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Modulation of muscle and neuromuscular junctions in invertebrates

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Neuromuscular modulation in invertebrates is widespread. Work on a few favorable neuromuscular preparations is reviewed here to illustrate emerging principles. Neuromuscular modulation operates at three levels, presynaptically, postsynaptically and by altering excitation-contraction coupling. Several modulators, small molecules and peptides, that are intrinsic to motor neurons, delivered by extrinsic modulatory neurons and/or circulating in the blood, act at one or more of these levels. Although analysis of neuromuscular modulation at the cellular level has progressed rapidly, the behavioral role of such modulation remains a significant challenge for future research.

Key words: modulation / muscle / neuromuscular junction / invertebrate

MODULATORY ACTION by neurally released factors and endocrine hormones was first observed in a muscle, the vertebrate heart,[1] and much of our present knowledge about the subcellular mechanisms of such modulation comes from studies on vertebrate cardiac muscle (see e.g. ref 2). Modulation of muscle contraction and transmission at neuromuscular junctions (here referred to simply as neuromuscular modulation) is now known to be widespread among invertebrates, and the advances made using a few particularly favorable invertebrate skeletal and cardiac muscle preparations are reviewed here. The general pattern that emerges is of several modulators in each system released by both the motor neurons themselves and extrinsic modulatory neurons. Their overall effect is to modify the amplitude, speed and duration of muscle contraction evoked by the fast transmitters released by the motor neurons. They do this by acting at any of three levels: (1) presynaptically on motor neuron terminals to alter transmitter release; (2) postsynaptically to alter the electrical properties and excitability of the muscle membrane; and (3) on the muscle fibers to alter excitation-contraction coupling. Although the contribution of this complex control for behavior in these animals is so far poorly understood, such studies in invertebrates can provide a theoretical framework for possible future studies of neuromuscular modulation in skeletal muscle of vertebrates.[3]

The nervous systems of many invertebrates comprise a relatively small number of uniquely identifiable neurons, many of which are large and accessible to a variety of cellular biochemical, anatomical, and electrophysiological techniques. Although the innervation of invertebrate muscles is often complex, each muscle is usually served by a small number of identifiable neurons. The identification of modulatory neurons, chemical characterization of modulators and physiological analyses of modulator action have been relatively easy.

Circulating neurohormones in Crustacea

Initial progress in the study of neuromuscular modulation in invertebrates came with the study of the action of amines, particularly octopamine and 5-hydroxytryptamine (5-HT; serotonin), in crustacea. In the lobster (*Homarus americanus*), for example, octopamine and 5-HT act at many levels to cause profound and apparently antagonistic changes in behavior.[4,5] Several other modulatory amines and peptides have now been reported, but their roles in behavior are less clear.

Octopamine is found in a group of neurosecretory cells associated with bifurcations of the second nerve roots of the thoracic ganglia.[6] These octopamine neurons have terminals near their cell bodies and axons that project into the periphery, many of which end in neurosecretory structures termed pericardial organs.[6,7] The region of the nerve root near the octopamine cell bodies contains numerous neurosecretory endings filled with dense core vesicles similar to those seen in the cell bodies.[6] Octopamine can be released into the extracellular fluid by high

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K^+ stimulation (calcium-sensitive) both from the vicinity of the cell bodies and from the pericardial organs.[7] As both these sites have free access to the haemolymph, octopamine presumably acts as a circulating neurohormone, and concentration in the blood is of the order of 10^{-9} to 10^{-8} mol l^{-1}.[8] Although some of the physiological properties and synaptic inputs of the octopamine neurons have been described,[4] the control and coordination of their activity is largely unknown.

5-HT was first detected chemically in crab pericardial organs[9] and has since been demonstrated histochemically to be present in crab CNS and in neuronal terminals in the pericardial organs.[10] 5-HT-like immunoreactivity has been detected in neurons and neuropil regions throughout the nerve cord in *Homarus*.[4] In spiny lobster (*Panulirus interruptus*) 5-HT and octopamine are synthesized in and released from the ligamental plexus, a morphological analog of pericardial organs.[11] Moreover, 5-HT, like octopamine, is a circulating neurohormone with a blood concentration of the order of 10^{-9} to 10^{-8} mol l^{-1} in *Homarus*.[8]

At concentrations found in the circulation both 5-HT and octopamine increase the frequency and strength of the heartbeat,[12] and both cause a dose dependent increase in the concentration of cyclicAMP (cAMP) in cardiac muscle.[4]

On skeletal muscle and neuromuscular junctions 5-HT and octopamine have many effects, although neither are effective at circulating concentrations. At 10^{-7} to 10^{-5} mol l^{-1} they have similar postsynaptic effects on skeletal muscle, producing prolonged contracture[13] and the appearance of Ca^{2+}-mediated action potentials in muscle fibers that are otherwise nonelectrogenic,[4] although these effects are not seen in all muscles, and both amines increase the amount of cAMP in skeletal muscle in a dose dependent manner.[4] Both amines also have presynaptic effects on motor neuron terminals that enhance transmitter release,[4,14] but octopamine acts more selectively on the innervation of particular muscles.[15] This presynaptic action appears to account for the ability of these amines to increase the strength of nerve-evoked contractions, although there is some evidence that 5-HT, at least, acts directly on muscle contractility.[15] On both heart and skeletal muscle, 5-HT is an order of magnitude more potent both in its physiological effects and in stimulating cAMP production.

More recently the peptide proctolin (see below) has been demonstrated in lobsters, where it is probably a circulating neurohormone.[16] Proctolin exerts effects similar to octopamine and 5-HT on lobster skeletal muscle.[4] Dopamine[11] and FMRF-amide-like peptides (see below)[17] have also been implicated as possible neurohormones in crustacea. In the striated muscle of the foregut (stomatogastric system) in the spiny lobster and crab (*Cancer*), dopamine enhances nerve evoked contractions by increasing the amplitude of excitatory junctional potentials (EJPs), at least partially by increasing the input resistance of the postsynaptic membrane.[18] Dopamine also induces steady depolarization and contracture in some muscle fibers and rhythmic plateau potentials and contractions in other muscle fibers.[18] In the cpv1 muscle of the stomatogastric system of the shrimp (*Palaemon serratus*), both dopamine and FMRFamide induce the production of rhythmic plateau potentials or single plateaus when muscle fibers are phasically depolarized, as they are by EJPs from motor neurons, depending on the duration of application.[19,20] Plateau potentials, which are essentially prolonged action potentials lasting hundreds to thousands of milliseconds, have been studied in detail in neurons (see articles by Dickinson and Sigvardt this volume), where they are associated with slowly inactivating inward currents carried by either Ca^{2+}, Na^+ or both. Plateau potentials evoke large all-or-none contractions in muscles of the stomatogastric system.[18-20]

Although the field of neuromuscular modulation was established as a result of these studies, they have been limited. Because of the neurohumoral nature of the modulatory systems, physiologically significant neuromodulator-target interactions have been difficult to define. With the discovery of modulatory neurons with defined transmitters and peripheral targets, first in locusts and *Aplysia* and then in other invertebrates including other crustaceans (crayfish), the study of modulation took another step forward.

Skeletal muscle of insects and crayfish

Work with two insect preparations, the extensor tibiae muscle of the locust hind leg and a slow coxal depressor muscle of the cockroach hind leg, has established the importance of octopamine and proctolin as neuromuscular modulators. More recent studies of the slow abdominal flexor muscles in crayfish confirm that proctolin which is coreleased from motor neuron terminals has its effects mainly on excitation contraction coupling. In the locust, octopamine acts mainly presynaptically but also

postsynaptically, emphasizing that several levels of control may be found in a single muscle.

The extensor-tibiae (ETi) muscle of the hind leg of locusts (for example, *Schistocerca*) is a large muscle that provides the thrust for jumping and kicking as well as extension of the tibia in walking and standing. It receives a rich innervation:[21,22] two excitatory motor neurons, one fast (FETi) and one slow (SETi), both of which probably use glutamate as a transmitter;[23] an inhibitory motor neuron, the common inhibitor, which is GABAergic;[23] and a specific modulatory neuron the dorsal unpaired median cell to the extensor-tibiae muscle (DUMETi), which is octopaminergic.[24-26] Evans and Myers[27] have thoroughly reviewed modulation in this system.

The ETi muscle is heterogeneous; slow muscle fibers innervated by SETi are found proximally and distally, whereas fast fibers innervated by FETi are found in central parts of the muscle and intermediate fibers innervated by both SETi and FETi are scattered throughout.[28] The common inhibitor innervates mainly proximal and distal slow and intermediate fibers.[28] At the proximal end, there is a small bundle of some twenty electrically coupled fibers that are innervated by SETi and the common inhibitor; this bundle produces a myogenic rhythm of contraction.[25,29] DUMETi appears to innervate the entire muscle, but its terminal endings, which contain both small clear vesicles and dense core vesicles, do not form conventional neuromuscular junctions.[22,30]

The work of Hoyle and coworkers,[24] indicated that DUMETi is octopaminergic and that both activity in DUMETi and octopamine could slow the myogenic rhythm of the ETi muscle, but it was the thorough work of Evans and O'Shea[25,31] that established the ETi muscle as a model system for the study of neuromuscular modulation. DUMETi contains about 0.1 pmol of octopamine in its cell body and about a four times higher concentration in its axon.[25] It releases octopamine when electrically stimulated.[26] Activity in DUMETi and superfused octopamine act directly on muscle to slow the rhythm of the myogenic bundle[25,31] and to increase the relaxation rate of twitch tension generated by both FETi and SETi.[25,31] Activity in DUMETi and superfused octopamine act presynaptically on SETi to increase the size of its excitatory junctional potentials in the muscle and thus to increase the rate of rise and amplitude of the contractions evoked by SETi.[31] Presynaptic action is indicated by the observation that superfused octopamine increases the frequency but not the amplitude of spontaneous miniature endplate potentials in muscle fibers innervated only by SETi and the common inhibitor.[31] The net effect of these actions is to increase the amplitude of individual twitches and the frequency at which individual twitches fuse, so that the muscle is poised for rapid changes in force.[27] Unfortunately, very little is known about the control of activity in DUMETi and its coordination with SETi and FETi.

Three different types of octopamine receptors 1, 2A and 2B have been distinguished in this system on the basis of the relative potency of a range of agonists and antagonists.[30] The type 1 receptor is associated with the myogenic bundle and seems to act through a second messenger system other than cAMP.[27] Type 2A receptors are associated with the presynaptic terminals of SETi, and type 2B with the nonmyogenic slow and intermediate type muscle fibers innervated by SETi respectively.[27] Both probably act through cAMP as a second messenger.[27]

The peptide proctolin (Arg-Tyr-Leu-Pro-Thr), first isolated and characterized from the gut of cockroaches,[32] has potent acceleratory effects on contraction of the myogenic bundle of the ETi muscle[33,34] when exogenously applied [threshold about 10^{-11} mol l^{-1}].[27] Pharmacological and biochemical evidence suggests that it increases cAMP in the fibers of the myogenic bundle.[27] Its effects on the myogenic bundle are thus antagonistic to those of octopamine. Biochemical and immunocytochemical evidence indicates that SETi contains and releases proctolin,[35] and stimulation of SETi has a proctolin-like acceleratory effect on the myogenic rhythm of the ETi muscle.[29,36]

Proctolin superfused over the ETi muscle also affects the nonmyogenic slow muscle fibers at the muscle's proximal end. It induces a dose dependent slow contraction of the muscle, and it enhances the force of tetanic contractions evoked by stimulating SETi.[34] The role of proctolin released by SETi in regulating tension of the nonmyogenic fibers of the ETi muscle remains to be explored. Other peptides, including FMRFamide-like peptides (see below)[27,37] and the adipokinetic peptides,[38,39] are also active on this preparation, and clarification of their sources and physiological roles may provide interesting contrasts to proctolin.

A pioneering series of studies in cockroach (*Periplaneta americana*) by O'Shea and coworkers has contributed much to our understanding of the role of peptide modulators as cotransmitters in motor

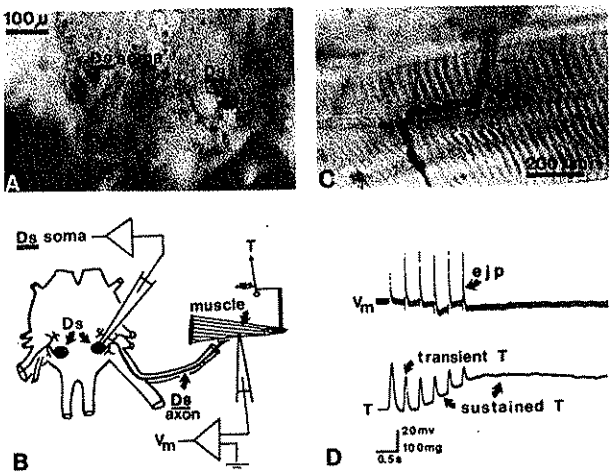

Figure 1. Proctolin, a cotransmitter in an identified motor neuron in the cockroach. A. The cell bodies of Ds motor neurons show proctolin-like immunoreactivity (dorsal view of metathoracic ganglion). B. The preparation used to generate the records shown in D is illustrated with microelectrodes placed in the Ds cell body and in a fiber of the slow coxal depressor muscle. C. Proctolin-like immunoreactivity in the axon (a) and terminals (t) of the Ds motor neuron on the slow coxal depressor muscle. D. When Ds is stimulated intracellularly to produce a burst of six action potentials, individual nonsummating EJPs are recorded in the muscle, but the tension output of the muscle is biphasic. The sustained tension produced probably results from the release of proctolin by Ds and is not associated with depolarization. Reprinted from O'Shea.[36]

neurons[36] (Figure 1). Proctolin is found in the presumed glutaminergic motor neuron, Ds, that innervates a postural muscle, the slow coxal depressor muscle of the hind leg. It can be released by electrical stimulation of Ds or with high K^+ depolarization (calcium dependent) of proctolin immunoreactive terminals on the muscle[40] (Figure 1A,B,C). Proctolin superfused over the coxal depressor muscle causes tonic contraction without depolarization of the muscle fiber membrane (5×10^{-9} mol l^{-1}), and the relaxation rate of Ds evoked twitches is prolonged (10^{-9} mol l^{-1}).[40] In individual muscle fibers, single action potentials in Ds give rise to fast EJPs that are associated with a fast twitch. When Ds is stimulated in a burst, the fast EJPs are unchanged (Figure 1D) but the mechanical response becomes biphasic.[40] Fast twitches one-to-one with the EJPs are superimposed on slow tension and the relaxation rate of each individual twitch is prolonged (Figure 1D). The slow tension and altered relaxation rate are consistent with the release of proctolin from Ds, in addition to glutamate that presumably gives rise to the EJPs. Thus, tonic activity in Ds can be expected to produce a sustained background tension on which slowly decaying twitches are superimposed, which fits well with the postural role of the slow coxal depressor. As only about 5% of cockroach motor neurons contain proctolin and fewer contain adipokinetic hormone-like peptides,[36] it will be interesting to see whether peptide cotransmitters are rare in insect motor neurons, or if there is a diversity of other peptide cotransmitters that have yet to be discovered.

Recently, proctolin has been found to be a cotransmitter in motor neurons innervating postural muscles, the slow abdominal flexor muscles, in the crayfish, *Procambarus clarkii*. In an elegant set of experiments, Bishop and coworkers[41,42] have also demonstrated that here too proctolin acting as a modulator contributes significantly to the development of tension. The slow flexor muscles are each innervated by five excitatory motor neurons (f1, f2, f3 and f4 which are probably glutaminergic and f6 which is probably cholinergic) and an inhibitory motor neuron (f5 which is probably GABAergic).[43,44,45] Cell bodies and axons of f1, f3 and f4 contain proctolin-like immunoreactivity, but those of f2, f5 and f6 do not. Proctolin-like immunoreactive processes are found on the muscle and extracts of the muscle contain true proctolin;[41] moreover, stimulation of f1 releases proctolin.[42] Proctolin (10^{-9} mol l^{-1}) superfused over the slow flexor muscle fibers seems to act on the excitation-contraction coupling mechanism to increase the tension generated by a given depolarization.[42] Proctolin alone has no apparent effect on resting muscle tension, membrane potential or on the size of EJPs evoked by f1 activity.[42]

Proctolin seems to be important for modulating the tension produced in the slow flexor muscle by neurons f1, f3 and f4. The proctolin containing f1 motor neuron produces reduced tension in slow flexor muscle fibers following prolonged low frequency stimulation, although the size of f1 EJPs does not change.[42] The reduction in tension is reversed by superfused proctolin (5×10^{-9} mol l^{-1}) and does not occur in the continued presence of superfused proctolin (10^{-8} mol l^{-1}). Motor neuron f6, which does not contain proctolin, does not show such use-associated tension reduction.[42] These results pose an important question: what functional role differentiates the proctolin-containing excitatory motor neurons from the excitatory motor neurons f2 and f6 that do not contain proctolin? Moreover, these experiments point to a potentially functionally

important aspect of neuromuscular modulation by peptides; depletion of peptides (synthesized in the cell body and transported to distant release sites) by intense but physiological levels of activity can have profound physiological and perhaps behavioral consequences.

Accessory radula closer muscle of *Aplysia*

The work of Weiss and coworkers has established that accessory radula closer (ARC) muscle of *Aplysia* as the preeminent preparation for studying neuromuscular modulation,[46,47] in which 5-HT and a plethora of peptides modulate the action of the neuromuscular transmitter, acetylcholine. The ARC muscle, which is involved in feeding, is innervated by three or four motor neurons located in the buccal ganglion, two of which (B15 and B16) can be repeatedly identified.[46] B15 and B16 seem to be cholinergic, and they produce fast monosynaptic EJPs in all muscle fibers of the ARC muscle. Depolarization associated with these EJPs produce graded contractions of the muscle. In addition, the ARC muscle (as well as several other buccal muscles) receives axonal branches from the giant metacerebral cell,[46] which contains 5-HT[48] and has a clear modulatory role.[46,47] Activity in metacerebral cell alone has no effect on muscle tension and does not cause any change in muscle membrane potential. Activity in the metacerebral cell or superfused 5-HT (10^{-8} mol l^{-1}) dramatically increases the strength of contractions in the ARC muscle evoked by stimulation of B15 or B16, without appreciably increasing the size of evoked EJPs.[46] The modulatory effect is also seen when the ARC muscle is activated by either direct application of acetylcholine or electrical stimulation,[46] and it is associated with an increase in cAMP in ARC muscle.[47] The modulatory action of the metacerebral cell thus seems to be mediated by 5-HT and cAMP and to act on excitation-contraction coupling in the muscle.

A rapidly increasing number of peptide modulators is known in the ARC muscle system. The first to be characterized were two small cardioactive peptides SCPa (Ala-Arg-Pro-Gly-Tyr-Leu-Ala-Phe-Pro-Arg-Met-NH_2) and SCPb (Met-Asn-Tyr-Leu-Ala-Phe-Pro-Arg-Met-NH_2) (SCPs), which are found in *Aplysia* and other molluscs.[49] The SCPs are found together in and released together from neurons,[49,50] and are cleaved from the same precursor molecule.[49] They have been detected biochemically in several identified motor cells, including the ARC motor neuron B15; the other identified ARC motor neuron B16 and the modulatory metacerebral cell do not contain SCPs.[51] SCPs have been immunocytochemically localized to dense core vesicles in the cell body of B15 soma and in neuronal varicosities on the ARC muscle.[51,52] Their effects on the ARC muscle are apparently identical to 5-HT, although they seem to act through separate receptors.[52] Although no direct modulatory action of B15 has yet been observed, the discovery of SCPs in this neuron opens the possibility that the ARC muscle is modulated by two pathways, one extrinsic (metacerebral cell/5-HT) and the other intrinsic (B15/SCPs) to the motor neurons.

Careful biochemical analyses of the neurons of the ARC muscle system has revealed several other peptide modulators. Myomodulin (Pro-Met-Ser-Met-Leu-Arg-Leu-NH_2) found in B16 but not in B15 or the metacerebral cell, has effects on the ARC muscle similar to those of 5-HT and the SCPs.[51,53] Buccalin (Asp-Ser-Leu-Ala-Phe-Ser-Gly-Gly-Leu-NH_2) is found in both B15 and B16, and when superfused on the ARC muscle, decreases the size of neurally evoked contractions apparently by acting presynaptically on B15 and B16 to reduce the size of their EJPs in the ARC muscle.[54,55] Extracts of the ARC muscle contain FMRFamide[50] and the novel peptide parabuccalin (Gly-Leu-Asp-Arg-Tyr-Gly-Phe-Val-Gly-Gly-Leu-NH_2)[56] and all of the peptides associated with the ARC muscle are synthesized by the buccal ganglion and transported to the muscle.[57] Parabuccalin has actions similar to buccalin,[56] and they may be colocalized. The source of the FMRFamide containing terminals on the ARC muscle is unclear, as FMRFamide is apparently not found in B15, B16, or the metacerebral cell,[50,51,53] and FMRFamide's action on the ARC muscle has not been defined.

The discovery of such a plethora of modulators substantiates the notion that several modulators may act on any given neuromuscular system, but begs the question of why this should be. Very little is known of the behavioral role of neuromuscular modulation in the ARC muscle system. Current research combining electrophysiological recording and behavioral analyses in a partially dissected feeding preparation promises to address this shortcoming in the near future.[58]

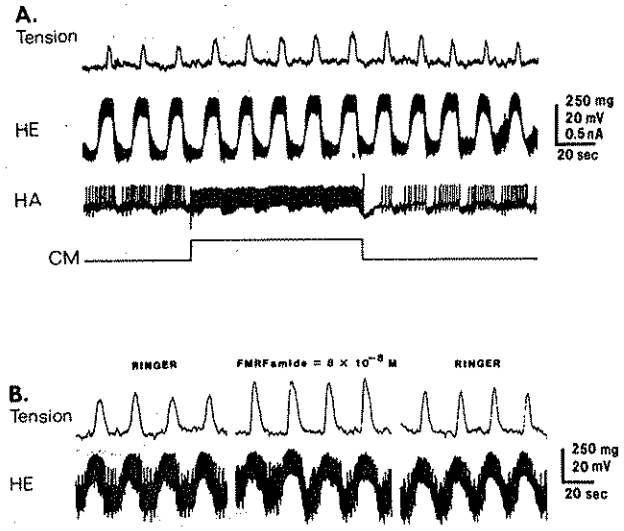

Figure 2. FMRFamide mimics the modulatory action of HA neurons on the leech heart. A. Increased activity (elicited by intracellular current injection) in an HA neuron increases the amplitude and duration of the beat tension, when the heart is normally entrained by rhythmic activity in an HE motor neuron. CM—monitors current injected into the HA cell body through the recording microelectrode. B. Superfusion of FMRFamide (8×10^{-8} mol l^{-1}) increases the amplitude and duration of the beat tension when the heart is normally entrained by rhythmic activity in an HE motor neuron. Four beats before, during and after FMRFamide application are illustrated. In both A and B, a heart was left innervated only in one segment and tension was monitored in that segment.

Cardiac muscle in leech and *Limulus*

The rhythmic beating of heart muscle in invertebrates, as in vertebrates, is modified by neuromodulators. In the leech the peptide FMRFamide, found in extrinsic modulatory neurons as well as in heart motor neurons, seems to be the main modulator whereas in *Limulus* octopamine, dopamine and proctolin are all involved.

The hearts of the leech (*Hirudo medicinalis*) are innervated by a cholinergic HE motor neuron in each body segment;[59-61] viewed in the electron microscope the neuromuscular junctions formed by the HE neurons contain both small clear vesicles and large dense core vesicles.[60] Each action potential in an HE motor neuron gives rise to a unitary EJP in heart muscle cells;[60] the EJPs are blocked by 10^{-4} mol l^{-1} curare.[61] The hearts are also intersegmentally innervated by HA modulatory neurons,[62] which have terminals on the heart muscle that are ultrastructurally potentials in HA neurons are not associated with EJPs in the muscle fibers.[60] HE motor neurons are normally rhythmically active and they entrain a myogenic rhythm of the heart by rhythmically depolarizing the muscle fibers.[63] When a heart is entrained by the HE neurons, stimulation of an HA neuron increases the amplitude and duration of the tension during the heartbeat[62] (Figure 2A).

The peptide FMRFamide (Phe-Met-Arg-Phe-NH_2), first isolated as a cardioexcitatory factor from the nervous system of a clam, *Macrocallista nimbosa*,[64] seems to be the modulator used by HA neurons. HA neurons contain FMRFamide-like immunoreactivity in their cell bodies and processes on the heart[65] (Figure 3). Extracts of hearts or of singly dissected HA cell bodies contain a FMRFamide immunoreactive peptide (about 30 fmol) that comigrates with synthetic FMRFamide in two different HPLC solvent systems.[66] Superfusion of FMRFamide increases the tension and duration of the heartbeat when the heart is normally entrained by rhythmic

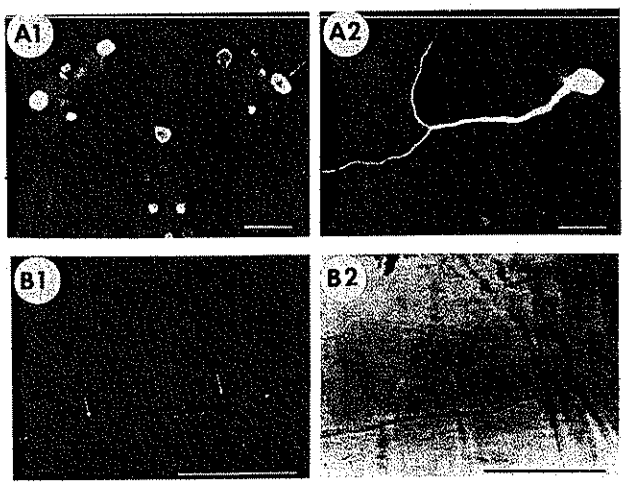

Figure 3. The HA cell body and its terminals on the heart show FMRFamide-like immunoreactivity. A. Labeling of a segmental ganglion with rhodamine conjugated secondary antibody to reveal FMRFamide-like immunoreactivity in neuron cell bodies (1) and with intracellularly injected Lucifer yellow to reveal the identified HA neuron (2) (ventral view). The arrow in (1) shows that the HA neuron is double labeled. B. Labeling of a heart with rhodamine conjugated secondary antibody to reveal FMRFamide-like immunoreactivity in nerve terminals (1) and with intracellularly injected horseradish peroxidase to reveal the HA cell's terminals (2). The arrows in (1) show that the terminals of the HA neuron on the heart are double labeled. Calibration bars = 0.1 mm.

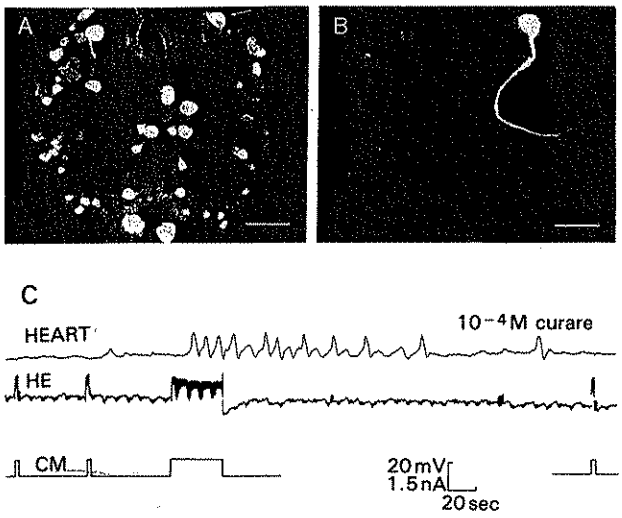

Figure 4. The HE cell body shows FMRFamide-like immunoreactivity. A & B. Labeling of a segmental ganglion with rhodamine conjugated secondary antibody to reveal FMRFamide-like immunoreactivity in neuron cell bodies (A) and with intracellularly injected Lucifer yellow to reveal the identified HE neuron (B) (ventral view). The arrow in (A) shows that the HE neuron is double labeled. Calibration bars = 0.1 mm. C. HE Motor neurons have modulatory effects on the leech heart. When cholinergic transmission is blocked, a few cycles of rhythmic activity in an HE neuron induces rhythmic electrical activity in the heart, but a brief burst of activity in an HE neuron has no effect. The heart was left innervated only in one segment and membrane potential was monitored in a heart muscle cell in that segment. Cholinergic transmission to the heart by the HE neuron was blocked with curare (10^{-4} mol l^{-1} d-tubocurarine) and normal rhythmic firing in the HE cell was suppressed with injected hyperpolarizing current. CM—monitors current injected into the HE cell body through the recording microelectrode.

HE motor outflow[67] (Figure 2B) and depolarizes isolated heart muscle cells; this depolarization requires external Ca^{2+},[68] suggesting that Ca^{2+} is involved in tension regulation.

Here, as in the ARC muscle, there are modulators intrinsic as well as extrinsic to the motor neurons. HE motor neurons also contain FMRFamide-like immunoreactivity in their cell bodies and terminals on the heart[65] (Figure 4A,B). When their EJPs in heart muscle are blocked with curare, prolonged activity (several seconds to minutes) in HE motor neurons induces myogenic activity in quiescent hearts[66] (Figure 4C). Superfused FMRFamide induces similar myogenic activity in quiescent hearts that have been deprived of normal input from HE motor neurons.[66,67] In the leech heart the same modulator may have a different role according to its location. Modulation by the HA neuron (extrinsic) appears predominantly to regulate beat tension, whereas modulation by the HE motor neuron (intrinsic) seems to regulate myogenic activity.

All the excitatory motor neurons that innervate the longitudinal muscles of the leech body wall and several other excitatory motor neurons are cholinergic[59,69] and contain FMRFamide-like immunoreactivity.[70] Superfusion of FMRFamide causes a tonic contraction of longitudinal muscle[70] and a Ca^{2+} dependent depolarization of the muscle membrane.[71] FMRF-amide acts on receptors other than those that mediate fast cholinergic EJPs,[69] as its effects are not blocked by curare.[70] In contrast to the cockroach where peptide cotransmitters in motor neurons may be relatively rare, in leech they seem to be ubiquitous.

In the horseshoe crab, *Limulus*, the amines dopamine and octopamine and the peptide proctolin all increase tension of the heartbeat by acting on excitation contraction-coupling.[72,73] Apparently, octopamine exerts its effects through the cAMP second messenger pathway, proctolin through the phosphotidylinostitol second messenger pathway and dopamine through both.[73] Dopamine also acts presynaptically on cardiac motor neurons to increase the size of their EJPs in heart muscle fibers.[72]

Concluding remarks

In the examples presented we have seen that neuromuscular modulation occurs at three levels, presynaptically, postsynaptically and by altering excitation-contraction coupling. Several modulators, small molecules and peptides, that are intrinsic to motor neurons, delivered by extrinsic modulatory neurons and/or circulating in the blood, act at one or more of these levels. Although these realizations are the result of considerable progress over the past 15 years, several gaps remain. The chemical identification of modulators and their cellular localization is proceeding rapidly, but our knowledge of the pharmacology and the nature of the receptors for these modulators is very limited. (Ironically, our lack of detailed knowledge of the nature and pharmacology of the classical neuromuscular transmitters often impedes our ability to study the action of neuromodulatory cotransmitters in isolation.) Our understanding of the subcellular mechanisms of modulator action is in a primitive state for neuromuscular systems, even though in

a few cases one or more second messengers have been identified. The occurrence of several modulators with seemingly identical effects in a single system has yet to be fitted into a theoretical framework that justifies their existence.

Much research is being pursued to address these issues, but the questions as to how neuromuscular modulation influences behavior and how neural networks are organized to take advantage of such modulation are receiving little attention. We have come to realize, as several of the other chapters of this issue demonstrate, that modulators acting on neuromuscular systems also act on the central nervous system and on sensory systems. We must integrate studies of modulation at the behavioral and neural network levels with studies of modulation at the cellular and molecular level if we are to achieve an understanding of the biological functions of neuromuscular modulation.

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