Activation of Embryonic Red and White Muscle Fibers During Fictive Swimming in the Developing Zebrafish

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INTRODUCTION

Studies on the locomotor behaviors of larval zebrafish have largely focused on the startle response (Eaton and DiDomenico 1986; Kimmel et al. 1974; Liu and Fetcho 1999; and references therein), while swimming behaviors have received less attention (Budick and O’Malley 2000; Buss and Drapeau 2001a; Fuiman 1986; Fuiman and Webb 1988; Muller et al. 2000; Saint-Amant and Drapeau 1998). Knowledge of the neural control of larval zebrafish swimming is restricted to motoneuron activity patterns and the synaptic drive to motoneurons in paralyzed zebrafish during fictive swimming (Buss and Drapeau 2001a). Whether the activity of motomuscle is set from the onset of development. The activity of zebrafish motoneurons is fundamentally similar to that observed in adult fishes during swimming. Our results indicate that the patterned activation of myotomal muscle is set from the onset of development.

METHODS

Experiments were performed on zebrafish (Danio rerio) larvae and embryos of the Longfin strain raised at 28.5°C and obtained from a breeding colony maintained according to Westerfield (1995). All procedures were carried out in compliance with the guidelines stipulated by the Canadian Council for Animal Care and McGill University. The experimental methodology has been described (Buss and Drapeau 2000). Results are taken from 25 paired and 28 single whole-cell patch-clamp recordings from ER and EW muscle fibers of zebrafish embryos aged 1.3–1.6 (day 1, length approximately 2.5 mm) and larvae aged 3.0–3.3 (day 3, length approximately 3.5 mm) days postfertilization. Fictive swimming and coiling occurred spontaneously or was evoked (day 3) by changes in illumination. The swimming was preceded by a lower frequency (1–13 Hz), more robust rhythmic drive resembling the “coiling” behavior of fish embryos. The motor activity observed in paralyzed zebrafish closely resembled the swimming and coiling behaviors observed in these developing fishes. At the early developmental stages examined in this study, myotomal muscle recruitment and coordination were similar to that observed in adult fishes during swimming. Our results indicate that the patterned activation of myotomal muscle is set from the onset of development.

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ming style of larval zebrafish changes from a sustained burst swimming pattern to a beat-and-glide pattern between day 2 (hatching) and day 4. Day 3 is a transition period where a beat-and-glide-like swimming pattern emerges yet burst swimming is still observed (Buss and Drapeau 2001a). Beat-and-glide-like fictive swimming was observed in all day 3 larvae examined (n = 32) and burst swimming was additionally observed in 10 preparations.

Experiments were performed at room temperature (approximately 22°C). The Evan’s fish saline recording solution (Buss and Drapeau 2001a; Drapeau et al. 1999) contained the following (in mM): 134 NaCl, 2.9 KCl, 2.1 CaCl₂, 1.2 MgCl₂, 10 HEPES, 3–10 glucose, 3 (day 3) or 15 (day 1) μM D-tubocurarine, osmolarity adjusted (with glucose) to 290 mOsm and pH 7.8. Patch-clamp electrodes (1.5–4 MΩ) contained a K-glucuronate solution consisting of the following (in mM): 2 MgCl₂, 10 HEPES, 10 EGTA, 10 D-gluconic acid sodium salt, and 6 KCl added to sufficient D-gluconic acid potassium salt to reach a final osmolarity of 290 mOsm, pH 7.2. All chemicals were purchased from Sigma Chemical (St. Louis, MO). A liquid junction potential of −12 mV was experimentally determined according to Barry and Lynch (1991) and Neher (1992) and records were corrected for this potential.

Recordings were performed with an Axoclamp-2A (0.1 headstage) and an Axopatch-1D (CV-4 headstage) amplifier. Data were low-pass filtered at 10 kHz and digitized at 1 kHz (day 1) or 10 kHz (day 3). Analyses were performed using pClamp 8 software (Axon Instruments). ER fibers were distinguished by their superficial distribution and longitudinal orientation, whereas EW fibers were deeper and had an oblique orientation (Buss and Drapeau 2000). Measurements of fictive swimming and coiling duration, rhythmic end plate potential (EPP) frequency, and rostral-caudal delay were made by eye (cursor measurement). Measurements on 50 consecutive EPPs were used to calculate mean fictive swimming and coiling EPP frequencies and rostral-caudal delay. Rostral-caudal delay was determined by measuring the fictive swimming rhythmic EPP delay (from EPP onset) between 9 and 12 segments centered on the anal segment. Rostral-caudal delay per segment was calculated by dividing the mean time delay by the number of separating segments. Percentage phase lag was calculated by dividing the rostral-caudal delay per segment by the mean cycle period (Wallen and Williams 1984). Paired recordings between ER and EW fibers located within a segment revealed differences in recruitment at day 3. When fictive swimming rhythmic EPPs were observed in EW fibers, synchronous activity was observed in ER fibers. However, ER fibers were often active in the absence of EW fiber activity. Instantaneous fictive swimming rhythmic EPP frequencies were measured during ER-EW fiber co-activity and ER fiber activity. Results are presented as mean ± SD throughout the text. The term significant denotes a relationship with P < 0.05 determined using the Student’s t-test.

RESULTS

Rostral-caudal delay and ipsilateral-contralateral alternation of synaptic drive to myotomal muscle during fictive swimming

The propulsive forces used in undulatory swimming are generated by an alternating rostral-caudal wave of myotomal muscle contraction, initiated by a synaptic drive originating from myotomal motoneurons, which interacts with the mechanical properties of body tissues (Bligh 1977; Grillner and Kashin 1975; Grillner et al. 1998; Hoff and Wassersug 2000; Lindsey 1978; Roberts 1981; Roberts et al. 1998; Wardle et al. 1995; Wassersug 1989). Therefore during a cycle of undulatory swimming, rostral myotomal muscle fibers receive synaptic drive prior to caudal fibers and within a segment the synaptic drive alternates between ipsilateral and contralateral sides of the musculature. To examine the synaptic drive to myotomal muscle fibers, embryos and larvae were paralyzed with a low concentration of the neuromuscular antagonist D-tubocurarine, which reduced, but did not abolish, neuromuscular synaptic drive. Thus the rhythmic synaptic drive (i.e., fictive swimming) underlying swimming was examined in immobilized larvae using the whole-cell patch-clamp technique.

Paired recordings revealed a synchronous synaptic drive to muscle fibers within an ipsilateral myotomal segment (Fig. 1; n = 11) and an alternating synaptic drive to contralateral muscle fibers (Fig. 2; n = 6). A rostral-caudal delay was observed in paired recordings from ipsilateral muscle fibers separated by 9–12 myotomal segments (Fig. 3; n = 7). On average, this delay was 0.55 ± 0.20 ms per myotomal segment.

**FIG. 1.** Synchronous co-activation of ER fibers within an ipsilateral myotomal segment during larval (day 3) fictive swimming (A, B). The region encompassing the black bar in A is shown on an expanded time scale in C.
and there was strong negative relationship (Fig. 5C) between the time delay per segment and the rhythmic EPP frequency (i.e., fictive tail beat frequency). Intersegmental phase lag per segment ranged from 0.8 to 2.7% and averaged 1.8 ± 0.6%. Synchronous activity within ipsilateral myotomal segments, alternation between ipsilateral and contralateral segments, and rostral-caudal delays were similarly observed in paired recordings between ER and ER (Figs. 1 and 3), ER and EW (Figs. 2 and 4), and EW and EW fibers (data not shown).

Differences in the recruitment of ER and EW muscle fibers during fictive swimming

Fishes that swim by undulatory propulsion generally use red muscle for slow swimming, recruit white muscle for faster swimming, and de-recruit red muscle at the fastest speeds of burst swimming (Bone 1978; Coughlin and Rome 1996; Jayne and Lauder 1996; Johnston 1981, 1983). Whether the embryonic forms of red and white muscle (ER and EW) found in larval fish are recruited as their adult forms has not been examined. Comparison of mean fictive swimming rhythmic EPP frequencies measured in ER (24 fibers, 1200 EPPs) and EW fibers (13 fibers, 650 EPPs) revealed a small but significantly different (P = 0.03) frequency of rhythmic EPPs in EW versus ER fibers (44 ± 7 vs. 39 ± 7 Hz). Both ER and EW fibers were recruited during fictive swimming (Figs. 2 and 4). However, within a swimming episode, there were periods, especially during the end of a fictive beat period, when ER fibers were active in the absence of EW fiber activation (Fig. 4). Fictive swimming rhythmic EPP frequencies were lower...
when ER fibers were active in isolation (Fig. 4D) of EW fibers. This is graphically illustrated in Fig. 5, A and B, where intrasegmental paired ER-EW recordings (n = 6) were used to measure rhythmic EPP frequencies during periods when only ER fibers were active (mean = 32 ± 3 Hz) and when both ER and EW fibers were active (mean = 44 ± 7 Hz). Furthermore, an attenuation of synaptic drive to ER fibers was observed during periods of robust high-frequency synaptic drive to EW fibers (Fig. 4D). The duration of fictive swimming, measured as long periods of repetitive brief swim episodes, was variable and ranged from <1 s to 2–3 min (Fig. 5D).

Fictive coiling and swimming behaviors prior to hatching

The rhythmic EPPs observed during fictive swimming strongly summed in ER fibers, whereas EPPs quickly decayed to baseline in EW fibers (Figs. 1–4). However, the time course of larval ER and EW muscle miniature EPPs are similar (Buss and Drapeau 2000; Nguyen et al. 1999), indicating that the rhythmic EPP summation was likely due to other factors. EPP summation could be due to EPPs originating from adjacent electrically coupled ER muscle fibers as EW fiber coupling is minimal at day 3, whereas ER fibers are extensively coupled (Buss and Drapeau 2000). To test whether the summation of rhythmic EPPs in ER but not EW fibers was due to electrical coupling, recordings were made from day 1 ER and EW muscle fibers which are both extensively electrically coupled at this age (Buss and Drapeau 2000).

Rhythmic EPPs summed similarly during fictive swimming in both ER and EW fibers, providing evidence that the summation was due to electrical coupling and not differences in EPP time course (Fig. 6, A and B). However, in addition to a rhythmic motor output expected for swimming, a slower and more robust rhythmic motor pattern, resembling the coiling behavior of embryonic fishes (Armstrong and Higgins 1971; Gideiri 1966, 1968a,b; Harris 1962; Richards and Pollack 1987; Whiting et al. 1992), was observed either alone or following fictive swimming (Fig. 6, A and B). A fictive coiling episode contained 1–13 rhythmic EPPs, occurring at a mean frequency of 5.1 ± 3 Hz (n = 21) (Fig. 6D), and was often (11/21) followed by rhythmic EPPs occurring at a faster frequency (Fig. 6C; mean = 24 ± 12 Hz) that resembled fictive swimming. A faster frequency of rhythmic EPPs (mean = 60 ± 3 Hz), characteristic of day 2 burst swimming (Buss and Drapeau 2001a), was observed in one EW fiber (Fig. 6C). When this value was excluded from the average, no significant difference in fictive swimming rhythmic EPP frequency was observed in ER (mean = 20 ± 2 Hz) and EW (mean = 22 ± 2 Hz) fibers. Fictive coiling/swimming episodes lasted from 0.3 to 10 s (mean = 2.2 ± 2 s) and occurred every 10–420 s (mean = 140 ± 80 s). At this age, changes in illumination were not effective at initiating fictive coiling or swimming behaviors.

Discussion

Coordinated synaptic drive to myotomal muscle during fictive swimming

Requirements for fictive undulatory swimming are appropriate cycle periods, alternating ipsilateral-contralateral motoneuron activity, a rostral-caudal delay in motoneuron activity, and a relationship between rostral-caudal delay and cycle period. By recording sub-threshold motoneuron-evoked EPP activity in myotomal muscle fibers, a coordinated motor output appropriate for swimming was revealed in paralyzed larval zebrafish
An appropriate motor coordination for swimming has previously been reported in paralyzed or isolated spinal cord preparations of lamprey (Cohen and Wallen 1980; Poon 1980; Wallen and Williams 1984), dogfish (Grillner et al. 1976), goldfish (Fetcho and Svoboda 1993), and amphibian tadpoles (Kahn and Roberts 1982; Kahn et al. 1982; Soffe and Perrins 1997; Soffe et al. 1983; however, see Blight 1977; Stehouwer and Farel 1980).

Phase lag per segment averaged 1.8 ± 0.6%, a value similar to the 2.1% reported in a related cyprinid (goldfish; Fetcho and
Svoboda (1993) but considerably larger than the value (1%) reported in the lamprey (Wallen and Williams 1984). Therefore a larval zebrafish with 30–34 myotomal segments will have approximately 58% of a full wave of undulatory activity along its body at any point in time. This compares with 63% in goldfish (29–30 segments) and a full wave (100%) in lamprey (approximately 100 segments). A full wave of activity is a characteristic of anguilliform swimming, while the briefer wave of activity observed in zebrafish and goldfish is a characteristic of subcarangiform swimming (Lindsey 1978). Thus

![Fictive coiling and swimming behaviors observed in EW (A) and ER (B) fibers of 2 different day 1 embryos. Insets show rhythmic fictive swimming EPPs of the regions encompassed by the black bar in A and B. Range of embryonic fictive swimming (C) and coiling (D) rhythmic EPP frequencies. Values below the black bar in C are from a single day 1 embryo that displayed fictive swimming rhythmic EPPs at a frequency characteristic of day 2 burst swimming. C: binwidth = 5 Hz; n = 559. D: binwidth = 1 Hz; n = 126. Graphed values are taken from fictive swimming episodes observed in 11 day 1 embryos and fictive coiling episodes observed in 21 day 1 embryos.](image-url)
although visual inspection of swimming larval zebrafish revealed an eel-like (anguilliform) style of swimming (Buss and Drapeau 2001a), examination of phase lag values reveal the subcarangiform style used by the adult. During carangiform swimming, anterior myotomes are active for a longer duration than caudal myotomes; i.e., some ipsilateral and contralateral muscle is synchronously active (Altringham and Ellerby 1999; Wardle et al. 1995). Whether a rostral-caudal variation in muscle activation exists in larval zebrafish was not determined. One prediction of this pattern of muscle recruitment would be different motoneuron output in the rostral and caudal spinal cord during fictive swimming.

**Larval locomotor muscle recruitment**

ER and EW muscle fibers were recruited for both burst and beat-and-glide swimming. At the highest rates of fictive undulatory swimming, ER fiber activity was reduced but not abolished, whereas at the slowest fictive swimming rates ER fibers could be active in isolation. These swimming rates were comparable to those observed in free swimming zebrafish (Budick and O’Malley 2000; Buss and Drapeau 2001a) as well as those previously observed during fictive swimming (Buss and Drapeau 2001a). Thus the pattern of muscle recruitment in larval zebrafish was organized as in adult fishes, where red muscle is recruited during slow undulatory swimming (Bone 1978; Coughlin and Rome 1996) and de-recruited at the fastest unsteady burst speeds when white muscle is recruited (Jayne and Lauder 1996). The neural basis for this muscle recruitment is unknown. However, the present findings and those of Bone (1966), Jayne and Lauder (1994), and Mos et al. (1990) indicate that two unique populations of motoneurons, that can be activated or inactivated independently of each other, innervate ER and EW muscle fibers.

A de-recruitment of slow (i.e., red muscle) muscle fibers during locomotion is not unique to fishes. In crabs, tonic firing of the common inhibitor neuron abolishes residual tension in slow tonic (but not fast phasic) muscle fibers during rapid walking and swimming (Bevengut and Clarac 1990; Rathmayer 1990; Wiens 1989).

The facilitation of fictive swimming EPPs in larval ER muscle fibers was likely due to summation of filtered and attenuated EPPs from adjacent electrically coupled ER fibers (Buss and Drapeau 2000). ER fibers have a low-contraction threshold (approximately −40 mV) and many similarities to vertebrate slow tonic muscle (i.e., outward rectification, a depolarized resting potential, and a low-chloride permeability; unpublished observations). During swimming, EPPs from neighboring ER fibers may summate to contraction threshold and provide a tonic level of muscle contraction, on which is superimposed the rhythmic swimming contractions. The locomotor function of tonic ER activation might include body stiffening, maintenance of posture, or steering, but its true function or existence remains to be determined. EW fibers have a high contraction threshold, which is likely reached in an all-or-none fashion via a regenerative voltage-activated current. Thus tonic muscle activation would not be expected in EW fibers which have characteristics more similar to vertebrate twitch muscle (i.e., a nearly linear I-V near the resting potential, a hyperpolarized resting potential, and a high-chloride permeability; unpublished observations). Recently Ono et al. (2001) observed action potentials in dissociated larval zebrafish muscle, a property not observed by Buss and Drapeau (2000). This was attributed to a combination of weak, undeveloped voltage-gated conductances and the inability to charge the membrane fast enough from a point source of current due to fiber cable properties. However, reexamination in vivo has revealed the presence of a TTX-sensitive action potential in some EW fibers (but not ER fibers) diazylated with a low-chloride patch-pipette solution and with resting potentials < −85 mV (unpublished observations), thus revealing further similarities between EW fibers and vertebrate twitch muscle. Thus although ER and EW fibers have many similar electrophysiological properties (i.e., input resistance, miniature EPP time course, and amplitude), they are functionally unique and receive different recruitment during swimming, and due to differences in electrical coupling, different degrees of synaptic facilitation.

**Embryonic fictive swimming and coiling**

Embryonic (day 1) ER and EW fibers both have extensive electrical coupling and were examined to provide evidence that the summed fictive swimming EPPs were due to electrical coupling and not EPP kinetics. As predicted, EPPs summated in both day 1 ER and EW fibers during fictive swimming. In contrast to larval fictive swimming (day 3), ER and EW fibers were recruited similarly in embryos (day 1). Rhythmic EPPs assumed to underlie fictive swimming occurred at a lower frequency in embryos than in larvae as observed by Buss and Drapeau (2001a) and Saint-Amant and Drapeau (1998) in behaving zebrafish. A second, slower and more robust fictive motor pattern that had the characteristics of the coiling behavior of embryonic fishes was also observed in embryos. However, the coiling observed in this study is not identical to the coiling and fictive coiling behavior described in younger embryos (Myers et al. 1997; Saint-Amant and Drapeau 1998, 2000) where single coils are observed. Rather they are more similar to the bursts of coils observed in zebrafish embryos >24 hpf (Saint-Amant and Drapeau 1998) and in the angelsh (Yoshida et al. 1996).

In conclusion, the motor output observed in paralyzed larval zebrafish is coordinated appropriately for generating undulatory swimming. Furthermore, at the early developmental stages examined in this study, muscle recruitment and swimming style (subcarangiform) were similar to that of adult fishes. Thus there is a considerable degree of sophistication in the organization of the locomotor circuitry at the onset of development of locomotion in the zebrafish.

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