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Functional and Structural Parallels in Crustacean and *Drosophila* Neuromuscular Systems[1]

HAROLD L. ATWOOD AND ROBIN L. COOPER

Department of Physiology, MRC Neural Group, University of Toronto, Toronto, Ontario M5S 1A8, Canada

SYNOPSIS. Comparison of morphological and physiological phenotypes of representative crustacean motor neurons, and selected motor neurons of *Drosophila* larval abdominal muscles, shows several features in common. Crustacean motor nerve terminals, and those of *Drosophila,* possess numerous small synapses with well-defined active zones. In crustaceans, neurons that are more tonically active have markedly varicose terminals; synapses and mitochondria are selectively localized in the varicosities. Phasic motor axons have filiform terminals, sometimes with small varicosities; mitochondrial content is less than for tonic axons, and synapses are distributed along the terminals. Tonic axons generate small excitatory potentials which facilitate strongly at higher frequencies, and which are resistant to depression. The phasic neurons generate large excitatory potentials which exhibit relatively little frequency facilitation, and depress rapidly. In *Drosophila,* counterparts of crustacean phasic and tonic motor neurons have been found, but the differentiation is less pronounced. It is inferred that cellular factors regulating the number of participating synapses and the probability of quantal release are similar in crustaceans and *Drosophila,* and that advantage can be taken of this in future to develop experiments addressing the regulation of synaptic plasticity.

INTRODUCTION

Crustacean neuromuscular systems have been well utilized as models for physiological and biochemical studies of synaptic transmission. Among the discoveries and concepts that have been developed through studies of crustacean motor neurons and their peripheral synapses, we can include: the concept of identified (and specified) neurons (Hoyle, 1977); mechanisms of synaptic inhibition, including presynaptic inhibition (Dudel and Kuffler, 1961*c*); mechanisms of quantal transmission (Dudel and Kuffler, 1961*a*) and short-term facilitation (Dudel and Kuffler, 1961*b*); physiological differentiation of neurons and their target muscle fibers (Atwood, 1965, 1976); mechanisms of long-term synaptic facilitation (Sherman and Atwood, 1971); modulation of transmission by neurohormones and neuromodulators (Kravitz *et al.,* 1980); physiological modification of neuronal phenotype (Lnenicka and Atwood, 1985); and definition of neurotransmitters, in particular gamma-aminobutyric acid (GABA) and glutamate (GLU) (Otsuka *et al.,* 1967; Kawagoe *et al.,* 1981; Atwood, 1982).

The utility of the crustacean preparations stems from the large size and small number of peripheral motor neurons, from the ready identification of specific neurons, and from the physiological and morphological similarities of the peripheral synapses to those found in central nervous systems. The large size and identifiability of the peripheral motor neurons has permitted experiments based upon isolation of specific neurons for stimulation, recording, and biochemical analysis. Pre-terminal axon branches can be visualized and penetrated with microelectrodes, which can be used to record and influence electrical events close to synapses, and to inject agents which modify specific aspects of synaptic transmission (Dixon and Atwood, 1989). Study of processes thought

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to be important in the central nervous system, such as short- and long-term facilitation, and synaptic depression, can be investigated in synapses that are readily accessible, and for which correlated fine structure can be obtained (Jahromi and Atwood, 1974).

Crustacean motor systems provide excellent material for physiological and biochemical studies, but have not been much investigated with modern techniques of molecular biology and genetics. The lack of crustacean-specific molecular probes for neuronal components—receptors, membrane channels, synaptic proteins—is a major drawback. Since physiological properties of synapses depend upon specific molecules, progress on the physiological topics requires finding ways to observe and manipulate synaptically significant molecules. Crustacean preparations would remain good material for investigation of synaptic physiology with more information about their synaptic molecules.

One of the organisms most heavily utilized for genetic and molecular biological studies, *Drosophila melanogaster,* belongs to an arthropod group which is phylogenetically relatively close to the crustaceans. *Drosophila* and crustacean molecules often appear to be more similar to each other than to those from other phylogenetic groups (Hariyama *et al.,* 1993). In the realm of physiology, it has long been known that insects share with crustaceans a similar neuromuscular organization (Hoyle, 1983) and the same peripheral neurotransmitters, GABA and GLU (Usherwood and Cull-Candy, 1975). Recent anatomical studies have concluded that some specific motor neurons in crustaceans and insects share a common origin (Wiens and Wolf, 1993).

The apparent phylogenetic closeness of crustaceans and insects leads to an interesting hypothesis, so far untested: synaptically important molecules found in *Drosophila* may have closely similar counterparts in crustaceans, and thus some of the molecular probes and antibodies arising from work in *Drosophila* could be utilized in crustacean systems. If this hypothesis proves valid, physiological investigations could be designed that would utilize the experimental advantages of crustacean neurons (in particular, their large size), in combination with the vast number of molecular products emerging from *Drosophila* investigations.

Previous physiological work on *Drosophila* has been undertaken both in adult flies (Ikeda, 1980) and in larval preparations (Jan and Jan, 1976). This work has shown that it is possible to use several physiological techniques, including voltage clamping of muscle fibers to measure ionic currents (Wu and Ganetzky, 1992), and recording of electrical events both in muscle fibers (Tanouye *et al.,* 1981) and in a few individual neurons (Koenig and Ikeda, 1983). However, the physiological work is more difficult in *Drosophila* than in crustaceans due to the much smaller size of the preparations. Nevertheless, in *Drosophila* it is possible to carry out physiological experiments, whereas in other popular models for study of neurogenetics, such as the nematode worm, *Caenorhabditis elegans,* physiological work has not been feasible to date.

In order to obtain more information about the possible physiological and morphological similarities and differences of crustacean and *Drosophila* preparations, we have applied the approaches previously found to be successful in crustaceans to larval *Drosophila* neuromuscular systems. The larval system provides the largest and most visible synapse-bearing nerve terminals. In this review, we provide a general comparison of crustacean and larval *Drosophila* neuromuscular systems from the standpoint of comparative physiology and morphology.

INNERVATION

Crustacean muscles

Effector neurons of crustacean muscles are either excitatory or inhibitory; within these broad categories, physiological subtypes can be distinguished (Atwood, 1976). A useful distinction between "phasic" and "tonic" types of motor neuron has been drawn for crayfish abdominal muscles, where slow postural muscles are separated from fast swimming muscles (Kennedy and Takeda, 1965*a, b*). In limb muscles, a spectrum of muscle fibers occurs. "Slow" or "tonic"

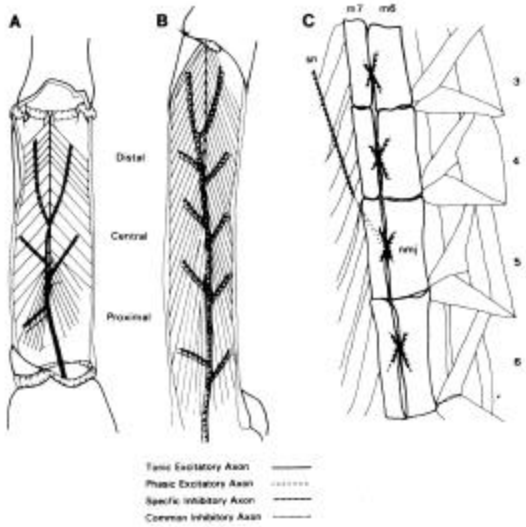

FIG. 1. Comparison of innervation for representative muscles of crayfish (A, B) and larval *Drosophila* (C). The opener muscle of the crayfish leg (A) receives one excitatory axon and a specific inhibitory axon (both distributed throughout the muscle), and a branch of the common inhibitory axon (distributed only to the proximal region of the muscle). The carpopodite extensor muscle, located in the meropodite, receives a tonic and a phasic motor axon, and a branch of the common inhibitory axon (all widely distributed). The longitudinal ventral abdominal muscles of larval *Drosophila* (muscles 6 and 7) receive two excitatory axons, one of which is physiologically more "tonic." Each segment (of which segments 3 to 6 are shown) receives its own axons via the segmental nerve (SN), and forms a limited neuromuscular junction. (The three drawings are not to the same scale).

axons of these muscles have extensive intramuscular distribution, but recruit slow-acting muscle fibers selectively at low impulse frequencies, while "fast" or "phasic" axons to the same muscles recruit fast-acting muscle fibers most readily. Normally, tonic motor axons are active at low frequencies and control postural or slow locomotor activities, while phasic axons are usually silent, and are activated when required for fast movement or escape. Synaptic differences are profound: the tonic axons to limb muscles exhibit facilitation without depression, while the phasic axons generally show depression with repetitive activation, and modest short-term facilitation (Hoyle and Wiersma, 1958; Lnenicka, 1991). Changes in neural activity alter the morphological and physiological phenotypes of these neurons (Lnenicka and Atwood, 1985; Lnenicka *et al.*, 1991).

The general innervation of two crustacean limb muscles is illustrated in Figure 1. The muscles illustrated are the opener muscle (abductor) of the dactylopodite, and the extensor muscle controlling the carpopodite. The opener muscle (Fig. 1A) receives one excitatory motor axon of the tonic type, and two inhibitory axons: a "specific" inhibitor (innervating only the opener muscle), and a branch of the "common" inhibitor (innervating all limb muscles). The "common" inhibitor branch to the opener muscle supplies only the most proximal fibers (Wiens, 1985). The extensor muscle (Fig. 1B) receives two excitatory motor axons, one clearly phasic, the other tonic, together with a branch of the common inhibitor axon.

Drosophila Larval Muscles

The larval abdominal muscles have been more often used for physiological studies than other muscles of *Drosophila,* though adult flight muscles are also studied (Ikeda, 1980). The two motor axons supplying the prominent ventral longitudinal muscles are best known. These two motor axons, both excitatory and glutamatergic (Johansen *et al.*, 1989; Keshishian *et al.*, 1992), form a circumscribed neuromuscular junction on muscle fibers 6 and 7 (Fig. 1C). This junction can be visualized in living specimens with Nomarski optics or confocal fluorescence microscopy (Atwood *et al.*, 1993). Other larval muscles are supported by small-varicosity peptide-containing neurons of unknown function (Keshishian *et al.*, 1992; Gorczyca *et al.*, 1993).

NERVE TERMINALS AND SYNAPSES

Crustacean muscles

Synapse-forming branches of the motor axons, visualized with fluorescent dyes such as 4-Di-2-Asp (Magrassi *et al.*, 1987), are characteristically varicose (Fig. 2). Electron microscopy has shown that each varicosity typically hosts 20–30 small synapses (Wojtowicz *et al.*, 1994). Both excitatory and inhibitory terminals of the opener muscle are well-endowed with mitochondria (Fig. 3A).

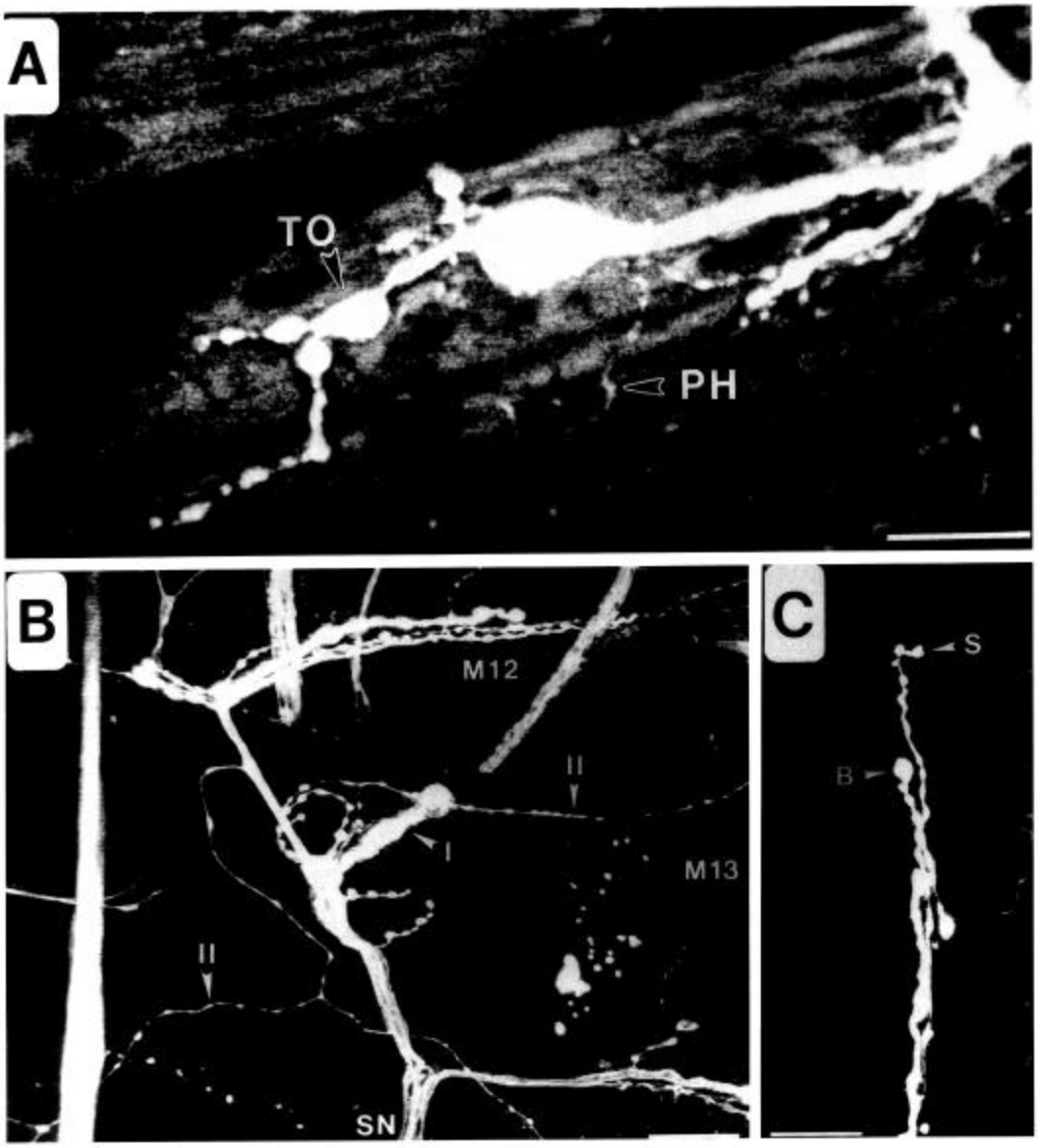

FIG. 2. Phasic (PH) and tonic (TO) nerve terminals in crayfish muscles and counterparts in *Drosophila* muscle, seen with 4-Di-2-Asp staining in confocal microscopy. (A). Associated phasic and tonic terminals on the surface of the leg extensor muscle. The tonic axon provides varicose terminals, sometimes with very large individual varicosities, while the phasic axon supplies very thin, thread-like terminals without pronounced varicosities. Scale: 25 μm. (B). Larval *Drosophila* muscle innervation. Overview of the region of muscles 6, 7, 12, and 13, showing Type I and Type II innervation originating from a branch of the segmental nerve (SN). (C). Example of individual nerve terminals on muscle 6, showing Type Is (small boutons, S) and Type Ib (big boutons, B). Scale: 25 μm.

Individual synapses usually contain one or more specialized "active zones," though a minority (10–20%) possess none (Jahromi and Atwood, 1974). Each active zone has a distinctive presynaptic dense body with closely associated synaptic vesicles (Fig. 3B). In freeze-fracture views of fixed material, large membrane particles, tentatively identified in other material as calcium and calcium-activated potassium channels (Rob-

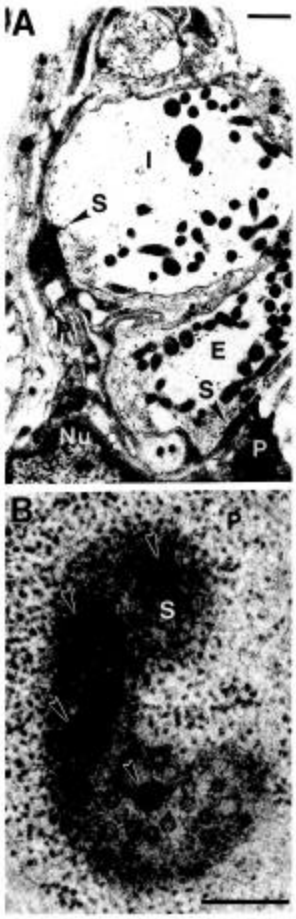

FIG. 3. Electron microscopy of nerve terminals in the crayfish opener muscle. (A). Low-power electron micrograph of nerve terminals. The excitatory (E) and inhibitory (I) terminals both form small synapses (S) on leaf-like postsynaptic processes (P) of the muscle fiber. A large muscle nucleus (Nu) underlies the postsynaptic apparatus. Scale: 1 μm. (B). High-power face view of a synaptic contact of complex type with four dense bodies, shown by the arrows. These structures are thought to represent "active zones" for transmission. Scale: 0.25 μm.

erts *et al.*, 1990), are clustered at the active zone, with images of vesicle release nearby (Pearce *et al.*, 1986; Govind *et al.*, 1994). In each excitatory varicosity, the number of synapses with multiple active zones (Fig. 3B) is small in relation to the total, at least in the crayfish opener muscle. A current hypothesis is that these complex synapses emit transmitter quanta more readily at low frequencies of stimulation, while the simpler synapses with only one active zone require higher frequencies of stimulation to emit quanta (Wojtowicz *et al.*, 1994). This could help to explain the striking frequency facilitation of synaptic transmission in these terminals.

Differentiation of the synapses of phasic and tonic neurons, long recognized at the physiological level, is seen also in their morphology (Fig. 2). In the carpopodite extensor muscle of the crayfish leg, terminals of the tonic axon are highly varicose, and individual varicosities often attain a large size (in Fig. 2A, approximately 25 μm by 15 μm). By contrast, the terminals of the phasic axon are much thinner and less varicose (*cf.* Atwood and Jahromi, 1977; Lnenicka *et al.*, 1986). The occurrence of two types of terminal on one muscle fiber indicates that the postsynaptic influence from the muscle fiber is not the sole factor responsible for nerve terminal differentiation; properties of the presynaptic neuron, and possible differences in timing of innervation during development (Atwood, 1973), must also be involved.

Drosophila

Morphological differences in nerve terminals supplying abdominal muscles of larval *Drosophila* are readily apparent (Fig. 2B, C). The varicosities elaborated by one of the axons to muscles 6 and 7 are significantly larger than those of the second axon. Ultrastructural features of the two axons also differ (Fig. 4A): the larger boutons (type Ib) contain more mitochondria, are associated with a more profuse subsynaptic reticulum, and possess more individual synapses than the smaller boutons (type Is). From serial reconstructions, it is evident that individual synapses on the two boutons do not differ in size, and the majority possess one or two presynaptic dense bodies (active zones); however, the total number of synapses supplied by the type Ib axon to muscles 6 and 7 is probably about twice that supplied by the type Is axon (Atwood *et al.*, 1993; Kurdyak *et al.*, 1994).

In several respects, the morphological differences of these *Drosophila* terminals are similar to those of crustacean phasic and tonic terminals (Fig. 4B). The crustacean

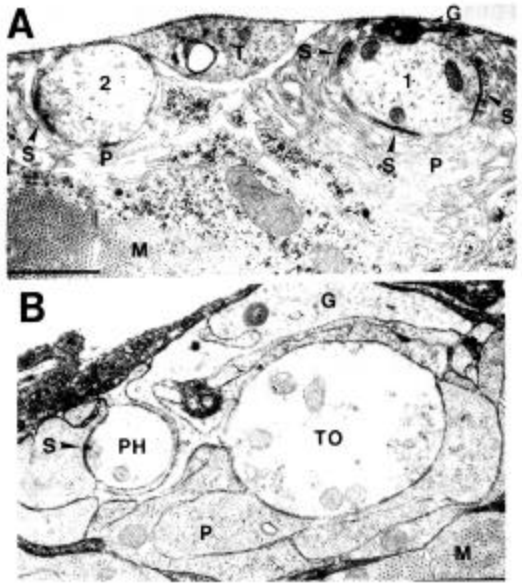

FIG. 4. (A). Electron micrograph of nerve endings on muscle 6 in larval *Drosophila*, with Type Ib (Terminal 1) and Type Is (Terminal 2) endings. Individual synapses (S) of similar size are present on both terminals. Synapses are made on specialized subsynaptic processes (P) derived from the muscle fiber (M). T, Tracheole. G, process thought to originate from a glial cell. Scale; 1 μm. (B). Electron micrographs of nerve endings in the crayfish closer muscle, showing phasic (PH) and tonic (TO) terminals. A synapse (S) with presynaptic dense body, is made by the phasic axons on a postsynaptic process of the muscle fiber (M). The nerve terminals are overlain by a glial cell (G). Scale: 1 μm.

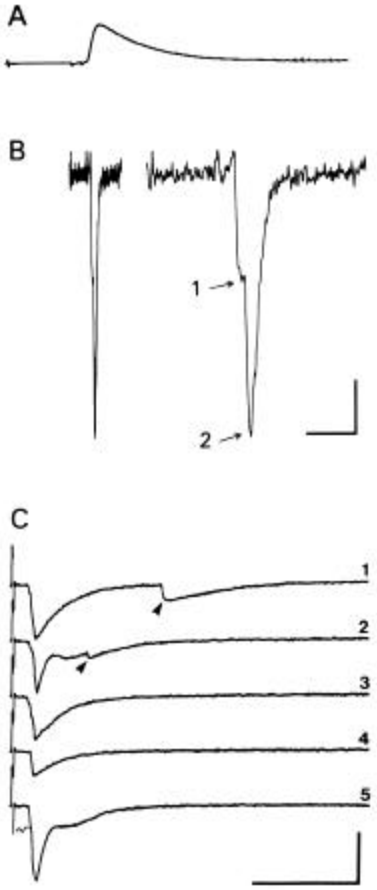

FIG. 5. EPSP (A) and synaptic current (B) in the crayfish opener muscle. Quantal events can be distinguished in the current recordings; two quanta are indicated in B. Scale: 1.2 Mv, 32 msec (A); 50 Pa (B), 32 msec (B, left trace), 6 msec (B, right trace). (C). Current recordings from a synaptic bouton of *Drosophila* in "standard" physiological solution; two spontaneous quantal events are seen (arrows), and multi-quantal evoked currents of variable shape are illustrated. Scale: 2 msec, 0.3 nA.

phasic terminals are smaller, and have fewer mitochondria, than the tonic terminals; they also have their synapses more evenly distributed along the terminal, rather than concentrated in varicosities (Atwood and Jahromi, 1977; Lnenicka *et al.*, 1986).

SYNAPTIC TRANSMISSION

Crustacean synapses

Information on the physiological performance of crustacean synapses comes from studies utilizing intracellular microelectrodes and extracellular recording of synaptic currents at active synapses. The first technique provides information about the overall response of the innervated target cell to concurrent activation of all of its responsive synapses, while the second approach permits better definition of the release of quantal units of transmitter at a well-defined terminal region (Fig. 5 A, B). Until recently, such extracellular recordings with "macropatch" electrodes were made without visualizing the structures from which recordings were obtained; but with the advent of improved vital fluorescent dyes, it is now possible to select the recording sites under visual control. We have found that the vital fluorescent dye 4-Di-2-Asp does not adversely affect quantal release at crayfish

opener excitor terminals (Cooper, *et al.*, 1995*a*).

In the crayfish opener muscle, recordings from visualized synaptic boutons of the single tonic excitatory axon generally show a low rate of quantal release at low frequencies of stimulation, in keeping with previous studies (Wernig, 1972). However, reconstruction of recorded boutons shows that they generally possess 20–30 individual synapses. Binomial statistical analyses of quantal release invariably indicate only a few responding units at low frequencies (Johnson and Wernig, 1971; Zucker, 1973; Wojtowicz *et al.*, 1988). If the responding units are active synapses (Zucker, 1973), the results suggest that only a small fraction of the many synapses on a bouton (possibly the "complex" synapses: Fig. 3B) are able to release transmitter quanta effectively at low frequencies of stimulation. As the frequency rises, quantal content, probability of release, and the estimated number of responding units all go up (Johnson and Wernig, 1971; Zucker, 1973; Wojtowicz *et al.*, 1994), suggesting that factors favouring increased release of transmitter lead to a higher rate of synapse participation. One obvious factor that could progressively alter release probability is build-up of intracellular calcium, which has been amply demonstrated (Delaney *et al.*, 1989).

In crustacean phasic neurons it is very likely that the rate of synapse participation at low frequencies is normally much higher, and that there is proportionately less recruitment of additional synapses at higher frequencies. This suggests that regulation of synapse release probability is set differently in the two types of neuron. The cellular regulatory components are not yet known.

Drosophila synapses

It is possible to record selectively *in situ* from visualized isolated boutons of the two motor axons innervating muscles 6 and 7 (Kurdyak *et al.*, 1994). Such recordings, initiated by Dr. A. Mallart and collaborators (Mallart *et al.*, 1991), show well-defined quantal units, as in crustaceans. The evoked release at low frequencies, even with quite low external calcium concentrations, appears to be multi-quantal (Cooper *et al.*, 1995*b*). Thus, synapse participation occurs at a higher rate than in the crayfish opener muscle boutons, unless release probability is reduced by lowering the external calcium concentration to an abnormally low value.

A problem with studies of evoked release in the commonly used "standard" *Drosophila* solution (Jan and Jan, 1976) and its low-calcium variants (Mallart *et al.*, 1991) is non-uniformity in time course of synaptic currents (Fig. 5C). Some evoked currents show a marked biphasic decay, while others (usually the smaller ones) show decay rates of simple exponential form, like those of spontaneously occurring quantal units. An explanation for the biphasic decay is that the recruitment of calcium-activated potassium conductance by sufficiently large synaptic potentials can deform the normal synaptic current by adding a large outward component (Mallart, 1993). An additional problem that frequently occurs when recordings are made in the "standard" solution is that the muscle cells become vacuolated and rapidly deteriorate (Stewart *et al.*, 1994).

These problems can be alleviated to some extent through use of haemolymph-like solutions, which contain lower sodium and higher magnesium than the "standard" solution (Stewart *et al.*, 1994). In haemolymph-like solutions, recordings from boutons can be made in calcium concentrations closer to the normal haemolymph value of 1–2 Mm, and the preparations are more stable. Synaptic currents recorded in such solutions generally have a simple exponential decay, even with multiquantal release.

Selective recording from terminals of type Is and type Ib have shown that the former generate a large EPSP, and the latter a somewhat smaller one. Currents from type Is boutons are usually larger at low frequencies, and generally show very little facilitation at higher frequencies of stimulation. In contrast, type Ib boutons usually generate currents that are initially smaller than those of type Is boutons, and more likely to show significant facilitation at higher frequencies (Kurdyak *et al.*, 1994).

From ultrastructural work, type Ib boutons are known to possess substantially more synapses than type Is boutons, yet they pro-

duce less current at low frequencies of stimulation. Thus, release probability and synapse participation rate appear to be differentially regulated in the two types of terminal.

DISCUSSION

This overview of comparative structure and function in crustacean and *Drosophila* neuromuscular innervation illustrates similarities and differences in the selected examples, which admittedly do not embrace all of the neuromuscular systems which have been investigated in these two types of arthropod. There is a parallel elaboration of morphological and physiological phenotypes of motor neurons in the two cases. The crustacean motor neurons known to be phasically active invariably develop slender, non-varicose terminals on the muscle. Transmitter release is substantial at low frequencies: a large EPSP is generated, even in abdominal swimming muscles where the postsynaptic input resistance is very low (Brown and Newby, 1980). We infer that participation rate for synapses in these terminals is high. However, EPSPs show relatively modest facilitation at higher frequencies of stimulation, and depress rapidly with continued stimulation. In contrast, terminals of tonic axons are markedly varicose; the varicosities are where most of the synapses occur (Lnenicka *et al.*, 1986). The EPSPs of these axons vary in amplitude in different regions of the limb muscles, but commonly are of small amplitude at low frequencies, exhibit very pronounced facilitation at higher frequencies, and do not show depression with maintained stimulation. It is likely that synapse participation rate is frequency-dependent, and this could account in part for short-term frequency facilitation.

In *Drosophila* abdominal muscles, the two major motor neurons to muscles 6 and 7 are morphologically and physiologically differentiated, though not as strikingly as in crustaceans. One of the neurons has larger boutons (type Ib), which possess more synapses and mitochondria, yet produce a smaller synaptic current with more pronounced short-term facilitation than is seen for the other neuron. Thus, the two neurons are differentiated morphologically and physiologically in a way that resembles the tonic-phasic dichotomy of crustacean motor neurons. We infer that synapse participation rate is lower for type Ib terminals at low frequencies. Whatever factors regulate quantal release probability in crustacean terminals probably are at work also in *Drosophila* terminals. Studies on both systems may advance understanding of basic mechanisms responsible for synaptic release properties.

ACKNOWLEDGMENTS

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