Peptide Neuromodulation of Synaptic Dynamics in an Oscillatory Network

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Although neuromodulation of synapses is extensively documented, its consequences in the context of network oscillations are not well known. We examine the modulation of synaptic strength and short-term dynamics in the crab pyloric network by the neuropeptide proctolin. Pyloric oscillations are driven by a pacemaker group which receives feedback through the inhibitory synapse from the lateral pyloric (LP) to pyloric dilator (PD) neurons. We show that proctolin modulates the spike-mediated and graded components of the LP to PD synapse. Proctolin enhances the graded component and unmasks a surprising heterogeneity in its dynamics where there is depression or facilitation depending on the amplitude of the voltage waveform of the presynaptic LP neuron. The spike-mediated component is influenced by the baseline membrane potential and is also enhanced by proctolin at all baseline potentials. In addition to direct modulation of this synapse, proctolin also changes the shape and amplitude of the presynaptic voltage waveform which additionally enhances synaptic output during ongoing activity. During ongoing oscillations, proctolin reduces the variability of cycle period but only when the LP to PD synapse is functionally intact. Using the dynamic clamp technique we find that the reduction in variability is a direct consequence of modulation of the LP to PD synapse. These results demonstrate that neuromodulation of synapses involves complex and interacting influences that target different synaptic components and dynamics as well as the presynaptic voltage waveform. At the network level, modulation of feedback inhibition can result in reduction of variability and enhancement of stable oscillatory output.

Introduction

Short-term synaptic dynamics such as facilitation and depression have been shown to play an important role in shaping the output of neuronal networks (Abbott et al., 1997; Tsodyks et al., 2000; Manor and Nadim, 2001; Zucker and Regehr, 2002; Deeg, 2009). Synaptic plasticity also ensues from modification of synaptic strength by neuromodulators (Ayali et al., 1998; Sakurai and Katz, 2003). Neuromodulation of the short-term dynamics, reported in many systems (Bristol et al., 2001; Baimoukhametova et al., 2004; Cartling, 2004; Sakurai and Katz, 2009), is considered a form of metaplasticity and can have complex network consequences (Fischer et al., 1997b); yet, little is known about neuromodulation of synaptic dynamics in the context of network oscillations. Synapses often involve distinct components that act at different time scales or involve spike-mediated, graded or asynchronous release (Warzecha et al., 2003; Otsu et al., 2004; Ivanov and Calabrese, 2006b). We explore how neuromodulation modifies distinct components of a synapse in an oscillatory network

and examine how these modulatory actions shape the combined synapse in the context of network activity.

The crustacean pyloric oscillations are generated in the stomatogastric nervous system (STNS) by a pacemaker group consisting of the gap-junction-coupled anterior burster (AB), pyloric dilator (PD), and (in crabs) lateral posterior gastric (LPG) neurons that burst in synchrony. The sole chemical feedback from the pyloric follower neurons to the pacemakers is the synapse from the lateral pyloric (LP) to the PD neurons, according to a key role for this synapse in the regulation of pyloric oscillations (Manor et al., 1997; Mamiya et al., 2003; Weaver and Hooper, 2003). A variety of neuromodulators in the STNS modify the intrinsic properties of individual pyloric neurons (Harris-Warrick et al., 1998; Swensen and Marder, 2000) and the strength and dynamics of synapses among these neurons (Johnson et al., 2005, 2011). We demonstrate the ability of the modulatory neuropeptide proctolin to alter the strength and unmask novel dynamics in the LP to PD synapse. Proctolin is released by projection neurons in the STNS (Nusbaum et al., 2001) and activates a voltage-gated ionic current in several pyloric neurons (Swensen and Marder, 2001). However, the effect of proctolin on the pyloric synapses has not been previously examined. The LP to PD synapse has both spike-mediated and graded components, as found in other systems (Angstadt and Calabrese, 1991; Pan et al., 2001; Warzecha et al., 2003; Ivanov and Calabrese, 2006b). We characterize the effect of proctolin on the strength and short-term dynamics of the two components of the LP to PD synapse separately. The graded component of this synapse, which depresses in control conditions (Manor et al., 1997), is enhanced

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and can show facilitation in proctolin, while the spike-mediated component is also strengthened by proctolin. To measure the combined effect of proctolin, we record voltage waveforms of the LP neuron during ongoing activity and use these realistic waveforms in the voltage-clamped LP neuron to unmask the contribution of these changes to total synaptic output in biologically realistic conditions. Finally, using the dynamic clamp technique to modify the synaptic current, we show that proctolin reduces variability in pyloric oscillations through its modulation of the LP to PD synapse.

Materials and Methods

Preparation and identification of the neurons.

Experiments were conducted on the STNS of male crabs *Cancer borealis*. Animals were obtained from local markets and maintained in filtered, recirculating seawater tanks at 10–12°C. The STNS was dissected out using standard procedures (Blitz et al., 2004; Tohidi and Nadim, 2009). Briefly, the complete isolated STNS [including the stomatogastric ganglion (STG), the oesophageal ganglion (OG), and the paired commissural ganglia (CoG); Fig. 1A] 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): 11 KCl, 440 NaCl, 13 CaCl₂, 26 MgCl₂, 11.2 Trizma base, 5.1 maleic acid, pH = 7.4–7.5. Proctolin (Sigma-Aldrich) 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 was made by dissolving the stock solution in *Cancer* saline immediately before use. The dose–response effect on the synaptic input–output curve was done by bath-applying proctolin from low to high concentration (10^{-9} – 10^{-5} M) in 20 min intervals. All other applications of proctolin were done at 10^{-6} M. Proctolin and other solutions were bath applied by means of a switching port in a continuously flowing superfusion system.

Extracellular recordings from identified motor nerves were made using stainless steel wire electrodes, inserted inside and outside of a petroleum jelly well built to electrically isolate a small section of the nerve, and amplified with a Differential AC amplifier (A-M Systems 1700). Intracellular recordings were made from the neuronal cell bodies with sharp glass microelectrodes containing 0.6 M K₂SO₄ and 20 mM KCl (final electrode resistance 20–30 MΩ). Microelectrodes were pulled using a Flaming-Brown P-97 micropipette puller (Sutter Instruments). All intracellular recordings were performed in single-electrode current-clamp or two-electrode voltage-clamp mode (Axoclamp 2B amplifiers; Molecular Devices). Pyloric neurons were identified according to their stereotypical axonal projections in identified nerves and interactions with other STG neurons (Weimann et al., 1991; Blitz et al., 2008).

Effect of proctolin on the strength and dynamics of the LP to PD synapse. Measurements of synaptic output were done by measuring maximum amplitude when there was little variability in the synaptic potential or current amplitudes. In cases where biological or other conditions resulted in variability of synaptic amplitude (such as during ongoing activity) we measured the total area of the IPSP or IPSC.

To study the graded component of the LP to PD synapse, the preparation was superfused with 10^{-7} M tetrodotoxin (TTX; Biotium) to block action potentials and therefore spike-mediated transmission. The LP neuron was two-electrode voltage clamped with a holding potential of –60 mV and stimulated with multiple square pulses of different amplitudes and fixed 500 ms duration (and different interpulse intervals as

Figure 1. Proctolin enhances the pyloric rhythm. **A**, Top, Schematic diagram of the stomatogastric nervous system. The paired commissural ganglia (CoGs) and oesophageal ganglion (OG) contain modulatory projection neurons that send axons to the STG through the stomatogastric nerve (stn). Bottom, Simplified diagram of the pyloric network shows the pacemaker group consisting of the AB, PD, and LPG neurons coupled strongly with electrical synapses (gap junctions) and synaptically inhibiting the follower neuron LP. The LP neuron in turn inhibits the two PD neurons, producing a reciprocally inhibitory subnetwork. **B**, Intracellular traces recorded in the normal ongoing pyloric rhythm with intact descending neuromodulatory inputs show the rhythmic alternation of the PD and LP neurons (Control). Blocking or cutting the stn removes all descending neuromodulatory input to the STG and disrupts the pyloric rhythm (Decentralized). Bath application of 10^{-6} M proctolin recovers the pyloric rhythm, increases the amplitude of the slow-wave oscillations in the PD and LP neurons and increases the spike frequency and number of spikes per burst (Proctolin). Line segments indicate the baselines for the membrane oscillations of PD (–56 mV) and LP (–59 mV) recorded in Control. stn, Stomatogastric nerve; lvn, lateral ventricular nerve; lpn, lateral pyloric nerve; pdn, pyloric dilator nerve.

noted in Results), as well as realistic waveforms of different amplitudes and frequencies. Application of realistic waveforms for synaptic measurements was done according to methods we have previously described (Mamiya et al., 2003; Tseng and Nadim, 2010). Graded synaptic responses were used to measure synaptic input–output relationships which were fit with Boltzmann-type equations $1/(1 + \exp((V_{LP} - V_{half})/k_{half}))$.

In almost all recordings, the amplitude of the graded IPSP (gIPSP) did not change subsequent to the fourth pulse and therefore we refer to the gIPSP in response to the fifth pulse as the steady-state gIPSP. To measure short-term synaptic dynamics, the ratio of the fifth to the first mean gIPSP amplitude (A_5/A_1) was used. To avoid outliers, the ratio was only calculated if the mean gIPSP amplitude was ≥ 0.5 mV. To measure spike-mediated transmission, the LP neuron was voltage clamped at a holding potential between –70 and –50 mV and one of the following two methods was used.

In the first method, short square voltage pulses of fixed 10 ms duration were used to elicit individual spikes and activate the spike-mediated component of the synapse without eliciting graded release. This was made possible because the voltage-clamp holding potential prevented graded release which depends on changes in the baseline membrane potential (we confirmed that 10 ms pulses were too short to elicit graded release by showing that no synaptic current was present in the presence of TTX) and, action potentials could be generated by the brief 10 ms pulses without any significant effect on the baseline membrane potential.

In the second method, antidromic spikes were elicited by stimulating the lateral pyloric nerve (lpn) using a pulse stimulator (A-M Systems isolated pulse stimulator 2100) using 0.5 ms, 3–10 V stimuli. The antidromic spikes (which cannot be clamped with somatic electrodes) invade the arborization of the LP neuron and result in spike-mediated but not graded synaptic release.

Measurement of putative Ca²⁺ currents was done in the presence of 10^{-7} M TTX and 10 mM TEA and the presynaptic electrodes were loaded with 2 M TEA and 2 M CsCl to block potassium currents (B. R. Johnson et al., 2003). To measure presynaptic Ca²⁺ currents, the experimental protocol was repeated in both normal saline and Mn²⁺ saline (where Ca²⁺ in the physiological saline is substituted with 12.9 mM Mn²⁺ and 0.1 mM Ca²⁺) and the difference between the presynaptic currents measured in normal saline and in Mn²⁺ saline was reported as a putative Ca²⁺ current (Golowasch et al., 1992). Calcium channel blockers Ni²⁺ and Cd²⁺

Figure 2. Proctolin strengthens the graded component of the LP to PD synapse. **A**, The LP neuron was voltage clamped at -60 mV and 500 ms depolarizing square pulses with increasing amplitudes up to 0 mV (V_{LP}) were used to activate the LP to PD synapse in Control, 10^{-6} M Proctolin and Wash. The resulting graded IPSP (gIPSP) recorded in the PD neuron (V_{PD}) increased as the amplitude of the V_{LP} pulse increased for all conditions and was always larger in Proctolin. The enhancement of the synaptic strength was reversible after a 45 min wash. The PD neuron was recorded at its resting potential which remains quite stable in TTX. Baseline V_{PD} (mV): Ctrl, -60.5 ; Proc, -59.5 mV; Wash, -60 mV. **B**, The peak amplitudes of gIPSPs were plotted against the V_{LP} pulse values to obtain the synaptic input–output curves in control and in different doses of proctolin. The amplitudes of gIPSPs were augmented in proctolin in a dose-dependent manner but saturated at $\sim 10^{-6}$ M. **C**, The synaptic input–output curve showed a sigmoidal dependency on the presynaptic voltage for both control and proctolin (10^{-6} M). Proctolin significantly strengthened gIPSPs of the LP to PD synapse for presynaptic amplitudes ≥ 35 mV compared with control and wash (two-way ANOVA *post hoc* Tukey analysis, $p < 0.01$ for $V_{LP} = -35$ to -10 mV; $n = 6$). **D**, The amplitude of the individual gIPSPs in each preparation was normalized to the maximum gIPSP in that condition. Each data point (black, control; red, proctolin; blue, wash) indicates a single measurement from six preparations, and these measurements were fit with Boltzmann type equations. Proctolin shifted the midpoint of the input–output curve by ~ 5.5 mV to more negative membrane potentials (V_{half} in mV—Ctrl, -29.0 ± 0.11 ; Proc, -35.4 ± 0.14 ; Wash, -28.0 ± 0.44) but did not significantly change the slope of this curve, although the slope became shallower after wash (k_{half} in mV—Ctrl, -3.11 ± 0.10 ; Proc, -2.83 ± 0.12 ; Wash, -6.36 ± 0.45).

were bath applied at concentrations of 1 and 0.2 mM, respectively (Golowasch et al., 1992; Hurley and Graubard, 1998; Ivanov and Calabrese, 2006a).

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, 1997; Rabbah et al., 2005; Rabbah and Nadim, 2005; Soto-Treviño et al., 2005). For clarity, the figures in this paper only show results from one PD neuron. The two PD neurons were recorded in several preparations for the various protocols to ensure that the responses were similar. In all measurements in this study only the response of a single PD neuron in each preparation is used.

Dynamic clamp. We used the dynamic clamp technique to modify the LP to PD synapse in control saline so as to mimic the effect of the enhancement of the synapse by bath application of proctolin. A NI PCI-6070-E board (National Instruments) was used for current injection in

dynamic clamp experiments. We use the dynamic clamp software developed in our laboratory (<http://stg.rutgers.edu/software>) on a Windows platform. In dynamic clamp, ionic currents are calculated using Hodgkin–Huxley-type equations as described below and continuously updated by recording the membrane potential (V) of the neurons in real time. To modify the synaptic current to mimic the proctolin effect, we injected the difference between the synaptic input–output relationships measured in proctolin and control into the postsynaptic PD neuron. The synaptic strength was calculated by adding the Boltzmann fits to the synaptic input–output curve measured in proctolin (Fig. 2C,D, red trace) and subtracting the fit measured in control (Fig. 2C,D, black trace):

$$I_{dyn} = g_{dyn} s(V_{LP})(V_{PD} - E_{syn})$$

$$\frac{ds}{dt} = \frac{s_{\infty}(V_{LP}) - s}{\tau_s}$$

$$s_{\infty}(V) = 1.4 \left(\frac{1}{1 + \exp\left(-\frac{V + 29.0}{2.83}\right)} \right) - 1 \left(\frac{1}{1 + \exp\left(-\frac{V + 35.4}{3.11}\right)} \right),$$

where $\tau_s = 10$ ms, $g_{dyn} = 100$ nS, and $E_{syn} = -80$ mV.

Recording, analysis, and statistics. Data were acquired using pClamp 9.2 software (Molecular Devices) or the Scope software (available at <http://stg.rutgers.edu/software> developed in the Nadim laboratory), sampled at 4–5 kHz, and saved on a PC using a Digidata 1332A (Molecular Devices) or PCI-6070-E data acquisition board (National Instruments). Statistical and graphical analyses were done using Sigmaplot 3.0 (Aspire Software) and Origin 8.0 (OriginLab). 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 SDs.

Results

During the ongoing pyloric rhythm, the LP and PD neurons fire in alternation (Fig. 1B, left). Removal of descending modulatory inputs to the STG (decentralization) results in slow and irregular pyloric oscillations or the oscillations are completely disrupted (Fig. 1B, middle; see also Nusbaum and Beenhakker, 2002). As shown in previous studies (Marder et al., 1986; Nusbaum and Marder, 1989a), bath application of proctolin enhances the pyloric rhythm by increasing the amplitude of the slow-wave oscillation of the LP and PD neurons and increasing the spike frequency and number of spikes per burst (Fig. 1B, right). 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). 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;

Figure 3. Proctolin modulates the short-term dynamics of the LP to PD graded synapse in response to different amplitude depolarizations. **A**, LP neuron was voltage clamped at -60 mV and stimulated with a series of five square pulses of 20 mV (V_{LP} , black trace) or 40 mV (V_{LP} , gray trace) amplitudes in control, proctolin, and wash. In control and wash, the LP to PD synapse was depressing. In proctolin, in response to 20 mV presynaptic depolarization, the gIPSPs in the PD neuron showed facilitation. Darker color traces in each condition correspond to the lower pulse amplitude of 20 mV. Baseline V_{PD} at -58 mV in control, -56.5 mV in proctolin and -58.5 mV in wash. **B**, The ratio of the fifth to the first mean gIPSP amplitude (A_5/A_1) calculated for all presynaptic depolarizations with an interpulse interval of 500 ms plotted against presynaptic pulse amplitudes (ΔV_{LP}). In control (black), the ratio A_5/A_1 was slightly less than one for all

Mamiya and Nadim, 2004, 2005). Our goal in this study is to characterize the neuromodulation of the LP to PD synapse by proctolin. An examination of the size of the IPSPs resulting from this synapse (gray regions marked in Fig. 1B) shows that during ongoing oscillations proctolin strengthens the synapse compared with control conditions (Control 1148 ± 155 mV $\cdot$ ms, Proctolin: 3468 ± 938 mV $\cdot$ ms; Student's t test, $p < 0.01$, $n = 5$).

The LP to PD synapse consists of two components: a spike-mediated component that manifests as unitary IPSPs in response to individual action potentials in the presynaptic LP neuron (clearly seen in the Decentralized PD neuron trace in Fig. 1B) and a graded (non-spike-mediated) component (Manor et al., 1997; Ayali et al., 1998; Mamiya and Nadim, 2004). We first examined the effects of proctolin on these two components of LP to PD synapse separately. The input resistance of the LP and PD (measured with 500 ms voltage-clamp steps from -60 to -70 mV) was not significantly altered by bath application of proctolin (PD: ctrl, 10.1 ± 3.7 M Ω ; proc, 11.0 ± 3.2 M Ω ; wash, 9.8 ± 3.3 M Ω ; LP: ctrl, 7.2 ± 3.0 M Ω ; proc, 8.3 ± 2.1 M Ω ; wash, 8.0 ± 2.6 M Ω ; $n = 7$; one-way RM-ANOVA, $p > 0.1$).

To measure the graded component of the LP to PD synapse, the preparation was bathed with 10^{-7} M TTX to remove action potentials and therefore block spike-mediated transmission. The LP neuron was voltage clamped to a holding potential of -60 mV and then injected with a series of 500 ms depolarizing square voltage pulses with increasing amplitudes, in control and during bath application of 10^{-6} M proctolin, and the resulting IPSPs were recorded in the PD neuron (Fig. 2). 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 gIPSPs were larger in proctolin, indicating the strengthening of the LP to PD synapse by proctolin. The enhancement of the synaptic strength was reversible after a 45 min wash although the synapse often appeared slower to activate at high presynaptic voltages after wash (Fig. 2A).

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,C). The amplitudes of gIPSPs were augmented in proctolin in a dose-dependent manner but saturated at 10^{-6} M (Fig. 2B, $n = 6$) which is the dose typically used in previous studies of proctolin in this system (Nusbaum and Marder, 1989a; Swensen and Marder, 2000). All subsequent data reported in this study are with 10^{-6} M proctolin.

Proctolin significantly strengthened gIPSPs of the LP to PD synapse for presynaptic amplitudes ≥ 35 mV (two-way ANOVA *post hoc* Tukey analysis, $p < 0.01$ for $V_{LP} = -35$ to -10 mV; $n = 6$). To examine the effect of proctolin on the synaptic input–output curve, the amplitude of the individual gIPSPs in each

values of presynaptic pulse amplitudes (ΔV_{LP}), indicating that the synapse was always depressing. In contrast, in the presence of proctolin (red), the A_5/A_1 ratios in response to pulse amplitudes between 20 and 24 mV were >1 , while the ratios in response to larger pulse amplitudes were <1 , indicating facilitation and depression, respectively (two-way ANOVA with *post hoc* Tukey test showing $p < 0.001$ between ctrl and proctolin and $p < 0.008$ between control and wash for $\Delta V_{LP} = 20, 22$, and 24 mV; not significant at other ΔV_{LP}). **C**, A_5/A_1 measured for different IPI durations in a train of presynaptic pulses applied at low (20 mV) or high (40 mV) amplitudes. Proctolin produced significant facilitation synapse at IPI values ≤ 1000 ms with low-amplitude presynaptic pulses (two-way ANOVA with *post hoc* Tukey test showing $*p < 0.01$; not significant at other IPI values). With high amplitude pulses there was no statistically significant difference in synaptic dynamics between control and proctolin, both of which resulted in depression (two-way ANOVA; $p > 0.5$; dashed green curve shows exponential decay fit $1 - 0.603 \exp(-t/434)$).

preparation was normalized to the gIPSP measured in response to the highest presynaptic amplitude (Fig. 2*D*). Each data point in Figure 2*D* indicates a single measurement in six preparations, and these measurements were fit with Boltzmann type equations (see Materials and Methods). We found that proctolin shifts the midpoint of the input–output curve by ~ 5.5 mV to more negative membrane potentials (V_{half} in mV—Ctrl: -29.0 ± 0.11 ; Proc: -35.4 ± 0.14 ; Wash: -28.0 ± 0.44 ; one-way ANOVA, $p < 0.001$) but did not significantly change the slope of this curve, although the slope became shallower after wash (k_{half} in mV—Ctrl: -3.11 ± 0.10 ; Proc: -2.83 ± 0.12 ; Wash: -6.36 ± 0.45 , $p = 0.2$ for Ctrl vs Wash).

To examine whether proctolin increased the variability in the synaptic amplitudes, we did a two-sample *F* test for differences in variance between the data measured in control and in proctolin. The test showed that for the non-normalized raw data, there was higher variability in the synaptic amplitude in proctolin than control ($F = 0.513$, $p < 10^{-9}$). However, when the data were normalized as shown in Figure 2*D*, there was no statistical difference between the two cases ($F = 1.029$, $p = 0.79$) suggesting that the variability was primarily due to changes in amplitude across preparations and not the voltage dependence of the synaptic activation.

The LP to PD synapse has been previously shown to display short-term depression (Manor et al., 1997). To examine whether proctolin modifies the dynamics of this synapse, we injected a train of voltage pulses with different amplitudes from a holding potential of -60 mV into the LP neuron and recorded the gIPSPs in the PD neuron in control saline and in the presence of 10^{-6} M proctolin. An example of the synaptic response to trains of pulses with two different amplitudes in control and proctolin is shown in Figure 3*A*. As seen in this figure, in response to 40 mV pulses, the steady-state gIPSP was always smaller than the first pulse gIPSP, indicating synaptic depression, in both control (gray) and proctolin (pink). In contrast, the gIPSPs elicited with 20 mV presynaptic pulses were qualitatively different: in this case, the gIPSPs in the PD neuron either did not change or showed slight depression in control, but facilitation in proctolin: the response to the first pulse was relatively small but became much larger by the third pulse. Figure 3*B* shows the ratio of the fifth to the first mean gIPSP amplitude (A_5/A_1 ; see Materials and Methods) calculated for all presynaptic depolarizations with an interpulse interval (IPI) of 500 ms. In control conditions, the ratio A_5/A_1 was slightly less than one for all values of presynaptic pulse amplitudes (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 pulse amplitudes between 20 and 24 mV were >1 while the ratios in response to larger pulse amplitudes were <1 , indicating facilitation and depression, respectively (two-way ANOVA with *post hoc* Tukey test showing $p < 0.001$ between control and proctolin and $p < 0.008$ between control and wash for $\Delta V_{\text{LP}} = 20, 22$ and 24 mV; not significant at other ΔV_{LP}). For ΔV_{LP} values <20 mV the gIPSP was typically too small (<1 mV, Fig. 2*C*) to reliably measure short-term dynamics which explains the large error bars seen at these voltages. 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 a low level of depression with high-amplitude depolarizations.

To examine the time dependence of synaptic dynamics, in a separate set of experiments, we changed the IPI duration (250–4000 ms) in trains of presynaptic pulses applied at low (20 mV) or high (40 mV) amplitudes (Fig. 3*C*). We found that proctolin

Figure 4. Proctolin caused a facilitation of the LP to PD graded IPSPs in response to low-amplitude presynaptic depolarizing pulses, which is associated with the activation of a Ca^{2+} -like inward current. **A**, The presynaptic currents in the LP neuron (I_{LP}) are measured in Ca^{2+} saline (green) and in Mn^{2+} saline (magenta) using a train of low-amplitude presynaptic voltage pulses. The difference between the two measured currents ($\Delta I_{\text{LP}} = I_{\text{LP, Ca}^{2+}} - I_{\text{LP, Mn}^{2+}}$) reveals a new inward current activated in proctolin (red). A series of low-amplitude presynaptic depolarizing pulses causes little response in postsynaptic neuron PD in control. Proctolin strengthened and caused facilitation in the PD neuron in response to low-amplitude presynaptic depolarizing pulses. ΔI_{LP} also increased with each pulse. V_{PD} is not shown in Mn^{2+} because synaptic transmission was blocked (baseline V_{PD} at -59 mV control, -60 mV proctolin; dotted line indicates 0 nA). **B**, The mean value (area divided by pulse duration) of the presynaptic inward current ΔI_{LP} in response to each pulse plotted against the mean value of the postsynaptic potentials V_{PD} (IPSP area divided by pulse duration) for measurements in Ca^{2+} saline in proctolin. Linear regression fit shows the correlation between the presynaptic inward current and the postsynaptic potentials ($r = 0.69$, $p < 0.001$, $n = 9$).

produced significant facilitation of the synapse at IPI values ≤ 1000 ms with low-amplitude presynaptic pulses (two-way ANOVA with *post hoc* Tukey test showing $p < 0.01$ between ctrl and proctolin for IPI = 250, 500, 1000 ms, not significant at other IPI values; Fig. 3*C*). With high-amplitude pulses there was no statistically significant difference in synaptic dynamics between control and proctolin, both of which resulted in depression (two-way ANOVA; $p > 0.5$).

We hypothesized that the appearance of synaptic facilitation with low-amplitude presynaptic voltage pulses in the presence of proctolin was due to additional presynaptic calcium currents. To examine this hypothesis, we applied 20 mV pulses in the LP neuron to correlate the synaptic response with presynaptic Ca^{2+} currents in the LP neuron. Each experimental protocol was first performed in normal saline and after blocking Ca^{2+} currents (and therefore synaptic transmission), in both control and proctolin, by substituting the Ca^{2+} with Mn^{2+} (see Materials and Methods). In all conditions, we made simultaneous measurements of the presynaptic current (Fig. 4*A*, I_{LP}) and the

postsynaptic potential (Fig. 4A, V_{PD}). The difference between the presynaptic currents measured in normal saline (labeled Ca^{2+}) and in Mn^{2+} saline was measured as a putative calcium current (Fig. 4A, ΔI_{LP}).

In normal saline, there was little synaptic response in control conditions and no apparent synaptic plasticity was observed (Figs. 3A, Ctl black trace; 4A, left). In contrast, the synaptic response was strengthened and showed facilitation in proctolin (Figs. 3A, Proc, dark red trace; 4A, right). For clarity, V_{PD} is not shown in Mn^{2+} saline because there was no synaptic transmission in either control or proctolin. The putative calcium current was small in control conditions and its amplitude showed no obvious variation among the different voltage pulses (Fig. 4A, left, ΔI_{LP}). In contrast, in the presence of proctolin, this current increased with each subsequent pulse, indicating accumulation of Ca^{2+} currents (Fig. 4A, right, ΔI_{LP}). We used these data to examine whether the putative Ca^{2+} current ΔI_{LP} measured in proctolin may underlie the increase in the amplitude of the gIPSPs thus resulting in the observed facilitation. The peak of the gIPSPs in response to low-amplitude presynaptic pulses was often highly variable or hard to discern, especially in control where the gIPSPs were small (Fig. 4A, V_{PD} trace). Due to the variability in the peak current and the peak gIPSPs and the fact that the measured presynaptic currents were at times contaminated with outward currents, we compared the mean values of ΔI_{LP} and gIPSP during each pulse. There was a modest positive correlation between the presynaptic inward current and the postsynaptic potentials (Fig. 4B, linear fit: $r = 0.69$, $p < 0.001$, $n = 9$), supporting the hypothesis that the facilitation in proctolin is due to a proctolin-induced accumulation of additional calcium current in the LP neuron.

We also examined whether proctolin affects the strength of the spiking-mediated component of the LP to PD synapse. To elicit single spike-mediated IPSPs (sIPSP), the presynaptic LP neuron was voltage clamped at a holding potential of -60 mV and stimulated with a very short voltage pulse of duration 10 ms and amplitude 30 mV to elicit a single action potential (Fig. 5A). The short voltage pulses ensured that there was no graded release (which we also verified by repeating the same protocol in TTX and observing no IPSP; see Materials and Methods). Bath application of proctolin significantly enhanced the amplitude of the unitary sIPSP values (Control: 5.6 ± 1.1 mV, Proctolin: 9.3 ± 1.0 mV; Student's t test, $p < 0.001$; $n = 6$).

It is known that the baseline potential of the presynaptic neuron can have a significant effect on the amplitude of the spike-mediated PSPs (Nicholls and Wallace, 1978; Ivanov and

Figure 5. The strength of unitary sIPSPs is enhanced by proctolin. **A**, The LP neuron was voltage clamped at -60 mV and stimulated with one short square pulse of duration 10 ms and amplitude 30 mV. This resulted in a single action potential in the LP neuron. The resulting sIPSP was recorded in PD neuron (V_{PD}) in control and in proctolin (10^{-6} M). Baseline V_{PD} was at -58 mV in both conditions. **B**, Increasing the presynaptic 10 ms square pulse amplitude (to peak voltages $V_{LP} = -40, -30, -20, -10$ mV; shown schematically) always resulted in a single action potential in the LP neuron which was recorded on the nerve lvn. The resulting sIPSPs recorded in PD neuron (V_{PD}) in control and in proctolin (10^{-6} M) depended on the amplitude of the presynaptic stimulus. **C**, The amplitude of sIPSPs in response to the 10 ms voltage pulse in control and proctolin increased if the amplitude of this short presynaptic voltage pulse was increased. Bath application of proctolin significantly increased the sIPSP amplitude for all values of the presynaptic pulse amplitudes (two-way ANOVA *post hoc* Tukey analysis, $p < 0.05$, $n = 8$). **D**, Schematic diagram showing how antidromic spikes were elicited in the LP neuron. The LP neuron (blue) soma was voltage clamped and the PD neuron (red) was recorded in current clamp. To elicit antidromic spikes, the lvn, which contains the axon of the LP neuron but not that of the PD neuron was stimulated. **E**, The LP neuron was voltage clamped at different holding potentials ($V_{LP} = -70, -60, -55$ and -50 mV). The sIPSPs were recorded in PD neuron in response to antidromic spikes in the LP neuron in control and proctolin (10^{-6} M). **F**, The peak sIPSP amplitude plotted against the holding potentials of the LP neuron in control and in proctolin. As the holding potential of the LP neuron was increased, the amplitude of sIPSPs in the PD neuron also increased in both control and 10^{-6} M proctolin. In the presence of proctolin, the sIPSPs were larger compared with control (two-way RM-ANOVA, $p < 0.05$, $n = 5$).

Calabrese, 2003). Although a 10 ms voltage pulse applied even at 40 mV amplitude in the presynaptic LP neuron never produced any graded synaptic release (IPSP amplitude 0 mV in TTX; $n = 3$), we observed that the amplitude of sIPSPs in response to single action potentials elicited by the 10 ms voltage pulse in normal saline increased if the amplitude of this short presynaptic voltage pulse was increased (to peak voltages $V_{LP} = -40, -30, -20, -10$ mV; Fig. 5B, C, Control). Additionally, bath application of proctolin significantly increased the sIPSP amplitude for all values of the presynaptic pulse amplitudes (Fig. 5B, C; two-way ANOVA *post hoc* Tukey analysis, $p < 0.01$, $n = 8$).

To completely exclude the possibility that these results were contaminated by the graded component of the LP to PD synapse, we also measured sIPSPs elicited by antidromic spikes while volt-

Figure 6. Proctolin does not have a significant effect on the short-term dynamics of the sIPSPs. **A**, The LP and PD neurons were voltage clamped at a holding potential of -50 mV. The resulting spike-mediated synaptic currents in the PD neuron in response to two successive antidromic spikes with a 100 ms interval were recorded and repeated 10 times. The averaged traces were shown in control (black) and proctolin (red). The sIPSC amplitude was significantly strengthened by proctolin (Ctrl, 0.49 ± 0.02 nA; Proc, 1.3 ± 0.1 nA; Student's t test, $p < 0.01$, $n = 4$). Baseline I_{PD} at 2.2 nA in control and 2.0 nA in proctolin. **B**, The ratio of the sIPSC amplitudes in response to the first two successive antidromic spikes plotted against interspike interval in control and in proctolin. The exponential fit curves ($y = 1 - Ae^{-x/B}$) were plotted in control (black: $A = -0.23$, $B = 125$) and in proctolin (red: $A = -0.15$, $B = 167$). Proctolin did not cause a significant change in the short-term dynamics compared with control (Student's t test on exponential fits, $p = 0.37$).

age clamping the LP neuron soma to constant holding potentials in the presence and absence of proctolin. To elicit antidromic spikes, we stimulated the lpn which contains the axon of the LP neuron but not that of the PD neuron (Fig. 5D). The stimulus-induced antidromic spikes propagated back to the STG where they resulted in synaptic release (Fig. 5E). We voltage clamped the LP neuron at different holding potentials below spike threshold ($V_{LP} = -70, -60, -55$, and -50 mV) and recorded the sIPSPs in the PD neuron in control and in the presence of bath-applied proctolin. We found that, as the holding potential of the LP neuron was increased, the amplitude of sIPSPs in the PD neuron also increased in both control and 10^{-6} M proctolin. Additionally, as in the protocol of Figure 5, *B* and *C*, the sIPSPs were larger in the presence of proctolin compared with control (Fig. 5F; two-way RM-ANOVA, $p < 0.05$, $n = 5$).

It has been suggested that calcium accumulation through low-threshold calcium channels activated by subthreshold membrane potential depolarization may result in enough background calcium in the presynaptic neuron to enhance spike-mediated release (Ivanov and Calabrese, 2003). Bath application of Ni^{2+} is known to block a number of low-threshold calcium channels (Lee et al., 1999; Perez-Reyes, 2003), including those responsible for graded transmission in leech heart interneurons (Ivanov and Calabrese, 2006b). We examined the effects of Ni^{2+} on the amplitudes of sIPSPs in the LP to PD synapse. We found that sIPSPs (elicited by 10 ms voltage pulses with different amplitudes similar to Fig. 5B) were weakened by bath application of Ni^{2+} by 40–75% (two-way ANOVA, $p < 0.05$, $n = 5$) but continued to be enhanced with baseline amplitude (data not shown; Ctrl: from 0.8 ± 0.6 at 10 mV amp to 8.5 ± 1.5 mV at 40 mV amp; Ni^{2+} : from 0.9 ± 0.4 at 10 mV amp to 4.8 ± 0.4 mV at 40 mV amp; $n = 5$). It was often difficult to assess the effect of Ni^{2+} on synaptic transmission because, with long duration bath application, it resulted in membrane potential oscillations reminiscent of an ongoing pyloric rhythm, even in the presence of TTX (Zirpel et al.,

1993). Yet, these results suggest that although an increase in the background calcium in the presynaptic neuron might cause the increase of the sIPSP amplitude as the baseline membrane potential increases, such background calcium is only partially due to Ni^{2+} -sensitive low-threshold calcium channels.

We also examined the effect of proctolin on the short-term dynamics of the sIPSPs. The LP neuron was clamped at a holding potential of -50 mV and the lpn was stimulated to produce two successive antidromic spikes, and the interspike intervals (ISIs) were varied (ISI: 30, 50, 100, 250, 500 ms). Because the stimulus artifact often interfered with the measurement of the sIPSPs with short ISIs, in this protocol we also voltage clamped the postsynaptic PD neuron with two electrodes at a holding potential of -50 mV and measured the spike-mediated synaptic currents (sIPSCs) which decay significantly more rapidly than the sIPSPs and are therefore less prone to contamination by the artifacts due to subsequent pulses (especially with short ISIs). The sIPSCs were measured and averaged for 10 repeated trials at each ISI value in each experiment and the resulting average amplitude was used as a single data point. As expected, the sIPSC amplitude was significantly strengthened by proctolin (Ctrl: 0.49 ± 0.02 nA, Proc: 1.3 ± 0.1 nA; Student's t test, $p < 0.01$, $n = 4$; Fig. 6A). To measure the extent of synaptic dynamics, we measured the ratio of the peaks of second to first sIPSC. The sIPSCs of LP to PD synapse exhibited short-term depression in both control and proctolin at shorter ISIs (Fig. 6B). However, there was no difference in depression between control and proctolin (exponential fit curves $y = 1 - Ae^{-x/B}$: Control $A = 0.23$, $B = 125$; Proctolin $A = 0.15$, $B = 167$; Student's t test, $p = 0.37$).

So far, our results demonstrated that proctolin can enhance both graded and spike-mediated components of the LP to PD synapse. A natural question is how the total IPSC is affected by proctolin. To explore this question we decentralized the preparation (Fig. 1B), voltage clamped the LP neuron at a holding potential of -60 mV and applied a voltage profile constructed using a prerecorded realistic LP neuron waveform as we have done in previous studies (Manor et al., 1997; Mamiya and Nadim, 2004; Johnson et al., 2005; Rabbah and Nadim, 2007). The LP waveform was applied to the voltage clamped LP soma periodically with a cycle period of 1 s and with a total amplitude of 26 mV (as measured in the recording of the waveform) or 39 mV (1.5 times the recorded amplitude). The scaling factor of 1.5 was used because it was the smallest scaling factor of this waveform that consistently resulted in synaptic depression in the gIPSP in both control saline and proctolin. The resulting IPSP was measured in the PD neuron in control saline or in the presence of proctolin (Fig. 7). When the measurements were done in normal saline (i.e., without TTX), the simulated action potentials of the realistic waveform resulted in real action potentials in the presynaptic LP neuron which could be seen on the extracellular recordings of the nerve lvn (Fig. 7A). As a result, the IPSP measured in these conditions was a combination of both graded and spike-mediated components. Additionally, the number and frequency of action

Figure 7. Proctolin strengthens the total LP to PD synaptic IPSP measured using prerecorded realistic waveforms in the LP neuron. **A**, After decentralizing the preparation, the LP neuron was voltage clamped at a holding potential of -60 mV and injected with a voltage profile constructed using a prerecorded realistic LP neuron waveform (V_{LP}) with a total amplitude 39 mV (1.5 times the recorded amplitude). The resulting IPSP was measured in the PD neuron (V_{PD}). The simulated action potentials of the realistic waveform resulted in real action potentials in the presynaptic LP neuron which were recorded on the nerve lvn. Baseline V_{PD} at -59 mV in Normal Saline control and -60 mV in both TTX control and TTX proctolin. Voltage scale bar applies to **A–D**. **B**, Application of the realistic waveform in TTX with total amplitude of 26 mV (same as recorded amplitude; gray V_{LP} trace) resulted in synaptic facilitation of the gIPSP in proctolin (red V_{PD} trace) but not control saline (black V_{PD} trace). In contrast, application of the waveform at 39 mV resulted in slight depression of the gIPSP both in control (gray) and in proctolin (pink). **C**, In normal saline, the LP waveform was applied periodically with total amplitude of 26 or 39 mV. The resulting IPSP, a combination of the graded and spike-mediated components, was measured in the PD neuron in control saline or in the presence of proctolin. The responses shown are after at least four cycles of the presynaptic waveform (circled in **A** and **B**) when the IPSP has reached its stationary state. **D**, In TTX, the same LP waveform was applied periodically with total amplitude of 26 or 39 mV. The resulting IPSP in this case consists only of the graded component. All responses shown in **C** and **D** were measured in the same preparation. **E**, Mean values of the IPSP as shown in **C** indicate a significant increase with both amplitude and Proc application (two-way RM-ANOVA; $p < 0.05$, $n = 5$; $p < 0.05$ in *post hoc* Tukey analysis). Ctrl indicates control saline. In **B–E**, darker colors in each condition correspond to the lower amplitude waveform (26 mV).

potentials were the same in control and proctolin because each (simulated) action potential of the prerecorded waveform elicited an action potential in the LP neuron.

To examine how much of the total IPSP was due to the graded component in the different conditions we repeated these mea-

surements in TTX. In these conditions, action potentials were blocked and therefore the spike-mediated IPSP was removed. Note that the simulated action potentials of the prerecorded waveform were still artificially played back in the voltage clamped LP neuron but, in TTX, these do not result in biological action potentials. Application of the realistic waveform in TTX with total amplitude of 26 mV (same as recorded amplitude) resulted in synaptic facilitation of the gIPSP in proctolin (Fig. 7B, red V_{PD} trace) but not control saline (black V_{PD} trace). In contrast, application of the waveform at 39 mV resulted in depression of the gIPSP both in control (gray) and in proctolin (pink). The effect of presynaptic waveform amplitude on synaptic dynamics is similar to that reported with the pulse waveforms shown in Figure 4.

We compared the IPSP amplitudes after at least five cycles of the realistic waveform application (circled region in Fig. 7A, B) to avoid measuring the transients. Increasing the amplitude of the presynaptic waveform resulted in a larger total IPSP in the PD neuron and proctolin always increased the synaptic amplitude in each preparation (Fig. 7C). On average, both waveform amplitude and the presence of proctolin significantly increased the total synaptic IPSP amplitude (Fig. 7E; two-way RM-ANOVA, $p < 0.05$, $n = 5$). In TTX, the realistic waveform at 26 mV amplitude did not produce a significant gIPSP in control saline but did so when the amplitude was increased. Additionally, proctolin increased the amplitude of the gIPSP measured with the realistic waveform (data not shown; two-way RM-ANOVA, $p < 0.05$, $n = 5$). Three conclusions can be derived from these results. First, under all conditions proctolin enhances the synaptic strength; second, in control conditions, but not in proctolin, synaptic release is mostly due to the spike-mediated component and; third, in proctolin compared with control saline, a large increase in the amplitude of the LP neuron waveform has a smaller effect on synaptic strength. This latter conclusion is consistent with the fact that in proctolin the gIPSP depresses with high-amplitude presynaptic input but facilitates with low-amplitude input (Fig. 7B).

Proctolin increases the amplitude of the LP waveform oscillations, but not by a factor of 1.5. To examine the effect on the synaptic output of the change in waveform amplitude and shape induced by proctolin under more realistic biological conditions, we used prerecorded LP neuron waveforms (Fig. 8A) recorded in control and proctolin to voltage clamp the LP neuron. Both waveforms were applied from a minimum voltage of -60 mV at the amplitudes they were recorded (Fig. 8A) and the resulting combined spike-mediated and graded IPSP was measured in the PD neuron in control saline and after bath application of proctolin. As expected, either waveform produced a significantly larger synaptic output in proctolin than control. However, the different waveforms produced similar synaptic outputs in control conditions but the proctolin waveform produced a significantly larger synaptic output in proctolin (Fig. 8B; two-way RM-ANOVA with *post hoc* Tukey analysis, $p < 0.02$, $n = 5$). The average increase of the IPSP amplitude by proctolin was $>400\%$ whereas the increase due to the change of the waveform shape and amplitude in proctolin was only 36%.

Although our characterization of the components of the LP to PD synapse under controlled conditions where we prescribe the presynaptic waveform provides insight into the actions of the modulator proctolin on this synapse, the effect of proctolin on this synapse should also be characterized under more natural biological conditions. As a first step in exploring these more biologically realistic effects, we measured the LP to PD synaptic currents during ongoing pyloric oscillations. To measure the

Figure 8. Proctolin effects on the LP to PD synapse in biological conditions. **A**, The LP neuron voltage waveform changes in shape and amplitude in proctolin. These waveforms were played back periodically at the original recorded amplitude (as shown) from a minimum holding potential of -60 mV and a cycle period of 1 s into the voltage-clamped LP neuron and the postsynaptic response (mean value) was measured in the PD neuron after reaching stationary state (Fig. 7). **B**, Bath application of Proc significantly enhanced the IPSP amplitude measured with either waveform (shown in **A**) compared with control saline (Ctrl). The proctolin-recorded waveform produced a modestly larger response only in the presence of proctolin but not in control saline (two-way RM-ANOVA with *post hoc* Tukey analysis; $*p < 0.02$; $n = 5$). **C**, Proctolin enhances the LP to PD synapse during the ongoing pyloric rhythm. The traces of extracellular recordings from lvn show the ongoing rhythm activity of the pyloric network in control and in proctolin (10^{-6} M). One of the two PD neurons was voltage clamped at a holding potential of -55 mV; the resulting synaptic currents were recorded in this PD neuron in control and in proctolin. Baseline I_{PD} at -1 nA in control and proctolin. **D**, The synaptic response in PD neuron was integrated from the end of a PD burst to the start of the following PD burst (vertical dashed lines in **A**). When necessary, DC current was injected in the second PD neuron to bring the cycle period to 1 s in all measurements. The IPSC area was significantly larger in proctolin than control or wash (one-way RM-ANOVA, $p < 0.01$, $n = 5$).

biological IPSCs, one of the two PD neurons was voltage clamped at -50 mV during ongoing pyloric activity and the IPSCs were measured in control and proctolin (Fig. 8C). Note that clamping the PD neuron at a holding potential of -60 mV stopped the ongoing pyloric rhythm because the PD neurons and the pacemaker AB neuron are electrically coupled. However, holding the voltage-clamped PD neuron at -50 mV did not disrupt the rhythm but often resulted in a slower cycle period. We matched the pyloric cycle period between control and proctolin conditions (compare the extracellular recordings from lvn) by injecting, when necessary, a small DC current in the second PD neuron to speed up or slow down the oscillation to a cycle period of 1 s. This ensured that the comparison in synaptic strength in the two conditions was not due to cycle-period-dependent factors such as short-term depression. The synaptic response in the PD neuron was measured by integrating I_{PD} from the end of the PD burst to the start of the consequent PD burst (Fig. 8C, vertical dashed line). As expected, the total biological IPSC strength was significantly

cantly larger in proctolin than control or wash (one-way RM-ANOVA, $p < 0.01$, $n = 5$, Fig. 8D).

An important functional question is how the enhancement of the LP to PD synapse by proctolin affects the pyloric rhythmic activity. It is difficult to dissect out the contribution of individual factors targeted by proctolin during an ongoing pyloric rhythm because, in addition to modulating the pyloric synapses, proctolin also modulates a voltage-gated current in pyloric neurons (Golowasch and Marder, 1992; Swensen and Marder, 2000). The effect of proctolin bath application on the pyloric cycle period and the activity phases of pyloric neurons has been previously reported (Nusbaum and Marder, 1989b). However, it is unknown to what extent these effects are due to the proctolin modulation of the LP to PD synapse. We were also interested in the effect of proctolin on the variability of the pyloric cycle period. In a previous study, we reported that the LP to PD synapse acts to stabilize the pyloric cycle period: the coefficient of variation (CV) of cycle period is significantly smaller in the presence of the synapse than when the synapse is blocked or functionally removed by hyperpolarizing the LP neuron (Nadim et al., 2011). The results of this previous report were obtained in control saline, but it is reasonable to assume that proctolin enhancement of the LP to PD synapse would further stabilize the pyloric rhythm.

A comparison between the CV of cycle period in control and after bath application of proctolin showed that the CV was indeed significantly decreased in proctolin (Fig. 9A). To find out the extent to which this decrease was due to the enhancement of the synapse, we introduced an extrinsic noisy input to the PD neuron and measured the CV of cycle period in control saline and proctolin during ongoing activity and after the LP to PD synapse was functionally removed by hyperpolarizing the LP neuron (-5 nA DC current to move the LP membrane potential below -80 mV). This noisy input was introduced as 0.5 nA, 20 ms current pulses injected into the PD neuron at discrete time intervals following a Poisson random distribution with a mean frequency of 5 Hz and was meant to mimic fast projection-neuron input to pyloric pacemaker neurons (Norris et al., 1996) or antidromic spikes from the secondary spike-generation zone of the pacemaker AB neuron (Blitz and Nusbaum, 2008). There was no significant difference in the pyloric cycle period in the presence of the noisy input in these four conditions (one-way RM-ANOVA, $n = 6$, $p > 0.1$; data not shown) and, as expected from our previous report, the CV of cycle period was significantly smaller when the LP to PD synapse was intact, both in control and in proctolin (Fig. 9B). Interestingly, the CV of cycle period was significantly smaller in proctolin compared with control but only when the LP to PD synapse was intact. There was no significant effect

Figure 9. The enhancement of the LP to PD synapse by proctolin reduces the variability in the pyloric cycle period. **A**, The CV of cycle period is significantly reduced in the presence of bath applied proctolin (Student's *t* test, $p = 0.030$, $n = 8$). **B**, The PD neuron was subjected to a noisy current input (0.5 nA, 20 ms voltage pulses applied at discrete time intervals following a Poisson distribution with mean frequency of 5 Hz). Functional removal of the LP to PD synapse (−5 nA DC current in LP) significantly increased the CV in both control and proctolin. The CV in proctolin was significantly smaller than control only when the LP to PD synapse was functionally intact (two-way RM-ANOVA with *post hoc* Tukey analysis; $*p < 0.05$; $n = 6$). **C**, The pyloric cycle period was perturbed with a single 2 nA, 50 ms pulse was injected into the PD neuron once every 10 or so cycles at different phases of the cycle. The absolute value of the change in cycle period ($|\Delta P/P|$), independent of the stimulus phase, was compared in different conditions. Functional removal of the LP to PD synapse significantly increased $|\Delta P/P|$ in both control and proctolin. The value of $|\Delta P/P|$ in proctolin was significantly smaller than control only when the LP to PD synapse was functionally intact (two-way RM-ANOVA with *post hoc* Tukey analysis; $*p < 0.05$; $n = 7$). **D**, Adding the proctolin-induced enhancement of the LP to PD synapse using dynamic clamp. Traces of extracellular recordings from *Ivn* show the ongoing pyloric rhythm activity. Intracellular traces recorded in LP and PD neurons show the rhythmic alternation of the PD and LP neurons. Left panel (black) shows pyloric activity in Control. Using dynamic clamp, the LP to PD synapse was enhanced (right panel, red; see Materials and Methods for equations). **E**, The CV of cycle period was calculated over 40 consecutive cycles from the nerve pdn to measure the effect of the dynamic-clamp enhanced synapse. The variability of the pyloric rhythm period was significantly reduced when the LP to PD synapse was enhanced (Student's *t* test, $p = 0.022$, $n = 5$; experiments in **A–C** and **E** are nonoverlapping).

on the CV in proctolin compared with control when the LP neuron was hyperpolarized (Fig. 9B; two-way RM-ANOVA, $n = 6$).

In a separate set of experiments, we used a different perturbation of the pyloric oscillation by injecting a single brief current pulse (2 nA, 50 ms) at different phases of the cycle and measured the change in cycle period compared with the average of the previous three cycles ($\Delta P/P$, as used to measure the phase-response curve). We found a similar result that this perturbation affected the cycle period significantly more ($|\Delta P/P|$ was larger) when the LP neuron was hyperpolarized (thus the LP to PD synapse was functionally removed) in both control saline and proctolin and the effect of the perturbation was significantly larger in proctolin compared with control saline only when the LP to PD synapse was intact (Fig. 9C). Together, these data imply that the effect of proctolin in stabilizing the pyloric cycle period is due to its enhancement of the LP to PD synapse.

To examine this prediction directly, we used the dynamic clamp technique to modify the LP to PD synapse by incorporating the enhancing effects of proctolin (see Materials and Meth-

ods) during ongoing oscillations in control saline. An example of the dynamic-clamp-enhanced LP to PD synapse is shown in Figure 9D. We found that the enhanced synapse did not significantly change the activity phase of pyloric neurons or the duty cycle or the PD neuron and resulted in a very small but significant increase in cycle period (from 720 ± 74 ms to 746 ± 72 ms; paired Student's *t* test, $p < 0.05$, $n = 5$). However, as expected, the enhancement of the synapse with dynamic clamp resulted in a significant reduction in the CV of the pyloric cycle period (Fig. 9B; paired Student's *t* test, $p < 0.05$, $n = 5$).

Discussion

Neuromodulation of synaptic output during ongoing network activity depends on modifications of the activity patterns, synaptic strength and its short-term dynamics. We show that the strength and short-term dynamics of the LP to PD synapse in the oscillatory crab pyloric network are modulated by the neuropeptide proctolin. By implementing the modification of the synaptic

strength using dynamic clamp we find that the enhancement of this feedback synapse to the pyloric pacemaker neurons reduces the variability in the pyloric cycle period, a result that is also supported by direct measurements of pyloric rhythm variability in the presence of proctolin.

Modulatory actions of proctolin

Several released neuromodulatory peptides are known to elicit distinct pyloric rhythmic patterns (Marder and Thirumalai, 2002) and their network actions have been found to be dose- and frequency-dependent (Nusbaum, 2002). Among the best studied of these neuropeptides is proctolin: all proctolinergic modulatory projection neurons in the crab STNS are known and the network actions of many of these neurons have been characterized (Nusbaum and Beenhakker, 2002). Proctolin strongly excites and modifies the pyloric rhythm (Marder et al., 1986; Hooper and Marder, 1987; Nusbaum and Marder, 1989b), an effect that is at least partly due to the activation of a voltage-dependent nonspecific cation channel in pyloric neurons (Golowasch and Marder, 1992; Swensen and Marder, 2001). However, the effect of proctolin on the pyloric synapses has not been previously examined.

Proctolin exerts its effects through binding to G-protein coupled receptors and subsequently activating downstream signaling pathways (E. C. Johnson et al., 2003). Studies in insect and crayfish muscle have shown that using second messengers IP_3 and cAMP, proctolin can increase intracellular calcium concentrations by modulating voltage-dependent or independent channels (Baines et al., 1990; Bishop et al., 1991; Wegener and Nässel, 2000). Proctolin, however, does not appear to cause an increase of cAMP levels in STG neurons (Flamm et al., 1987; Hempel et al., 1996) and little is known about the second messenger pathways underlying the modulatory actions of proctolin in the STNS.

Neuromodulation of short-term synaptic dynamics

Proctolin unmasks an amplitude-dependent heterogeneity in the short-term dynamics of the LP to PD synapse: the graded component remains depressing with high-amplitude presynaptic stimuli but becomes facilitatory with low-amplitude stimuli (Fig. 3). While the effects of modulators on synaptic dynamics are documented (Fischer et al., 1997a; Gil et al., 1997; Bristol et al., 2001; Logsdon et al., 2006; Sakurai et al., 2006; Giocomo and Hasselmo, 2007; Parker and Gilbey, 2007; Sakurai and Katz, 2009), only a few studies have shown a modulator to switch the direction of synaptic dynamics from depression to facilitation (Parker, 2003; Baimoukhametova et al., 2004; Bevan and Parker, 2004; Barrière et al., 2008). The switch in synaptic dynamics in these studies has been proposed to be a modulator-dependent change in the initial probability of release p from high (depression) to low (facilitation). The proctolin-induced facilitation of the LP to PD synapse is different from these examples of classical synaptic facilitation which depend on the timing of the presynaptic activity and not its amplitude. Yet a similar mechanism may be at work in the proctolin modulation of the LP to PD synapse as well. Due to the graded nature of this synapse, however, the value of p in the presence of proctolin may depend on the amplitude, not frequency, of the presynaptic stimulus.

Short-term facilitation is generally governed by the properties of the presynaptic neuron (Zucker and Regehr, 2002). Our results also support a partly presynaptic mechanism because the increase in the synaptic strength in response to a train of pulses is correlated with the slow activation of a presynaptic Mn^{2+} -sensitive inward current (Fig. 4). This current is likely to be a calcium

current activated by proctolin, although a definitive proof requires additional experiments beyond the scope of this study. Proctolin actions on the LP to PD synapse may be due to the modulation of a low-threshold voltage-gated calcium current. Yet, other mechanisms may underlie the appearance of the putative calcium current. For example, as mentioned above, proctolin activates a nonspecific cation channel in pyloric neurons which is presumed to have a pore block by calcium ions (Golowasch and Marder, 1992; Swensen and Marder, 2000). It has been shown that ions that produce pore block—such as Mg^{2+} in NMDA channels—can also permeate into the cytoplasm (Stout et al., 1996). This allows the possibility that the proctolin-activated channel has some permeability for calcium, whose accumulation leads to the observed synaptic facilitation. These two hypothetical mechanisms could be distinguished by imaging calcium entry in the presynaptic LP neuron in the presence or absence of calcium channel blockers.

The role of baseline membrane potential on spike-mediated transmission

The strength of the spike-mediated component of the LP to PD synapse is dependent on the baseline presynaptic membrane potential from which action potentials are generated (Fig. 5). Such a relationship has been shown in many systems including mammalian central synapses (Nicholls and Wallace, 1978; Ivanov and Calabrese, 2003; Alle and Geiger, 2006; Ivanov and Calabrese, 2006b; Shu et al., 2006). In leech heart interneurons, it is known that low-threshold calcium currents activate the graded component of synaptic release, whereas spike-mediated synaptic transmission is triggered by high-threshold calcium currents (Angstadt and Calabrese, 1991; Lu et al., 1997). In these neurons, an increase in the background calcium levels at the presynaptic site due to the membrane potential depolarization is correlated with the increase in the amplitude of the spike-mediated IPSPs (Ivanov and Calabrese, 2006b). We suspect a similar mechanism to be at work for the LP to PD synapse although, unlike the leech synapses where Cd^{2+} specifically blocks the high-threshold calcium current and thus only spike-mediated release, Cd^{2+} blocks both components of the LP to PD synapse and cannot be used to test this prediction. The modulatory enhancement of the spike-mediated component by proctolin without any effect on the short-term dynamics may be due to an increase in the background calcium levels by proctolin modulation of voltage-gated channels (see above) or calcium release from internal sources.

The effect of proctolin on combined transmission using realistic waveforms

Graded transmission is the major form of synaptic communication among pyloric neurons (Raper, 1979; Graubard et al., 1983; Hartline et al., 1988; Manor et al., 1997). Yet, many pyloric synapses have a strong spike-mediated component. Interestingly, when we played back the LP neuron realistic waveform at the amplitude which it was recorded, we saw little graded transmission in control conditions yet this synaptic component was clearly present in proctolin (Fig. 7C). It is therefore possible that under different modulatory conditions one or the other component of synaptic transmission is dominant in affecting the total synaptic strength.

It is common for neuromodulators to change the activity waveform of bursting neurons (Marder and Thirumalai, 2002). Proctolin, for example, increases the amplitude of the LP neuron burst waveform and its spike frequency (Hooper and Marder, 1984; Nusbaum and Marder, 1989b). The LP to PD synapse is

significantly enhanced if the amplitude of the LP waveform is increased, independent of any direct actions of proctolin on the synapse (Fig. 7). This enhancement is due both to the increase in strength of the graded component with presynaptic voltage amplitude and because the spike-mediated component is enhanced when the baseline membrane potential is increased. However, proctolin also directly enhances these two synaptic components. Thus, during ongoing oscillations, the proctolin enhancement of the LP to PD synapse results from a combination of direct modulation as well as enhanced release due to the change in the presynaptic waveform amplitude and shape.

Network consequences of proctolin enhancement of the LP to PD synapse

Many oscillatory networks involve pacemaker neurons that receive rhythmic inhibitory feedback (Ramirez et al., 2004; Marder and Bucher, 2007). Previous experimental studies of the pyloric network have shown that the inhibitory LP to PD feedback synapse may have little effect on the average pyloric cycle period (Mamiya and Nadim, 2004; Nadim et al., 2011) even if the synapse is drastically strengthened by a neuromodulator (Thirumalai et al., 2006). These studies proposed that such feedback inhibition may act to stabilize the pyloric cycle period in response to perturbing inputs. Here we show that the variability of the pyloric cycle period is significantly decreased by proctolin and that this stabilizing effect is a direct consequence of the pyloric modulation of the LP to PD synapse (Fig. 9). Feedback inhibition plays an important role in generating and regulating oscillatory networks (Hentall and White, 1997; Mann et al., 2005; Martinez, 2005; Blitz and Nusbaum, 2008) and has been shown to be involved in promoting stability and synchronization in hippocampal gamma oscillations (Stenkamp et al., 2001; Bartos et al., 2007). Our findings therefore indicate that neuromodulation of feedback inhibition may be a regulatory mechanism for producing stable oscillations in a variety of networks ranging from the respiratory network to thalamocortical oscillations during sleep.

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