Neuromodulation unmasks short-term synaptic facilitation in a depressing synapse

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Abstract

Network output depends crucially on the strength of synapses among the network neurons. Synaptic strength is a dynamic variable and is subject to modifications due to short- and long-term plasticity and the actions of neuromodulatory substances. We show that a neuromodulator can act to reverse the direction of short term synaptic dynamics by changing depression to facilitation. Moreover, depression or facilitation in the presence of the neuromodulator depends on the amplitude of the slow voltage waveform of the presynaptic neuron, which is an important component of synaptic release in bursting neurons. We examined the effects of the neuropeptide proctolin on the short term dynamics of the inhibitory synapse from the lateral pyloric (LP) to the pyloric dilator (PD) neuron in the crab pyloric network. This synapse is the only chemical feedback to the pacemaker group of neurons in this central pattern generating network. In response to periodic injection of high amplitude (≥40 mV) waveforms in the voltage-clamped LP neuron, the LP to PD synapse showed depression in both control and in the presence of proctolin. In contrast, low-amplitude (≤30 mV) waveforms resulted in depression in control and facilitation in proctolin. These results show that a synapse may change from depressing to facilitating depending only on the amplitude and not the frequency of the presynaptic signal. Such a neuromodulator-induced switch in short-term dynamics may allow the synapse to maintain a steady level of release despite variations in the presynaptic bursting amplitude, thus contributing to network stability.

Introduction

Short-term synaptic dynamics such as depression and facilitation are considered to be increasingly important in shaping the output of networks in the central nervous system. Synaptic dynamics have been implicated in synchronization (Tsodyks et al., 2000) and bistability (Manor and Nadim, 2001) in recurrent networks, sound localization (Cook et al., 2003), motor pattern selection (Combes et al., 1999) and development (Fedirchuk et al., 1999). In the context of network oscillations, short-term dynamics enables the synapse to adjust its output levels as a function of the rhythm frequency. For instance, the efficacy of a depressing synapse decreases if the presynaptic activity increases in frequency, allowing the synapse to act as a low-pass filter (Rose and Fortune, 1999). Thus, short term dynamics enable synapses to produce an increasing degree of network plasticity.

It is well-known that neuromodulation of intrinsic neuronal properties plays an important role in network flexibility (Marder and Thirumalai, 2002). Several studies have shown that synaptic dynamics are also subject to neuromodulation (Dudel, 1965; Byrne and Kandel, 1996; Kreitzer and Regehr, 2000; Mitchell and Silver, 2000; Sakurai and Katz, 2003; Baimoukhametova et al., 2004). However, the consequences of the neuromodulation of synaptic dynamics for network function remain unexplored. In this study, we demonstrate the ability of exogenous neuromodulators to unmask novel synaptic dynamics in an oscillatory network. The rhythmically-active pyloric network is located in the crustacean stomatogastric ganglion (STG) and is among the most extensively researched neural systems for studying the effects of neuromodulation (see

Nusbaum and Beenhakker, (2002) and Skiebe, (2001) for reviews). The pyloric rhythm is generated by a pacemaker group consisting of the anterior burster (AB) and pyloric dilator (PD) neurons. The co-active AB and PD neurons inhibit all other (follower) pyloric neurons and receive chemical feedback from the follower neurons only through a synapse from the lateral pyloric (LP) neuron to the PD neurons. Thus, the LP to PD synapse plays an important role in the regulation of pyloric activity(Manor et al., 1997; Weaver and Hooper, 2003; Mamiya and Nadim, 2004).

Neuromodulators have been shown to affect intrinsic properties of pyloric neurons (Golowasch and Marder, 1992; Harris-Warrick et al., 1998; Swensen and Marder, 2000) and the strengths ofpyloric synapses (Johnson and Harris-Warrick, 1997; Ayali et al., 1998), thus producing a variety of network outputs (Swensen and Marder, 2001; Marder and Thirumalai, 2002). Yet, the effect of neuromodulation on short term synaptic dynamics is mostly unexplored (but see Johnson et al (2005). We investigate the effect of the neurally released modulatory peptide proctolin (Nusbaum, 2002) on the short-term dynamics of the LP to PD synapse. It was previously shown that the unmodulated synapse exhibits short-term depression in response to both low- and high-amplitude repetitive presynaptic stimuli (Manor et al., 1997; Mamiya and Nadim, 2004, 2005). Here we show that proctolin enhances the strength of the LP to PD synapse and unmasks a surprising heterogeneity in its dynamics depending on the magnitude of the presynaptic input. Specifically, in the presence of bath-applied proctolin, the synapse remains depressing in response to high-amplitude presynaptic stimuli, yet, in response to low-amplitude (<40 mV) stimuli, the synapse demonstrates short-term facilitation. This, to

our knowledge, is the first demonstration of a shift in synaptic dynamics that is due to either the presynaptic signal amplitude or neuromodulation. Such a shift in the direction of short-term synaptic dynamics may have important functional implications for this and other oscillatory networks.

Materials and Methods

Preparation and identification of the neurons

Experiments were conducted on the stomatogastric nervous system (STNS) of the crab *Cancer borealis*. Animals were obtained from local markets and maintained in filtered, re-circulating seawater tanks at 10-12 °C. The STNS was dissected out using standard procedures (Selverston et al., 1976; Bartos and Nusbaum, 1997). The complete isolated STNS (including the stomatogastric ganglion, STG; the esophageal ganglion, OG; and the paired commissural ganglia, CoG) was pinned down on a Sylgard-coated petri dish. The STG was desheathed to facilitate penetration of the pyloric neuron cell bodies. All preparations were continuously superfused with chilled (10-13°C) physiological *Cancer* saline containing (in mM) KCl; 11, NaCl; 440, $CaCl_2$; 13, $MgCl_2$; 26, Trizma base; 11.2, Maleic Acid; 5.1, pH=7.4-7.5. Proctolin (Sigma-Aldrich, St. Louis, MO) was dissolved as stock solution in distilled water to a final concentration of 10^{-3} M, divided into aliquots and frozen at –20 °C. The final concentration of 10^{-6} M was made by dissolving the stock solution in *Cancer* saline immediately before use. Proctolin was bath applied by means of a switching port in a continuously flowing superfusion system.

Extracellular recordings from identified nerves were made using stainless steel wire electrodes and amplified with a Differential AC amplifier(A- M systems 1700, Carlsborg, WA). Intracellular recordings were made from the neuronal cell bodies with sharp glass microelectrodes containing 0.6 M K_2SO_4 and 20 mM KCl (final electrode resistance 20-40 MΩ). Microelectrodes were pulled using a Flaming-Brown micropipette puller (Sutter Instruments, CA). All intracellular recordings were performed in single-

electrode current clamp or two-electrode voltage clamp mode (Axoclamp 2B amplifiers; Molecular Devices, Foster City, CA). Pyloric neurons were identified according to their stereotypical axonal projections in identified nerves using conventional techniques (Selverston et al., 1976; Harris-Warrick et al., 1992).

Neuromodulatory inputs to the STG

More than 20 different neuromodulators (including many peptides) have been identified in the STNS (Marder and Bucher, 2001). These neuromodulators are released from neurons whose cell bodies reside in anterior ganglia (OG, CoG) and nerves, and project to the STG via the stomatogastric nerve (*stn*) (Skiebe, 2001). Several of these neuromodulatory peptides were shown to elicit distinct versions of the pyloric rhythm (Marder and Thirumalai, 2002) and their actions of at the network level have been found to be dose- and frequency-dependent (Nusbaum, 2002). In this manuscript, we focused on the neuromodulatory effects of a single, well characterized neuropeptide, proctolin (Hooper and Marder, 1984; Siwicki and Bishop, 1986; Hooper and Marder, 1987; Golowasch and Marder, 1992; Blitz et al., 1999; Swensen and Marder, 2001). To block all endogenous neuromodulatory inputs to the STG, either the *stn* was cut for current clamp recordings or the preparation was superfused with *Cancer* saline containing 10^{-7} M tetrodotoxin (TTX; Biotium, CA) for voltage clamp recordings. Bath application of TTX also allowed for better control of the membrane potential of the neurons in voltage clamp recordings by blocking spontaneous rhythmic activity.

Effect of proctolin on the strength and dynamics of the LP to PD graded synapse

In an ongoing rhythm, the pyloric neurons show smooth envelopes of slow-wave depolarizations that give rise to bursts of action potentials (e.g., see Fig. 1). The slow wave oscillations are responsible for graded synaptic transmission, the major form of synaptic communication among pyloric neurons (Raper, 1979; Graubard et al., 1983; Hartline et al., 1988), where neurotransmitter release is dependent on the level of presynaptic depolarization. Graded transmission was recorded after blocking spike-mediated transmission by superfusing the preparation with *Cancer* saline containing 10^{-7} M TTX.

The LP neuron was voltage clamped with two electrodes to a holding membrane potential (V_{hold}) of –60 mV. The postsynaptic PD neurons were impaled with one electrode each and the elicited graded inhibitory postsynaptic potentials (gIPSPs) were recorded in current clamp. The two PD neurons are anatomically identical and functionally similar; they exhibit similar intrinsic properties and make and receive similar synaptic connections (Miller and Selverston, 1982; Eisen and Marder, 1984; Hooper, 1997a; Rabbah et al., 2005; Rabbah and Nadim, 2005; Soto-Trevino et al., 2005). For clarity, the figures in this manuscript only show results from one PD neuron. All voltage-clamp manipulations were first done in *Cancer* saline containing 10^{-7} M TTX (henceforth referred to as control) followed by saline containing TTX and 10^{-6} M proctolin (henceforth referred to as proctolin).

To study the effects of the neuromodulator proctolin on the strength of the LP to PD synapse, the LP neuron was stimulated with single 2-second square pulses of increasing amplitudes from $V_{LP} = -60$ to 0 mV in both control and proctolin.
To reveal the effect of proctolin on the dynamical properties (extent and recovery from depression) of the LP to PD synapse, westimulated the presynaptic LP neuron with a train of 5 square pulses with a fixed duration of 500 msec each. We repeated this stimulation with different interpulse intervals (IPIs) from 250 to 4000 msec and different presynaptic amplitudes from 20 to 60 mV.

To measure synaptic activity under conditions more similar to the ongoing rhythm, we stimulated the voltage clamped LP neuron with sinusoidal or pre-recorded realisitc waveforms with various amplitudes (ranging from 20-60 mV and frequencies (0.25 to 10 Hz; cycle period 100 to 4000 ms) in control and proctolin. To create unitary realistic LP waveforms, voltage traces of the LP neuron were recorded during the ongoing rhythm and divided into individual cycles that were then averaged. The resulting unitary realistic LP waveform was either played back into the voltage-clamped LP neuron as is (i.e. with oscillation and action potentials; referred to as non-filtered) or after low-pass filtration at 10 Hz. Filtration removes the action potentials riding on the membrane potential oscillations of the LP neuron, allowing for the isolation of the graded (voltage-dependent) component of synaptic transmission (Manor et al., 1997). The unfiltered waveforms were always applied so that the underlying slow oscillation amplitudes matched those of the filtered waveforms (20, 40, 60 mV).

Recording, Analysis and Statistics

Data were acquired using the Scope software (available at http://stg.rutgers.edu/software/index.htm developed in the Nadim laboratory), sampled at 4 kHz and saved on a PC using a PCI-6070-E data acquisition board (National Instruments, Austin, TX). Statistical and graphical analyses were done using Sigmastat 3.0 (SPSS, Chicago, IL) and Origin 6.1 (OriginLab, Natick, MA). Reported statistical significance indicated that the achieved significance level p was below the critical significance level $\alpha=0.05$. All error bars shown and error values reported denote SEM.

Results

During the ongoing pyloric rhythm, the LP and PD neurons fire in alternation (Fig. 1; left panel). Removal of descending modulatory inputs to the STG disrupts the pyloric rhythm as the alternating oscillation of the LP and PD neuron becomes slow and irregular (Fig. 1; middle panel) (see also (Nusbaum and Beenhakker, 2002). As shown in previous studies (Marder et al., 1986; Nusbaum and Marder, 1989), bath application of proctolin enhances the pyloric rhythm by increasing the amplitude of the slow wave oscillation of the LP and PD neurons, increasing the spike frequency and number of spikes per burst and decreasing the cycle-to-cycle variability (Fig. 1; right panel). It is known that proctolin enhances the bursting activity of the LP and pacemaker neurons by eliciting a voltage-gated inward current (Golowasch and Marder, 1992; Swensen and Marder, 2000). As seen in the right panel of Fig. 1, it also appears that the reciprocal synaptic inhibition between the LP and PD neurons was strengthened in proctolin, compared to control.

The LP to PD synapse is the only chemical synaptic feedback to the pyloric pacemaker neurons. As such, this synapse is in a key position to affect the frequency and phase relationships of the pyloric network (Eisen and Marder, 1982; Weaver and Hooper, 2003; Mamiya and Nadim, 2004, 2005). It is possible that the enhancement of this synapse in the presence of proctolin is an indirect result of the increased bursting activity in the presynaptic LP neuron and not the synaptic machinery itself. We therefore examined whether the dynamics and strength of the LP to PD synapse are directly modulated by proctolin. If they were, we expected to find thatafter blocking spontaneous network activity, thatdepolarization of the presynaptic LP neuron would elicit larger and

possibly kinetically distinct IPSPs in the PD neuron in proctolin compared to control conditions.

We first examined whether synaptic strength changed in the presence of proctolin. This was done by voltage-clamping the LP neuron to a holding potential V_{hold} = –60 mV and injecting a series of two-second depolarizing square voltage pulses with increasing amplitudes (V_{LP}= –50 to 0 mV) in control and proctolin (Fig. 2). Figure 2A shows the response in the PD neuron as recorded in current clamp. The IPSP amplitude increased as the amplitude of the LP neuron depolarizations increased in both control and proctolin, as expected from a graded synapse. The amplitudes of the graded IPSPs (gIPSPs) were larger in proctolin, indicating the strengthening of the LP to PD synapse. The synaptic input-output curve constructed using the peak amplitudes of the gIPSPs showed a sigmoidal dependency on the presynaptic voltage for both control and proctolin (Fig. 2B). However, proctolin significantly strengthened the LP to PD synapse for presynaptic amplitudes ≥ 35 mV(two -way ANOVA post-hoc Tukey analysis, $p < 0.004$ for V_{LP} = –35 to 0 mV; N=6).

The LP to PD synapse has been previously shown to exhibit short-term depression (Manor et al., 1997). To examine whether proctolin modifies the dynamics of this synapse, we voltage-clamped the LP neuron to a holding potential V_{hold} = –60 mV and injected a train of five pulses with varying interpulse intervals (IPI= 250, 500, 1000, 2000, 4000 ms) and amplitudes (ΔV_{LP}= 20, 30, 40, 60 mV) in control and proctolin. Presynaptic stimuli with amplitudes less than 20 mV elicited a postsynaptic response that

was less than 4 mV in either condition (see Fig. 2). In all recordings, the amplitude of the gIPSP did not change subsequent to the 3rd or 4th pulse and therefore we refer to the gIPSP in response to the 5th pulse as the steady-state gIPSP. Figure 3A shows an example of this protocol with IPI 250 ms and amplitudes of 20 and 40 mV. In response to 40 mV pulses, the steady-state gIPSP was always smaller than the 1st pulse gIPSP, indicating synaptic depression, in both control (right panel) and proctolin (left panel). In contrast, the gIPSPs elicited with 20 mVpresynaptic pulses were qualitatively different: in this case, the gIPSPs in the PD neuron showed depression in control, but facilitation in proctolin: the response to the 1st pulse was relatively small but became much larger by the 3rd pulse. Figure 3B shows the ratio of the 5th to the 1st peak gIPSP amplitudes (A_5/A_1) calculated for all presynaptic depolarizations at IPI = 250 ms. In control conditions, the ratio A_5/A_1 was less than one for all values of presynaptic depolarization (ΔV_{LP}), indicating that the synapse was always depressing. In contrast, in the presence of proctolin, the A_5/A_1 ratios in response to ΔV_{LP} of 20 and 30 mV were greater than 1 while the ratios in response to ΔV_{LP} of 40 and 60 mV were less than 1, marking facilitation and depression, respectively. These experiments showed that proctolin acts at the level of the synapse, causing this depressing synapse to become facilitating in response to low-amplitude presynaptic depolarizations, while maintaining depression with high-amplitude depolarizations.

To examine the extent and recovery from synaptic plasticity in control and proctolin, we plotted A_5/A_1 as a function of interpulse interval for presynaptic amplitudes of 20 and 40 mV (Fig. 3C). In proctolin, the ratios calculated for 20 mV waveforms (red)

for all interpulse intervals were greater than 1, indicating synaptic facilitation. This was not seen in control (black). On the other hand, in response to 40 mV waveforms and either control (grey) or proctolin (magenta), the synapse showed depression. The extent of depression for these two cases was not significantly different (two-way ANOVA, $p > 0.05$). Both facilitation and depression were most prominent at the shortest period (IPI=250 ms) whereas there was almost full recovery at IPI=4000 ms (Fig. 3C). With 30 mV waveforms, the short-term dynamics observed were qualitatively similar to 20 mV waveforms, i.e. depressing in control and facilitating in proctolin (two way ANOVA, $p > 0.05$; data not shown). With 60 mV waveforms, the short term dynamics were statistically similar to 40 mV waveforms (two-way ANOVA, $p > 0.05$; data not shown). This trend was similar independent of the presynaptic waveform type used (square pulses, sine waves, realistic waveforms) and henceforth only data from 20 and 40 mV presynaptic waveforms are shown.

The switch from short-term facilitation to short-term depression seen in proctolin occurred, on average, between ΔV_{LP} = 30 and 40 mV presynaptic stimulations. We attempted to isolate the exact voltage of the presynaptic LP neuron at which the switch occurs. There was variability across preparations in this set of experiments. In some cases, the synapse abruptly changed from facilitation to depression within ~1 mV (facilitation at ΔV_{LP} =38 mV and depression at ΔV_{LP} =39 mV) while in other cases the change was gradual from facilitation, to no short term dynamics, to depression within a few millivolts (N=4; data not shown).

In vivo and *in vitro* studies have shown that the pyloric rhythm can operate across a wide frequency range from ~0.1 to ~2.5 Hz, while maintaining the phase relationships between the constituent neurons (Turrigiano and Heinzel, 1992; Hooper, 1997a, b). In order to study the frequency dependence of the strength and time course of the LP to PD synapse in proctolin, we stimulated the LP neuron with sinusoidal waveforms of various amplitudes and periods. We voltage clamped the LP neuron, then applied sinusoidal waveforms of amplitudes 20-60 mV, from a baseline of –60 mV, with frequency ranging from 0.25 to 10 Hz (cycle period 100 to 4000 ms) and measured the postsynaptic response from the PD neuron. Figure 4A shows an example of the gIPSPs recorded from the PD neuron when 20 and 40 mV presynaptic sinusoidal waveforms of frequency 4 Hz (period 250 ms) were injected in control (middle panel) and in proctolin (bottom panel). As with trains of voltage pulses, in control conditions, the gIPSPs showed depression for both 20 and 40 mV amplitudes of the sinusoidal waveform. In proctolin, the gIPSPs in response to 40 mV waveforms showed depression, while the 20 mV waveform response showed facilitation.

Figure 4B plots A_5/A_1 as a function of the period of the waveform injected in the LP neuron, for both ΔV_{LP}=20 and 40 mV. Both forms of plasticity tended towards recovery as the stimulation period increased (A_5/A_1 approached 1). Interestingly, as the period increased, in ΔV_{LP}=20 mV control conditions, the ratio increased to above 1 (black symbols), indicating facilitation. This suggests that the potential ability of the synapse to facilitate with relatively small presynaptic depolarizations may also be present in control conditions but masked by short-term depression at fast cycle periods. Thus, the

proctolin may function to enhance this underlying facilitation mechanism at all cycle periods.

Figure 4C shows superimposed steady-state gIPSPs recorded in the PD neuron when sinusoidal waveforms of a short (250 ms) and long (2000 ms) period of 40 mV were used to activate the LP to PD synapse in control conditions. These waveforms were normalized to cycle period. The longer period sinusoidal waveform elicited a larger amplitude gIPSP, consistent with previously published reports on the LP to PD synapse in *Panulirus interruptus* (Mamiya and Nadim, 2004). The amplitudes of the steady-state PD gIPSPs elicited with ΔV_{LP}=20 and 40 mV depolarizations as a function of the period of the sinusoidal waveform injected in the LP neuron in control and proctolin were quantified and plotted in Figure 4D. This figure shows three results. First, for each period used, the steady-state gIPSP amplitudes increased as ΔV_{LP} was increased (from 20 to 40 mV), in control and in proctolin, reflecting the graded nature of the synapse (two-way ANOVA, $p < 0.034$ for Control20 vs. Control40; $p < 0.019$ for Proctolin20 vs. Proctolin40; N= 6). Second, for each ΔV_{LP}, the amplitude of the steady-state gIPSP in proctolin was always larger than those recorded in control (two-way ANOVA post-hoc Tukey analysis, 20 mV: $p < 0.045$ for Period= 1000 and 4000 ms; 40mV: $p < 0.028$ for Period= 500 to 4000 ms; N=6). This indicated that proctolin enhanced the efficacy of the LP to PD synapse at all amplitudes. Third, proctolin significantly increased the dependence of the steady-state IPSP amplitudes on the stimulation period in response to high (40 mV) ΔV_{LP}; i.e. the amplitudes increased as the stimulation period frequency was increased (one-way ANOVA for the effect of frequency, $p = 0.002$; N=6).

However, with ΔV_{LP}=20 mV, the period-dependent effect of proctolin on the steady-state IPSP amplitudes was non-monotonic. For periods ≤ 1000 ms, the IPSP amplitudes recorded in proctolin increased and approached the values of the IPSP amplitudes recorded in control ΔV_{LP}=40 mV (Fig. 4D, compare red and grey traces). At period = 1000 ms, the steady-state IPSP amplitude recorded in Proctolin20 and Control40 were statistically similar (two-way ANOVA post-hoc Tukey analysis, $p > 0.05$; N=6). For periods > 1000 ms, the IPSP amplitudesin Proctolin 20 decreased and became significantly smaller than those recorded in Control40 (two-way ANOVA post-hoc Tukey analysis, $p < 0.035$ for Period 2000 and 4000 ms; N=6).

We also measured the changes in the steady-state gIPSP peak phase (Φ) at different presynaptic periods, amplitudes and neuromodulatory conditions (control and proctolin). We defined Δt as the difference between the beginning of the LP waveform and the peak of the gIPSP in the PD neuron. Φ was then calculated as Δt/Period. When the LP neuron was voltage clamped with sinusoidal waveforms, Φ advanced as a function of cycle period (from Control20: 0.9 ± 0.05 and Proctolin20: 1.04 ± 0.67 at Period 100 msec to Control20: 0.47 ± 0.02 and Proctolin20: 0.56 ± 0.05 at Period 4000 msec; from Control40: 0.93 ± 0.04 and Proctolin40: 0.82 ± 0.03 at Period 100 msec to Control40: 0.48 ± 0.01 and Proctolin40: 0.5 ± 0.02 at Period 4000 msec), independent of presynaptic waveform amplitude or neuromodulatory conditions (two way ANOVA, $p > 0.05$; N=6; data not shown).

Stereotyped stimuli, such as square pulses and sinusoidal waveforms, are useful for characterizing synaptic parameters such as peak and steady-state amplitudes, extent of depression and time-to-peak. However, a full description of synaptic dynamics requires the characterization of synaptic output in the context of network activity. This can be done by using realistic waveforms recorded during ongoing network activity to stimulate the presynaptic neuron (Olsen and Calabrese, 1996; Manor et al., 1997; Mamiya et al., 2003; Johnson et al., 2005; Rabbah and Nadim, 2005). Thus, we used pre-recorded realistic LP waveforms to drive synaptic transmission from the LP neuron to the PD neuron, in control and proctolin conditions. Periodic applications of both non-filtered (Fig. 5) and filtered (not shown, but similar to non-filtered waveform results) realistic LP waveforms were used to voltage-clamp the LP neuron while recording the response in the PD neuron. The amplitudes of the non-filtered waveforms were amplified such that the envelope of the slow oscillations matched that of the filtered waveforms (see Methods); the amplitudes reported are those of the slow oscillations. Figure 5 showsrepresentative non-filtered LP realistic waveforms applied in 5 consecutive cycles with a cycle period of 1 second and two different amplitudes, 20 and 40 mV. Also shown are the gIPSPs recorded in the PD neuron in control and proctolin conditions. proctolin caused an increase in gIPSP amplitudes at both presynapticamplitudes tested, similar to what was reported for square pulses and sinusoidal waveforms. Also similarly, proctolin switched the direction of short-term plasticity from slightly depressing to strongly facilitating at low (20 mV), but not high (40 mV), presynaptic amplitudes. Interestingly, in proctolin, the amplitude of the steady-state gIPSPs in response to both low and high amplitude presynaptic depolarizations were similar in amplitude. This was in contrast to control

conditions where there was a large difference in the amplitude of the steady-state gIPSPs (Fig. 5; arrows). The inset shows an overlay of the steady-state IPSP recorded in control conditions and high presynaptic waveform amplitudes (grey trace) and the IPSP recorded in proctolin and low presynaptic waveform amplitudes (red trace). At this cycle period, the IPSP amplitudes were qualitatively similar, consistent with Figure 4D.

We also used realistic waveforms to examine whether the effects of proctolin on the synapse depended on the waveform shape. In particular, we varied the cycle frequency at which the LP realistic waveform was injected into the LP neuron (from 4-10 Hz) and quantified all parameters of the gIPSPs as we had done for the square pulses and sinusoidal waveforms. The results obtained from the realistic waveforms were similar to those obtained with the sinusoidal waveforms and thus are not shown. The findings strongly indicate that the switch in the direction of short-term plasticity elicited by proctolin is only dependent on the amplitude of the presynaptic LP waveform and independent of the presynaptic waveform shape (square pulse, sine wave or realistic waveforms; $N \geq 6$ for each waveform).

Discussion

Short and long term processes have been shown to regulate nearly all synapses, some leading to an enhancement (facilitation) and others to a decrease (depression) in synaptic strength. A variety of synapses show both short-term facilitation and depression (simultaneously, or at different times), albeit one effect may be masked by the other in a manner dependent on the timing of synaptic activity (Lev-Tov et al., 1983; Zador and Dobrunz, 1997; Dittman et al., 2000; Saviane et al., 2002; Zucker and Regehr, 2002; Sippy et al., 2003). For example, hippocampal CA3 to CA1 Schaffer Collateral synapses facilitate at low frequencies and depress at high frequencies (> 50 Hz) (Dittman et al., 2000). This time dependence of synaptic dynamics, commonly referred to as frequency-dependence, has so far been shown to be true for spike-mediated synapses where neurotransmitter release is all or none. In contrast, dynamics of graded synapses can be affected by changes in the amplitude or shape of presynaptic depolarizations as well as by the cycle frequency. Such changes can be brought about by the actions of neuromodulators or sensory input. Many motor and sensory networks have been found to use graded synaptic transmission, including central pattern generators (Marder and Calabrese, 1996), the retina and other sensory networks (Laughlin et al., 1987; von Gersdorff et al., 1996; Burrone and Lagnado, 2000; Prescott and Zenisek, 2005) and, more recently, circuits of the cortex and hippocampus (Alle and Geiger, 2006; Shu et al., 2006). Thus, when investigating synaptic plasticity in neural networks that employ graded synapses, it is important to ask whether the amplitude of the presynaptic input, as well as its frequency, can cause a change in the dynamical properties of a synapse.

How important is it to characterize the effects of the neuropeptide proctolin on synaptic dynamics?

We focused on the neuromodulatory effects of a single, well characterized neuropeptide, proctolin, using the crab STNS as our model system (Golowasch and Marder, 1992; Nusbaum and Beenhakker, 2002). Our choice was motivated by several factors. First, although there are several reports of the actions of neuromodulators on intrinsic neuronal properties and synaptic strength in this system (see (Nusbaum and Beenhakker, 2002) for a review), there is little documentation of neuromodulatory effects on the short-term dynamics of synaptic transmission in the STNS (Johnson et al., 2005). Second, many synapses in the mammalian CNS are known to release peptides from large dense-core vesicles (Hokfelt et al., 2000), but most studies of neuromodulation in mammalian CNS focus on acetylcholine and monoamines (Kaczmarek and Levitan, 1987; McCormick, 1989). Much less is known about the modulatory effects of neuropeptides on neurons and synapses of the mammalian CNS, or how such effects result in modulatory reconfigurations at the network level. Third, all proctolinergic modulatory projection neurons have been identified in the crab STNS and the actions of many of these neurons at the network have been characterized (Nusbaum and Beenhakker, 2002). Finally, the effects of several neuropeptides, including proctolin, on the intrinsic properties of STG neurons have been documented. In particular, it has been shown that, in the STG neurons of the crab *Cancer borealis*, the actions of neuropeptides are mainly due to the activation of a single voltage-gated inward current (Swensen and Marder, 2000). Characterizing the effects of the neuropeptide proctolin on synaptic dynamics would enable us to combine

the results on proctolin modulation of cellular and synaptic components and explain its actions at the network level.

Functional consequences of facilitation and depression in the same synapse

We have shown that a single synapse can exhibit two forms of short-term plasticity dependent on the waveform amplitude of the synaptic input it receives. To our knowledge, this is the first report of a neuromodulator or amplitude dependent shift in the direction of short-term synaptic dynamics. Such a drastic shift in dynamics can have significant consequences for the role of the synapse in network activity. We suggest that the presence of depression at high presynaptic amplitudes and facilitation at low presynaptic amplitudes acts to buffer synaptic strength from any extrinsic input that would change the amplitude of oscillations of the presynaptic neuron. For example, in the case of the LP neuron, activation of modulatory commissural neuron 5 (MCN5) located in the commissural ganglia, has been shown to reduce LP activity (Norris et al., 1996). Because the LP neuron works to stabilize cycle period (Weaver and Hooper, 2003; Mamiya and Nadim, 2004) through a negative feedback synapse to the PD neurons, it is reasonable to assume that dampening of its oscillations would weaken that synapse, thus minimizing its effect on cycle period (see for example Fig. 5, control trace). The presence of proctolin, however would allow the dampened LP oscillations to remain effective at changing the cycle period by facilitating the LP to PD synapse, hence restoring its efficacy.

Divergent actions of neuromodulatory peptides on synaptic dynamics

Pyloric network activity is subject to modulation from over 20 neuromodulatory inputs from anterior ganglia (Marder and Bucher, 2001). In this work, we reported the effect of one such neuromodulator, proctolin, on the short-term dynamics of a key synapse in the pyloric network. We also investigated whether another neuropeptide, red-pigment concentrating hormone (RPCH), can modulate this synapse in the same amplitude dependent manner. Although we found that RPCH can enhance the amplitude of the LP to PD synapse at all presynaptic amplitudes (Atamturktur et al., 2003), it does not produce an amplitude-dependent switch from depression to facilitation (N=8; data not shown). The distinct effects of RPCH and proctolin on the dynamics of this synapse are particularly interesting in light of the fact that these neuropeptides activate the same intrinsic ionic current in the LP neuron (Swensen and Marder, 2001). This suggests that different neuromodulators may have convergent actions on the intrinsic properties of a neuron yet have differential effects on the dynamics of its synapses.

Possible mechanisms underlying facilitation of the graded LP to PD synapse in the presence of proctolin

Elucidating the mechanisms that control short-term plasticitywill provide insight on how neurons process incoming information. Typically, depletion of releasable vesicles from the presynaptic terminals (Rosenmund and Stevens, 1996; Wang and Kaczmarek, 1998; Oleskevich et al., 2000) and postsynaptic receptor desensitization (Otis et al., 1996; Neher and Sakaba, 2001) are thought to account for short term depression. On the other hand, facilitation is commonly believed to result from the residual calcium hypothesis (Zucker, 1996; Neher, 1998) and local saturation of calcium buffers (Rozov et al., 2001;

Blatow et al., 2003). However, it is unclear how a neuromodulator can determine the direction (facilitation versus depression) of plasticity at a given synapse in a manner that is completely dependent on the strength of synaptic input. Does the neuromodulator target any of the above mentioned preexisting pre- and postsynaptic mechanisms or does it regulate an altogether separate process? Recent results from our laboratory indicate that a switch to facilitation is correlated with the slow activation of a presynaptic Mn^{2+}-sensitive inward current, suggesting that it is a slowly-accumulating Ca^{2+} current activated by proctolin (Zhou et al., 2006). Using a mechanistic model of synaptic release, Zhou et al (2006) show that the actions of proctolin on the LP to PD synapse can be explained by the activation of a low-threshold calcium currentthat has slow activation and inactivation kinetics.

Conclusion

Neuromodulation can reconfigure a network to produce a multitude of outputs thought to be important for both functions and disorders of the nervous system (Chin, 1989; Hasselmo, 1995; Katz and Frost, 1995). Characterizing the neuromodulatory effects on synaptic dynamics will provide a more comprehensive description of the actions of neuromodulators on cellular and synaptic components. This understanding can be used to elucidate the underlying mechanisms for the actions of neuromodulators at the network level.

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Figure Legends

Figure 1: *Proctolin enhances the pyloric rhythm*. Intracellular traces recorded in the normal ongoing pyloric rhythm with intact descending neuromodulatory inputs show the rhythmic alternation of the LP and PD neurons (left panel). Cutting the stomatogastric nerve *stn* removes all descending neuromodulatory inputs to the STG and disrupts the pyloric rhythm (middle panel). Bath application of 10^{-6} M proctolin recovers the pyloric rhythm, increases the amplitude of the slow wave oscillations in the LP and PD neurons and increases the spike frequency and number of spikes/burst (right panel). The baselines for the membrane oscillations of LP (−58 mV) and PD (−62 mV) recorded in control conditions are marked by arrows. Recordings in the three coniditions are shown at the same scale.

Figure 2: *Proctolin strengthens the LP to PD synapse.* **A.** The LP neuron was voltage-clamped to V_{hold} = −60 mV and two-second depolarizing square pulses with increasing amplitudes up to 0 mV (traces labeled V_{LP}) were used to activate the LP to PD synapse in control and proctolin. The resulting IPSP amplitudes recorded in the PD neuron (traces labeled V_{PD}) increase as V_{LP} increases for both control and proctolin but they were larger in proctolin. **B.** Synaptic input/output curve shows all ΔV_{PD} values (measured from membrane potential baseline to peak IPSP amplitude) plotted against V_{LP} values (mean ± SEM). Proctolin significantly increases IPSP amplitudes for all V_{LP} values above −45mV

Figure 3: *Proctolin causes a depressing synapse to become facilitating in response to low amplitude presynaptic depolarizations.* **A.** The LP neuron was stimulated with a series of 5 square pulses of 20 and 40 mV amplitudes from V_{hold} = −60 mV with IPI 250

ms in control (left panel) and proctolin (right panel). In control, the LP to PD synapse is always depressing, i.e. the amplitudes of the 2nd and subsequent IPSPs are smaller than the 1st. In proctolin, in response to 20 mV presynaptic depolarizations (red traces), the amplitudes of the 2nd and subsequent IPSPs are larger than the 1st, indicating facilitation. The resting membrane potential was −57 mV for PD. **B.** The ratio of the 5th to the 1st peak IPSP amplitudes (A_5/A_1) plotted vs. all the presynaptic depolarizations used (20, 30, 40, 60 mV) at IPI 250 ms in control (black) and proctolin (red) (mean ± SEM). In proctolin, when pulses of 20 and 30 mV amplitudes were used to activate the LP to PD synapse, the ratio was greater than 1, indicating facilitation. **C.** The extent and recovery from short-term plasticity, measured as the ratio of A_5/A_1 at each IPI (mean ± SEM) is similar for control40 (grey) and proctolin40 (magenta). With 20 mV amplitude presynaptic depolarizations, proctolin causes facilitation of the LP to PD synapse at all IPIs (red).

Figure 4: *Proctolin affects the frequency dependence of the strength of the IPSPs and of the short-term plasticity of the LP to PD synapse.* **A.** The LP neuron was voltage-clamped with a train of sinusoidal waveforms of amplitudes 20 and 40 mV and cycle period 250 ms (frequency 4 Hz) in control and proctolin. All of the resulting IPSPs recorded in the PD neuron exhibited depression except for the IPSPs recorded in proctolin during 20 mV presynaptic depolarizations (red traces). **B.** Ratio of A_5/A_1 plotted vs. input periods 100 to 4000 ms (frequency 0.25 to 4 Hz) for control and proctolin (mean ± SEM). Note that at long periods, control20 (black) showed weak facilitation (ratio greater than 1) while proctolin20 (red) showed strong facilitation at all periods tested. **C.** Two superimposed

traces recorded from the PD neuron in control in response to 250 ms (short period) and 2000 ms (long period) and 40 mV presynaptic depolarizations of the LP neuron normalized in phase (by dividing the time from the beginning of the LP sinusoidal waveform to the peak of the IPSP by cycle period). The IPSP in response to the short period stimulation is smaller in amplitude and peaks later in phase. **D.** The amplitudes of the 5^{th} pulse IPSPs (referred to as steady-state) elicited during low and high presynaptic stimulations were measured and plotted vs. cycle period for control and proctolin (mean ± SEM). The steady-state amplitudes show frequency-dependence. Specifically, as cycle period increases (low frequency), the amplitudes increase for Control20, Control40 and Proctolin40 while it increases then decreases for Proctolin20. Note that at cycle period 1000 ms (frequency 1 Hz), the amplitude for Proctolin20 approaches that of Control40.

Figure 5: *A consequence of proctolin-induced facilitation in the normally depressing LP to PD synapse.* Realistic LP waveforms recorded in the normal ongoing rhythm were played back into the voltage-clamped LP neuron at 1 Hz (cycle period 1000 ms) with 20 and 40 mV amplitude depolarizations. The response of the PD neuron (V_{PD}) was recorded in control and proctolin. In control, there is a large difference in the two IPSP amplitudes at steady-state (arrow). In proctolin, the IPSP is depressing in response to the high amplitude waveform but facilitating in response to low amplitude waveform, so that at steady-state the two IPSPs are similar in amplitude (arrow). Moreover, the steady-state IPSPs recorded in Control40 (inset, grey trace) and in Proctolin20 (inset, red trace) are also of similar amplitudes.

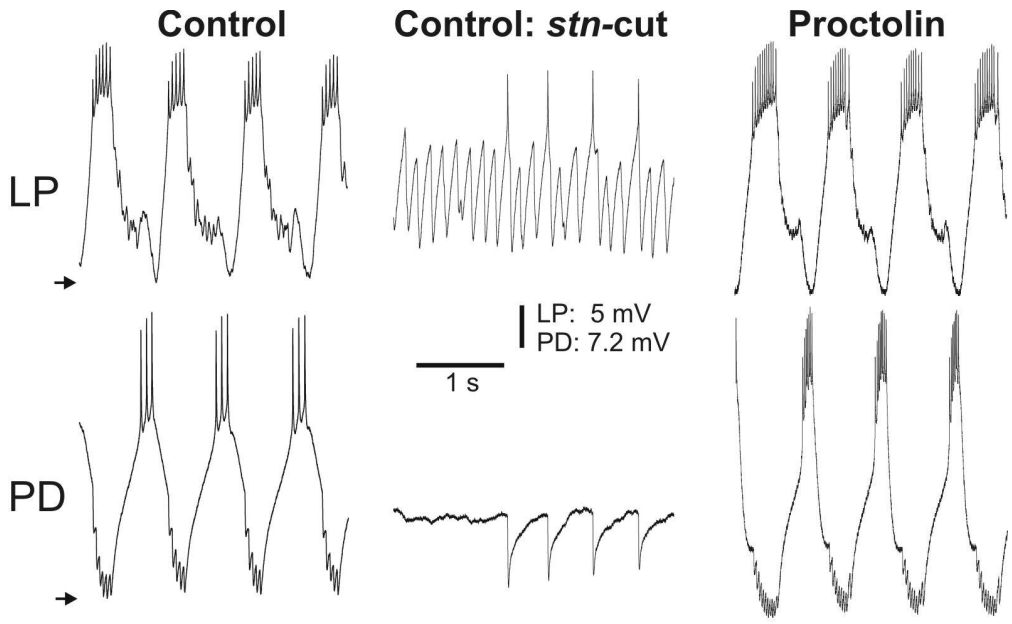


Figure 1

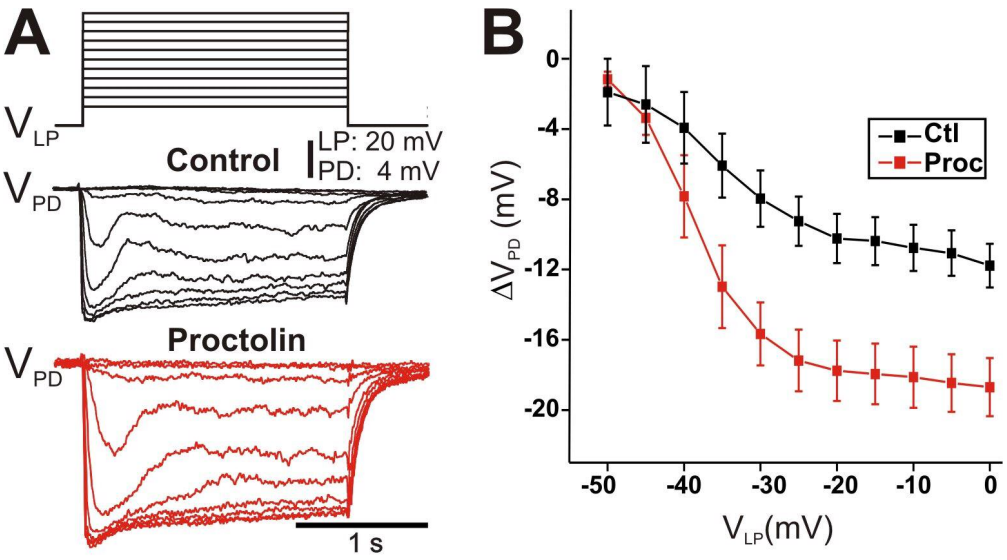
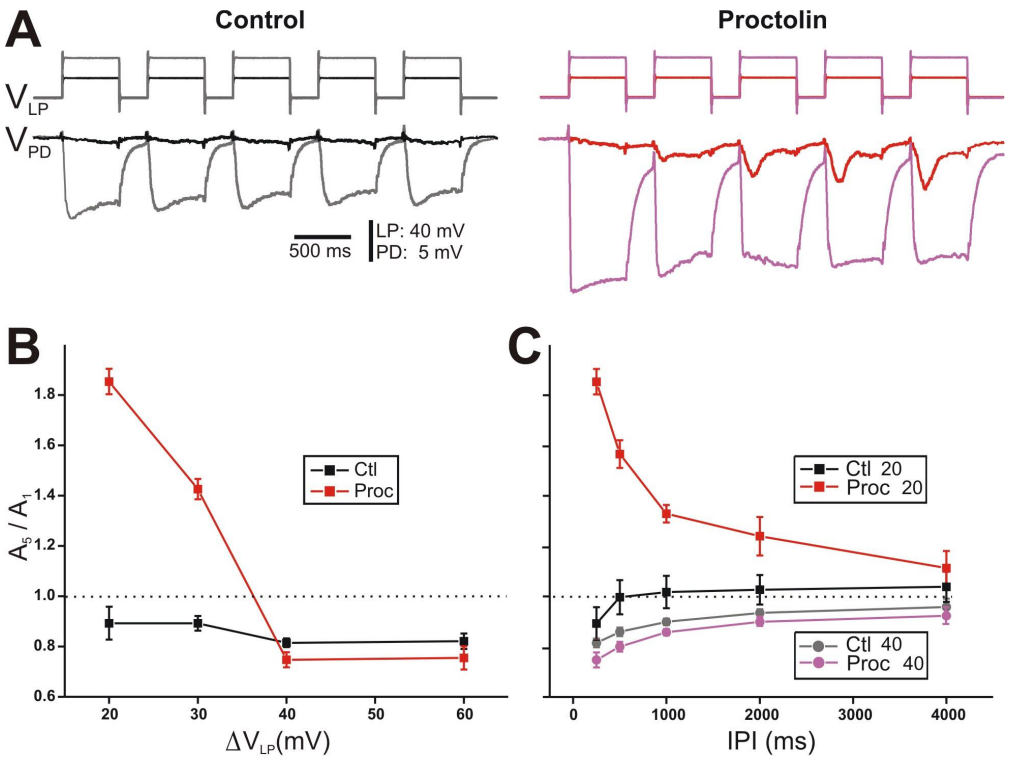

A
V LP
LP: 20 mV
PD: 4 mV
Control
V PD
Proctolin
V PD
1 s
B
0
-4
-8
-12
-16
-20
ΔV PD (mV)
-50
-40
-30
-20
-10
0
V LP(mV)
Ctl
Proc

Figure 2


A
Control
Proctolin
V LP
V PD
500 ms
LP: 40 mV
PD: 5 mV
B
C
Ctl
Proc
Ctl 20
Proc 20
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Proc 40
A5 / A1
ΔV LP(mV)
IPI (ms)

Figure 3

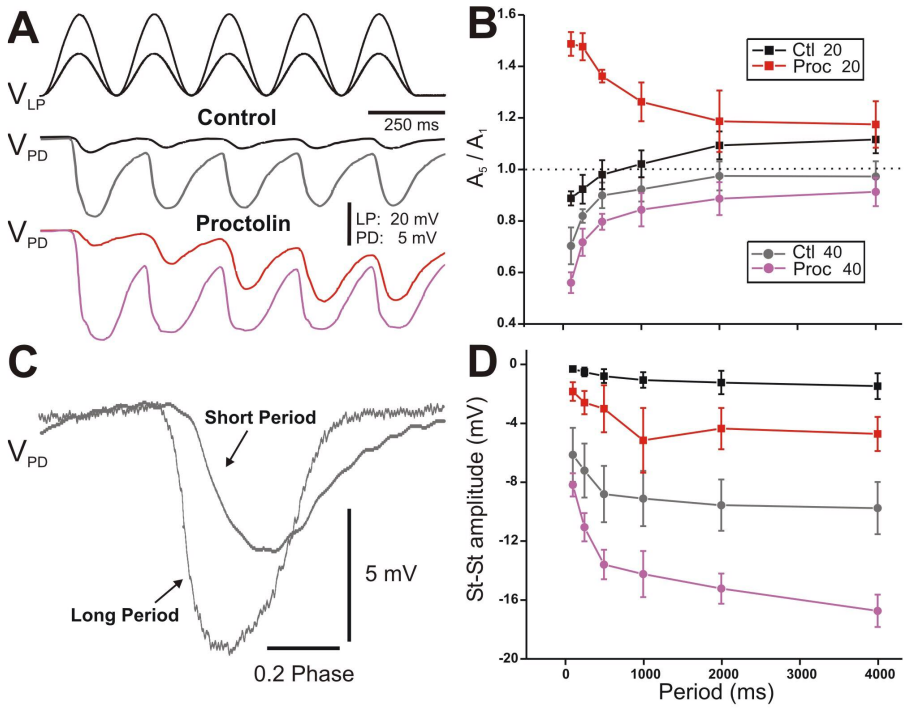
A
V LP
Control
250 ms
V PD
Proctolin
LP: 20 mV
PD: 5 mV
V PD
B
Ctl 20
Proc 20
Ctl 40
Proc 40
A5 / A1
1.6
1.4
1.2
1.0
0.8
0.6
0.4
C
V PD
Short Period
Long Period
5 mV
0.2 Phase
D
St-St amplitude (mV)
0
-4
-8
-12
-16
-20
0
1000
2000
3000
4000
Period (ms)

Figure 4

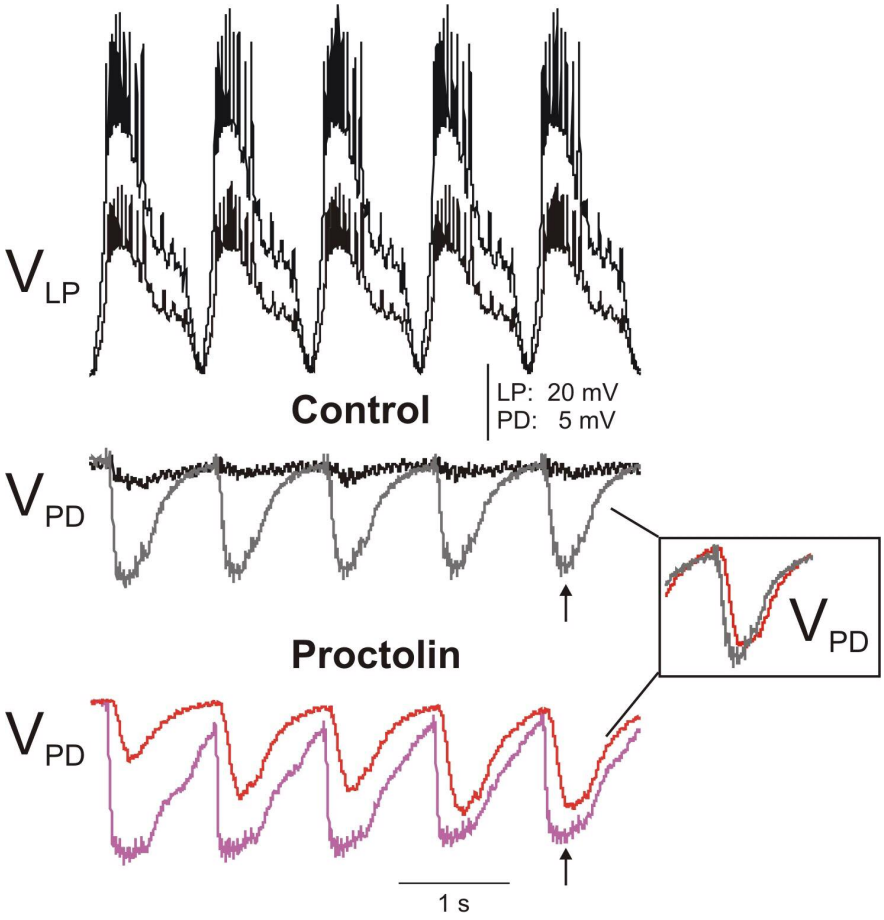

Figure 5