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# Excitatory Synapses of Blue Crab Gastric Mill Muscles*

H.L. Atwood, C.K. Govind, and S.S. Jahromi**
Department of Zoology, University of Toronto, Toronto, Ontario, Canada

**Summary.** Physiological and ultrastructural studies were made of neuromuscular synapses in stomach muscles, especially two gastric mill muscles of the blue crab innervated by neurons of the stomatogastric ganglion. These muscles depolarized and contracted with application of glutamate, but not acetylcholine, whereas the dorsal dilator muscles of the pyloric region depolarized and contracted in acetylcholine, but not in glutamate. Large excitatory postsynaptic potentials (EPSP's) of 5–20 mV were recorded in the gastric mill muscles. At low frequencies of activation, individual synapses released on average about 2 quanta of transmitter for each nerve impulse. Facilitation of EPSP's after a single nerve impulse could be detected for at least 10 s. Synapses were found on enlarged terminals of the motor axon; their contact areas ranged from 0.2 $\mu m^2$ up to 3 $\mu m^2$. Both electron-lucent, round synaptic vesicles and dense-cored vesicles occurred near these synapses. A possible correlation between contact area of a synapse and output of transmitter, is discussed.

**Key words:** Neuromuscular synapse – Crustacean – Ultrastructure – Physiology.

## Introduction

Crustacean neuromuscular synapses show a wide range of physiological performance: facilitation, depression, and the amount of transmitter released differ greatly from one synapse to another, even among those formed by a single

*Send offprint requests to:* Dr. H.L. Atwood, Department of Zoology, University of Toronto, Toronto, Ontario, Canada

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** Present address: Biology Department, Pahlavi University, Shiraz, Iran

motor axon (Hoyle and Wiersma, 1958; Atwood, 1965, 1967, 1973; Bittner, 1968; Frank, 1973; and others).

A few attempts have been made to correlate the ultrastructural features of these synapses with physiological performance (Atwood and Johnston, 1968; Sherman and Atwood, 1972). Preliminary evidence suggested that in motor axon terminals of crab leg muscles, synapses releasing comparatively little transmitter are smaller than those releasing larger amounts of transmitter (Sherman and Atwood, 1972). However, these observations were made without serial sectioning, so that the total area of the synapses could not be measured.

Synapses of the central part of the crayfish opener muscle, which release very little transmitter except at high frequencies of stimulation (Bittner, 1968), have been analyzed in serial sections (Jahromi and Atwood, 1974; Atwood and Kwan, 1976). Excitatory neuromuscular synapses occur thickly on the nerve terminals, but they are rather small, seldom exceeding 1 $\mu m^2$ in contact area. Inhibitory neuromuscular synapses, which release more transmitter substance than the excitatory synapses on the same muscle fiber at low frequencies of excitation (Atwood and Bittner, 1971) are less numerous but attain a larger maximum size (1.5–2 $\mu m^2$).

Crustacean stomach muscles afford advantages for comparative studies of synaptic structure and function. One advantage is simplicity of innervation: unlike crustacean leg muscles, the majority of the stomach muscles are innervated by a single motor axon, and there is apparently no inhibitory innervation (Maynard, 1972; Maynard and Atwood, 1969; Govind et al., 1974). Thus, the problem of identifying synapses of different axons at the ultrastructural level does not arise in most of the stomach muscles. A further advantage is uniformity of muscle fibers and of excitatory post-synaptic potentials (EPSP's) within individual muscles (Govind et al., 1974; Jahromi and Govind, 1976). Most of the EPSP's in these muscles are large at low frequencies of stimulation, suggesting a synaptic physiology different from that encountered in the central region of the crayfish opener muscle, and inviting ultrastructural comparisons.

The motor neurons supplying crustacean stomach muscles form synapses (mostly inhibitory) in the stomatogastric ganglion. These have recently been studied in detail by King (1975, 1976a, b) in the spiny lobster, and it is of interest to compare ultrastructural features of ganglionic and neuromuscular synapses of these neurons. Also, some of these neurons are apparently cholinergic, while others may utilize glutamate or other substances as transmitters (Marder, 1974a, b). Correlation of ultrastructural features with the type of transmitter substance produced by individual neurons is feasible in this system.

The present study correlates physiological and ultrastructural features of synapses in two gastric mill (GM) muscles of the blue crab. These synapses differ in several respects from those described in crustacean leg muscles.

## Materials and Methods

Specimens of the blue crab, *Callinectes sapidus*, obtained live from a local supplier, were maintained in aerated 75% sea water at 15–20° C until used. The stomach muscles and their innervation

have been described in previous publications (Govind et al., 1974; Maynard and Dando, 1975; Jahromi and Govind, 1976). Gastric mill muscles GM8b and GM9, innervated together by a single excitatory neuron, were studied in detail. Comparative pharmacological observations were made on one of the pyloric muscles (Pl) and on the dorsal dilator muscle (CPV 1a).

Preparation of the muscles for microelectrode penetration, stimulation of the relevant axons, and recording of EPSP's with intracellular microelectrodes followed the procedures described previously (Govind et al., 1974). The preparations were kept in marine animal solution (Atwood and Dorai Raj, 1964) at 15° C during the experiment. Recordings with extracellular microelectrodes at individual synaptic terminals followed procedures used for other crustacean neuromuscular preparations (Dudel and Kuffler, 1961a; Atwood and Johnston, 1968). Because of muscle contraction, records were obtained only at low frequencies of stimulation.

Responses of muscles to drugs were studied by mounting isolated muscles in a small perfusion chamber in which the solution could be changed completely in about 5 s. During perfusion, membrane potentials of individual muscle fibers were monitored by means of intracellular microelectrodes. In some experiments, isometric tension was recorded by a small strain gauge attached to the tendinous region at one end of the muscle.

For electron microscopic observations, specimens were prepared as described in Jahromi and Govind (1976). Serial sections were made through regions containing synapse-bearing nerve terminals. Reconstructions of the nerve terminals, and measurements of synaptic contact areas from serial sections, were done as described by Jahromi and Atwood (1974) and Atwood and Kwan (1976).

## Results

### *Physiological Properties*

Single stimuli applied to the motor axon evoked excitatory postsynaptic potentials (EPSP's) of 5–15 mV in most fibers of GM8b and GM9 (Fig. 1). The responses were usually quite uniform throughout the muscles, indicating uniformity of synapses and of muscle fibers. However, in a few preparations smaller EPSP's (2–5 mV) were recorded in some of the fibers of GM8b. It was suspected that partial denervation of the muscle during dissection may have produced this result in at least some cases.

With continued stimulation at a very low frequency (0.2–0.5 Hz), EPSP's facilitated appreciably in size and many of them became large enough (15–20 mV) to generate a slow, graded secondary electrical response in the muscle membrane (Fig. 1A), accompanied by a slow, weak contraction. At frequencies of 1 Hz or greater, more rapid facilitation of EPSP's ensued, accompanied by stronger contractions. The long duration of EPSP's (500–600 ms total duration, with time constants of decay of 200–300 ms) led to summation of successive EPSP's, and to large levels of depolarization and strong contractions, at frequencies of stimulation above 1–2 Hz. Because of the problem of contraction, extracellular recordings at individual synapses could be made only at relatively low frequencies.

Preliminary observations on time course of facilitation of the EPSP's were obtained by the use of paired stimuli separated by a variable time interval (Fig. 1B). A large amount of facilitation was apparent when short interstimulus intervals were used. In Figure 1, $B_1$ at an interstimulus interval of 10 ms the second EPSP is 5 times the amplitude of the first. As the interstimulus interval

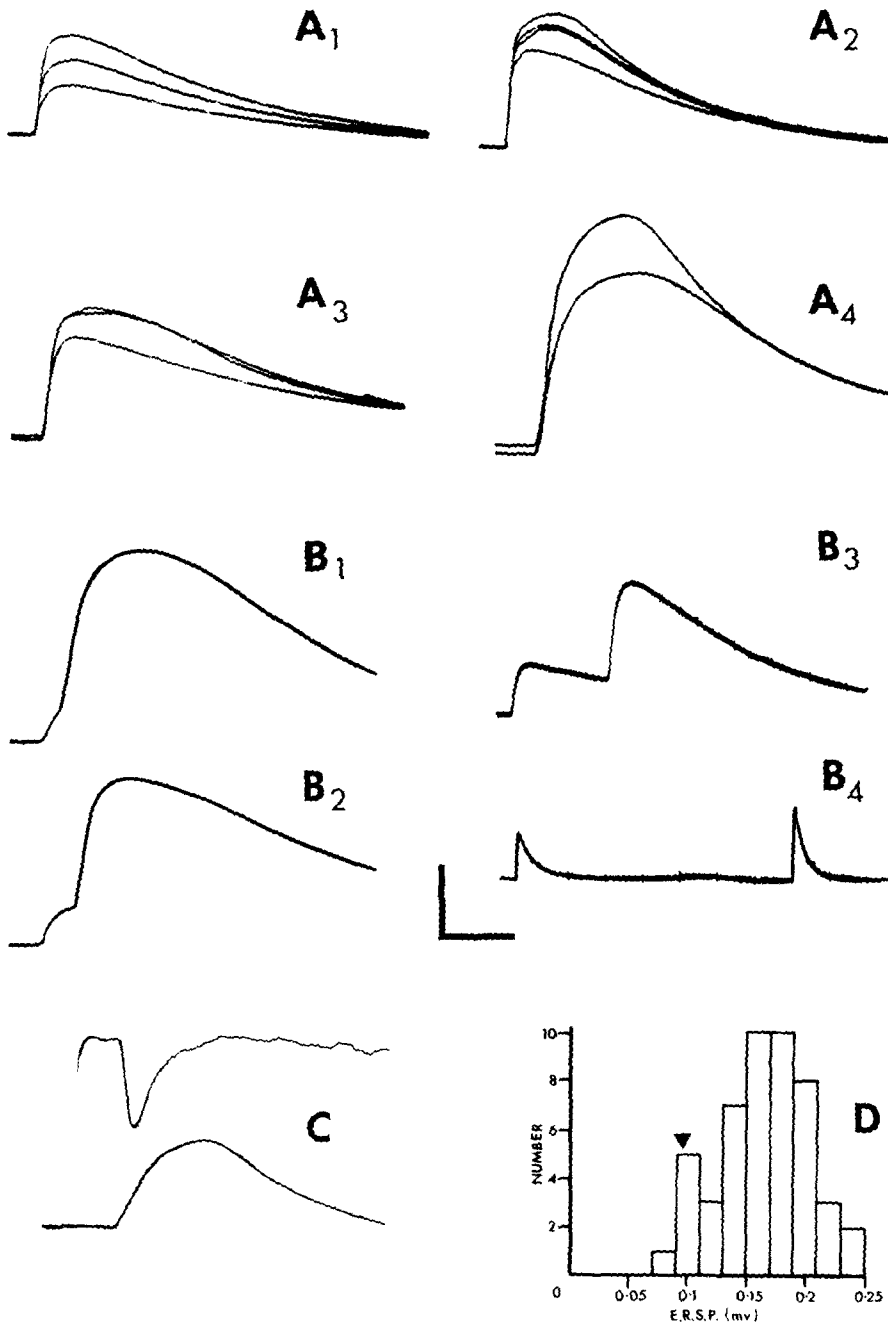


**Fig. 1A–D.** Electrophysiology of GM8b synapses. **A** EPSP's recorded at 0.5 Hz from two muscle fibers ($A_{1-2}$ and $A_{3-4}$), showing initial size of EPSP's and growth with repeated stimulation. In $A_2$, $A_3$ and $A_4$, graded secondary membrane responses are generated by EPSP's after a few stimuli. **B** Facilitation of EPSP's with interstimulus intervals of 10 ms ($B_1$), 20 ms ($B_2$), 250 ms ($B_3$), and 4000 ms ($B_4$). **C** Simultaneous recording of ERSP (upper trace) and EPSP (lower trace) at 0.5 Hz, with signal averaging (64 sweeps). **D** Histogram of ERSP amplitudes, showing average value for unitary responses (arrow) and a predominance of values at about twice the amplitude of the unitary response. No failures of transmission occurred. Calibrations: Vertical, 10 mV; horizontal, $A_{1-4}$, $B_{1-2}$ 40 ms; $B_3$ 100 ms; $B_4$, 1 s; $C$, 20 ms.

was increased, the second EPSP showed progressively less facilitation (Fig. 1 B), but facilitation could still be demonstrated with interstimulus intervals of 10 s. (In Fig. 1, $B_4$, facilitation of the EPSP with an interval of 4 s is shown.) It is apparent that both magnitude and duration of facilitation at these synapses are greater after one impulse than at other crustacean neuromuscular synapses so far described, and certainly greater than for any vertebrate neuromuscular synapses which have been examined (e.g., Mallart and Martin, 1967). More detailed study of the time course of facilitation at these synapses is presently underway (M.C. Charlton, unpublished observations).

Since the amplitude of an intracellularly recorded EPSP depends both on the amount of transmitter released by the nerve terminals, and on the membrane properties of the muscle fiber, and since we wished to compare the transmitter-releasing capabilities of different crustacean nerve terminals, we measured the quantal content of transmission ($m$) at low frequencies of stimulation, at several individual synaptic sites with extracellular microelectrodes.

Synaptic foci proved difficult to locate in these muscles, at least in comparison with crustacean leg muscles; we infer that there are relatively few synaptic terminal regions along each muscle fiber. Externally recorded synaptic potentials (ERSP's) were typically 15–20 ms in duration (Fig. 1 C), terminating at the time the intracellularly recorded EPSP reached peak amplitude. The durations of the ERSP's in these muscles were considerably longer than those reported for crayfish opener muscles (3–8 ms: Dudel and Kuffler, 1961 a, b) but within the range of values recorded in lobster leg muscles (Atwood and Parnas, 1968). Nerve terminal potentials were difficult to detect, even with signal averaging at what were judged to be good electrode placements.

Quantal content of transmission was estimated by recording a number of ERSP's at each synaptic focus, measuring their amplitudes, and dividing the ERSP mean amplitude by the mean amplitude of unitary spontaneous ERSP's or of unitary ERSP's observed during asynchronous release of transmitter (see Johnston and Wernig, 1971; Zucker, 1973). A representative result is shown in Figure 1 D for muscle GM9. At this synapse, as at others in GM8b and GM9, no failures of transmission occurred at a stimulation rate of 0.5 Hz, and most of the releases appeared to be of 2 quantal units. Quantal content was estimated at 1.84. At two other synapses, closely similar values of 1.82 and 2.1 were calculated. Incomplete recordings at other locations showed no major variations in performance.

### *Pharmacological Properties*

Marder's (1974a, b) reports of cholinergic synapses in several stomach muscles of the spiny lobster prompted us to test acetylcholine and glutamate on the gastric mill muscles (GM8b, GM9) and also on pyloric (Pl) and dorsal dilator (CPV 1a) muscles.

Acetylcholine, in concentrations ranging from $10^{-5}$ M to $10^{-4}$ M, was made up in marine animal solution containing physostigmine ($10^{-5}$ M). It was perfused rapidly over isolated muscles at 15° C. No effects, other than occasional

movement artefacts, were observed in GM8b or GM9 (five preparations) or in pyloric muscle Pl (one preparation). In contrast, at acetylcholine concentrations above $0.5 \times 10^{-4}$ M, a marked depolarization was recorded in CPV 1a, along with contractions which developed slowly over about 30 s and thereafter gradually relaxed. The positive result for muscle CPV 1a confirms Marder's (1974a) report of an acetylcholine effect in the homologous muscle of the spiny lobster.

D-tubocurare ($10^{-4}$ M) did not reduce the amplitudes of EPSP's in GM8b or GM9, even after prolonged soaking.

Glutamate, at concentrations of $0.5 \times 10^{-4}$ to $10^{-4}$ M, invariably caused a vigorous contraction of GM8b and GM9, but had no effect on CPV 1a. Concentrations of glutamate between $10^{-5}$ and $0.5 \times 10^{-4}$ M did not always cause contraction in GM8b, but these solutions did depolarize the muscle fibers by 10 to 30 mV, and reduced EPSP amplitudes to less than 1/3 initial amplitude. EPSP's were not eliminated completely in these solutions. The effects were fully reversible with restoration of the normal solution.

### *Nerve Terminals*

The ultrastructural features of the nerve terminals in muscles GM8b and GM9 are similar. Synapse-bearing nerve terminals are embedded in folds of granular sarcoplasm at the surfaces of the muscle fibers (Fig. 2) as in other crustacean muscles (Jahromi and Atwood, 1967, 1974). They were difficult to locate by random sectioning, which confirms the impression gained from extracellular recording of the restricted distribution of the synapses.

The axons leading to the synaptic terminals are very thin (1–2 μm, tapering down to 0.1–0.3 μm just before the synaptic regions), and well protected by a surrounding glial sheath (Fig. 2D) everywhere except at synaptic locations.

Three dimensional reconstructions of synapse-bearing terminals were made for 3 terminal regions in GM8b, and for 2 terminal regions in GM9 (Fig. 3). It became apparent that the thin presynaptic axons expand into synaptic terminals which are many times the diameter of the axons themselves. The enlarged terminals usually show a few prominent synapses, along with others of smaller size. Along any particular axon branch, two or more enlarged synaptic terminals can often be shown in series, provided the number of sections is large enough (Fig. 3A, C). Occasional small projections and side branches come off the main terminal (Fig. 3C). Terminal secondary branching is less evident in these muscles than in the crayfish opener muscle (Jahromi and Atwood, 1974; Atwood and Kwan, 1976).

In order to quantify the sizes of the synapses in these terminals, synaptic contact areas of 26 synapses were calculated from serial sections of two terminal regions in GM8b. A histogram of synapse sizes was plotted (Fig. 4B) for comparison with previous results from the crayfish opener muscle (Jahromi and Atwood, 1974). The size range of synapses in GM8b is greater than in the crayfish opener muscle. In particular, synapses in GM8b attain a larger maximum size (3 $\mu m^2$) than in the crayfish opener muscle (1 $\mu m^2$).

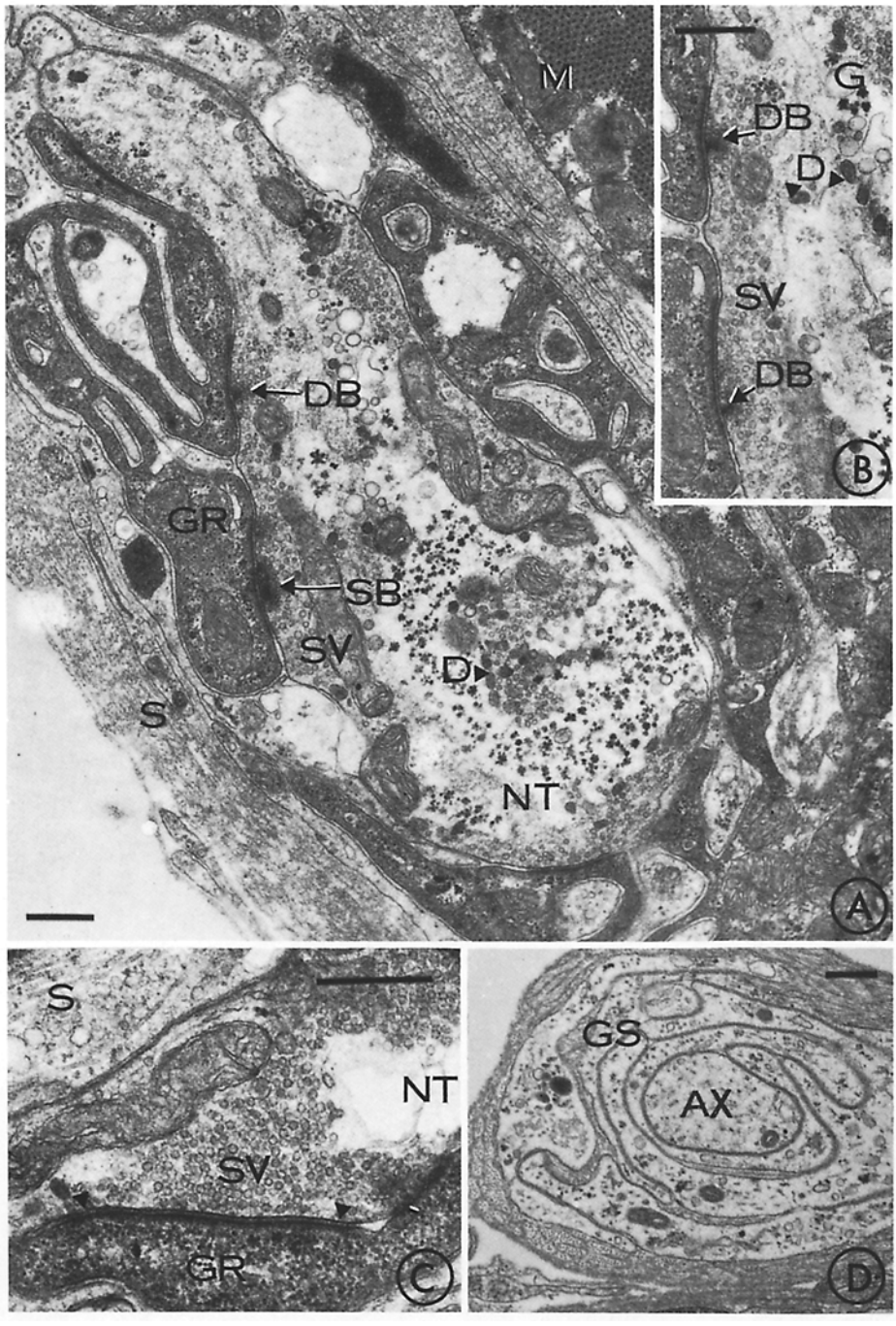


**Fig. 2A–D.** Synapses and nerve terminals of GM9. **A** Nerve terminal (*NT*) embedded beneath sarcolemmal layer (*S*) with several synapses. *GR* granular region of muscle; *M* myofilaments; *SV* synaptic vesicles; *D* dense vesicles; *DB* presynaptic dense body; *SB* presynaptic bar. **B** Part of another terminal showing two synapses with dense bodies, dense vesicles, and putative glycogen granules (*G*). **C** Large synapse and vesicles of another terminal. **D** Subterminal axon (*AX*) on the muscle's surface, with its associated glial sheath (*GS*). Scale marks: 0.5 μm

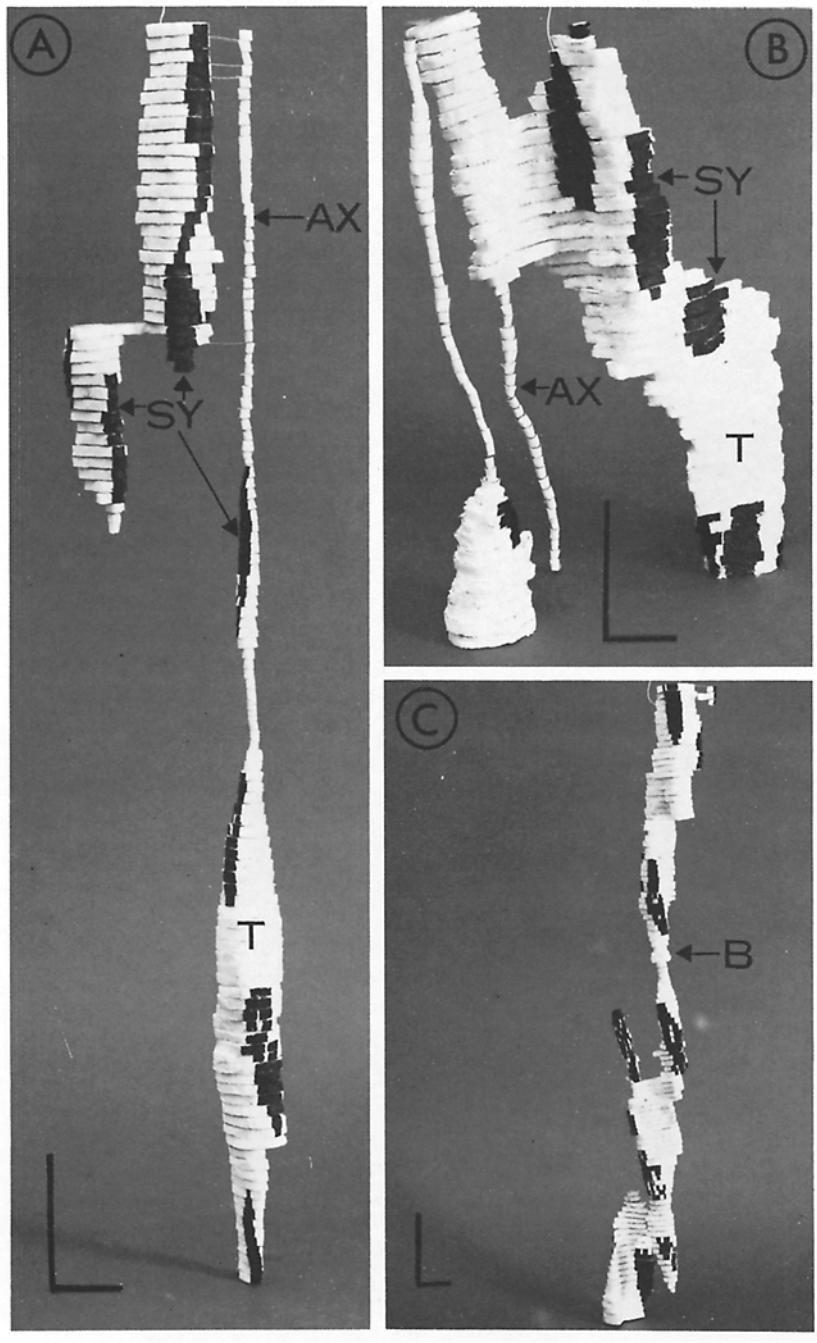


**Fig. 3A–C.** Reconstructions of terminals in GM8b (**A**, **C**) and GM9 (**B**) from serial sections. Synapses (*SY*) black. Axons (*AX*) leading to terminals (*T*) very thin. In **C**, a bottleneck (*B*) separates two expanded terminals. Scale marks: 1 μm

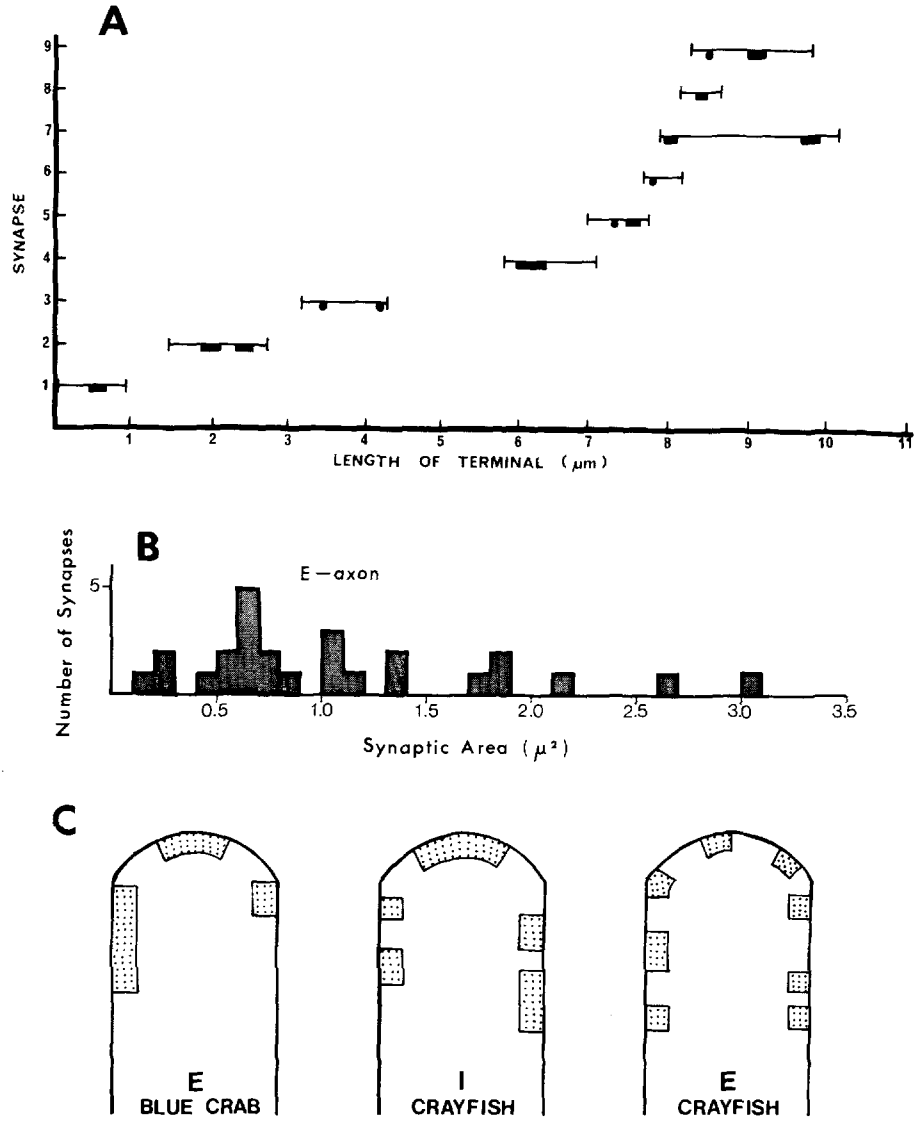


**Fig. 4. A** Occurrence of synapses (horizontal lines) and presynaptic dense bodies (dots and bars) in serially-sectioned GM8b terminal, showing variation in number and length of dense bodies. **B** Histogram of synaptic areas in two serially-sectioned GM8b terminals. **C** Diagram to compare relative numbers and sizes of synapses in representative terminals of: GM8b, blue crab, excitatory: opener muscle's central region in crayfish inhibitory (*I*) and excitatory (*E*). Fewer synapses of larger maximum size occur in GM8b

*Synaptic Ultrastructure*

Synapses are clearly recognizable from their electron-dense pre- and post-synaptic membranes, separated by a uniform gap of about 20 nm; from the clusters of synaptic vesicles near the presynaptic membrane; and from the occurrence of presynaptic "dense bodies" or "tufts" on the presynaptic membrane (Fig. 2A, B; cf. Jahromi and Atwood, 1974; King, 1976a).

The presynaptic dense bodies are tuft-like or hemispherical (40–60 nm across) and limited to one or two sections in many cases. However, many of them extend over two or more sections and resemble bars rather then tufts (Fig. 2A; Fig. 4A). Almost all completely sectioned synapses possess at least one presynaptic dense body, and many show two (Fig. 4A). In a sample of 29 completely sectioned synapses, 15% have no dense body, 56% have one, and 29% two.

The vesicles near the presynaptic membrane are mostly of the clear-cored type encountered at excitatory synapses of crustacean leg muscles (Atwood and Morin, 1970; Lang et al., 1972; Atwood et al., 1972). They are generally round, with an admixture of less regular entities. Typically these vesicles ("synaptic vesicles") fall in the 35–50 nm diameter range.

A smaller number of electron-opaque vesicles or granules is also present, usually more remote from the presynaptic membrane than the clear-cored synaptic vesicles. Some resemble the dense-cored vesicles of other synapses of both vertebrates and arthropods: they are typically 65–90 Å in diameter. They have been reported for ganglionic synapses of the stomatogastric ganglion of the spiny lobster (King, 1976a) and also for neuromuscular synapses of spiny lobster stomach muscles (Atwood et al., 1971).

Some of the electron-opaque vesicles appear to lack a distinct dense core, and instead seem to be uniformly dense (Fig. 2A, B). Often, they are oblong in shape, measuring approximately 70 by 150 nm. We are not certain whether these are the same as the other dense-cored vesicles, only fixed differently.

Other structures near the synapse include small dark granules, often clustered, possibly of glycogen, and numerous mitochondria and microtubules (Fig. 2A, B, C).

## Discussion

The present results, viewed in the light of previous studies on crustacean neuromuscular and intra-ganglionic synapses, show that physiological differences in synaptic performance are accompanied by ultrastructural differences. The only other studies to date in which quantitative information on synaptic contact areas and presynaptic dense bodies of crustacean neuromuscular synapses was obtained are those of Jahromi and Atwood (1974) and Atwood and Kwan (1976) on the crayfish opener muscle's central region. Comparison of the present data with those from the crayfish opener muscle (Jahromi and Atwood, 1974), shows several differences.

First, the synaptic contact areas of GM8b and GM9 attain a larger maximum size (3 $\mu m^2$) than do those of the crayfish opener muscle's central region (1 $\mu m^2$). (Inhibitory synaptic contact areas in the crayfish opener muscle also attain

a larger maximum size than the excitatory synaptic contact areas of the same muscle fiber).

Second, there are fewer synapses in the GM muscles lacking a presynaptic dense body, and perhaps more significantly, some of the presynaptic dense bodies in GM synapses are larger than those in the crayfish opener muscle's excitatory synapses. (Inhibitory synapses of the crayfish opener muscle also have a significant number of bar-shaped presynaptic dense bodies; Atwood and Kwan, 1976).

Third, the number of synapses per length of terminal sectioned is smaller for GM muscles than for the crayfish opener muscle, i.e. 1.2 per μm and 3.5 per μm respectively. (Inhibitory terminals of the crayfish opener muscle have fewer synapses per length of terminal sectioned than do excitatory terminals of the same muscle fibers; i.e., 2.1 per μm). A diagrammatic representation of the differences in number and size of synapses of blue crab GM excitatory terminals, crayfish excitatory terminals, and crayfish inhibitory terminals, is shown in Figure 4C.

Comparison of physiological performance shows that GM synapses release more transmitter at low frequencies of activation, and show more pronounced and longer-lasting facilitation after a single nerve impulse than do excitatory synapses of the crayfish opener muscle's central region. Quantal contents of crayfish opener muscle synaptic foci are usually much less than 1 at a frequency of stimulation of 1 Hz (e.g., Dudel and Kuffler, 1961a, b), whereas for GM muscles the values we obtained were close to 2 at 0.5 Hz. (In both cases, it is likely that the electrode recording ERSP's is sampling several ultrastructural synapses; Atwood and Johnson, 1968; Atwood and Parnas, 1968). In GM muscles, facilitation by a single nerve impulse can cause at least 5 times as much transmitter to be released by the next impulse at close interstimulus spacing (Fig. 1B), whereas in the crayfish opener muscle, facilitation in the central region of the muscle builds up more gradually with repetitive stimulation and the duration of facilitation after a single impulse is much shorter (Linder, 1974).

Physiological differences have also been reported between excitatory and inhibitory synapses of the crayfish opener muscle; the inhibitory synapses release more transmitter onto the muscle during low frequency activation than the excitatory synapses (Atwood and Bittner, 1971).

A tentative hypothesis can be formulated to link the physiological and ultrastructural observations. On average, each ultrastructurally defined "synapse" in a GM terminal is more effective in releasing transmitter with a single nerve impulse than its counterpart in the central region of the crayfish opener muscle. The effectiveness could be linked either to the larger presynaptic dense bodies of GM synapses, or to a larger synaptic area, which could allow more transmitter to leave during a nerve impulse, and/or more $Ca^{++}$ to enter through synaptic calcium channels (Katz and Miledi, 1969) to effect excitation-secretion coupling.

If in fact larger synapses with more developed presynaptic dense bodies release more transmitter, then it could well be that only the largest synapses of a terminal release transmitter for a single impulse, and that the others are "quiescent" until recruited by facilitation induced by a preceding nerve impulse,

or by maintained high-frequency stimulation. On this reasoning, the crayfish opener nerve terminal has a larger reserve of "quiescent" synapses than the GM terminal, and can potentially increase its output of transmitter to a greater extent, with maintained high frequency stimulation. Physiological evidence indicates that facilitation at high frequencies of stimulation can lead to a 10 to 30-fold output of transmitter at crayfish neuromuscular synapses, even though facilitated output after a single impulse is rather modest (Bittner, 1968). In GM muscles, a single impulse produces more facilitation, but it is likely that the relative increase in output of transmitter at high frequencies of stimulation is much less than for crayfish neuromuscular synapses, since the output of transmitter probably becomes saturated at a much lower frequency of stimulation in GM terminals.

This hypothesis also predicts a difference in physiological performance between neuromuscular and intraganglionic synapses of stomatogastric neurons. Although the intraganglionic synapses for the neuron innervating GM8b and GM9 in the blue crab have not been studied, the work of King (1976a, b) on the lobster stomatogastric ganglion has shown that the intraganglionic synapses are much smaller and more numerous than the neuromuscular synapses we studied. Our hypothesis predicts that each of the intraganglionic synapses is less likely to release transmitter for a single nerve impulse than the average neuromuscular synapse. Thus, the postsynaptic potentials recorded in the ganglion during operation of these neurons may represent discharge of only a small fraction of the total pool of synapses, which is large.

The pharmacological evidence on transmitter identity in muscles GM8b and GM9, though preliminary and incomplete, suggests that acetylcholine is not involved, but glutamate may be. In crustacean leg muscles, where the case for glutamate as the excitatory transmitter is stronger (Takeuchi and Takeuchi, 1972), synaptic vesicles of excitatory terminals are predominantly round when fixed in glutaraldehyde in a buffer which is not too hypo- or hyper-osmotic (Atwood and Morin, 1970; Tisdale and Nakajima, 1976). The fact that synaptic vesicles in GM8b and GM9 are predominantly round, is consistent with the observations on crustacean leg muscles.

In the stomatogastric ganglion of the spiny lobster, one type of terminal has round clear-cored vesicles ("Type B" of King, 1976a). It is possible that glutamate is the transmitter in the neurons with which these terminals are associated. If so, the correlation of round synaptic vesicles with glutamate-releasing terminals may be further extended in crustaceans.

In the present study, the putative glutamate-releasing synapses had both clear-cored and dense-cored vesicles associated with them, whereas in the lobster ganglion, Type B synapses had predominantly clear-cored vesicles. Whether this difference is species-dependent, location-dependent, or transmitter-dependent, remains to be shown.

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