FINE STRUCTURE OF THE SWIMMING PADDLE OPENER MUSCLE AND ITS INNERVATION IN THE BLUE CRAB, *Callinectes sapidus*

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science in Zoology Graduate Department of Zoology, University of Toronto

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0-612-63237-7
ABSTRACT

The fifth pereiopod of the blue crab is a specialized swimming limb, the dactyl of which acts as a rowing paddle. Abduction of this paddle is brought about by contractions of the opener muscle that has assumed a specialized role as a fairly equal antagonist to the closer muscle (the adductor of the dactyl). Electron microscopic examinations of the central region of the opener muscle revealed typically slow fibres with long sarcomere lengths and a high actin to myosin ratio. Muscle fibres were highly innervated with a majority having multiple innervation sites each with an inhibitory and excitatory terminal profile. This large amount of innervation may play a role in ensuring fatigue resistance and fine control for the specialized muscle. Inhibitory terminals originated synapses that were two thirds neuromuscular, inhibiting the muscle directly, and one third axo-axonal, inhibiting the excitatory axon itself. This is an unusually high degree of presynaptic inhibition possibly associated with the fine control of the paddle limb. The overwhelming majority of synapses formed by the excitatory terminal were neuromuscular, in keeping with its primary function of making the muscle contract. A very unusual synapse was also recorded in every serially sectioned area examined: axo-axonal synapses polarized from the excitatory terminal to the inhibitory terminal, suggesting putative presynaptic excitation of the inhibitory nerve terminals. Altogether, the innervation of the blue crab swimming paddle opener muscle indicated specializations for its function as a highly active muscle rapidly contracting for prolonged periods of time.
ACKNOWLEDGEMENTS

It is with great gratitude that I thank Dr. C.K. Govind for giving me the guidance and opportunity to teach, learn and grow beyond what I had ever anticipated. To Joanne Pearce for her incredible patience and brilliance that has helped to make this experience a wonderful one. To Raymond who was there when something wasn’t quite working, but was always able to fix it. To the people who were with me in the lab, Nadine, Ros, Asheer and Rahim, who listened and shared. Also, to other fellow students who made me smile and laugh, Najeeb, Allison, Lori, Joanne, Manav, Christine, Andrea, Andrea, and Sandi. And to Herman, Scott, CC, Rhoda, Shintaro, and Richard who helped me grow spiritually. Thank-you also to the students that I was so fortunate to teach, I have learned many things from you.

To Jaykob, what sunshine you have brought me; thank-you Roberta and Yola for trusting me with your beautiful (great-) grandson. To my absolutely terrific friends who I love, thank-you for everything, without you, laughter would not come so easily, Meaghan, Todd, Carlene, Stacey. Shane, Sam, Thane, Kevin, Kevin, and Ferris, and the beautiful Angela and Laura who have loved me for 20 years. To the people I have shared a house with Amy, Ray, Jess, Tina, Kevin, Cristiane and Leigh, thank-you. Also, thank-you to the Welch’s for always listening, and to Chris and Scott for taking such good care of me.

To my beautiful family who have shown me so much and have listened to me dream. Thank-you especially mum, dad, Ali and Graham, you are incredible, and brilliant and so much more patient than I can ever hope to be. And thank-you God for answering my many prayers. Thank-you for absolutely everything.
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Introduction

A. Crustacean Neuromuscular Systems

Crustacean neuromuscular systems have been extensively used to investigate chemical transmission properties as most muscles are innervated by relatively few motoneurons, typically six or less (Atwood, 1976), that often display a diverse range of transmitter release capabilities. For this reason numerous studies have focused on examining the ultrastructural basis that would account for this physiological diversity (Govind et al., 1994; King et al., 1996). In addition, unlike vertebrate muscle that only receives excitatory neuromuscular contacts, most invertebrate muscles receive input from an inhibitory motoneuron, which acts as an antagonist to the excitor. These antagonistic motoneurons form numerous synaptic contacts along the length of a muscle fiber, i.e. multiterminal innervation, which allows for fine graded contractions of the muscle. Although the main target tissue for both types of motoneurons is the muscle fibre, they may also form axo-axonal contacts with each other. Multiterminal innervation and axo-axonal contacts are features shared with the mammalian central nervous system, however the invertebrate peripheral nervous system, with few, identifiable motoneurons, is more advantageous for ultrastructural examination. This study investigates the fine structure of the motoneurons innervating the swimming paddle opener muscle of the blue crab, Callinectes sapidus in order to describe ultrastructural features of a tonically active muscle innervated by excitatory and inhibitory axons. The following introduction will briefly review properties of the crustacean neuromuscular system by considering the
component parts, viz. muscle fibres, motoneurons, and neuromuscular and axo-axonal synapses.

**B. Muscle Fibres**

Crustacean muscles, like mammalian skeletal muscles, are striated. Myofibrils composed of proteins are arranged as sarcomeres that repeat themselves along the length of the muscle fibre. Upon muscle fibre excitation, contraction and shortening along the long axis of the fibre takes place. Based on contractile, physiological, biochemical and structural properties, muscle fibres are highly differentiated and broadly classified into fast and slow types (Atwood, 1976). It is important to note that within this broad classification system fibres with intermediate properties exist as well. Crustacean muscle can be heterogeneous in muscle fibre composition, or uniformly composed of one muscle fibre type (Atwood, 1976).

1. **Fast Muscle Fibres**

Fast-acting, or phasic, muscle fibres have short sarcomeres (2-4 μm in length), thin, straight and well-aligned Z bands, and a low number of thin-to-thick filaments (6:1) (Atwood, 1976; Govind and Atwood, 1982). Furthermore, the tubule system and the sarcoplasmic reticulum are well developed. These characteristics are thought to allow for rapid contraction and rapid relaxation. Rapid contraction however, is achieved at an expense: fast muscle routinely generates less total force and tension than its slow
counterpart (Huxley and Niedergerke, 1954; Jahromi and Atwood, 1969). Moreover, fast fibres are poorly designed to maintain periods of tension (Hoyle and McNeill, 1968a, b).

The muscle membranes of fast acting muscle fibres are often electrically excitable and are capable of generating large graded spikes, or all-or-nothing impulses (Atwood, 1976). Additionally, fast fibres display high excitation-contraction coupling values, requiring depolarizations of around 20 to 30 mV for a contraction to occur (Atwood, 1965; Parnas and Atwood, 1966).

With the above structural and physiological features fast fibres are well developed for rapid, powerful contractions, like an escape response, but are not designed for sustained postural control, or locomotion. Examples of fast muscle fibres are found in the crayfish and lobster deep abdominal flexor and extensor muscles where the entire muscle is composed of fast fibres (Kennedy and Takeda, 1965a; Parnas and Atwood, 1966). Other muscles, such as the claw closer muscle in lobsters, have a mixture of fast and slow types (Atwood, 1965).

2. Slow Muscle Fibres

Structurally, slow or tonic muscle fibres have long sarcomeres (10 to 15 μm), long A-bands, thick and wavy Z-bands that are not regularly aligned, and a high number of thin-to-thick filaments (10-14:1) (Atwood, 1976; Govind and Atwood, 1982). The long sarcomeres in the slow muscle fibres allow for slow contraction and graded tension development.

Slow muscle fibres characteristically exhibit slow or graded muscle contraction and relaxation even with rapid depolarization, with relaxation of the muscle fibres taking
up to several seconds (Atwood 1965; Jahromi and Atwood, 1971; Parnas and Atwood 1966). The threshold for excitation-contraction coupling in the slow muscle fibres is low and can be within a few millivolts of the resting potential (Atwood, 1965; Reuben et al., 1967), allowing for weak tonic contractions to occur (Hoyle, 1968).

The ability of slow muscle fibres to generate and sustain tension (Hoyle, 1968) is indicative of its role as muscle used for postural control and repetitive locomotor movements. Examples of slow muscle fibres are found in the crayfish and lobster superficial abdominal flexor and extensor muscles (Kennedy and Takeda, 1965b; Parnas and Atwood, 1966), in the crayfish limb opener muscle, and the stretcher muscles of crabs and crayfish (Bittner, 1968; Sherman and Atwood, 1972).

C. Motoneurons

Unlike mammalian muscle, crustacean muscle can be innervated by both excitatory and inhibitory axons (Atwood, 1976) providing very fine control of muscle contraction. Although not all crustacean muscles receive inhibitory innervation, the total number of excitatory and inhibitory axons innervating different muscles is variable, with each muscle receiving efferent innervation by generally less than six axons (Atwood, 1976). Below is a brief outline of the excitatory and inhibitory neurons.

1. Excitatory Neurons

Every crustacean muscle fibre is innervated by at least one excitatory motoneuron (Atwood, 1976). Not all excitatory axons exhibit the same properties and, as a result, excitatory motoneurons may be classified as either phasic or tonic. Innervation of a
muscle fibre can occur through phasic motoneurons, tonic motoneurons or a combination of the two. For example, the crayfish limb opener muscle is innervated by a single tonic axon (Wilson and Davis, 1965), while the crayfish limb extensor muscle is innervated by both a tonic and a phasic motoneuron such that single muscle fibres receive input from both axons (Wiersma, 1961; Bradacs et al., 1997).

Phasic axons are generally large in diameter, up to 50 \( \mu \text{m} \), (Atwood, 1976) that are electrically inactive most of the time and are recruited for rapid activity. Such rapid activity is typical of an escape response (Govind and Atwood, 1982). Following a single action potential, phasic axons generate large excitatory postsynaptic potentials causing a large depolarization in the innervated muscle membrane (Hoyle and Wiersma, 1958; Bradacs et al., 1997). These currents are many times larger than those of tonic axons (Msghina et al., 1998). Though phasic axons produce large excitatory postsynaptic potentials, they show poor facilitation and are rapid to fatigue even at low frequency stimulation (Hoyle and Wiersma, 1958; Atwood, 1973).

Tonic axons tend to be smaller in diameter than phasic axons and have smaller cell bodies (Atwood, 1976). These axons are electrically active most of the time and fire impulses for prolonged periods. Postural control and locomotion are controlled by innervation of tonic motoneurons, which continuously produce impulses during these processes (Kennedy and Takeda, 1965b; Atwood and Wojtowicz, 1986).

2. Inhibitory Neurons

Unlike excitatory innervation, not all crustacean muscles receive inhibitory innervation (Atwood, 1976). Muscles lacking inhibitory innervation tend to be involved in stereotypic movement patterns where the regulation of muscle contraction is
adequately provided by variations in excitatory impulse output. Crustacean muscles that do receive inhibitory innervation are generally innervated by one or two inhibitory axons. When two inhibitory axons innervate a given muscle, the physiological properties of the two differ. Like excitatory axons, inhibitory axons express phasic and tonic properties. Inhibitory axons supplying the fast abdominal muscles display phasic properties, as do the excitatory axons innervating the same muscles (Atwood, 1976), while the specific inhibitory axon of the limb opener muscle in crayfish and crabs shows tonic properties, as does its excitatory axon (Wilson and Davis, 1965; Bush, 1962).

**D. Neuromuscular Synapses**

Excitatory and inhibitory axons travel to the muscle and innervate individual muscle fibers by contacting them at multiple sites along the length of the fibre, reflecting multiterminal innervation. A nerve terminal is identified when an axonal branch is located beneath the muscle sarcolemma and is not completely encased in glial cells or connective tissue. It is possible to differentiate between inhibitory and excitatory terminals at the ultrastructural level, based on the shape and size of clear synaptic vesicles. Inhibitory terminals have synaptic vesicles that are elliptical in shape and tend to be slightly smaller than the spherical and larger vesicles found within excitatory terminals (Atwood et al., 1972; Jahromi and Atwood, 1974). This difference in vesicle morphology is actually an artifact of the fixation with aldehydes (Tisdale and Nakajima, 1976), however this does not detract from its usefulness in differentiating between the two terminal types. Furthermore, the pre- and postsynaptic membranes of excitatory synapses are more densely stained than those of inhibitory synapses (Atwood et al., 1972).
Transmission electron microscope examinations reveal that neuromuscular synapses appear as electron dense areas at contact points between a nerve terminal and a muscle membrane. These contact points are actually separated by a 20 to 50 nm synaptic cleft between the rigidly aligned pre- and postsynaptic membranes (Korn, 1998). The neuromuscular connections have been studied extensively and have revealed that synapses are highly specialized for tailoring behavioural responses. Some of these specializations include excitatory neuromuscular synapses that are differentiated into phasic and tonic types, and inhibitory neuromuscular connections.

1. Excitatory Synapses

Release of neurotransmitter from excitatory neuromuscular synapses causes muscle contraction to occur via depolarization of the innervated muscle fibre. The excitatory neuromuscular synapse is the most commonly found synapse, although studies on the opener muscle of crayfish have revealed that these synapses have a smaller surface area than inhibitory neuromuscular synapses (Atwood and Kwan, 1976; Govind et al., 1994).

Excitatory terminals and neuromuscular synapses vary ultrastructurally and physiologically with properties ranging from phasic to tonic. Structurally, the terminal regions of phasic axons are thinner, and lack the varicosities that are present on tonic terminals (Lnenicka et al., 1986). They have fewer synapses per terminal length than tonic terminals (King et al., 1996; Bradacs et al., 1997; Msghina et al., 1998), but have more complex synapses containing two or more closely spaced dense bars. A smaller vesicle population and lower concentration of the transmitter glutamate were found in phasic terminals by Shupliakov et al. (1995) and King et al. (1996) who compared
phasic terminal regions with tonic terminal regions from muscle fibres innervated by both types of axons. Phasic terminals have fewer synaptic vesicles close to the presynaptic membrane (Jahromi and Atwood, 1967), although the large excitatory postsynaptic potentials are preceded by a rapid initial release of large quantities of neurotransmitter from the phasic terminal (Hoyle and Wiersma, 1958; Bradacs et al., 1997; Sherman et al., 1976; Kennedy and Takeda, 1965a; Atwood and Wojtowicz, 1986). The neuromuscular synapses of phasic terminals rapidly fatigue (Atwood, 1976), as seen in the “motor giant” axon of the crayfish abdominal flexor muscle, where a single impulse is followed by marked depression (Bruner and Kennedy, 1970). Meshina et al. (1998) studied the physiological responses of crayfish phasic motoneurons, and found that with maintained stimulation at low frequency a slowly developing decrease in the evoked current was generally produced; this decline was not seen in the terminals of the tonic axon. The fact that phasic terminals rapidly fatigue could be partly attributable to exhaustion of readily available stored transmitter. With fewer vesicles, ergo a lower transmitter concentration, and a large initial transmitter release it is not surprising that phasic axons are prone to rapid fatigue. Also, the mitochondrial content is much lower in phasic axons, and the mitochondria are smaller and less complex than those found in the terminals of tonic axons (King et al., 1996).

The impulses from tonic motoneurons are longer in duration than phasic impulses and initially cause very little transmitter release. This low amount of initial transmitter release generates smaller excitatory postsynaptic potentials that show small responses at low frequencies of stimulation (Hoyle and Wiersma, 1958; Bradacs et al., 1997; Bittner, 1968). At higher frequencies of stimulation, facilitation occurs allowing for more
neurotransmitter to be released and an increase in the excitatory postsynaptic potential in tonic synapses (Sherman et al., 1976). Thus unlike phasic terminals where the initial transmitter release was high, the initial neurotransmitter release is lower in tonic synapses but greater over prolonged bursts of impulses. Also, tonic axons fire over a greater frequency range than phasic axons (Dudel and Kuffler, 1961). Bittner and Kennedy (1970) found that with maintained stimulation at relatively high frequencies, neuromuscular synapses of tonic axons were not fatigued. These results indicate a key feature of the tonic axons; that is that they are fatigue resistant. This is reflected ultrastructurally by a higher mitochondrial content and a greater synaptic vesicle content than found in phasic motoneurons. As well, the mitochondria of tonic terminals are larger and more complex. A hypothesis proposed by Mshina et al. (1995) states that variations between phasic and tonic terminals could also be a result of differences in active zones and Ca\textsuperscript{2+} channels.

Differences between phasic and tonic terminals have given rise to the structure-function hypothesis. The structure-function hypothesis states that the probability of a quantum of neurotransmitter being released is related to the quantity of active zone material on the presynaptic membrane (Atwood and Lnenicka, 1986). Active zones are identified as dense bars surrounded by synaptic vesicles and are the putative site of calcium channels (Atwood and Lnenicka, 1986). A dense bar is an electron dense region that is 50 to 70 nm wide found on the cytoplasmic side of presynaptic membranes. Calcium influx at the active zone dense bar triggers neurotransmitter release to occur (Atwood and Lnenicka, 1986). The larger or more prevalent the dense bars the greater the calcium influx. Hence, for excitatory neuromuscular synapses the number and length
of dense bars present indicated the strength of transmitter output (Atwood and Marin, 1983; Govind and Walrond, 1989; Walrond et al., 1993). Also, with larger synapses, the number of dense bars per synapse tends to increase (Jahromi and Atwood, 1974) allowing for greater release of neurotransmitter. Synapses containing zero or one dense bar are referred to as simple synapses, while synapses containing two or more dense bars are referred to as complex. In complex synapses it is postulated that calcium clouds from two closely associated active zones overlap to increase the internal calcium concentration. As the distance between the active zones decreases, the degree of interaction increases. With a distance less than or equal to 200 nm the maximum internal calcium concentration is highest at the midpoint between the adjacent active zones and at the center of each one (Cooper et al., 1996). This calcium concentration is higher than at homologous positions for single dense bars. The overlapping of calcium clouds and increases in internal calcium concentration would be expected to increase neurotransmitter release. Furthermore, terminals releasing large amounts of neurotransmitter contain more synapses that are complex, with multiple dense bars (Cooper et al., 1996). Generally, complex synapses are recruited at lower frequencies, while simple synapses do not fire until firing frequencies are higher (Cooper et al., 1996). Synapses containing zero dense bars are typically silent, however with prolonged periods of high frequency stimulation, long-term potentiation can occur, causing these synapses to be recruited for neurotransmitter release (Atwood and Wojtowicz, 1999).

Phasic terminals in crayfish have been found to have a greater amount of calcium entry when compared with tonic terminals in the same muscle (Mshghina et al., 1995; Mshghina et al., 1998). Because dense bar length and number were not consistently larger
in the phasic terminals, it would seem that physiological aspects account for a large amount of the disparity between transmitter release and calcium channels. It is postulated that synapses of phasic terminals have a higher probability of calcium channel opening or a greater amount of available calcium channels (Msghina et al., 1998). This may be manifest by the larger percentage of complex synapses located in synapses of phasic terminals (Msghina et al., 1998).

At synapses of crustacean neuromuscular systems evidence of two types of excitatory neurotransmitter have been found: acetylcholine and glutamate. The neurotransmitter glutamate is released by excitatory motoneurons innervating the limb muscles (Sorenson, 1973). Four to six glutamate ions are required to activate a glutamate receptor on the muscle fibre and open a conductance channel (Dudel, 1975). With the opening of the conductance channel, sodium ions (Dudel, 1974), and to a small extent calcium ions, are able to enter the muscle fibre membrane channels and cause a depolarization of the muscle fibre at the synaptic site (Takeuchi and Takeuchi, 1963). If the depolarization reaches the excitation-contraction threshold of the muscle fibre, contraction of the muscle fibre will occur (Robbins, 1959; van Harreveld and Mendelson, 1959).

Studies by Marder (1974, 1976) have indicated that pyloric dilator muscles of the lobster stomach are depolarized by acetylcholine and not glutamate, yet no conclusive data has shown that acetylcholine is released in any of the limb muscles of crustaceans.

2. Inhibitory Synapses

In crustacean neuromuscular systems inhibitory neuromuscular synapses are the second most common form of synapse (Atwood, 1976). Post-synaptic inhibition occurs
when a synapse is polarized from an inhibitory terminal to a muscle fibre. Release of neurotransmitter from the inhibitory terminal causes hyperpolarization of the muscle fibre, resulting in reduction or inhibition of muscle fibre contraction.

At low frequencies of stimulation, the inhibitory neuromuscular synapses are found to have a greater quantal release of neurotransmitters than excitatory terminals in the same muscle. This may be manifest by the fact that inhibitory neuromuscular synapses are greater in surface area and have more active zones than excitatory neuromuscular synapses (Jahromi and Atwood, 1974). When high-output and low-output excitatory neuromuscular synaptic regions of the lobster distal accessory flexor muscle were compared, increased synapse size and number were found for the inhibitory axon in the high-output regions (Walrond et al., 1993; Govind et al., 1995). Also active zone number was higher at these inhibitory synapses indicating a relationship between transmitter release and neuromuscular synaptic structure.

When stimulated, inhibitory nerve terminals release the neurotransmitter gamma-aminobutyric acid (GABA) (Otsuka et al., 1966) into the synaptic cleft where it binds to GABA receptors found on the muscle membrane. Four molecules of GABA are required to bind to a receptor site in order to cause a single membrane channel to open (Feltz, 1971). GABA acts to hyperpolarize the muscle by increasing the muscle fibre membrane conductance to chloride ions and other anions (Takeuchi and Takeuchi, 1967, 1972; Motokizawa et al., 1967, 1969) thus causing a gradual decrease in contraction strength of the muscle (Atwood, 1976).


**E. Axo-axonal Synapses**

Muscle contraction in crustaceans is also controlled by axo-axonal synapses made between excitatory and inhibitory terminals. In general, axo-axonal synapses are made by the inhibitory axon onto the excitatory axon. There are also however, axo-axonal synapses made by the excitor axon onto the inhibitor axon. Each of these axo-axonal synapses are described below.

1. Excitatory Synapses

The excitatory axo-axonal synapse is polarized from an excitatory terminal to an adjacent inhibitory terminal and is rarely found in crustacean neuromuscular systems. In the three instances that excitatory axo-axonal synapses have been documented in crustacean limb muscles, the excitatory axo-axonal synapses were actually one of two correlates of a reciprocal synapse (Atwood and Kwan, 1979; Pearce and Govind, 1993). Although common in vertebrate central nervous systems (Wiersma, 1961), reciprocal synapses are rarely found in crustacean neuromuscular systems. A reciprocal synapse is an arrangement between neural elements such that chemical transmission is in opposite directions at two adjacent synapses (a reciprocal pair), or at two separate synapses (a reciprocal arrangement) (Shepherd, 1974). A reciprocal synapse thus requires two axo-axonal synapses of opposite polarities, excitatory and inhibitory, be adjacent to or near each other. Although excitatory axo-axonal synapses have only been documented in three muscles in crustacean neuromuscular systems, inhibitory axo-axonal synapses are very common.

Evidence for reciprocal synapses have been found in the limb stretcher muscle of the spider crab *Hyas areneus* (Atwood and Kwan 1979), in the limb closer muscle in the
crab *Eriphia spinifrons* and the distal accessory flexor muscle in the crayfish *Procambarus clarkii* (Pearce and Govind, 1993). In the limb stretcher muscle, which receives inhibitory innervation by both a common inhibitor and a specific inhibitor, it is not known which of the two axons are involved. Since the limb closer muscle in the crab *Eriphia spinifrons* and the distal accessory flexor muscle in the crayfish receive inhibitory innervation from only the common inhibitory axon, reciprocal synapses occur only between the excitatory and the common inhibitory terminals (Pearce and Govind, 1993). To determine if reciprocal synapses occur with specific inhibitory terminals it would be necessary to examine a muscle that receives inhibition by the two inhibitory axons in separate regions. The opener muscle is one such example, the common inhibitory axon is found only in the proximal region of the muscle, while innervation by the specific inhibitory axon is widespread (Wiens, 1984). Pearce and Govind (1993) did an extensive examination of the crayfish opener muscle and did not find any examples of axo-axonal synapses occurring from the excitatory terminal onto either the specific or the common inhibitory terminals. This is in keeping with findings of other examinations of the crayfish opener muscle where examples of inhibitor-to-excitor synapses have been found, however no examples of excitor-to-inhibitor synapses have been documented for either the common or the specific inhibitory terminals (Atwood and Morin, 1970; Atwood *et al.*, 1972; Jahromi and Atwood, 1974). Thus to date, it has not been determined with certainty if excitatory axo-axonal synapses are restricted solely to terminals of the common inhibitory axon or if they also occur onto terminals of the specific inhibitory axon.
The purpose of the reciprocal synapse in the crustacean neuromuscular system is not fully understood. It is unknown if the excitatory axo-axonal synapse has an excitatory or inhibitory effect on the inhibitory axon; in order to state for certain it would be necessary to determine if the receptor sites themselves on the inhibitory axon are inhibitory or excitatory. A few possible roles for the reciprocal synapse have been discussed in the literature. Atwood and Kwan (1979) suggest a metabotropic function for this neural arrangement while Pearce and Govind (1993) suggest the function could be to decrease the inhibitory effect of the common inhibitory axon by activating K⁺ channels within the inhibitory membrane. Further analysis would be necessary to conclusively determine the function.

2. Inhibitory Synapses

Inhibitory axo-axonal synapses, compared with the presence of excitatory axo-axonal synapses, is common in crustacean neuromuscular systems (Atwood, 1976). Inhibitory axo-axonal synapses have been found to make up 20% of the inhibitory synapses in the crayfish opener muscle (Govind et al., 1995). The release of neurotransmitter from the inhibitor decreases the effectiveness of the excitor axon by reducing the action potential or depolarization within the excitatory terminal and thereby decreases release of excitatory transmitter (Dudel and Kuffler, 1961; Atwood, 1982). This is referred to as presynaptic inhibition. Dudel (1965) and Takeuchi and Takeuchi (1966) showed that with the application of GABA to excitatory terminals mimics the effects of presynaptic inhibition. In other words, the nerve terminal potential of the excitatory terminal following application of GABA decreased as did quantal output of the excitatory neurotransmitter. Quite often, an axo-axonal synapse will occur at a bottleneck
in the excitatory terminal; a strategic location for presynaptic inhibition (Jahromi and Atwood, 1974; Atwood and Kwan, 1976). At this location presynaptic inhibition could block the propagation of the excitatory impulse and thus inhibit a small region of the excitatory terminal.

F. Limb Opener Muscle

Two inhibitory axons and an excitatory axon provide fine control of the crustacean limb opener muscle (Wiersma, 1961). The two inhibitory axons to the opener muscle are the common inhibitor and the specific inhibitor. The common inhibitory axon innervates every muscle in the crustacean limb (Rathmayer and Bevengut, 1986) and preferentially inhibits slow muscle fibres used for postural control (Atwood, 1973; Rathmayer and Erxleben, 1983; Wiens and Rathmayer 1985). This axon innervates only a restricted proximal region of the opener muscle, yet its innervation is much more widespread in other limb muscles (Bevengut and Cournil, 1990). When the limb is in a standing posture or performing very slow movements the common inhibitory axon is virtually silent, allowing for an economical maintenance of the postural positions by utilizing tonic tension. It fires when the limb begins locomotory motions and remains tonically active during locomotion eliminating tension within the muscle fibres (Ballantyne and Rathmayer 1981). Rathmayer and Bevengut (1986) suggest that the function of the common inhibitor is to prevent impeding slow muscle fibres from participating in rapid contraction and relaxation cycles during locomotion. This is accomplished by decreasing residual muscle tension in tonic contractions, allowing for more rapid alternations of muscle contractions (Wiens, 1989).
Two specific inhibitory axons also innervate the limb muscles (Wiersma, 1941) the purpose of which is to provide fine control of muscle contraction (Wiens, 1984). The stretcher and opener muscles each has its own specific inhibitory axon that acts to decouple the two muscles since both muscles share a single excitatory axon. The specific inhibitory axon of the stretcher muscle and the specific inhibitory axon of the opener muscle each can completely inhibit contraction in their respective muscles when firing at half the frequency of the common excitatory motoneuron. The speed and force of contraction in the opener and stretcher muscles are graded by variations in the ratios of excitatory axon impulses and of the specific inhibitory axon impulses. The specific inhibitory axon, by hyperpolarizing the muscle membrane, prevents contraction via postsynaptic inhibition. This axon also prevents muscle contraction by synaptically contacting terminals of the excitatory axon and decreasing its transmitter output via presynaptic inhibition.

The excitatory innervation for the opener muscle is a tonic excitatory motor axon that is shared with the stretcher muscle of the same leg (Bittner, 1968; Wiens, 1984). In the opener muscle, excitation of the tonic motoneuron causes depolarization of the muscle membrane and abduction, or opening, of the dactyl as well as contraction of the stretcher muscle. In order for contraction of the opener to occur without contraction of the stretcher, the two muscles are decoupled via action of the specific inhibitory axon of each muscle. The specific inhibitory axon of the opener fires to allow for contraction of the stretcher muscle without abduction of the dactyl. This role of decoupling is evident in the development of the specific inhibitory and excitatory axons where the two grow simultaneously into the opener muscle fibre allowing for a close association between the
two (Atwood and Kwan, 1976). In opener muscles of adult crayfish the two axons are found in parallel and closely associated, however the two do travel apart for several microns at a time and separate terminals can be found.

**G. Swimming Paddle Opener Muscle of Blue Crabs**

*Callinectes sapidus*, the blue crab, is a species known for its swimming abilities. The blue crab has several exoskeletal adaptations that have allowed it to become an excellent swimmer. For example, the body of the blue crab is streamlined, allowing efficient movement through the water (Cochran, 1935). Also, and perhaps more relevant to the present study are the changes seen in the fifth pereiopod, or the swimming limb. In other crustaceans, such as the crayfish, the fifth pereiopod is a walking limb and its dactyl is elongated and narrow, shaped much like the dactyls of the second, third and fourth pereiopods. In the blue crab, the fifth pereiopod has been adapted into a swimming paddle that is also used in males in courtship displays (Teytaud, 1971; Wood and Derby, 1995). The propus of the fifth pereiopod is flattened while the dactyl is enlarged, flat, and oval-shaped to function as a paddle.

Movement of the dactyl occurs with contractions of the opener and closer muscles, which have their origins on the propus and insertions on the dactyl. Abduction or opening of the dactyl occurs with contraction of the opener muscle, while adduction or closing occurs with contraction of the closer muscle. In the walking limb, opening and closing of the dactyl occurs intermittently during locomotion and to maintain postural control. In the swimming paddle, opening and closing of the dactyl occurs for prolonged periods with rapid alternations during sideways swimming (Teytaud, 1971). The base of the swimming paddle articulates with the thorax such that it is rotated 90° relative to the
walking legs. Abduction brings the dactyl in line with the other limb segments and in this way increases the effectiveness of the dactyl to function as a paddle. Furthermore, adduction brings the dactyl closer to the propus and hence decreases its effectiveness as a swimming paddle.

**H. Objectives**

Amongst non-swimming crustaceans such as the crayfish, the limb opener muscle is small compared to the antagonistic closer muscle and contracts intermittently during walking. In contrast, the blue crab opener muscle in the swimming paddle is as large as the antagonistic closer muscle and contracts rapidly for prolonged periods of time during swimming. The limb opener muscle is innervated by one excitatory axon, as well as a common inhibitory axon with restricted innervation, and a specific inhibitory axon with innervation that is widespread (Wiens, 1984). The neuromuscular system of the crayfish walking limb opener muscle has been extensively investigated, but little is known about the homologous muscle in the swimming paddle of blue crabs.

Using thin section electron microscopy I intend to describe the morphological features of the inhibitory and excitatory motoneurons innervating the central fibres of the opener muscle in the swimming paddle. I will begin by describing the qualitative features of nerve terminals, their synapses and active zone dense bars. Next, in an attempt to correlate structure with function, this description will include a quantitative analysis of the components of nerve terminals, of neuromuscular and axo-axonal synapses, and of their active zone dense bars. Such a quantitative analysis for the excitor and inhibitor axons to the swimming paddle opener muscle may reveal some features that are an adaptation for the unique function of this muscle in swimming.
Materials and Methods

Adult female blue crabs, Callinectes sapidus, were purchased from a local fish market and held in artificial seawater at 22°C. The fifth pereiopod or swimming paddle was autotomized and the opener muscle was exposed by removing the overlying exoskeleton and the adjacent closer muscle. The dissection was carried out in a marine saline solution composed of 472 mM NaCl, 10 mM KCl, 16 mM CaCl₂, 7 mM MgCl₂·6H₂O, 10 mM glucose, and 10mM Hepes at pH 7.4 (Govind and Lang, 1974). The exposed opener muscle was held at approximately rest length by pinning out the dactyl and merus. The muscle was then prepared for electron microscopy as outlined below. In total, four muscles from two animals were fixed.

A. Electron Microscopy

The exposed opener muscle was flooded with primary fixative initially at 4°C and fixed in situ for one hour. The primary fixative consisted of 2.5% glutaraldehyde with 0.2% formaldehyde, 2 mM calcium chloride, 0.3 M sucrose and 0.06 M sodium chloride carried in a 0.15 M sodium cacodylate buffer with pH 7.5 (Govind and Pearce, 1982). Small groups of muscle fibers were dissected free and fixed for an additional hour in fresh fixative at 4°C. The tissue was rinsed in several changes of 0.15 M cacodylate buffer that contained 0.3 M sucrose, 0.06 M sodium chloride and 2 mM calcium chloride for 30 minutes. The tissue was postfixd for one hour in cacodylate-buffered 2% osmium tetroxide. The fibers were washed briefly in the buffer solution for 10 minutes with two changes and dehydrated in a graded series of ethanol, 70%, 80%, 90%, 95% and 100%,
for 10 minutes with two changes in each of the series. The tissue was next cleared in propylene oxide for 30 minutes with three changes. The tissue was left overnight in a 50% propylene oxide – 50% Epon-Araldite mixture to allow for gradual infiltration of the resin. The following day, the tissue samples were placed in moulds containing fresh Epon-Araldite and left at room temperature for five hours after which the moulds were placed in an oven at 60°C for 24 hours.

Thin sections (50 to 95 nm) of several of the blocks containing muscle fibres from the central region of the opener muscle were cut using a diamond knife mounted in a Reichert OMU 2 ultramicrotome. These thin sections were examined for nerve terminal regions and suitable selected regions were subsequently cut in serial thin sections. The sections appeared as ribbons of thin sections floating in the distilled water in the boat of the diamond knife. These sections were straightened using chloroform fumes. After the ribbon of thin sections was straightened, the interference colours were recorded in order to determine section thickness. The colours of the thin sections ranged from grey to gold, corresponding to section thicknesses of 50 to 95 nm. A single slot copper grid was placed on top of a floating thin section ribbon. With the sections suspended on a drop of water inside the grid each grid was placed on a formvar coat stretched over an aluminum grid rack. The thin sections were left to dry on the grid rack allowing the sections to adhere to the formvar, and the formvar to adhere to the single slot grid. The grids were then removed from the rack and stained in aqueous uranyl acetate for 30 minutes, rinsed with distilled water, and stained with lead citrate for 2 minutes, then washed and dried.

Thin sections of the tissue were viewed with a Siemens102 or Hitachi H7500 electron microscope. Images taken with the Siemens 102 electron microscope were
photographed routinely at 8,000x and were printed with a final magnification of 26,000x. These prints were used for qualitative examination and analysis of structural features of the innervation. Specific areas of interest were also photographed at a higher magnification of 20,000x and were made into photographic prints of varying magnifications.

Thin sections of innervation were also examined with a Hitachi H7500 electron microscope equipped with a digital camera (MegaView II). Digital images with a final magnification of 31,260x were viewed on the computer screen and initial quantitative data was obtained using the AnalySIS computer program. Subsequently these images were printed using a Lexmark printer.

In order to examine the fine structure of the muscle fibre, a few thin sections of the opener muscle were examined with the Siemens 102 electron microscope. Longitudinal sections of opener muscle fibres were examined using photographs with a final magnification of 6,000x. To measure sarcomere lengths in these photographs, distances between adjacent Z-lines were measured. The A-band lengths within these sarcomeres were also measured. Cross-sections of the opener muscle fibres at a final magnification of 150,000x were examined to determine the number of thin-to-thick filaments. Several thick filaments were selected on the basis of clarity and ability to identify individual surrounding thin filaments, and in these the number of thin filaments surrounding each of the thick filaments was counted. The average number of thin filaments surrounding one thick filament was then calculated.
B. Quantitative Analysis

Micrographs of the thin serial sections of the nerve terminals in the opener muscle were used to obtain quantitative data on features of the neuromuscular innervation. Individual nerve terminals were traced through these serial micrographs and within these terminals synapses and presynaptic dense bars were identified and labeled numerically. Nerve terminal length was determined by summing the thickness of each section in which the nerve terminal was present.

The number of synapses was determined by counting only completely sectioned synapses in the serial micrographs. The surface area of these synapses was calculated by first measuring the length of the synapse on individual micrographs using calipers preset at 2 mm. The length was multiplied by the magnification factor to convert to microns then multiplied by section thickness to obtain the surface area of the synapse on a single micrograph. These individual areas from each micrograph were added together to obtain the surface area for a completely serially sectioned synapse. Synaptic area per terminal length was measured using the sum of the synaptic area for incomplete and complete synapses and dividing this total by terminal length.

Dense bars were identified on the presynaptic membrane as electron dense dumbbell-shaped regions, approximately 60 nm in width. The number and distribution of presynaptic dense bars was calculated. The total number of dense bars in the terminal regions was determined by adding together all of the dense bars found in both incomplete and complete synapses. The length of dense bars cut in cross-section was determined by summing the thickness of each section in which a dense bar appeared in both complete and incomplete synapses. The length of a longitudinally cut dense bar was determined by
measuring the length of the dense bar on a micrograph using calipers and multiplying this length by the magnification factor. The number of dense bars per synapse was calculated by adding together the number of dense bars found in complete synapses and dividing this sum by the number of complete synapses. Dense bar length per area of synapse was calculated using the sum of dense bar lengths from incomplete and complete synapses and dividing this value by the synaptic area of incomplete and complete synapses.

**C. Volumetric Analysis**

Serial micrographs of the nerve terminals allowed me to calculate the volume percent of the terminal occupied by different components making up the nerve terminal. These included clear synaptic vesicles, dense core vesicles, mitochondria, glycogen granules, membrane bound sacs, and axoplasm. A dot-grid system used routinely in our laboratory was used in the volumetric analysis (King *et al.*, 1996). An acetate sheet with dots marked 0.375 cm apart was placed over top of every fifth micrograph in a series and the number of dots falling on the different components of each nerve terminal was counted. This method also gave the total number of dots falling within entire nerve terminal profiles. In order to calculate percent composition of the terminals the number of dots counted for each component was divided by the total number of dots counted in each terminal. To calculate terminal volume the number of dots in each terminal was totaled and multiplied by \((0.375 \times \text{magnification factor in microns})^2\) to give a value for surface area of the terminal in each section analyzed. This surface area was then multiplied by the length of the terminal, using section thickness in microns, from one section to the next analyzed section. This procedure was repeated for all of the analyzed sections and summed to give a total terminal volume.
The opener muscle from the swimming paddle of two separate animals was examined in this study. Samples from both animals were used for the qualitative description of the neuromuscular apparatus. Quantitative analysis using thin serial sections was restricted to one of the animals that showed a better preservation of the tissue. Data for synapse size and dense bar length between excitatory and inhibitory terminals were treated to statistical testing, using the student’s t-test (two-tailed), with an alpha level set at 5 percent (p < .05).
Results

A. Opener Muscle

Movements of the swimming paddle (Fig. 1) are brought about by a pair of antagonistic muscles located in the propus of the fifth pereiopod. The opener and closer muscles have origins at the proximal end and insertions at the distal end of the propus. The tendons of these muscles insert on the dactyl so that contraction of the opener muscle abducts the dactyl while contraction of the closer addsucts it. The size relationship between the paired muscles, as seen in cross section (Fig. 1B), demonstrates that the opener is as large, if not larger, in diameter than the closer in the central region of the propus. Measurements of the muscle perimeter taken at five cross-sectional sites show that the opener is at least 30% larger than the closer with the average perimeter being 1.5 times larger than that of the closer.

Previous studies in our laboratory have shown that the opener muscle is composed of slow fibres, based on sarcomere lengths of 6 to 12 µm that were measured using light microscopy (Dawson and Govind, unpublished observations). I was able to confirm these results with electron microscopy. Longitudinal sections of the muscle (Fig. 2A) show that the sarcomeres are not regularly aligned and that the Z-lines are relatively thick and wavy, qualitative features that are characteristic of slow crustacean muscle (Atwood, 1976).

To confirm the light microscopic measurements the lengths of twelve sarcomeres were measured and the average length (±sd) was calculated to be 8.43 (±0.62) µm with a range of 7.05 to 9.23 µm. To ensure that these average lengths were not due to
Figure 1. A: The dactyl of the fifth pereiopod in the blue crab, *Callinectes sapidus*, is flat and oval shaped allowing for use as a swimming paddle. Abduction and adduction of the dactyl occurs respectively with contraction of the opener and closer muscles situated within the propus.

B: The opener muscle is larger in girth than the closer muscle in most regions as seen in the frozen cross section of the propus stained for NADH-diaphorase.

Scale bar: 2 mm. Magnification: B x5.5
Figure 2. A: Longitudinal section of the opener muscle reveal long-sarcomeres (between Z-lines), A-bands (AB), I-bands (IB), and thick, wavy Z-lines (Z). B: Cross-sections of the opener muscle show 13 thin filaments surrounding a single thick filament.

Scale bars: A 2 μm; B 0.2 μm. Magnification: A x6,000; B x150,000.
sarcomeres becoming unduly stretched during fixation, the A-band lengths were measured as these are not prone to artificial stretching. Twelve A-bands were measured and the mean length (±sd) was calculated to be 6.18 (±0.53) μm with a range of 5.20 to 6.88 μm. The A-bands comprised approximately 60 to 80% of the sarcomere lengths, thus indicating that the sarcomeres measured were fixed at rest length.

Cross-sectional views of the muscle fibres reveal the alignment and number of thin and thick filaments (Fig. 2B). Twelve to fourteen thin filaments were found to surround one thick filament, with a mean value of thirteen thin filaments surrounding one thick filament (n=12). These counts give a thin-to-thick filament ratio of 6.5:1, as each thin filament is shared by two thick filaments. Since the muscle fibres had a thin to thick filament ratio in the range of 4-7:1 (Atwood, 1976), these findings also indicate that the muscle fibres of the opener muscle are slow.

B. Innervation

The main objective of this study was to describe the fine structure of the axons innervating the opener muscle in the blue crab paddle limb, using thin section electron microscopy. To assess the quality of the preserved tissue and locate innervation sites, sample sections were cut at random from fibres removed from the central region of the opener muscle in two different animals. An initial examination of two adjacent muscle fibres viewed in a single cross-section revealed that both fibres had three separate sites of innervation located around their perimeter. General features of an innervation site are described in detail later. Figure 3 illustrates a single large fibre that has six sites of
**Figure 3.** A cross-section of a single fibre from the blue crab opener muscle displaying multiple innervation sites (arrows). Here the six individual sites happen to be congregated on one face of the fibre.

*Scale bars: 200 µm. Magnification: x400.*
innervation, all of which happen to be restricted to one face of the muscle. A random survey of single cross-sections examining 15 muscle fibres from the two animals, revealed that all 15 fibres had at least one innervation site, while 10 fibres had multiple (2 to 6) innervation sites. In total 31 innervation sites were located on the fifteen fibres, with a mean of two innervation sites per muscle fibre. A sampling of other limb muscles (Pearce and Govind, unpublished observations) and stomach muscles (Patel 1997) in the blue crab did not reveal such profuse innervation of muscle fibres.

Sites of innervation are typically located around the periphery of a muscle fibre beneath the sarcolemma and are made up of profiles of nerve terminals and associated glial cells (Fig. 4). The terminals are embedded in muscle granular sarcoplasm, a specialized region of the muscle devoid of actin and myosin filaments. It is recognized by the granular appearance of the cytoplasm that is usually populated with mitochondria, and displays an intricate network of tubules or channels that serve to increase the surface area of the muscle membrane (Fig. 4). Innervation sites displayed a variable number of terminal profiles (2 to 4), however the most common situation encountered was two profiles in fairly close proximity to one another as seen in Figure 5A. Mitochondrial profiles are a typical component encountered within most terminals. Some of the mitochondria appear as single, circular or elliptical profiles, while others are highly branched complex structures as seen in a more longitudinal view of terminal regions (Fig. 5A). Also evident in the terminals were single membrane-bound sacs that were without any apparent content. These sacs were highly variable in diameter ranging from 100 to 400 nm. Terminals also contained glycogen granules (Fig. 5A) recognized as very
Figure 4. Cross-section of an innervation site showing four terminal profiles, two excitatory (E), and two inhibitory (I). The innervation site is located beneath the muscle sarcolemma (ms) in a region devoid of contractile elements. Glial cells (gc) and granular sarcoplasm (gs) are located adjacent to the terminals and muscle mitochondria (mm) are in close proximity. The terminals are irregularly shaped and form numerous synaptic contacts with the muscle membrane (arrows). Inside the terminals, mitochondria (m), and single membrane bound sacs (mb) are interspersed. Located within this cross section is an example of an interdigitation (arrowhead), where a finger of an excitatory terminal has branched into an adjacent inhibitory terminal.

Scale bar: 2 μm. Magnification: x22 320.
**Figure 5.** A: Nerve terminals showing spherical synaptic vesicles characteristic of an excitatory (E) motoneuron and elliptical vesicles characteristic of an inhibitory (I) motoneuron. Both terminals are populated mainly by clear synaptic vesicles (c), a few dense-core vesicles (d), mitochondria (m), and glycogen granules (g). Synaptic contacts (between arrows) appear adjacent to muscle granular sarcoplasm (s) and are recognized by densely stained opposing membranes separated by a uniform synaptic cleft filled with electron dense material. Presynaptic dense bars with clustered synaptic vesicles denote an active zone (arrowhead). B: An innervation site on the swimming paddle opener muscle, showing two axon profiles, an excitatory (ae) and inhibitory (ai), that give rise to excitatory (e) and inhibitory (i) nerve terminals situated within the granular sarcoplasm (s) of the muscle fiber.

Scale bars: A 1 μm; B 5 μm. Magnification: A x33, 750; B x6, 000.
electron dense, irregularly shaped particles, scattered throughout the cytoplasm. There were also a few small (80 to 100 nm diameter) vesicles with a dense core.

A very prominent and characteristic component of all nerve terminals are the small (40 to 50 nm diameter), clear, synaptic vesicles (Figs. 4, 5A). The shape of these synaptic vesicles allowed me to identify the terminals as excitatory or inhibitory. Due to the aldehyde fixation process, excitatory vesicles are spherical, and inhibitory vesicles are elliptical in shape (Atwood et al., 1972; Jahromi and Atwood, 1974). The difference in vesicle shape is striking between the two axonal types (Fig. 5), a feature more pronounced in marine crustaceans than in freshwater species (personal observation).

In a few cases, it was possible with thin serial sectioning to trace excitatory and inhibitory terminals to where they branch from the pre-terminal axons (Fig. 5B). In these examples there were only two axonal profiles, an excitatory and an inhibitory axon, which is in keeping with the finding usually two profiles in any one innervation site. On this basis, it was tentatively determined that two axons innervate the central region of the blue crab opener muscle, one excitatory and one inhibitory.

In both inhibitory and excitatory terminals, clear synaptic vesicles were found to congregate at the site of a synapse (Figs. 5A, 6A). For the purpose of this study, the synapse was defined as a specialized region where the more densely stained pre- and postsynaptic membranes were separated by a uniform gap that was filled with electron dense material (Fig. 6A). Dense bars were found on most of the synapses and were identified as electron dense, dumb-bell shaped, structures on the presynaptic membrane of the terminal surrounded by an accumulation of synaptic vesicles. In several instances, adjacent to the dense bar, omega shaped indentations of membrane into the terminal
**Figure 6.** A: Excitatory nerve terminal showing a synapse (between arrows), recognized by the electron dense pre- and postsynaptic membranes, and the presynaptic dense bar, shows evidence of exocytosis. Exocytosis was identified at this synapse by an omega-shaped figure (double-arrow) on the presynaptic membrane adjacent to an active zone (arrowhead). B, C: Excitatory nerve terminal showing evidence of endocytosis (arrows) by semi-circular indentations into the presynaptic membrane. The indentation has a fuzzy coat indicating vesicle endocytosis. Endocytosis was located within a synapse (B), as well as at the end of a synapse (C).

Scale bar: 0.4 μm. Magnification: x100 000.
were seen, representing a synaptic vesicle that has fused to the presynaptic membrane. Based on the location and shape of these omega-shaped figures they were identified as sites of exocytosis. Therefore dense bars represent active zones where vesicle exocytosis occurs (Pearce et al., 1986). Another interesting qualitative feature, found in several terminals, on the cytoplasmic side are small semi-circular indentations of the membrane (Fig. 6B, C). These semi-circular indentations are surrounded by a halo of fuzzy material. Based on the shape and location, these indentations were identified as sites of endocytosis and the presence of a fuzzy coating is indicative of clathrin coating.

As previously mentioned, the majority of innervation sites encountered around the periphery of muscle fibres displayed a single excitatory and inhibitory profile in close proximity to each other, with the majority of fibres having more than one innervation site. To illustrate the spatial relationship between excitor and inhibitor, as well as emphasize the amount of synaptic contacts formed onto the muscle via the excitor, a 3-dimensional reconstruction of just one of three sites located on a single fibre is shown in Figure 7. Here the small diameter excitor axon expands into a large synapse-bearing terminal which branches sending a long terminal (72 μm in length) down the side of the fibre. The main inhibitory axon runs adjacent to the excitor (not shown in figure) and branches in parallel with the excitor, out of the plane of section. In the drawing, the inhibitory terminals have been separated from the excitatory, in order to provide a better view of the two. The arrows indicate the actual sites of abutment between the two axons. Both terminals are populated with synapses (shaded areas) and display numerous dense bars (heavy black dots). These two axons continue down the fibre and are viewed again in the second innervation site (Fig. 4) where the two profiles of both axons are present and
Figure 7. A three-dimensional reconstruction of primarily the excitatory axon found in just one of three innervation sites around the periphery of a single muscle fibre from the central region.

Here the small diameter excitatory axon enlarges into a synapse bearing terminal and branches, sending a long lateral synapse-bearing terminal down the side of the fibre. The inhibitory axon runs parallel with the excitatory and produces synapse-bearing terminals that have been disassociated from the excitor for easier viewing. The arrows indicate the points of lateral abutment between the two axons. Synapses on the terminals are shown by shaded regions and dense bars as black dots within the shaded regions.

Scale bars: longitudinal 10μm; horizontal 2μm
where a small profile (arrowhead) filled with clear round vesicles appears to be inside the inhibitory axon, it is at this site where an interesting interdigitation of inhibitory and excitatory terminals is seen. Figure 8 shows a series of 6 micrographs focusing on this pair where a small profile of the excitatory terminal appears to be within the inhibitory terminal (Fig 4, Fig 8A), but when followed in serial micrographs, this excitatory profile is seen to arise via a small branch of the excitor protruding into the inhibitor. Figure 9 shows another example of an interdigitation where an inhibitory terminal sends a small finger into an excitatory terminal.

At the interdigitations shown in Figures 8 and 9, synapses were seen between the two axons i.e. axo-axonal synapses. Thus, branches of the excitatory and inhibitory axons not only made synapses on the muscle membrane but also with each other. The axo-axonal synapses were not restricted to interdigitations of the two axons but also occurred where the two axons were adjacent to each other (Fig.10). Axo-axonal synapses had densely stained pre- and postsynaptic membranes that ran parallel forming an evenly spaced synaptic cleft, but typically lacked the electron dense material in the cleft. These synapses had dense bars that showed a congregation of clear vesicles on the presynaptic membrane. Most of the axo-axonal synapses were polarized from the inhibitor onto the excitor, judging from the presynaptic dense bar on the inhibitory terminal (Fig. 8, 9A-C). However, a few axo-axonal synapses were seen from the excitatory to the inhibitory terminal (Fig. 9D-F, 10). Both types of axo-axonal synapses occurred at separate locations although in one case the two were adjacent to each other. This is shown in Figure 9 where an inhibitory axo-axonal synapse (Fig. 9A-C) is followed directly by an excitatory axo-axonal synapse (Fig. 9D-F).
Figure 8. Axo-axonal synaptic contact (between arrows) from an inhibitory (I) terminal onto an excitatory (E) terminal (recognized respectively by their elliptical and spherical synaptic vesicles) extending through 10 serial micrographs of which only six (A-F) (numbers 3, 5, 6, 7, 9, 10) are shown. The axo-axonal synaptic contact is made onto a small branch of the excitatory terminal that projects into the inhibitory terminal; the presynaptic dense bars (arrowheads), occurring on the inhibitory membrane, indicates the polarity of the synapse. Three different dense bars are shown in this figure, two extend through one section, and the other is long extending through seven thin serial sections.

Scale bars: 0.5 μm. Magnification: x50 000.
Figure 9. A-C: A branch of the inhibitory terminal (I) adjacent to the excitatory terminal (E) (identified respectively by their elliptical and spherical synaptic vesicles) where an axo-axonal synapse is located from the inhibitory terminal to the excitatory terminal (arrows). The polarity of the synapse is indicated by the presence of two dense bars on the presynaptic membrane (arrowheads) of the inhibitory terminal. D-F: The branch of the inhibitory terminal projects into the excitatory terminal where an axo-axonal synapse is located from the excitatory terminal to the inhibitory branch (arrows). The polarity of this synapse is indicated by the presence of a dense bar (arrowhead) on the presynaptic membrane of the excitatory terminal.

A reciprocal synapse is thus located at this interdigitation of the two axons as synapses of opposite polarities are present within the same terminals in this consecutive series of six micrographs (A-F).

Scale bar: 0.5 μm. Magnification: x30 120.
Figure 10. A, B: Axo-axonal synaptic contact (between arrows) from an excitatory (E) onto an inhibitory (I) terminal, each characterized respectively by their spherical and elliptical synaptic vesicles. The dense bar (arrowhead), indicative of synapse polarity from the excitor, is nestled in a prominent trough formed by both pre- and postsynaptic membranes. Note the absence of electron dense material in the synaptic cleft that is so characteristic of neuromuscular synapses, but is lacking in the axo-axonal junctions.

Scale bars: 0.3 μm. Magnification: x100 000.
C. Nerve Terminals

In order to provide a quantitative analysis of the innervation to the blue crab opener muscle, four sites that were fairly representative of those observed in survey sections were serially sectioned for lengths of 4.5 to 11.6 μm. The resulting serial micrographs of the innervation showed that there was much more excitatory innervation than inhibitory innervation. This was seen in the total length of the two terminal types (Table 1). The total excitatory terminal length was calculated to be 118.2 μm, while the total inhibitory terminal length was calculated to be 41.4 μm. Thus, within the sampled innervation sites, the excitatory axon had approximately three times the terminal length as the inhibitory axon. Another measurement that showed the difference in amount of innervation between excitatory and inhibitory terminals was a volumetric analysis of two of the serially sectioned sites. The total volume was 5969.3 μm³ for the excitatory terminals and 3932.0 μm³ for the inhibitory terminals representing a 1.5 fold greater volume for the excitatory than the inhibitory terminal. Thus, the excitatory terminal was greater in terminal length and volume than the inhibitory terminal in the sample examined in my study.

Both types of nerve terminals were populated by clear synaptic vesicles, dense core vesicles, mitochondria, glycogen, and membrane bound sacs. The percent volume of nerve terminal occupied by these different components was calculated by doing a quantitative analysis of the serial micrographs of the four innervation sites and the results
Table 1. Quantitative analysis of nerve terminals, neuromuscular synapses from excitatory and inhibitory axons to the opener muscle in the blue crab swimming paddle.

<table>
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<th>Excitatory (E)</th>
<th>Inhibitory (I)</th>
<th>E/I ratio</th>
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<tr>
<td>Terminal length (μm)</td>
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<td>Total number of synapses</td>
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<td>Number of complete synapses</td>
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<tr>
<td>Mean synaptic area (μm²) for complete synapses</td>
<td>0.804±0.571</td>
<td>1.040±1.430</td>
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Table 2. Percent composition of the nerve terminal occupied by cellular constituents found in excitatory and inhibitory nerve terminals in the blue crab swimming paddle opener muscle.

<table>
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<th>Excitatory (E)</th>
<th>Inhibitory (I)</th>
<th>E/I ratio</th>
</tr>
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<tr>
<td>% clear vesicles</td>
<td>22.5</td>
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</tr>
<tr>
<td>% dense vesicles</td>
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<td>% membrane bound sacs</td>
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<td>% axoplasm</td>
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Figure 11. Histogram of percent composition of nerve terminals occupied by organelles and cellular constituents in excitatory and inhibitory nerve terminals in the blue crab swimming paddle opener muscle
are presented in Table 2 and Fig. 11. The most abundant component of the nerve terminals was the clear synaptic vesicles.

I found that just less than one quarter of the volume percentage for each terminal type was composed of clear vesicles. Dense core vesicles, thought to contain neuromodulatory substances (Thureson-Klein et al., 1988) were found sporadically throughout the two terminal types, and were the least prevalent of the components.

The energy substrate content of the excitatory and inhibitory terminal types was found in the form of mitochondria and glycogen granules. The mitochondrial content was similar between the two types of nerve terminals at slightly less than 10% (Table 2, Fig. 11). The glycogen content differed between the two terminal types with the excitatory terminal showing a higher content than the inhibitory terminal. When the total energy substrate content was added together, it was found that the excitatory terminals had a higher energy substrate content of 20.8% while the inhibitory terminals were found to have a lower content of 16.3%.

Also found within the two terminal types were membrane bound sacs, which are of unknown function but may represent some aspect in the recycling of membranes within the terminal. These sacs were located throughout both terminal types with a content of slightly less than 5% (Table 2, Fig. 11).

Thus the most prevalent constituent of each terminal type are clear synaptic vesicles followed in decreasing order by glycogen, mitochondria, membrane bound sacs and dense core vesicles (Table 2, Fig. 11). The volume percent of the different components is similar between excitatory and inhibitory terminals with a ratio of around 1.
Neuromuscular Synapses

Arising from the large "off-shore axons" (Fig 5B) are finer branches that travel beneath the muscle basal lamina and expand into large synapse-bearing nerve terminals. Neuromuscular synapses located from both terminal types were identified on the serial micrographs from the four innervation sites and analyzed for their size (Table 1). In terms of the number of synapses there were in total 175 excitatory and 56 inhibitory synapses. Excitatory neuromuscular synapses occurred every 0.675 μm of terminal length, while inhibitory neuromuscular synapses occurred every 0.739 μm of terminal length. However, of the total number of synapses identified, many occurred at the beginning or end of the serial sections and were regarded as being incompletely sectioned. Therefore only 86 synapses were completely serially sectioned for the excitatory axon and 39 for the inhibitory axon (Table 1). There were twice as many complete excitatory synapses as complete inhibitory synapses and this is seen in the ratio of these two types. The size of the complete synapses was also measured. The excitatory synapses had a range in surface area of 0.006 to 3.847 μm² with a mean area of 0.804 μm². The inhibitory neuromuscular synapses ranged in size from 0.022 to 7.50 μm² with a mean area of 1.040 μm². Inhibitory neuromuscular synapses were therefore on average 25% larger than excitatory neuromuscular synapses, with the largest inhibitory neuromuscular synapse being approximately twice the size of the largest excitatory neuromuscular synapse. When the surface area of synapses is normalized to the terminal
length sampled, the excitatory terminals had a much smaller synaptic area per terminal length than the inhibitory terminals as reflected in the ratio between the two types.

On examination of the synapses it was apparent that many of the excitatory and inhibitory neuromuscular synapses contained dense bars (Table 3, Fig. 12), representing active zones for transmitter release (Atwood and Lnenicka, 1986). Dense bars were present at most of the synapses although 37% of excitatory and 36% of inhibitory synapses that were completely sectioned lacked a dense bar.

Excitatory neuromuscular synapses contained 190 dense bars ranging in length from 0.04 to 0.195 μm with a mean length of 0.092 μm (Table 3). However only 114 of these dense bars were located in complete synapses. The number of dense bars per complete synapse ranged from zero to six with the majority having at least one dense bar and 21% of these having three or more (Fig. 12).

Inhibitory neuromuscular synapses also contained dense bars, however these tended to be longer than excitatory neuromuscular dense bars (Table 3). In inhibitory neuromuscular synapses, 74 dense bars were located ranging in length from 0.04 to 0.295 μm with a mean length of 0.131 μm, however only 53 of these dense bars were located in complete synapses. The number of dense bars per synapse ranged from zero to eight with the majority of inhibitory neuromuscular synapses having at least one dense bar and 18% of these having three or more (Fig. 12).

In conclusion, more dense bars were found in the excitatory neuromuscular synapses than in inhibitory neuromuscular synapses, however, these had a smaller range in size and were 30% smaller than the dense bars from inhibitory neuromuscular synapses (Table 3). When comparing the number of dense bars per synapse for the
Table 3. Quantitative analysis of dense bars from excitatory and inhibitory axons to the opener muscle in the blue crab swimming paddle.

<table>
<thead>
<tr>
<th></th>
<th>Excitatory (E)</th>
<th>Inhibitory (I)</th>
<th>E/I ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal length (µm)</td>
<td>118.177</td>
<td>41.400</td>
<td>2.855</td>
</tr>
<tr>
<td>Total number of dense bars</td>
<td>190</td>
<td>74</td>
<td>2.568</td>
</tr>
<tr>
<td>Total dense bar length</td>
<td>17.425</td>
<td>9.755</td>
<td>1.786</td>
</tr>
<tr>
<td>Mean dense bar length (µm)</td>
<td>*0.092±0.040</td>
<td>*0.131±0.060</td>
<td>0.702</td>
</tr>
<tr>
<td>Dense bar length/ µm terminal</td>
<td>0.147</td>
<td>0.236</td>
<td>1.787</td>
</tr>
<tr>
<td>Number of dense bars in complete synapses</td>
<td>114</td>
<td>53</td>
<td>2.151</td>
</tr>
<tr>
<td>Dense bar number/complete synapse</td>
<td>1.326±0.816</td>
<td>1.359±1.393</td>
<td>0.975</td>
</tr>
<tr>
<td>Dense bar length/µm² of synapse</td>
<td>0.146</td>
<td>0.165</td>
<td>0.886</td>
</tr>
</tbody>
</table>

* statistically significant (p < .05)
Figure 12. Histogram of percentage of complete synapses containing different numbers of dense bars from 0 to 5 in excitatory and inhibitory neuromuscular synapses of the blue crab swimming paddle opener muscle.
Number of dense bars per complete synapse

Percent of complete synapses

- Excitatory
- Inhibitory
excitatory neuromuscular and the inhibitory neuromuscular synapses the two had similar values of 1.33 and 1.36 respectively. Also, when dense bar length per terminal length was compared the value was 12% smaller in the excitatory terminals than in the inhibitory terminals (0.146 μm for excitatory neuromuscular terminals and 0.165 μm for inhibitory neuromuscular terminals).

**E. Axo-axonal Synapses**

Two types of axo-axonal synapses were documented, the more common type being the inhibitory axo-axonal synapse. At inhibitory axo-axonal synapses, the synapse is polarized from the inhibitory terminal while the target cell is the excitatory axon. Inhibitory axo-axonal synapses occurred in each of the inhibitory terminals examined. A very few of these synapses occurred on interdigitations of the excitatory axon into the inhibitory axon (Fig. 8). The majority of inhibitory axo-axonal synapses occurred when the two terminals were simply adjacent to each other. The second type of axo-axonal synapse was the excitatory axo-axonal synapse and occurred from the excitatory terminal onto the inhibitory terminal (Fig. 9D-F, 10). At least one excitatory axo-axonal synapse was found in each of the four serially-sectioned innervation sites examined.

Quantitative analysis was done for the inhibitory and excitatory axo-axonal synapses from the serial micrographs of the four innervation sites (Table 4). There were 5 excitatory and 25 inhibitory axo-axonal synapses. Of the synapses that were identified, 4 synapses were completely serially sectioned for the excitatory axon and 22 for the inhibitory axon. The excitatory synapses ranged in size between 0.013 to 0.109 μm² with a mean area of 0.016 μm². The inhibitory axo-axonal synapses ranged in size from 0.008
Table 4. Quantitative analysis of excitatory and inhibitory axo-axonal synapses in the opener muscle to the blue crab swimming paddle

<table>
<thead>
<tr>
<th></th>
<th>Excitor</th>
<th>Inhibitor</th>
<th>E/I ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal length (μm)</td>
<td>118.177</td>
<td>41.4</td>
<td>2.855</td>
</tr>
<tr>
<td>Total number of synapses</td>
<td>5</td>
<td>25</td>
<td>0.200</td>
</tr>
<tr>
<td>Total synaptic area (μm²)</td>
<td>0.171</td>
<td>6.166</td>
<td>0.028</td>
</tr>
<tr>
<td>Synaptic area/terminal length</td>
<td>0.00144</td>
<td>0.1489</td>
<td>0.007</td>
</tr>
<tr>
<td>Number of complete synapses</td>
<td>4</td>
<td>22</td>
<td>0.182</td>
</tr>
<tr>
<td>Mean synaptic area (μm²) for complete synapses</td>
<td>*0.016±0.017</td>
<td>*0.136±0.207</td>
<td>0.117</td>
</tr>
</tbody>
</table>

* statistically significant (p < .05)
to 2.41 μm² with a mean area of 0.136 μm². Inhibitory axo-axonal synapses were therefore on average 6.5 times larger than excitatory axo-axonal synapses with the largest inhibitory axo-axonal synapse approximately twenty times the size of the largest axo-axonal synapses from the excitatory terminal. When the surface area of synapses was normalized to the terminal length sampled, the excitatory terminals had a very much smaller synaptic area per terminal length (0.001 μm) than the inhibitory terminals (0.15 μm) as reflected in the ratio between the two types (0.007).

Following the definition of Shepherd (1974), that a reciprocal synapse refers to an arrangement between neural elements in which chemical transmission is in opposite directions at two adjacent or separate synapses, the excitatory axo-axonal synapses were identified as one half of a reciprocal synapse, since each of the inhibitory terminals had synaptic contact onto the excitatory terminals. Thus at each site of excitatory axo-axonal synapses, a reciprocal synapse was occurring.

Like neuromuscular synapses, the majority of axo-axonal synapses contain dense bars. These dense bars are similar in appearance to the neuromuscular dense bars, however respective of terminal types the dense bars tend to be longer in the axo-axonal synapses. Thirty dense bars were located in inhibitory axo-axonal synapses with a range in length from 0.07 to 0.41 μm and a mean length of 0.181 μm (Table 5). Although 32% of the inhibitory axo-axonal synapses lacked a dense bar, 33% had between two and five dense bars per synapse (Fig. 13). In the much smaller excitatory axo-axonal synapses four dense bars were found that ranged in length from 0.05 to 0.15 μm and had a mean length of 0.116 μm. The range and mean dense bar length is smaller for the excitatory axo-axonal synapses than the inhibitory axo-axonal synapses. Twenty-five percent of the
excitatory axo-axonal synapses lacked a dense bar with the remainder having either one or two (Fig. 13). Moreover, the dense bar number per synapse is twenty-eight percent smaller in the excitatory terminals than the inhibitory terminals, with a value of 1.00 for the excitatory terminals and 1.38 for the inhibitory terminals (Table 5). Also the dense bar length per terminal length is 10 times smaller in the excitatory terminals, with a value of 0.011 for the excitatory terminals and 0.131 for the inhibitory terminals. Synaptic strength based on dense bar distribution was four times greater in excitatory synapses, with dense bar length per synapse values of 2.73 for excitatory synapses and 0.88 for inhibitory synapses.

Although the axo-axonal synapses are smaller than the neuromuscular synapses, the dense bar data indicates that the axo-axonal synapses have a high capacity for transmitter release.
Table 5. Quantitative analysis of dense bars in excitatory and inhibitory axo-axonal synapses in the opener muscle to the blue crab swimming paddle

<table>
<thead>
<tr>
<th></th>
<th>Excitor</th>
<th>Inhibitor</th>
<th>E/I ratio</th>
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<tr>
<td>Terminal length (µm)</td>
<td>118.177</td>
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<td>2.855</td>
</tr>
<tr>
<td>Total number of dense bars</td>
<td>4</td>
<td>30</td>
<td>0.133</td>
</tr>
<tr>
<td>Total dense bar length (µm)</td>
<td>0.465</td>
<td>5.420</td>
<td>0.086</td>
</tr>
<tr>
<td>Mean dense bar length (µm)</td>
<td>0.116±0.055</td>
<td>0.181±0.00</td>
<td>0.641</td>
</tr>
<tr>
<td>Dense bar length/ µm terminal</td>
<td>0.0112</td>
<td>0.1309</td>
<td>0.086</td>
</tr>
<tr>
<td>Number of dense bars in complete synapses</td>
<td>4</td>
<td>30</td>
<td>0.133</td>
</tr>
<tr>
<td>Dense bar number/synapse</td>
<td>1.00±1.483</td>
<td>1.38±1.678</td>
<td>0.725</td>
</tr>
<tr>
<td>Dense bar length/µm² of synapse</td>
<td>2.7252</td>
<td>0.8790</td>
<td>4.176</td>
</tr>
</tbody>
</table>
Figure 13. Histogram of percentage of complete synapses containing different numbers of dense bars from 0 to 3 in excitatory and inhibitory axo-axonal synapses of the blue crab swimming paddle opener muscle.
Number of dense bars per complete synapse

- Excitatory
- Inhibitory

Percent of complete synapses

0  1  2  3+  
Number of dense bars per complete synapse

56-a
Discussion

A. Opener Muscle

In the crayfish walking limb, the opener muscle is smaller than the antagonistic closer muscle, indicating that the focal role in movement of the dactyl is played by the closer and not the opener. On examination of the size of the opener and closer muscles in the blue crab swimming paddle, it was found that when the two run concurrently, the opener was 1.5 times larger in girth than the closer. Hypertrophy of the opener muscle is an indication of the function it plays as a fairly equal antagonist in the abduction/adduction of the swimming paddle. This is seen in the swimming motion of the blue crab where the dactyl is rapidly abducting and adducting for prolonged periods of time. Electron microscopy analysis on these fibres revealed properties typical of slow muscle: long sarcomeres, thick, wavy and irregularly aligned Z-lines, and a high thin-to-thick filament ratio. These findings corroborate unpublished enzyme histochemistry and light microscopy data (Govind and Pearce, unpublished). Enzyme histochemistry with NADH-diaphorase and ATPase on the opener fibres revealed staining intensities typical of slow fibres, while light microscopy examinations revealed long irregularly aligned sarcomeres in the opener fibres.

That the opener muscle in the blue crab swimming paddle is a slow muscle is not surprising. Previously studied opener muscles from other crustaceans have typically showed slow properties (Bittner, 1968). This is an indication that the muscle fibres of the blue crab swimming paddle opener muscle are well-suited for their function in prolonged contraction. Slow muscle fibres are typically found in muscle used in postural control
and locomotion as they have the ability to support extended periods of tension. Furthermore, slow fibres fire over a large range of frequencies displaying graded contraction strength.

**B. Innervation**

The innervation pattern and synaptic morphology of the crayfish opener muscle have been thoroughly examined using freeze-fracture microscopy (Govind et al., 1994; Govind et al., 1995), transmission electron microscopy (Atwood and Morin, 1970; Jahromi and Atwood, 1974) and electrophysiology (Atwood, 1967; Atwood and Bittner, 1971; Wiens and Atwood, 1975). The opener muscle in crayfish is innervated by three axons and the blue crab opener has the same innervation pattern (Wiens, 1984; Rathmayer and Bevengut, 1986). A common inhibitory axon that innervates all of the limb muscles is located in the proximal region of the opener muscle, while the specific inhibitory axon innervates every muscle fibre in the opener. The excitatory innervation is a shared axon that innervates every muscle fibre in the opener muscle and in the stretcher muscle (Wiens, 1984). Although the opener muscle is innervated by three axons, the central and distal regions receive innervation by only two, the excitor and the specific inhibitor. This then allows for positive identification of the inhibitory axon when examinations are restricted to the central and distal regions. As a result, the data from the present study was collected from central fibres.

In crustacean systems, such as the crayfish opener muscle, the excitatory and inhibitory axons are located close together as offshore axons and under the muscle basal lamina as terminal regions (Msghina and Atwood, 1997). Atwood and Kwan (1976)
found that the excitatory and inhibitory axons grow simultaneously into the crayfish limb opener muscle during early development. Further support for the parallel branching of the inhibitory and excitatory axons comes from a study by Atwood and Bittner (1971). These researchers examined both the crayfish opener muscle and crab stretcher muscle and confirmed that parallel branching of the inhibitory and excitatory axons occurred within both animal systems. My study of the blue crab swimming paddle opener muscle has produced the same conclusion. When off-shore axons were located, one excitatory and one inhibitory axon profile were found together, giving further evidence that two axons innervate the central region of the opener muscle. Furthermore, at most terminal regions, both inhibitory and excitatory innervation could be identified with the majority of innervation sites showing one excitatory and one inhibitory terminal. This would indicate that two axons innervate the central region of the opener muscle, one excitatory and one inhibitory.

In crayfish and other crustacean pereiopod muscles it is uncommon to locate terminal regions on muscle fibres in the first viewing of a sampled section (Govind and Wiens, 1985; Read and Govind, 1993; Govind et al., 1995). In the blue crab swimming paddle opener muscle however, innervation sites were found on every viewing of thin sections taken from randomly sampled muscle fibres. More importantly, fibres viewed in a single thin section displayed an average of two sites per fibre, suggesting that the axons branch profusely. Thus the opener muscle in the blue crab is very highly innervated in comparison with other crustacean muscles. This may be an indication of the high level of fine muscle control and prolonged contraction required in this muscle in order to maintain rapid abduction of the dactyl for prolonged periods of time. Quantitative
comparisons of the terminals showed that the excitatory terminals had 3 times the
terminal length and 1.5 times the terminal volume of the inhibitory terminals, indicating
widespread excitatory innervation in the blue crab opener muscle. This rich innervation
by the excitatory motoneuron may ensure that fatigue or exhaustion of neurotransmitter
does not occur in the highly active opener muscle. Thus this feature of the blue crab
opener muscle may be a specialization to its derived function (swimming).

In my examination of the blue crab swimming paddle opener muscle, three
instances occurred where excitatory and inhibitory terminals had branches into each
other. That is, a finger of the excitatory terminal was found in an adjacent inhibitory
terminal and fingers of inhibitory terminals were located in adjacent excitatory terminals.
Some of these ‘fingers’ possessed synapses, suggesting direct communication between
these terminals. This type of branching could be similar to axo-axonal synapses located
at bottlenecks or axonal branch points where the diameter of the terminal is small
(Atwood, 1976). These axo-axonal synapses are theoretically well placed to effectively
block transmission of an impulse. The interdigitations in the blue crab muscle could
serve a similar purpose. A small synapse with at least one dense bar that contacts or is
contacted by an interdigation may affect the transmission of an impulse differently from
a synapse located on a branch that is adjacent to (as opposed to within) a given terminal.
Further studies would be required to determine the effect and purpose of this type of
branching.

C. Terminal Components

Analysis of the innervation to the blue crab swimming paddle opener muscle
indicated structural adaptations that contribute to the high performance activity of the
neurons. Specialized sites of exocytosis and endocytosis were identified on several synapses in the muscle indicating that the terminals are very active with membrane recycling occurring. Exocytosis is the release of neurotransmitter from presynaptic vesicles into the synaptic cleft. Exocytosis of clear vesicles occurs adjacent to active zones and is Ca$^{2+}$ dependent. In highly active nerve terminals, membrane recycling is accomplished via endocytosis, which allows for efficient disposal of the vesicle membrane. During endocytosis, coated vesicles are brought into the nerve terminal, and transported in membrane bound sacs to cisternae within the golgi apparatus where the vesicles are recycled and filled with neurotransmitter. The vesicles are then released and reused.

During clear vesicle recycling, as opposed to coated vesicle recycling, vesicle membranes are retrieved and taken up into membrane bound sacs, or cisternae. The vesicle membranes are then recycled and filled with glutamate from the axoplasm. During high levels of activity and neurotransmitter release, there is an increase in the number of vesicles to be recycled and therefore a demand for vesicle recycling pathways. In the blue crab swimming paddle opener muscle membrane bound sacs constituted 4% of the cytoplasm in excitatory and inhibitory terminals, and may represent a step in vesicle recycling which reflects a high level of vesicle release.

Another indicator that the terminal regions were specialized for high-energy expenditures was the large amount of mitochondria and glycogen present in both inhibitory and excitatory terminals. Like the tonic terminals of the crayfish limb extensor muscle (King et al. 1996), the mitochondria in the present study were complex and branched.
In a study by Sharman et al. (2000), the mitochondrial content of four stomach muscles in the blue crab was examined and it was found that inhibitory terminals had a lower content (11%) than the excitatory terminals (25%). In my study, where the total energy substrate content was 20.8% for the excitatory terminal and 16.1% for the inhibitory terminal, the mitochondrial content was 8.7% (excitatory) and 7.7% (inhibitory) while the glycogen content was 12.1% (excitatory) and 8.6% (inhibitory). Although the values for mitochondrial content were lower than those obtained for tonic excitatory axons in the crayfish limb extensor muscle (10% to 27%) (King et al., 1996), and in blue crab stomach muscles (16% to 21%) (Patel and Govind, 1997), the total high-energy content was similar between the excitatory axon to the opener muscle and these other two excitatory tonic axons.

Glycogen is included in the high-energy content and provides a much more rapid source of energy than mitochondria (Morin and McLaughlin 1973). Although glycogen is common in the terminals of the blue crab opener, very little has been found in the terminals of the stomach muscle of the blue crab swimming paddle and it is typically absent in terminals of freshwater crayfish. The relative abundance of the glycogen in the nerve terminals of the blue crab opener muscle in comparison to nerve terminals of other rhythmically moving muscles strongly suggests that the opener fires rapidly for sustained periods of time. This suggestion is verified by the findings of Wood and Derby (1995), that the frequency of firing in the motoneurons of the swimming paddle opener muscle is between 50-100 Hz while the motoneurons of the stomach muscles fire at a much slower frequency (5-10Hz) (Selverston and Moulins, 1987). Therefore, in order to maintain the high frequency of firing, a high percentage of the terminal volume would have to be
composed of rapidly usable energy substrates and glycogen is better suited to this role than are mitochondria (Morin and McLaughlin, 1973).

Generally, tonic terminals contain more clear vesicles at the presynaptic membrane than phasic terminals (Atwood, 1976). This higher availability of neurotransmitter allows the tonic terminals to respond to elevated levels of motoneuron activity for long periods of time without becoming fatigued. King et al. (1996) found that the crayfish limb extensor muscle had a higher percentage of clear vesicles in phasic terminals (21%) than in tonic terminals (16%), yet the overall clear vesicle content was higher in the tonic terminals however, because the tonic terminals were greater in volume that the phasic terminals. In the tonic muscles of the blue crab stomach muscle the clear vesicle content ranges from 22% to 34% (Patel and Govind, 1997). A similar content was found in the inhibitory terminal of the blue crab stomach muscle (Sharman et al., 2000). In the swimming paddle opener muscle, my data show the percent composition of clear vesicles in the tonic terminal was slightly lower than that found in the stomach muscles, but higher than the value obtained in the crayfish tonic limb extensor motoneuron. Like the stomach muscles, the inhibitory and excitatory terminals had similar compositions of clear vesicles in the blue crab swimming paddle opener muscle (22.5% for the excitatory terminals and 24.5% for the inhibitory terminals). The high clear vesicle content indicates that a large amount of neurotransmitter is available at the site of the synapse, as is common in tonic axons. Therefore clear vesicle content indicates that the terminals of the blue crab opener muscle are adapted to high levels of prolonged activity.
Dense core vesicles are generally thought to contain neuromodulatory hormones that, when released, can enhance the efficacy of excitatory neuromuscular synapses (Lloyd, 1986). Dense core vesicles are commonly found in the nerve terminals of stomach muscles (Atwood et al., 1978; Patel and Govind, 1997; Sharman et al., 2000) and are thought to play a role in the repetitive contractions of the stomach muscles. In the crayfish limb extensor muscle, less than 1% of the tonic and phasic terminals were composed of dense core vesicles (King et al., 1996). In blue crab stomach muscles dense core vesicle content ranged from 2% to 3.6% (Patel and Govind, 1997), while Sharman et al. (2000) found that the blue crab stomach muscles had a dense core vesicle content of 47% in neuromodulatory terminals, 9% in excitatory terminals and 17% in inhibitory terminals. In my examination of the blue crab swimming paddle opener muscle, the percent composition of dense core vesicles was approximately 1% in both the inhibitory and excitatory terminals. This is slightly lower than the findings of the crayfish limb muscle and much lower than values obtained for the blue crab stomach muscles. The low percentage of dense core vesicles would indicate that regulation of muscle fibre contraction is not brought about by neuromodulatory mechanisms, but rather through precise control of the inhibitory and excitatory terminals.

D. Neuromuscular Synapses and Dense Bars

The analysis of synapse size and distribution revealed that the majority of the synapses were excitatory neuromuscular, this is in accordance with previous studies on other crustacean muscles (Atwood and Morin, 1970; Jahromi and Atwood, 1974; Atwood and Kwan, 1976). Though there were more than twice as many excitatory neuromuscular synapses than inhibitory neuromuscular synapses the excitatory neuromuscular synapses
were on average twenty-five percent smaller than the inhibitory neuromuscular synapses. Previous studies on the crayfish opener muscle have also indicated that the excitatory neuromuscular synapses are smaller than the inhibitory neuromuscular synapses (Atwood and Kwan, 1976; Jahromi and Atwood, 1974). The excitatory neuromuscular synapses found on the adult crayfish opener by Atwood and Kwan (1976) had a mean size of 0.39 \( \mu m^2 \) while the inhibitory neuromuscular synapses had a size of 0.50 \( \mu m^2 \). These findings were replicated by Jahromi and Atwood (1974), as excitatory neuromuscular synapses had a mean size of 0.39\( \mu m^2 \) and the inhibitory neuromuscular synapses had a mean size of 0.46 \( \mu m^2 \). In the blue crab swimming paddle opener muscle, I found excitatory neuromuscular synapses were 0.804 \( \mu m^2 \) on average, while inhibitory neuromuscular synapses were 1.040 \( \mu m^2 \) on average. This difference in size between neuromuscular synapses of the blue crab and the crayfish would make sense as the opener muscle in the blue crab is larger, and therefore larger synapses would be required to have depolarizing and hyperpolarizing effects on the larger muscle fibres.

Active zones are the probable sites of Ca\(^{2+}\) channels required for transmitter release (Atwood and Lnenicka, 1986). Though the presence of active zones does not ensure that neurotransmission occurs at that site, dense bar distribution is one method used to determine synaptic strength. There is a positive correlation between the number and size of dense bars and the potency of a synapse. Simple synapses, containing zero or one dense bar, are generally recruited at higher frequencies than synapses with more dense bars (Cooper et al., 1996). When dense bars are within 0.19 \( \mu m \) of each other, the close proximity of the Ca\(^{2+}\) channels permit more Ca\(^{2+}\) to enter the terminal (Cooper et al., 1995a). The greater influx of Ca\(^{2+}\) allows a greater release of neurotransmitter into
the synaptic cleft (Cooper et al., 1995a). Cooper et al., (1996) suggested that complex synapses, with two or more closely associated dense bars, are recruited preferentially to non-complex synapses.

Govind et al. (1995) showed that there was at least one dense bar in every synapse of the specific inhibitory terminal in the crayfish opener muscle, and in 85-95% of the excitatory synapses (Govind et al., 1994). These findings are in contrast to my examination of the innervation to the opener muscle in the blue crab. Thirty-six percent of the inhibitory and 39% of the excitatory neuromuscular synapses lacked a dense bar. This may indicate that, though innervation is profuse in the blue crab opener muscle, each of the synapses may not be as powerful as in other muscle systems. This also may indicate that many of the synapses analyzed were silent synapses, effectively recruited only at very high impulse frequencies (Atwood and Wojtowicz, 1999), thus allowing for synaptic plasticity within the muscle.

In the crayfish opener muscle the proximal region consists of high output terminals with synapses that are almost all complex, while the low output terminals in the central region mainly contain synapses that are simple, possessing zero or one dense bar (Govind et al., 1994). An examination of the crayfish opener muscle by Cooper et al. (1995a) found that terminals containing a larger number of complex synapses had presynaptic Ca^{2+} signals and a quantal content that were higher than in terminals containing fewer or no complex synapses. Only 0.6% of the synapses from the central terminals of the crayfish opener muscle contained three or more dense bars (Cooper et al., 1995b). King et al., (1996) found that in the crayfish extensor muscle 2.1% of the synapses in tonic terminals contained three or more dense bars. In my study 21% of the
excitatory neuromuscular synapses contained three or more dense bars, suggesting elevated presynaptic Ca\(^{2+}\) signals and quantal content. This may be an adaptation of the blue crab opener muscle to maintain high levels of activity for prolonged periods, by ensuring that elevated neurotransmitter release can occur.

In synapses with longer or multiple dense bars, the Ca\(^{2+}\) influx is larger with a single impulse than it is in simple synapses (Zucker et al., 1991). In a study by Wojtowicz et al. (1994) neuromuscular terminals of the crayfish opener muscle were subjected to high frequency stimulation and long term facilitation of neurotransmitter release resulted. When crayfish that had undergone long-term facilitation were compared with control animals, a greater number of complex synapses were discovered in the adapted terminals, along with a larger number of synapses that were active at transmitter release during low frequency stimulation. It was also found that as the frequency of stimulation was increased the number of active synapses releasing neurotransmitter also increased. With an increase in the internal Ca\(^{2+}\) concentration, the interaction of dense bars further from each other is enhanced, allowing for an additional way in which synaptic output can be altered by frequency (Cooper et al., 1996). In my study on the blue crab opener muscle, approximately one third of the synapses contained two or more dense bars. This high incidence of complex synapses could be a result of the high frequency of firing of the nerve terminals. Also, larger terminals tend to contain more dense bars (Atwood et al., 1978) and those of the blue crab opener muscle were 2.5 times larger than those of the crayfish opener muscle.

Govind et al. (1994) found that synapses that produce larger excitatory postsynaptic potentials had longer mean dense bar lengths. A larger number of Ca\(^{2+}\)
channels, as found in longer dense bars, allowing for a greater influx of Ca\(^{2+}\) for a single impulse (Cooper et al., 1996). In previous studies tonic synapses were found to possess dense bars with a mean length of 0.1 \(\mu m\) (Cooper et al., 1995b; King et al., 1996; Coulthard, 1998). Studies on the opener muscle of crayfish walking limbs indicate that inhibitory synapses had mean dense bar lengths that were longer than those of the excitatory synapses (Govind et al., 1994; Govind et al., 1995). Govind et al. (1994) found that the mean length of excitatory dense bars in the central region of the crayfish opener muscle was between 0.08 \(\mu m\) and 0.09 \(\mu m\), while inhibitory synapses in the central region of the crayfish opener muscle had a mean dense bar length of 0.11 \(\mu m\) (Govind et al., 1995). These findings are very similar to those in my study of the central region of the blue crab swimming paddle opener muscle where the dense bars of the neuromuscular synapses had a mean length of 0.09 \(\mu m\) for the excitatory terminals and 0.13 \(\mu m\) for the inhibitory terminals. In my examination, excitatory neuromuscular synapses had two and a half times as many dense bars as inhibitory neuromuscular synapses for the same length of serial examined. Dense bars were thirty percent smaller in the excitatory neuromuscular synapses than in the inhibitory neuromuscular synapses, however because there were so many more dense bars in the excitatory neuromuscular synapses, the dense bar area was still greater in the excitatory terminals. Although the synapses in the blue crab swimming paddle opener muscle had a large synaptic area, the dense bar lengths were similar between the crayfish opener muscle and the blue crab opener muscle. The difference in dense bar distribution may lie in the fact that one-third of the blue crab synapses were complex, containing two or more dense bars.
E. Axo-axonal Synapses and Dense Bars

Axo-axonal synapses are also common in crustacean neuromuscular systems. Inhibitory axo-axonal synapses have been found to comprise 10-20% of the synapses of the specific inhibitory axon in the crayfish opener muscle (Govind et al., 1995). Presynaptic inhibition is used for precise control of muscle movement. Moreover, once the presynaptic inhibition is removed from the excitatory terminal a strong excitatory response is produced as a result of the availability of a large readily releasable pool of neurotransmitter (Atwood and Walcott, 1965). In the blue crab swimming paddle opener muscle 36% of the inhibitory synapses were axo-axonal, this is in contrast to the 10-20% found in the crayfish opener muscle (Govind et al., 1995). The high incidence of presynaptic inhibition in the opener muscle of the blue crab swimming paddle may be an adaptation to the highly specialized swimming motion. The high percentage of presynaptic inhibition would indicate that precise control of the opener muscle might be necessary for it to function effectively as a fairly equal antagonist to the closer muscle.

Like neuromuscular synapses, not all of the inhibitory axo-axonal synapses contained dense bars. This is contrary to what was previously reported by Govind et al. (1995) in the crayfish opener muscle, and could indicate that though there are a greater percentage of axo-axonal synapses in the blue crab, many may be either silent and could be recruited at much higher frequencies as is seen in synapses that do not possess dense bars, or they may be immature and not fully developed (Atwood 1976; Atwood and Wojtowicz, 1999).

The surprising finding was the presence of excitatory axo-axonal synapses. Though there were not a large number of these, they were found at least once in every
area sampled and were thus determined not to be an artifact. The excitatory axo-axonal synapses were much smaller than any of the other types of synapses, and three quarters contained at least one dense bar. These dense bars were smaller than those of the inhibitory axo-axonal synapses, but longer than those of the excitatory neuromuscular synapses. Because the synapses are small, but contain long dense bars, the value for dense bars per synaptic area is largest in the excitatory axo-axonal synapse. Based on dense bar distribution, synaptic strength per synaptic area is highest in the excitatory axo-axonal synapses.

The excitatory axo-axonal synapse is actually one of two correlates of a reciprocal synapse, the second being the inhibitory axo-axonal synapse. In my study, each of the excitatory axo-axonal synapses were polarized onto an inhibitory terminal that had adjacent synapses onto the excitatory terminal. Within the serial sections, the excitatory axo-axonal synapses were generally located 50 to 250 nm away from an inhibitory axo-axonal synapse. Shepherd (1974) defined a reciprocal synapse as an arrangement between neurological elements in which chemical transmission is in opposite directions at two adjacent or separate synapses. Four cases of reciprocal synapses were seen in the blue crab opener muscle.

Reciprocal synapses have only been documented in three other instances in crustacean limb muscles (Atwood and Kwan, 1979; Pearce and Govind, 1993). Atwood and Kwan (1979) found the first evidence of a reciprocal synapse in the stretcher muscle of the spider crab, *Hyas areneus*. In their ultrastructural study, it was not possible to conclude whether or not the reciprocal synapses were between the excitatory terminal and the common or specific inhibitory axon since the two inhibitory axons innervate the same
regions of the stretcher muscle. Pearce and Govind (1993) also examined the presence of reciprocal synapses but found it in muscles only receiving inhibition from the common inhibitory axon; the limb closer muscle of the crab *Eriphia spinifrons* and the distal accessory flexor muscle in the crayfish *Procambarus clarkii*. One third of the axo-axonal synapses located in the study by Pearce and Govind (1993) were identified as reciprocal, while 10% of the axo-axonal synapses in the study by Atwood and Kwan (1979) were identified as reciprocal. In the present study, 15% of the axo-axonal synapses in the blue crab opener muscle were found to be reciprocal, thus within the range of those previously reported.

The crayfish opener muscle has been extensively studied and no evidence of reciprocal synapses has been documented. Following their finding of reciprocal synapses in the closer of the crab *Eriphia spinifrons* and distal accessory flexor muscle of crayfish, Pearce and Govind (1993) examined the crayfish opener muscle for the presence of reciprocal synapses. The opener muscle receives inhibitory innervation by both the common and the specific inhibitory axons, however, the common inhibitory axon only innervates a very restricted proximal region of this muscle, whereas the specific inhibitory axon shows much more widespread innervation (Bevengut and Cournil, 1990; Wiens, 1984). For this reason, if excitatory axo-axonal synapses were discovered in the distal or central regions of the opener muscle it would be possible to state that excitatory axo-axonal synapses were polarized onto the specific inhibitory axon. Thorough examinations have found no evidence of excitatory axo-axonal synapses, although inhibitory axo-axonal synapses are a common occurrence in the crayfish opener muscle.

When the entire muscle was sampled in juvenile crayfish, excitatory axo-axonal synapses
were not seen (Pearce and Govind, 1993). The presence of reciprocal synapses in the blue crab swimming paddle opener muscle suggests that a specialized innervation pattern is utilized in the swimming motion of the blue crab paddle.

The function of the reciprocal synapse in the crustacean neuromuscular system is as yet unknown. Atwood and Kwan (1979) suggested a metabotropic function for this neural arrangement and Pearce and Govind (1993) suggested that the function could be to decrease the inhibitory effect of the common inhibitory axon where the reciprocal synapse is occurring by activating K⁺ channels within the inhibitory membrane. In order to state for certain the purpose of the excitatory axo-axonal synapse it would be necessary to determine whether or not the receptors on the postsynaptic inhibitory terminal membrane are excitatory (GABA_B) or inhibitory (GABA_A) (Miwa et al., 1990).
Summary

The fifth pereiopod in the blue crab exhibits many exoskeletal and muscular adaptations in its function as a swimming paddle. Fibres of the fifth pereiopod opener muscle were analyzed to examine the presence of neuromuscular adaptations. Ultrastructural analysis of muscle fibres and terminal regions were used to obtain quantitative and qualitative data.

In cross-sections of the central regions of the opener and closer muscles, the opener muscle was found to be 1.5 times greater in girth than the closer. The hypertrophy of the opener muscle is indicative of the role that it plays as an equal antagonist in the abduction/adduction of the blue crab opener muscle. With ultrastructural examination, the blue crab swimming paddle opener muscle was determined to be composed of slow muscle fibres. This was found in data collected using electron microscopy where the sarcomere length measured between 7 to 9 μm, the number of actin filaments surrounding one myosin filament was high (13:1), the sarcomeres were irregularly aligned, and the Z-lines were thick and wavy. Unpublished light microscopy and NADH-diaphorase and ATPase enzyme histochemistry results confirmed that the blue crab opener was composed of slow muscle fibres.

An examination of the opener muscle innervation revealed that this muscle is very profusely innervated. Every viewing of thin sections taken from randomly sampled muscle fibres revealed innervation sites, with fibres containing an average of two sites. Each site was composed of one excitatory and one inhibitory terminal profile that were closely associated.
Off-shore axons, as well as terminal regions, of the inhibitory and excitatory neurons were found in parallel. An interesting method of branching was located at several innervation sites. A branch of one terminal had protruded into an adjacent antagonistic terminal. On one of these branches was located a reciprocal synapse where the inhibitory branch had a synapse polarized onto the excitatory terminal, and adjacent to this the excitatory terminal had a synapse polarized onto the inhibitory branch.

An analysis of the percent composition of the inhibitory and excitatory terminals revealed that the two terminal types had similar organelle composition. The greatest percent composition for terminal types was the clear vesicles, occupying approximately one-quarter of the terminals. The high-energy content was also a major component of the terminals and was composed of mitochondria and glycogen. Membrane bound sacs and dense cored vesicles were located within the terminals as well, but represented only a small percentage of the total composition.

Further analysis of the terminals revealed that neuromuscular and axo-axonal synapses occurred in the inhibitory and excitatory terminals. Like in other crustacean neuromuscular systems, the excitatory neuromuscular synapses were the greatest in quantity, but the inhibitory neuromuscular synapses had the largest area. Inhibitory axo-axonal synapses were present throughout sampled regions and composed 36% of the inhibitory synapses. Axo-axonal synapses polarized from the excitatory terminal to the inhibitory terminal were also located. These are very rare in crustacean neuromuscular systems and have been documented in only three other muscles. The inhibitory and excitatory axo-axonal synapses are the two correlates necessary to make up a reciprocal
synapse and reciprocal synapses were located in each of the four serially sectioned areas examined in the present study of the blue crab swim paddle opener muscle.
References


