Physiological Properties of Zebrafish Embryonic Red and White Muscle Fibers During Early Development

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Buss, Robert R. and Pierre Drapeau. Physiological properties of zebrafish embryonic red and white muscle fibers during early development. J Neurophysiol 84: 1545–1557, 2000. The zebrafish is a model organism for studies of vertebrate muscle differentiation and development. However, an understanding of fish muscle physiology during this period is limited. We examined the membrane, contractile, electrical coupling, and synaptic properties of embryonic red (ER) and white (EW) muscle fibers in developing zebrafish from 1 to 5 days postfertilization. Resting membrane potentials were $-73$ mV in 1 day ER and $-78$ mV in 1 day EW muscle and depolarized 17 and 7 mV, respectively, by 5 days. Neither fiber type exhibited action potentials. Current-voltage relationships were linear in EW fibers and day 1 ER fibers but were outwardly rectifying in some ER fibers at 3 to 5 days. Both ER and EW fibers were contractile at all ages examined (1 to 5 days) and could follow trains of electrical stimulation of up to 30 Hz without fatigue for up to 5 min. Synaptic activity consisting of miniature endplate potentials (mEPPs) was observed at the earliest ages examined (1.2–1.4 days) in both ER and EW fibers. Synaptic activity increased in frequency, and mEPP amplitudes were larger by 5 days. Miniature EPP rise times and half-widths decreased in ER fibers by 5 days, while EW fiber mEPPs showed fast kinetics as early as 1.2–1.4 days. ER and EW muscle fibers showed extensive dye coupling but not heterologous (red-white) coupling. Dye coupling decreased by 3 days yet remained at 5 days. Somites were electrically coupling, and this allowed filtered synaptic potentials to spread from myotome to myotome. It is concluded that at early developmental stages the physiological properties of ER and EW muscle are similar but not identical and are optimized to the patterns of swimming observed at these stages.

INTRODUCTION

Two types of muscle fibers are easily recognized in most fishes (Greer-Walker and Pull 1975) by their characteristic red and white coloration in fresh specimens (Bone 1978; Johnston 1981). A typical teleost myomere, zebrafish included, has a superficial band of red muscle fibers that runs parallel to the rostral-caudal axis of the body and deeper layers of white muscle that run at an oblique angle to this axis (Alexander 1969; Van Raamsdonk et al. 1979). Microscopic, immunological, and histochemical examination reveals a greater diversity of fish muscle fiber types. The myotome of the adult zebrafish is composed of five distinguishable layers of muscle cells: 1) the superficial adult red layer, 2) the intermediate pink layer, 3) the deep white layer, 4) the scattered dorsal and ventral fiber layer, and 5) the red muscle rim layer (Van Raamsdonk et al. 1978, 1980, 1982a, 1987; Waterman 1969). At one extreme, superficial red fibers have small diameters, contain many mitochondria, have a rich blood supply, and have metabolic specializations, making them suitable for sustained activity but not rapid contractions. At the other extreme, each of these characteristics is the opposite for the deep white muscle fibers, suggesting a rapidly fatiguing muscle capable of fast and powerful contractions. How these different muscle fibers develop and their precise functional roles in the embryo are less well understood.

Morphological development of muscle fibers has been studied in considerable detail in the zebrafish, although the physiological roles of different muscle fibers have not been directly examined. A functional characterization of muscle development is important to understand motor control during normal development and to assess dysfunction in the numerous interesting locomotor mutants that have been isolated in the first large genetic screens of vertebrate development (Granato et al. 1996).

At a morphological level, a simpler pattern of muscle fibers is observed in embryonic and young larval zebrafish. Embryonic white (EW) fibers (lateral presomitic cells) form mediolateral to the notochord and do not migrate (Du et al. 1997). When embryonic myotomal segments first form, they are block-shaped and composed entirely of EW fibers running parallel to the notochord. Within a few hours the EW fibers take on their characteristic oblique chevron-shaped orientations (Van Raamsdonk et al. 1974). Embryonic red (ER) fibers (adaxial cells) later form at the midline next to the notochord and then migrate to the surface of the muscle (Du et al. 1997); their differentiation is regulated by the Hedgehog and TGF-β gene families (Blagden et al. 1997; Currie and Ingham 1996; Du et al. 1997). A thin band of muscle consisting of approximately 30 ER fibers per segment blankets these deep fibers and retains their parallel orientation. An anatomical and histochemical division into these two fiber types at early developmental stages is present in a diversity of fish species (Batty 1984; Forstner et al. 1983; Matsuoka and Iwai 1984; O’Connell 1981; Proctor et al. 1980; Stoiber and Sänger 1996; Veggetti et al. 1993), including a number of cyprinid species related to...
zebrafish (El-Fiky et al. 1987; Stoiber and Sänger 1996). During the first week of development, the ER and EW fibers are the only muscle types present. El-Fiky and Wieser (1988) suggest the ER fibers are the main organs of gas exchange prior to gill development and that the deeper muscle layers are involved in locomotion. In later larval development the ER fibers divide to ultimately form the red muscle rim fibers (Van Raamsdonk et al. 1979, 1982b; Waterman 1969).

At a physiological level, the first movements (17 h) (Saint-Amant and Drapeau 1998) are observed shortly after neuromuscular innervation commences (Liu and Westerfield 1992), which is when muscle contractile properties emerge (Van Raamsdonk et al. 1977). Muscle pioneers are the first fibers contacted by primary motoneurons, the first to form functional synapses, and the first to contract (Melancon et al. 1997). Secondary motoneurons begin to innervate muscle at ~26 h (Myers et al. 1986), and swimming is first observed soon after at 27 h (Saint-Amant and Drapeau 1998). At this period, all fibers have metabolic properties similar to adult white muscle, i.e., they are fatigable, and it is not until near the fifth day that the superficial fibers begin to acquire the metabolic characteristics of red muscle (Van Raamsdonk et al. 1978). However, even at this early age, both fibers have distinct red and white myofibrillar properties (Bladgen et al. 1997), but their functional roles are unknown. A rapidly fatigable muscle may be one reason why brief periods of burst swimming, followed by extended periods of rest (Van Raamsdonk et al. 1974), are only observed at early larval stages. Rapidly fatiguing swimming in early larval life appears to be a consequence of muscle metabolic properties in a variety of cyprinids (El-Fiky et al. 1987) and whitefish (Forstner et al. 1983).

The goal of this study is to compare the physiological properties of, and synaptic inputs to, developing ER and EW muscle fibers of the zebrafish. Developmental changes in the properties of these muscles are examined from when swimming behavior is first manifested (day 1) to when zebrafish actively swim to capture prey (day 5).

**METHODS**

**Preparation**

Experiments were performed on zebrafish (*Danio rerio*) embryos and larvae of the Longfin strain raised at 28.5°C and obtained from a breeding colony maintained according to Westerfield (1993). Results are taken from recordings made on 84 muscle fibers. All procedures were carried out in compliance with the guidelines stipulated by the Canadian Council for Animal Care and McGill University. Zebrafish were anesthetized in 0.02% tricaine (MS-222) dissolved in physiological extracellular Evans (1979) solution consisting of (in mM) 134 NaCl, 2.9 KCl, 2.1 CaCl₂, 1.2 MgCl₂, 10 HEPES, and 10 glucose (Drapeau et al. 1999), osmolarity adjusted to ~290 mOsm, pH 7.8. The animal was then 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. The preparation was moved to the recording setup and then continuously perfused with a tricaine-free Evans solution containing 15 μM d-tubocurarine or 1 μM tetrodotoxin (TTX) to paralyze the animals. In dye coupling measurements (*n* = 25) a high magnesium and low calcium extracellular Evans solution (in mM: 123 NaCl, 2.9 KCl, 0.7 CaCl₂, 10 MgCl₂, 10 HEPES, and 10 glucose, osmolarity adjusted to ~290 mOsm, pH 7.8) was used instead of a paralyzing agent. Although these fibers were not used for electrophysiological measurements, robust spontaneous synaptic activity was observed in all 25 cells. Recordings made in this solution were very stable due to an absence of muscle contractions, and for this reason this solution was used during paired recordings (*n* = 5).

**Whole cell recordings**

Standard whole cell recordings (Hamill et al. 1981) were performed on the superficial ER fibers and the first two layers of EW fibers (van Raamsdonk et al. 1979, 1982b; Waterman 1969). Muscle pioneers are the first fibers contacted by primary motoneurons, the first to form functional synapses, and the first to contract (Melancon et al. 1997). Secondary motoneurons begin to innervate muscle at ~26 h (Myers et al. 1986), and swimming is first observed soon after at 27 h (Saint-Amant and Drapeau 1998). At this period, all fibers have metabolic properties similar to adult white muscle, i.e., they are fatigable, and it is not until near the fifth day that the superficial fibers begin to acquire the metabolic characteristics of adult red muscle (Van Raamsdonk et al. 1978). However, even at this early age, both fibers have distinct red and white myofibrillar properties (Bladgen et al. 1997), but their functional roles are unknown. A rapidly fatigable muscle may be one reason why brief periods of burst swimming, followed by extended periods of rest (Van Raamsdonk et al. 1974), are only observed at early larval stages. Rapidly fatiguing swimming in early larval life appears to be a consequence of muscle metabolic properties in a variety of cyprinids (El-Fiky et al. 1987) and whitefish (Forstner et al. 1983).

**TABLE 1. Developmental changes in the properties of ER and EW muscle fibers**

<table>
<thead>
<tr>
<th>Day</th>
<th>ER</th>
<th>EW</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Vₑ, mV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rₑ, MΩ</td>
<td></td>
</tr>
<tr>
<td>Tau, ms</td>
<td>Hyperpolarizing Depolarizing</td>
<td>Tau, ms</td>
</tr>
<tr>
<td>tₑ</td>
<td>% Amplitude</td>
<td>tₑ</td>
</tr>
<tr>
<td>Dye coupling</td>
<td>Number of cells</td>
<td>Number of segments</td>
</tr>
<tr>
<td>1</td>
<td>31 ± 2 (5)</td>
<td>33 ± 6 (5)</td>
</tr>
<tr>
<td>2</td>
<td>71 ± 3 (20)</td>
<td>76 ± 4 (18)</td>
</tr>
<tr>
<td>3</td>
<td>23 ± 2 (13)</td>
<td>77 ± 1.3 (10)</td>
</tr>
<tr>
<td>4</td>
<td>24–25 (2)</td>
<td>14–18 (2)</td>
</tr>
<tr>
<td>5</td>
<td>5 (5)</td>
<td>25 (5)</td>
</tr>
<tr>
<td>6</td>
<td>5 (5)</td>
<td>7 (3)</td>
</tr>
<tr>
<td>7</td>
<td>2 (5)</td>
<td>9,12 (4)</td>
</tr>
<tr>
<td>8</td>
<td>19</td>
<td>4.4</td>
</tr>
<tr>
<td>9</td>
<td>2 (5)</td>
<td>14,42</td>
</tr>
<tr>
<td>10</td>
<td>1 (2)</td>
<td>26</td>
</tr>
<tr>
<td>11</td>
<td>6 (5)</td>
<td>3 (10)</td>
</tr>
</tbody>
</table>

Values are means ± SE; number in parentheses is number of fibers. ER and EW, embryonic red and white, respectively. * No twitch contractions observed with depolarization up to ~35 mV. Paired superscript numbers indicate a significant difference of means (Student’s *t*-test or Mann-Whitney rank sum test), and comparisons were made both horizontally and vertically in the table.
Raamsdonk et al. (1982b). ER and EW fibers correspond to the adaxial and lateral presomitic cells of Devoto et al. (1996) and Du et al. (1997). All physiological measurements were performed on dorsal and ventral fibers located one to two segments rostral or caudal to the anus. A patch pipette controlled by a micromanipulator was used to tease off one or two overlying ER fibers to expose the underlying EW fibers. Muscle fibers were visualized with Hoffman modulation optics (×40 water immersion objective). Experiments were performed at room temperature (22°C). Patch-clamp electrodes were pulled from thin-walled Kimax-S1 borosilicate glass and were filled with either a K-gluconate (for physiological measurements), Cs-gluconate (for paired recordings), or CsCl (for dye coupling measurements) solution to yield electrodes with resistances of 1.5–3 MΩ as described previously (Drapeau et al. 1999). The K-gluconate solution was composed of (in mM) L-glutamic acid-potassium salt, 16 KCl, 2 MgCl₂, 10 HEPES, 10 EGTA, and 4 Na₂ATP, osmolarity adjusted to 290 mOsm, pH 7.2. K-gluconate and KCl were replaced with Cs-gluconate and CsCl in the Cs-gluconate solution or with CsCl in the Cs-chloride solution. The liquid junction potential was −5 mV for K-gluconate and Cs-gluconate electrodes and −2 mV for CsCl electrodes when measured in Evans solution, and records were corrected for this potential. Recordings were made with an Axoclamp-2A amplifier (Axon Instruments) in bridge mode and were low-pass filtered at 10 kHz and digitized at 20–30 kHz. In paired recordings an Axopatch 1D (Axon Instruments) was used as a second amplifier. Cells were discarded if the resting membrane potential was more depolarized than −50 mV. Measurements were made 3–5 min after obtaining the whole cell configuration to ensure cell dialysis.

**Dye coupling between muscle fibers**

All fibers reported in this study were filled with 0.2% fluorescent sulforhodamine B to examine the extent of dye coupling between muscle fibers, and electrophysiological measurements were taken from these same fibers. Images were captured with a Panasonic BP510 charge-coupled device (CCD) camera and a Scion Corporation LG3 frame grabber using Scion/NIH Image software. Whole cell configuration was maintained for 10–15 min in each fiber to allow complete equilibration of the dye. Dye coupling was quantified by counting the number of fluorescent fibers and the number of segments that contained fluorescent fibers.

**Current-voltage relationships and membrane time constants**

Recordings were made in d-tubocurarine to eliminate CNS evoked muscle contractions. Current steps were manually incremented through an A-M Systems isolated pulse stimulator, captured using Axon Instruments and measurements were made in Axoscope 7 software (Axon Instruments). Current injections of 300-ms duration were made once every 2–4 s. A small hyperpolarizing or depolarizing current was injected and constantly adjusted to maintain a membrane potential of −65 mV in 3- and 5-day embryos.

**TABLE 2. Developmental changes in the properties of mEPPs**

<table>
<thead>
<tr>
<th>Muscle Fiber</th>
<th>Amplitude, mV</th>
<th>Rise Time, ms</th>
<th>Half-Width, ms</th>
<th>Frequency, Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 ER</td>
<td>0.87 ± 0.04</td>
<td>2.3 ± 0.2</td>
<td>24 ± 2</td>
<td>0.38 ± 0.04</td>
</tr>
<tr>
<td>Day 1 EW</td>
<td>0.80 ± 0.10</td>
<td>0.58 ± 0.06</td>
<td>3.8 ± 0.1</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Day 5 ER</td>
<td>2.3 ± 0.4</td>
<td>0.39 ± 0.04</td>
<td>3.6 ± 0.2</td>
<td>0.57 ± 0.11</td>
</tr>
<tr>
<td>Day 5 EW</td>
<td>3.7 ± 0.8</td>
<td>0.33 ± 0.03</td>
<td>3.5 ± 0.2</td>
<td>1.0 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE; n is number of fibers. mEPPs, miniature endplate potentials. Day 1 embryos were 29–33 h post-fertilization. Paired superscript numbers indicate a significant difference of means (Student’s t-test or Mann-Whitney rank sum test), and comparisons were made both horizontally and vertically in the table.

**FIG. 1.** Coupling between embryonic red (ER) and white (EW) muscle fibers. A1 and B1: bright field illumination images corresponding to the fluorescent images shown in A2 and B2. Dye coupling in sulforhodamine B filled day 1 ER (A2) and EW (A3) muscle fibers and in day 5 ER (B2) and EW (B3) muscle fibers. The middle brightest cell in A3 was filled.

Some embryonic muscle fibers were unstable at −65 mV, so all recordings were performed at −75 mV, which is closer to the resting membrane potential at this age. During recordings, the electrode response was constantly monitored at high gain and high sweep speed on an analog oscilloscope and the electrode balanced using the bridge circuitry. Electrode resistance was generally low (3–5 MΩ) but was as high as 10 MΩ in some recordings, and the electrode capacitance was <3 pF. Both the electrode resistance and capacitance were an order of magnitude smaller than those for the muscle membrane, resulting in small and fast transients that were easily compensated and unlikely to contribute to measurement errors.

Membrane time constants were determined by fitting the voltage response to a hyperpolarizing current injection (15- to 25-mV deflection) with a sum of exponential curves. The presence of one or two exponential components was tested by comparing the sum of squared errors of the fits.

**Muscle contraction rates**

In many muscle fibers, it was possible to inject sufficient current in the center of the fiber to evoke a muscle contraction and still maintain the whole cell configuration. Following voltage recordings, the current pulse duration was reduced to 15–20 ms and the intensity increased to 2–7 nA, which was sufficient to evoke a muscle contraction. A duration of 15–20 ms was chosen to mimic the cycle duration of motoneuron output that must be occurring for the larvae to swim at
the observed tail beat frequencies of approximately 30–60 Hz (Budrick and O’Malley 1999; Eaton et al. 1977; Saint-Amant and Drapeau 1998). Briefer durations of current were usually ineffective regardless of the intensity of stimulation up to 10 nA. The contraction of the muscle was observed on a video monitor and recorded on a VCR (Panasonic S-VHS) along with stimulus parameters, which were recorded on Axotape for later analysis. Inter-pulse interval was gradually decreased until the individual contractions fused together, which is referred to as the tetanus fusion rate. Long (300 ms) depolarizing current injections were used to determine the rheobasic contraction threshold, which was recorded as the membrane potential at which the first twitch contraction was observed.

Analysis of miniature endplate potentials (mEPPs)

mEPPs were recorded in Evans solution containing TTX to block CNS-evoked muscle contractions but not spontaneously occurring synaptic vesicle release. A small hyperpolarizing or depolarizing current was injected into the fiber to hold the membrane potential at −75 mV (day 1) or −65 mV (days 3–5). Five-minute recordings were made in Fetchex (Axon Instruments), and analysis was performed off-line using Axograph 4.0 (Axon Instruments). Events were detected using the template function set at 4–5 SD above baseline noise. It was not possible to discriminate events <0.5 mV in amplitude due to the background noise even with Cs-gluconate pipettes. Small events were excluded followed by visual examination and deletion of erroneous events. The remaining events were used to calculate peak amplitudes, 20–80% rise times, half-widths, and mEPP frequencies.

Paired recordings

Paired recordings were performed in a high magnesium (10 mM) and low calcium (0.7 mM) solution, which abolished muscle contractions and allowed stable recordings with two electrodes. A Cs-glucuronate intracellular solution was used to potentiate mEPPs and facilitate their detection. These fibers were not used for calculating any parameters listed in Tables 1 or 2. All paired recordings were made from longitudinally adjoining ER fibers of day 3 animals. Positive or negative current was ejected through the recording electrodes to equalize the membrane potential of each cell.
The goal of this experiment was to determine whether the small and slow mEPPs occurred simultaneously with large and fast mEPPs in adjacent fibers, the small and slow events being due to filtering through intercellular junctions. Digitized data were examined by scrolling through 50-ms windows in Axoscope 7. Whenever an event was detected (>0.5 mV) in one fiber, a simultaneous corresponding event was searched for in the other fiber. This preliminary analysis revealed that events large enough to be detectable, i.e., >0.5 mV, always occurred simultaneously in both fibers with one mEPP appearing as a filtered and attenuated version of the other mEPP. Time-to-peak and peak amplitude measurements were then made by eye for 50 consecutive paired mEPPs. The peak amplitude and time-to-peak values of the larger mEPP was divided by the peak amplitude and time-to-peak value of the smaller mEPP and was normalized to 100% to express the extent of filtering between each pair of mEPPs.

Results are presented as means ± SE, and significant denotes a significant relationship ($P < 0.05$) determined using the Student’s t-test or Mann-Whitney rank sum test. Correlations were tested using the Pearson Product Moment Correlation, and a significant relation was noted when $P < 0.05$. Sulforhodamine B was purchased from Molecular Probes (Eugene, OR) and all other chemicals from Sigma Chemical (St. Louis, MO).

RESULTS

To avoid the errors inherent with voltage-clamp recording from electrically coupled muscle cells (e.g., Broadie 1999; Nguyen et al. 1999), experiments were performed in current-clamp mode. Muscle fibers were examined at three time periods of development (Kimmel et al. 1995): in embryos (day 1.2–1.5; referred to as day 1) near the time swimming-like behavior is first observed (Saint-Amant and Drapeau 1998), in quiescent posthatching larvae (day 3.4–3.6; referred to as day 3) and in active free-swimming larvae (day 4.9–5.8; referred to as day 5). As the appearance of the two types of muscle fibers did not change over the brief (4 day) interval examined, we refer to them as ER or EW muscle fibers. Resting membrane potentials recorded with K-gluconate intracellular solutions (Table 1) were −73 ± 2 (SE) mV in ER and −78 ± 1 mV in EW fibers and depolarized by 7 mV (EW) and 17 mV (ER) during development to the free swimming larval stage (day 5).

At all stages examined, the resting membrane potentials of the EW fibers were more negative than those of the ER fibers. By day 5 the difference in resting membrane potential between EW and ER fibers was 15 mV.

Dye coupling

Dye coupling was observed in 69 of 74 muscle fibers examined in this study, the exceptions being 5 EW fibers at 3–5 days. No obvious differences in dye coupling were observed in rostral or caudal segments (±10 segments from the anus) or in dorsally or ventrally located fibers. Dye coupling in ER fibers spanned up to five segments (only 3 segments are shown in Fig. 1) at all stages, which included up to 38 labeled fibers at day 1 and 12 at days 3–5. The average number of dye-coupled segments decreased by 38%, and the number of fibers filled decreased by 73% (Table 1) during the transition from day 1 to day 3, and there was no further change by day 5. Fibers were dye-coupled in both dorso-ventral and rostro-caudal directions (Fig. 1, A2 and B2).

Dye coupling was less extensive in the EW than in ER fibers at all corresponding stages examined (Fig. 1; Table 1) but showed a similar decrease in segmental coupling (by 39%) and decrease (by 70%) in the number of fibers coupled from day 1 to day 3, and, similar to the ER fibers, no further change in coupling was observed by day 5. Unlike ER fibers, coupling in the EW fibers was generally restricted (21 of 26 fibers) to the rostro-caudal axis in larvae where 1–4 segments could contain dye-filled fibers. Dorso-ventral coupling in addition to rostro-caudal coupling was observed in day 1 EW fibers where 3–13 fibers were found coupled over 3–5 segments at later stages. Coupling between EW fibers running in different oblique orientations (Fig. 1A3) was observed occasionally. However, coupling was never observed between ER and EW fibers.

Current-voltage (I-V) relationships

The I-V relationship of each type of muscle fiber was examined to reveal any differences or changes in the membrane properties during development of the ER and EW fibers. At day 1, ER fibers had mean input resistances of 31 ± 2 MΩ (Table 1) and showed no rectification, and the time course of the voltage response was similar in both depolarizing and hyperpolarizing directions (Fig. 2A1). The mean input resistance of day 3 ER fibers increased to 74 ± 10 MΩ (Table 1) when hyperpolarizing currents were injected. In some cells outward rectification was observed with depolarizing currents, and the initial voltage response was steeper in the depolarizing direction (Fig. 2A2). Interestingly, the input resistance at day 5 decreased to 30 ± 5 MΩ, a value similar to that observed at day 1. It is likely that the initial increase in input resistance from day 1 to day 3

![Embryonic Red](Day 1)

![Embryonic White](Day 1)

![Day 5](2 mV)

![Day 5](7 mV)
was principally due to muscle fiber uncoupling, and the decrease in input resistance observed from day 3 to day 5 was due to insertion of voltage-gated ion channels into the membrane. Similar to day 3, some day 5 fibers rectified and showed a steeper initial voltage response in the depolarizing direction (Fig. 2A; Table 1). Action potentials were not observed in any ER fibers.

Day 1 EW fibers had a mean input resistances of 33 ± 6 MΩ (Table 1) and showed a similar voltage response in both depolarizing and hyperpolarizing directions (Fig. 2B). Similar to ER fibers, the input resistance in day 3 EW fibers was slightly higher (46 ± 8 MΩ) and then decreased to 27 ± 5 MΩ by day 5 (Table 1). Rectification was not observed in any EW fibers. The initial voltage response was steeper in the depolarizing direction than the hyperpolarizing direction in day 3 EW fibers (Fig. 2B2), but this was not apparent in day 5 EW fibers (Fig. 2B3). No action potentials were observed in any EW fibers.

**Membrane time constants**

Membrane voltage responses to 300-ms hyperpolarizing current injections were fit with the sum of two exponential curves with membrane time constants $\tau_{fast}$ and $\tau_{slow}$. At all ages and in both ER and EW fibers, $\tau_{fast}$ mean values ranged from 0.5 to 4.4 ms, and $\tau_{slow}$ means ranged from 16 to 76 ms (Table 1). At day 1, EW fibers had a faster $\tau_{fast}$ (0.5 ± 0.2 ms) than that of ER fibers (3.2 ± 0.8 ms). The $\tau_{fast}$ exceeded the membrane time constant and may reflect an active conductance, or the presence of unfused myoblasts as observed previously (Nguyen et al. 1999). The $\tau_{slow}$ was very similar (ER = 71 ± 3 ms vs. EW = 76 ± 4 ms; Table 1). However, $\tau_{slow}$ contributed to 75% of the response amplitude in ER fibers, whereas in EW fibers only 21% of the response amplitude was due to $\tau_{slow}$. This difference might be explained by the more extensive dye coupling observed in ER fibers. At day 5, $\tau_{slow}$ decreased similarly in EW and ER fibers to 40 ± 9 ms and 39 ± 11 ms. The contribution of $\tau_{slow}$ to the peak amplitude changed little (21 to 19%) in EW fibers, while it decreased from 75 to 36% in ER fibers. These changes in membrane time constants closely parallel the changes in dye coupling observed at these same ages. Accordingly, $\tau_{fast}$ likely corresponded to the membrane response of the fiber recorded from and perhaps immediately adjacent fibers (immediate compartment), whereas $\tau_{slow}$ was the response of more distant coupled muscle fibers (remote compartment). However, changes in fiber size and channel composition, which were not examined in this study, are also likely to influence the changes in time constants observed. By day 5, $\tau_{slow}$ changed little in EW fibers (36 ± 7 vs. 40 ± 9 ms) and only contributed to 12 ± 1% of the amplitude of the voltage response. At this age $\tau_{slow}$ contributed to 49% of the amplitude of the voltage response in ER fibers, which by this time had developed a significantly faster $\tau_{slow}$ (16 ± 3 vs. 36 ± 7 ms) than day 5 EW fibers.
Contraction thresholds and maximal contraction rates

During the I-V recordings (300-ms current pulses) the membrane depolarization required for muscle to twitch was noted. The voltage threshold for muscle contraction changed little in day 1–5 ER fibers [means varied from $-24.2 \pm 6.4 \text{ mV}$ to $-24.8 \pm 6.7 \text{ mV}$ (Table 1)]. In contrast, EW fibers did not contract in response to long depolarizing current that depolarized the membrane to $-35 \text{ mV}$. Larger current injections that depolarized the membrane well above $0 \text{ mV}$ resulted in robust muscle twitches and the loss of whole cell configuration. It was apparent that EW muscle did not respond well to focal stimulation, which was not unexpected in these unexcitable and multiply innervated muscle fibers.

Following I-V recordings, stimulus parameters were adjusted to resemble more closely the currents the muscle fibers would presumably receive during swimming at maximally observed contraction rates. The electrode seal was lost in several fibers once muscle contractions started, but recordings were relatively stable in others. The local (point) current necessary to evoke a muscle contraction depolarized the membrane well above $0 \text{ mV}$ resulting in robust muscle twitches and the loss of whole cell configuration. It was apparent that EW muscle did not respond well to focal stimulation, which was not unexpected in these unexcitable and multiply innervated muscle fibers.

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Of the successful recordings, ER and EW fibers at all stages were capable of following trains of 15–20 ms stimulation delivered at frequencies up to $30 \text{ Hz}$ (observed in a day 5 ER fiber), which predicts a sustainable upper limit of $30 \text{ Hz}$ for alternating tail beat. Thirty hertz is below the maximum tail beat frequencies ($\sim 50 \text{ Hz}$) (Budick and O’Malley 1999) reported in larval zebrafish. This discrepancy is likely because our study measured sustained muscle contraction rates and cannot rule out the possibility that more rapid muscle contraction rates, which fatigue within a few muscle contractions, are possible. The average muscle tetanus fusion rates (Table 1) were fastest at day 5 and appear slower at day 1, which correlates well with the gradual increase in swimming tail beat frequency observed in developing zebrafish (Budick and O’Malley 1999; Eaton et al. 1977; Saint-Amant and Drapeau 1998). Stimulation was generally delivered for 2–5 min, during which time muscle fatigue was not observed.

$\text{mEPPs}$

Figure 3, A–D, shows representative examples of mEPPs recorded in day 1 and day 5 muscle fibers. Readily apparent are the higher frequency of events at day 5 versus day 1 and the prominence of large amplitude events at day 5 compared with smaller event amplitudes at day 1. Representative mEPPs are shown on an expanded time scale in Fig. 3, A1–D1. The same mEPPs used to construct Fig. 4 are used here.
increased in both ER and EW fibers from day 1 to day 5, but this increase was greatest in EW fibers where it increased 10 times compared with ER fibers where the frequency increased only 1.5 times. Specifically, day 1 ER fibers received a higher frequency of mEPPs (0.38 ± 0.04 Hz) than EW fibers (0.10 ± 0.02 Hz), but this difference was reversed by day 5 when ER fibers received mEPPs at 0.57 ± 0.11 Hz compared with frequencies of 1.00 ± 0.24 Hz observed in EW fibers. Differences in passive membrane properties did not account for slow events in day 1 ER fibers. Even though day 1 ER fibers had slower time constants than day 1 EW fibers, these values (as well as the resistances) were more similar to those of both types of fibers at later stages (Table 2). Therefore the kinetics of mEPPs recorded in day 1 ER fibers were fundamentally slower than those of day 1 EW fibers and of both fiber types at later stages.

The properties of mEPPs were further examined by comparing the distributions of event amplitudes, rise times, and half-widths. Representative mEPP distributions from a day 1 ER and EW fiber are shown in Fig. 4. The amplitude distributions were very similar in the day 1 ER and EW fibers and appeared to be normally distributed, but the rise time and half-width distributions are clearly wider and displaced to the right in the ER fibers. Scatter plots of event rise times versus amplitude and half-widths (Fig. 5) do not reveal more than one population of events and further illustrate the difference between mEPPs in day 1 ER and EW fibers.

mEPP distributions appeared normally distributed and were similar in day 5 ER and EW fibers. Thus amplitude, rise time, and half-width distributions are only shown for EW fibers (Fig. 6). Unlike day 1 fibers, some day 5 fibers displayed one population of mEPP amplitude, rise time, and half-width distributions (Fig. 6A), while others displayed two distributions (Fig. 6B). This was also apparent in scatter plots of rise times versus amplitudes (Fig. 7, A1 and B1) or half-widths (Fig. 7, A2 and B2). The distribution common to all fibers was composed of events with large amplitudes (>1–2 mV), fast rise times (<0.2–0.4 ms), and short half-widths (<4–5 ms; Fig. 7A). In the majority of cells (3 of 5 ER; 3 of 4 EW) a second population of events with smaller amplitudes (<1–2 mV), slower rise times (>0.2–0.4 ms), and longer half-widths (>4–5 ms) were observed (Fig. 7B). Closer examination of the fibers where this second population of small and slow events was observed revealed a lower baseline noise in these fibers. Thus two populations of events may be present in all day 5 fibers, but baseline noise prevents the detection of these smaller and slower events. No clear differences could be detected in the amplitude distributions of day 5 ER and EW fibers, and mean mEPP amplitudes were not significantly different in EW (3.7 ± 0.84 mV) and ER (2.3 ± 0.43 mV) fibers.

Evidence for the filtered propagation of mEPPs between coupled muscle fibers

A simple explanation for the two types of events is that the population of large and fast mEPPs originated from synapses
located on the cell recorded from, whereas the population of smaller and slower mEPSSs would represent mEPSSs originating in electrically coupled neighboring fibers that were recorded as filtered mEPSSs. To test this possibility, simultaneous recordings were made from adjacent cells, and the presence of a large, fast event in one cell occurring simultaneously with a small, slow event in the other fiber was investigated. A total of five paired recordings were performed on adjacent ER fibers in day 3 larvae. A pair of dye-coupled fibers is shown in Fig. 8, A and B, and the corresponding paired recording is shown in Fig. 8C. Apparent is the presence of a large and fast event recorded form one electrode paired with a smaller and slower event recorded with the second electrode (Fig. 8, C and D). The larger and faster event was observed with equal occurrence in either of the electrodes. Fifty consecutive mEPSSs were observed in each of the five paired recordings and time-to-peak and peak amplitude measured. In every instance, a larger and faster event occurred simultaneously with a smaller and slower event. On average the amplitude of filtered events was half (45 ± 7%) as small and the time-to-peak was twice (2.0 ± 0.1) as slow. In addition, a trend observed (r = 0.64) was that the events with the least attenuated amplitudes also showed the...
smallest increase in time-to-peak. These were events where the lowest amplitude was small to begin with (e.g., Fig. 8, C and D, 3 and 5). Conversely, events with the greatest amplitude attenuation showed the greatest increase in time-to-peak. These events resembled 2 of Fig. 8, C and D, where the large amplitude event was big to begin with. Thus the electrical coupling acted as a low-pass filter, strongly attenuating the synaptic events from immediately adjacent fibers while only weakly attenuating synaptic events from more distant cells.

**Discussion**

This is the first study examining the physiological development of red and white fish muscle. At all stages examined, the resting membrane potential of the ER fibers was more depolarized than that of the EW fibers. A more depolarized resting potential in fish red muscle has been reported in a variety of fishes (Andersen et al. 1963; Hidaka and Toida 1969a; Stanfield 1972; Takeuchi 1959; Teräväinen 1971). The absolute membrane potential of the EW muscle at the latest stage examined (day 5) was $-71 \text{ mV}$, which contrasts with a mean resting potential of $-82 \text{ mV}$ in zebrafish adult white muscle (Westerfield et al. 1986).

The input resistance of adult red muscle is generally lower than that of white muscle (Alnaes et al. 1964; Hidaka and Toida 1969a; Klein and Prosser 1985; Nicolaysen 1976a,b; Stanfield 1972; Teräväinen 1971), while ER and EW fiber resistances were similar at the same stages. The red and white muscle of adult fish shows rectification in some species of ray finned fishes but not in the silver carp (Eugene and Baret 1982; Hagiwara and Takahashi 1967; Hidaka and Toida 1969a), cartilaginous (Hagiwara and Takahashi 1967), or jawless fishes (Alnaes et al. 1964). The I-V curves of day 5 EW muscle were linear, while some day 3 and 5 ER muscle showed outward rectification. The more extensive coupling in ER fibers may have contributed to the rectification as injected current could have spread to other cells.

Action potentials were not observed in the ER or EW muscle fibers. However, the absence of action potentials does not rule out the existence of voltage-gated channels, which is suggested by the presence of a faster rising membrane response to positive, compared with negative, current injections in both ER and EW fibers (Fig. 2). The white fibers of adult fish generate action potentials that may or may not be overshooting, whereas the red fibers may have contributed to the rectification as injected current could have spread to other cells.

Intercellular coupling between developing muscle cells has been reported in mammals, birds, amphibia, and fish and is lost with developmental maturation (Dennis 1981). Intercellular junctions have been observed in ultrastructure studies of embryonic zebrafish muscle (Waterman 1969) and in rainbow trout (Nag and Nursall 1972). There was a clear trend toward fiber uncoupling with age in this study (Fig. 1), although fibers were not fully uncoupled at the latest stages examined. It cannot be assumed that all fibers are fully uncoupled in adult zebrafish because in other fish muscle fibers remain coupled into adulthood (e.g., the lamprey) (Teräväinen 1971).

If we assume that the extent of detectable fiber coupling was limited by diffusion barriers, then it appears that the cytoplasm of all myotomal muscle (ER and EW) is connected at these developmental stages. The absence of coupling between ER and EW fibers could restrict diffusion of intracellular factors involved in muscle differentiation and growth to these distinct fiber types. No evidence was found to suggest electrical coupling between motor axons and muscle (Dennis 1981) as dye did not pass from muscle into motoneuron axons and the bursting patterns of action potentials observed in motoneuron recordings (Drapeau et al. 1999) were not observed as patterns of depolarization in curarized muscle.

With the exception of the lamprey (Teräväinen 1971), the white muscle fibers of the typical teleost will contract in response to sub-threshold synaptic stimuli (Bone 1964; Hagiwara and Takahashi 1967; Hidaka and Toida 1969a). The maximal contraction rates (mean rates of 23–27 Hz, Table 1) of both the ER and EW fibers changed little during day 3 to day 5, although contraction rates may have been slower at day 1. These contraction rates are within the range of contraction rates observed in other fish myotomal muscle that range from 5 to 60 Hz (Altringham and Johnston 1988; Johnston 1980). The maximal contraction rates observed in the ER and EW muscle are consistent with swimming cycle periods observed behaviorally (Budick and O’Malley 1999; Eaton et al. 1977; Saint-Amant and Drapeau 1998) and show that neural excitation is optimized to match the contractile apparatus. This finding shows that ER muscle could be co-active with EW muscle at all swimming speeds at the stages examined. However, this would contrast with the situation in adult zebrafish muscle where red muscle is believed to be active at slow swimming speeds and white muscle is recruited during short bursts of rapid swimming (Liu and Westerfield 1988).

There have been a limited number of studies examining synaptic properties in fish muscle. Comparable mEPP amplitudes were observed in ER and EW fibers at the same stages ($-1 \text{ mV}$ at day 1 and $-2 \text{ mV}$ at day 5, Table 2). mEPP amplitudes are generally $<2.5 \text{ mV}$ in silver carp, lamprey, and hagfish (Alnaes et al. 1964; Balezina and Gulyaev 1985; Hidaka and Toida 1969b), while events of 7 mV were found in the fast contracting sonic muscle of the toadfish (Gainer and Klancher 1965; Skoglund 1959). Synaptic events recorded in fish muscle have rapid decay kinetics, with current half-widths or time constants no more than 2 ms in duration and mEPP half-widths ranging from 5 to 10 ms (Alnaes et al. 1964; Balezina and Gulyaev 1985; Gainer and Klancher 1965; Hidaka and Toida 1969b; Macdonald 1983; Macdonald and Montgomery 1986; Mileti and Reiser 1983). The decay kinetics of day 5 zebrafish mEPPs were at least as fast having mean half-widths of $<4 \text{ ms}$ (Table 2) due to mEPCs with decay time constants of $<2 \text{ ms}$ (Legendre et al. 2000; Nguyen et al. 1999) and were comparable to the mEPPs recorded in 2-day-old zebrafish myotomal muscle shown in Fig. 3 of Felsenfeld et al. (1990). Rapid mEPC kinetics are consistent with the fast contraction rates during swimming as the zebrafish is one of the fastest swimming teleosts of its size (Plaut 2000).

In the vast majority of teleosts, including the zebrafish, red
and white muscle receive numerous distributed endings from a plexus of nerves spread over the surface of the muscle fibers (Altringham and Johnston 1981; Barets 1961; Bone 1964; Gainer 1969; Gainer and Klancher 1965; Hudson 1969; Ogata 1988; Shenk and Davidson 1966; Takeuchi 1959; Westerfield et al. 1986), which contrasts with the terminal innervation of white muscle fibers in non- and basal-teleost groups (Best and Bone 1973; Bone and Ono 1982; Ono 1983). The function of this multiterminal innervation may be to allow the presynaptic motoneuron action potential to reach several points of the muscle fiber faster and with greater efficiency than would be possible by active or passive conduction along the muscle fiber if activated by a single terminal junction (Shenk and Davidson 1966). Short-duration (<10 ms) current injected through the patch electrode was ineffective at eliciting muscle contraction, and in EW fibers long duration (300 ms) current injections did not evoke a muscle twitch until the membrane potential approached or overshot 0 mV, which is a seemingly unphysiological potential for the membrane to reach.

In the zebrafish, the biophysical properties of individual acetylcholine receptor molecules at the neuromuscular junctions are also optimized to maximize current spread. At these junctions, the endplate current is generated on reversal from open channel block following removal of acetylcholine (Legendre et al. 2000). This results in a delayed closing of the acetylcholine receptors and consequently a transient, rebound synaptic current. The net consequence is a larger charge distributed over a broader time course, presumably enhancing the distributed depolarization leading to muscle contraction.

Distinct changes were observed in the properties of mEPPs over the stages examined. mEPP amplitudes increased in ER and EW fibers, suggesting an increase in acetylcholine receptor density postsynaptically or an increase in the acetylcholine content of a transmitter quantum or both. The increase in mEPP frequencies could be due to an increase in the number of synaptic sites and/or probability of vesicle release. Liu and Westerfield (1992) reported an increase in the number and size of acetylcholine receptor clusters during synaptogenesis. mEPP kinetics were similar in day 1 EW fibers and day 5 EW and ER fibers but were distinctly slower in the day 1 mEPP kinetics were similar in of acetylcholine receptor clusters during synaptogenesis. The large difference in mEPP kinetics as observed in ER fibers in this study, thus making it likely that the superficial ER fibers were the fibers examined at the embryonic stages. The large difference in synaptic kinetics and frequency of occurrence in ER and EW fibers at the embryonic stages could be due to immaturity of the ER fibers or differential innervation by ER and EW muscle by different populations of motoneurons or both.

In adult zebrafish, populations of motoneurons with dorsal and ventral positions in the spinal cord have been found to innervate white and red muscle, respectively, with high specificity (Myers 1985; Van Asselt et al. 1993; Van Raamsdonk et al. 1983). The dorsal population of motoneurons undoubtedly include the primary motoneurons described by Myers et al. (1986), which are the first motoneurons to exit the spinal cord and form synapses on muscle fibers. In the adult zebrafish there is a greater diversity of fiber types and most secondary motoneurons innervate more than one class of muscle, e.g., white and intermediate (De Graaf et al. 1990). It is possible that the outgrowing secondary motoneurons are forming synapses on ER fibers as well as EW fibers in this period. We suggest that the primary motoneurons of Myers et al. (1986) only innervate EW muscle fibers, and having started synaptogenesis at 17 h (Liu and Westerfield 1992), have undergone upward of 10 h of synaptic development by the time of recording. We therefore hypothesize that both the ER and EW fibers are innervated by the population of ventral secondary motoneurons (Myers et al. 1986), which do not exit the spinal cord until ~26 h. Recordings made on ER fibers could potentially be at the initial period of synaptogenesis when synaptic currents are expected to have slow kinetics (Kullberg and Owens 1986; Kullberg et al. 1977; Nguyen et al. 1999; Schuetze and Role 1987).

Paired recordings performed on day 3 ER fibers clearly demonstrate that small amplitude mEPPs with slow kinetics do not all arise as events with distinct kinetics but are due to low-pass filtering through an electrically coupled synctium of muscle fibers. All small and slow mEPPs are not necessarily filtered mEPPs, and we would expect that an unknown fraction of these events (including undetected events <0.5 mV in amplitude) would arise from newly formed synapses. Electrical coupling between adjacent muscle fibers undoubtedly places a limit on the possible movements of the zebrafish embryo and larva. During swimming, propulsion is generated by alternating, neural-mediated waves of contractions progressing down the body of the fish (Lindsey 1978). Electrical coupling would spread (limited by low-pass filtering) the neurally evoked mEPPs to adjacent myotomes, depolarizing the membrane closer to contraction threshold. However, the electrical coupling would limit the degree of curvature in the body of the larvae that swim with shallow eel-like movements (unpublished observations), an energetically efficient means of movement for small larval fish (Batty 1984). Thus the physiological properties of larval zebrafish muscle complement the most energetically favorable form of swimming in this developing fish.

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