*Molecular Neurobiology*

ISSN0893-7648/93/7(3-4): 335–347/$3.60

# Functional Roles of Peptide Cotransmitters at Neuromuscular Synapses in *Aplysia*

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## Contents



## Abstract

Neuromuscular synapses in *Aplysia* have been used as model systems to study peptidergic cotransmission. Here we describe neuromuscular preparations in which it has been possible to investigate the physiological consequences of peptide transmitter release in detail. In the first preparation, the release of peptide cotransmitters from identified motor neuron B15 has been shown to be sensitive to the pattern of stimulation. High frequencies and long burst durations evoke peptide release that modulates muscle contractions in a manner similar to that produced by exogenous cotransmitter. By contrast, the release of the same peptide transmitters from motor neuron B1 show little dependence on pattern. We conclude that there are no stimulation patterns that are prerequisites for peptide release. Peptide cotransmitter release from motor neuron B47 has also been studied. B47, depending on the stimulation pattern, uses either ACh, which acts as a conventional inhibitory transmitter, or ACh plus neuropeptides, which act as excitatory modulatory cotransmitters. Thus, neuropeptide cotransmitters have the capability to greatly increase synaptic plasticity at neuromuscular synapses.

**Index Entries:** Peptides; neuromuscular junction; transmitter release; motor neurons; modulation; *Aplysia.*

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## Introduction

Neuropeptides are intercellular messengers commonly used in the modulation of behavior (Bartfai et al., 1988; Arch and Berry, 1989; Calabrese, 1989). Peptide transmitters can act over varied distances and time scales, thereby increasing the potential diversity of their effects (Jan and Jan, 1983; Mayeri et al., 1985; Kow and Pfaff, 1988). Mounting evidence suggests that in many, and perhaps even in a majority, of neurons, one or more peptide transmitters coexist with a conventional (typically fast-acting) transmitter. Peptide cotransmitters have been found widely in both invertebrate and vertebrate neurons (O'Shea and Bishop, 1982; Gibson et al., 1984; Ch'ng et al., 1985; Schwarz et al., 1984; Kuhlman et al., 1985). This wide distribution suggests that coexistence of transmitters may have considerable physiological importance.

Knowledge of the factors governing the release of cotransmitters as well as their modulatory effects is crucial to our understanding of the physiological roles of each transmitter. Whereas a good deal of research has concentrated on conventional transmitters, much less is known about the biology of neuropeptides at all stages from release to physiological function. However, we know that important differences exist between the processing of conventional and peptide transmitters. Conventional transmitters are synthesized enzymatically throughout the cytoplasm of a neuron, but particularly at synaptic terminals. Once released, these transmitters usually have high affinity uptake systems that permit reuptake of the transmitter or its metabolites into the presynaptic terminal. Thus, there is a local cellular machinery dedicated to maintaining homeostatic levels of conventional transmitter content. In contrast, peptide transmitters are processed from precursors translated in the neuronal cell body, a site that is often remote from the terminals, and there is little evidence for efficient reuptake of released peptide cotransmitters.

Although it is accepted that a single action potential is sufficient to release conventional transmitters, in surprisingly few cases has it been shown that physiological patterns of activity actually release neuropeptides. Although it may seem unlikely that neurons would synthesize transmitter-like molecules and neglect to release them, the demonstration of release is of fundamental importance (Kupfermann, 1991). It is commonly assumed that high frequency firing is required to release peptide transmitters (*see* Bartfai et al., 1988; DeCamilli and Jahn, 1990). However, in very few cases is there experimental evidence to support this proposition. Once released, peptides rarely evoke rapid postsynaptic electrical responses but more commonly produce slow modulatory actions (Mulle et al., 1988; Dale and Kandel, 1990; Simmons et al., 1990). Whereas much progress has been made in documenting the postsynaptic effects of exogenously applied peptides, less is known about the functional roles of released peptides. Because effective peptide antagonists are still relatively rare, it is difficult to separate the relative contributions of simultaneously released cotransmitters. Thus, it is not clear under what conditions peptide cotransmitters are released and produce physiological effects.

In order to gain insight into the precise functional roles played by peptide cotransmitters, we suggest that four experimental requirements should be satisfied. First, peptide cotransmitters must be characterized and localized to individual, identified neurons. Second, methods must be developed to monitor release of the peptide cotransmitters. Included in this requirement is the need to precisely control neuronal activity. Third, the physiological consequences of the released cotransmitter must be assessed. Fourth, the activity of the neurons must be measured during ongoing behaviors to establish whether the stimulation conditions used in the second and third experimental requirements are physiologically relevant. To address these points we describe model systems from *Aplysia* consisting of identified motor neurons and the muscles they innervate. In this review we will discuss how information about each of the above requirements has led to our current view of the functional roles played by neuropeptides in some of these motor neurons. Finally, for the purposes of this review we follow the terminology of Kupfermann (1991) in regarding cotrans-

mission as involving the coexistence and release of two or more bioactive substances from the terminals of a single neuron.

## Characterization and Localization of Peptide Cotransmitters to Identified Motor Neurons

The motor neurons described in this review are located in the buccal ganglia, the part of the central nervous system that regulates the rhythmic biting and swallowing movements produced by muscles of the buccal mass during feeding behaviors. Members of at least four different peptide families are synthesized by motor neurons in the buccal ganglia These peptide families include the small cardioactive peptides (SCPs; Morris et al., 1982; Lloyd et al., 1987b), the buccalins (Cropper et al., 1988), the myomodulins (Cropper et al., 1987b), and the FMRFamide-related peptides (Price and Greenberg, 1977; Weiss et al., 1986; Cropper et al., 1991). The amino acid sequences of the precursor molecules for several of these peptides have been inferred from cDNA cloning, and the existence of additional family members thereby proposed (Mahon et al., 1985; Miller et al., 1989, 1991b; Lopez et al., 1993). Peptides from each of these families are transported by fast axonal transport via peripheral nerves to the neuronal terminals in different muscles of the buccal mass (Lloyd, 1988). Peptides are not uniformly distributed among all buccal muscles; instead different muscles receive different complements of peptides. Initially, peptides were localized to the motor neurons using immunocytology with antisera raised to the peptides (Lloyd et al., 1985, 1987a; Miller et al., 1991a, 1992). Neuropeptides could be localized to previously characterized neurons by marking physiologically identified neurons with injected dyes and subsequently staining the ganglia with specific antisera. In the last few years, a more direct and quantitative approach has also been used to examine peptide expression. In this procedure, peptides synthesized in identified neurons are labeled with $^{35}$S-methionine. Peptides are then extracted from individual cell bodies and separated by several modes of HPLC. This procedure not only identifies multiple peptides expressed by individual neurons, but also quantifies their relative rates of synthesis. In a survey of over 20 identified motor neurons, each neuron was found to express a subset of the peptides described above. Many neurons expressed several peptides, either processed from a single precursor, or in some cases processed from multiple precursors (Cropper et al., 1987a,b, 1988; Lloyd et al., 1987a, 1988; Lotshaw and Lloyd, 1990; Church and Lloyd, 1991; Church et al., 1993). Exogenous application of peptides from each of the four families has been shown to modulate the efficacy of muscle contractions. However, the underlying mechanisms of modulation vary for different buccal muscles (*see* The I5 Neuromuscular System and the I3a Neuromuscular System). Other neuropeptides have been localized by immunocytology to neurons in the buccal ganglia but have not yet been implicated in motor neuron function (Alevizos et al., 1991; Taussig et al., 1985).

## Two Neuromuscular Preparations

As a result of the surveys described above, two preparations were selected for detailed study; the Intrinsic 5 (I5; also called the ARC muscle; Cohen et al., 1978) neuromuscular system and the Intrinsic 3 anterior (I3a) neuromuscular system. Since the buccal ganglia/buccal mass are bilaterally symmetrical, both preparations have contralateral homologs, a fact that can be experimentally advantageous. The I5 system was chosen because the I5 muscle is innervated by only two excitatory motor neurons and is readily accessible to biochemical and physiological analyses. Using this preparation it has been possible to meet all the requirements that we suggested earlier were needed to gain insight into the precise functional roles played by peptide cotransmitters. The I3a system was chosen because the I3a muscle is innervated by an inhibitory motor neuron (B47)

as well as two excitatory motor neurons (B3 and B38; Fig. 1B). Each of these motor neurons expresses peptide cotransmitters (*see* Fig. 1). Inhibitory motor neurons are relatively rare in *Aplysia*.

## The I5 Neuromuscular System

### Release of the SCPs from the Terminals of Motor Neuron B15

I5 is a discrete buccal muscle in *Aplysia* that is innervated by two excitatory cholinergic motor neurons (termed B15 and B16; Fig. 1A). The electrophysiological properties of these neuromuscular synapses have been extensively studied (Cohen et al., 1978; Weiss et al., 1978, 1979). Studies of peptide release have concentrated on B15, which synthesizes the SCPs and members of the buccalin peptide family (Cropper et al., 1987a, 1988). The SCP family is composed of two peptides, $SCP_A$ (11 amino-acids; Lloyd et al., 1987b) and $SCP_B$ (9 amino acids; Morris et al., 1982). There is abundant evidence indicating that the SCPs serve a transmitter-like function in B15. Both peptides are transported from the buccal ganglion to the I5 muscle (Lloyd, 1988) and are located in dense-core vesicles in the soma and in the terminal varicosities of B15 on the I5 muscle (Lloyd et al., 1984; Cropper et al., 1987a). Finally, the SCPs are released from B15 neurons in culture in a stimulation- and calcium-dependent manner (Whim and Lloyd, 1993). There is also considerable evidence that the SCPs function as neuromodulators at the I5 muscle. Application of exogenous SCPs at low concentrations increases the amplitude and relaxation rate of I5 muscle contractions evoked by stimulation of either motor neuron (Fig. 2A). This modulation involves a cAMP-mediated enhancement of the excitation–contraction coupling mechanism (Lloyd et al., 1984). Motor neuron B16 innervates the same I5 muscle fibers as B15 but does not contain the SCPs (Cropper et al., 1987a).

The I5 neuromuscular preparation was used to examine whether stimulation of B15 released the SCPs, and subsequently to study what fre-

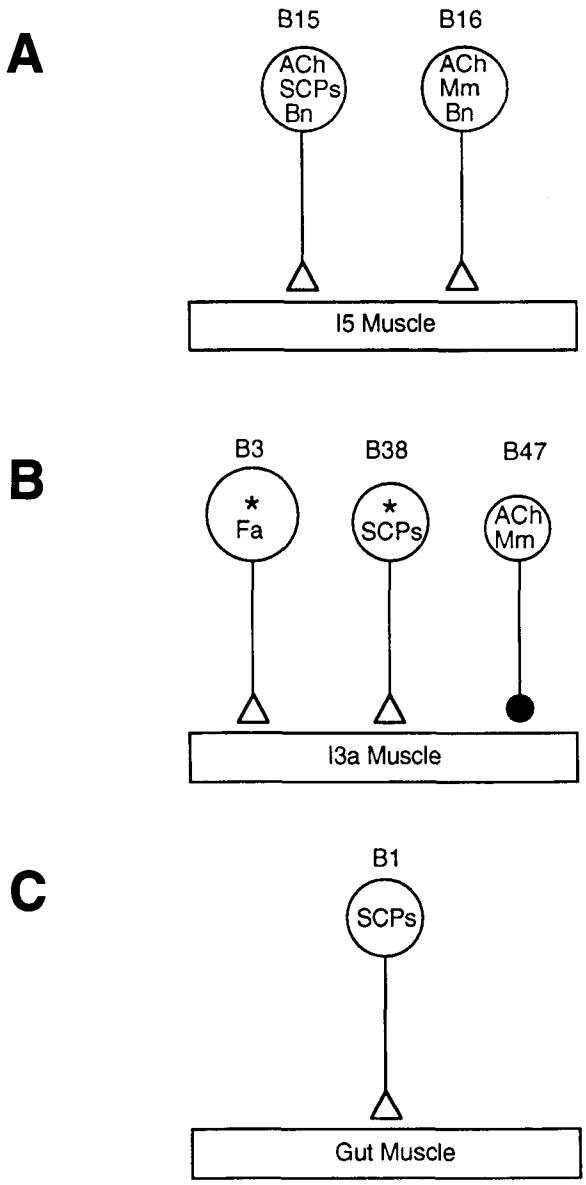


Fig. 1. Schematic illustration of the neuromuscular preparations discussed in the text. Each motor neuron synthesizes peptide cotransmitters. (**A**) The I5 muscle is innervated by two excitatory cholinergic motor neurons. (**B**) The I3a muscle is innervated by an inhibitory cholinergic motor neuron (B47) and two excitatory motor neurons whose conventional transmitters is unknown (★) but is likely to be an excitatory amino acid. (**C**) Muscles of the posterior crop region of the gut are innervated by neuron B1, which synthesizes the SCPs. Peptides: Buccalin A (Bn), FMRFamide (Fa), Myomodulin A (Mm), and SCPs.

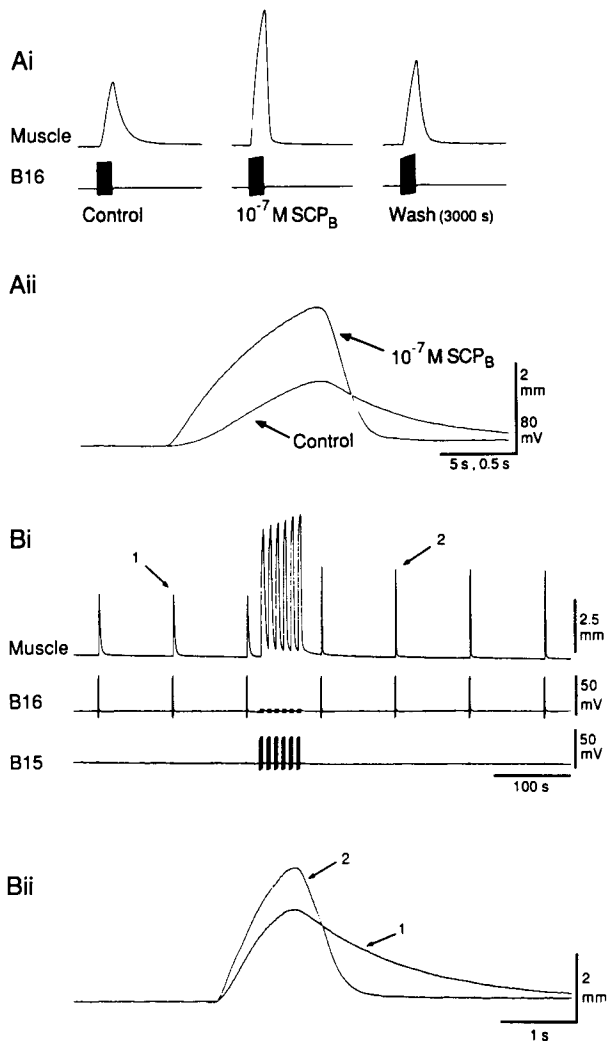


Fig. 2. (**Ai**) I5 muscle contractions produced by stimulation of B16 are reversibly increased by selective superfusion of the muscle with $10^{-7}M$ $SCP_B$. B16 was stimulated at 20 Hz for 1 s to elicit contractions. (**Aii**) Expansion of the contractions in (**Ai**) illustrates the increase in relaxation rate produced by $SCP_B$. Traces were aligned so that the motor neuron bursts (not shown) were superimposed. (**Bi**) Modulation of B16-evoked contractions by stimulation of B15. Motor neuron B16 was stimulated as in **A** at 100 s intervals; B15 was stimulated to fire six 4- s bursts at 25 Hz, a stimulation pattern known to release endogenous SCPs *between* B16 bursts. (**Bii**) B16-contractions on a faster time scale. Note that the large increase in relaxation rate following B15 stimulation is very similar to that produced by application of the SCPs to the muscle in **Aii**.

quencies and patterns of stimulation were necessary to evoke this release. Using a combination of HPLC and a sensitive bioassay, extracts of the bilaterally symmetrical I5 muscles were found to contain nearly identical levels of the SCPs (Whim and Lloyd, 1989). In common with many other peptides, there is no efficient uptake system for the SCPs in the muscle or nerve terminals, and transport rates indicate that <1% of the SCPs in the nerve terminals can be replaced in 4 h (Whim and Lloyd, 1989). B15 was unilaterally stimulated and the levels of SCPs in the stimulated muscle were then compared to those in the contralateral, unstimulated, control muscle. B15 was stimulated using three paradigms, each of which had an average frequency of 5 Hz. When stimulating tonically at 5 Hz, or at 25 Hz for 2 s every 10 s, there was no significant depletion of the SCPs in the stimulated muscles. However, stimulation at 50 Hz for 1 s every 10 s caused a marked depletion of the peptides from stimulated muscles. Tonic hyperpolarization of B15 or stimulation of B16 with the 50 Hz paradigm (to cause muscle contractions), did not alter the SCP content of the stimulated muscle. Although 50 Hz is rather rapid firing for most *Aplysia* motor neurons (Cropper et al., 1990a) by increasing the burst duration (to 4 s), stimulation at 25 Hz was found to deplete peptide to a similar degree to the 50 Hz paradigm. It was concluded that the SCPs could be released from B15 terminals and that this release depended on the precise stimulation pattern (Whim and Lloyd, 1989). Similar results have been reported by Cropper et al. (1990b).

The observations regarding the importance of stimulation pattern were supported by a second, independent, experimental approach. These experiments relied on the fact that the SCPs are the only major substances present in B15 that elevate I5 muscle cAMP levels when applied exogenously (Lloyd et al., 1984; Whim and Lloyd, 1989). If stimulation of B15 did release the SCPs, one would expect a subsequent elevation of muscle cAMP levels. It was found that B15 stimulation did increase cAMP levels in a fashion that accurately paralleled the frequency dependence

found in the depletion experiments. Because of the substantial agreement in the results produced by both methods it was possible to use the stimulation-dependent increase in cAMP levels as a way to more clearly define which parameters were important in determining peptide release. Lowering the stimulation frequency while keeping all other parameters constant revealed a "cut-off" frequency for the elevation of cAMP levels that varied with the burst interval. Clearly, if the change in muscle cAMP levels is an accurate reflection of the release of the SCPs, the threshold for peptide release in this system must be sharply regulated by the stimulation parameters.

We believe that these experiments indicate that the release of the SCPs from the terminals of B15 can be monitored, and that this release is steeply dependent on stimulation frequency, burst duration, and burst interval. Since cholinergic EJPs can be evoked with single spikes, it seems likely that during low-frequency firing B15 releases predominantly ACh, but that both ACh and the SCPs are released during higher frequency firing.

### *Physiological Consequences of the Release of the SCPs*

The effects of the SCPs released from the terminals of B15 were assayed on muscle contractions evoked by stimulation of motor neuron B16. This experimental design was possible because B15 and B16 innervate the same muscle fibers, and the SCPs modulate muscle contractions mainly via a postsynaptic mechanism (Cohen et al., 1978; Weiss et al., 1979; Cropper et al., 1988). Using the three stimulation paradigms previously described for the depletion experiments, it was observed that firing B15 with the 50 Hz paradigm produced an increase in the amplitude and relaxation rate of B16-evoked contractions that slowly reversed. Consistent with previous results, the other two stimulation paradigms (tonic 5 Hz or 25 Hz for 2 s every 10 s), which did not evoke the release of the SCPs, produced no significant change in B16-evoked contractions (Whim and Lloyd, 1990). By increasing the burst duration and decreasing the interburst interval, the effect of B15 on B16-evoked contractions could be elicited with stimulation frequencies as low as 10 Hz. The effect of B15 on B16 evoked contractions was very persistent, often requiring as long as an hour to return to baseline. The enhancement of contraction amplitude and relaxation rate seen with B15 stimulation was very similar to that induced by the application of exogenous SCPs (Fig. 2B). The effect is unlikely to be caused by the release of ACh from B15, since transiently blocking ACh receptors with a cholinergic antagonist during stimulation of B15 did not affect the subsequent modulation of B16-evoked muscle contractions. In common with the actions of exogenous peptide application, the B15-induced modulation of muscle contractions did not involve a change in the amplitude of the excitatory junction potentials (EJPs), but rather a change in the efficiency of excitation–contraction coupling in the muscle fibers (Whim and Lloyd, 1990). Thus, stimulation paradigms that evoke release of the SCPs also modulate I5 muscle contractions in a manner very similar to application of exogenous peptide. An interesting observation is that although the increase in contraction amplitude could also be produced by additional motor neuron spikes (to increase ACh release), this has no effect on muscle relaxation rate. Thus, the release of the peptide cotransmitters modifies the dynamics of the neuromuscular system. Modeling of muscle interactions in the buccal mass suggests that the peptide cotransmitters may function to adjust the relationship between the frequency, magnitude, and relaxation rate of muscle contractions to maintain optimal phase relationships between muscles during feeding (Weiss et al., 1992).

From experiments such as these we propose the following sequence of events: When B15 fires at a relatively high frequency or at lower frequencies with a relatively long burst duration, the SCPs are released from the terminals of B15 in the I5 muscle. Subsequent binding of the SCPs to receptors on the muscle results in the elevation of muscle cAMP levels, producing an increase in contraction amplitude and relaxation rate via mechanisms that are currently under investigation (Brezina et al., 1992; Hooper et al., 1992; Probst et al., 1992). Whether a B15 firing pattern

evokes a release of the SCPs depends crucially on the interplay between stimulation frequency, burst duration, and interburst interval.

### Are the SCPs Released by Physiological Firing Patterns?

Although the consequences of evoked peptide release can be dramatic, the behavioral relevance is less clear. This is primarily because of the difficulty in determining whether the firing patterns that evoke peptide release in reduced preparations actually occur in the animal. Such information can be obtained by recording the activity of identified buccal neurons in a behaving animal. Cropper and colleagues have taken advantage of the fact that the I5 muscle is innervated by only two motor neurons, each of which evoke characteristic EJPs. By implanting a wire electrode in the I5 muscle, firing patterns in B15 and B16 have been measured during a variety of behaviors, including biting, swallowing, and rejection (Cropper et al., 1990a,b). During ingestion both B15 and B16 are active, with B15 firing at up to 12 Hz for 3.5 s with an interburst interval of about 3.5 s. During egestion B16, but not B15, fires. Similar patterns have also been recorded intracellularly from B15 and B16 in more reduced preparations (Church and Lloyd, 1993). Therefore, the stimulation patterns that evoke release of the SCPs are within the range that B15 fires during feeding (Whim and Lloyd, 1989, 1990; Cropper et al., 1990b).

## The I3a Neuromuscular System

### Release of the SCPs from B38

Figure 1B summarizes the motor innervation of buccal muscle I3a. Note that this neuromuscular preparation differs significantly from I5. First, the two excitatory motor neurons (B3 and B38) are not cholinergic and are likely to use an amino acid as their conventional transmitter. Second, I3a is innervated by an inhibitory motor neuron (B47) that is cholinergic. All three motor neurons express peptide cotransmitters (Lotshaw and Lloyd, 1990, Church and Lloyd, 1991; Church et al., 1993; Fox and Lloyd, 1993). To examine whether the peptides expressed in these motor neurons could be released in a stimulation- and $Ca^{2+}$-dependent manner, neurons B3, B38, and B47 were maintained in primary culture and newly synthesized peptides were labeled with $^{35}S$-methionine. The cells were stimulated via intracellular electrodes, and the superfusate was analyzed using HPLC and liquid scintillation counting. Peaks of radioactivity corresponding to previously sequenced peptides were identified. In culture, each motor neuron continued to synthesize those peptides that they synthesized in the ganglia, and released them in a $Ca^{2+}$- and stimulation-dependent manner, indicating that they are likely to be cotransmitters (Church et al., 1993).

All three of the peptides expressed in the I3a motor neurons (the SCPs, myomodulin A, and FMRFamide) have been found to increase the amplitude of B3-evoked EJPs and I3a muscle contractions. In contrast to the I5 neuromuscular system, these peptides also function presynaptically in I3a (Fox and Lloyd, unpublished observations). Because both B38 stimulation and exogenous SCPs increase cAMP levels in the I3a muscle fibers, the stimulation-dependent increase in cAMP levels was used as a monitor of the release of the SCPs from B38. Stimulation frequencies as low as 10 Hz produce a severalfold increase in cAMP levels, presumably reflecting the release of the SCPs. To test whether the released SCPs could modulate B3-evoked I3a muscle contractions, B38 was stimulated in patterns that release the SCPs during a B3 interburst interval. Subsequent B3-evoked EJPs and contractions were enhanced in amplitude. These results suggest that peptide cotransmitter release occurs from the terminals of the excitatory motor neuron B38 and this release has physiological consequences.

### Release of an "Excitatory" Modulatory Peptide from an Inhibitory Motor Neuron

B47 is an inhibitory motor neuron of the I3a muscle. It uses ACh as a conventional transmitter to evoke IJPs and to inhibit muscle contractions evoked by stimulation of either of the

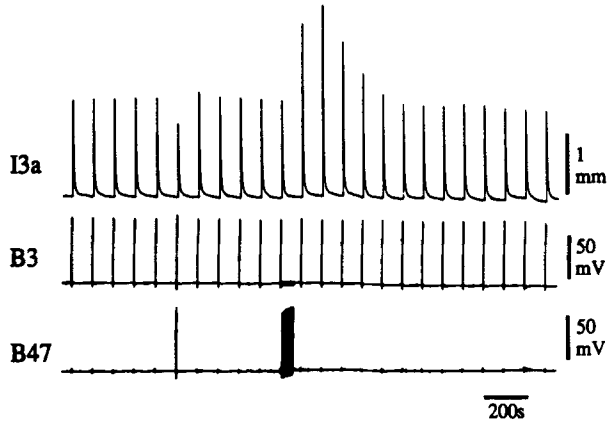


Fig. 3. Modulation of B3-evoked contractions by stimulation of B47. Motor neuron B3 was stimulated at 16 Hz for 1.6 s to elicit contractions. When B47 was stimulated at 20 Hz *simultaneously* with B3, the muscle contraction was reduced in amplitude. When B47 was stimulated to fire six 4 s bursts at 20 Hz *between* B3 bursts, subsequent B3-evoked contractions were increased in amplitude.

excitatory motor neurons (Fig. 3; Church et al., 1993). As discussed above, B47 also expresses the myomodulin A peptide and releases it in a $Ca^{2+}$- and stimulation-dependent manner. However, the exogenous application of low concentrations of myomodulin A enhances B3- or B38-evoked EJPs and muscle contractions. Thus B47, an inhibitory motor neuron, contains a peptide that could be considered excitatory. The physiological consequences of the release of conventional and peptide cotransmitters were investigated. As noted above, when B47 was stimulated simultaneously with B3, the evoked contraction was reduced in amplitude. However, when B47 was stimulated in extended bursts designed to release its peptide cotransmitters during the B3 interburst interval, subsequent B3-evoked contractions (Fig. 3) and EJPs were enhanced in amplitude. These observations suggest that B47 may utilize its conventional transmitter, ACh, as an inhibitory transmitter and its peptide cotransmitters, including myomodulin A, as an excitatory modulatory transmitter at the same neuromuscular synapse (Church et al., 1993). Thus, depending on the stimulation paradigm, a motor neuron can selectively inhibit or enhance the effects of a second motor neuron at the same neuromuscular junction.

### *Are the Modulatory Peptides Released by Physiological Firing Patterns?*

Because muscle I3a has a more complex innervation pattern than I5, characteristic EJPs cannot be easily measured in a behaving animal. However, in reduced preparations the activity of identified neurons can be monitored during evoked feeding-like buccal motor programs (Rosen et al., 1991; Morton and Chiel, 1993). These motor programs can be associated with specific aspects of feeding behavior, based on the firing patterns recorded in B15 and B16. Using a reduced preparation consisting of the lips, buccal mass, and the buccal and cerebral ganglia, it has been possible to record intracellularly from B38, B3, and B47 simultaneously with B15 and B16 during evoked feeding-like buccal motor programs. During ingestion, B3, B38, and B47 fire in bursts lasting up to 8 s. B3 and B38 fire at up to 15 Hz, and B47 fires at frequencies exceeding 25 Hz (Church and Lloyd, 1993). These frequencies overlap with those that are sufficient to evoke peptide cotransmitter release from these motor neurons.

## Is Peptide Release Always Sensitive to Stimulation Pattern?

The release of the SCPs from B15 and B38 is particularly sensitive to the stimulation paradigm. To determine whether this was a characteristic common to other SCP-containing neurons, we cultured identified neurons and directly measured the release of the SCPs from B15, and B1, a buccal motor neuron that innervates muscles of the gut (Fig. 1C). The release of the SCPs from cultured B15 cells still retained a pattern sensitivity in culture. When comparing the tonic 5 Hz paradigm to stimulating the cell at 50 Hz for 1 s every 10 s, approx sixfold more peptide was released per spike by the latter stimu-

lation paradigm. Although the threshold for peptide release was lower in the cultured cells than the threshold found in neuromuscular preparations, when *physiological* patterns of bursting activity were compared to tonic firing, more peptide was still released by the bursting pattern in culture (Whim and Lloyd, 1993). Thus, a neuron that exhibits a pattern sensitivity to peptide release at a neuromuscular synapse retains similar characteristics in culture. In contrast, the release of the SCPs from B1 was pattern-independent. Comparing the release of the SCPs by tonically stimulating the cells (over the range 1–5 Hz) indicated that each spike released the same amount of peptide regardless of the stimulation frequency. Similarly, there was no difference in release when the neuron was stimulated to fire the same number of spikes but in two patterns (one at 6 Hz for 0.5 s every 3 s, and one with tonic 1 Hz stimulation). Thus, in contrast to the release of the SCPs from B15, the release of the SCPs per spike from B1 appears completely independent of frequency or pattern.

Using an extracellular electrode implanted on a peripheral nerve, the firing pattern of B1 in intact feeding animals has been determined (Lloyd et al., 1988). The neurons fire specifically during swallowing movements at frequencies up to 3 Hz in short bursts. Stimulating B1 in a reduced preparation consisting of the buccal ganglion and gut muscle produces an increased contractile activity that is identical to that produced by an exogenous application of the SCPs. The threshold frequency required to produce this contractile activity is below 1 Hz. Thus, these neurons release the SCPs at very low firing rates (Whim and Lloyd, 1992, 1993). From such experiments it can be concluded that there are no stimulation patterns or frequencies that are universal prerequisites for peptide release.

## Discussion

It appears that there are no obvious rules that indicate *a priori* the stimulation paradigms that result in peptide release. In some motor neurons (such as B15) the release of peptides is extremely sensitive to the pattern of stimulation, and the release of peptide cotransmitters requires higher frequencies or longer burst durations than those required for release of the conventional transmitters. Similar conclusions have been drawn regarding peptide release from a variety of experimental preparations, including the vertebrate neurohypophysis, cultured myenteric plexus neurons, and frog sympathetic ganglia (Dutton and Dyball, 1979; Cazalis et al., 1985; Willard, 1990; Peng and Horn, 1991). Nevertheless, exceptions do occur since peptide release from motor neuron B1 is simply proportional to the number of spikes and not their pattern or frequency. Perhaps it is best to think of peptide secretion as a continuum, with release of the SCPs from B15 exhibiting an extreme form of pattern-dependence and release of the SCPs from B1 demonstrating a pattern-independence. It may be that no clear distinction can be made in this regard between conventional and peptide transmitters. The release of the SCPs from B15 is so tightly coupled to the stimulation pattern that it is possible that the cell can function as two different neurons: one that is cholinergic and one that is both cholinergic and peptidergic. A complex situation also exists for neuron B47. Here the conventional transmitter is inhibitory, whereas the peptide cotransmitter increases the effectiveness of excitatory motor neurons that innervate the same muscle fibers as B47. The capability of independently releasing multiple transmitters confers extreme plasticity on individual neurons.

The release of peptides (in common with conventional transmitters) can also be regulated by factors other than firing pattern. Exogenous transmitters, including enkephalin and norepinephrine, inhibit the release of substance P from chick sensory neurons (Mudge et al., 1979; Holz et al., 1989) and serotonin inhibits the release of the SCPs from neuron B1 (Whim and Lloyd, 1992). In the latter case serotonin actually increases the excitability of B1 but decreases peptide release, indicating that changes in peptide release are not necessarily correlated with changes in a neuron's activity state.

Further complexity arises when we consider that transmitters that have opposing effects can be colocalized and coreleased. For example, members of two peptide families, the buccalins and the SCPs, are coreleased from B15 together with the conventional transmitter ACh (Vilim et al., 1991; Whim and Lloyd, 1993). Although this result is not unexpected given that the buccalins and the SCPs are thought to be contained in the same vesicle (Vilim et al., 1990), one might question the utility of their colocalization since the SCPs enhance, whereas buccalin A depresses muscle contractions (Cropper et al., 1988). It may be significant that the I5 muscle is not innervated by an inhibitory motor neuron, this role perhaps being functionally assumed by buccalin A in the excitatory motor neuron B15. Since the SCPs potentiate both contraction amplitude and relaxation rate, whereas buccalin A affects only contraction amplitude, it has been suggested that the corelease of the SCPs and buccalin from B15 may be important in maintaining the correct phase relationships between antagonistic muscles in times of increased muscular demand (Weiss et al., 1992). Presumably the potentiating effect of B15 stimulation on I5 contractions results from a balance between the actions of buccalin and the SCPs, with the effects of the SCPs predominating (e.g., Fig. 2B).

In the future it will be interesting to determine if neurons that contain multiple neuropeptides in separate vesicle populations are capable of independently releasing subsets of these peptides. Indeed, there are precedents for the differential packaging and localization of neuropeptides within a cell (Fisher et al., 1988; Sossin et al., 1990). This raises the possibility that coexisting peptides may not be coreleased with a fixed stoichiometry, but rather that their secretion may be differentially controlled by particular patterns of stimulation.

Finally, although there has been a fairly good match between the actions of peptides when applied exogenously and when released by neuronal stimulation, unexpected observations, such as the prolonged time course of action of the released SCPs, underlines the need for a full description of the bioactive compounds synthesized by a neuron, the conditions under which they are released, as well as their postsynaptic effects.

## Summary

Peptide cotransmitters can be released from motor neurons by physiological patterns of stimulation and can have marked effects on muscle physiology. However, the stimulation patterns that release a particular peptide from one neuron need not be those that release the same peptide from another neuron. Thus the stimulation parameters controlling peptide release must be determined on a neuron by neuron basis.

Although the actions of most peptides on *Aplysia* buccal muscle have involved a modulation of muscle contractions, trophic effects, perhaps involving the differentiation and maintenance of the neuromuscular junction, may also occur (*see* Hall and Sanes, 1993). It is likely that the general characteristics of peptide release as demonstrated by *Aplysia* motor neurons will be shared with other cells. For example, there are striking parallels with a vertebrate peptide, calcitonin gene-related peptide, which is present in motor neurons and potentiates muscle contractions, primarily via a postsynaptic action that is likely to involve a change in excitation–contraction coupling (Ohhashi and Jacobwitz, 1988; Laufer and Changeaux, 1989).

## Acknowledgments

We thank W. L. Pearson for critical reading of the manuscript. This work was supported by grants from NIH, NSF, and the Brain Research Foundation.

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