

Temporal Dynamics of Convergent Modulation at a Crustacean Neuromuscular Junction

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Jorge-Rivera, Juan Carlos, Kamal Sen, J. T. Birmingham, L. F. Abbott, and Eve Marder. Temporal dynamics of convergent modulation at a crustacean neuromuscular junction. *J. Neurophysiol.* 80: 2559–2570, 1998. At least 10 different substances modulate the amplitude of nerve-evoked contractions of the gastric mill 4 (gm4) muscle of the crab, *Cancer borealis*. Serotonin, dopamine, octopamine, proctolin, red pigment concentrating hormone, crustacean cardioactive peptide, TNRNFLRFamide, and SDRNFLRFamide increased and D-allatostatin-3 and histamine decreased the amplitude of nerve-evoked contractions. Modulator efficacy was frequency dependent; TNRNFLRFamide, proctolin, and D-allatostatin-3 were more effective when the motor neuron was stimulated at 10 Hz than at 40 Hz, whereas the reverse was true for dopamine and serotonin. The modulators that were most effective at high stimulus frequencies produced a significant decrease in muscle relaxation time; those that were most effective at low stimulus frequencies produced modest increases in relaxation time. Thus modulator actions that appear redundant when examined only at one stimulus frequency are differentiated when a range of stimulus dynamics is studied. The effects of TNRNFLRFamide, serotonin, proctolin, dopamine, and D-allatostatin-3 on the amplitude and facilitation of nerve-evoked excitatory junctional potentials (EJPs) in the gm4 and gastric mill 6 (gm6) muscles were compared. The EJPs in gm4 have a large initial amplitude and show relatively little facilitation, whereas the EJPs in gm6 have a small initial amplitude and show considerable facilitation. Modulators that enhanced contractions also enhanced EJP amplitude; D-allatostatin-3 reduced EJP amplitude. The effects of these modulators on EJP amplitude were modest and showed no significant frequency dependence. This suggests that the frequency dependence of modulator action on contraction results from effects on excitation–contraction coupling. The modulators affected facilitation at these junctions in a manner consistent with a change in release probability. They produced a change in facilitation that is inversely related to their action on EJP amplitude.

INTRODUCTION

A surprisingly large number of neuromodulatory amines and neuropeptides can alter the strength of synaptic connections and the intrinsic properties of neurons and muscles (Brezina et al. 1996; Harris-Warrick et al. 1992; Marder and Calabrese 1996). Many of these substances can act on the same target tissue. For example, the crustacean stomatogastric ganglion (STG) is the target of ≥ 15 different neuromodulators (Marder and Calabrese 1996; Marder and Weimann 1992; Marder et al. 1995), and the single lateral pyloric

(LP) neuron of the STG responds to almost all of these (Flamm and Harris-Warrick 1986b; Hooper and Marder 1987; Nusbaum and Marder 1988; Swensen and Marder 1997; Weimann et al. 1993, 1997).

The neuromuscular preparations studied here include 2 of the >40 striated muscles that are innervated by motor neurons of the STG (Maynard and Dando 1974). Several previous studies demonstrated neuromodulation of stomach muscles and neuromuscular junctions by an amine or peptide (Jorge-Rivera 1997; Jorge-Rivera and Marder 1996, 1997; Lingle 1981; Meyrand and Marder 1991; Meyrand and Moulins 1986; Weimann et al. 1997), but this is the first study in which the actions of numerous potential neuromodulators were studied on the same stomach neuromuscular junction. We show here that the amplitude of nerve-evoked contractions of one of the muscles of the crab stomatogastric nervous system is modulated by ≥ 10 different substances.

There is significant precedent for convergent modulation at invertebrate neuromuscular junctions and muscles. The extensor-tibiae muscle of the locust (Evans 1984, 1994; Evans and Meyers 1986; Evans and Siegler 1982), crustacean muscle (Worden et al. 1995), and the accessory radula closer (ARC) muscle of *Aplysia californica* (Brezina et al. 1996; Cropper et al. 1987, 1990; Weiss et al. 1992, 1993) are all modulated by multiple substances. Is extensive convergent modulation of one target neuron, neuromuscular junction, or muscle functionally redundant? In the case of both the extensor tibiae and the ARC muscles, it appears that one or several neuromodulators may be more effective at some frequencies of motor neuron discharge (Brezina et al. 1996; Evans 1984, 1994; Evans and Meyers 1986; Evans and Siegler 1982; Weiss et al. 1993). We also find that the extent of modulation depends on the frequency of stimulation of the presynaptic neuron and that the dependence is different for different modulators.

The mechanisms of modulation at many invertebrate neuromuscular junctions were studied extensively (Calabrese 1989; Marder and Calabrese 1996; Weiss et al. 1992). Modulators can alter the intrinsic properties of motor neurons (Weimann et al. 1993, 1997), transmitter release at motor neuron terminals (Dudel 1965; Glusman and Kravitz 1982; Skerrett et al. 1995; Worden et al. 1995), or the contractile machinery of the muscle fiber itself (Brezina et al. 1994a,b). While attempting to understand why the action of modulators depends on the frequency of presynaptic stimulation, we noted modulator effects on the facilitation of the muscle EJPs. Analysis of these effects suggests that the change in facilitation seen in the presence of modulators is inversely related to their effects on EJP amplitude.

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METHODS

Animals and solutions

All experiments were performed on male *Cancer borealis* purchased from local fishermen in Boston, MA, and held in aerated saltwater aquaria at 12°C until used. Physiological saline had the following composition (mM): 440 NaCl, 11.3 KCl, 13.3 CaCl₂, 26.3 MgCl₂, 11.0 Trizma base, 5.2 maleic acid, pH 7.4–7.5.

Crustacean cardioactive peptide (CCAP) was purchased from Bachem. D-Allatostatin-3 (AST), red pigment concentrating hormone (RPCH), SDRNFLRFamide, and TNRNFLRFamide were obtained from American Peptide. Proctolin, histamine, serotonin, octopamine, and dopamine were obtained from Sigma. Each substance was dissolved in distilled water at 10⁻² or 10⁻³ M and stored at -20°C. Aliquots were diluted in saline at desired concentrations immediately before use.

Physiology

Neuromuscular preparations [identified according to the nomenclature of Maynard and Dando (1974)] were isolated from the foregut of the crab and pinned out in 5-ml superfusion chambers. Preparations were superfused continuously with a gravity-fed system at 10–15 ml/min. Modulators were bath applied by means of a switching port at the inflow of the superfusion system. Bath volume was ~3 ml. The saline temperature was held between 10 and 12°C by a Peltier cooling system and was continuously monitored with a thermoelectric probe in the bath.

Contraction measurements

Muscle contraction recordings were obtained with a movement transducer (FT03, Grass Instruments). One of the muscle insertions was pinned down in the dish. The other insertion was tied to a 6-in. (15.24 cm) string that was attached to the transducer. The muscle was stretched to its resting length. Stimulation of the motor nerve in this configuration produced muscle shortening. We designated the time taken for the muscle to relax from peak contraction to 10% of the maximum as the relaxation time.

EJP measurements

EJP recordings from the singly innervated gastric mill 4 (gm4) and gastric mill 6 (gm6) muscles were obtained with conventional 2.5 M KCl-filled microelectrodes, with resistances of 10–15 MΩ. Motor nerves were stimulated with suction electrodes. EJP data were collected and analyzed as described by Sen et al. (1996). EJP amplitude was defined as the peak membrane potential of an EJP relative to the exponentially extrapolated baseline from the previous EJP to subtract the effects of summation and membrane capacitance.

To describe the amplitudes of EJPs in response to an arbitrary spike train we used a modification of the synaptic decoding method of Sen et al. (1996). The EJP amplitude A was written as a product of two factors, $A = A_0 F$ where A_0 was a constant and F was a dynamic variable that represented facilitation. Initially F was 1. With each presynaptic spike F was incremented by a constant $f \geq 0$, $F \rightarrow F + f$ representing the amount of facilitation per spike. Between spikes F recovered exponentially to 1 with time constant τ_F , obeying $\tau_F dF/dt = 1 - F$. The root-mean-square (RMS) difference between predicted and measured amplitudes was used as a measure of accuracy. The set of model parameters that minimized the RMS error was found by an automated search algorithm (Varela et al. 1997).

RESULTS

The experiments in this paper study the gm4 and gm6 muscles of the crab stomach. The gm4 muscle is innervated by the single dorsal gastric (DG) excitatory motor neuron, and the gm6 muscle is innervated by the single lateral gastric excitatory motor neuron. Both muscles participate in the gastric mill motor pattern and move the medial and lateral teeth of the gastric mill, respectively. Unlike many motor neurons that contain peptide cotransmitters (Bishop et al. 1987; Cropper et al. 1990; Norris and Calabrese 1987), the motor neurons of the adult STG are not known to contain any modulatory substances other than the neurotransmitters acetylcholine or glutamate (Lingle 1980; Marder 1976, 1987). Thus it appears that most neuromodulators act on the stomatogastric muscles and neuromuscular junctions as neurohormones, released from the pericardial organs and other neurosecretory structures into the circulating hemolymph (Christie et al. 1995; Keller 1992). The 10 peptides and amines examined here are found in the neurosecretory structures of the crab (Christie et al. 1995).

Contraction amplitude is modulated by 10 substances

Figure 1 shows nerve-evoked contractions of the gm4 muscle in control saline and during bath application of 10 modulators. Note that serotonin, dopamine, octopamine, TNRNFLRFamide, SDRNFLRFamide, CCAP, proctolin, and RPCH all strongly potentiated the contraction evoked by stimulation at 20 Hz for 1 s, whereas AST and histamine decreased the amplitude of the nerve-evoked contractions. Figure 2 summarizes these effects for data pooled from many experiments. In the experiments shown in Figs. 1 and 2 serotonin, dopamine, octopamine, TNRNFLRFamide, RPCH, proctolin, and CCAP were applied at 10⁻⁷ M, and SDRNFLRFamide, AST, and histamine were applied at 10⁻⁶ M. These concentrations are approximately those required to give maximal effects. Threshold concentrations were in the 10⁻¹⁰ to 10⁻⁹ M range (Jorge-Rivera and Marder 1996, 1997; Weimann et al. 1997).

To avoid complications produced by long-lasting effects of the modulators and the possibility of synergistic interactions between them, the experiments were performed by applying only one modulator to a given preparation. In three preparations, we bath applied each modulator after long washes between them and confirmed that the same gm4 muscle was modulated by all of these substances (not shown).

Modulator action depends on frequency of motor neuron stimulation

All of the substances shown in Figs. 1 and 2 also alter the motor patterns (the temporal pattern of discharge of the motor neurons) produced by the *C. borealis* stomatogastric nervous system (Marder 1987; Marder and Hooper 1985; Marder and Weimann 1992; Weimann and Marder 1994; Weimann et al. 1993, 1997). Because contraction amplitude depends critically on the discharge pattern of the motor neuron (Jorge-Rivera and Marder 1996; Weimann et al. 1997), the amplitude of muscle contraction in vivo will also be altered by modulation of the motor pattern. We had pre-

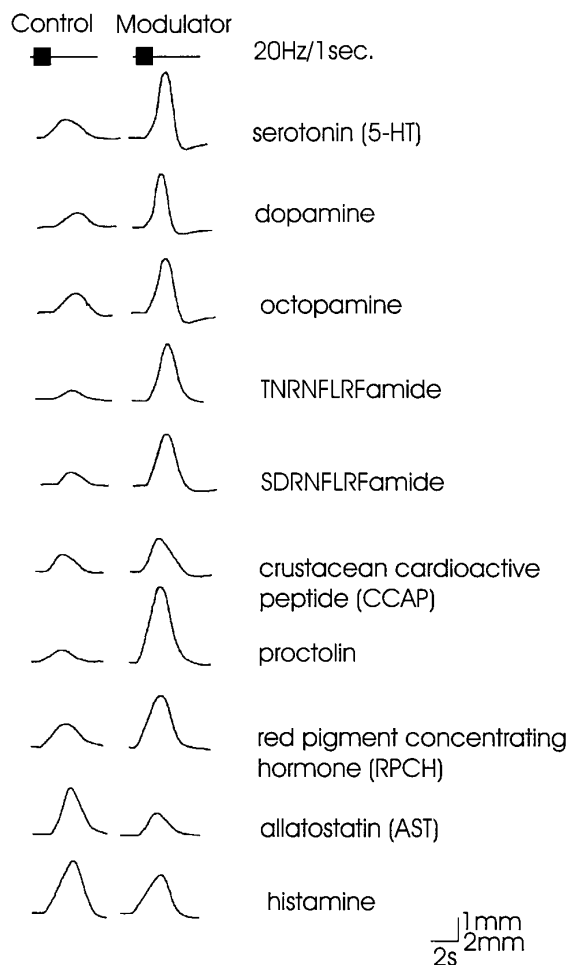


FIG. 1. Effect of 10 modulators on nerve-evoked contractions of the gastric mill 4 (gm4) muscle. The dorsal gastric nerve (dgn) was stimulated at 20 Hz for 1 s. Muscle contraction was monitored with a movement transducer in control saline and in saline containing the modulator indicated. Each modulator was tested on a separate neuromuscular preparation. Serotonin, dopamine, octopamine, TNRNFLRFamide, red pigment concentrating hormone, proctolin, and crustacean cardioactive peptide were applied at 10^{-7} M, and SDRNFLRFamide, D-allatostatin-3 (AST), and histamine were applied at 10^{-6} M. Vertical scale bar: 1 mm AST and histamine; 2 mm all others.

viously shown for a muscle innervated by the LP neuron that CCAP was more effective in increasing contraction amplitude at low frequencies of motor neuron discharge than at high frequencies (Weimann et al. 1997). Therefore we wanted to determine the extent of modulation of muscle contraction at different frequencies of motor neuron stimulation.

We studied the effects of TNRNFLRFamide, proctolin, serotonin, dopamine, and AST on gm4 contractions evoked by nerve stimulation in 0.5 s trains at 10, 20, 30, and 40 Hz (Fig. 3). Proctolin and TNRNFLRFamide were more effective at potentiating the contractions at 10 Hz than at 40 Hz. In contrast, serotonin was more effective at 40 Hz than at 10 Hz. AST was more effective at decreasing the contraction amplitude at 10 Hz than at 40 Hz. A two-way analysis of variance (ANOVA) showed a strong interaction between modulators and frequency of stimulation [$F(9,63) = 3.61$, $P \leq 0.001$]. The modulatory actions at 40 Hz were signifi-

cantly different from those at 10 Hz (Student's *t*-test, TNRNFLRFamide, $P \leq 0.01$; serotonin, $P \leq 0.01$; proctolin, $P \leq 0.05$; AST, $P \leq 0.05$; significance is not indicated for the difference between 10 and 40 Hz for dopamine because the one-way ANOVA failed to demonstrate a significant interaction between frequency and percent modulation because of the variance in the 30 Hz data).

To eliminate possible artifacts caused by the time taken for modulators to act, and to rule out long-lasting interactions among different stimuli, we randomized the order of presentation of the different frequencies. The amplitude of the contraction for low frequencies (10 Hz) did not change after the whole sequence of nerve stimulations.

Effect of modulators on muscle relaxation

In a number of preparations, exogenously applied modulators alter not only the amplitude of muscle contraction but also the rate of muscle relaxation (Evans 1984, 1994; Evans and Meyers 1986; Evans and Siegler 1982; Weiss et al. 1992, 1993). Because the time of muscle relaxation plays a critical role in determining whether a muscle relaxes fully before its next contraction in response to repeated trains of presynaptic activation (Morris and Hooper 1997), we asked if the time needed for the gm4 muscle to relax is altered by modulatory substances. Figure 4A shows overlays of the contraction profiles seen at 20 and 40 Hz, with the 20-Hz response scaled to match the amplitude of the 40-Hz response. This allows a direct comparison of the time of contraction and relaxation. Note that the 20- and 40-Hz waveforms are superimposable. This indicates that the change in contraction amplitude caused by stimulation frequency does not modify the contraction and relaxation rates. Figure 4A

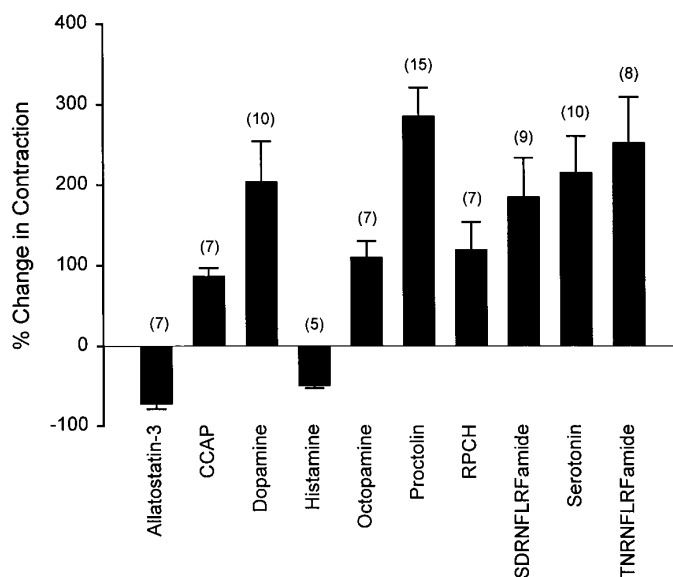


FIG. 2. Effect of modulators on nerve-evoked contractions. Modulators were bath applied at 10^{-7} M except for SDRNFLRFamide, histamine, and AST, which were bath applied at 10^{-6} M. These concentrations were selected to elicit maximum effects on contractions. These data summarize results of experiments in which the preparations were stimulated at 10–20 Hz for 1–2 s. Results are expressed as percent change in peak contraction for each preparation, values are expressed as means ± SE. The number of preparations is indicated in parentheses above each bar.

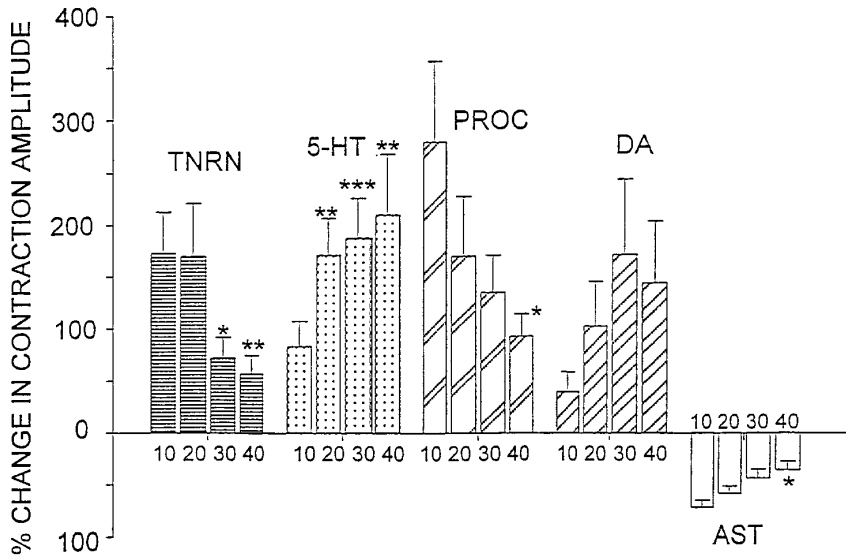


FIG. 3. Frequency dependence of percent change in contraction for 5 different modulators. The dgn was stimulated from 10–40 Hz for 0.5 s, and muscle contraction was monitored. TNRNFLRFamide (TNRN), serotonin (5-HT), proctolin (PROC), dopamine (DA), or AST were bath applied at 10^{-7} M in separate neuromuscular preparations. Histograms show pooled data for 7 preparations for each modulator. Error bars are SE. *: statistically significant differences in mean values (by paired *t*-tests subsequent to analysis of variance) when compared with 10 Hz. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

also shows that, when the responses to serotonin and proctolin are compared with their controls (by scaling their amplitudes), neither serotonin nor proctolin altered the time course of contraction, but serotonin decreased the relaxation time and proctolin increased it. Data for other modulators (Fig. 4B) indicate that dopamine, like serotonin, decreased the relaxation time significantly, and TNRNFLRFamide, like proctolin, increased it. The effects of AST were variable and did not reach significance.

Changes in relaxation time are usually attributed to direct effects of modulators on the excitation–contraction coupling processes in muscle, suggesting that at least some of the frequency-dependent modulator actions were direct effects on the muscle itself. However, we wished to determine whether the modulation of EJP amplitude also depended on the frequency of nerve stimulation and, if so, whether this might contribute to the frequency dependence of the effects of modulators on the amplitude of the peak contraction (Fig. 3). To this end,

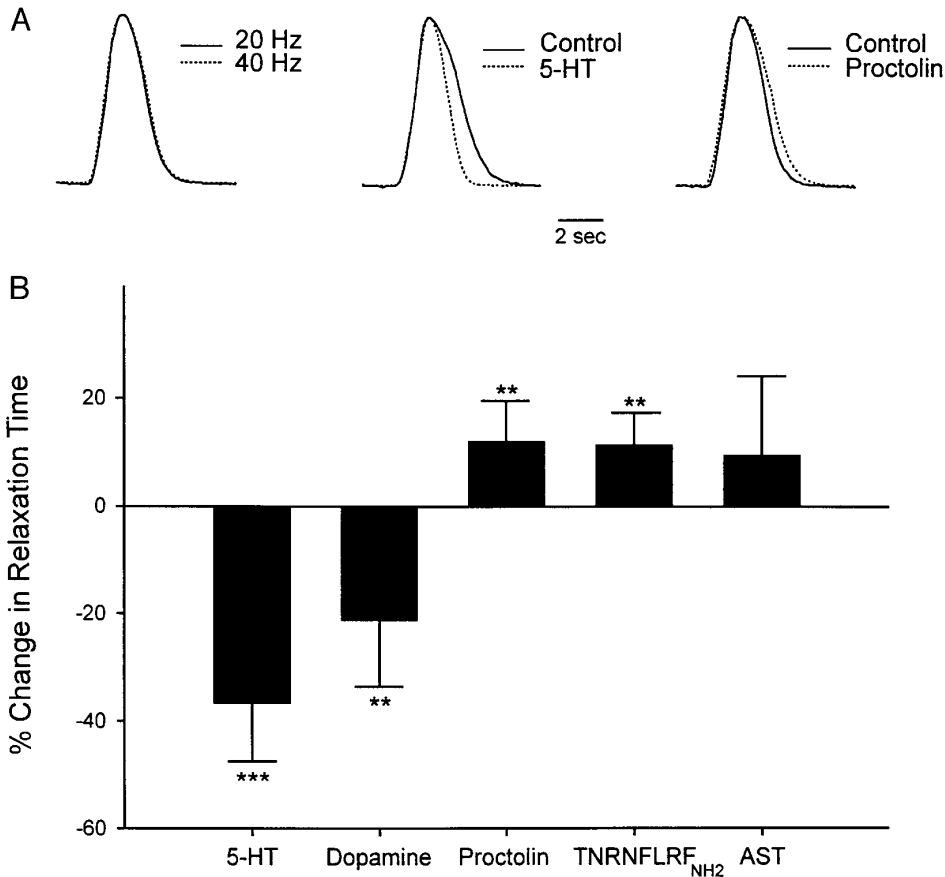


FIG. 4. Modulation of gm4 muscle relaxation time. A: pairs of traces were scaled so that they have the same amplitude to allow comparison of the waveforms. *Left*: muscle contractions produced by stimulation at 20 Hz and 40 Hz for 1 s show no change in contraction or relaxation time. In the presence of serotonin (*middle*) there is a decrease in relaxation time, whereas in proctolin (*right*) there is an increase in relaxation time. B: percent change in gm4 relaxation time caused by application of TNRNFLRFamide ($n = 6$), serotonin ($n = 7$), proctolin ($n = 8$), dopamine ($n = 9$), and AST ($n = 6$). The muscle contractions were generated by 1 s of 20-Hz stimulation. The relaxation time is defined as the time for the muscle to relax from the maximum to 10% of the maximum contraction length. Error bars are SE. Paired *t*-tests were used to compare the control and modulated relaxation times for each substance to determine significance (symbols as in Fig. 3).

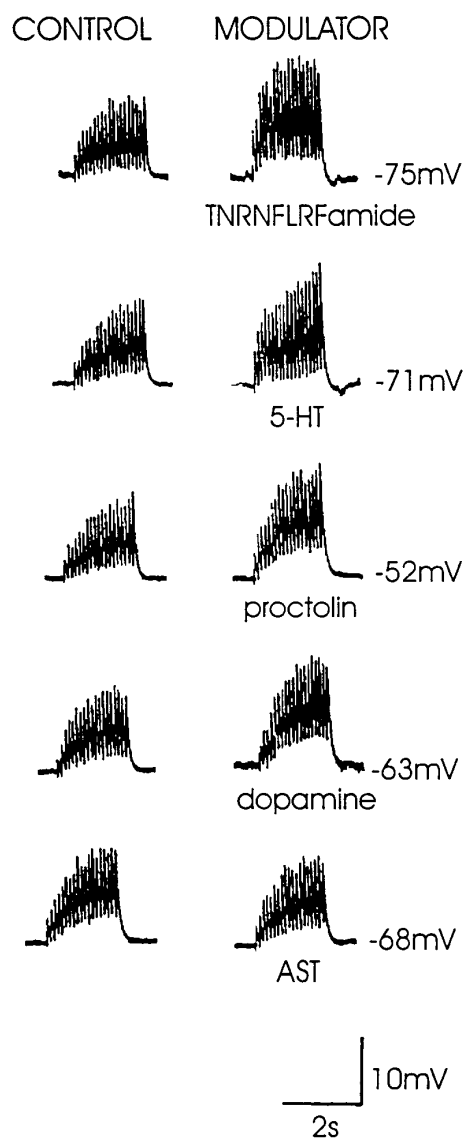


FIG. 5. Effect of 5 different modulators on nerve-evoked excitatory junctional potentials (EJPs). *Left*: nerve-evoked EJPs at 10 Hz for 2 s under control saline. *Right*: EJPs in response to the same train with the modulator indicated.

we studied the effects of modulators on the amplitude of EJPs and on short-term plasticity (in this case facilitation).

Modulators alter the amplitude of EJPs

Figure 5 shows the effect of five different modulators on nerve-evoked EJPs in the gm4 muscle. TNRNFLRFamide, serotonin, proctolin, and dopamine increased the amplitude of the peak depolarization evoked by the train of stimuli, and AST slightly decreased the peak membrane potential. These changes are smaller than the percentage changes seen in the contraction recordings (Figs. 1 and 2) but are in the same direction. Because the relationship between muscle depolarization and contraction is not necessarily linear, this does not rule out the possibility that the effects of the modulators on contraction are a direct consequence of the altered change in EJP amplitude. Therefore we measured the effects

of proctolin and serotonin on the amplitude of EJPs evoked at frequencies from 10 to 40 Hz.

Figure 6 shows the percent increase in amplitude produced by serotonin and proctolin on peak depolarizations evoked by 0.5-s trains at 10, 20, 30, and 40 Hz. In neither case was there a statistically significant difference in the effects of the modulators as a function of the frequency of stimulation. Figure 3 shows that serotonin is most effective on contraction amplitudes at high frequencies of stimulation, whereas Fig. 6 shows a tendency in the opposite direction. Proctolin enhances contraction amplitude more effectively at low frequencies (Fig. 3), and, although Fig. 6 shows a tendency in the same direction, the extent of this tendency (if it is real) is relatively small. Therefore we conclude that the frequency-dependent effects of the neuromodulators on contraction (Fig. 3) are unlikely to result from frequency-dependent effects of the modulators on EJP amplitude.

Do the modulators directly influence facilitation?

Some of the stomatogastric neuromuscular junctions display considerable facilitation. As part of our study of the effects of modulators on the gm4 EJPs, we noted that some of the modulators appeared to influence the degree of facilitation of the EJPs. Figure 7, *top panel*, shows the effects of serotonin on EJPs evoked at 5 and 10 Hz in the gm4 muscle. The ratios of the first EJP in the train to the last EJP in the train in the absence and presence of serotonin are different, suggesting a

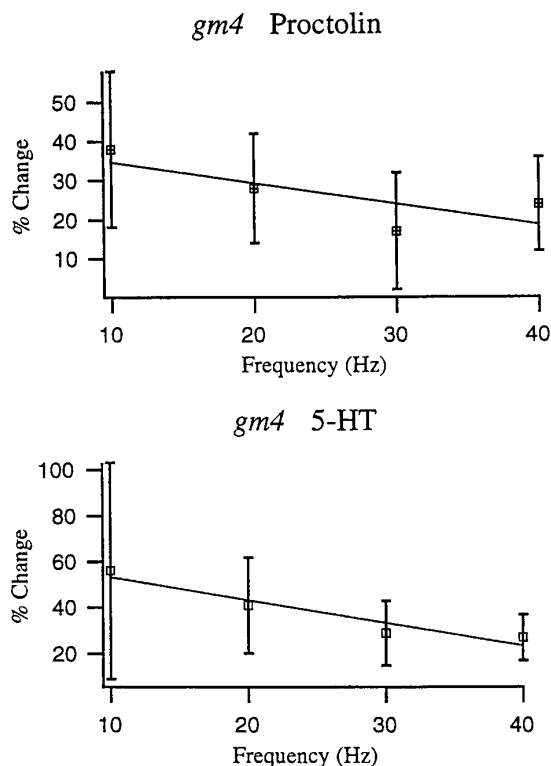


FIG. 6. Effect of stimulation frequency on modulation of peak postjunctional depolarization. The dgn was stimulated for 1 s at 10, 20, 30, and 40 Hz in control saline or in the presence of either proctolin (*top*) or serotonin (*bottom*). The plot shows the mean percent increase in peak postjunctional depolarization for 5 experiments. The line is the best linear fit to these points.

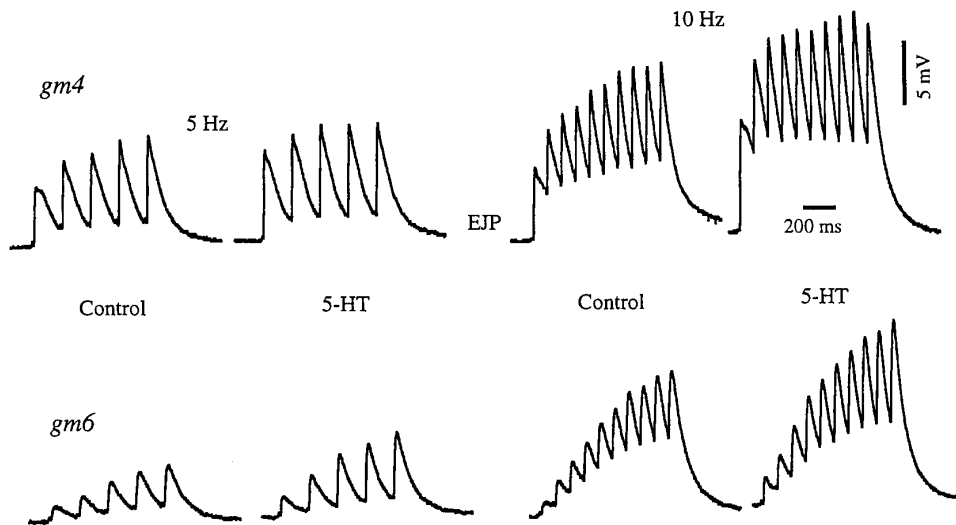


FIG. 7. Effect of serotonin on gm4 and gm6. *Top traces*: nerve-evoked EJPs in the gm4 muscle in control saline and with 10^{-7} M serotonin at 5 and 10 Hz for 1 s. The baseline membrane potentials for the traces are (from left to right) -60 , -63 , -60 , and -61 mV. *Bottom traces*: same sequence for the gm6 muscle. The baseline membrane potential for all traces was -61 mV.

modulation of facilitation. For example, in the 5-Hz traces in Fig. 7, the ratio of the last to first EJPs in the train was 1.6 in control saline and 1.1 in the presence of serotonin. We wished to study this further, but noted that the extent of facilitation seen in the gm4 junction is relatively modest compared with that seen in other neuromuscular junctions of the stomatogastric nervous system. Therefore for the following analyses we chose to examine neuromuscular junctions of both gm4 (which shows modest facilitation) and gm6. Figure 7 shows that gm6 displays considerable facilitation (compare the amplitude of the first and last EJPs in the train) and also responds to serotonin.

The method (Sen et al. 1996) used to characterize quantitatively the effects of the modulators on facilitation in these muscles is illustrated in Fig. 8 and described in the METHODS. Figure 8, *top traces*, shows the responses of a gm4 muscle fiber evoked by a train of impulses at 5 and 10 Hz. In response to the 5-Hz stimulus, the EJPs show significant facilitation (the EJP amplitude at the end of the train is larger than the EJP amplitude at the beginning of the train) but relatively little temporal summation. In contrast, the train of stimuli at 10 Hz produced both facilitation and temporal summation (the individual EJPs ride on a significant baseline depolarization). To extract the amplitude of the EJPs themselves independently of the effects of temporal summation, the EJP amplitude was measured on a vertical line dropped from the peak of the EJP to the extrapolated exponential decay of the previous EJP. These amplitudes are plotted as the vertical lines below the EJP traces.

To study the EJPs in response to a train of spikes with a variety of interspike intervals, we stimulated the neuromuscular junction with a Poisson spike train. The approach presented in METHODS provided an excellent fit of the EJP amplitudes in response to the Poisson train (Fig. 8). The parameter A_0 provides a measure of the baseline strength of the synapse, and f and τ_F characterized the amplitude and time course of facilitation, respectively. The curve bounding the shaded region in Fig. 8 shows the facilitation evoked by a single presynaptic action potential ($F = 1 + f = 1.05$) and the time course of recovery from this facilitation (exponential with $\tau_F = 3.36$ s). A single measure of the total degree

of facilitation, reflecting both the amplitude and time course, is the area under the facilitation curve, the shaded region in Fig. 8. This area provides us with a facilitation index equal to $f\tau_F$. Note that this is a different definition of the facilitation index than is often used. This definition is preferable because it is independent of a specific stimulus paradigm.

Figure 9 shows the use of this approach to quantify the effect of serotonin on the strength of the initial EJP and on the facilitation for both gm4 and gm6. In both control and modulated conditions, gm4 showed a large unitary EJP amplitude and a small facilitation index. In contrast, gm6 showed a small unitary EJP amplitude with much larger facilitation index. In both muscles, serotonin increased the unitary EJP amplitude and decreased the facilitation index. In pooled data from gm4, serotonin ($n = 6$) increased synaptic strength 30% ($P \leq 0.005$) and decreased the facilitation index 43% ($P \leq 0.003$). In gm6, serotonin ($n = 6$) increased the synaptic strength 49% ($P \leq 0.03$) and decreased the facilitation index 17% ($P \leq 0.01$).

We also studied the effects of AST and proctolin on these two muscles. AST ($n = 11$) had no significant effect on either synaptic strength ($P = 0.2$) or the facilitation index ($P = 0.4$) in gm4. In contrast, AST ($n = 8$) significantly decreased the synaptic strength 45% ($P \leq 0.02$) and increased the facilitation index 64% ($P \leq 0.004$) of EJPs in the gm6 muscle. In gm4, proctolin ($n = 9$) increased the synaptic strength 35% ($P \leq 0.01$) and but did not have a significant effect on the facilitation index ($P = 0.09$). In gm6, proctolin ($n = 3$) had no significant effect on either synaptic strength ($P = 0.4$) or the facilitation index ($P = 0.3$).

As noted before, gm4 had a large unitary EJP amplitude and a small facilitation index, whereas gm6 had a small unitary EJP amplitude and a larger facilitation index. Large changes in the unitary EJP amplitude produced by modulators were usually accompanied by a change in the facilitation index, with increases in synaptic strength accompanied by a decrease in the facilitation index and the converse. These observations suggest an inverse relationship between the facilitation index and the unitary EJP amplitude. Figure 10 is a plot of the facilitation index versus synaptic strength, for both the gm4 and gm6 muscles, in the presence and absence

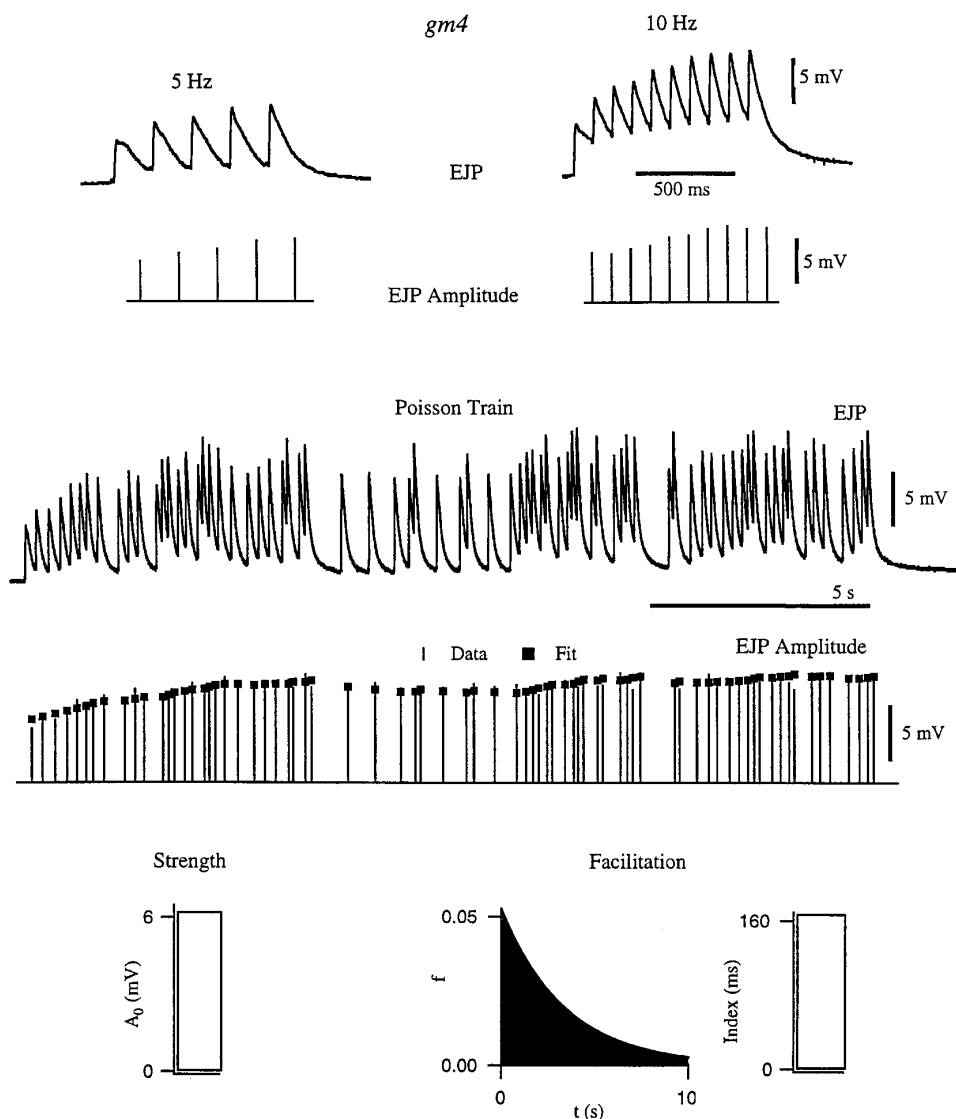


FIG. 8. Measuring and modeling facilitation. *Top panel:* nerve-evoked EJPs in a gm4 muscle at 5 Hz (baseline membrane potential, $V_m = -60$ mV) and 10 Hz ($V_m = -60$ mV) for 1 s. Amplitudes of the EJPs extracted from these traces are shown below them. *Middle panel:* nerve-evoked EJPs in response to a 20 s long 4-Hz Poisson train ($V_m = -60$ mV). Below that are the extracted EJP amplitudes (sticks) and the fit of these amplitudes (squares). *Bottom panel:* strength parameter A_0 and the facilitation function $\text{fexp}(-t/\tau_F)$. The area under the facilitation function (shaded region) in milliseconds is shown on the right and is used as an overall measure of the degree of facilitation. The parameters of the fit were $A_0 = 6.23$ mV, $f = 0.05$, and $\tau_F = 3.36$ s. The root-mean-square (RMS) error in the fit was 5.6%.

of the modulators. This plot shows the inverse relationship between the facilitation index at a neuromuscular junction and the strength of EJP. A striking feature of this relationship (Fig. 10) is that values corresponding to control groups as well as groups with modulators for both muscles appear to lie on the same curve.

DISCUSSION

When are multiple modulators redundant?

It is remarkable that at least eight different neuromodulatory substances increase the amplitude of nerve-evoked contractions in the gm4 muscle. At first glance, it is reasonable to assume that these substances are in some sense redundant, which may provide, as is often posited, a kind of biological safety net in case of loss of one or several of these modulators. However, when modulator effects are studied in the frequency domain, much of the apparent redundancy disappears. The frequencies we employed represent a subset of the complete range of activity patterns of the DG motor neuron. It is possible that, if the complete range of motor

neuron discharges were studied, each modulator would be found to be optimally effective for a specific pattern of pre-synaptic activity.

Movements of the crab stomach are controlled by >40 pairs of muscles, each of which shows different frequency-dependent properties (Govind et al. 1975; Katz et al. 1993). Our present knowledge of the modulation of stomach muscles is incomplete but suggests that each of these muscles may respond to a different subset (Jorge-Rivera 1997; Jorge-Rivera and Marder 1996, 1997; Lingle 1981; Meyrand and Marder 1991) of the >15 substances that are likely to be released into the hemolymph (Christie et al. 1995; Cooke and Goldstone 1970; Keller 1992). Therefore, even if two substances produced truly redundant actions on a single muscle, it is likely that by acting on different subsets of muscles they could produce widely different alterations of stomach movements.

Modulator interactions

In the experiments reported here, the modulators were studied separately. This was done to remove any potential

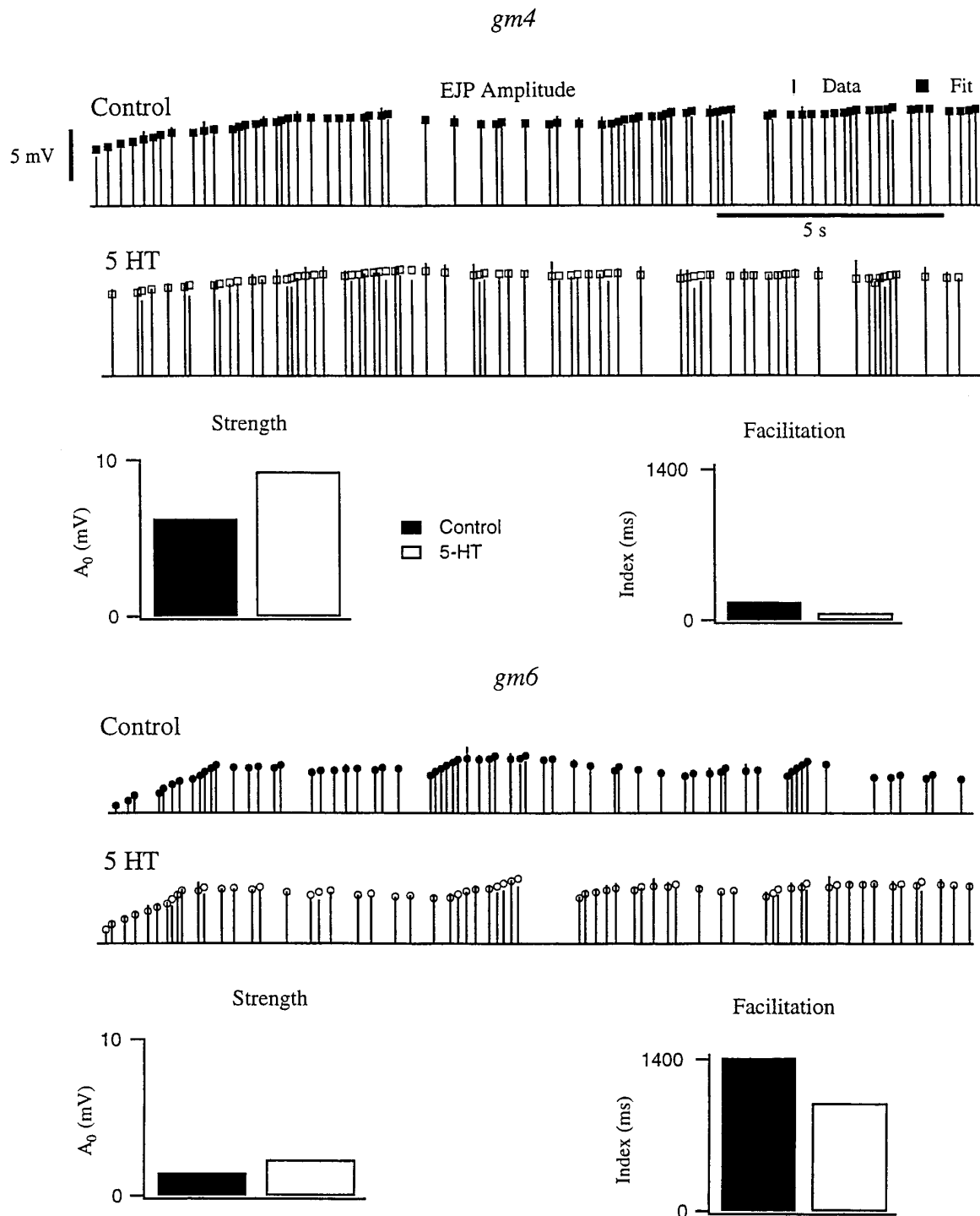


FIG. 9. Comparison of the effect of serotonin on gm4 and gm6. *Top traces*: EJP amplitudes in a gm4 muscle (sticks) in response to a 4-Hz Poisson train for 20 s under control saline ($V_m = -60$ mV) and serotonin ($V_m = -60$ mV) and the model fit (squares). The parameters of the fit for the control trace were $A_0 = 6.23$, $f = 0.05$, and $\tau_F = 3.36$ s. The RMS error in the fit was 5.6%. The parameters for the fit with serotonin were $A_0 = 9.29$, $f = 0.025$, and $\tau_F = 2.83$ s. The RMS error in the fit was 6.9%. *Bottom traces*: EJP amplitudes in a gm6 muscle (sticks) in response to a 4-Hz Poisson train for 20 s under control saline ($V_m = -69$ mV) and serotonin ($V_m = -67$ mV) and the fit (circles). The parameters of the fit for the control trace were $A_0 = 0.97$, $f = 0.66$, and $\tau_F = 2.16$ s. The RMS error in the fit was 6.7%. The parameters for the fit with serotonin were $A_0 = 1.58$, $f = 0.42$, and $\tau_F = 2.40$ s. The RMS error in the fit was 8.7%.

complications produced by long-lasting modulatory actions that could then alter the effects of modulators applied subsequently or that could make the exact order of modulator

application critical. These experiments used high concentrations of modulators to produce almost saturating responses. However, previous work on many of these same substances

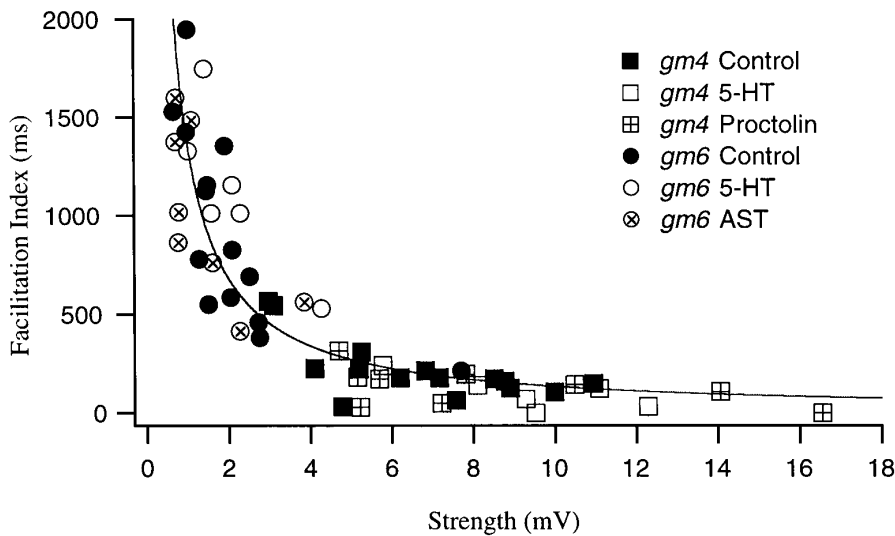


FIG. 10. Plot of facilitation index as a function of the amplitude of the initial EJP, the synaptic strength factor A_0 . Control gm4, filled squares; control gm6, filled circles; serotonin gm4, open squares; proctolin gm4, crossed squares; serotonin gm6, open circles; AST gm6, crossed circles. Only the modulators that had a statistically significant effect on the 2 muscles are shown. The line is the best fit of an inverse function of synaptic strength.

has shown that their threshold concentrations for physiological actions on neuromuscular junctions are in the 10^{-10} to 10^{-9} M range (Jorge-Rivera 1997; Jorge-Rivera and Marder 1996, 1997; Weimann et al. 1997). As each modulator has a unique dose dependence of its action, and some may converge on either receptors or second messenger systems (Brezina et al. 1996), it is difficult to assess how mixtures of low concentrations of modulators might act. Low concentrations of one peptide may alter the response to a second peptide (Dickinson et al. 1997). Therefore, in the animal, long-lasting responses to one modulator may shape the response of these neuromuscular junctions to other modulatory substances. It is likely that several of these substances are simultaneously in the hemolymph. In the blue crab *Callinectes sapidus* unique combinations of proctolin, dopamine, and octopamine shape three different rhythmic motor behaviors produced by the same appendages (Wood 1995).

Coordinating modulator actions on motor patterns and movements

The motor patterns that are produced by the STG undergo dramatic changes in the presence of modulators (Bal et al. 1994; Blitz et al. 1995; Christie et al. 1997; Elson and Selverston 1992; Flamm and Harris-Warrick 1986a; Hooper and Marder 1987; Marder and Calabrese 1996; Marder and Hooper 1985; Marder et al. 1986; Nusbaum and Marder 1988, 1989a,b; Skiebe and Schneider 1994; Weimann and Marder 1994; Weimann et al. 1993, 1997). All of the modulatory substances studied in this paper, except for CCAP, are also found in modulatory projection neurons that send processes into the crab STG (Marder et al. 1986; 1987; Nusbaum and Marder 1988, 1989a,b; Skiebe and Schneider 1994; Weimann et al. 1993), and all of these substances, including CCAP (Weimann et al. 1997), alter the motor patterns produced by the STG.

Some modulators act directly on both the motor neuron within the pattern-generating networks of the STG and its neuromuscular junction. For example, SDRNFLRFamide activates plateau potentials in the DG neuron (Weimann et al. 1993) and acts on the gm4 muscle innervated by the DG

neuron. CCAP acts directly on the LP neuron and on its neuromuscular junctions (Weimann et al. 1997). When a neuromodulator changes the discharge pattern of a motor neuron, this produces an immediate change in movement because movement depends on the number and duration of the impulse bursts in the motor neuron (Jorge-Rivera and Marder 1996; Morris and Hooper 1997; Weimann et al. 1997). The effects of neuromuscular modulation must then be superimposed onto the changes in contraction that result directly from the alterations in motor pattern.

The frequency dependence of modulators on muscle contraction is not simply a consequence of the effects of modulators on EJP amplitudes

Numerous studies documented modulation of motor function at multiple sites, including the presynaptic terminals, postsynaptic membranes, and contractile apparatus (Brezina et al. 1994a,b, 1996; Calabrese 1989; Evans and Meyers 1986; Worden et al. 1995). Our data argue that direct effects of the modulators on excitation–contraction coupling (or some postsynaptic action downstream from EJP amplitude) are responsible for the strong frequency dependence of the modulators on contraction amplitude because the effects on muscle contraction are significantly larger than would be expected from the changes in EJP amplitude produced by modulators. The apparent discrepancy between the frequency dependence of the actions of serotonin on EJPs and evoked contractions further supports the assertion that serotonin acts directly on the process of excitation–contraction coupling.

Measuring facilitation

We used a descriptive modeling method (Sen et al. 1996) to predict the amplitude of any EJP evoked by an arbitrary pattern of presynaptic activation. To make the comparisons of facilitation, we computed a facilitation index that effectively collapses into one number the amplitude and time course of the measured facilitation. This allowed us to see the relationship between initial EJP amplitude and facilitation (Fig. 10).

Relationship between facilitation and response amplitude

Crustacean neuromuscular junctions have long been used to study synaptic integration (Atwood and Wojtowicz 1986; Dudel and Kuffler 1961). The EJPs recorded here are the result of neurotransmitter released over a number of spatially separated sites from branches of a single motor neuron over the surface of the large muscle fiber. In this organization, the multiterminal innervation replaces active membrane properties of vertebrate muscles to ensure that the entire muscle fiber is depolarized and therefore will contract. Focal recordings from some stomatogastric muscles close to individual terminals show considerable quantal fluctuation and failures at low stimulus rates (Lingle et al. 1981; Marder 1976). At higher stimulus frequencies, the number of failures decreases as the amplitude of the focally recorded potentials increases, arguing that, as is the case for many crustacean synapses, facilitation is associated with increases in the probability of neurotransmitter release (Tank et al. 1995; Worden et al. 1997). Our data are consistent with earlier reports (Atwood and Marin 1983; Cooper et al. 1995; Govind et al. 1975; Msghina et al. 1998) that crustacean neuromuscular junctions with small initial amplitude EJPs show considerable facilitation and those with larger initial amplitude EJPs show less facilitation or even depression.

One surprising result is that the relationship between amplitude of the synaptic potential and facilitation holds for two different muscles, innervated by different motor neurons, and in the presence and absence of several different modulators. It may be coincidental that the data for the two muscles seem to fall on the same curve because elegant studies combining serial electron microscope reconstructions of synaptic sites and macropatch quantal analysis (Cooper et al. 1995) found that different presynaptic terminals of the same motor neuron have widely different initial quantal amplitudes and that this appears to be due in part to differences in Ca^{2+} dynamics in the two sets of varicosities. Therefore it would be interesting to compare the data presented here with data on other stomach muscles to see if the relationship in Fig. 10 is preserved.

There is ample evidence that modulators such as serotonin or peptides can act presynaptically at crustacean neuromuscular junctions (Dudel 1965; Glusman and Kravitz 1982; Worden et al. 1997). As a first approximation, we would expect that substances that acted primarily postjunctionally on the amplitude of the receptor-activated current would move points horizontally off the curve in Fig. 10 and substances that acted primarily to alter the probability of transmitter release would follow the inverse relationship of Fig. 10. This suggests that the modulators studied here act presynaptically. However, AST may also have postsynaptic actions (Jorge-Rivera and Marder 1997).

We interpret the data in Fig. 10 to mean that, as a first approximation, the modulators we studied do not appear to influence selectively the dynamics of facilitation; their modification of facilitation is a consequence of their effect on the initial synaptic potential amplitude through changes in release probability. Other examples in which modulators alter synaptic short-term plasticity (e.g., Abraham and Bear 1996; Huang et al. 1997; Kang and Schuman 1995; Varela

et al. 1997) may be consequences of an alteration in release probability, not changes in the kinetics of facilitation.

The relationship plotted in Fig. 10 has interesting functional consequences. When the amplitude of the unitary synaptic potential is approximately ≥ 5 mV, there will be relatively few frequency-dependent changes caused by facilitation. However, for synaptic potentials this large, at high frequencies of stimulation, the effect of summation will be considerable. Therefore, after the first few impulses, the membrane potential produced by a train of impulses will depend more on the motor neuron firing rate than on the duration of the train (Morris and Hooper 1997). In contrast, when the initial unitary synaptic potential is < 2 mV, the effects of facilitation and firing frequency will be dramatic, but the effects of summation will be smaller. In this case, the final membrane potential will depend critically on train duration as well as on its frequency.

This work was supported by National Institute of Neurological Disorders and Stroke Grant NS-17813, National Science Foundation Grant IBN-9421388, the Sloan Center for Theoretical Neurobiology, and the W. M. Keck Foundation. J. C. Jorge-Rivera was a recipient of a Ford Foundation Dissertation Fellowship.

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Received 24 March 1998; accepted in final form 10 August 1998.

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