Three Types of Nerve Terminals based on Vesicle Morphology in the Blue Crab Stomach Pyloric Muscles

By

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

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THREE TYPES OF NERVE TERMINALS BASED ON VESICLE MORPHOLOGY IN
THE BLUE CRAB STOMACH PYLORIC MUSCLES

An abstract submitted in conformity with the requirements
For the degree of Master of Science in Zoology
In the Graduate Department of Zoology
University of Toronto

Asheer Sharman, 1999

ABSTRACT

Movements of the gastric mill and pylorus in the stomach of the blue crab
Callinectes sapidus are brought about by a series of striated muscles which receive
exclusively excitatory input from neurons in the stomatogastric ganglion. Electron
microscopic examination of the pyloric muscles of the p11, p12, p13 and p14 motor unit
revealed the presence of two other putative types of innervation, namely inhibitory and
neuromodulatory, based on vesicle morphology. The first type, excitatory, was readily
identified by the spherical shape of its small, clear synaptic vesicles. The second type,
inhibitory, was identified by the presence of elliptically shaped clear synaptic vesicles.
Both of these terminal types were observed in all four muscles examined. The third
type, neuromodulatory, seen in only the p14 muscle was characterized by the presence
of primarily large dense core vesicles containing eccentrically located cores. All three
nerve terminal types formed synaptic contacts, with dense bars, with the pyloric muscle
membrane. Altogether, the existence of excitatory, inhibitory and neuromodulatory
innervation of the pyloric muscles implies a level of modulation at the periphery that is
as complex as that found in the stomatogastric ganglion itself.
Acknowledgements

It is with the sincerest words that I write this final piece of my thesis. Dr. Govind, your diligence and unparalleled perfectionism is superceded only by your good nature and compassion. I thank you for many things, guidance, advice, and criticisms, but most of all I thank you for inspiring me to do my best. Joanne Pearce, your perpetually smiling face provided a welcomed contrast to the ominous atmosphere that is usually present in any scientific laboratory. Thank you for your patience and technical advice, without you my work here would have been immeasurably more difficult. Rahim Hirji, for the past 13 years we’ve worked together. I hope our time spent together is as memorable for you as it is for me, and if it is written that we go our separate ways from here on in, I hope we will remain as good friends as ever. You will always remain one of my most valued friends. Sheel Parekh and Suneesh Gambhir, your continual advice over the last two years has been invaluable. Thank you both for your support and your willingness to always listen to my problems, no matter how obtuse and convoluted they seemed. I hope all of your ambitions will one day be fulfilled. To all of my closest friends, your friendship is one of the biggest reasons I find the courage to attempt new things. I am very grateful that all of you are in my life, and your loyalty over the years has been much appreciated. Finally, and most importantly, my parents Ravinder and Pushpa, and my brother Shalin, thank you for so many things. It is no understatement to say that all of my accomplishments in life were made possible because of your love, encouragement and support.

In pursuit of this degree I learned many things, but perhaps the most important lesson I learned was to never be afraid to attempt new things. I think this lesson was most eloquently put by the poet Whittier who once wrote:

For of all sad words of tongue or pen,
The saddest are these: “It might have been.”
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I. INTRODUCTION

A. Crustacean Neuromuscular Systems

Crustacean neuromuscular systems have proven to be invaluable in the study of synaptic mechanisms and neuronal plasticity. This is due, in part, because they are composed of relatively few motoneurons, often only one or two, each of which is individually identifiable (Atwood, 1976; Atwood and Wojtowicz, 1986). Furthermore, the motor nerve terminals present in crustacean neuromuscular systems are readily accessible for experimentation. Additionally, crustacean neuromuscular synapses resemble those of the vertebrate central nervous system more so than the synapses seen at the vertebrate neuromuscular junction. For example, unlike vertebrate neuromuscular junctions, the presence of inhibitory nerve fibers innervating crustacean muscles is well noted. Not only are such fibers present within some crustacean neuromuscular systems, but there is evidence that they regulate muscles directly (postsynaptic inhibition) and indirectly by way of limiting the output of excitatory terminals (presynaptic inhibition). Such interplay between excitatory and inhibitory forms of innervation is one characteristic that crustacean neuromuscular systems share with the vertebrate central nervous system. Secondly, like the vertebrate CNS, where multiterminal innervation is a prevalent characteristic, single crustacean muscle fibers have many synapses of different sizes along their entire length (Govind and Walrond, 1989; Atwood and Govind, 1990). Finally, unlike synapses of the vertebrate central nervous system, crustacean neuromuscular synapses are relatively accessible and easy to work with. Thus, the allure of crustacean neuromuscular systems is that they provide an accessible model to study the characteristics of the vertebrate central nervous system.
1. **Excitatory Innervation**

Each crustacean muscle is innervated by at least one excitatory motor neuron; however, not all of the motor neurons exhibit the same properties. In fact, there are many types of excitatory motor neurons. The broadest form of classification separates all excitatory motor neurons into either phasic motor neurons or tonic motor neurons.

Examples of phasic motor neurons include the fast axon of the crayfish claw closer muscle (Wiersma, 1961), the fast axon of the leg closer muscle in the crab *Pachygrapsus* (Hoyle and Wiersma, 1958a; Wiersma, 1961; Atwood and Walcott, 1965; Atwood et al., 1967), and the fast flexor and fast extensor neurons of crayfish and lobster abdominal muscles (Kennedy and Takeda, 1965a, b; Parnas and Atwood, 1966; Selverston and Remler, 1972). Typically, neuromuscular synapses of phasic axons show a large excitatory postsynaptic potential (EPSP) upon stimulation. These synapses characteristically show rapidly developing depression or fatigue when stimulated with a train of impulses. It is believed that these axons fire impulses in brief bursts during periods of intense activity but are silent most of the time (Atwood, 1976).

Tonic motor neurons, on the other hand, show a comparatively smaller EPSP than their phasic counterparts, but are generally more fatigue resistant (Atwood, 1976). In fact, these axons are active much more often than their phasic counterparts, and usually maintain a low-frequency background discharge of impulses. It is believed that tonic axons are responsible for controlling posture or routine locomotion, and the frequency of their firing shifts to bring about small adjustments. Examples of tonic axons include those supplying the slow flexor muscles of the crayfish abdomen (Kennedy and Takeda, 1965a, b; Evoy et al., 1967), and some of the axons of crustacean limb muscles,
particularly the opener-stretcher motor axon of the crayfish claw and leg (Wiersma, 1961; Wilson and Davis, 1965; Bittner, 1968).

A number of studies have attempted to correlate the electrophysiological characteristics of phasic and tonic neurons with their respective morphological characteristics. There are two broad types of morphological differences that can account for the observed physiological differences: (1) differences present at postsynaptic sites, and (2) differences present at presynaptic sites. It is the contribution of the presynaptic site that is thought to be the predominant factor in giving rise to the physiological characteristics. Thus, researchers tend to focus on the presynaptic region. Examinations of the crayfish claw closer muscle have shown that tonic terminals house more synapses than phasic terminals (Hill and Govind, 1981; Florey and Cahill, 1982). This is contrary to what one might expect given the physiological characteristics of the two types of neurons. Therefore, there must be some other presynaptic factor contributing to the difference. One difference between phasic and tonic terminals of the crayfish limb extensor muscle is the amount of active zone material, or particles, found at the synapses (King et al., 1996). Active zones are thought to be represented by prominent densely stained bodies, termed dense bars (DBs), on the cytoplasmic side of the presynaptic membrane of synapses. Phasic synapses have more active zone material than tonic synapses. The phasic synapses house a greater number of dense bars, which were longer than those seen on tonic synapses.

Such findings regarding differences between phasic and tonic synapses at the active zone level have given rise to the "structure-function" hypothesis. The structure-function hypothesis states that the probability of a quantum of transmitter being released is correlated to the amount of active zone material present (Atwood and Lnenicka, 1986). This hypothesis works in conjunction with the calcium release hypothesis. Freeze fracture studies show active zones as being comprised of arrays of intramembrane
particles, on the p-face, which are often surrounded by putative exocytotic pits thought to represent vesicular fusion. The close proximity of these intramembranous particles to the exocytotic vesicles, and the brief delay between calcium influx and transmitter release, suggests that these particles may be calcium channels (Pumplin et al, 1981). Thus, in comparison with tonic synapses, it is believed that the greater number of dense bars present on phasic synapses will result in a greater amount of calcium influx, which subsequently will lead to a greater amount of vesicle release, thus yielding a larger EPSP (Atwood and Wojtowicz, 1986).

However, recent studies involving the motor neurons supplying the extensor muscle in the crayfish leg have refuted the findings of King et al. (1996). Msghina et al. (1998) found no significant difference in active zone number per synapse or active zone size between phasic and tonic synapses. This recent study refutes the convention that differences in EPSP size can be partially explained by differences at the level of the active zone. The researchers speculated that the differences in EPSP size demonstrated by the phasic and tonic neurons may be explained by either a difference in the density of calcium channels at the active zone, or a difference in the rate of calcium entry into the terminal. Msghina et al. (1999) subsequently employed the techniques of freeze-fracture and calcium imaging to test these two possibilities. Calcium signals were typically 2-3 times larger in phasic terminals than tonic terminals at low frequencies of stimulation. The freeze-fracture images of synapses revealed similar numbers of prominent presynaptic active zone particles (putative ion channels) for both neurons, and a 2-4 fold phasic:tonic ratio of active zones per terminal volume. Msghina et al. (1999) speculated that the difference between the two terminal types with respect to the number of active zones per terminal volume may account for the larger calcium signals seen in the phasic terminals.
In addition to synaptic differences exhibited between different excitatory axons, single tonic axons have also shown to exhibit differentiation of their motor nerve terminals. One example of this is the single excitor axon innervating the crayfish limb opener muscle. The terminals stemming from this axon are regionally differentiated into high- and low-output terminals which are located in the proximal and central regions of the muscle, respectively (Bittner, 1968; Govind et al., 1994). Proximal synapses house longer dense bars than the central synapses. Furthermore, the proximal synapses, on average, had a greater number of active zones. These differences at the active zone level are thought to account for the disparity in EPSP amplitude exhibited by synapses on the proximal and central muscle fibers. Thus, the causation implied by the structure-function hypothesis, with regard to the amount of active zone material and transmitter output, is further supported by these findings. In fact, differences at the active zone level may be a general mechanism for regulating the transmitter output of a terminal.

a. Excitatory Transmitters

Currently there is evidence for at least two types of excitatory transmitters occurring at crustacean neuromuscular systems: acetylcholine (Ach) and glutamate. Of the two excitatory transmitters, acetylcholine, is the less prevalent at the crustacean neuromuscular junction. One claim for Ach activity in crustacean muscles comes from studies of the crayfish abdominal slow flexor muscles (Futamachi, 1972). There are five motor axons supplying these muscles, the largest of which is thought to release Ach. The synaptic foci of this axon responds to iontopheretic application of Ach, generating a depolarization of the muscle fiber. Furthermore, the application of curare depressed the EPSPs of the largest axon. However, the claim for Ach activity at crustacean slow abdominal flexor muscles has been weakened by a report that in lobsters, neither Ach
nor choline acetyltransferase can be detected in these muscles or the nerves supplying them (Hildebrand et al., 1974).

A second example of Ach activity at crustacean neuromuscular synapses comes from studies involving the pyloric dilator muscles of the lobster stomach (Marder, 1974a, b). These muscles are depolarized by Ach, and not by glutamate, whereas some of the other pyloric muscles responded to glutamate exclusively. Additionally, the enzyme choline acetyltransferase is present in the dilator motorneurons, and not in certain other motor neurons of the stomatogastric ganglion.

Glutamate is the primary excitatory transmitter substance in limb muscles of crustaceans (Atwood, 1976). Low concentrations of glutamate, when perfused into limb muscles, can elicit contractions (Robbins, 1959; van Harreveld and Mendelson, 1959). Furthermore, studies involving the crayfish opener muscle fibers have shown that glutamate is effective in producing depolarizations at regions where synaptic terminals are located (Takeuchi and Takeuchi, 1963, 1964). Alternately, glutamate can be collected from perfused crustacean muscle preparations, and the amount collected increases upon stimulation of excitatory, but not inhibitory, axons (Kerkut et al., 1965; Kravitz et al., 1970). It is thought that the amount of extra glutamate released during stimulation of the excitatory axon is sufficient to account for the size of the EPSPs recorded in the muscle (Takeuchi and Takeuchi, 1972).

The primary postsynaptic effect of glutamate binding to receptors is to open channels mainly for sodium ions (Taraskevich, 1971; Takeuchi and Onodera, 1973; Dudel, 1974). Calcium ions may, to a small extent, also enter these channels but chloride and potassium ions do not. Dose response curves indicate that four to six glutamate molecules are required to react with its receptor in order to activate a single membrane channel. Pharmacologically, the glutamate receptors can be blocked by
various analogs of glutamate, the most effective of which is the gamma-methylester of glutamic acid (Lowagie and Gerschenfeld, 1974).

After receptor binding, glutamate may be removed from the synaptic cleft by means of uptake by the presynaptic terminal. Studies of lobster nerve terminals utilizing the tracer horseradish peroxidase reveal that it accumulates more rapidly in the nerve terminals after stimulation than at rest (Holtzman et al., 1971). This would indicate that a greater uptake of extracellular material is occurring during stimulation.

2. Inhibitory Innervation

Many crustacean muscles do not receive any inhibitory innervation. For example, it is believed that none of the stomach muscles innervated by neurons from the stomatogastric ganglion receive innervation from an inhibitor (Maynard and Atwood, 1969; Govind et al., 1975). Those muscles which are regulated by inhibitory innervation may receive one or two inhibitory axons (Wiersma, 1961; Atwood, 1967, 1968). Electrophysiological studies reveal that when an inhibitory axon is stimulated, a recording of an inhibitory postsynaptic potential can be obtained from the muscle. Like their excitatory counterparts, it appears as though inhibitory axons can display either phasic or tonic like activity. For example, discharge patterns of inhibitory axons to the fast abdominal muscles in the crayfish exhibit phasic characteristics (Atwood, 1976), whereas the discharge of the inhibitory axon of the crayfish opener muscle is fairly tonic (Wilson and Davis, 1965). Furthermore, the discharge patterns of the inhibitory axon to the slow flexor muscles seem to be neither phasic nor tonic, but rather an intermediate of the two types (Kennedy and Takeda 1965a, b; Evoy et al., 1967). This particular axon may be the correlate to the high-output tonic terminals seen with excitatory axons.
At the ultrastructural level, inhibitory terminals can easily be distinguished from excitatory terminals by the unique appearance of their synaptic vesicles. It has been found that fixation with aldehydes and osmium tetroxide cause the gamma-aminobutyric acid (GABA) containing synaptic vesicles of inhibitory terminals to assume an elliptical shape (Uchizono, 1967; Atwood et al., 1972), as opposed to the spherical shape assumed by the glutamate containing synaptic vesicles of excitatory terminals. Although there is no doubt that the shape of the inhibitory synaptic vesicles is influenced by fixation, and thus represents a type of fixation artefact, this does not limit their usefulness as a tool for determining the identity of different terminals. In addition to housing irregularly shaped vesicles, systems which contain one excitor and one inhibitor have shown inhibitory synapses to be greater in size than excitatory synapses, thereby providing another means to distinguish the two terminal types at the ultrastructural level (Govind and Pearce, 1989; Atwood and Kwan, 1976).

a. Inhibitory transmitters

In crustacean muscles, GABA is thought to be the sole inhibitory transmitter. There are a number of studies that support GABA as being an inhibitory transmitter. In the lobster nerve preparation, GABA is released when the inhibitory axon is stimulated, but not when the excitatory axon is stimulated (Otsuka et al., 1966). As expected from a neurotransmitter, this release is reduced when the preparation is placed in a solution with low calcium concentration. Furthermore, glutamate decarboxylase, the enzyme responsible for generating GABA from glutamate, is present in higher levels in inhibitory neurons. Thus, the enzymatic machinery necessary for the synthesis of GABA appears to be better developed in inhibitory neurons than in sensory or motor neurons (Molinoff and Kravitz, 1968; Hall et al., 1970).
GABA receptors are localized on muscle fibers at inhibitory synapses, and are distinct from those of excitatory synapses (Takeuchi and Takeuchi, 1965, 1972). The main postsynaptic effect exerted by GABA is to increase the muscle fiber membrane conductance to chloride ions and other anions (Takeuchi and Takeuchi, 1967, 1971; Motokizawa et al., 1967, 1969). Dose response curves obtained by Feltz (1971) using the crayfish opener muscle reveal that four GABA molecules are needed to bind to a receptor in order to activate a single membrane channel.

After receptor binding, inactivation of GABA is brought about, not by enzymatic degradation as in the case of acetylcholine at the vertebrate neuromuscular synapse, but by diffusion away from the synapse and uptake into cellular components. Iversen and Kravitz (1968) found that uptake of radioactively labeled GABA by the lobster abdominal slow flexor muscles occurred by a sodium-dependent saturable transport mechanism. This uptake mechanism could be inhibited by drugs such as chlorpromactive zoneine and β-GP.

3. Neuromodulatory innervation

This third, and rather novel, type of innervation differs from the excitatory and inhibitory forms in that these axon terminals are primarily constituted of large and dense core vesicles (DCV) as opposed to the smaller clear vesicles. Because DCVs are typically involved in slow acting neuromodulatory type functions, these neurons are thought to have a neuromodulatory function (Rane et al., 1983).

To date, this type of innervation has been identified more often in the CNS of invertebrates than at the neuromuscular junction. However, a study by Rheuben (1995) identified three types of neuromodulatory innervation at the neuromuscular junction of the skeletal muscles of various insects. Two of the three types (types II and III) of
neuromodulatory innervation involve nerve terminals that are composed primarily of DCVs. The type II axons were found to be in direct contact with the muscle fiber membrane of the skeletal muscles. The type III axons also contained primarily DCVs but were not found to make direct contact with the muscle fiber membrane. This type is similar to the neurohemal organs often seen in the CNS of many invertebrate species (Rheuben, 1995).

Neurohemal organs have been found in the corpus cardiactum of insects (Normann, 1965), the neurohypophysis of vertebrates (Normann, 1976), and the central body of the crayfish brain (Schurmann et al., 1991). The specific function of this form of innervation is still under considerable debate. Schurmann et al. (1991) speculate that these unique axons of the central body of the crayfish brain respond to various sensory modalities. In fact, similar studies on a similar structure of the honey bee brain have also shown that these neurohemal organs as being responsive to sensory input (Homberg, 1985).

a. Neuromodulatory Transmitters

To date, there is evidence for two types of substances that can constitute the dense core material of DCVs: biological amines, and neuropeptides. One of the most studied amine constituents of DCVs is octopamine, which is a phenol analogue of norepinepherine. One study involving the CNS of the horseshoe crab *Limulus polyphemus* used the technique of immunolabeling with gold particles to identify octopamine as being a constituent of the large and unusually shaped dense core granules present in the nerve terminals (Lee and Wyse, 1991). Furthermore, morphological studies of octopaminergic nerve terminals in the firefly light organ have shown that these terminals contain a mixture of small clear synaptic vesicles and large
DCVs, thereby adding further evidence that DCVs likely contain octopamine (Oertel et al., 1975). Octopamine can function as either a neurotransmitter, a neurohormone or a neuromodulator in invertebrates (Axelrod and Saavedra, 1977; Orchard, 1982; David and Coulon, 1985). As a neuromodulator, octopamine potentiates neuromuscular transmission in several arthropod preparations (Florey and Rathmayer, 1978; May et al., 1979; O'Shea and Evans, 1979; Watson and Hoshi, 1981; Watson and Augustine, 1982). Rane et al. (1983) found that octopamine exerted a neuromodulatory effect on the skeletal muscle contractions of Limulus. Specifically, octopamine was responsible for increases in EPSP size. However, a similar effect of EPSP potentiation was not obtained when glutamate or aspartate, the neurotransmitters, were applied exogenously; thus, it is believed that octopamine potentiates neuromuscular transmission primarily by presynaptic means, by facilitating the release of the neuromuscular transmitter (Rane et al., 1983). Studies involving the lobster neuromuscular preparation, however, have indicated that octopamine may act to potentiate neuromuscular transmission at postsynaptic sites (Kravitz et al., 1980; Giusman and Kravitz, 1982). In these studies, octopamine induced muscle spikes although EPSP size was not affected. Although the consensus is that octopamine, along with other biogenic amines, can act in a neuromodulatory capacity to potentiate neuromuscular transmission, the specific location of its effect may be affected by species variation.

Like their biological amine counterparts, neuropeptides can also act as both neurotransmitters and slower acting neuromodulators. One of the most studied neuropeptides is the pentapeptide proctolin. Proctolin acts as a neurotransmitter in a skeletal motorneuron of the cockroach (O'Shea and Bishop, 1982). As a neuromodulator, proctolin enhances myogenic and neurally-evoked contractions as well as increases the frequency of myogenic contractions in several insect species (Brown, 1975; Cook and Holman, 1978, 1979; May et al., 1979; Irving and Miller, 1980).
arthropods, such as *Homarus* and *Limulus*, proctolin is capable of potentiating neurally-evoked contractions (Schwarz et al., 1980; Kravitz et al., 1980; Benson et al., 1981). In skeletal muscles of the horseshoe crab *Limulus*, proctolin potentiated muscle contractions even in preparations where the neurotransmitter glutamate was exogenously applied (Rane et al., 1983). This would suggest that proctolin exerts its effect directly on the skeletal muscle and hence acts postsynaptically. Additionally, studies of the tonic flexor muscles of the crayfish, *Procambarus clarkii*, abdomen also hinted at a possible postsynaptic role of proctolin (Bishop et al., 1987). When proctolin was superfused into the muscles, it enhanced neurally induced muscle tension. However, Bishop et al. (1987) were unable to detect any change in the size of EPSP that could account for the change in tension. Thus, it is likely that proctolin is exerting its effect postsynaptically.

4. **Types of Crustacean Muscle Fibers**

Crustacean muscles can vary enormously in structure, electrical properties, and contractile properties (Atwood, 1976). Furthermore, unlike their mammalian muscle counterparts where the contractile and biochemical properties of motor units are uniform, some crustacean motor units have very different types of muscles fibers innervated by the same motor axon. Crustacean muscle fibers can be functionally classified as “fast” acting muscle fibers, and “slow” acting muscle fibers. However, it is important to note that these two types are at opposite ends of the spectrum and there is a wide range of intermediate fiber species.

Fast-acting crustacean muscle fibers generally have short sarcomeres, relatively thin and straight Z-lines, and a low ratio of thin filaments to thick filaments in the contractile apparatus (Atwood, 1976). Both the excitatory transverse tubule system and
the sarcoplasmic reticulum are well developed. It is believed that these characteristics provide a mechanism for the rapid contraction and relaxation that is characteristic of these muscle fibers. Additionally, fast-acting muscle fibers generally contain electrically excitable membranes which generate large graded spikes. Furthermore, the excitation-contraction coupling level is usually high, and depolarizations of 20-30 mV are typically sufficient to generate a contraction (Atwood, 1965; Parnas and Atwood, 1966). The ability to support prolonged tension in these muscles is poorly developed (Atwood and Dorai Raj, 1964). Examples of fast-acting muscle fibers are those in the phasic abdominal flexor and extensor muscles of the crayfish, shrimp and rock lobster abdomens (Kennedy and Takeda, 1965a; Parnas and Atwood, 1966; Parnas and Dagan, 1969), as well as those in fast-acting regions of certain leg muscles (Atwood, 1967).

In contrast to fast-acting crustacean muscle fibers, slow-acting fibers have longer sarcomeres, longer A-bands, thick and wavy Z-lines, and a higher ratio of thin filaments to thick filaments (Atwood, 1976). The transverse tubule system is less well developed than in the fast muscle fibers, but there is little difference in the degree of sarcoplasmic reticulum development. The threshold for excitation-contraction coupling is usually low, typically within a few millivolts of the resting potential (Atwood et al., 1965; Reuben et al., 1967). The contractions of these fibers develop very gradually (Atwood and Dorai Raj, 1964; Atwood et al., 1965; Jahromi and Atwood, 1971), and the relaxation is slow (Parnas and Atwood, 1966; Evoy et al., 1967). These fibers display an ability to support prolonged tension (Atwood and Dorai Raj, 1964; Hoyle, 1968a, b), which makes them suitable for the control of posture and repetitive locomotory activity. Examples of slow muscle fibers include those in the slow abdominal flexor and extensor muscles of crayfish and lobsters (Kennedy and Takeda, 1965b; Parnas and Atwood, 1966), and many of the limb muscles including the opener and stretcher muscles of crabs and crayfish (Bittner, 1968; Sherman and Atwood, 1972).
In comparison with mammalian and other vertebrate skeletal muscles, those of crustaceans show a wide diversity of structure and of contractile and electrical properties. Crustacean fibers tend to exploit differences in sarcomere length as a means of getting different types of performance to a greater extent than vertebrate muscle fibers. Sarcomere length is uniform and short among vertebrate muscle fibers, even in the tonus fibers, the functional equivalent to crustacean slow-acting fibers, the sarcomere length is only slightly larger than in the twitch fibers, the functional equivalent to the fast acting crustacean fibers (Atwood, 1976).

B. Crustacean Stomatogastric System

The crustacean stomatogastric system is a valuable tool in the field of neurobiology. Analysis of the neuronal mechanisms controlling crustacean stomach behavior provided insights into the production of rhythmic motor patterns for locomotion. The crustacean intestinal tract can be separated into three components: the foregut, the midgut and the hindgut. Foregut movements are primarily responsible for swallowing, chewing and internal sorting of food particles for further chewing, assimilation, or processing as waste material (Claiborne and Ayers, 1987). The foregut consists of the esophagus and the stomach chamber. The stomach chamber can further be divided into the cardiac sac, the gastric mill and the pylorus.

After food has traveled down the esophagus via peristaltic movements, it arrives at the cardiac sac (Claiborne and Ayers, 1987). The cardiac sac functions primarily as a storage area for food to be macerated and as a mixing area for food and gastric juice from the digestive gland. Next, the mixture of food and gastric juices arrives at the gastric mill, which functions to chew the food and separate it into many food particles.
This chewing action is achieved by the presence of three specialized ossicles called teeth. Two lateral teeth are responsible for gripping the food while the third medial tooth chews and degrades the food. When this process is complete, the now smaller food particles travel to the pylorus for sorting.

The pylorus is the most complicated region of the foregut as it acts as a press and filter apparatus for sorting food particles (Claiborne and Ayers, 1987). Large and coarse food particles are sent directly to the midgut for excretion, whereas the finer food particles and liquid are sent to the pyloric filter. The pyloric filter consists of overlapping rows of hairs matted together to form a filter press whose pumping action strains and further divides food particles on the basis of size. The smallest particles (<1 μm) enter the digestion gland for final digestion and absorption. Intermediate sized particles are sent directly to the midgut for expulsion. The large particles are directed back to the gastric mill for further chewing.

1. Muscle Fibers

The muscles of the crustacean stomach can be divided into two types: extrinsic and intrinsic. Extrinsic muscles originate on the thoracic skeleton and the intrinsic muscles originate on the stomach itself. Both muscle types insert on the stomach (Maynard and Dando, 1974; Govind et al., 1975; Meiss and Norman, 1977). The fibers of all stomach muscles are striated and have an ultrastructure typical of crustacean muscles. Within a single muscle, the fine structure of its fibers is homogenous; however, there is considerable variation in the fine structure between muscles, especially with respect to sarcomere length and the ratio of thin to thick myofilaments. In blue crab stomach muscles, the sarcomere length can vary from 3 to 10 μm with the pyloric muscles generally at the lower end of this range, and the gastric mill muscles at the higher end (Govind et al., 1975). Additionally, the thin to thick filament ratio can be as
low as 6 :1, or as high as 12 : 1. Unlike stomach muscles, crustacean limb and abdominal muscles exhibit a fairly reliable correlation between the thin to thick filament ratio and sarcomere length. Limb and abdominal muscles which generally have a low thin to thick filament ratio (6:1) also have relatively short sarcomere lengths (<4 μm), whereas those muscles that have a larger thin to thick filament ratio (>10:1) also have relatively long sarcomere lengths (>6 μm) (Atwood 1976, Govind and Atwood 1982).

2. Neuromuscular innervation and Transmitters

To date, all of the motor axons to crustacean stomach muscles have been found to be excitatory (Maynard and Atwood, 1969; Maynard and Dando, 1974; Govind et al., 1975; Hooper et al., 1986). Among the intrinsic muscles in the stomach of blue crabs, those of the gastric mill and cardio-pyloric valve receive single axons which are shared among them, resulting in individual motor units of several muscles. In contrast, 6 of the 14 pyloric muscles receive more than one axon. The difference in the numbers of axons innervating gastric and pyloric regions of the stomach may be related to the functional requirements of these two regions. The longer-lasting gastric mill cycle with its slow powerful contractions can be regulated by single axon outputs to muscle, while the more rapid pyloric cycle demands faster action of the muscles which can be achieved by summation of the synaptic potentials from several axons.

Two main types of neurotransmitters have been identified in the crustacean stomatogastric system: glutamate and Ach. In general, intrinsic muscles receive glutamatergic innervation whereas extrinsic muscles receive cholinergic innervation (Lingle, 1980). However, there are some exceptions. Notably, the pyloric muscles of Cancer irroratus receive innervation from 2 - 4 cholinergic neurons (Hooper et al., 1986). Furthermore, intrinsic muscles of P. argus, P. interruptus, C. magister, C. irroratus and H. americanus exhibit an excitatory response to Ach application (Lingle 1980). Although no
inhibitory innervation to the stomatogastric muscles has been shown, inhibitory conductances are found on stomatogastric muscles. Specifically in *Panulirus*, the gastric mill muscle gm6b has a GABA-activated chloride conductance (Albert et al., 1986). Also, the gm1 muscle of *H. americanus* and *C. magister* has glutamate-activated chloride conductance (Lingle and Marder, 1981). However, neither immunological nor histological studies for the presence of inhibitory transmitters within the stomatogastric muscles have been carried out.

In addition to the traditional fast transmitters glutamate and acetylcholine, four neuromodulatory agents have been shown to affect the muscular contraction of crustacean stomatogastric muscles. The four neuromodulators dopamine, octopamine, proctolin and serotonin all increase the amplitude of contractions evoked by stimulation of the excitatory motor nerve to particular foregut muscles. Dopamine produces muscle contracture and induces spontaneous rhythmic contractions at concentrations as low as 5nM in the cpv1a muscle of *P. interruptus*, and the gm6b muscle of *P. interruptus*, *C. magister* and *C. irroratus* (Lingle, 1981). Proctolin, a pentapeptide, also produces enhancement of foregut muscle contractions at concentrations as low as $5 \times 10^{-9}$ M in the gm6b, cpv1ab, and gm1 muscles (Lingle, 1979). The gm6b and cpv1ab muscles of *Panulirus* both show increases in the amplitude of nerve-evoked contractions upon application of serotonin and octopamine. Typically, slight responses to either serotonin or octopamine occur at concentrations as low as $5 \times 10^{-8}$ M, and significant responses are reliably observed at concentrations of $10^{-7}$ M serotonin and $5 \times 10^{-7}$ M (Lingle, 1979).

To date, there is evidence suggesting that some of the peripheral nerves intrinsic to the stomatogastric system may contain proctolin and serotonin (Marder et al., 1986; Beltz et al., 1984). Both of these substances are good candidates for the
C. Objectives

The crustacean foregut is involved in the ingestion of food via the esophagus, the maceration of this food in the cardiac sac, and the channelling of the triturated food into the midgut via the pyloric filter. To accomplish this task, motor patterns generated in the stomatogastric ganglion drive muscles in the foregut. Control of these motor patterns resides largely within the stomatogastric ganglion via numerous synaptic inputs and interactions. While these patterns are largely responsible for regulating the timing and intensity of muscle contraction, some degree of control of these contractions is also present in the neuromuscular innervation because of the differentiated nature of this innervation. It is this aspect of the stomatogastric system that is the focus of the present study. In particular, I plan to examine the innervation of the pyloric muscles using electron microscopy in order to ascertain whether the differentiation of neuromuscular systems in these muscles may involve not only excitatory, but also inhibitory and neuromodulatory forms of innervation.

There is reason to suspect that the pyloric muscles may receive more than just excitatory innervation because their function in filtering food requires much finer control than functions such as the maceration of food. This fine control of pyloric muscles is also reflected in the fact that each muscle is typically innervated by 2-3 axons compared to the single axons innervating the gastric mill and cardiac muscles.

The specific aim of my study was to analyze the ultrastructure of the innervation of four pyloric muscles p11, p12, p13, and p14, in the blue crab Callinectes sapidus, that have been electrophysiologically characterized as comprising a single motor unit (Govind et al., 1975). Their location at the end of the pylorus before the food passes into the midgut or hepatopancreas suggests that they would play a role in selectively filtering the food into these alternate pathways. For such a role, it would be helpful to have fine control of muscle contraction in the form of differentiation of the nerve terminals of individual axons, as well as differentiation of axons into excitatory, inhibitory and possibly neuromodulatory types.
II. MATERIALS AND METHODS

Adult female blue crabs, *Callinectes sapidus*, were purchased from a local fish store and were briefly maintained in a marine aquarium containing Instant Ocean at 22°C prior to dissection.

A. Electron Microscopy

1. Tissue Preparation

   The foregut was removed from the animal by chipping away the dorsal exoskeleton. It was immersed in marine animal saline for further dissection according to procedures established in our laboratory (Govind et al. 1975). The intrinsic muscles of the cardiac and pyloric parts of the foregut were exposed and subsequently immersed in primary fixative, consisting of 4% sucrose, 1mM CaCl₂, 2.5% glutaraldehyde and 0.5% formaldehyde in a 0.1 M sodium cacodylate buffer (pH=7.4), for half an hour at room temperature to allow for easier dissection of the relevant muscles (Govind et al., 1975). The pyloric muscles of interest were then dissected from the animal and immersed in fresh fixative for a further one hour before being washed several times in a buffer containing 0.1 M sodium cacodylate, 4% sucrose and 1mM CaCl₂. After that the tissue was fixed in a secondary fixative containing 2% OsO₄ in a 0.1 sodium cacodylate buffer (pH=7.4) for one hour. Next, the tissue was rinsed several times in buffer for 15 minutes, and dehydrated in a graded ethanol series before clearing in propylene oxide for 30 minutes. The tissue was left overnight in a 50% propylene oxide – 50% Epon-Araldite mixture to allow gradual infiltration of the resin. The following day, the tissue was embedded in moulds containing fresh Epon-Araldite and left for at least 4 hours at room temperature before placing the moulds in the oven at 60°C for a 48 hour period.
and left for at least 4 hours at room temperature before placing the moulds in the oven at 60°C for a 48 hour period.

2. Tissue Surveying and Serial Sectioning

The resin block with embedded tissue was mounted on a Reichert OMU 2 Ultramicrotome for cutting thin (approximately 70 nm) sections. The block face was trimmed into a trapezoidal shape in such a manner so that only the embedded tissue was present. The tissue was surveyed at approximately 10 μm intervals and at each of these stations a diamond knife was used to cut approximately 20-30 thin sections. Once cut, the sections were captured from the water-holding trough of the diamond knife using a copper grid. The sections were subsequently stretched using chloroform vapors and mounted on Formvar-coated single slot grids. The thickness of each section was estimated from its interference colour. Once dried, the grids were stained for 30 minutes with uranyl acetate. Next, the grids were washed with double distilled water and dried for approximately 30 minutes. This was followed by staining with lead citrate for 3 minutes, after which the grids were again washed and dried. Using a Zeiss 9S electron microscope, the survey sections were examined for the presence of nerve terminals, and if located they were photographed at a magnification of 8,000x. The negatives were enlarged 3.25x to yield photographic prints with a final magnification of 26,000x.

Examination of these survey prints alerted us to promising areas of innervation which could be examined with serial thin sectioning of 5 to 10 μm lengths of tissue using techniques established in our laboratory (Patel and Govnd, 1996). The actual place of a section in a serial was identified by virtue of the fact that the sections typically adhered to one another forming a ribbon. Each ribbon was then captured from the water trough using a grid, stretched, and placed on a Formvar-coated single slot grid to dry. Each grid in the serial then underwent a staining regimen similar to that performed on the
survey sections. Those areas containing nerve terminals were photographed, developed and then printed. The electron micrographs obtained had a final magnification of 26,000x, which enabled both quantitative and qualitative analysis of the nerve terminals.

B. Quantitative Analysis

1. Synaptic Features

Synapses were identified on a terminal by the presence of a densely stained pre- and postsynaptic membrane. The area of every complete synapse was determined by using a three step procedure. First, calipers were used to measure the length of every synaptic profile of a given synapse. Next each of these lengths was multiplied by the thickness of the section upon which it appeared to obtain the individual surface area. Finally, to calculate the total surface area of the synapse, the individual surface areas of the profiles, calculated in the previous step, were all added together.

Dense bars were identified as dense bodies situated on the presynaptic membrane often surrounded by a cluster of vesicles. The total number of dense bars included those found in both complete and incomplete synapses. The number of dense bars per synapse was determined by dividing the total number of dense bars within complete synapses by the number of complete synapses. If a dense bar appeared to be cut in cross section, the dense bar length was obtained by adding together the thickness of each of the contributing sections. However, if the dense bar appeared to be cut longitudinally, the length of the dense bar was ascertained by using calipers to measure the length of the dense bar on the electron micrograph, which was then multiplied by the magnification factor to obtain a length in μm.
2. **Vesicle Size and Shape**

In order to measure the size of vesicles in the nerve terminals, selected thin sections were rephotographed under the electron microscope at a higher magnification (20,000x). The resulting negatives were then enlarged into prints with a final magnification of 67,000x for measuring the maximum and minimum diameters of vesicles. This measurement was then multiplied by the magnification factor in order to calculate the actual size of the two measurements in nm.

The shape of the vesicles was determined by dividing the measured maximum diameter by the measured minimum diameter. This yielded what was referred to as the shape index. A shape index close to 1 indicates a spherically shaped vesicle, whereas values greater than 1 indicated a more elliptical shaped vesicle (Atwood and Morin, 1970). Vesicles selected for measurement were usually taken from nerve terminals that were making synaptic contact with the muscle membrane. All of the vesicles of the terminal profiles selected were used to calculate the mean size and shape of the vesicles.

C. **Volumetric Analysis**

A volumetric analysis of the nerve terminal was undertaken to determine what percent of a given terminal's volume was occupied by clear vesicles, dense core vesicles, mitochondria, and axoplasm (King et al., 1996). An acetate grid with dots placed 0.375 cm apart was superimposed on every fifth section in order to determine what percentage of the terminal profile was occupied by each of the terminal constituents. The total number of dots falling within the double membrane boundary of the terminal was counted. The number of dots which were in contact with the aforementioned constituents was then also counted. Eventually, the total number of dots within the terminal from every fifth section was added together to give a total
number of dots counted for a given terminal. In a similar fashion, the total number of dots contacting clear vesicles, dense core vesicles, mitochondria, and axoplasm was calculated. Finally the proportion of the terminal that contained the aforementioned constituents was ascertained by dividing the total number of dots in contact with a particular structure by the overall total number of dots counted for the given terminal.

D. Sample size

Pyloric muscles from four female blue crabs were initially examined for innervation. Two blue crabs, termed animal #1 and animal #3, were selected for further study because the tissue was better preserved, and because nerve terminals were more readily located. In these two animals, the innervation of seven pyloric muscles were scrutinized with extensive surveying; three muscles (p11, p12, and p13) from animal #1, and four muscles (p11, p12, p13, and p14) from animal #3 were chosen. In addition, a more intense examination using thin serial sectioning for 5-10 μm lengths was performed on five of the seven muscles viz. muscles p11, p12 and p13 from animal #1 and muscles p12 and p14 from animal #3. Statistical significance was calculated by means of a student’s t-test.
III. RESULTS

Examination of the p11, p12, p13 and p14 motor unit revealed the presence of potentially three forms of innervation. The first type, excitatory innervation, was expected as Govind et al., 1975 showed that the blue crab pyloric muscles exhibit EPSPs. The second form of innervation seen in my study was thought to be inhibitory. As will be described, the evidence for the presence of inhibitory innervation is based primarily on morphological features. The presence of elliptically shaped clear vesicles was the primary means of identifying the inhibitory terminals. Finally, a third and rather unconventional type of innervation was observed in the p14 muscle. This terminal was different from the aforementioned two terminal types in that it was primarily composed of dense core vesicles which were dramatically different in appearance from the dense core vesicles seen in the two more conventional terminal types. Because of these dense core vesicles, this terminal may represent some form of neuromodulatory innervation. Each type of terminal will be described below.

A. Excitatory Nerve Terminals

1. Identification

The presence of excitatory innervation in the pyloric muscles of blue crabs was first demonstrated electrophysiologically by Govind et al. (1975). In that study, all of the 14 pyloric muscles were seen to exhibit EPSPs. The electrophysiological demonstration of excitatory innervation was corroborated in the present ultrastructural study by observing nerve terminals populated by uniformly spherical synaptic vesicles (Fig. 1A), the characteristic shape assumed by excitatory vesicles with aldehyde and
Figure 1.

A. Cross section of the blue crab pyloric muscle p12 showing the presence of two excitatory terminals. Both terminals are filled with spherically shaped clear (c) synaptic vesicles, few dense core (d) vesicles, and some mitochondria (o). Synaptic contacts (between bars) are present on the terminal membrane adjacent to the muscle granular sarcoplasm (s). Two presynaptic dense bars (arrowheads), each surrounded by a cluster of clear vesicles, are seen on two of the synapses.

Magnification: 36 600x. Scale bar: 0.5 um

B. Cross section of an excitatory terminal from the blue crab pyloric muscle p13 recognized by the spherical clear (c) synaptic vesicles. Present within the terminal are mitochondrial (o) profiles and some scattered dense core (d) vesicles and a cluster of dense core vesicles enclosed within a double membrane (arrow).

Magnification: 48 000x. Scale bar: 0.5 um.
osmium fixation (Uchizono, 1967; Atwood and Morin, 1970). All of the muscles of the p11, p12, p13, and p14 motor unit had excitatory nerve terminals. The shape of the synaptic vesicles was assessed by measuring the major and minor diameter of individual vesicles and then taking their ratio as an index of shape. A measurement of the major diameter, minor diameter, and shape index was obtained from nerve terminals of p11-p13 muscles from animal #1, and p11-p14 muscles from animal #3 (Table 1). The excitatory vesicles were typically 30-40 nm in diameter and electron-lucent or clear, and exhibited a shape ratio close to 1.2.

2. Synaptic Features

The excitatory terminals made synaptic contact with the pyloric muscles (Fig. 1A). Typically, these terminals and their synaptic contacts were found on the periphery of a muscle fiber, although intramuscular terminals along with synaptic contacts were occasionally observed. Excitatory synapses were characterized by opposing membranes which were both densely stained and parallel. Additionally, the presynaptic membrane of excitatory synapses was also seen to house dense bars, which were surrounded by clear vesicles.

Since previous studies of pyloric (Atwood et al., 1978) and gastric mill (Atwood et al., 1977; Patel and Govind, 1997) muscles of blue crabs have quantitatively characterized excitatory synapses and dense bars, I undertook a similar analysis for comparison. As well, I wanted to compare synaptic features among the four muscles comprising this pyloric motor unit. Hence, a quantitative analysis of the synapses and dense bars was carried out for muscles p11, p12, and p13 of animal #1 and muscles p12, and p14 of animal #3 (Table 2). The mean synaptic area of excitatory synapses exhibited a wide range. The smallest
Table 1. Analysis of size and shape for clear vesicles of excitatory terminals of the blue crab pyloric muscles p11, p12, p13, and p14 (values for maximum diameter, minimum diameter and shape are given as x ± sd).

<table>
<thead>
<tr>
<th>Animal #1</th>
<th>Excitatory Terminals</th>
<th>Number of Vesicles</th>
<th>Diameter (nm)</th>
<th>Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Max</td>
<td>Min</td>
</tr>
<tr>
<td>p11</td>
<td></td>
<td>55</td>
<td>38.0±7.5</td>
<td>31.9±6.8</td>
</tr>
<tr>
<td>p12</td>
<td></td>
<td>52</td>
<td>39.4±7.3</td>
<td>34.0±7.2</td>
</tr>
<tr>
<td>p13</td>
<td></td>
<td>181</td>
<td>41.4±8.3</td>
<td>35.0±6.5</td>
</tr>
</tbody>
</table>

| Animal #3 |                      |                    | Max           | Min   | Max/Min |
|-----------|----------------------|--------------------|---------------|-------|
|           |                      |                    |               |       |         |
| p11       |                      | 48                 | 39.3±11.6     | 31.3±7.2 | 1.3±0.5 |
| p12       |                      | 127                | 45.5±7.0      | 38.4±7.6 | 1.2±0.3 |
| p13       |                      | 80                 | 43.3±8.8      | 37.9±8.4 | 1.2±0.3 |
| p14       |                      | 44                 | 45.5±7.8      | 38.8±6.1 | 1.2±0.2 |
Table 2. Quantitative analysis of synapses and dense bars of excitatory terminals of the blue crab pyloric muscles p11, p12, p13 and p14 (values for synapse size and dense bar length are given as x ± sd).

<table>
<thead>
<tr>
<th></th>
<th>Animal #1</th>
<th></th>
<th>Animal #3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p11</td>
<td>p12</td>
<td>P13</td>
<td>p12</td>
</tr>
<tr>
<td>Number of Synapses</td>
<td>10</td>
<td>9</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Size of Synapses (μm²)</td>
<td>0.38±0.38</td>
<td>0.64±0.44</td>
<td>0.89±0.51</td>
<td>1.57±0.85</td>
</tr>
<tr>
<td>Number of Dense bars</td>
<td>14</td>
<td>10</td>
<td>8</td>
<td>23</td>
</tr>
<tr>
<td>Number of Dense bars per Synapse</td>
<td>1.4</td>
<td>1.1</td>
<td>1.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Length of Dense Bars (μm)</td>
<td>0.144±0.089</td>
<td>0.091±0.035</td>
<td>0.090±0.045</td>
<td>0.118±0.052</td>
</tr>
<tr>
<td>Dense Bar length per μm² Synaptic Area (μm)</td>
<td>0.530</td>
<td>0.157</td>
<td>0.101</td>
<td>0.109</td>
</tr>
</tbody>
</table>
mean synaptic area was observed for the p14 muscle of animal #3 (0.18 \( \mu m^2 \)) and the largest mean synaptic area was observed for the p12 muscle of animal #3 (1.57 \( \mu m^2 \)).

The majority of synapses possessed a dense bar representing the active zone for transmitter release (Fig. 1). The average length of the dense bars seen within the excitatory synapses was fairly consistent among the muscles examined (Table 2). All of the muscles displayed an average dense bar length close to 0.1 \( \mu m \). The number of dense bars per synapse exhibited a wide range among the muscles examined. The largest average number of dense bars per synapse (2.3) was seen for the p12 muscle of animal #3, and the least average number of dense bars per synapse (0.7) was seen in the p14 muscle of the same animal. In order to compensate for differences in synaptic size among the four muscles, I computed the length of dense bar per unit synaptic area, and this showed a large amount of variance with the p13 muscle from animal #1 exhibiting the smallest mean dense bar length per unit synaptic area (0.101 \( \mu m \)) and the p11 muscle from the same animal showing the largest (0.530 \( \mu m \)). In this very limited sample size, the four muscles comprising this motor unit vary in synaptic strength based on dense bar length per synapse area, with p11 the strongest, followed by p14, p12, and finally p13.

Interestingly, the p12 muscles from animal #1 and animal #3 displayed remarkably dissimilar mean synaptic areas and a dissimilar average number of dense bars per synapse. In fact, only the average length of dense bars parameter was seen to be similar for these two muscles.

3. Dense Core Vesicles

Another feature of the excitatory terminals was the presence of DCV (Fig. 1A). Unlike the clear vesicles, these DCVs are either completely electron dense or have an electron dense core. Furthermore, these vesicles were larger than their clear
counterparts and exhibited a diameter of approximately 70 nm. They appeared scattered in the nerve terminals and fewer in number than the clear vesicles. A volumetric analysis revealed that DCVs are typically less prevalent in excitatory terminals than clear vesicles (Table 3). In fact the DCVs occupy between two to five times less terminal volume than the clear vesicles.

As mentioned above, the usual arrangement of the DCVs was scattered within the excitatory terminals but, in one case, the DCVs of an excitatory terminal in the p13 muscle of animal #1 were contained within a membranous sac (Fig. 1B). This sac extended through several thin serial sections and was approximately 0.5 μm in length.

Previous studies of crustacean nerve terminals provide evidence for exocytosis of DCVs exclusively at either extrasynaptic sites (Schurmann et al., 1991), or at synpatic sites (Patel and Govind, 1996). In the blue crab pyloric muscles, however, I find evidence for exocytosis at both synaptic (Fig. 2) as well as at extrasynaptic sites of the same terminal (Fig. 3A, B). Exocytosis in these terminals is characterized by a small infolding of the presynaptic membrane within which single, double, or multiple dense cores are observed. In cases of single DCV exocytosis, the presynaptic membrane showed distinct omega shaped infoldings which usually were seen to incorporate one irregularly shaped dense object which resembled the core of a DCV. The cases where two or more electron opaque cores were located between the omega shaped infolding of the presynaptic membrane and the unaffected postsynaptic membrane were taken to represent evidence for multiple DCV exocytosis.

Although exocytosis of DCVs was observed at both synaptic and extrasynaptic sites, it appeared more prevalent at the extrasynaptic sites. Hence, counts of exocytotic figures were made at both sites for the five pyloric muscles that were serially thin
Table 3. Percent composition of clear and dense core vesicles and mitochondria determined from a volumetric analysis of the excitatory terminals from the blue crab pyloric muscles p11, p12, p13, and p14.

<table>
<thead>
<tr>
<th></th>
<th>Animal #1</th>
<th>Animal #3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p11</td>
<td>p12</td>
</tr>
<tr>
<td>Clear Vesicles (%)</td>
<td>33.3</td>
<td>46.1</td>
</tr>
<tr>
<td>Dense Core Vesicles (%)</td>
<td>10.9</td>
<td>5.3</td>
</tr>
<tr>
<td>Mitochondria (%)</td>
<td>25.8</td>
<td>5.7</td>
</tr>
<tr>
<td>Axoplasm (%)</td>
<td>29.9</td>
<td>42.9</td>
</tr>
</tbody>
</table>
Figure 2. Excitatory terminals from blue crab pyloric muscles depicting examples of dense core vesicle exocytosis (arrows) at synaptic contacts. These terminals are filled with spherically shaped clear (c) synaptic vesicles, few dense core (d) vesicles, and mitochondria (o), and also form synapses (between bars) with the adjacent muscle granular sarcoplasm (s).

A. An excitatory terminal from the p13 muscle showing a dense core enclosed within a slight infolding of the presynaptic membrane (arrows) indicative of exocytosis of a dense core vesicle.
Magnification: 66 300x. Scale bar: 0.5 um.

B. An excitatory terminal from the p13 muscle showing a dense core enclosed within a presynaptic infolding (arrow) indicative of exocytosis which in this case is adjacent to a presynaptic dense bar (arrowhead).
Magnification: 56 400x. Scale bar: 0.5 um.

C. An excitatory terminal from the p11 muscle showing several dense cores enclosed within a large infolding of the presynaptic membrane (arrow) indicative of exocytosis of several dense core vesicles.
Magnification: 56 400x. Scale bar: 0.5 um.
Figure 3.

A. An excitatory terminal from the p13 muscle of the blue crab forming synaptic contacts (between bars) with the adjacent muscle granular sarcoplasm (s). The extrasynaptic terminal membrane shows infoldings enclosing single (short arrow) and double (long arrow) dense cores indicative of exocytosis.
Magnification: 58 000x. Scale bar: 1 um.
Inset: An excitatory terminal from the blue crab pyloric muscle p13 showing a dense core (d) vesicle possibly attached to nerve terminal membrane indicating an early step in the exocytosis of a dense core vesicle.
Magnification: 73 600x. Scale bar: 0.1 um.

B. An excitatory terminal from the blue crab p13 pyloric muscle, containing mitochondria (o), showing possible endocytosis of a dense core vesicle (double arrow) via a dense core housed within a presynaptic membrane infolding which is decorated by a halo of short filaments. Note synaptic contact (between bars) with the adjacent muscle granular sarcoplasm (s) and exocytosis of multiple dense core vesicles (arrows).
Magnification: 58 000x. Scale bar: 0.5 um.
sectioned. The resulting comparison of the frequency of exocytosis was clearly greater for the extrasynaptic site compared to the synaptic site (Table 4). The difference of occurrence of exocytosis was approximately two to six fold higher at the extrasynaptic sites.

While evidence for exocytosis of DCVs was a prevalent feature of the excitatory terminals of blue crab pyloric muscles, I also observed, in a single instance, evidence for DCV endocytosis (Fig. 3C). Endocytotic profiles appeared in the form of a dense core contained within an infolding of the presynaptic membrane which was decorated with a halo of evenly spaced thin filaments. The endocytotic profiles were found to be approximately the same diameter (70 nm) as the DCVs found within the terminal.

4. Mitochondria

A prominent feature of these excitatory nerve terminals was the presence of mitochondria (Figs.1, 2, 3). They were seen as simple or complex branched structures scattered throughout the terminal. In order to assess their relative density within the terminal, a volumetric analysis was undertaken (Table 3). Mitochondria make up almost a quarter of the terminal volume in all of the muscles except for one muscle, p12 of animal #1. Mitochondria therefore constitute a major component of these pyloric motor terminals.

B. Putative Inhibitory Nerve Terminals

1. Identification

In the electrophysiological study by Govind et al. (1975) mentioned earlier, only excitatory innervation of the pyloric muscles was seen. Inhibitory innervation of the pyloric muscles in the form of inhibitory postsynaptic potentials was not demonstrated.
Table 4. Comparison of the number of dense core vesicle fusion sites, synaptic (syn) and extra-synaptic (ext), of excitatory terminals of blue crab pyloric muscles p11, p12, and p13.

<table>
<thead>
<tr>
<th></th>
<th>Number of Extrasynaptic Fusion Sites</th>
<th>Number of Synaptic Fusion Sites</th>
<th>Frequency Ratio (Ext/Syn)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p11 (Animal #1)</td>
<td>7</td>
<td>3</td>
<td>2.3</td>
</tr>
<tr>
<td>p12 (Animal #1)</td>
<td>11</td>
<td>2</td>
<td>5.5</td>
</tr>
<tr>
<td>p12 (Animal #3)</td>
<td>47</td>
<td>27</td>
<td>1.7</td>
</tr>
<tr>
<td>P13 (Animal #1)</td>
<td>88</td>
<td>40</td>
<td>2.2</td>
</tr>
</tbody>
</table>
My study, however, provides morphological evidence for an inhibitory presence within the pyloric muscle system of the blue crab (Fig. 4). Unlike excitatory vesicles which assume a spherical shape after fixation with aldehyde and osmium, inhibitory vesicles assume an elliptical shape (Uchizono, 1967; Atwood et al., 1972). As with vesicles present in the excitatory terminals, vesicle shape of these putative inhibitory terminals was assessed by obtaining a ratio of major to minor diameter as an index of shape. Because inhibitory vesicles are more elliptical in shape than excitatory vesicles, it would be expected that the major to minor diameter ratio for these vesicles would be significantly greater than 1. For these measurements, adjacent excitatory and inhibitory nerve terminals were selected to ensure that fixation and section thickness were consistent. These measurements were carried out on the p11, p12 and p13 muscles from animal #1, and the p11, p12, p13, and p14 muscles from animal #3 (Table 5). The inhibitory vesicles exhibited ratios between 1.81 and 2.25. Since a ratio of 1 is indicative of a perfect sphere, ratios exhibited by the putative inhibitory terminals indicate vesicles that were markedly more elliptical in shape as compared to their excitatory counterparts, which exhibited ratios of approximately 1.2 (Table 1). The differences in the ratios seen in Tables 1 and 5 are graphically displayed in Figure 5 which shows that for each muscle examined, the inhibitory vesicles exhibited a higher ratio than the excitatory vesicles. Each muscle examined for inhibitory vesicles had a mean shape index which was significantly greater than the mean shape index of the excitatory vesicles of the same muscle (p < 0.05). The significant differences in the vesicle shape index confirm our initial suspicion that at least two terminal types exist, those that house spherical vesicles, viz. excitatory terminals, and those that house elliptical vesicles, viz. putative inhibitory terminals.
Figure 4.

A. Adjacent nerve terminals in the p11 muscle of the blue crab, one of which has spherically shaped clear (c) synaptic vesicles, marking it as excitatory (E), and the other has elliptically shaped clear vesicles, marking it as inhibitory (I). Both terminals make synaptic contact (between bars) with the muscle granular sarcoplasm (s). Note the scattered dense core (d) vesicles and mitochondria (o) seen in the inhibitory terminal. Magnification: 51 800x. Scale bar: 0.5 um.

B. A putative inhibitory terminal (I) recognized by elliptically shaped clear (c) synaptic vesicles in the blue crab pyloric muscle p12. Synaptic contacts (between bars) are observed between the inhibitory terminal and the adjacent muscle granular sarcoplasm (s). A single dense bar (arrowhead) is present on one of the synapses. Note the presence of scattered dense core (d) vesicles and mitochondria (o). Magnification: 49 500x. Scale bar: 0.5 um.
Table 5. Analysis of size and shape for clear vesicles of the putative inhibitory terminals from blue crab pyloric muscles, p11, p12, p13, and p14 (values for maximum diameter, minimum diameter and shape are given as x ± sd).

<table>
<thead>
<tr>
<th>Animal #1</th>
<th>Number of Vesicles</th>
<th>Diameter (nm)</th>
<th>Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Max</td>
<td>Min</td>
</tr>
<tr>
<td>p11</td>
<td>125</td>
<td>42.9±10.9</td>
<td>26.0±8.7</td>
</tr>
<tr>
<td>p12</td>
<td>48</td>
<td>40.1±11.2</td>
<td>23.4±8.6</td>
</tr>
<tr>
<td>p13</td>
<td>157</td>
<td>51.5±17.9</td>
<td>29.7±9.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Animal #3</th>
<th>Number of Vesicles</th>
<th>Diameter (nm)</th>
<th>Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Max</td>
<td>Min</td>
</tr>
<tr>
<td>p11</td>
<td>52</td>
<td>45.6±15.5</td>
<td>25.8±6.9</td>
</tr>
<tr>
<td>p12</td>
<td>46</td>
<td>49.2±13.6</td>
<td>25.5±9.1</td>
</tr>
<tr>
<td>p13</td>
<td>59</td>
<td>47.1±9.6</td>
<td>26.9±8.7</td>
</tr>
<tr>
<td>p14</td>
<td>34</td>
<td>52.0±17.8</td>
<td>28.7±9.3</td>
</tr>
</tbody>
</table>
Figure 5. Graphical comparison of the mean shape index values of excitatory and inhibitory synaptic vesicles. Note that for all muscles from both animals, the shape index for the inhibitory vesicles is markedly higher than for the excitatory vesicles.
Clear Vesicle Analysis For Excitatory and Inhibitory Terminals

Shape Index

Animal #1

Animal #3

Muscle

p11

p12

p13

p14

Inhibitory Vesicles

Excitatory Vesicles

398
2. **Synaptic Features**

Like excitatory terminals, the putative inhibitory terminals were present at the periphery of a muscle fiber, and usually in close proximity to excitatory terminals. The inhibitory terminals made synaptic contacts with the muscle membrane (Fig 4). These synaptic contacts were characterized by densely-stained presynaptic and postsynaptic membranes which were virtually parallel. Additionally, these synapses were often decorated with presynaptic dense bars surrounded by a cluster of clear elliptical vesicles.

A quantitative analysis of inhibitory synapses and their dense bars was carried out for the p11, p12, and p13 muscles of Animal #1, and the p12, and p14 muscles of Animal #3 (Table 6). Unlike the excitatory synapses examined for the same muscles, the mean synaptic area for the inhibitory synapses exhibited a narrow range. In fact, only the p14 muscle exhibited a mean synaptic area that was substantially smaller than the other examined muscles. The number of dense bars per synapse as well as their average length varied considerably among the four muscles. The p14 muscle of animal #3 exhibited the least average number of dense bars per synapse (1.09), whereas the p12 muscle of animal #1 exhibited the largest (4.14). Interestingly, the inhibitory synapses examined from animal #3 showed a lower average number of dense bars per synapse than those examined from animal #1, even in cases where the same muscle was examined for both animals. Average dense bar length varied from 0.111 μm to 0.179 μm; however, there was no significant difference between the muscles with regards to dense bar length. To compare synaptic strength based on structural features, we calculated the dense bar length per synaptic area (Table 6). It was interesting to note that the inhibitory synapses of the p12 muscle from animal #1
Table 6. Quantitative analysis of synapses and dense bars of the putative inhibitory terminals of blue crab pyloric muscles p11, p12, p13, and p14 (values for synapse size and dense bar length are given as x ± sd).

<table>
<thead>
<tr>
<th></th>
<th>Animal #1</th>
<th></th>
<th>Animal #3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p11</td>
<td>p12</td>
<td>p13</td>
<td>p12</td>
</tr>
<tr>
<td><strong>Number of Synapses</strong></td>
<td>4</td>
<td>7</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td><strong>Size of Synapses (μm²)</strong></td>
<td>0.39±0.11</td>
<td>0.38±0.23</td>
<td>0.31±0.09</td>
<td>0.35±0.17</td>
</tr>
<tr>
<td><strong>Number of dense bars</strong></td>
<td>13</td>
<td>29</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td><strong>Number of Dense bars per synapse</strong></td>
<td>3.25</td>
<td>4.14</td>
<td>3.00</td>
<td>1.86</td>
</tr>
<tr>
<td><strong>Length of Dense Bars (μm)</strong></td>
<td>0.179±0.151</td>
<td>0.170±0.098</td>
<td>0.138±0.061</td>
<td>0.130±0.070</td>
</tr>
<tr>
<td><strong>Dense bar length per μm² Synaptic Area (μm)</strong></td>
<td>1.491</td>
<td>1.853</td>
<td>1.335</td>
<td>0.689</td>
</tr>
</tbody>
</table>
demonstrated the greatest synaptic strength (1.853 μm), and the same muscle from animal #3 the least synaptic strength (0.689 μm).

3. Dense Core Vesicles

The presence of DCVs was also a characteristic of the putative inhibitory terminals (Fig. 4). Like the excitatory terminals, the DCVs were approximately 70 nm in diameter, and usually fewer in number than their clear counterparts. A volumetric analysis of the inhibitory terminals revealed that the volume of terminal occupied by the DCVs was approximately half that of the volume occupied by the clear vesicles (Table 7). However, in the p13 muscle of animal #1, the DCVs occupied a greater proportion of the terminal volume than the clear vesicles.

The volumetric analysis also revealed that, with the exception of the p14 muscle, the p11, p12, and p13 muscles all displayed a higher proportion of DCVs in the inhibitory terminals (Table 7) than the excitatory terminals (Table 3) of the same muscle. The proportion of DCVs in the inhibitory terminals was approximately double that of the proportion present within the excitatory terminals (16.5% versus 8.7%). The p12 muscle from animal #3 was exceptional in that the proportion of DCVs in the inhibitory terminal was almost quadruple that of the excitatory terminals.

The DCVs were usually scattered among the clear vesicles within the inhibitory nerve terminals, although in some cases they formed a fairly homogenous population separated from populations of clear vesicles (Fig. 6). This regionalization of the two types of vesicles occurred within one section of the terminal or in widely separated sections. An example of the latter was particularly well illustrated in the p11 muscle of animal #3 where different regions of the same terminal contained either a mixture of both vesicles types or predominantly one of the two types (Fig. 7).
Table 7. Percent composition of clear and dense core vesicles and mitochondria determined from a volumetric analysis of the putative inhibitory terminals from the blue crab pyloric muscles p11, p12, p13, and p14.

<table>
<thead>
<tr>
<th></th>
<th>Animal #1</th>
<th>Animal #3</th>
<th></th>
<th></th>
<th></th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p11</td>
<td>P12</td>
<td>P13</td>
<td>p12</td>
<td>p14</td>
<td></td>
</tr>
<tr>
<td>Clear Vesicles (%)</td>
<td>33.2</td>
<td>25.3</td>
<td>15.7</td>
<td>46.1</td>
<td>32.6</td>
<td>30.6</td>
</tr>
<tr>
<td>Dense Core Vesicles (%)</td>
<td>19.5</td>
<td>11.2</td>
<td>23.2</td>
<td>22.5</td>
<td>6.3</td>
<td>16.5</td>
</tr>
<tr>
<td>Mitochondria (%)</td>
<td>12.5</td>
<td>8.7</td>
<td>11</td>
<td>9.6</td>
<td>14.9</td>
<td>11.3</td>
</tr>
<tr>
<td>Axoplasm (%)</td>
<td>35.0</td>
<td>54.8</td>
<td>50.1</td>
<td>21.9</td>
<td>46.2</td>
<td>41.6</td>
</tr>
</tbody>
</table>
Figure 6. Cross section of the blue crab pyloric muscle p11 showing excitatory (E) and inhibitory profiles (I). The four inhibitory terminals arise from a single axon and show different proportions of clear (c) and dense core (d) vesicles. Profile (I1) is filled primarily with elliptically shaped clear synaptic vesicles and few dense core vesicles. Another profile (I2) is filled with both clear synaptic vesicles and dense core vesicles in approximately equal proportions; note the synaptic contact (between bars). A third type of profile (I3, I4) is filled primarily with dense core vesicles and few clear synaptic vesicles. These three profile types are taken as evidence for the regionalization of dense core vesicles in inhibitory terminals.

Magnification: 39 000x. Scale bar: 0.5 um.
The putative inhibitory terminals of the blue crab pyloric muscles exhibited evidence for exocytosis of DCVs occurring both at synaptic (Fig 8a) and extrasynaptic (Figs. 8b) sites. Like the excitatory terminals, exocytosis in the inhibitory terminals occurs in the form of a small omega shaped infolding of the presynaptic membrane containing single, double, or multiple, dense cores. In the case of synaptic exocytosis, the exocytotic profile was frequently in close proximity to a dense bar. A quantification of the exocytotic profiles at both synaptic and extrasynaptic sites was conducted for the inhibitory terminals of the five muscles that were serially sectioned (Table 8). Like the excitatory terminals, the inhibitory terminals showed extrasynaptic DCV exocytosis to be more prevalent than synaptic DCV exocytosis.

4. Mitochondria

Simple and complexly branched mitochondria were also present in these putative inhibitory terminals (Fig. 4) but they appeared to be less prominent than other constituents such as clear and dense core vesicles. A volumetric analysis confirmed this qualitative observation; mitochondria constituted approximately 11% of the volume of the terminal compared to 30% for clear vesicles and 17% for DCVs (Table 7).

C. Putative Neuromodulatory Nerve Terminals

1. Identification

In addition to the excitatory and inhibitory terminals in the pyloric muscles, a novel type of innervation was seen in the p14 muscle of Animal #3
Figure 7. Selected micrographs from serial sectioned 3 µm length of a single inhibitory terminal of the blue crab pyloric muscle p11 showing changes in the composition of clear (c) and dense core (d) vesicles. In (A) clear vesicles predominate while in (B) there is a mixture of clear and dense core vesicles and in (C) dense core vesicles predominate. In (A) note the presence of synaptic contacts (between bars), one with a presynaptic dense bar (arrowhead). In (B, C) note the presence of mitochondria (○).

Magnification: 44 200x. Scale bar: 1 µm.
Figure 8. Cross sections of a putative inhibitory nerve terminal from the blue crab pyloric muscle p13 taken from different parts of a serial. In (A) a synaptic contact (between bars) between the terminal membrane and the adjacent granular sarcoplasm (s) is seen. At one edge of the synaptic contact is an infolding of the presynaptic membrane (arrow) enclosing dense cores and indicative of exocytosis of dense core vesicles. In (B) an infolding of the nerve terminal (arrow) membrane within which is a dense core indicative of exocytosis at an extrasynaptic site. Also note the presence of clear (c) vesicles, dense core (d) vesicles, and mitochondria (o).

Magnification: 66 300x. Scale bar: 0.5 um.
Table 8. Comparison of the number of dense core vesicle fusion sites, synaptic (syn) and extra-synaptic (ext), of putative inhibitory terminals of blue crab pyloric muscles p11, p12, and p13.

<table>
<thead>
<tr>
<th></th>
<th>Number of Extrasynaptic Fusion Sites</th>
<th>Number of Synaptic Fusion Sites</th>
<th>Frequency Ratio (Ext/Syn)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p11 (Animal #1)</td>
<td>9</td>
<td>3</td>
<td>3.0</td>
</tr>
<tr>
<td>p12 (Animal #1)</td>
<td>7</td>
<td>2</td>
<td>3.5</td>
</tr>
<tr>
<td>p12 (Animal #3)</td>
<td>8</td>
<td>3</td>
<td>2.6</td>
</tr>
<tr>
<td>p13 (Animal #1)</td>
<td>32</td>
<td>10</td>
<td>3.2</td>
</tr>
</tbody>
</table>
based on its DCVs (Fig. 9). As a result, I have tentatively identified this type of innervation as representing a putative neuromodulatory terminal. The DCVs in this putative neuromodulatory terminal were remarkably different from those in the excitatory and inhibitory terminals in several respects. First, they were much larger in size than their counterparts in the other two types of terminals. The DCVs from the neuromodulatory terminals were between 90-100 nm in diameter, whereas those of excitatory and inhibitory terminals were between 70-80 nm in diameter (Table 9). This difference in size was found to be significant via a student's t-test (p < 0.05).

Second, the location of the dense core within these DCVs was more variable, being located as often eccentrically as centrally. The DCVs in the excitatory and inhibitory terminals appeared more consistently in a central location. In the excitatory and inhibitory terminals, the dense core is not much smaller than the entire vesicle, whereas in the putative neuromodulatory terminals the dense core is much smaller than the entire vesicle. The variable location of the dense core within the vesicle gives a distinctly different appearance to these vesicles compared to their counterparts in the other two types of terminals.

Third, the proportion of DCVs in this putative neuromodulatory terminal was unusually high (Table 10). A volumetric analysis revealed that DCVs make up approximately half the terminal volume (47%) in the neuromodulatory terminal, compared with 9% in the excitatory terminals, and 17% in the inhibitory terminals.

2. Synaptic Features

Equally surprising as finding a putative neuromodulatory terminal was finding that it actually made neuromuscular synaptic contacts (Fig. 10). The pre- and postsynaptic membranes stain slightly more intensely than elsewhere but are not strictly parallel giving a less well-defined appearance to the synapse. Alternately, a
Figure 9. Three adjacent profiles from the blue crab pyloric muscle p14 depicting excitatory (E), putative inhibitory (I) and putative neuromodulatory (N) nerve terminals.

The neuromodulatory terminal is distinguished from the other two terminal types by its population of large dense core (I0) vesicles that have eccentrically located cores.

Excitatory and inhibitory profiles are distinguished by the shape of the small, clear (c) synaptic vesicles which are spherical and elliptical, respectively. The excitatory terminal shows a synaptic contact (between bars) with a presynaptic dense bar (arrowhead) and the presence of mitochondria (o).

Magnification: 41 200x. Scale bar: 0.5 um.
Table 9. Differences in the shape and size of dense core vesicles present in excitatory, putative inhibitory, and neuromodulatory terminals of the blue crab pyloric muscle p14.

<table>
<thead>
<tr>
<th>p14 (Animal #3)</th>
<th>Num</th>
<th>Diameter (nm)</th>
<th>Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Max</td>
<td>Min</td>
</tr>
<tr>
<td>Excitatory</td>
<td>11</td>
<td>82.5±10.9</td>
<td>74.5±10.1</td>
</tr>
<tr>
<td>Inhibitory</td>
<td>16</td>
<td>81.9±9.4</td>
<td>71.2±9.3</td>
</tr>
<tr>
<td>Neuromodulatory</td>
<td>44</td>
<td>105.8±31.9</td>
<td>93.6±29.8</td>
</tr>
</tbody>
</table>
Table 10. Percent composition of clear and dense core vesicles and mitochondria determined from a volumetric analysis of the neuromodulatory terminal from the blue crab pyloric p14 muscles compared to those in excitatory and putative inhibitory terminals of muscles p11, p12, p13, and p14.

<table>
<thead>
<tr>
<th></th>
<th>Excitatory Terminals (Mean)</th>
<th>Putative Inhibitory Terminals (Mean)</th>
<th>Putative Neuromodulatory Terminal of the p14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear Vesicles (%)</td>
<td>33.8</td>
<td>30.6</td>
<td>7.3</td>
</tr>
<tr>
<td>Dense Core Vesicles (%)</td>
<td>8.7</td>
<td>16.5</td>
<td>46.8</td>
</tr>
<tr>
<td>Mitochondria (%)</td>
<td>25.2</td>
<td>11.3</td>
<td>5.9</td>
</tr>
<tr>
<td>Axoplasm (%)</td>
<td>31.9</td>
<td>41.6</td>
<td>39.9</td>
</tr>
</tbody>
</table>
Figure 10. Three consecutive thin serial section micrographs (A-C) of a neuromodulatory terminal from the blue crab pyloric muscle p14 characterized by large dense core (ld) vesicles. Features typical of a synaptic contact (between bars) with muscle granular sarcoplasm (s) are denoted by the densely stained pre- and postsynaptic membranes, presynaptic dense bars (arrowheads), and prominent postsynaptic density (arrow). In (A, B) dense core vesicles above the presynaptic membrane are indicative of docking of these vesicles.

Magnification: 87 400x. Scale bar: 0.2 um.
distinct heavily stained band appears attached to the postsynaptic membrane, denoting a well-defined receptor zone which is only occasionally seen in conventional synapses (Atwood and Wojtowicz, 1986). In addition to seeing the putative neuromodulatory terminals making synaptic contact, it was also seen that these synapses are often decorated with dense bars, indicating active zones for transmitter release (Fig. 10). The close juxtaposition of dense core vesicles to the presynaptic membrane suggests vesicle docking and putative exocytotic sites adjacent to dense bars.

A quantitative analysis of the three synaptic contacts found in the putative neuromodulatory terminal revealed the mean synaptic area to be relatively small (0.11 ± 0.07 μm²) in comparison to the excitatory (Table 2) and inhibitory (Table 5) synapses of the same and other examined muscles. These neuromodularic synapses were seen to have a greater number of dense bars per synapse (2.33) compared to the excitatory (Table 2) and inhibitory (Table 5) terminals innervating the same p14 muscle. The mean length of the 7 dense bars present was found to be 0.27±0.12 μm, which was larger than the mean dense bar lengths present on the excitatory (Table 2) and inhibitory (Table 5) synapses.

3. Mitochondria

Mitochondria was a characteristic component of these neuromodulatory terminals, appearing as simple and branched structures, scattered within the terminal (Fig. 9). They were, however, far less prevalent than the DCVs (Table 10). A volumetric analysis showed that mitochondria made up approximately 6% of the volume of the terminal compared to 47% for DCVs.
IV. DISCUSSION

A. Excitatory Innervation

1. Ultrastructural Confirmation of Previous Physiological Work.

Previous work involving the pyloric muscle system of the blue crab *Callinectes sapidus* detailed the spatial anatomical relationships (Maynard, 1972) and the electrophysiological characteristics of the fourteen muscles and the nerves that innervate them (Govind et al. 1975). Only one of the pyloric muscles (p1) of the blue crab has been scrutinized at the electron microscope level (Atwood et al., 1978). The electrophysiological study by Govind et al. (1975) recorded the presence of EPSPs exclusively from all of the fourteen pyloric muscles. Only one of the pyloric muscles (p13) demonstrated a large (33 mV) EPSP, whereas the remaining five muscles for which EPSPs were quantified (p1, p6, p10, p11, p12) yielded EPSPs within the range of 0.5 mV to 3.0 mV. Govind et al. (1975) determined a facilitation index for four of the pyloric muscles (p1, p6, p11, p13). Of these four muscles only the p13 muscle showed a comparatively low facilitation index (0.9), while the other muscles demonstrated facilitation indices that ranged from 5.0 to 12.6. One idea proposed by Govind et al. (1975) was that the fourteen pyloric muscles could be divided into functional subgroups based on two criteria: close proximity, and a common source of innervation. On the basis of these criteria, three functional subgroups were proposed. The first of such groups involved the p1, cpv6, and cpv4ab muscles, the second involved the p2, p8, and p10 muscles, and the third involved the p11, p12, p13, and p14 muscles.

Corroboration of the contention that the pyloric muscles of the blue crab receive excitatory innervation came from the electron microscopic study of the p1 muscle (Atwood et al., 1978), where nerve terminals with clear spherical synaptic vesicles were
present. The presence of these round synaptic vesicles served as morphological evidence for excitatory innervation, since spherical clear vesicles are typically taken to indicate excitatory nerve terminals (Uchizono, 1967, Atwood et al., 1972). The present study revealed nerve terminals in the p11, p12, p13, and p14 pyloric muscles which housed uniformly spherical clear synaptic vesicles, and thus provides a morphological correlate for the electrophysiological results obtained by Govind et al. (1975).

2. Synaptic Features

The present study of the blue crab pyloric muscles involved a quantification of the size of synapses and the size and abundance of dense bars. Previously Atwood et al. (1978) intensively examined the synaptic parameters of the excitatory terminals of the p1 muscle of the blue crab. The synapses of the p1 muscle were considerably less uniform in size than those in many other crustacean muscles. Individual synapses ranged in size from less than 0.5 \( \mu \text{m}^2 \) in area to just under 10 \( \mu \text{m}^2 \). Coincidentally, dense bars were more likely to be found on the larger synapses than on smaller synapses, suggesting a relationship between synapse size and the presence of presynaptic dense bodies. My study also revealed the excitatory terminals to possess synapses of varied size. All of the muscles for which excitatory synapses were measured displayed a rather large variance. Furthermore, the excitatory synapses of the p12 muscle, which were the largest of all those examined, displayed the largest average number of dense bars per synapse. In contrast, the p14 muscle excitatory synapses, which were the smallest, displayed the least average number of dense bars per synapse. This finding corroborates the relationship between synaptic size and presence of dense bodies previously found (Atwood et al., 1978).

Similar studies of the synaptic characteristics of other stomach muscles of the blue crab, namely the gastric mill muscles gm8b and gm9, did not reveal a large
variance of synaptic size (Atwood et al., 1977). Thus, the pyloric muscles have excitatory terminals which house synapses that exhibit a wide range of sizes compared to those of the gastric mill muscles.

3. Dense Core Vesicles

a. Distribution

DCVs are a characteristic component of nerve terminals of the stomach muscles. They have been described in studies of the pyloric muscle p1 of the blue crab (Atwood et al., 1978), as well as in studies of the gastric mill muscles gm8b, gm9 (Atwood et al., 1977), and gm5a (Patel and Govind, 1997). I found them in the excitatory terminals in all four pyloric muscles that were examined. My study, however, provided a quantitative measure of their density by estimating the percent of the terminal volume occupied by them. A volumetric analysis revealed that DCVs occupied considerably less of the terminal volume than their clear vesicle counterparts in all of the muscles examined. This is consistent with other studies of stomach muscles of the blue crab which note the clear vesicles to be more prevalent than DCVs (Atwood et al, 1977; Atwood et al., 1978; Patel and Govind, 1997).

In the excitatory terminals examined, the distribution of DCVs was usually scattered within the terminal. However, in one case, the p13 muscle of animal #1, the DCVs were enclosed in a membraneous sac in addition to being scattered within the terminal. The functional importance of such a sac like structure is at best speculative. One possibility is that this structure represents a mechanism for transporting a quantity of DCVs within the terminal. Another more likely possibility is that the sac represents a stage in the recycling of DCVs within the nerve terminal. Following DCV exocytosis, the
membrane may be retrieved via presynaptic sacs which detach to form cisternal sacs. These cisternal sacs may in turn bud off DCVs. This is an endocytotic mechanism which would result in a sac filled with DCVs similar to the one observed in the present study. Sacs filled with small, clear synaptic vesicles are present in motor nerve terminals of larval fruit flies (Hill, 1996) where it has been proposed that these sacs represent a stage in the replenishment of vesicles within the nerve terminals.

b. Evidence for Exocytosis

Previous studies have shown DCV exocytosis to occur exclusively at extrasynaptic sites, or non-specialized sites, within neurohemal organs such as the corpus cardaicum of insects, the neurohypophysis of vertebrates (Normann, 1976), and the central body of the crayfish brain (Schurmann et al., 1991). Another study which dealt with the neuropiles of invertebrates was the first to show that extrasynaptic DCV exocytosis was not restricted to neurohemal organs where DCVs are in abundance, but also occurred in a terminal where there is a majority of clear synaptic vesicles (Golding and Bayraktaroglu, 1984).

Alternately, a study of the blue crab gastric mill muscles showed DCV exocytosis to occur exclusively at synaptic sites (Patel and Govind, 1997). Single, double, and multiple forms of DCV release were seen at specialized synaptic sites of the terminal membrane. Multiple DCV release occurred via a sac like structure which invaginated into the postsynaptic membrane. Multiple dense cores were present within this sac. When visualized in three dimensions, the postsynaptic sac appeared to be a rectangular sheet which was connected at some point to a synapse. The authors suggested that the postsynaptic sac may elaborate into a finger-like invagination of the postsynaptic membrane which would eventually break up into small membrane bound particles. Such finger-like complex profiles were typically in close association with dense bars.
present on the synapse. The more simple forms of DCV release seen in the study (single and double) were found also to occur within synaptic areas of a terminal, but not necessarily in close association with a dense bar (Patel and Govind, 1997).

My study is the first where both types of DCV release, synaptic and extrasynaptic, was seen to occur. Like the studies that show DCV exocytosis at extrasynaptic sites (Normann, 1976; Schurmann et al., 1991; Golding and Bayraktaroglu, 1983), and studies that show DCV exocytosis at synaptic sites (Patel and Govind, 1997), my study also revealed the presence of multiple or compound DCV exocytosis as indicated by the presence of more than one dense core in the extracellular space. The central body of the crayfish brain also has a wide variety of forms of multiple DCV exocytosis (Schurmann et al., 1991). Multiple DCV exocytosis could occur in tandem, in packets and in rows lined up along non-specialized terminal membrane. Like the simple synaptic fusion sites seen in other muscles of the crab gut (Patel and Govind, 1997), the synaptic DCV exocytosis in the pyloric muscles was not restricted to sites on the synapse that were in close proximity to a dense bar. This may be interpreted to indicate that within the excitatory nerve terminals of the blue crab pyloric muscles, DCV release works via a mechanism independent of the active zone, and hence independent of an area of high concentration of calcium channels. Further corroboration for this idea comes from the fact that DCV exocytosis was frequently encountered on areas of non-specialized terminal membrane which are distant from dense bars and the high density of calcium channels they possess.

c. Differences in Frequency of Exocytosis

In a recent study of a blue crab stomach motor unit comprised of four separate muscles, the initial release of transmitter was high in two muscles (cv2 and cv3) and low in the other two muscles (gm5a and cpv7a) (Patel and Govind, 1997). The high-output
type displayed a greater amount of synaptic DCV exocytosis compared to that found in the low-output type. A similar correlation can be made for the pyloric muscles based on the results of an earlier study in which synapses of p11 and p12 were characterized as low-output and those of p13 as high-output (Govind et al., 1975), and the present study in which the high-output terminals of the p13 displayed a greater amount of both extrasynaptic and synaptic DCV exocytosis than the low-output terminals of the p11 and p12 muscles. These differences in the frequency of DCV release at the synapse may contribute to the differences in EPSP size.

DCV release at the synapse may result in a type of intrinsic modulation of muscle receptor sensitivity (Campbell, 1987), and thus may amplify the effects of normal fast acting transmitters. One example of a neuromodulatory substance acting to potentiate the postsynaptic response by altering muscle receptor sensitivity is the finding that small cardioactive peptides (SCPs) can enhance the excitatory efficacy of neuromuscular synapses at the accessory radula closer muscle of *Aplysia* (Lloyd, 1986). It is believed that SCPs increase the levels of the secondary messenger cAMP within the postsynaptic muscle. This in turn results in greater activation of cAMP dependent kinases which serve to alter the phosphorylation of the muscle receptors rendering them more sensitive to transmitter binding. The ultimate result of SCP presence is a potentiated contractile response.

SCPs in the abdominal ganglia of *Aplysia* have been shown to modulate, or potentiate, synaptic transmission by presynaptic means. SCP release results in a subsequent increase in cAMP levels in presynaptic terminals (Lloyd, 1986), resulting in the closure of specific K+ channels. When an action potential reaches the terminal, the closed K+ channels will prolong the time period required for the terminal membrane to repolarize back to its resting potential. Thus, the terminal will experience a longer duration of depolarization, a phenomenon known as spike broadening which, in turn will
lead to a greater amount of Ca\textsuperscript{2+} and enhanced transmitter release (Robitaille and Charlton, 1992). A similar mechanism of spike broadening is thought to underlie a type of learning known as sensitization of the gill withdrawal reflex seen in Aplysia (Abrams et al., 1984).

A further example of how neuromodulatory substances can affect the output of the postsynaptic response comes from studies involving the abdominal flexor muscle in crayfish (Bishop et al., 1987). The abdominal flexor muscle is innervated by motorneurons which contain a conventional excitatory fast transmitter, presumably glutamate, as well as the slow acting neuromodulatory substance proctolin (Bishop et al., 1984). When proctolin is released, it acts postsynaptically to potentiate the contraction of the depolarized muscle fiber.

It is possible that the observed differences in the frequency of DCV release observed in my study contribute to the differences in EPSP size. It is tempting to speculate that the synaptic DCV release affects EPSP size by postsynaptic means, while the extrasynaptic DCV release contribute to the differences in EPSP size by exerting an effect on the presynaptic terminal. Also of interest is the observation that the p13 excitatory terminal which demonstrated the greatest amount of DCV exocytosis also showed the greatest proportion of DCVs under volumetric analysis as compared to the p11, and p12 excitatory terminals. Presumably, a larger pool of DCVs in p13 terminals compared to the other muscles would contribute to the p13’s ability to produce larger EPSPs.

d. Evidence for Endocytosis

My investigation provides the first images of DCV formation by way of endocytosis. Figure 3C depicts a granule that is enclosed by an omega shaped
membrane enclosure that is coated by evenly spaced thin filaments. Typically, endocytotic profiles are coated by a similar appearing clathrin coat. DCV formation by way of endocytosis would suggest a mechanism for DCV recycling analogous to that of clear synaptic vesicles. One hypothesis regarding membrane retrieval after DCV exocytosis comes from studies involving the crustacean sinus gland (Bunt, 1969). After, or during, the release of granule contents by exocytosis, a coated pit is formed in the plasma membrane where the exocytosis just occurred, or is occurring. This coated pit is believed to induce membrane vesiculation, giving rise to a coated synaptic vesicle, called an acanthosome. The acanthosome may eventually shed its coat and become a smooth synaptic vesicle (Nagasawa, 1977; Nagasawa et al., 1971; Nagasawa and Douglas, 1972; Whittaker et al., 1966), or undergo a process of lysosomal degeneration (Normann, 1976). Thus, the coated pits seen at sites of exocytotic granule release on crustacean sinus gland terminals suggest that a portion of the membrane which initially enclosed the secretory granule may subsequently be returned to the interior of the terminal as a coated vesicle (Bunt, 1969).

Further evidence for this hypothesis comes from studies using extracellular tracers. One study involved the use of the tracer horseradish peroxidase which was found to be incorporated into the coated microvesicles of the neurosecretory terminals of the mammalian posterior pituitary gland (Nagasawa et al., 1971). Another study employing the use of the extracellular tracer Thorotrast yielded similar findings (Bunt, 1969). Thorotrast was found to be incorporated into both smooth and coated vesicles of the crayfish sinus gland. Both of these studies suggest that these vesicles find their origin in the process of micropinocytosis. Since both of these studies examined terminals which were primarily filled with DCVs or granules, they suggest that one method of compensating for granular exocytosis is the formation of coated and smooth vesicles by the process of micropinocytosis.
The presence of a coated omega shaped membrane surrounding a dense core in the p13 excitatory terminal may be representative of a DCV in the process of exocytosis with the surrounding membrane preparing for micropinocytosis, or it may be representative of a dense core being endocytosed. The former possibility is unlikely as other examples of DCV exocytosis observed in the same and other pyloric muscles did not show this bristle like halo around the membrane surrounding the dense core. In fact, other exocytotic profiles that appear further advanced (i.e. dense core was actually in the extrasynaptic cleft) than the one in question did not display any type of coating. Thus, it is likely that the coated omega shaped membrane surrounding a dense core represents an event which is distinct from exocytosis, most likely a DCV being formed via a process of endocytosis.

Therefore, the presence the DCV endocytotic profile may indicate a recycling mechanism similar to that for clear synaptic vesicles to replenish the supply of DCVs in pyloric muscle nerve terminals of the blue crab.

B. Putative Inhibitory Innervation

1. Identification Based on Synaptic Vesicle Shape

Identification of inhibitory and excitatory motorneurons on the basis of shape of their clear synaptic vesicles is a firmly established criterion. Uchizono (1967), studying the stretch receptor neuron in the crayfish abdomen, was the first to point out that fixation with aldehydes and osmium rendered inhibitory vesicles elongated or pleomorphic in shape. It was previously known that the stretch receptor neuron receives inhibitory innervation which is localized to the dendritic zone of the receptor neuron, and typically not a source of direct innervation to the receptor muscle (Kuffler and Eyzaguirree, 1954). Conversely, the excitatory input is mostly restricted to the receptor
muscle and rarely found on the dendritic zone of the receptor neuron. Under electron microscopic investigation, the population of inhibitory terminals differed from the excitatory terminal population primarily on the basis of vesicle composition (Uchizono, 1967). The inhibitory terminals housed elongated elliptical vesicles whereas the excitatory terminals contained round, or spherically, shaped vesicles.

Subsequent studies have reiterated the finding by Uchizono (1967). Utilizing the crayfish opener muscle, it was possible to selectively deplete either round synaptic vesicles or the elongate synaptic vesicles by prolonged simulation of excitatory and inhibitory axons, respectively, in the presence of a metabolic inhibitor 2,4-dinitrophenol (Atwood et al., 1972). 2,4-dinitrophenol penetrates nerve cells and uncouples oxidative phosphorylation, thereby depleting the energy reserves of the cell. The ultimate effect of 2,4-dinitrophenol is to affect sodium extrusion and all other adenosine triphosphate utilizing reactions. Thus, in the presence of 2,4-dinitrophenol, the energy supply necessary for resynthesis or replenishment of synaptic vesicles under a prolonged stimulation regimen is exhausted. Electron microscopic examination revealed that when the excitatory axon underwent prolonged stimulation in the presence of 2,4-dinitrophenol, terminals that contained round vesicles were markedly depleted. Similarly, in preparations where it was the inhibitory axon that underwent prolonged stimulation in the presence of the metabolic inhibitor, it was found that terminals containing irregular, or elliptically, shaped vesicles were markedly depleted (Atwood et al., 1972). The shape index ratios of the excitatory and inhibitory vesicles in the study ranged between 1.00 - 1.18 and 1.3 - 1.45, respectively. These ratios were lower than the ratios found for the excitatory and inhibitory synaptic vesicles in my study, namely 1.2 and 1.9 respectively (Tables 1 and 5). However, the putative inhibitory terminals of all four pyloric muscles displayed synaptic clear vesicles that had significantly higher
shape indices than the synaptic clear vesicles seen within the excitatory terminals of the same muscle.

Although the shape of synaptic vesicles serves as an indicator of excitatory or inhibitory innervation in crustacean muscles (Atwood, 1976), other more convincing means for identification of terminal type has to be employed before the pyloric muscles in blue crab are shown to be innervated by an inhibitory axon. One such method is the immunohistochemical demonstration of GABA, in which an antibody to GABA is detected with fluorescence microscopy. Using these techniques, Birmingham and Marder (personal communication) have tentatively detected GABA staining fibers in the nerves of the pyloric muscles in Cancer crabs. These preliminary studies support my electron microscopic evidence for inhibitory innervation in the pyloric muscles of blue crabs.

2. Synaptic Features

Like their excitatory counterparts, the inhibitory terminals made synaptic contact with the pyloric muscles. Previous studies comparing excitatory and inhibitory synapses have shown inhibitory synapses to be larger in surface area (Govind and Pearce, 1989; Atwood and Kwan, 1976). Specifically in the distal accessory flexor muscle of the adult lobster, inhibitory synapses were two times greater in surface area than the excitatory synapses, and the proximal accessory flexor muscle exhibited inhibitory synapses that were fifteen times greater in surface area than the excitatory synapses (Govind and Pearce, 1989). Similar findings regarding the differences in synaptic area between inhibitory and excitatory synapses were shown in the crayfish opener muscle (Atwood and Kwan, 1976). In addition to differences in synaptic surface area, it is generally thought that when a muscle receives innervation from a single excitor and a single inhibitor, the inhibitory innervation will be less prevalent. Studies on both the lobster
accessory flexor muscle and the crayfish opener muscle revealed that although inhibitory synapses tend to be larger in size, they are also fewer in number when compared with excitatory synapses (Govind and Pearce, 1989; Atwood and Kwan, 1976).

Similar to previous findings, my study showed inhibitory innervation to be less prevalent than excitatory innervation. For all of the muscles examined, the inhibitor formed fewer synapses than the excitor. Furthermore, there were some sites which displayed only excitatory terminals. At sites where both types of terminals were witnessed, the excitatory terminals always outnumbered the inhibitory terminals. However, in contrast to previous findings, my study did not show inhibitory synapses to be greater in size than excitatory synapses. This may in part be due to the relatively small number of inhibitory synapses that were quantified.

One interesting feature revealed by my study was that the inhibitory synapses of the p11, p12, and p13 muscles from animal #1 were very similar in synaptic area and displayed a similar mean number of dense bars per synapse. This similarity suggests the possibility that the muscles of this motor unit are innervated by a common inhibitor.

Interestingly, for all of the muscles surveyed, not one example of presynaptic inhibition was found. The accepted morphological correlate of presynaptic inhibition, polarized axo-axonal contacts from an inhibitory terminal to an excitatory terminal, has been observed in the crayfish opener muscle (Atwood, 1982). However, similar to my investigation, studies involving the lobster accessory flexor muscle, which is innervated by an excitor and an inhibitor, failed to show evidence for presynaptic inhibition.

3. Functional Importance

In addition to the Govind et al. (1975) study, there are a number of electrophysiological studies of the innervation of crustacean stomach muscles (Maynard
and Atwood, 1969; Hooper et al., 1986). All of these studies revealed recordings of either spontaneous or evoked synaptic potentials from the stomach muscles that were exclusively depolarizing. Thus, these stomach muscles have been thought to receive only excitatory innervation. In addition to the physiological studies, electron microscopic studies of many stomach muscles in blue crabs have revealed the presence of solely excitatory nerve terminals (Atwood et al., 1977; Patel and Govind, 1997). My study reveals that inhibitory innervation may be present in the p11-14 motor unit, and that such the inhibitory innervation may be restricted to the pyloric muscles of the blue crab.

The pyloric muscles exhibit a three phase activity pattern that is sometimes repeated within a period of 0.5 seconds (Rezer and Moulins, 1983), a period that is considerably shorter than that seen for other foregut motor patterns, such as the gastric mill rhythm which has a period of 5 seconds (Hartline and Maynard, 1975). It is likely that the pyloric muscles will exhibit more precise control of muscular contraction when compared with the muscles of the gastric mill or the cardiopyloric valve. This need for fine muscle control is perhaps satisfied by the presence of an inhibitory neuron. Using this line of reasoning, the absence of inhibitory innervation seen in the Atwood et al. (1978) study of the p1 muscle can be explained by the fact that the p1 muscle is part of a motor unit involving muscles of the cardiopyloric valve, and not other pyloric muscles. Thus, the need for fine motor control of the p1 muscle is likely not as great as that of the other pyloric muscles.

4. Dense Core Vesicles

a. Distribution

Similar to their excitatory counterparts, the DCVs occupied considerably less of the terminal volume than clear synaptic vesicles. However, the inhibitory terminals had
a greater amount of their terminal volume devoted to DCVs than their excitatory counterparts. Typically, the distribution of dense core vesicles in the inhibitory terminals, like their excitatory counterparts, was scattered. In a few cases, there was a high degree of regionalization of DCVs and clear vesicles within the inhibitory terminals. This was best exemplified in the inhibitory terminal of the p11 muscle from animal #1, where the DCVs were highly regionalized within certain areas of a single terminal. Whether such regionalization has functional utility is difficult to ascertain, but it would be worth investigating as it has the potential for very localized control of release of fast and slow acting transmitters.

b. Differences in Frequency of Exocytosis

Similar to their excitatory counterparts, the inhibitory terminals displayed both synaptic and extrasynaptic forms of DCV exocytosis, and the extrasynaptic variety was again the more prevalent of the two types. A further similarity between the inhibitory terminals and excitatory terminals was the correlation between the amount of both synaptic and extrasynaptic DCV exocytosis and EPSP size. Inhibitory terminals of the p13 muscle displayed a greater amount of extrasynaptic and synaptic DCV exocytosis in comparison to the inhibitory terminals of the p11 and p12 muscles. Furthermore, as evidenced by volumetric analysis, the inhibitory terminals of the p13 muscle contained a greater proportion of DCVs than those of the p11 and p12 muscles. Thus the relationship between DCV exocytotic frequency and DCV volume that was observed for the excitatory terminals was again witnessed in the inhibitory terminals. Assuming the function of synaptic DCV exocytosis is to potentiate the postsynaptic response to transmitter binding, as seen with investigations into the effect of SCP release of transmitter output performed on the radula closer muscle and abdominal ganglia of the Aplysia (Lloyd, 1986), it would follow that the p13 muscle should be more strongly
inhibited than the p11, and p12 muscles. This is a tempting scenario because if the function of the inhibitory innervation is to add to the precision of muscle control of the pyloric muscles, it would follow that the muscle that is capable of large excitatory output should be inhibited to a larger extent than muscles that are less capable of generating a large excitatory response. Such matching of excitatory and inhibitory inputs was shown for synapses of the crayfish limb muscle (Atwood and Bittner, 1971).

C. Putative Neuromodulatory Innervation

1. Identification Based on Dense Core Vesicle Morphology

   The present study showed an unusual type of nerve terminal in the p14 muscle which was composed primarily of unique large DCVs with eccentrically located cores. Because DCVs contain neuromodulatory substances, I tentatively identified this type of terminal in the p14 muscle as a type of neuromodulatory innervation. However, there are a number of reservations surrounding this tentative identification.

   The first reservation is that the large size and eccentrically located core of these vesicles may represent regular DCVs which have become swollen during fixation for electron microscopy. Such fixation artefacts are common in neuromuscular tissue prepared with relatively slow acting chemical fixatives (Bodian, 1970). This seems unlikely since only a very small select group of nerve terminals was affected in this manner. In fact, the excitatory and putative inhibitory nerve terminals in the same p14 muscle contained conventional appearing DCVs. Similar fixation methods in other blue crabs revealed the conventional type of DCVs in p11, p12 and p13 muscles, and not this unusual swollen type. A more convincing argument that the p14 putative neuromodulatory terminal is not a fixation artefact was the recent finding in our
laboratory (unpublished observations) of this swollen type of DCV in a p2 muscle taken from two separate blue crabs fixed on two separate occasions. The p2 muscles in these animals also displayed nerve terminals with conventional looking DCVs. The finding of a putative neuromodulatory terminal on three separate occasions tends to refute the view that it represents a fixation artefact.

The second reservation is that this identification is based on a single terminal of a single p14 muscle. Clearly, a larger sample size, for both numbers of muscles and number of animals, has to be obtained before a more definitive conclusion can be made.

The third reservation is that the putative neuromodulatory terminal observed in this study is a segment of a more conventional fast transmitter type axon specialized to store and release DCVs, as seen with the p11 inhibitory terminal. However, serial sectioning of the putative neuromodulatory terminal in the p14 muscle revealed no connection between the neuromodulatory terminal and a more typical terminal for a 5-10 μm length. Because tracing these terminals for such short lengths is not a guarantee that they are separate axons, it is possible that our putative neuromodulatory terminal is a specialized region of a more conventional fast transmitter type axon.

2. Dense Core Vesicle Composition

Because of the unique appearance of the DCVs in the neuromodulator, it is fair to assume that they house substances that are different than the DCVs found in the excitatory and inhibitory terminals. A study of an area of dense neuropile in the central body in the median protocerebrum of the brain of the crayfish *Cherax destructor* identified the presence of three types of vesicles (Schurmann et al., 1991). First, the electron lucent synaptic vesicles identified were approximately 35 nm in diameter. Second, DCVs which were approximately 64 nm in diameter, and finally what were referred to as large structured DCVs were 98 nm in diameter. Similarly our investigation
revealed three types of vesicles with diameters similar to those found in the Schurmann study (1991). While the median protocerebrum of the crayfish brain revealed terminals that housed all three of the vesicle types, my study identified the third type, large structured DCVs, to be restricted to a separate terminal. There is evidence that large DCVs differ from their smaller counterparts by housing two substances as opposed to just one. It is generally thought that both biogenic amines and peptides are found to be localized within the larger variety of DCVs, whereas the smaller DCVs contain solely biogenic amines. In the mammalian CNS, the peptide substance P coexists with the amine 5-HT, and enkephalins (peptides) coexist with catecholamines within the dense cored vesicles found within chromaffin cells of the adrenal medulla (Pelletier et al., 1981). Immunological and histological assays of the protocerebral body of the crayfish brain have revealed the presence of both peptidergic and amminergic fibers (Schurmann et al., 1991). Perhaps the neuromodulatory DCVs seen in my study differ in composition from those seen in the more conventional terminals by housing two substances (e.g. biogenic amines and peptides) as opposed to just one (e.g. amines). This may be the cause of their unique appearance after fixation with aldehydes and osmium tetroxide.

One ultrastructural study of the interneural, neuronematocyte and neuromuscular junctions of the hydromedusan Gonionemus vertans showed the presence of DCVs that were similar in appearance to those seen in the neuromodulatory terminal of my study (Westfall, 1970). Almost all of the DCVs were orbital in appearance with an electron lucent area between the dense core and the encapsulating membrane. It is possible that the contents of these vesicles in the hydromedusan are similar to the contents of the neuromodulatory terminal DCVs in blue crab stomach muscles. Unfortunately, neither an immunological nor a histological assay was performed on the hydromedusan
preparation; thus, the specific contents of the DCVs of the neuromodulatory terminal remains speculative.

3. **Synaptic Features**

My study provides some of the first images of synaptic contacts between a putative neurosecretory terminal and its postsynaptic target, in this case the pyloric muscle. Furthermore, these synaptic contacts housed presynaptic dense bars. Previous investigations into neurohemal organs has shown DCV release to occur exclusively at non-synaptic sites (Normann, 1976; Golding and Bayraktaroglu, 1983; Schumann et al., 1991). Thus, my study provides the first example of a neurosecretory terminal where DCV release occurs at a specialized region of the terminal membrane. The presence of presynaptic dense bodies indicates that this release may be calcium dependent, and thus similar to the mechanism of fast transmitter release in more conventional terminals. It is important to note the synaptic contacts observed between the neuromodulatory terminal and the p14 pyloric muscle differed from those of the more conventional terminal types with respect to intensity of staining. The neuromodulatory synaptic contacts were characterized by a distinct heavily stained band attached to the postsynaptic membrane, and an electron lucent extracellular space. These attributes would suggest that these synaptic contacts are characterized by a well-defined receptor zone and an absence of the extracellular proteins typically seen at synaptic contacts. Although the neuromodulatory terminal showed areas of the terminal membrane that were specialized for DCV release, these areas appear to be less specialized than those commonly seen between crustacean muscles and the more traditional terminal types.
V. **Summary**

1. Based on electrophysiological evidence, stomach muscles in crustaceans receive exclusively excitatory innervation. In the present electron microscopic examination of the pyloric muscles p11, p12, p13, and p14 of the blue crab *Callinectes sapidus*, two other forms of innervation are revealed on the basis of vesicle morphology, namely inhibitory and neuromodulatory.

2. Excitatory terminals were identified based on the spherical shape of their clear synaptic vesicles. The ratio of the maximal and minimal diameter of these vesicles gave a shape index of 1.2, which is almost a sphere. These clear synaptic vesicles constituted 38% of the nerve terminal volume.

3. Serial sectioning of the excitatory terminals revealed typical neuromuscular synaptic contacts which were varied in size among the four muscles. These synaptic contacts possessed presynaptic dense bars indicative of active zones for transmitter release.

4. Excitatory terminals contained dense core vesicles which made up 9% of the terminal volume and were randomly distributed. Exocytosis of dense core vesicles was observed at synaptic sites and more frequently at extrasynaptic sites. An example for endocytosis of a dense core vesicle was also observed and this, in conjunction with the exocytotic images, suggests that the recovery and release of dense core vesicles may be similar to that for clear vesicles.

5. Mitochondria made up a substantial volume (25%) of excitatory nerve terminals.

6. Putative inhibitory nerve terminals were identified on the basis of the elliptical shape of their clear synaptic vesicles; their shape index of 1.9 was significantly different from the 1.2 index of the excitatory vesicles. Synaptic vesicles constituted 31% of the inhibitory terminal volume.
7. Inhibitory terminals, which were less prevalent than their excitatory counterparts in all four muscles, displayed characteristic neuromuscular synaptic contacts with presynaptic dense bars.

8. Dense core vesicles made up 17% of the volume of the inhibitory terminals, which is substantially higher than that of the excitatory terminals. Although randomly distributed in most inhibitory terminals, dense core vesicles were distinctly regionalized in one case. Exocytosis of dense core vesicles in inhibitory terminals was observed at synaptic sites and more frequently at extrasynaptic sites.

9. Mitochondria made up a relatively smaller proportion of the inhibitory terminal volume at 11%.

10. Putative neuromodulatory terminals were identified only in the p14 muscle, and this identification was based on their dense core vesicles which were significantly larger than those of the excitatory and inhibitory terminals, and had eccentrically located cores. They constituted almost half (47%) of the terminal volume.

11. These neuromodulatory terminals formed synaptic contacts with the muscle membrane, which were less well defined and smaller than those of the excitatory and inhibitory terminals. Those neuromodularic synapses displayed presynaptic active zones with adjacent docked dense core vesicles, implying active zones for release.

12. Mitochondria made up a substantially smaller volume of these neuromodulatory terminals at 6%, suggesting comparably lower activity levels than excitatory and inhibitory terminals.

13. The presence of these three separate forms of innervation, viz. excitatory, inhibitory, and neuromodulatory, provide fine control of contraction of the pyloric muscles which are responsible for filtering food. These multiple forms of innervation of the pyloric muscles denote a level of modulation that is as complex as that found in the stomatogastric ganglion itself.
VI. REFERENCES


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