout the text. The term significant denotes a relationship with P < 0.05 determined using the Student’s t-test, Mann-Whitney rank sum test, or paired t-test.

(Natick, MA). Sulforhodamine B was purchased from Molecular Probes (Eugene, OR) and all other chemicals from Sigma Chemical (St. Louis, MO).

Fig. 1. Swimming is sustained and continuous (burst swimming) in 2-day-old larvae and intermittent (beat-and-glide swimming) in 4-day-old larvae. A segment of burst swimming (A) and beat-and-glide swimming (B) is shown. The larva in A is gradually turning throughout the period shown. In B, a gliding larva initiates the following tail-beat period with a small turn to the right and then glides until the next tail-beat period, which begins without a change in direction. High-speed video imaging was performed at 500 Hz (every 5th image is shown). Insets: propulsion is generated in both cases by a wave of body movement propagating in a rostral to caudal direction; individual images are shown every 2 ms (i.e., at maximal camera rate). Inset scale bars are 2 mm and 2 ms.

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rhythmic action potentials with a temporal pattern consistent with the motoneuron output expected for swimming. This activity, believed to be fictive swimming, was further examined and compared with the free-swimming behavior.

The fictive swimming consisted of a tonic depolarization and rhythmic postsynaptic potentials. At day 2 (Fig. 2A), the tonic depolarization driving the fictive swimming was sustained for tens of seconds (mean = 11 ± 2 s). In day 4 motoneurons (Fig. 2C), the tonic depolarization lasted only a few hundred milliseconds and was followed by a repolarization of the membrane toward the resting potential. At day 4, these periods of depolarization and repolarization could occur repeatedly often for several minutes or as long as the recordings (in this case making it impossible to quantify the duration of beat-and-glide swimming) but sometimes for only a few cycles. Each of these fictive swimming patterns corresponded closely to the burst swimming observed in day 2 larvae and the beat-and-glide swimming observed in day 4 larvae. Day 3 (Fig. 2D) motoneurons behaved similarly to day 2 motoneurons. However, in some recordings, an episode of fictive burst swimming was followed by activity resembling beat-and-glide fictive swimming although there was an incomplete membrane repolarization during the glide period. The addition of the beat-and-glide-like activity could prolong the day 3 swimming episodes for 2–3 min (mean = 33 ± 11 s). As day 3 animals developed further, the swimming style became increasingly more like beat-and-glide swimming. However, even at day 4, short lasting periods of burst like swimming could occur preceding a much longer period of beat-and-glide fictive swimming.

The swimming parameters examined were related to those that were measurable in patch-clamped motoneurons during fictive swimming. The parameters included swim duration, number of tail beats, tail-beat frequency, and duration of either phase of beat-and-glide swimming. Tables 1 and 2 present the values for these and other parameters during free and fictive swimming. The average membrane potential (Table 2) reached during the tonic depolarization phase of fictive swimming was −54 ± 1 mV during both fictive burst swimming (day 2–3) and fictive beat-and-glide swimming (day 4). The rhythmic postsynaptic potentials (i.e., the network output) could reach action potential threshold (−41 ± 0.8 mV) at all stages. The action potential threshold values were measured as the membrane potential at which rhythmic EPSPs initiated action potential firing. Rhythmic EPSPs during fictive burst swimming (day 2–3) occurred with a mean frequency of 52 ± 2 Hz, while the frequency during beat-and-glide fictive swimming was significantly lower (35 ± 3 Hz). All rhythmic EPSPs did not evoke action potentials, nor did all motoneurons fire action potentials during fictive swimming, even though a tonic depolarization and rhythmic EPSPs were clearly visible. Some rhythmic EPSPs could evoke one to four action potentials on their rising phase in all larval age groups. Action potentials elicited during fictive swimming reached an average peak membrane potential of −14 ± 2 mV that was similar at all stages but could on occasion be overshooting in an individual motoneuron. Action potentials did not have an afterhyperpolarization.

A smaller PSP, occurring between the rhythmic EPSPs, was observed occasionally, in 20 of the 38 motoneurons examined. When present (Fig. 2, A and B, * in insets), these other PSPs did not occur regularly throughout the fictive swimming episodes. The irregular PSPs were more clearly resolved under voltage clamp (described in the following text). The largest and most clearly defined irregular PSPs usually occurred when the excitatory synaptic drive was greatest during a fictive swimming episode. During periods of weak rhythmic synaptic drive, especially during day 4 beat-and-glide fictive swimming, the irregular PSP was not observed (Fig. 2C, inset).

In day 2 and day 3 larvae, bursts of fictive swimming were sometimes followed by a small 0.5- to 3-mV hyperpolarization that was sustained for 10–40 s (Fig. 2). At day 2, fictive swimming was followed by a 2.1 ± 0.2-mV hyperpolarization lasting 14 ± 3 s in 12 of 15 motoneurons. Five of 16 day 3 motoneurons displayed a hyperpolarization (mean amplitude = 1.3 ± 0.3 mV; mean duration = 17 ± 3 s) following a burst of fictive swimming. Membrane hyperpolarization was not observed following beat-and-glide swimming in day 4 motoneurons (Fig. 2C).

From these recordings it was not possible to determine the nature of the depolarizing drive to the motoneurons during fictive swimming. The depolarizing drive appeared synaptic in origin and current pulses sufficient to depolarize or hyperpolarize the membrane potential 20–40 mV (for tens to hundreds of milliseconds) during episodes of fictive swimming did not disrupt the following motoneuron output (not shown). Bath application of TTX (n = 4) abolished fictive swimming. However, some motoneurons may have intrinsic oscillatory properties as small (<2 mV), long-lasting (~1 s) membrane depolarizations were observed (3 of 4 motoneurons) in the presence of TTX (see also Ali et al. 2000a).
To gain insight into the pharmacology of the fictive swimming synaptic drive, receptor antagonists of the major zebrafish spinal cord synaptic neurotransmitters, glutamate (Ali et al. 2000a) and glycine (Ali et al. 2000b), were bath applied to the preparation. Addition of the glutamate receptor antagonist kynurenic acid or a combination of the specific AMPA/kainate and N-methyl-D-aspartate (NMDA) receptor antagonists (CNQX and AP-5) to the fish saline abolished spontaneous or light-induced fictive swimming \((n = 4)\). The resting membrane potential was unaffected by the glutamatergic antagonists, and the remaining spontaneous synaptic activity was blocked by the glycine receptor antagonist strychnine. Cholinergic synaptic drive was not critical for the production of fictive swimming as no noticeable difference in motoneuron activity was observed if \(\alpha\)-bungarotoxin or \(\beta\)-tubocurarine was used to paralyze the preparations.

In contrast, blocking glycinergic transmission \((n = 6)\) by bath application of strychnine did not abolish rhythmic activity.
even though it causes spasms of bilateral contractions in intact larvae (Granato et al. 1996). Strychnine did not significantly affect the frequency of rhythmic EPSPs (strychnine 52 ± 6 Hz, control 52 ± 6 Hz) or the tonic synaptic drive (253 ± 6 vs. 253 ± 6 mV) during a fictive swimming episode observed in single motoneurons. There was an abolition of the irregular PSP and a distinct increase in motoneuron spiking during fictive swimming. Significantly more action potentials occurred per second of fictive swimming (6.5 ± 2 vs. 2.4 ± 1); this was attributable to the initiation of an extra one or two action potentials by many of the rhythmic EPSPs. Action potential threshold significantly decreased by 5 mV (240 ± 2 vs. 245 ± 1 mV, P = 0.019), and action potential height significantly decreased by 13 mV (225 ± 4 vs. 212 ± 2 mV) in the presence of strychnine. Fictive swimming duration and resting membrane potential were not noticeably affected by strychnine, while a hyperpolarization following fictive swimming was revealed in two cells that did not display it prior to strychnine application.

**Properties of fictive swimming synaptic drive**

The preceding current-clamp recordings revealed that motoneurons depolarized in both a phasic and tonic pattern during fictive swimming, although the nature of this depolarization was not positively identified. Bath application of glutamatergic and glycinergic antagonists either abolished or changed, respectively, the fictive swimming motor pattern, a result consistent with a glutamatergic and glycinergic synaptic drive underlying the fictive swimming depolarization. However, bath-applied antagonists do not act solely on motoneurons, and the changes observed could be due to indirect actions on other neurons active during fictive swimming. To overcome this shortfall in the current-clamp analysis of motoneuron activity patterns, motoneurons were voltage clamped at the reversal potential for chloride ion to reveal isolated cation currents (presumably glutamatergic) or at the cation reversal potential to reveal isolated chloride ion currents (presumably glycinergic) without pharmacological perturbation of network activity. Furthermore due to the small size of larval zebrafish neurons, an effective space clamp is achieved for synaptic currents (Ali et al. 2000a,b; Drapeau et al. 1999), allowing for a more quantitative index of synaptic activity.

Motoneurons that were voltage clamped at the chloride reversal potential displayed spontaneous or light-evoked bursts of inward synaptic currents composed of a tonic inward current and rhythmic EPSCs (Fig. 4). The chloride ion reversal potential was set in each motoneuron by determining the reversal potential of the spontaneously occurring glycinergic synaptic currents, i.e., the reversal potential was not calculated but determined experimentally (about −42 mV). In general, the same frequency of activity was observed in voltage-clamped motoneurons as was observed in the current-clamped motoneurons (Table 2). Day 2 and 3 motoneurons (Fig. 4, A and B) displayed activities consistent with the motor output required to produce burst swimming and day 4 motoneurons (Fig. 4C) that required to produce beat-and-glide swimming. These findings indicated that the depolarizing drive underlying fictive swimming was synaptic in nature and that the rhythmicity...
producing the motor output was due to a cationic conductance (presumably glutamatergic). The tonic inward current averaged 41 ± 6 pA and tended to vary from cell to cell but did not change significantly with development (Table 2). Rhythmic EPSC amplitudes sometimes exceeded 200 pA (mean = 49 ± 6 pA) and increased significantly with development (Table 2). The frequency of the rhythmic EPSCs (Table 2) were higher during day 2 and 3 fictive burst swimming (mean = 45 ± 2 Hz) than during the fictive beat-and-glide swimming (mean = 37 ± 3 Hz).

If the rhythmic EPSCs were glutamatergic, they should have kinetic characteristics resembling those of the spontaneous, quantal glutamatergic synaptic currents previously characterized in zebrafish motoneurons (Ali et al. 2000a). That study revealed biexponential decay time constants for both AMPA/kainate (faster $\tau = 0.5–0.8$ ms, slower $\tau = 3–6$ ms) and NMDA (faster $\tau = 5–8$ ms, slower $\tau = 30–45$ ms) components of spontaneous synaptic currents. To examine the possible contributions of these two types of glutamate receptors, decays were fit to a single exponential function to approximate roughly the time course of decay of the rhythmic EPSCs. Fits to more complex time courses were not possible due to interruption of the summated evoked events. Rhythmic EPSCs varied in amplitude and the largest EPSCs appeared to have a
faster rate of decay. To verify this apparent difference, the rhythmic EPSCs were divided into two size groups containing small (10–25 pA) or large (>50 pA) EPSCs. The decay time course of the rhythmic EPSCs varied from 2.8 ms (day 2 large EPSCs) to 5.9 ms (day 4 small EPSCs). The time course of the large EPSCs was faster than that of the small EPSCs at all stages examined, and this difference was statistically significant in day 2 and 3 motoneurons (Table 3). Furthermore, in low-noise, high-resolution recordings (Fig. 6C), the rhythmic EPSCs were found to be composed of many small currents that closely resembled the miniature AMPA/kainate synaptic currents described by Ali et al. (2000a).

The preceding current-clamp recordings revealed the presence of an occasional small, irregular PSP at days 2 and 3 that was presumably a glycinergic chloride ion conductance as it was not observed in the presence of strychnine. To gain a greater understanding of this PSP, motoneurons were voltage clamped at a potential (about −28 mV) intermediate to the chloride reversal potential and the cation reversal potential to reveal a mixture of inward cation currents and outward chloride ion currents.

At the intermediate holding potential, rhythmic inward currents were superimposed on a sustained tonic chloride ion current. This was observed in day 2–3 motoneurons (Fig. 5B) displaying fictive burst swimming as well as in day 4 motoneurons (Fig. 6B) displaying fictive beat-and-glide swimming. To study the chloride current in isolation, the holding potential was depolarized further to ~5 mV, the cation reversal potential (Ali et al. 2000a). At this potential, it was readily apparent that there was mostly a sustained, tonic chloride current occurring during fictive swimming (Figs. 5A and 6A). Closer examination revealed that the chloride current appeared to be composed of

### Table 3. Developmental changes in the time course (τ) of decay of large and small amplitude rhythmic EPSCs occurring during fictive swimming

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small EPSC</td>
<td>5.8 ± 0.2*</td>
<td>4.5 ± 0.4*</td>
<td>5.9 ± 0.7</td>
</tr>
<tr>
<td>Large EPSC</td>
<td>2.8 ± 0.2*</td>
<td>3.0 ± 0.3*</td>
<td>5.1 ± 0.7</td>
</tr>
</tbody>
</table>

Values shown are means ± SE. The numbers of motoneurons examined to calculate the presented mean values were 5, 3, and 4, respectively, for days 2, 3, and 4. * Significant difference of means (paired *t*-test); comparisons were made vertically in the table.

A Cation Reversal Potential

B Intermediate Holding Potential

C Chloride Ion Reversal Potential

**Fig. 5.** The synaptic drive underlying fictive burst swimming is composed of a tonic and slightly rhythmic chloride current (A) that occurs concurrently (B) with a tonic and highly rhythmic cation current (C). Whole cell voltage-clamp recordings were made with a cesium-glucosate-based intracellular solution. Membrane voltage is clamped near the cation reversal potential (5 mV) to reveal isolated chloride currents (A) and at the chloride ion reversal potential (~42 mV) to reveal isolated cation currents (C). A mixture of inward cation and outward chloride ion currents is revealed at an intermediate holding potential (~28 mV, B). The day 3 fictive burst swimming is followed by a period of beat-and-glide like swimming. Insets: enlargements of the portions of the fictive swimming episodes marked with ■. The vertical scale bar in C represents 50 pA in C and 100 pA in A and B. Calibrations shown for the inset in C are the same for the insets shown in A and B.
many individual synaptic currents that closely resembled the glycineergic synaptic currents described in larval zebrafish motoneurons (Buss et al. 1999) and reticulospinal neurons (Ali et al. 2000b). The peaks of these synaptic currents showed rhythmicity, which was likely to account for the occasional irregular PSPs observed in the current-clamp recordings. Because it was difficult to voltage clamp motoneurons at such a depolarized level, the rhythmicity of the chloride synaptic current peaks was only examined in 11 cells (day 2 = 4; day 3 = 3; day 4 = 4).

Closer examination of the chloride ion PSCs revealed that they did not always display (Fig. 5A, inset, vs. 6A) the same high degree of rhythmicity as the rhythmic cationic EPSCs described in the preceding text. The timing of the chloride ion PSC peaks varied greatly, and during a single episode of fictive swimming inter-peak frequencies could range from 20 to 500 Hz. Although the rhythmic cationic EPSCs were invisible at this potential, it was reasonable to conclude that some of the chloride current peaks would be occurring midway between the rhythmic cationic EPSCs. However, many of these chloride current peaks could also be occurring synchronously with rhythmic cationic EPSCs. The frequency of the chloride current peaks was measured and normalized, on a cell-by-cell basis, to the frequency of the rhythmic cationic EPSCs believed to underlie the timing of tail beats in the free swimming larvae. On average, the chloride current peaks occurred at a frequency two to three times higher than the rhythmic cationic EPSCs (2.6 ± 0.2 times, day 2; 2.0 ± 0.5 times, day 3; 2.7 ± 0.6 times, day 4). This finding supports the occurrence of rhythmic synaptic chloride current peaks (irregular PSPs) occurring with rhythmic cationic EPSCs. However, the additional

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**FIG. 6.** The synaptic drive underlying fictive beat-and-glide swimming is composed of a tonic and slightly rhythmic chloride current (A), which occurs concurrently (B) with a tonic and highly rhythmic cation current (C). Whole cell voltage-clamp recordings were made with a cesium-gluconate-based intracellular solution. Format as in Fig. 5 except that single episodes of synaptic activity are shown. Voltage clamp near the cation (A; 5 mV) and chloride ion reversal potential (C, -42 mV) and at an intermediate potential (B, -28 mV). Clearly defined synaptic currents closely resembling fast AMPA/kainate synaptic currents are visible in the rhythmic cation currents shown in C. Time calibrations shown in C are the same for the traces shown in A and B, while the vertical scale bar in C represents 20 pA in B and C and 100 pA in A.
Conductance added by the peak of the chloride current was small compared with the magnitude of the tonic chloride conductance occurring during fictive swimming, and the physiological significance of the chloride current most likely lies in its tonic component. Noteworthy is the fact that the system-wide antagonism of glycinegic currents did not disrupt rhythmic synaptic activity, while it had clear affects on the firing properties of the motoneuron during this activity.

Comparison of fictive swimming and free swimming

Motoneurons displayed the same fictive swimming activity whether they were examined using current- or voltage-clamp recording techniques (Figs. 2 vs. 4–6). The measured parameters were not significantly different so all fictive swimming data were pooled and compared with the parameters measured in free swimming larvae. In both fictive and free swimming, tail-beat/rhythmic EPSP frequency was higher during burst swimming than during beat-and-glide swimming (Table 2). The mean tail-beat/rhythmic EPSP frequency during beat-and-glide swimming was identical (35 Hz) during fictive and free swimming, although during burst swimming it was significantly higher during the behavior than during fictive swimming (Table 1). During day 4 beat-and-glide swimming (Tables 1 and 2), the mean beat periods were very similar, while the mean glide periods were significantly longer during fictive swimming. Although there were differences in the preceding mean values, all values recorded during fictive swimming were within the range of values observed in the free swimming behavior. The higher observed free swimming values could be due to the presence of active sensory feedback during free swimming or could simply be because the most active (and fastest swimming) larvae swam through the field of view during the high speed video recording. Another difference between free and fictive beat-and-glide swimming was the occurrence of about two fewer tail beats per beat period (when compared with the mean number of rhythmic EPSPs/EPSCs) occurring during fictive swimming (Table 1). This difference is likely because of the inclusion of small sub-threshold EPSPs/EPSCs when calculating the number of rhythmic EPSPs (i.e., fictive tail beats) in the fictive beat period. The addition of these sub-threshold EPSPs would also explain the longer mean burst periods observed during fictive beat-and-glide swimming (Table 1).

Dye coupling between motoneurons and other neurons

Dye coupling between the patched motoneuron and the axon of another neuron was clearly observed in 3 of the 66 dye-filled motoneurons examined in this study. In two instances, the dye-coupled axon could be traced to a dye-filled cell body that was always an ipsilateral descending interneuron. Whether the other dye-filled axon was a descending interneuron, propriospinal neuron, or descending axon from the brain stem could not be determined. Dye coupling between motoneurons and other motoneurons was never observed.

Discussion

Free swimming

Beat-and-glide swimming has been previously examined in larval zebrafish (Budick and O’Malley 2000; Fuiman and Webb 1988) while the transition from burst swimming to beat-and-glide swimming has not. In addition, Saint-Amant and Drapeau (1998) have described the earliest embryonic swimming. From the onset of swimming at 28–36 h, a period when the embryo remains encapsulated in the egg, the tail-beat frequency increases from 7 to 27 Hz. The present study shows a continued twofold increase in tail-beat frequency during the next day of development.

The mean tail-beat period (180 ms) and glide period (390 ms) described in this study matched closely with the 130-ms tail-beat period and the range of glide periods (400–2,000 ms, which average toward the lower range values) described by Fuiman and Webb (1988). As described in this other study, episodes of beat-and-glide swimming regularly began with a turn and swimming in a new direction. The mean tail-beat frequency observed in this study (35 Hz) corresponds to the spontaneously initiated swimming described by Budick and O’Malley (2000). A similar transition from burst swimming to beat-and-glide swimming has been observed in anchovies by Hunter (1972). Similar to zebrafish, at hatching, anchovies remain motionless except for brief (1–2 s) spontaneous bursts of swimming characterized by continuous tail beating at rates ≤50 Hz. Within a few days, a new dominant mode of intermittent swimming emerges consisting of alternating periods of swimming and gliding using lower tail-beat frequencies. For hydrodynamic reasons, it is advantageous for small larval fish to swim continuously and rapidly as gliding is not physically possible. Larger larval and adult fish swim and glide, which is then possible due to their larger, more streamlined shape (Webb and Weihls 1986; Weihls 1980). Much later in development, the anguilliform swimming of larval zebrafish (the common mode of swimming in larval fish) is replaced by the subcarangiform mode of the adults, which is characterized by a reduced side-to-side eel-like motion in the anterior end of the fish (Lindsey 1978).

Fictive swimming

The spontaneous or light-evoked episodes of depolarization and rhythmic action potential firing in motoneurons are consistent with a fictive motor pattern that would activate myotomal muscle in a pattern appropriate for swimming in a non-paralyzed preparation. The frequency of rhythmic EPSPs, EPSCs, and action-potential firing recorded in paralyzed preparations closely matched the free swimming tail-beat frequencies (Tables 1 and 2). A developmental change from burst to beat-and-glide swimming was similarly observed in the free swimming and paralyzed preparations, and the underlying structure of the beat-and-glide swimming was similar.

A tonic depolarization and rhythmic EPSPs capable of initiating action potentials characterize fictive swimming in day 2–4 zebrafish. The tonic depolarization arises from cationic synaptic currents, which likely sum with tonic chloride ion synaptic currents, due to the depolarizing nature of chloride ions in these developing motoneurons (Buss et al. 1999; Saint-Amant and Drapeau 2000). Synaptic cation currents are hypothesized to be glutamatergic based on four observations: glutamatergic antagonists abolished fictive swimming, glutamatergic antagonists abolished all cationic miniature EPSCs in zebrafish motoneurons (Ali et al. 2000a), summing synaptic currents with the properties of AMPA/kainate mEPSCs were...
observed in the rhythmic EPSCs, and cholinergic synaptic currents were never observed in larval zebrafish motoneurons (Buss and Drapeau 2000b).

We hypothesize that the rhythmic EPSPs are formed by fast ($\tau = 0.5$–0.8 ms and 3–6 ms) AMPA/kainate synaptic currents, combined with the faster component (with $\tau = 5$–8 ms) of the NMDA synaptic currents (Ali et al. 2000a). Table 3 shows that the decay time constant of the largest rhythmic EPSCs ($\sim$3 ms) is faster than the faster decay time constant of NMDA synaptic currents. This indicates that the faster AMPA/kainate channels could carry much of this current. The smaller rhythmic EPSC currents ($\sim$6 ms) are close to the value of the slow time constant of AMPA/kainate synaptic currents as well as the fast time constant of NMDA synaptic currents and may thus be due to a combination of inputs from these receptors. The prolonged decay time course of NMDA synaptic currents (slower $\tau = 30$–45 ms) (Ali et al. 2000a) arising from either mixed NMDA/AMPA synapses or pure NMDA synapses, could provide much of the sustained tonic depolarization. However, an additional tonic drive mediated by slow acting metabotropic glutamate receptor or muscarinicholinic receptor activated channels cannot be ruled out. The glutamatergic synaptic transmission is mediated by action-potential-evoked synaptic release as fictive swimming was abolished by TTX application.

Chloride-mediated, glycinergic synaptic currents occurred concurrently with the glutamatergic currents. These chloride-ion-mediated synaptic currents were concluded to be glycinergic based on three observations: strychnine abolished the irregular PSP, glycinergic antagonists abolished all chloride ion mediated mPSCs (except for rare, infrequently occurring bicuculline-sensitive GABAergic mPSCs observed in a small percentage of motoneurons) in larval zebrafish motoneurons (Buss et al. 1999) and resticulospinal neurons (Ali et al. 2000b), and the decay time course and appearance of the chloride mediated synaptic currents observed during fictive swimming resembled glycinergic synaptic currents described by Buss et al. (1999) and Ali et al. (2000b).

The peaks of these synaptic chloride currents formed the irregular PSPs, which were present in approximately half of the motoneurons examined but represented only a third of all the peaks observed. Moreover the chloride currents were largely tonic in nature and were not essential to the patterning of the locomotor rhythm since eliminating glycinergic synaptic currents with strychnine had no significant affect on the frequency of the rhythmic EPSPs. However, strychnine did affect motor output, causing a significant increase in the frequency of action potentials during fictive swimming as well as a decrease in action potential amplitude and threshold.

The tonic chloride conductance occurring during fictive swimming could act to decrease the input resistance and consequently the membrane length and time constants of the motoneurons. Strychnine caused a decrease in action potential threshold and amplitude, and these effects may reflect an increased motoneuron input resistance, length, and time constants. As the action potential of larval zebrafish motoneurons occur during the decay of the membrane depolarization evoked by short (2 ms) current injections (unpublished observations), the spikes are likely initiated in the axon and not the soma. A longer membrane length constant, after application of strychnine, would lessen the attenuation of the membrane depolarization from the spike initiation zone to the soma, resulting in a perceived lowering of the action potential initiation threshold measured at the soma.

Blocking the chloride conductance with strychnine would increase the membrane time constant and reduce the recording bandwidth, resulting in filtered action potentials of smaller amplitude. Shortening of the membrane time constant due to a sustained, glycinergic chloride conductance will serve to shorten the time course of synaptic potentials. Larval zebrafish motoneurons have input resistances an order of magnitude larger than reported in adult fish but have to swim with much faster undulations to propel themselves through the water. Glutamatergic synaptic currents (AMPA/kainate) have very fast kinetics (0.5–0.8 ms) (Ali et al. 2000a) and motoneurons produce a coordinated rhythmic synaptic output sometimes reaching 100 Hz during swimming.

**Electrical transmission**

Although the sources of synaptic drive to motoneurons during fictive swimming are attributed to chemical synapses (glutamatergic and glycinergic), it is probable given the results of dye-coupling experiments that electrical synapses provide an additional source of synaptic drive. In the zebrafish embryo, electrical synaptic transmission appears to play a critical role in the production of the spontaneous motor activity occurring during the first day of development prior to the appearance of chemical synaptic transmission in embryonic motoneurons (Saint-Amant and Drapeau 2000). Electrical synapses have been extensively examined in adult fishes as well (Batueva 1987; Bennett 1966, 1997; Pappas and Bennett 1966; Rovainen 1979). A number of the descending axons that were dye coupled to motoneurons originated from segmental descending interneurons that are likely homologous to a class of descending interneurons described in the goldfish (Fetcho 1992).

**Developmental changes**

The most obvious developmental change to occur, the switch from burst swimming to beat-and-glide swimming, was associated with a lowering of tail-beat frequency. However, at a cellular level, there were few changes. The hyperpolarization following fictive swimming common at day 2 was not observed in day 4 beat-and-glide fictive swimming. This could be due to the loss of this conductance, to it being obscured by synaptic activity following the fictive swimming, or to a lower input resistance of day 4 motoneurons. Rhythmic EPSC amplitudes increased from day 2 to day 4, which could compensate for the reduction in input resistance and is likely due to the increase in size of the unitary AMPA/kainate synaptic events described by Ali et al. (2000a). There were no significant changes in most cellular properties including resting membrane potential, fictive swimming tonic depolarization, action potential amplitude or threshold, or rhythmic EPSC decay time course.

**Comparison with fictive swimming described in other fish**

The neural control of swimming has been extensively examined at the cellular level in the lamprey (reviewed in Grillner et al. 1991, 1998; Rovainen 1979) through the use of the isolated or paralyzed spinal cord preparation. The activity of motoneurons and unidentified interneurons has been examined.
during fictive swimming in dogfish (Mos et al. 1990a,b) and stingray (Williams et al. 1984) with intracellular techniques. However, most studies, in fishes other than the lamprey, have been limited to extracellular recording techniques (goldfish, Fetcho and Svoboda 1993; carp, Uematsu et al. 1994; angel-fish, Yoshida et al. 1996; stingray, Leonard 1986; dogfish, Roberts 1981).

During glutamate-induced fictive locomotion in lamprey, phasic excitation alternates with a phasic inhibition that is mediated by a glycinergic chloride conductance (Alford and Williams 1989; Dale 1986; Kahn 1982; Russell and Wallen 1983). These alternating excitatory and inhibitory oscillations are superimposed on a tonic depolarization when locomotion is evoked by sensory stimuli (Alford and Williams 1989). Fictive swimming is antagonized by glutamatergic antagonists (Brodin and Grillner 1985) and the phasic excitation is mediated by glutamatergic synaptic inputs having both AMPA and NMDA components (Alford and Sigvardt 1989; Alford and Williams 1989; Dale 1986; Dale and Grillner 1986; Hagevik and McClellan 1994; Moore et al. 1987). Spontaneously occurring fictive swimming recorded in stingray motoneurons (Williams et al. 1984) is characterized by a tonic depolarization with superimposed rhythmic PSPs, with little sign of alternating inhibition. Similarly, fictive swimming recorded from dogfish motoneurons does not reveal alternating inhibition (Mos et al. 1990a).

Blocking glycinergic inhibition, by bath application of strychnine, increases the rate of fictive swimming in the lamprey (Cohen and Harris-Warrick 1984; Grillner and Wallen 1980), but not in the zebrafish, and synchronizes normal alternating ipsilateral/contralateral fictive motor output in the lamprey (Alford and Williams 1989; Cohen 1987; Cohen and Harris-Warrick 1984). Thus Cohen and Harris-Warrick (1984) concluded that the neuronal network generating the rhythmic excitatory oscillations, observed during fictive swimming, operates independently of glycinergic inhibitory connections. The lack of effect of strychnine on the rhythmicity of zebrafish fictive swimming supports this conclusion. Furthermore, strychnine does not disrupt the rostral-to-caudal phase lag of motor output that underlies the propulsion for undulatory swimming in the lamprey (Alford and Williams 1989; Cohen 1987). The present study examined the activities of individual motoneurons and could not determine whether the rostral-to-caudal phase lag of motoneuron activity was affected. The ipsilateral/contralateral alternation during swimming is perturbed by strychnine, resulting in bilateral contractions resembling the phenotype of accordion mutants (Granato et al. 1996).

The synaptic drive to zebrafish myotomal motoneurons is very similar to that observed in other fishes. The principal difference is the presence of a tonic glycinergic drive, which we hypothesize to be an adaptation to the high frequencies of motor output required for undulatory locomotion in these small larval fish. The patterning of rapid undulatory movements by the nervous system may underlie fundamental features in the vertebrate nervous system because this form of locomotion is observed in phylogenetically distant organisms having a prevertebrate chordate ancestry (e.g., Lancers; Stokes 1997). Phasic glycinergic inhibition during fictive swimming is not prominent in the motoneurons of the two elasmobranch fish examined (Mos et al. 1990a; Williams et al. 1984), suggesting that phasic glycinergic inhibition of motoneurons is not critical for locomotor activity. The apparent necessity for the involvement of glycinergic inhibition in ipsilateral/contralateral alternation likely lies at a premotoneuronal level.

Similarities with mammalian locomotion

The properties of the synaptic drive to zebrafish motoneurons during fictive swimming have many similarities with the synaptic drive to feline motoneurons during fictive locomotion. In both preparations, motoneuron output is determined largely by a rhythmic excitatory synaptic drive (Jordan 1983; Pratt and Jordan 1987). In the cat, reciprocal relationships underlying extensor and flexor motoneuron output during locomotion are believed to be determined at a premotoneuronal level by interactions among interneurons forming the flexor reflex afferent pathways (Jankowska 1992; Jankowska et al. 1967a,b; Schomburg et al. 1998; Shefchyk and Jordan 1985a). Similarly, motoneuron rhythmic activity remains after glycinergic inhibition is antagonized by strychnine (Pratt and Jordan 1987) or nicotinic cholinergic transmission is antagonized with mecamylamine (Noga et al. 1987) but not when glutamatergic antagonists are administered (Douglas et al. 1993). The synaptic drive to motoneurons is due to a sequential excitatory and inhibitory synaptic drive that overlap during the step cycle (Orsal et al. 1986; Perret and Cabelguen 1980; Shefchyk and Jordan 1985a,b). Two classes of identified interneurons have been shown to provide this inhibitory synaptic drive to motoneurons during fictive locomotion. Renshaw cell activity provides an inhibitory drive concurrent with the excitatory phase of the locomotor drive potential and Ia inhibitory interneurons provide an inhibitory drive alternating with the excitatory phase (i.e., midcycle inhibition) of the locomotor drive potential (Feldman and Orlovsky 1975; McCrea et al. 1980; Noga et al. 1987; Pratt and Jordan 1987). Although both interneurons provide inhibitory drive to motoneurons during fictive locomotion, neither are components of the spinal rhythm generating network (Pratt and Jordan 1987). It is possible that analogous (or homologous) inhibitory interneurons, which are not elements of the spinal rhythm generator, provide the glycinergic drive to zebrafish motoneurons during fictive swimming. We conclude that there are many similarities between zebrafish and mammals, in the properties of the synaptic drive to motoneurons during fictive locomotion, and that the larval zebrafish is a useful preparation for gaining new insights into the neural control of vertebrate locomotion.

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