JOURNAL OF EXPERIMENTAL ZOOLOGY 286:36–48 (2000)

Muscle Type–Specific Myosin Isoforms in Crustacean Muscles

WILLIAM A. LaFRAMBOISE,[1] BRUCE GRIFFIS,[2] PHILIP BONNER,[2] WENDY WARREN,[2] DEBORAH SCALISE,[1] ROBERT D. GUTHRIE,[1] AND ROBIN L. COOPER[2]*

[1]*Department of Pediatrics, Laboratory of Cellular and Molecular Myology, Allegheny University of the Health Sciences, Pittsburgh, Pennsylvania 15024*

[2]*Nerve-Muscle Group, Thomas Hunt Morgan School of Biological Sciences, University of Kentucky, Lexington, Kentucky 40506-0225*

ABSTRACT Differential expression of multiple myosin heavy chain (MyHC) genes largely determines the diversity of critical physiological, histochemical, and enzymatic properties characteristic of skeletal muscle. Hypotheses to explain myofiber diversity range from intrinsic control of expression based on myoblast lineage to extrinsic control by innervation, hormones, and usage. The unique innervation and specialized function of crayfish (*Procambarus clarkii*) appendicular and abdominal musculature provide a model to test these hypotheses. The leg opener and superficial abdominal extensor muscles are innervated by tonic excitatory motoneurons. High resolution SDS-PAGE revealed that these two muscles express the same MyHC profile. In contrast, the deep abdominal extensor muscles, innervated by phasic motoneurons, express MyHC profiles different from the tonic profiles. The claw closer muscles are dually innervated by tonic and phasic motoneurons and a mixed phenotype was observed, albeit biased toward the phasic profile seen in the closer muscle. These results indicate that multiple MyHC isoforms are present in the crayfish and that differential expression is associated with diversity of muscle type and function. *J. Exp. Zool. 286:36–48, 2000.*

Striated muscle exhibits an evolutionarily preserved sarcomeric architecture for the transduction of chemical energy into mechanical force. The dominant molecule underlying this fundamental contractile structure is the myosin heavy chain (MyHC), a motor protein housing the myosin ATPase and nucleotide binding site, the actin binding site for cross-bridge formation, and a coiled-coil alpha-helical region for self-association and thick filament formation (Rayment et al., '93). Within the context of this highly conserved sarcomeric and molecular organization, considerable heterogeneity has been described both within and across muscle groups from a variety of morphological, histochemical, enzymatic, biochemical and physiological assays (Burke, '81). This diversity among muscle fibers is recognized largely by the differential expression of members of the MyHC gene family, molecules that play central roles in determining critical physiological (velocity of shortening and power output), histochemical (myosin ATPase pH stability), and enzymatic (ATPase rate) properties of muscle tissue (Moss et al., '95).

Muscles have the potential to display a remarkable range of MyHC expression. For example, flight muscles of the little brown bat (*Myotis lucifugus*) exhibit unusual homogeneity in that all pectoralis myofibers are of the same phenotype, expressing a single MyHC isoform, reflected by uniform histochemical staining throughout the muscle (Hermanson et al., '91). At the other extreme, extraocular muscles of the blue marlin (*Makaira nigracans*) exhibit an immunohistochemical MyHC heterogeneity consistent with the presence of at least six distinct fiber types within the superior rectus muscle alone. These divergent phenotypes likely reflect adaptation to activation pattern and usage (Tullis and Block, '97). The molecular specialization of the bat pectoralis muscle is consistent with its singular commitment to pow-

Grant sponsor: University of Kentucky Research and Graduate Studies; Grant Sponsor: National Science Foundation; Grant numbers: IBN-9808631 and ILI DUE-9850907.

William A. LaFramboise's current address: Tissue Informatics, 3636 Blvd. of the Allies, Pittsburgh, PA 15213.

*Correspondence to: Robin L. Cooper, Nerve-Muscle Group, School of Biological Sciences, University of Kentucky, Lexington, KY 40506-0225. E-mail: RLCOOP1@pop.uky.edu

Received 20 January 1999; Accepted 23 April 1999

ering the downstroke of chiropteran flight. In contrast, the extraocular muscles reflect the diverse contractile demands ranging from high-velocity, ballistic movements to slow-tonic gaze fixation. In fact, this heterogeneity may extend to single myofibers as regional co-expression of multiple MyHC species within rodent extraocular myofibers is thought to reflect an intracellular adaptation to "dampen" the impact of contractile forces from high velocity movements and to minimize fiber trauma (Porter and Baker, '96).

Several hypotheses exist to explain the basis for myofiber diversity, including intrinsic control based on myoblast lineage and extrinsic determination by a host of factors including innervation pattern, hormonal influences, and physiological use. The crayfish (*Procambarus clarkii*) provides an interesting model for analysis of MyHC phenotype and physiological profile due to the unique innervation and highly specialized function of its appendicular and abdominal musculature (Wiens, '89, '93; Bradacs et al., '97; Cooper et al., '98). The walking leg opener and superficial abdominal muscles are innervated by tonic excitatory motor neurons, in contrast to the deep abdominal extensor muscles, which are innervated by phasic motor neurons. The claw closer and leg extensors are notable for their dual innervation by both tonic and phasic motor neurons. The present study was undertaken to determine the impact of this specialized innervation on the physiological properties and myosin phenotype of these muscles.

MATERIALS AND METHODS

Animals and dissection

All experiments were performed using the crayfish *Procambarus clarkii*, measuring 6 to 10 cm in body length (Atchafalaya Biological Supply Co., Raceland, LA). Animals were housed in an aquatic facility and fed fish food.

The *opener muscle* of the first walking legs was prepared via standard dissection (Dudel and Kuffler, '61; Cooper et al., '95a). Only the ventral aspect of this muscle was viewed for terminal structure and physiological measures (Fig. 1A). The central region of this muscle was removed for myosin heavy chain analysis. The medial surface of the *leg extensor muscle* of the first pair of walking legs was stained and viewed to measure synaptic responses (Fig. 2A). The muscle was exposed by removing the cuticle on the lateral aspect of the meropodite along with the entire flexor muscle and the main leg nerve. The motor nerve of the extensor muscle separates from the main leg nerve as it enters the meropodite and is left in place on the muscle after removal of the main leg nerve (Bradacs et al., '97). The entire muscle was used for myosin heavy chain analysis. The *closer muscle* was exposed by standard dissection (Lnenicka and Atwood, '85a,b) and the dorsal surface was used for protein extraction and morphological and physiological measurements (Fig 3A). The ventral surface of the deep *abdominal extensor muscles* was exposed by removing the ventral side of the abdomen after cutting along the length of the abdomen at the midline on each side. The dorsal half was pinned down and residual flexor muscles were removed (Fig. 4A). This allows excellent visual identification of the deep extensor muscles (L1, L2, and M) and the superficial lateral extensor muscle (SEL). The superficial medial extensor muscle (SEM) can be seen after removal of the L1 and L2 musculature (Cooper et al., '98). The SEL and L muscles were used for physiological studies and protein analysis.

To visualize living nerve terminals, freshly dissected muscles were incubated for 2 to 5 min in 2 to 5 μM 4-Di-2-ASP (4-[4-(diethylamino)styryl]-N-methylpyridinium iodide; Molecular Probes, Eugene, OR) (Magrassi et al., '87), followed by a wash in saline. The tissues were positioned in a Sylgard dish for viewing and photographing with a Nikon Optiphot-2 epifluorescence microscope with a 40× (0.55 NA) Nikon water-immersion objective. Dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (in mM: 205, NaCl; 5.3, KCl; 13.5, $CaCl_2$ $2H_2O$; 2.45, $MgCl_2$ $6H_2O$; 5, HEPES) at 14°C and adjusted to pH 7.4.

Excitatory postsynaptic potentials (EPSPs)

Intracellular recordings were performed with 30–60 MΩ resistance microelectrodes filled with 3 M KCl. Responses were recorded with a 1× LU head stage and an Axoclamp 2A amplifier. Electrical signals were recorded on VHS tape (Vetter, 400), and on-line to a Power Mac 9500 via a MacLab/4s interface. EPSPs were recorded at 10 kHz. To determine the facilitation index for the train facilitation, the amplitude of the 10th EPSP is divided by the amplitude of the 1st EPSP pulse and the result is subtracted by one. The amplitudes of the EPSPs are obtained by the difference in the peak value to the baseline preceding the train of stimulation. All events were appropriately scaled to known test pulses applied through the electrode and directly measured on an oscilloscope. The corrected scale was then adjusted with

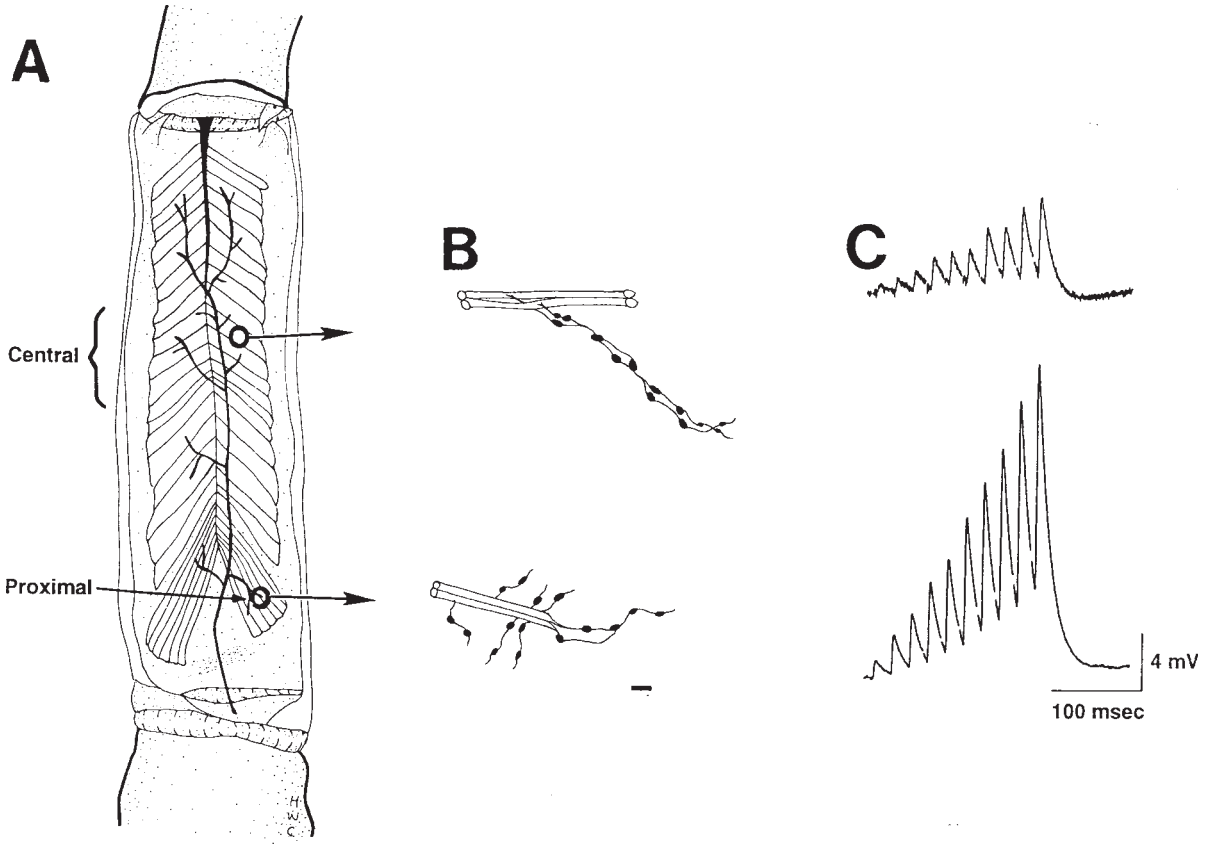

Fig. 1. Anatomy and physiological responses common to a tonic neuromuscular preparation. The ventral view of the opener muscle is shown with the general layout of the neural innervation (**A**). Note that there are two distinct regions of the muscle based on muscle fiber dimensions. The proximal fibers are thin and long whereas the central fibers are thicker and generally shorter. The opener excitor and opener inhibitor motor neurons parallel each other across the preparation. This is evident at the terminals as well. In (**B**), traces of terminals stained with 4-Di-2-Asp are shown for the two regions of the muscle. The central region commonly has terminals that form long strings of varicosities while in the proximal fibers the terminals form shorter strings with clusters of varicosities. The physiological responses also are varied between the two regions: intracellular recordings of EPSPs induced by a train of stimuli at 50 Hz show pronounced differences (**C**). Note that the initial EPSP is smaller in the central fibers than for proximal fibers. Scale bar = 10 μm for both top and bottom panels of B.

MacLab Scope software (version 3.5.4). A Grass S-88 stimulator and stimulus isolation unit (Grass) with leads to a standard suction electrode set-up (Cooper et al., '95a) were used to stimulate the excitatory nerve.

Opener muscle

Excitatory postsynaptic potentials (EPSPs) were recorded simultaneously in the proximal and central fibers of the opener muscle to illustrate the difference between the two distinct regions (Atwood et al., '94; Cooper and Ruffner, '98). Selective stimulation of the excitatory axon was carried out as described by Dudel and Kuffler ('61). The axon was stimulated with trains of ten stimuli at 30 Hz with a train interval of 10 sec. Averages of 10 to 20 trains were used for measurement.

Leg extensor and closer muscle

EPSPs were measured when stimulating selectively the phasic or tonic excitatory motor neurons at various frequencies. Selective stimulation of the two excitatory axons was carried out by using a 'macro-patch' electrode with an inner diameter of 15 to 20 μm placed directly on the phasic or tonic axon (Bradacs et al., '97). The axon type is easily identified after staining because 4-Di-2-ASP stains the tonic axon more brightly, due to the larger number of mitochondria within it (Atwood and Nguyen, '95).

Abdominal extensor muscles

The entire nerve root to the superficial and deep extensor muscles was stimulated with a suction

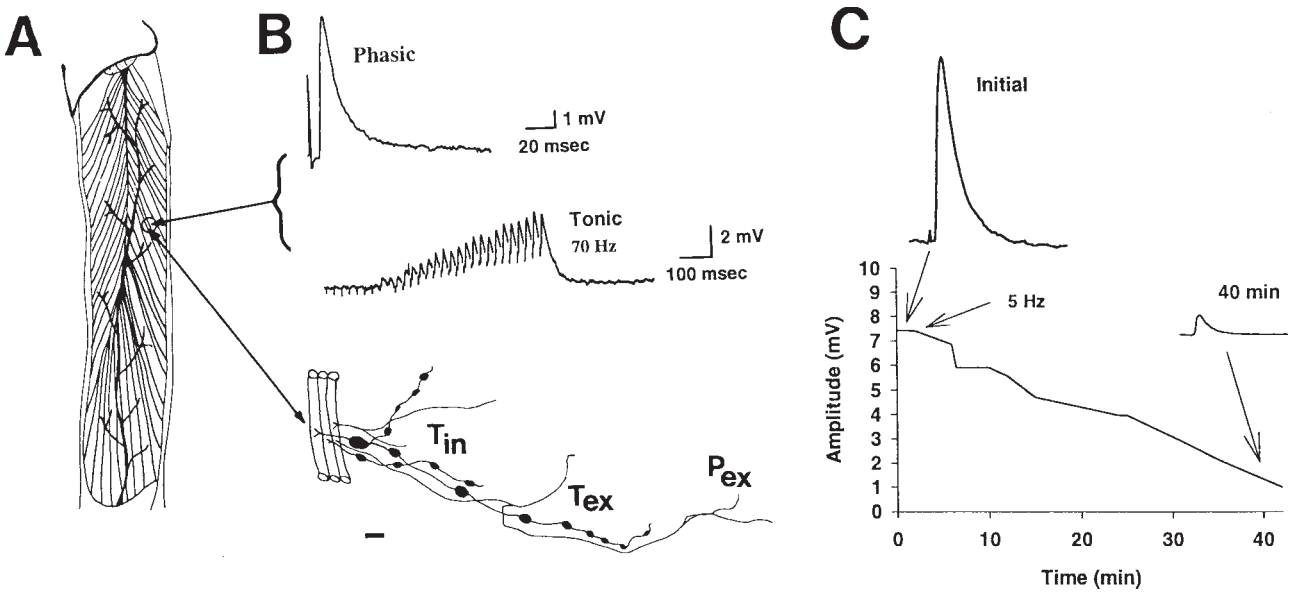

Fig. 2. The leg extensor neuromuscular preparation in which each muscle fiber is innervated by both a phasic and a tonic excitatory motor neuron. In (**A**), the medial view of the extensor muscle is shown with the general layout of the innervation. Illustrations of 4-Di-2-ASP stained terminals show the three types of terminal structure. In (**B**), the physiological responses measured by intracellular recording of EPSPs within a single muscle fiber show the difference in the size of the tonic and phasic responses. Note that the initial EPSP is smaller for the tonic response and is really only measurable after facilitation. The preparation is innervated by a tonic excitor, a phasic excitor, and an inhibitor motor neuron. The terminals of the tonic excitor (Tex) and the inhibitor (Tin) are both varicose in nature whereas the phasic terminals (Pex) are thin and filiform (B). In (**C**), the phasic response will show fatigue rapidly upon a 5 Hz continuous stimulation, which results in the EPSP amplitude decreasing in size. There is substantial variation among preparations and differences among crayfish of different size (Bradacs et al., '97). Scale bar = 10 μm for panel B.

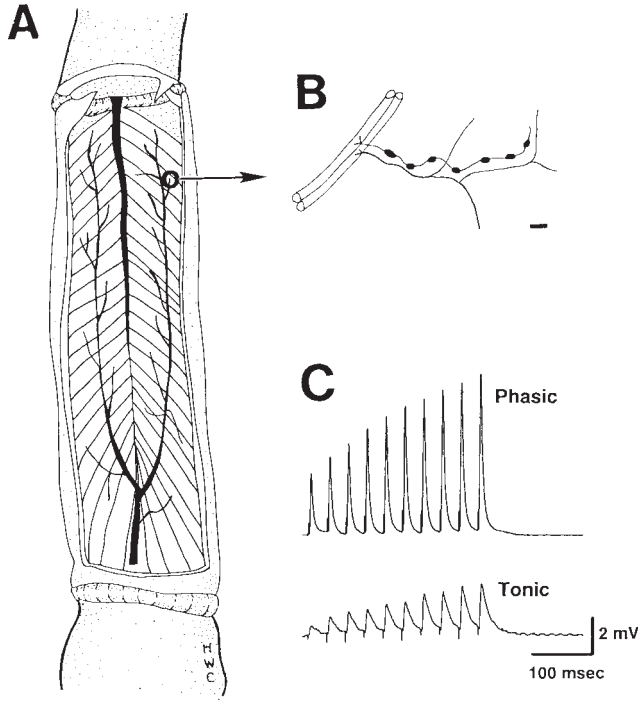

Fig. 3. The muscle fibers of the closer are innervated by a phasic and tonic excitatory motor neuron. The dorsal view of the closer muscle is shown with the general layout of the innervation (**A**). Illustrated is the innervation by the tonic and phasic excitor neurons. In (**B**), traces of terminals stained with 4-Di-2-Asp are shown (scale bar = 10 μm). As with the leg extensor motor nerve terminals, the tonic terminals are varicose in nature whereas the phasic terminals are filiform (B). The physiological responses measured by intracellular recordings within a single muscle fiber of EPSPs show the differences in the size of the tonic and phasic responses (**C**). Note that the tonic initial EPSP is smaller than the phasic but is much larger in closer muscle than in the leg extensor (compare Fig. 3C and 2C).

electrode. Because this approach can stimulate the inhibitory motor neuron along with the excitatory motor neurons, picrotoxin (1 mM) was used to block activity of the inhibitory neuron (Atwood et al., '67; Mercier and Atwood, '89). While stimulating the nerve root in segment 2, responses were measured in the L1 and SLE muscles of the next posterior segment (segment 3). By recording in the adjacent posterior segment, responses from one of the five excitatory neurons can be selectively measured (Atwood and Parnas, '68; Mercier and Atwood, '89).

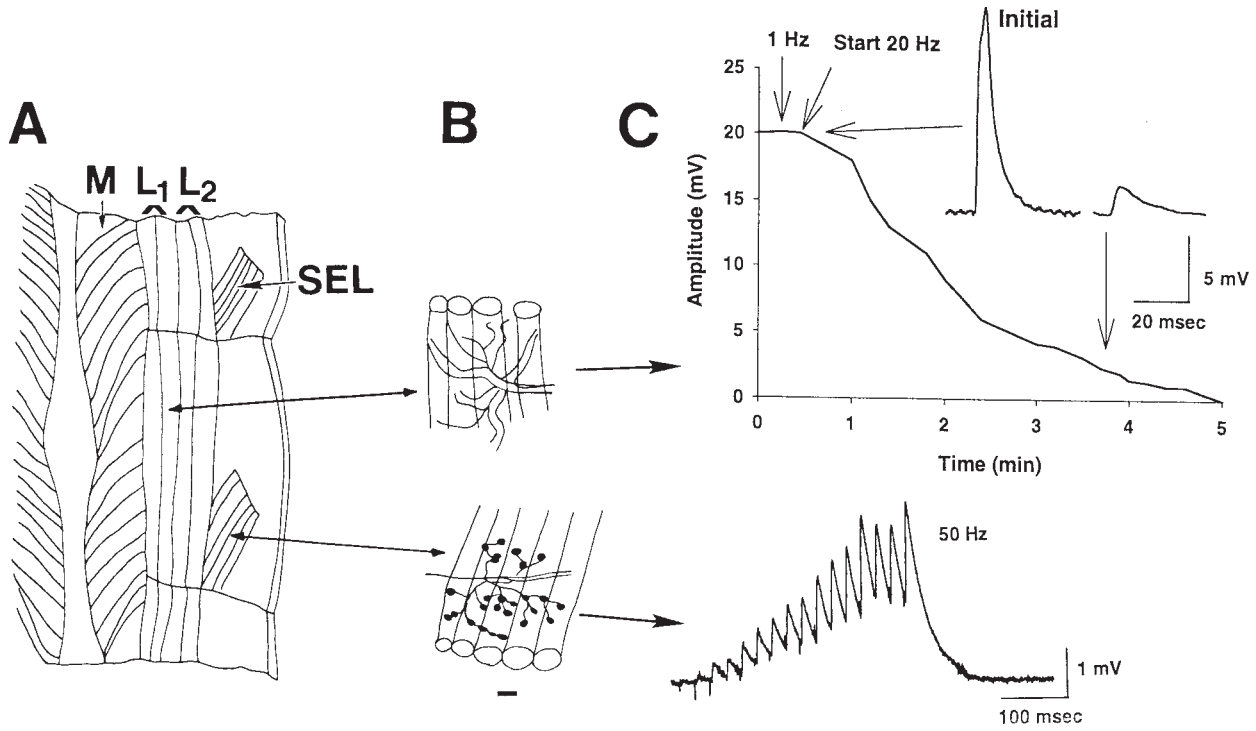

Fig. 4. Differences in terminal structure and function in the phasic and tonic abdominal extensor neuromuscular preparations. (**A**) Ventral view of the deep abdominal extensor muscles as traced from photographs of a dissected preparation; one half of the preparation is shown. The muscle closest to the dorsal midline is the M muscle with its characteristic spiral fiber pattern. L1 and L2 are the first two laterals of the deep extensors. The most lateral bundle of fibers is the superficial lateral extensor muscle (SEL). M, L1, and L2 are phasic and the SEL is a tonic neuromuscular preparation. In (**B**), traces made from photographs of stained terminals indicate the differences between phasic and tonic terminals: varicose terminals on the tonic SEL, filiform on the phasic L1 muscles. In (**C**), the physiological responses of the tonic and phasic terminals are shown to be similar to those recorded in the leg extensor preparation, in that the phasic gives a large response but fatigues quickly and the tonic terminals give rise to small responses which facilitate. Each segment (S1, S2, and S3) in the abdomen repeats this pattern of muscle arrangement. Scale bar = 10 μm for panel B.

Myosin extraction and electrophoresis

Dissected cuticle and attached muscles were rinsed twice in cold 0.5 M NaCl and 5 mM sodium phosphate (pH = 7.4), quickly frozen in liquid nitrogen then stored on dry ice in microfuge tubes until MyHC analysis. Muscle samples were obtained for analysis from two crayfish for the claw muscles and four animals in the case of the deep extensors. Their small size led us to combine the SEL/SEM complex as well as the L1 and L2 muscles from individual animals, but in subsequent samples the L1 was extracted separately. Myosin was extracted and purified as previously described except for minor adjustments for small sample size (Butler-Browne and Whalen, '84). Iridectomy scissors were used to mince samples on ice in 4 volumes of high-salt buffer (300 mM NaCl, 100 mM NaH_2PO_4, 50 mM Na_2HPO_4, 12 mM $MgCl_2$, 10 mM EDTA, and 0.1% β-mercaptoethanol, pH = 6.5) for 40 min. A minimum of 20 μl buffer was used for small samples i.e., <0.1 mg wet weight. Extracts were centrifuged at 12,000*g* for 30 min, and the supernatants were recovered for suspension in 9 volumes of low-salt buffer (1 mM EDTA, 0.1% β-mercaptoethanol). Myosin filament precipitation took place overnight at 4°C. Filaments were visible the next morning in all but the smallest samples which were subjected to a second round of filament precipitation to increase myosin yield. After centrifugation (12,000*g* for 30 min) the pellets were dissolved in myosin sample buffer (0.5 mM NaCl, 10 mM NaH_2PO_4 pH = 7.0) and diluted 1:1 in sodium dodecyl sulfate sample buffer. Samples were boiled for 2 min and then stored at –80°C.

Electrophoresis parameters were varied to obtain optimal separation of crayfish MyHC bands while retaining separation of the four mouse muscle MyHC isoforms. The reproducible rodent

isoform separation pattern served as a quality control for the procedure. The mouse vastus intermedius, a red type muscle, was used since it expresses four well known rodent MyHC isoforms: I, 2B, 2X, and 2A. SDS-PAGE was performed in separating gels using a range of acrylamide from T = 4.8–7% and C = 2.8–5%. Highest resolution was achieved with 4.8 and 5% separating gels (C = 5%) containing 30% glycerol (v/v) but with the addition of 0.1% b ME to the upper running buffer (LaFramboise et al., '90). No glycerol was added to the stacking gel. Electrophoresis was performed for 22 hr at 15°C and constant 120 volts for 5% gels but the time was extended to 24 hr for 4.8% gels. The separating gels were silver-stained by ammonium hydroxide stabilization of silver diamine complexes according to established procedures (Oakley et al., '80).

Immunoblot analysis was performed after overnight electrophoretic transfer (1 amp, 15°C) of unstained gels to PVDF filters (Millipore, Bedford MA). Membranes were blocked for 60 min to 24 hr with 5% dried milk in TBS (500 mM Tris, 20mM NaCl, pH: 7.4) via a "wet" blocking technique. The filters were then incubated overnight (4°C) with primary antibodies in TBS followed by a 1-hr incubation at 37°C. An alternate hydrophobic "dry" technique was also utilized whereby the immunotransfer was air-dried for a minimum of 2 hr and then subjected to primary antibody incubation. Monoclonal antibodies specific for epitopes common to the MyHC family including MF-20 (Bader et al., '82) and MY-32 for fast isoforms (Sigma, St. Louis, MO) were utilized to define the presence of various myosin isoforms in rodent and crayfish samples. Secondary antibodies were anti-mouse alkaline phosphatase conjugate (whole molecule IgG) which were subsequently visualized with a Western Blue stabilized substrate (Promega, WI).

Quantitative analysis of gels and immunoblots involved acquisition of high resolution images on a color three-chip CCD camera (DXC-950P, Sony Corp.), image capture with a Matrox frame grabber card and analysis with Optimas 6 software (Bothell, WA) utilizing a Micron Millenia Pro2 PC (Micron, Nampa, ID). Custom macros were written to calculate band intensity and molecular size based on comparison with a myosin standard dilution curve and molecular weight markers.

RESULTS

Anatomy

Opener muscle

The opener muscle is composed of fast fibers proximally and slow fibers centrally (Günzel et al., '93) and is innervated by a single tonic excitatory motor neuron and two inhibitory motor neurons (Wiens, '89; Wiens and Wolf, '93; Cooper et al., '96b). The specific inhibitory motor neuron parallels the excitatory motor neuron whereas the common inhibitory motor neuron innervates only the very proximal fast fibers; its terminals do not follow the specific inhibitor or excitor terminals in any obvious manner. The main axons of the excitor and specific inhibitor branch about midway along the muscle to form a "Y," each branch of the Y innervating the most distal fiber bundles of the muscle. The ventral surface of the opener muscle is shown in Figure 1A: the branching of the main axons and the regional divisions of the muscle are depicted. Differences in secondary and tertiary branching patterns of the opener excitatory motor nerve terminals are observed across the muscle. These differences depend on the region of the muscle: the central, slow muscle fibers have terminals in the shape of long strings with intermittent varicosities, and the proximal, fast muscle fibers have more highly branched terminals (Cooper et al., '95a, '96b). The branching differences in the proximal and central terminals are shown in Figure 1B. The terminals were traced from photographs taken at various focal planes in order to make a composite of an entire terminal in each muscle region.

Leg extensor muscle

A unique feature of this muscle, as compared to vertebrate muscle, is that single fibers are innervated by both phasic and tonic motor neurons. The general layout of the inner surface of the muscle and the innervation pattern is illustrated in Figure 2A. The centrally located motor nerve is separated into two lateral branches supplying the two sides of the distal end of the muscle, in a manner similar to the opener muscle. Two types of terminals are seen on the muscle: large-varicosity tonic terminals in which individual varicosities range substantially in size and thin, non-varicose phasic terminals of relatively uniform size. When stained with 4-Di-2-ASP, the terminals of the tonic axon fluoresce more brightly than those of the phasic axon, suggesting a higher mitochondrial content for the tonic terminals.

Figure 2B shows the pattern of terminals on the medial surface of the muscle. No distinct regional differences in terminal type have been observed. Thin sections of phasic and tonic terminals revealed that individual synapses are generally similar in appearance among the two terminals except for the number of active zones per synapse (Bradacs et al., '97). However, this muscle does display differences of fiber type based on ATPase staining along its length and lateral-medial orientation; there is a distinct region in the proximal wedge which is composed predominantly of fast fibers (Bradacs et al., '97).

Closer muscle

Like the leg extensor, individual fibers of the closer muscle each receive both tonic and phasic excitatory innervation. Upon entering the propus segment, the main axon branches in numerous directions to innervate this relatively large muscle. Two axon branches run along the dorsal surface sending out secondary branches as they proceed to innervate the most distal fibers (Fig. 3A). The structures of the phasic and tonic terminals are the same as those of the leg extensor, with the tonic containing more robust varicosities than the phasic (Fig. 3B).

Abdominal extensor muscles

The purely phasic muscles L1, L2, and M of the deep extensors display only thin, filiform, excitatory terminals characteristic of phasic motor neurons. The purely tonic SEM and SEL muscles show varicose terminals. Figure 4B depicts the differences in the terminals between the L1 and the SEL muscles. The tonic terminals of the SEL are highly branched with compact clusters of varicosities.

Physiological recordings

Opener muscle

Iravani ('65) showed differences in the postsynaptic response between central and proximal region fibers, and a number of subsequent reports have elaborated on the differential response (Bittner, '68; Govind et al., '94; Cooper et al., '95b, '96b,c). The amplitudes of the EPSPs of the most proximal fibers are larger than the central fiber EPSPs (Cooper et al., '95a). This is illustrated by responses in the train of responses in Figure 1C.

EPSP facilitation also differs between the two regions. The response shown in the central recording of Figure 1C shows a facilitation value of 2.5 whereas the response of the proximal fiber has a facilitation value of 14, indicating that under this stimulation paradigm the neuromuscular junctions in the proximal region have a greater ability to facilitate. The tonic nerve can be repetitively stimulated at 20 Hz for 30 min without fatigue or depression of the EPSP amplitudes.

Leg extensor muscle

Low frequency stimulation of the tonic nerve induces barely detectable EPSPs (Fig. 2B). Terminals must be stimulated at high frequencies to facilitate an observable response. In contrast, repetitive 5 Hz stimulation of the phasic nerve innervating the same muscle fiber gives rise to large EPSPs that become greatly depressed within a few minutes. This type of depression is common in arthropod phasic neuromuscular junctions (Atwood and Cooper, '96b).

Closer muscle

Much like in the leg extensor, the phasic (fast closer excitor; FCE) and tonic (slow closer excitor; SCE) motor nerves of the closer muscle give rise to different postsynaptic responses (Wiens and Atwood, '78; Wiens, '93). The EPSP differences between the tonic and the phasic axons are not as striking as in the leg extensor, but the general pattern is similar—that the responses from the tonic axon show more pronounced facilitation, but the facilitated EPSPs elicited by the tonic axon are still smaller than facilitated EPSPs elicited by the phasic axon (Fig. 3C). A difference between closer and extensor muscles is that the tonic EPSP amplitudes in the closer are much larger and can usually be recruited with a single presynaptic evoked event.

Abdominal extensor muscles

The phasic response of the L1 and the tonic response of the SEL are typical of phasic and tonic arthropod neuromuscular junctions. The L1 gives rise to a large EPSP which fatigues within a few minutes of repetitive stimulation (Fig. 4C), and the SEL shows a small initial response which can facilitate with short trains of stimulation (Fig. 4D).

Myosin heavy chain analysis

Initial analytic studies utilized high resolution 5% SDS-PAGE to compare crayfish myosin heavy chains to a mixed phenotype rodent appendicular muscle (red vastus intermedius) containing four well characterized myosin isoforms based on electrophoretic mobility (MyHC: β/slow > 2B > 2X > 2A). This technique afforded maximum separation

of mammalian myosins based on molecular weight and provided sufficient resolution of the crayfish myosins to reveal a diversity of phenotypes. At this level of resolution, the phasic M, L1, and L2 (not shown) muscles consistently displayed a common phenotype of two bands while the tonic SEL/SEM complex contained three distinct bands. The exact number of MyHC bands comprising the phenotypes of the claw muscles was difficult to confirm since (1), the individual myosins were restricted to a limited molecular weight range extending from rodent $MyHC_{2B}$ to the level of $MyHC_{\beta/slow}$ (Fig. 5A), and (2), some muscles exhibited bands in only trace amounts.

In order to resolve MyHC composition of claw muscles, it was necessary to reduce the acrylamide concentration to T = 4.8% and extend the electrophoresis running time to 24 hr. This procedure results in the bands migrating an additional 2 centimeters into the gel compared with the 5% SDS-PAGE in which the MyHC bands migrated 4.5 to 5.5 centimeters into the separating gel. The 4.8% SDS-PAGE technique increases separation among the bands but at the cost of obtaining less focused bands. However, it is possible to compensate for this effect by increasing the loading volume which allows detection of bands present at less than 5% of the total myosin (LaFramboise et al., '90). Distinct MyHC phenotypes were identified by juxtaposing individual sample profiles with coelectrophoresis studies in which samples were pooled from different muscles and run in the same lane (data not shown). Based on these comparisons, it was possible to delineate four distinct crayfish MyHC bands with distinct electrophoretic properties present in various combinations among the array of crayfish muscles (Fig. 5B). These four bands are given preliminary assignment based on gel mobility as C1 > C2 > C3 > C4. Only the fastest migrating band, C1, shared electrophoretic properties with a previously identified rodent isoform, $MyHC_{\beta/slow}$ in that these two bands demonstrated comparable electrophoretic mobility in adjacent lanes and were indistinguishable in coelectrophoresis studies of rodent vastus and SEL/SEM samples.

There were three general myosin phenotypes distributed among the crayfish muscles based on 4.8% SDS-PAGE. The three bands present in the superficial abdominal extensor complex (SEL/SEM) were identified by coelectrophoresis studies as C1, C3, and C4 and were also typical of the central region of both of the opener muscle specimens (Fig. 5B). This distribution of myosin heavy chains appeared to correlate with the presence of tonic muscles. Densitometric analysis of the SEL/

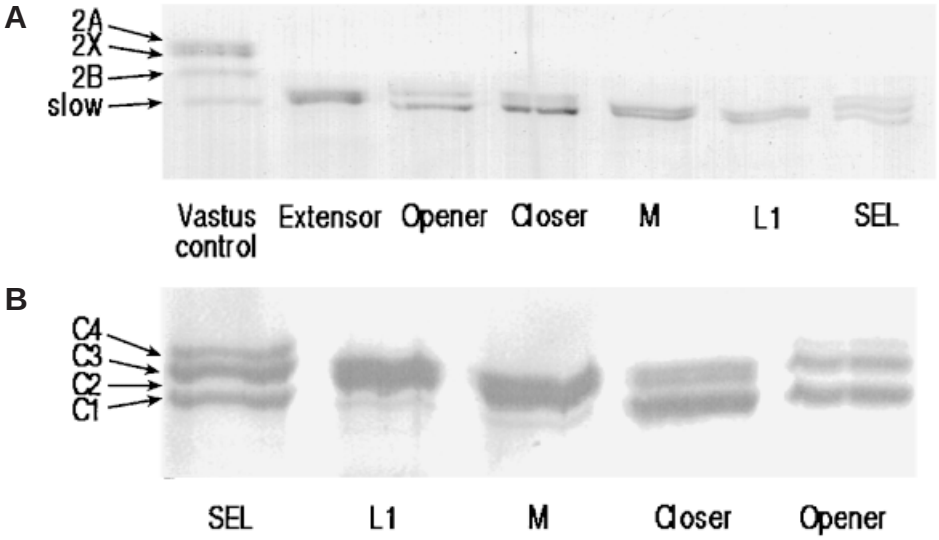

Fig. 5. SDS-PAGE of crayfish MyHCs. A representative 5% gel is displayed in (**A**). Vastus intermedius muscle of the mouse contains the standard rodent MyHCs run as a reference for vertebrate isoforms including three fast isoforms (2A, 2X, and 2B) and the slow isoform which is also referred to as the β/ventricular slow MyHC. The superfical extensor muscles are presented together in A, as the tonic superficial lateral extensor (SEL), the phasic lateral deep extensor (L1) muscle, and the phasic midline (M) abdominal extensor. The results in (**B**) are from a 4.8% gel with increased protein loads in order to detect bands present in small amounts. This allows resolution of bands at trace levels such as the C4 band in the opener muscle, but companion bands in large amounts tend to collapse together as occurred for the C2 and C3 bands of the L1 and M samples.

SEM indicated that these three bands were distributed in different proportions with a predominance of C3 (43% of total MyHC in gel depicted in Fig. 5B) but large amounts of C1 (33%) and C4 (24%). The central opener muscle contained the same three bands but was comprised mostly of C1 (56%) and C3 (37%) with a small representation of the C4 (7%) isoform.

The second phenotype was typical of the phasic deep abdominal extensors, including the single medial and two lateral muscles which were independently characterized as M, L1, and L2. Each of these muscles contained a band C2, not present in the opener or SEL, which comprised approximately 50% of the total MyHC of each muscle. The remaining MyHC was distributed primarily in band C3, but a trace amount of the C1 isoform (<5%) was detected in some, but not all, of the samples by increasing the volume load. The C2 isoform was also present in the leg extensor muscle, which contains nearly equal amounts of the C2 and C3 bands. Thus, although C1 and C3 were common among all muscles tested, two general phenotypes were defined by the exclusive expression of band C2 in phasic muscles or band C4 in tonic muscles.

The claw closer muscle displayed a third phenotype inasmuch as it exhibited properties of both of the previously described types. This muscle contained the C2 isoform typical of the phasic deep abdominal extensors, albeit in an uncharacteristically low amount (16%) compared with L and M muscles, which contained >50% of the C2 isoform. However, unlike these extensors which expressed barely detectable, if any, C1, the closer muscle contained a preponderance of band C1 (66%), typical of muscles of the tonic phenotype such as the superficial abdominal extensors and the claw opener. Thus, the claw closer muscle represents a heterogenous mix of both phasic and tonic MyHC phenotypes typical of crayfish muscles.

Western blots with MF-20 did not indicate antibody binding to the crayfish isoforms utilizing the "wet" blocking technique or the "dry" hydrophobic technique at primary antibody concentrations that stained each vastus band. The results with MY-32 were less definitive. At a dilution of 1:400 this antibody stained the crayfish bands with low intensity compared to the rodent control sample while meeting the criterion of a fast-specific myosin antibody (i.e., staining the rodent fast 2B, 2A, and 2X isoforms). Increased concentrations of MY-32 enhanced staining of the crayfish myosin but engendered non-specific activity amongst the rodent isoforms. Extensive blocking improved the specificity of MY-32 to the rodent fast forms but reduced the relative staining of all of the myosin isoforms. These findings suggest that the mammalian derived MyHC monoclonal antibody, MY-32, may bind to an epitope common to crayfish myosin heavy chains but with a much lower avidity compared to the control rodent vastus muscle. Likewise, immunocytochemistry on frozen sections of abdominal M, L1, and L2 muscles showed MY-32 positive immunoreactivity, although with high background.

DISCUSSION

This study is a survey of crayfish muscle types having diverse physiological and anatomical functions. The opener muscle of the crayfish leg and the abdominal SEL/SEM muscles are innervated by purely tonic motor neurons while the abdominal M, L1, and L2 muscles are innervated by purely phasic motor neurons. In contrast to these singly innervated muscles, tonic and phasic motoneurons co-innervate the leg closer and extensor muscles. These three types of preparations (pure tonic, pure phasic, and mixed) provide experimental models to explore questions about the development, maintenance, and plasticity of muscle and nerve phenotypes. The correlative analysis of the myosin heavy chain composition among these muscles suggests that a relationship exists between the types of neurons innervating crayfish muscles and the muscle myosin heavy chain composition.

There are prominent differences in nerve terminal morphology between tonic and phasic motor neurons. Tonic terminals have varicosities connected by narrow "bottleneck" regions along their lengths; the majority of synaptic sites are found within the varicosities (Florey and Cahill, '82; Cooper et al., '95a, '96b), and synaptic output is low. Additionally, there is intraterminal variation of synaptic efficacy in tonic terminals (Cooper et al., '95a, '96b,c; Cooper and Ruffner, '98) as measured by focal, macro-patch recordings over terminals, and interterminal variation among tonic and phasic terminals (King et al., '96; Bradacs et al., '97; Msghina et al., '98;). Phasic terminals contain only slight swellings along their lengths; synaptic sites are located all along the terminal, and synaptic output is high. There are ultrastructural differences that can account, at least in part, for differing degrees of synaptic efficacy between tonic and phasic terminals. Direct correlations of synaptic ultrastructure and electrophysiological parameters measured at defined

regions of terminals revealed that the higher the synaptic output, the more prevalent were synapses containing multiple active zones and less prevalent were blank, inactive synapses (Cooper et al., '95a, '96a,b,c; King et al., '96; Msghina et al., '98). These structure-function relationships arose from work with the leg opener, closer, and extensor muscles of crayfish (see review Atwood and Cooper, '96a,b) and other crustacean species (Atwood, '67, '73b; Atwood and Marin, '83) as well as from various *Drosophila* NMJs (Atwood and Cooper '95; Stewart '96; Stewart et al., '96; Ruffner et al., '99). Detailed structural analysis of L1 and SEL muscles has not been completed.

Exceptions to the general rule of phasic and tonic terminal morphology occur when the terminals are undergoing a phenotype transformation induced by altered electrical activity of the neurons (Lnenicka and Atwood, '85a; Nguyen and Atwood, '90a). Tonic activity can induce phasic motor neurons to convert to a tonic-like state in both terminal morphology and physiology. These presynaptic adaptations depend upon protein synthesis and axonal transport (Nguyen and Atwood, '90a,b) and can persist for hours or days (Lnenicka and Atwood, '85a). This type of phasic terminal transformation was shown to occur in the claw closer and the abdominal extensor muscles (Lnenicka and Atwood, '85a; Mercier and Atwood, '89). Muscles innervated by transformed phasic neurons are characterized by reduced initial EPSP amplitude and lower fatigue resistance (Lnenicka and Atwood, '85a,b, '89; Mercier and Atwood, '89; Atwood and Nguyen, '95). The terminal transformation from thin-phasic to varicose-tonic is accompanied by increased mitochondrial cross-sectional area and branching (Lnenicka et al., '86; Lnenicka and Zhao, '91). A previous investigation has shown that the postsynaptic target muscle does not show evidence of a phenotype switch until three weeks of electrical conditioning. At that time a number of phenotype-specific muscle proteins began to be replaced by their tonic-muscle counterpart isoforms (Cooper et al., '98). These findings are consistent with studies in mammalian muscles which commonly show biochemical and structural changes consequent to alteration of synaptic activity (Burke, '81; Kirschbaum et al., '90; Pette and Vrbova, '92; Jarvis et al., '96).

An evolutionary strategy among avian and mammalian organisms is the generation of multiple sarcomeric myosin heavy chain (MyHC) isoforms which exist independently or collectively within individual muscles and myofibers to meet their unique biochemical and physiological demands (Schiaffino et al., '89; Weiss and Leinwand, '96). Crustacea are no different in this regard. Their striated muscles exhibit three main types of fibers based on sarcomere length (SL): long-SL, intermediate-SL, and short-SL fibers (>8 μm, 6–8 μm, and 4–6 μm, respectively) (Atwood, '73a,b; Crabtree and Sherman, '81). In adult lobsters, the slow, crusher-claw muscle is composed almost entirely of long-sarcomere fibers whereas the fast, cutter-claw muscle contains a small majority of short sarcomere fibers, the rest being intermediate- and long-SL fibers (Goudey and Lang, '74). The long-SL and intermediate-SL fibers may represent, respectively, fibers with slow-twitch and tonic contractile properties (Mykles, '85, '88, '90). In addition, it is clear that fibers within the superficial and deep abdominal extensor muscles, which are homogeneous in phenotype (Cooper et al., '98), show a good correlation with sarcomere length and contraction velocities (Jahromi and Atwood, '69). Likewise the correlation of sarcomere lengths and ATPase activity with speed of contraction has been shown for the claw closer muscle (Ogonowski et al., '79). The differences in MyHC composition among whole crayfish muscles reported in the present study may reflect fiber type-specific diversity at the level of the individual myofiber as has been established in mammalian and avian muscles. However, physiological and biochemical properties obtained from single myofibers classified according to sarcomere length will be necessary to determine if these structural differences correlate with distinct contractile properties and expression of a specific MyHC composition.

There is scant information on the number of myosin heavy chain protein isoforms or genes among the crustaceans. Mykles has identified two distinct gene sequences in lobster (*Homarus americanus*) by their reactivity with myosin heavy chain nucleic acid probes derived from lobster fast and slow muscle libraries (Cotton and Mykles, '93; Mykles, '97). The present study utilizing high-resolution SDS-PAGE suggests the presence of distinct profiles of phasic, mixed, and tonic crayfish muscle myosin phenotypes derived from variations in expression of at least four separate isoforms. Further studies at the molecular level will be necessary to determine if these isoforms correspond to either of the MyHCs identified by Mykles' probes or to isoforms peculiar to particular sarcomere-length fibers. In many species studied to date, including arthropods and nematodes, fish, amphibians, birds, and mammals, multiple MyHC

genes and proteins have been identified. Four sarcomeric MyHC isoform genes have been identified in the nematode *C. elegans* (Emerson and Bernstein, '87). In some species, single genes may give rise to multiple MyHC isoforms as in the scallop, *A. irradians*, and the fruit fly; in *Drosophila*, all the sarcomeric myosin isoforms are produced by alternative splicing (Nyitray et al., '94; Morgan, '95). It is not known whether the multiple isoforms of crayfish MyHC arise from discrete genes or from some degree of alternative splicing.

Varied fiber types of muscle and concomitant muscle dynamics serve the crayfish well in its natural environment. The tonic, walking leg opener muscle is used for feeding, grooming, and static behavioral posturing, but only sparingly during locomotion. Likewise, the tonic SEL muscle appears to be used continually during abdominal posturing with possibly a higher intrinsic activity. The intrinsic activity may also be the explanation for the very small, highly facilitating postsynaptic potentials observed in the opener in comparison to the SEL (see Figs. 1 and 4). The mixed leg extensor muscle is used for walking and claw closing during display behavior and feeding, whereas the purely phasic, L1 muscle is used in rapid abdominal extensions. Investigation of the physiological status of these muscles in saline-superfused, semi-intact preparations provides only a partial understanding of the dynamics that take place during short- and long-term influences occurring within the animal. This is at least partly because such influences are based on behavior and developmental stage, factors which are peculiar to the individual history of each animal (Atwood '76, '92; Cooper, '98; Neckameyer and Cooper, '98; Cooper and Neckameyer, '99; Ruffner et al., '99; see review by Pette and Staron, '97). For example, a neuromodulator that is elevated for a long time in an individual could grossly offset the normal activity pattern for particular muscles and potentially alter muscle phenotype (Cooper et al., '98; Cooper and Ruffner, '98; Ruffner et al., '99). These peculiarities in levels of activity, developmental stage, and influence of neuromodulators set the stage for future investigations in the model neuromuscular systems presented in this study.

ACKNOWLEDGMENTS

We are grateful to Dr. T.J. Wiens (Univ. of Manitoba, Canada) for helpful communications and discussion on the responses of the closer muscle. Illustrations were provided courtesy of Hye Won Cooper. Funding was provided by University of Kentucky Research and Graduate Studies (R.L.C.), NSF grants IBN-9808631 and ILI DUE-9850907 (R.L.C.).

LITERATURE CITED

Atwood HL. 1967. Variation in physiological properties of crustacean motor synapses. Nature Lond 215:57–58.

Atwood HL. 1973a. Crustacean motor units. In: Stein RB, Pearson KG, Smith RS, Redford JB, editors. Control of posture and locomotion. New York: Plenum Press. p 87–104

Atwood HL. 1973b. An attempt to account for the diversity of crustacean muscles. Am Zool 13:357–378.

Atwood HL. 1976. Organization and synaptic physiology of crustacean neuromuscular systems. Prog Neurobiol 7:291–391.

Atwood HL. 1992. Age-dependent alterations of synaptic performance and plasticity in crustacean motor systems. Exp Gerontol 27:51–61.

Atwood HL, Parnas I. 1968. Recording from the crayfish abdominal extensor muscle preparation with microelectrodes. In: Kerkut GA, editor. Experiments in physiology and biochemistry, Vol. 1. New York: Academic Press. p 307–330.

Atwood HL, Marin L. 1983. Ultrastructure of synapses with different transmitter-releasing characteristics on motor axon terminals of a crab, *Hyas areneas*. Cell Tissue Res 231:103–115.

Atwood HL, Cooper RL. 1995. Functional and structural parallels in crustaceans and *Drosophila* neuromuscular systems. Am Zool 35:556–565.

Atwood HL, Nguyen PV. 1995. Neural adaptation in crayfish. Am Zool 35:28–36.

Atwood HL, Cooper RL. 1996a. Assessing ultrastructure of crustacean and insect neuromuscular junctions. J Neurosci Meth 69:51–58.

Atwood HL, Cooper RL. 1996b. Synaptic diversity and differentiation: crustacean neuromuscular junctions. Invert Neurosci 1:291–307.

Atwood HL, Parnas I, Wiersma CGA. 1967. Inhibition in crustacean phasic neuromuscular systems. Comp Biochem Physiol 20:163–177.

Atwood HL, Cooper RL, Wojtowicz JM. 1994. Non-uniformity and plasticity of quantal release at crustacean motor nerve terminals. In: Stjärne L, Greengard P, Grillner SE, Hökfelt TGM, Ottoson DR, editors. Advances in second messenger and phosphoprotein research: molecular and cellular mechanisms of neurotransmitter release. New York: Raven Press. p 363–382.

Bader D, Masaki T, Fischman DA. 1982. Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. J Cell Biol 95:763–770.

Bittner GD. 1968. The differentiation of crayfish muscle fibers during development. J Exp Zool 167:439–456.

Bradacs H, Cooper RL, Msghina M, Atwood HL. 1997. Differential physiology and morphology of phasic and tonic motor axons in a crayfish limb extensor muscle. J Exp Biol 200:677–691.

Burke R. 1981. Motor units: anatomy, physiology, and functional organization. In: Anonymous, editor. Handbook of physiology: the nervous system: motor control, Sect. 1, Vol. 2. Bethesda, MD: American Physiological Society. p 345–422.

Butler-Browne GS, Whalen RG. 1984. Myosin isozyme transitions occurring during the postnatal development of the rat soleus muscle. Dev Biol 102:324–334.

Cooper RL. 1998. Development of sensory process during limb regeneration in adult crayfish. J Exp Biol 201:1745–1752.

Cooper RL, Ruffner ME. 1998. Depression of synaptic efficacy at intermolt in crayfish neuromuscular junctions by 20-Hydroxyecdysone, a molting hormone. J Neurophys 79:1931–1941.

Cooper RL, Neckameyer WS. 1999. Dopaminergic neuromodulation of motor neuron activity and neuromuscular function in *Drosophila melanogaster*. Comp Biochem Physiol B 122:199–210.

Cooper RL, Marin L, Atwood HL. 1995a. Synaptic differentiation of a single motor neuron: conjoint definition of transmitter release, presynaptic calcium signals, and ultrastructure. J Neurosci 15:4209–4222.

Cooper RL, Stewart BA, Wojtowicz JM, Wang S, Atwood HL. 1995b. Quantal measurement and analysis methods compared for crayfish and *Drosophila* neuromuscular junctions and rat hippocampus. J Neurosci Meth 61:67–79.

Cooper RL, Hampson D, Atwood HL. 1996a. Synaptotagmin-like expression in the motor nerve terminals of crayfish. Brain Res 703:214–216.

Cooper RL, Harrington C, Marin L, Atwood HL. 1996b. Quantal release at visualized terminals of crayfish motor axon: Intraterminal and regional differences. J Comp Neurol 374:1–18.

Cooper RL, Winslow J, Govind CK, Atwood HL. 1996c. Synaptic structural complexity as a factor enhancing probability of calcium-mediated transmitter release. J Neurophys 75:2451–2466.

Cooper RL, Warren WM, Ashby HE. 1998. Activity of phasic motor neurons partially transforms the neuronal and muscle phenotype to a tonic-like state. Muscle Nerve 21:921–931.

Cotton JLS, Mykles DL. 1993. Cloning of a crustacean myosin heavy chain isoform: exclusive expression in fast muscles. J Exp Zool 267:578–586.

Crabtree RL, Sherman RL. 1981. Ultrastructural characterization of superficial and deep flexor muscles in the crayfish thorax. Can J Zool 59:357–363.

Dudel J, Kuffler SW. 1961. The quantal nature of transmission and spontaneous miniature potentials at the crayfish neuromuscular junction. J Physiol Lond 155:514–529.

Emerson CP, Bernstein SI. 1987. Molecular genetics of myosin. Annu Rev Biochem 56:695–726.

Florey E, Cahill MA. 1982. The innervation pattern of crustacean skeletal muscle. Cell Tissue Res 224:527–541.

Goudey LR, Lang F. 1974. Growth of crustacean muscles: asymmetric development of the claw closer muscle in the lobster *Homarus americanus*. J Exp Zool 189:421–427.

Govind CK, Pearce JM, Wojtowicz JM, Atwood HL. 1994. "Strong" and "weak" synaptic differentiation in the crayfish opener muscle: structural correlates. Synapse 16:45–58.

Günzel D, Galler S, Rathmayer W. 1993. Fibre heterogeneity in the closer and opener muscles of crayfish walking legs. J Exp Biol 175:267–281.

Hermanson JW, LaFramboise WA, Daood MJ. 1991. Uniform myosin isoforms in the flight muscles of little brown bats, *Myotis lucifugus*. J Exp Zool 259:174–180

Iravani J. 1965. Membrandepolarisation der Muskelfasern des Offnermuskels des Flusskrebses auf Nervenreiz und Kaliumapplikation. Experientia 21:609–610.

Jahromi SS, Atwood HL. 1969. Correlation of structure, speed of contraction and total tension in fast and slow abdominal muscles of the lobster (*Homarus americanus*). J Exp Zool 171:25–38.

Jarvis JC, Sutherland H, Mayne CN, Gilroy SJ, Salmons S. 1996. Induction of a fast-oxidative phenotype by chronic muscle stimulation: mechanical and biochemical studies. Am J Physiol 270:C306–C312.

King MJ, Atwood HL, Govind CK. 1996. Structural features of crayfish phasic and tonic neuromuscular terminals. J Comp Neurol 372: 618–26.

Kirschbaum BJ, Schneider S, Izumo S, Mahdavi V, Nadal-Ginard B, Pette D. 1990. Rapid and reversible changes in myosin heavy chain expression in response to increased neuromuscular activity of rat fast-twitch muscle. FEBS Lett 268:75–78.

LaFramboise WA, Daood MJ, Guthrie RD, Moretti P, Schiaffino S, Ontell M. 1990. Electrophoretic separation and immunological identification of type 2X myosin heavy chain in rat skeletal muscle. Biochim Biophys Acta 1035:109–112.

Lnenicka GA, Atwood HL. 1985a. Long-term facilitation and long-term adaptation at synapses of a crayfish phasic motoneuron. J Neurobiol 16:97–110.

Lnenicka GA, Atwood HL. 1985b. Age-dependent long-term adaptation of crayfish phasic motor axon synapses to altered activity. J Neurosci 5:459–467.

Lnenicka GA, Atwood HL. 1989. Impulse activity of a crayfish motoneuron regulates its neuromuscular synaptic properties. J Neurophysiol 61:91–96.

Lnenicka GA, Zhao Y. 1991. Seasonal differences in the physiology and morphology of crayfish motor terminals. J Neurobiol 22:561–569.

Lnenicka GA, Atwood HL, Marin L. 1986. Morphological transformation of synaptic terminals of a phasic motoneuron by long-term tonic stimulation. J Neurosci 6:2252–2258.

Magrassi L, Purves D, Lichtman JW. 1987. Fluorescent probes that stain living nerve terminals. J Neurosci 7:1207–1214.

Mercier AJ, Atwood HL. 1989. Long-term adaptation of a phasic extensor motoneurone in crayfish. J Exp Biol 145:9–22.

Morgan NS. 1995. The myosin superfamily in *Drosophila melanogaster*. J Exp Zool 273:104–117.

Moss RL, Difee GM, Greaser ML. 1995. Contractile properties of skeletal muscle fibers in relation to myofibrillar protein isoforms. Rev Physiol Biochem Pharmacol 126:1–62.

Msghina M, Govind CK, Atwood HL. 1998. Synaptic structure and transmitter release in crustacean phasic and tonic motor neurons. J Neurosci 18:1374–1382.

Mykles DL. 1985. Heterogeneity of myofibrillar proteins in lobster fast and slow muscles: variants of troponin, paramyosin, and myosin light chains comprise four distinct protein assemblages. J Exp Zool 234:23–32.

Mykles DL. 1988. Histochemical and biochemical characterization of two slow fiber types in decapod crustacean muscles. J Exp Zool 245:232–243.

Mykles DL. 1990. Calcium-dependent proteolysis in crustacean claw closer muscle maintained in vitro. J Exp Zool 256:16–30.

Mykles DL. 1997. Crustacean muscle plasticity: molecular mechanisms determining mass and contractile properties. Comp Biochem Physiol 117B:367–378.

Neckameyer WS, Cooper RL. 1998. GABA transporters in *Drosophila melanogaster*: developmental expression, behavior, and physiology. Invert Neurosci 3:279–294.

Nguyen PVN, Atwood HL. 1990a. Expression of long-term adaptation of synaptic transmission requires a critical period of protein synthesis. J Neurosci 10:1099–1109.

Nguyen PVN, Atwood HL. 1990b. Axotomy-induced temporal dissociation of long-term adaptive changes at neuromuscular synapses of a crayfish phasic motoneuron. Brain Res 533:107–112.

Nyitray LA, Jansco Y, Ochiai L, Graf AB, Szent-Gyorgyi A. 1994. Scallop striated and smooth muscle myosin heavy-chain isoforms are produced by alternative RNA splicing from a single gene. Proc Natl Acad Sci USA 91:12686–12690.

Oakley BR, Kirsch DR, Morris NR. 1980. A simplified, ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal Biochem105:361–363.

Ogonowski MM, Lang F. 1979. Histochemical evidence for enzyme differences in crustacean fast and slow muscle. J Exp Zool 207:143–151.

Pette D, Staron RS. 1997. Mammalian skeletal muscle fiber type transitions. Int Rev Cytol 170:143–223.

Pette D, Vrbova G. 1992. Adaptation of mammalian skeletal muscle fibers to chronic electrical stimulation. Rev Physiol Biochem Pharmacol 120:115–202.

Porter JD, Baker RS. 1996. Muscles of a different "color": the unusual properties of the extraocular muscles may predispose or protect them in neurogenic and myogenic disease. Neurology 46:30–37.

Rayment I, Holden HM, Whittaker M, Yohn CB, Lorenz M, Holmes KC, Milligan RA. 1993. Structure of the actin-myosin complex and its implications for muscle contraction. Science 261:58–65.

Ruffner ME, Cromarty SI, Cooper RL. 1999. Depression of synaptic efficacy in high- *and* low-output *Drosophila* neuromuscular junctions by the molting hormone (20-HE). J Neurophysiol 81:788–794.

Schiaffino S, Gorza L, Sartore S, Saggin L, Ausoni S, Vianello M, Gundersen K, Lomo T. 1989. Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. J Muscle Res Cell Motil 10:197–205.

Stewart BA. 1996. Neuromuscular physiology in *Drosophila*: Nerve terminal morphology and synaptic strength. PhD dissertation. Toronto: University of Toronto.

Stewart BA, Schuster CM, Goodman CS, Atwood HL. 1996. Homeostasis of synaptic transmission in *Drosophila* with genetically altered nerve terminal morphology. J Neurosci 16:3877–3886.

Tullis A, Block BA. 1997. Histochemical and immunohistochemical studies on the origin of the blue marlin heater cell phenotype. Tissue Cell 29:627–642.

Weiss A, Leinwand L. 1996. The mammalian myosin heavy chain gene family. Annu Rev Cell Dev Biol 12: 417–439.

Wiens TJ. 1989. Common and specific inhibition in the leg muscles of decapods: sharpened distinctions. J Neurobiol 20:458–469.

Wiens TJ. 1993. The closer muscle is a second target for the stretcher inhibitor motoneuron of the crayfish's thoracic limbs. J Comp Physiol 173:435–444.

Wiens TJ, Atwood HL. 1978. Motorneuron interactions in crayfish claw control: evidence from intracellular recording. J Comp Physiol 124:237–247.

Wiens TJ, Wolf H. 1993. The inhibitory motoneurons of crayfish thoracic limbs: identification, structures, and homology with insect common inhibitors. J Comp Neurol 335:1–18.