# Myofibrillar protein isoform expression is correlated with synaptic efficacy in slow fibres of the claw and leg opener muscles of crayfish and lobster

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## Summary

In the crayfish and lobster opener neuromuscular preparations of the walking legs and claws, there are regional differences in synaptic transmission even though the entire muscle is innervated by a single excitatory tonic motor neuron. The innervation of the proximal fibres produced larger excitatory postsynaptic potentials (EPSPs) than those of the central fibres. The amplitudes of the EPSPs in the distal fibres were intermediate between those of the proximal and central regions. These differences in EPSP amplitudes were correlated with differences in short-term facilitation between the three regions. When given a 10- or 20-pulse train of stimuli, the proximal fibres showed greater short-term facilitation initially, often followed by a maximization of short-term facilitation towards the end of a train. In contrast, the central fibres showed a linear increase in short-term facilitation throughout a stimulus train. The distal fibres showed intermediate short-term facilitation compared with the other two regions. Analysis of myofibrillar isoforms showed that levels of troponin-$T_1$ ($TnT_1$), a 55 kDa isoform expressed in slow-tonic ($S_2$) fibres, were correlated with synaptic properties. Proximal fibres had the highest levels of $TnT_1$, with lower levels in distal fibres; central fibres lacked $TnT_1$, which is characteristic of slow-twitch ($S_1$) fibres. In addition, differences in troponin-I isoforms correlated with $TnT_1$ levels between the proximal, central and distal regions. The correlation between slow fibre phenotype and strength of innervation suggests a relationship between synaptic structure and expression of troponin isoforms.

Key words: muscle, troponin, troponin-I, troponin-T, myofibrillar protein, isoform, innervation, neuromuscular junction, synapse, crayfish, lobster, Crustacea, Arthropoda, *Homarus americanus*, *Procambarus clarkii*.

## Introduction

The claw and leg opener muscles in the crayfish and lobster provide preparations in which regional differences in synaptic strength and muscle fibre phenotype can be compared, since the postsynaptic responses differ among regions of the muscle even though it is innervated by a single excitatory tonic motor neuron. The nerve terminals on the central muscle fibres give rise to small excitatory postsynaptic potentials (EPSPs) compared with the large responses measured in proximal fibres and the intermediate responses in the distal muscle fibres. The large EPSPs on proximal fibres in crayfish are due to more effective synaptic transmission per length of nerve terminal at low stimulation frequencies (Cooper et al., 1995b). Differences in short-term facilitation (STF) are present between these regions on the opener muscle (Linder, 1974; Günzel et al., 1993; Govind et al., 1994). STF is demonstrated by an enhancement of the postsynaptic response within a short train of action potentials induced in the motor nerve (Dudel and Kuffler, 1961; Katz and Miledi, 1968; Atwood and Wojtowicz, 1986).

A facilitation index (FI) is commonly used to quantify differences in STF; this is based on the use of ratios of the amplitudes of the postsynaptic potentials within the train of responses. In the intact animal, the slow muscles involved in limb movements are recruited into action by bursts of activity from the motor neurons. This type of activity results in summation and facilitation of the postsynaptic graded responses to elicit muscle contraction, which can be reproduced with isolated preparations to investigate the mechanisms of synaptic function and muscle properties (Crider and Cooper, 2000).

Differences in the terminal structure and synaptic strength of the excitatory motor neuron that innervates the various regions on the opener muscle in crayfish are well established (Günzel et al., 1993; Govind et al., 1994; Cooper et al., 1995a,b,c, 1996a), but how the differences arise is unknown. Local retrograde influences from intrinsic differences among the muscle fibres may alter terminal synaptic structure or synaptic

properties early in development may influence the muscle fibres differentially, which then feed back on maintaining the nerve terminal structure and function (for a review, see Atwood, 1973). Using improved methods for identification of fibre phenotypes in crustaceans (for a review, see Mykles, 1997), we have investigated whether the opener muscle is homogeneous or heterogeneous in muscle fibre phenotype with respect to the differences in nerve terminal function.

The pinnate arrangement of fibres in the opener muscle of the blue crab (*Callinectes sapidus*) leg generates differential forces and selective recruitment of tension receptors that monitor muscle force (Tryba and Hartman, 1997). These regional differences, together with the differences in terminal structure and function described in the crayfish, suggest that there are phenotypic differences in the opener muscle fibre type. Three types of fibre have been biochemically characterized in muscles from a variety of crustacean species: fast-twitch, slow-twitch ($S_1$) and slow-tonic ($S_2$) (for a review, see Mykles, 1997). These fibres differ in their assemblages of myofibrillar protein isoforms, which confer unique contractile properties consistent with their physiological functions.

Fibre-type-specific isoforms of myosin heavy and light chains, tropomyosin, paramyosin and troponin-I, -T and -C are expressed in lobster and crayfish muscles (Mykles, 1985a,b; Kobayashi et al., 1989; Li and Mykles, 1990; Miyazaki et al., 1990, 1992, 1993; Nishita and Ojima, 1990; Garone et al., 1991; Miegel et al., 1992; Cotton and Mykles, 1993; Neil et al., 1993; Galler and Neil, 1994; Sakurai et al., 1996; Ishimoda-Takagi et al., 1997; Cooper et al., 1998; Mykles et al., 1998; Holmes et al., 1999; LaFramboise et al., 1999; Sohn et al., 2000). However, for an isoform to serve as a suitable diagnostic marker, it must (i) be exclusively expressed in a specific fibre type in various decapod crustacean species and (ii) be easily distinguished in SDS–polyacrylamide gels. Only two proteins meet these criteria: P75 for fast-twitch and troponin-$T_1$ ($TnT_1$) for slow-tonic ($S_2$); neither P75 nor $TnT_1$ is expressed in slow-twitch ($S_1$) fibres (for a review, see Mykles, 1997). $TnT_2$ is expressed only in fast fibres, but it is not easily distinguished from $TnT_3$, which is expressed in $S_1$ and $S_2$ fibres (Mykles, 1985b; Sohn et al., 2000). The presence or absence of the TnT and troponon-I (TnI) isoforms was used in the present study to distinguish the two slow fibre phenotypes in the regions of the opener muscles of crayfish and lobsters.

This paper compares the physiological properties of innervation efficacy by a single tonic motor neuron with muscle fibre phenotype in the claw and leg opener muscles of crayfish and lobster. Regional differences in slow fibre phenotype were correlated with the strength of innervation from the motor neuron, suggesting a relationship between synaptic efficacy and expression of troponin isoforms.

## Materials and methods
### Animals
Mid-sized crayfish *Procambarus clarkii* (Girard), measuring 6–10 cm in body length, were obtained from Atchafalaya Biological Supply Co. (Raceland, LA, USA). Animals were housed in an aquatic facility within the laboratory in individual tanks. Only male crayfish in their intermolt stage were used. Live lobsters *Homarus americanus* (Milne-Edwards), measuring 25–30 cm in body length, were purchased from a local supermarket and dissected within 1 h.

### Electrophysiology
The excitatory axon was selectively stimulated by placing a branch of the leg nerve (from the merus segment) into a suction electrode connected to a Grass stimulator (Dudel and Kuffler, 1961). Intracellular recordings were performed with microelectrodes filled with $3 \, \text{mol} \, \text{l}^{-1}$ KCl. The resistance of the electrode in the bath was $30–60 \, \text{M}\Omega$. Responses were recorded with an Axoclamp 2A intracellular electrode amplifier (Axon Instrument). Signals were simultaneously recorded using a Vetter PCM VHS tape recorder and digitized using MacLab/s v3.5. The crayfish and lobster salines used were the same as described by Sohn et al. (2000).

Short-term facilitation was induced by giving a train of 10 or 20 pulses every 10 s. Each pulse consisted of stimulation at either 30 or 50 Hz. To determine FI, 1 was subtracted from the ratio of the amplitude of a preceding pulse (first, third, fifth, tenth or fifteenth) to the amplitude of the last pulse (either tenth or twentieth); FI was zero if no facilitation occurred (Crider and Cooper, 1999; Sohn et al., 2000). The amplitudes of the EPSPs were measured using the MacLab/s v3.5 programs Scope or Chart. The signals recorded on the computer were calibrated to the raw traces observed on the oscilloscope.

### Analysis of myofibrillar proteins
Muscle fibres were glycerinated and analyzed by SDS–PAGE and western blotting as described previously (Mykles, 1985b; Sohn et al., 2000). Briefly, fibres were incubated in glycerination buffer ($20 \, \text{mmol} \, \text{l}^{-1}$ Tris-HCl, pH 7.4, $0.1 \, \text{mol} \, \text{l}^{-1}$ KCl, $1 \, \text{mmol} \, \text{l}^{-1}$ EDTA, 0.1 % Triton X-100 and 50 % glycerol) and solubilized overnight in 25–100 µl of SDS sample buffer ($62.5 \, \text{mmol} \, \text{l}^{-1}$ Tris-HCl, pH 6.8, 12.5 % glycerol and 1.25 % SDS). Myofibrillar proteins (4–6 µg) were separated in 10 % SDS–polyacrylamide gels (Laemmli, 1970). Proteins in gels were either fixed in 10 % glutaraldehyde and stained with silver (Wray et al., 1981; Mykles, 1985a,b) or transferred to PVDF membrane for western blotting (Towbin et al., 1979). After blocking with 5 % non-fat milk in Tris-buffered saline (TBS; $20 \, \text{mmol} \, \text{l}^{-1}$ Tris-HCl, pH 7.5, $0.5 \, \text{mol} \, \text{l}^{-1}$ NaCl), blots were incubated for 1 h with polyclonal antibodies to P75 ($7.3 \, \mu\text{g} \, \text{IgG} \, \text{ml}^{-1}$), troponin-$I_3$ (1:5000 dilution of antiserum) or troponin-T ($0.8 \, \mu\text{g} \, \text{IgG} \, \text{ml}^{-1}$) in 0.05 % Tween-20 in TBS (TTBS) containing 3 % non-fat milk. After washing in TTBS, blots were incubated with biotinylated anti-rabbit IgG (1:10 000 dilution in TTBS) for 1 h followed by avidin/biotinylated horseradish peroxidase complex (Vectastain ABC reagent, 1:1000 dilution in TTBS) and chemiluminescent detection (Covi et al., 1999).
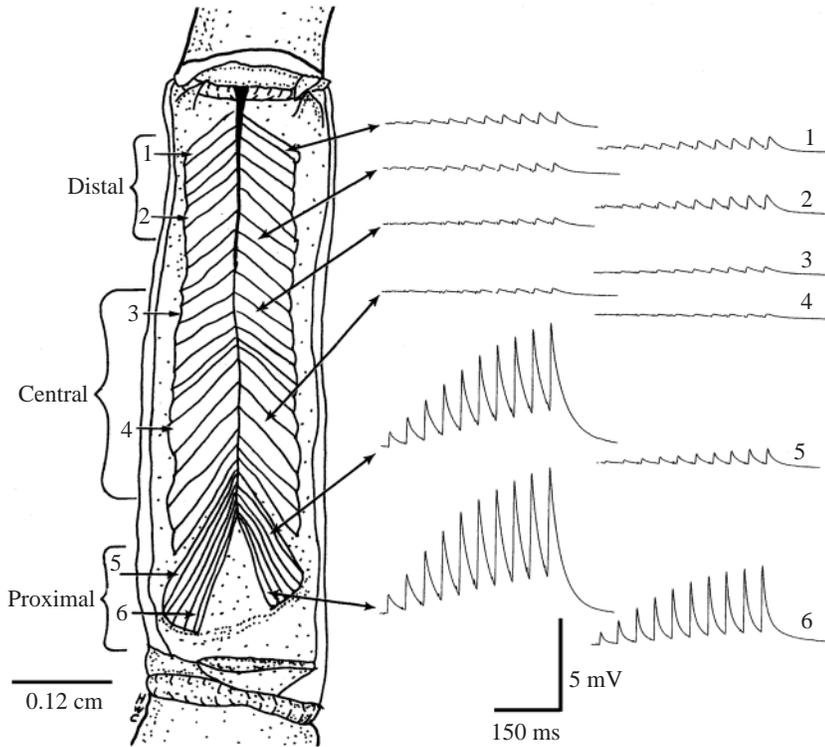
Fig. 1. Opener muscle in the crayfish walking leg showing regional variation in excitatory postsynaptic potential (EPSP) amplitudes. The excitatory motor neuron was stimulated at 30 Hz (10 stimuli per train). The largest potentials were measured in proximal fibres, with smaller potentials in distal fibres; the smallest potentials occurred in central fibres. The double-headed arrows indicate where the medial EPSP traces originated on the muscle. The numbered arrows indicate where the lateral EPSP traces were sampled and correspond to the numbered traces on the right. The regional differences are similar for both lateral and medial halves of the muscle.

event with various earlier EPSPs to obtain an overview of STF responses rather than relying on a single comparison. The short-term FIs are summarized in Tables 1 and 2 for all preparations used in this study.

As demonstrated previously (Iravani, 1965; Bittner 1968a; Cooper et al., 1995b), the opener muscle of the first walking leg of the crayfish is divided into three anatomical regions: distal, central and proximal (Fig. 1). These three regions showed distinct differences in the amplitudes of the EPSPs. There were also consistent differences between regions in the degree of STF when fibres were stimulated with a 10- or 20-pulse train. The most proximal fibres showed a rapid increase in the amplitudes of the EPSPs within the first couple of stimuli within the train, often followed by a smaller increase towards the end of the train (Fig. 1, lower traces). This was reflected in the greater FI for the tenth/first comparison than for the tenth/fifth comparison for the proximal fibres (Table 1). This pattern was quite different for the central fibres, in which the

## Results

### Electrophysiology

A variety of methods have been used to quantify STF within short stimulus trains (Linder, 1974). With trains of stimuli, EPSP amplitudes are measured from the resting membrane potential to the peak amplitude of the EPSPs or from the trough preceding each EPSP; the latter method was used in the present study. Depending on the method used, various FIs are obtained (Crider and Cooper, 1999). Here, we compared the last EPSP

Table 1. *Short-term facilitation in the three regions of opener muscle in the crayfish first walking leg stimulated at 30 Hz*

| Preparation | FI=(tenth/first)−1 | | | FI=(tenth/fifth)−1 | | |
|---|---|---|---|---|---|---|
| | Distal | Central | Proximal | Distal | Central | Proximal |
| 1 | 5.94 | 5.83 | 15.65 | 2.66 | 2.33 | 0.95 |
| 2 | 4.63 | 5.12 | 5.20 | 1.07 | 0.95 | 0.47 |
| 3 | 4.63 | 5.52 | 7.27 | 1.12 | 0.84 | 0.71 |
| 4 | 9.38 | 12.62 | 12.56 | 1.24 | 1.79 | 0.99 |
| 5 | 9.30 | 11.38 | 15.71 | 1.40 | 1.33 | 0.98 |
| Mean±S.E.M. | 6.77±1.07 | 8.09±1.61 | 11.28±2.16 | 1.50±0.30 | 1.45±0.28 | 0.82±0.10 |
| % Change from central | −14 | | +48 | +7 | | −36 |

In four out of five preparations for tenth/first, the proximal region facilitated more than central region.

In five out of five preparations for tenth/first comparison, the proximal region facilitated more than the distal region ($P<0.05$, Wilcoxon rank order).

In five out of five preparations for tenth/fifth comparison, the distal region facilitated more than the proximal region ($P<0.05$, Wilcoxon rank order).

For the tenth/fifth comparison, the central region was not consistently different from the other two regions. The percentage change from the central region was calculated for each preparation and then averaged.

FI, facilitation index, determined as 1 substracted from the ratio of the amplitude of the tenth pulse to that of either the preceding first (tenth/first) or the preceding fifth (tenth/fifth) pulse.

Fig. 2. Opener muscle in the crayfish claw showing regional variation in excitatory postsynaptic potential (EPSP) amplitudes. The excitatory motor neuron was stimulated at 50 Hz (20 stimuli per train). The largest potentials were measured in proximal fibres, with smaller potentials in distal fibres; the smallest potentials occurred in central fibres. The double-headed arrows indicate where the medial EPSP traces originated on the muscle. The numbered arrows indicate where the lateral EPSP traces were sampled and correspond to the numbered traces on the right. The regional differences are similar for both lateral and medial halves of the muscle.

EPSPs continued to facilitate throughout the stimulus train (Fig. 1).

Although the opener muscle in the claws of the crayfish lacked the proximal tuft of muscle fibres present in the walking legs, there were still regional differences in the EPSP amplitude and STF (Fig. 2; Table 2). Since the opener muscle in the crayfish claw had smaller EPSPs than the walking leg at 30 Hz stimulation, a 50 Hz, 20-pulse stimulus train was used to discriminate better differences between the three regions. The proximal fibres facilitated more quickly (within the first five EPSPs) than did the central and distal fibres (Fig. 2). By the fifth EPSP, the amplitudes no longer increased linearly in the proximal fibres, so that FIs calculated from the fifth and tenth EPSPs showed that the distal and central fibres facilitated more than the proximal fibres at the twentieth/fifth and twentieth/tenth comparisons (Fig. 3; Table 2). FI decreased closer to the twentieth pulse (i.e. twentieth/fifteenth comparison), because the responses reached a plateau in their amplitudes close to the fifteenth event within the train (Fig. 3). This was also observed for 30 Hz, 10-pulse stimulation trains.

The opener muscles in the first walking leg and claw of lobsters were anatomically similar to those of the crayfish, with the exception of minor differences due to the dimorphic claws of the lobster. Regional physiological differences of the opener muscles also occurred in lobster, although the responses were smaller in the walking legs of the lobster for the same stimulation paradigm (Fig. 4).

*Analysis of myofibrillar proteins in opener muscles*

Regional differences in the isoform compositions of claw and leg opener muscle fibres were correlated with synaptic

Table 2. *Short-term facilitation in the three regions of opener muscle in the crayfish claw stimulated at 50 Hz*

| Preparation | FI=(twentieth/fifth)–1 | | | FI=(twentieth/tenth)–1 | | | FI=(twentieth/fifteenth)–1 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Distal | Central | Proximal | Distal | Central | Proximal | Distal | Central | Proximal |
| 1 | 2.27 | 2.64 | 0.96 | 0.53 | 0.81 | 0.24 | 0.16 | 0.16 | 0.09 |
| 2 | 2.73 | 3.27 | 2.60 | 0.69 | 1.01 | 0.64 | 0.18 | 0.25 | 0.18 |
| 3 | 2.02 | 2.76 | 1.04 | 0.52 | 0.68 | 0.25 | 0.09 | 0.21 | 0.06 |
| 4 | 2.79 | 3.02 | 0.88 | 0.76 | 0.88 | 0.20 | 0.22 | 0.23 | 0.06 |
| 5 | 1.89 | 2.09 | 1.68 | 0.45 | 0.72 | 0.41 | 0.10 | 0.12 | 0.08 |
| Mean±S.E.M. | 2.34±0.18 | 2.76±0.20 | 1.43±0.33 | 0.59±0.06 | 0.82±0.08 | 0.35±0.08 | 0.15±0.15 | 0.20±0.04 | 0.09±0.02 |
| % Change from central | −15 | | −47 | −28 | | −58 | −21 | | −50 |

Only the central and proximal regions were different for each case when using a Student *t*-test.

For twentieth/fifth and twentieth/tenth comparisons, the central region facilitated more than the proximal or distal regions.

In all cases (twentieth/fifth, twentieth/tenth, and twentieth/fifteenth comparisons), the distal region facilitated more than the proximal region.

The percentage change from the central region was calculated for each preparation and then averaged.

F1, facilitation index, calculated as 1 subtracted from the ratio of the amplitude of the twentieth pulse to that of the fifth (twentieth/fifteenth), tenth (twentieth/tenth) or fifteenth (twentieth/fifteenth) pulse.
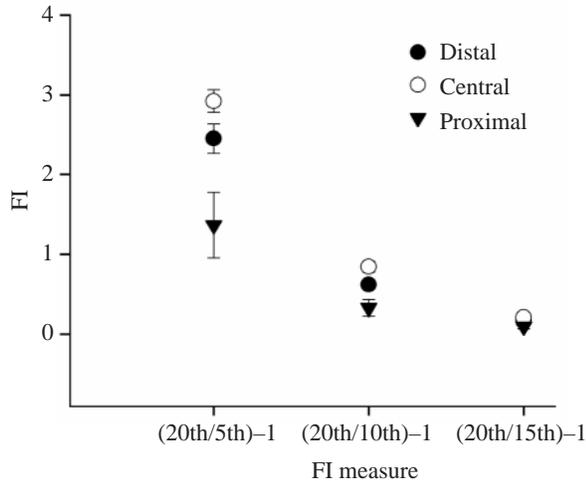
Fig. 3. Facilitation indices (FIs) for crayfish claw opener muscle. Trains of stimuli at 50 Hz were given to produce 20 events, which were used to calculate FIs from the ratios of the amplitudes of the fifth, tenth, fifteenth and twentieth excitatory postsynaptic potentials. Values of FI are means ± S.E.M.

properties. All fibres in the openers were of the slow type, since they lacked the P75 regulatory protein characteristic of fast fibres (Fig. 5C; data for crayfish not shown). This is consistent with physiological and ultrastructural studies of lobster and crayfish opener muscles (Velez et al., 1981; Günzel et al., 1993). In general, the proximal region contained $S_2$ fibres and the central region contained $S_1$ fibres; in three of the four muscles, the distal region also contained $S_2$ fibres.

The complete analysis with the three antibodies is shown for the lobster claw opener (Fig. 5). The identification of $S_1$ and $S_2$ fibres was based on the expression of $TnT_1$ and TnI isoforms. Central fibres were classified as $S_1$, since they lacked $TnT_1$ (55 kDa) and had a similar TnI isoform composition to that of the $S_1$ fibres from crusher claw closer muscle (Fig. 5B,D; compare lanes f–h with lane b). As described previously (Mykles, 1988; Sohn et al., 2000), the level of $TnT_1$ varied reciprocally with the level of $TnT_3$ (Fig. 5B, compare lanes c, d, e and i). Furthermore, the proximal fibres (lanes d, e) had higher levels of $TnT_1$ than the most distal fibre (lane i). The TnI isoform composition also varied between $S_2$ fibres.
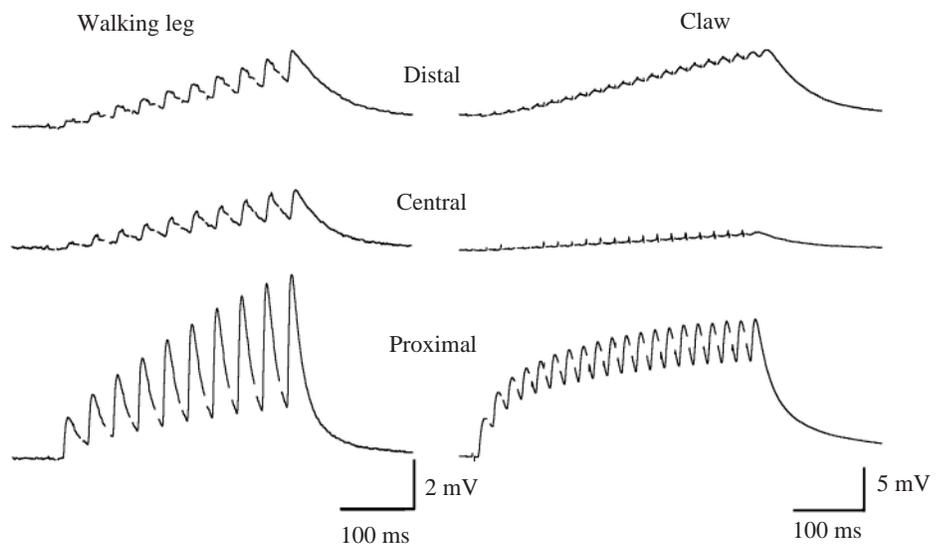
Since the results of the analysis of opener muscles in lobster leg and crayfish claw and leg were similar to those obtained on the lobster claw opener muscle, only the blots probed with the anti-TnT antibody are shown in Fig. 6. In all three muscles, the proximal region consisted of $S_2$ fibres (Fig. 6A, lanes d, e; Fig. 6B, lanes d, e; Fig. 6C, lanes d, e), while the central region consisted of $S_1$ fibres (Fig. 6A, lanes f, g; Fig. 6B, lanes f, g; Fig. 6C, lane f). The distal region of crayfish leg (Fig. 6B, lanes h, i) and claw (Fig. 6C, lane g) openers contained $S_2$ fibres, whereas $TnT_1$ was not detected in the distal fibres of lobster leg opener (Fig. 6A, lane h). As observed in lobster claw opener (Fig. 5), the distal $S_2$ fibres in the crayfish opener muscles expressed lower levels of $TnT_1$ than the most proximal fibres.

**Discussion**

Crustacean tonic motor neurons generally induce muscle contraction by eliciting a train of action potentials in a presynaptic motor neuron to generate a graded response in a muscle fibre. The rapid burst of activity within the presynaptic nerve may result in a measured facilitated postsynaptic response, which is due to an increase in transmitter release as a result of previous activity at the synapse. Facilitated responses are readily observed in crustacean neuromuscular junctions (Atwood and Wojtowicz, 1986), which provide accessible and viable preparations in which to investigate the underlying mechanisms and regional variation in synaptic strength. The larger the facilitation, the greater is the degree of muscle contraction for these slow skeletal muscle fibres.

The synaptic strength or efficacy is correlated with muscle phenotype in various invertebrate and vertebrate species. In crayfish and lobster in particular, phasic motor neurons are associated with a fast muscle phenotype and tonic motor neurons are associated with slow muscles (Atwood, 1976; Günzel et al., 1993; Atwood and Cooper, 1996b; LaFramboise et al., 1999; Griffis et al., 2000). Since troponin-I and -T expression was correlated with regional variation in synaptic



Fig. 4. Regional variation in excitatory postsynaptic potential amplitudes in the opener muscles in the lobster walking leg (left) and claw (right). The excitatory motor neuron was stimulated at either 30 Hz/10-pulse train (walking leg) or 50 Hz/20-pulse (claw) train. The largest potentials were measured in proximal fibres, with smaller potentials in distal fibres; the smallest potentials occurred in central fibres.

Fig. 5. Analysis of the myofibrillar protein isoform composition of lobster claw opener muscle by SDS–PAGE and western blotting. Fast (F), slow-twitch ($S_1$) and slow-tonic ($S_2$) fibres from lobster cutter claw closer, crusher claw closer and superficial abdominal muscles are provided for reference (lanes a–c, respectively). Claw opener fibres are arranged according to their position from most proximal (lane d) to most distal (lane i). (A) Silver-stained polyacrylamide gel showing myofibrillar proteins (MHC, myosin heavy chain; P, paramyosin; P75, 75 kDa protein; TnT, troponin-T; Ac, actin; Tm, tropomyosin; TnI, troponin-I). (B) Western blot probed with anti-TnT antibody. $S_2$ fibres (lanes c–e, i) possess varying amounts of $TnT_1$ (arrow), with higher levels in proximal fibres. (C) Western blot probed with anti-P75 antibody. Only fast fibres possess the P75 protein (lane a). (D) Western blot probed with anti-TnI antibody. Although not all five isoforms were detected at this loading, there were clear differences between F (lane a), $S_1$ (lanes b, f–h) and $S_2$ (lanes c–e, i) fibres. On the basis of this analysis, the proximal (lanes d, e) and distal (lane i) fibres were classified as $S_2$, while central fibres (lanes f, g) were classified as $S_1$. The positions of molecular mass standards are indicated on the left.

properties, we propose that a presynaptic-induced mechanism leads to the enhanced postsynaptic facilitation, which in turn results in a $Ca^{2+}$-induced activation of genes encoding slow-tonic fibre isoforms. This does not exclude the possibility of a retrograde factor altering the nerve terminal properties or even the possibility of mutual interactions between the synapse and the muscle fibre phenotype.

Our results are in general agreement with earlier work examining regional differences in the phenotype and physiological properties of the crayfish opener muscle. Günzel et al. (1993) distinguished three fibre types (A, B and C) in three species of crayfish on the basis of the stability of myofibrillar ATPase at basic pH values. Fibres with low and intermediate ATPase activities (A and B fibres, respectively) were most abundant in the proximal region, while fibres with the highest ATPase activity (C fibres) were most abundant in the central region. Since all fibres in the opener muscle have long sarcomeres (Günzel et al., 1993), the A/B fibres probably corresponded to $S_2$ fibres and the C fibres to $S_1$ fibres. The regional differences in the EPSP amplitudes were the same as we show here, although the absolute amplitudes of the EPSPs of the proximal fibres were significantly smaller.

The opener muscle in crayfish has provided a great deal of insight into the fundamental mechanisms of synaptic transmission (Biedermann, 1887; Wiersma, 1933; Dudel, 1965; Atwood, 1967, 1973, 1976, 1982; Atwood et al., 1994;

# A

Lobster leg opener



# B

Crayfish leg opener



# C

Crayfish claw opener
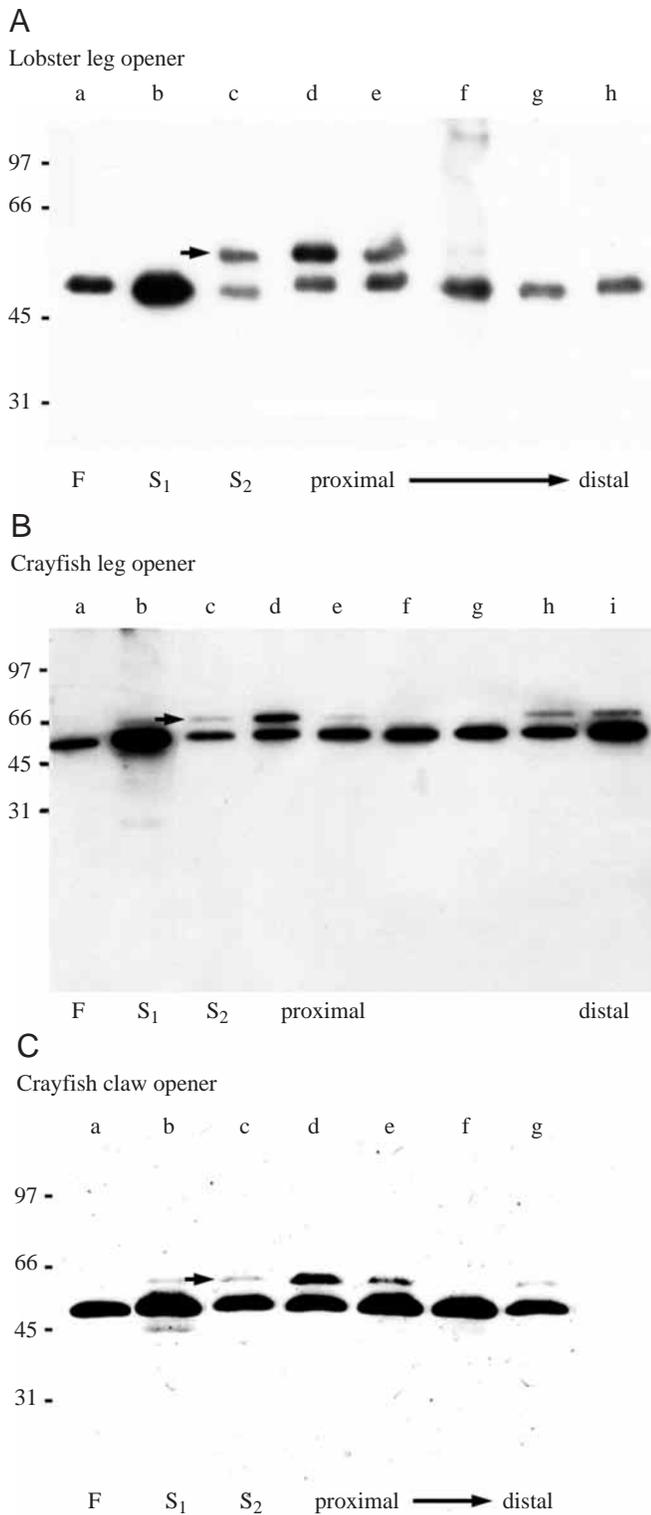


Fig. 6. Western blots of crayfish and lobster opener muscle fibres probed with an antibody to troponin-T (TnT) . Fast (F), slow-twitch ($S_1$) and slow-tonic ($S_2$) fibres from lobster cutter claw closer, crusher claw closer and superficial abdominal muscles are provided for reference (lanes a–c, respectively); the reference fibres used for the lobster blot (A) were different from the reference fibres used for the crayfish blots (B,C). In lobster, only the proximal fibres ($S_2$) in the leg opener muscle expressed $TnT_1$ (A, lanes d, e). The proximal and distal fibres ($S_2$) expressed variable amounts of $TnT_1$ (arrow) in the crayfish leg opener (B, lanes d, e, h, i) and crayfish claw opener muscles (B, lanes d, e, g), with higher levels of $TnT_1$ in proximal fibres. The central region of crayfish opener muscles (B, lanes f, g; C, lane f) and the central and distal regions of lobster leg opener (A, lanes f–h) contained $S_1$ fibres. The positions of molecular mass standards are indicated on the left.

1982a; Zucker and Haydon, 1988; Dudel, 1989a,b,c,d). It is now known that the majority of the differences result from local presynaptic changes in synaptic structure and physiology (Atwood et al., 1994; Atwood and Cooper, 1995, 1996a,b; Cooper et al., 1995b, 1996a,b). However, the basis for the regional differentiation is not understood.

Frank (1973) proposed that the target muscle determines the release properties of the motor nerve terminals in lobsters. For example, it appears that in the cricket *Acheta domesticus* a retrograde factor from target neurons can locally alter nerve terminal function (Davis and Murphey, 1993). Similarly, for a motor neuron in the stomatogastric system of the lobster, a similar observation was made for target influences on a single presynaptic neuron for two different terminals (Katz et al., 1993). It has also been proposed that motor neurons influence muscle differentiation (for reviews, see Atwood, 1973; Grinnell, 1995). The proposed mechanisms have focused primarily on activity patterns and neurotrophic substances of the neurons. In crustacean systems, the timing of innervation during fibre differentiation may determine the final properties of the fibre (for a review, see Atwood, 1973).

Such local differences in the terminals also occur in the crayfish. The central region of the opener muscle contains nerve terminals arranged in long chains of varicosities (swellings), whereas the nerve terminals are grouped into clusters of varicosities in the proximal region (Atwood and Cooper, 1996b). Ultrastructural analysis has shown that the varicosities contain the majority of the synaptic contacts (Florey and Cahill, 1982; Cooper et al., 1995b). It has also been shown that the efficacy of synaptic transmission decreases along the length of a single terminal (Cooper et al., 1996a). The varicosities on the proximal fibres have high synaptic outputs compared with the relatively low output of varicosities in the central region. High-output varicosities along a terminal contain more active sites per synapse than do low-output varicosities, which contain a high percentage of synapses with only one or no active sites on the synapse (Govind et al., 1994; Cooper et al., 1996a). This indicates that there are more silent synapses in the low-output varicosities (Atwood and Wojtowicz, 1999). The differences in the synaptic structure among the varicosities may in part explain the differences in

Zucker, 1973, 1974a,b; Bittner and Sewell, 1976; Parnas et al., 1982a,b,c,d; Dudel et al., 1983; Vyshedskiy and Lin, 1997a,b,c). The regional differentiation in synaptic strength and facilitation, which arises from the single motor neuron, has led to many hypotheses regarding the underlying presynaptic mechanisms (Iravani, 1965; Atwood, 1967; Bittner, 1968a,b; Sherman and Atwood, 1972; Zucker, 1974a; Parnas et al.,

the $Ca^{2+}$ influx during stimulation at various frequencies (Cooper et al., 1995b, 1996b). This synaptic complexity within the varicosities appears to account for much of the difference in EPSP amplitudes between the proximal and central fibres (Cooper et al., 1996a).

Presynaptic structural elements that control the amount of neurotransmitter release may indeed drive muscle phenotype, but then what drives the regional differences of a single motor neuron to display structural differences in terminal length and degree of branching? Perhaps the regional differences in a developmentally regulated muscle phenotype determine and maintain the regional differences of the motor neuron. This suggests that the phenotype is developmentally fixed, either by a fibre's location during development or by some property of timing in innervation with development (for a review, see Atwood, 1973).

An alternative hypothesis is that, given the pinnate arrangement of the fibres, some fibres are stretched more than others and thus have a greater amount of force exerted on them per fibre mass, resulting in differential cellular regulation of muscle phenotype. This, in turn, may cause the muscle to induce changes in synaptic structure during development. Alterations in the levels of proteins involved with synaptic vesicle docking and release change the release characteristics of motor neurons in crustaceans (He et al., 1999). A factor secreted from the muscle or localized on the surface could produce regional differences in synaptic properties. For example, it is known that a reduced expression of Fasciclin II in *Drosophila melanogaster* neuromuscular junctions produces shorter motor nerve terminals but, as a result, the nerve compensates by altering synaptic structure to produce an enhanced synaptic efficacy (Stewart et al., 1996). In another *Drosophila melanogaster* mutation, 'Highwire', lower-output synapses are associated with greatly expanded terminals on the muscle fibres (Wan et al., 2000). Such evidence in insects and crustaceans suggests a tight regulation in the communication between the nerve and muscle as for classical Hebbian models and homeostatic mechanisms (for a review, see Turrigiano, 1999).

The strong correlation between synaptic properties and expression of troponin-T and -I isoforms suggests that neurotransmission patterns regulate or maintain gene expression and, thus, determine slow-twitch ($S_1$) and slow-tonic ($S_2$) phenotypes. In mammals, motor neuron activity controls the expression of fibre-type-specific isoforms of myosin heavy and light chains, tropomyosin and troponin-I, -C and -T (for reviews, see Buonanno et al., 1998; Pette, 1998). Differences in muscle phenotype, identified by biochemical properties, and characteristics in the strength of synaptic transmission correlate well in the abdominal superficial flexor muscle in lobsters (Neil and Fowler, 1990). However, this particular muscle is innervated by five excitatory neurons and one inhibitory neuron, so numerous mechanisms may give rise to fibre type differentiation. Chronic electrical stimulation of crayfish abdominal muscle induces a shift from fast to slow-tonic myofibrillar protein composition, although the identities

of the isoforms have not been established (Cooper et al., 1998). Fibre transformation during claw differentiation in lobsters and snapping shrimp *Alpheus heterochelis* requires an intact nerve supply (Govind and Kent, 1982; Govind et al., 1988). In mammalian and avian muscles, elevated intracellular $[Ca^{2+}]$, driven by motor neuron activity, stimulates $Ca^{2+}$-dependent signal-transduction enzymes (e.g. calcineurin, calmodulin kinase and protein kinase C) that regulate slow fibre genes (Antipenko et al., 1999; Naya et al., 2000; DiMario, 2001).

In conclusion, regional differences in synaptic transmission and myofibrillar protein isoform composition were observed in a muscle that is innervated by a single excitatory tonic motor neuron. The high-output terminals on the proximal region of the muscle produced larger EPSPs than the low-output terminals on the central region of the muscle. The distal region behaved electrophysiologically as being intermediate between the central and proximal regions. There were also differences in STF between these regions. The differential responses to facilitation depended on the type of stimulation paradigm used, so a range of stimulation conditions from trains of 10 to 20 stimuli given at frequencies of 30–50 Hz was used to characterize regional differences in STF.

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