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Effects of neuromodulator RPCH on short-term dynamics of a key synapse in an oscillatory network of crab *Cancer borealis*

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Abstract

Central pattern generators (CPGs) generate the rhythmic movements in vertebrates and invertebrates. CPGs must be able to produce stable patterns to produce the rhythmic movement, and be flexible to adapt to the changing environment. CPGs are subject to activity-dependent plasticity and their activity is modified by neuromodulatory substances. Neuromodulators alter the intrinsic properties and synaptic strength. This alteration may then modulate the flexibility of the motor patterns.

The pyloric network of the crustacean stomatogastric nervous system is a well-studied CPG that is subject to extensive neuromodulation. Effects of neuromodulators on intrinsic properties of neurons have been studied in great detail, but the effects of neuromodulators on the short-term plasticity of the synapses are overlooked. Therefore, the main goal of this study was to examine the role of neuromodulator RPCH on the short-term dynamics of pyloric LP to PD synapse, a key synapse, by using the crab *Cancer borealis* as a model system. Our results showed that, RPCH strengthened the LP to PD synapse in a dose-dependent manner, depressed the synapse, but did not affect the peak phase.

Results of this study promote the understanding of how modulation of short-term synaptic dynamics of a key synapse affects the generation and the control of the pyloric rhythm. These results will help to better understand the mechanisms underlying pattern generation by CPGs in general. Moreover, such an understanding of modulation of a circuitry used in rhythmic behaviors should lead to treatments of certain illnesses or injuries such as spinal cord injury.

Keywords: synaptic plasticity, depression, stomatogastric, crustacea, central pattern generator, neuromodulator

INTRODUCTION

Background and Significance

Central pattern generators (CPGs) are networks of neurons that generate the timing and phasing cues for rhythmic movements in vertebrates and invertebrates. CPGs must be able to produce stable patterns to produce the rhythmic movement, but at the same time they must be flexible to adapt to the changing environment. The dynamics of CPGs are regulated by the intrinsic properties of the neurons and the strength, time course, and time- dependent properties of the synapses they form [1]. The synapses in a CPG are dynamic and respond to different patterns of input with short- and long-term changes in strength [2].

Long-lasting alterations in synaptic strength, lasting hours to days are thought to underlie learning and memory formation. In addition to long-lasting plasticity, most synapses show some form of short-term plasticity lasting from milliseconds to seconds. This latter form of synaptic plasticity may be important for the network to produce the correct output in response to changes in activity and possibly to initiate the changes necessary for long-term plasticity [1]. Short-term depression and short-term facilitation are the two forms of short-term plasticity. Short-term depression is a process that may affect synaptic strength rapidly in a use dependent manner. It is generally believed that the synapses with an initial low release probability are more likely to show facilitation whereas synapses with high release probability are more likely to show depression consistent with a depletion of vesicles from the readily releasable pool [3, 4].

Neuromodulators such as monoamines and peptides play a central role in determining the output of a CPG by altering the synaptic strengths, therefore synaptic plasticity, and intrinsic cellular properties of the circuit neurons, thereby having an important role in achieving both a stable and a flexible operation [5, 6, and 7]. It has been determined that the actions of neuromodulators are also important for learning and memory formation sensory processing [8], and motor pattern generation [8, 9, and 10]. The stomatogastric nervous system (STNS) of the crab *Cancer borealis* is an excellent system to study the effects of neuromodulators on motor pattern generation. Despite a few studies about the effects of neuromodulators on the synapses [11, 12], effects of neuromodulators on the synaptic dynamics and their functional role in shaping the network output in crustaceans remain unknown. Therefore, the main goal of this study was to investigate the role of neuromodulators

in altering short-term synaptic plasticity using the pyloric circuit of the crab *Cancer borealis* as a model system. The working hypothesis is, "neuromodulators, specifically RPCH may affect the short-term dynamics of the LP to PD synapse and these effects, if any, in turn may change the pyloric network output".

Overview of the pyloric circuit

The pyloric circuit

The stomatogastric nervous system of the crab *Cancer borealis* consists of four ganglia: the paired commisural ganglia (CoG), the esophageal ganglion (OG) and the stomatogastric ganglion (STG). The set of CPGs within these ganglia control the movement of the stomach and different feeding behaviors of the animal. The best understood CPGs in this set are the pyloric and the gastric mill circuits. These CPGs control the movements of the pylorus (filtering chewed food), and the gastric mill (chewing). The pyloric and gastric mill rhythms are generated within the STG, which contains only 25-26 neurons. The pyloric circuit is composed of 11 neurons in crab *Cancer borealis*. It is responsible for the movement of the pylorus, which is a part of the stomach that filters the food after it has been chewed [13, 14]. The pyloric rhythm is a tri-phasic rhythm that is composed of alternate bursting of neurons that dilate (the Pyloric Dilator or PD and the Ventricular Dilator or VD neurons) and constrict (the Lateral Pyloric or LP, the Pyloric Constrictor or PY, and the Inferior Cardiac or IC neurons) the pyloric chamber. Fig.1 shows a schematic drawing of the pyloric circuit and the extracellular recordings of lvn and intracellular recordings of the two neurons, LP and PD. The AB neuron is the only interneuron in the pyloric network. It is electrically coupled to the two PD neurons. These three neurons burst in phase, and are named the "pacemaker ensemble". Each burst of impulses in AB-PD defines the beginning of a new cycle of the pyloric rhythm. The AB and the PD neurons directly inhibit all other pyloric neurons and suppress their activity during the AB-PD burst. Following the AB-PD burst, other pyloric neurons rebound from inhibition. The LP neuron bursts earlier in phase than the PY neurons and the LP burst is terminated by the PY bursts. The PY burst is then terminated by the next AB-PD burst and the cycle repeats. The IC neuron is coactive with the LP neuron, whereas the VD neuron is coactive with the PY neurons and the PD neuron is coactive with the LPG neuron. In general, the cycling frequency of the pyloric rhythm is in the range of 0.5 to 2 Hz and can maintain a similar tri-phasic rhythm over this range [13, 14].

Pyloric synapses

The neurons in the pyloric circuit are coupled through both electrical and chemical synapses. Many of these chemical synapses are independent of action potentials and they work by graded transmission [15, 16]. In these synapses, the threshold for transmitter release is close to the resting membrane potential of these neurons [15]. The pyloric pacemaker ensemble is inhibited by the follower neuron LP. It is the sole chemical feedback synapse to the pacemaker group. Although the graded synapses are the predominant form of transmission among central synapses of the STG, the pyloric network output is transferred to the muscles and to the other central ganglia by action potentials. All the synapses in the circuitry are glutamergic except the synapses from PD and VD that are cholinergic. All the chemical synapses within the pyloric circuitry are inhibitory. In this study, the AB and PD neurons were treated as one unit. Although the synapse from the LP neuron to the PD neuron is the sole chemical synapse to the pacemakers, other synapses that affect the shape of the LP waveform may have indirect effects in frequency regulation of the pyloric rhythm. These indirect effects were not studied in this study.

In this study, graded synaptic transmission is studied for several reasons. First, many invertebrate sensory and motor networks use graded synaptic transmission as the main source of the inhibition [2, 17, 18]. Second, it has previously been shown that in the absence of the spike-mediated component, it is possible to produce a triphasic pyloric rhythm [19]. Third, in the pyloric network, spikes always occur on top of plateau potentials that cause graded synaptic transmission. Fourth, it has been shown that the postsynaptic response is almost the same in response to a waveform, which has spikes and the filtered version of the same waveform [2].

All the synapses in the pyloric circuitry show short-term depression. In the STG, regulation of synaptic depression may play a pivotal role in the control of pyloric cycle period. Under steady state conditions, the LP to PD synapse is depressed and the pacemaker ensemble controls the pyloric frequency. However, it has been postulated that in some modulatory conditions the feedback synapse may be strengthened to control the pyloric cycle frequency [20]. Although a few studies have looked at the dynamics of depression in detail [2, 21], none of these studies have examined the effects of neuromodulators on the short-term synaptic dynamics. Very few studies have examined the effects of the neuromodulation on the synapses in the pyloric circuit [11, 16]. Therefore, the functional role of neuromodulators on short-term synaptic dynamics in the pyloric circuit remains to be investigated.

The advantages of working on the pyloric circuit

One very well explored system for the study of behavioral choice and pattern generation has been the pyloric circuit of the STG. There are great features of this system that make it an ideal preparation to study the functional role of the neuromodulators on short-term synaptic dynamics in the CPG. First, the anatomy of the system and its functional properties, such as neurotransmitters, reversal potentials, threshold, are known and all STG neurons are readily recorded and identified in extra- and intracellular recordings, thus allowing for long-term monitoring of the circuit activity. Second, when the nervous system is removed from the animal, the circuits of the STG continue to generate the

rhythmic patterns that underlie motor programs. This allows us to hypothesize the role of short-term dynamics of the synapse in vivo based on the results gathered from the *in vitro* preparation. Third, the small number of neurons and synapses, the extensive neuromodulation and the great number of activity that results from neuromodulation allow us to study the effects of neuromodulation on short-term dynamics of a synapse easily. Fourth, the neurons in the STG have large diameters (25-120 mm), allowing for long and simultaneous recordings from several neurons at the same time. Finally, the synapses in the pyloric circuit show short-term synaptic depression.

The characteristics listed above make it possible to manipulate a specific synapse in a controlled manner to study its dynamics, neuromodulation and subsequent role in shaping the network output, using the in vivo like pattern. Therefore, the insights obtained from this small neuronal network can be extended to any neuronal network where synaptic dynamics are functionally significant.

Neuromodulation in the pyloric circuitry

The stomatogastric nervous system of the crab *Cancer borealis* is subject to extensive neuromodulation [14, 22]. The two CoGs and the single OG provide most of the modulatory inputs to the STG. Researchers have identified approximately 19 different neuromodulatory substances in the STNS. These neuromodulators fall into two categories: neuropeptides and neuroamines, and are found in approximately 20 pairs of modulatory axons in the STNs. Bath application of these neuromodulators can initiate or modulate the pyloric or gastric rhythm in the isolated STG, and the presence of many neuromodulators in the system raises the possibility of modulation of the short-term synaptic dynamics in this system.

The neuromodulator RPCH

RPCH (red pigment concentrating hormone) is an octapeptide (pGlu-Leu-Asn-Phe-Ser-Pro-Gly-TrpNH2) isolated from the shrimp *Pandulus borealis* [23]. The sequence of RPCH is quite similar to the adipokinetic hormones (AKHs) in insects. The AKH/RPCH family is mainly involved in influencing energy metabolism in insects regulating carbohydrate and lipid breakdown in the fat body. RPCH has also been shown to have actions on crustaceans. These actions include: pigment concentration in one or more types of chromothophores, dark-adaptational screening pigment movement in distal eye pigment cells, increase of retinal sensitivity and neuromodulation [24]. RPCH-like immunoreactivity has been found in the stomatogastric nerve stn and in processes throughout the neuropil of the STG. RPCH-like immunoreactivity has also been detected in the CoGs and the OG [25]. The sinus gland (a neurohemal organ in the crustacean eyestalk), the brain, the thoracic ganglia and the commisural ganglia are the main sources of RPCH.

RPCH was shown to have effects both as hormone and neuropeptide. RPCH has been shown to have functions as a circulating hormone [13] and circulating hormones can have effects on the movements triggered by the stomatogastric nervous system by altering the neurally evoked motor patterns. Migration of pigments in the erythrophores of crustaceans is regulated by RPCH via increasing calcium influx. RPCH was later shown to have functions other than its hormonal function. First, it was shown that RPCH immuno- positive cells of the brain and thoracic ganglia did not appear to be classical neurosecretory cells in the crab *Carcinus maenas* [26]. Later, it was shown that RPCH-like immunoreactivity existed in the neuropilar processes of the STG of the crab *Cancer borealis*, and furthermore these investigators found that RPCH increased the cycle frequency of the rhythm [25]. It was also shown that RPCH activated the LP and the PD neurons in *Cancer borealis, Panulirus interruptus* and *Homarus americanus* [25, 27].

State- and dose-dependence of neuromodulation in the pyloric circuit

The activity mode of a CPG may also depend on different concentrations of neuromodulators. In early studies it has been shown that the gastric mill activity depends on the different doses of proctolin and octopamine in *Panulirus interruptus* [28]. Injections of 10^{-6} M proctolin elicited chewing in the squeeze mode and 10^{-4} M triggered chewing in the cut-and-grind mode. Very few studies have examined the dose-dependent effects of neuromodulators on the individual components (such as neurons and the synapses between them) and at the network level [29, 30]. These studies showed that the pyloric activity pattern changed in a dose-dependent manner in octopamine and dopamine and the follower LP neuron was excited in different-doses of proctolin. The effects of neuromodulators in a dose–dependent manner on the individual neurons and synapses are yet to be studied in detail.

Therefore, this study focused on the dose-dependent effects of the neuromodulator RPCH on the short-term dynamics of the LP to PD synapse, and intended to increase the understanding of the roles of neuromodulators in generating network flexibility. The reason of studying the dose-dependent effects is that neuromodulators are released from the neurons at very high concentrations and when they reach to the neuropil their concentration may be low due to the size of the neuropil.

This approach contrasts with previous studies, which have examined neither the effects of neuromodulators on short-term synaptic plasticity, nor how these changes in short-term plasticity, if any, affect the overall network output. Also, ideas from this study may be applicable to any system in which short-term synaptic plasticity and neuromodulation exist.

MATERIALS AND METHODS

Isolation of the complete STNS and identification of the neurons

Experiments were conducted on the stomatogastric nervous system of the crab *Cancer borealis*. Animals were obtained from local markets (Newark, NJ) and maintained in filtered, re-circulating seawater tanks at 10-12 °C until used. The stomatogastric nervous system (STNS), which included the stomatogastric ganglion (STG) and the esophageal and paired commisural ganglia that provide modulatory input to the STG, was dissected out using standard procedures [31]. The isolated complete STNS was pinned down on a Sylgard-coated Petri dish and desheathed to allow penetration of the cell bodies. All preparations were continuously super-fused with chilled (11-13°C) physiological saline (containing in mM: KCl; 11, NaCl; 440, $CaCl_2.2H_20$; 13, $MgCl_26H_2O$; 26, Trizma base; 11.2, Maleic Acid; 5.1, pH=7.4-7.5).

Modulators

RPCH (Bachem) was dissolved as stock solution in distilled water to give a final concentration of 10^{-3} M and frozen in smaller quantities. The desired concentration was made from the stock solution immediately before use. RPCH was bath applied by means of a switching port in a continuously flowing superfusion system at the concentrations of 10^{-9} M to 10^{-5} M for dose response experiments. RPCH was applied at concentration of 10^{-6} M for the rest of the experiments.

Electrophysiology

Extracellular recordings from nerves were made using stainless steel wire electrodes from identified nerves. The signals were amplified using a Differential AC amplifier model 1700 (A-M systems, Carlsborg, WA). Intracellular recordings were made from the soma of the cells using Axoclamp 2B amplifiers (Axon Instruments, Foster City, CA) in either single-electrode current clamp, or two-electrode voltage clamp (TEVC) modes. Glass microelectrodes for intracellular recordings were pulled by Flaming-Brown micropipette puller (Sutter Instruments, CA) and filled with 0.6 M K2SO4 and 20 mM KCl (potassium sulfate electrode). Electrode resistances ranged from 20-40 MW. All signals were digitized at 4 kHz using a PCI-MIO-16E-1 board (National Instruments, Houston, TX) and a custom made recording software written in CVI (National Instruments, Houston, TX) and stored on the PC. Pyloric neurons were identified according to their stereotypical axonal projections in identified nerves using conventional techniques [31].

Construction of realistic waveforms. The membrane potential oscillations of the LP

neurons were recorded in normal saline and used both as is (non-filtered realistic waveforms) to study spike mediated synaptic transmission and after low-pass filtration of the action potentials at 10 Hz to remove action potentials (filtered realistic waveforms) to study graded synaptic release. This low-pass filter will limit the bandwidth of the data by rejecting signals (action potentials) and noise above 10 Hz. This procedure was repeated for different LP neurons in numerous preparations and a library of these realistic waveforms was constructed. A representative waveform was chosen to test the temporal dynamics of the synapse. This waveform was chosen because it was qualitatively similar to LP waveforms recorded in other experiments.

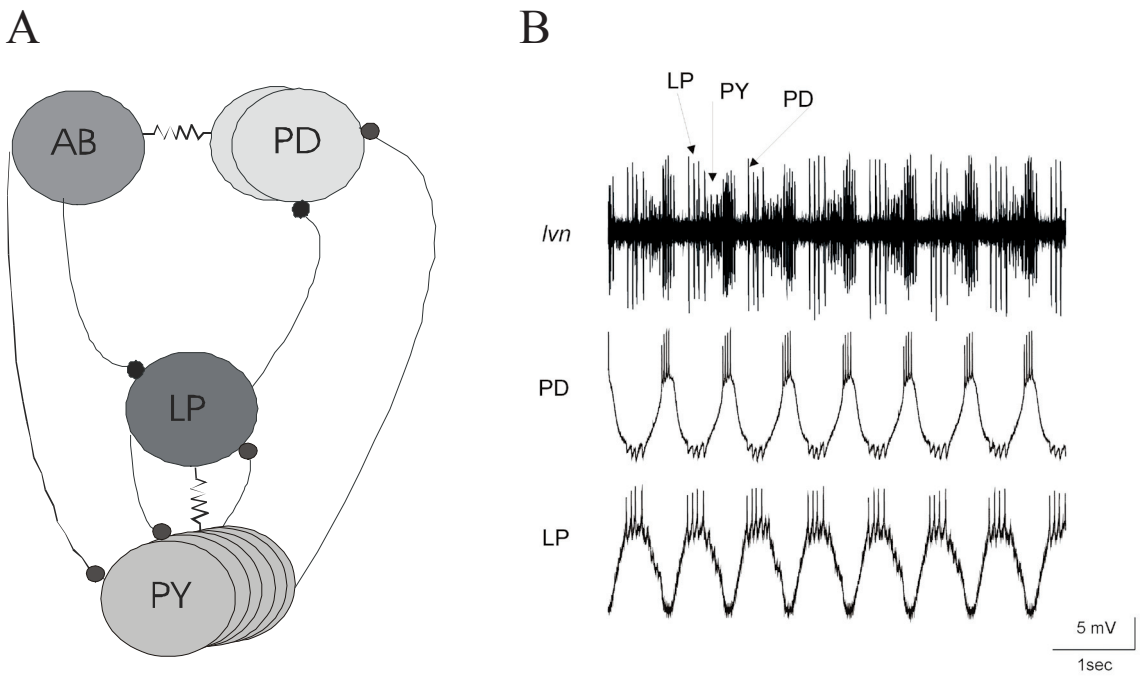

Fig 1. The pyloric network. A. Schematic drawing of the reduced pyloric circuit in crab, Cancer borealis. The IC, VD and LPG neurons are not shown. B. An example of tri-phasic pyloric rhythm. Extracellular recording from the lateral ventricular nerve (lvn) is shown on the top trace. Intracellular recordings of the LP and PD neurons are shown on the bottom traces.

Non-filtered and filtered waveforms were played back into the voltage-clamped LP neuron from a holding potential of –60 mV. The amplitudes of the non-filtered waveforms were amplified such that the envelope of the slow oscillations matched that of the filtered waveforms. A reference point was picked at the envelope, and the amplitude of the waveform was divided by the amplitude of the reference point. This was done both for the filtered and non-filtered waveforms, and the value obtained from the filtered waveform was divided by the value obtained from the non-filtered waveform. The amplitude of the waveform that would be used then multiplied by this value. For example, the amplitude of the representative filtered-waveform was 32 mV and the reference point was 16 mV. The amplitude of the non-filtered –waveform was 37 mV and the reference point was 25 mV. The values obtained from dividing the amplitude by reference point were 2 (non-filtered) and 1.68 (filtered). Then it 2 was divided by 1.68. This value then multiplied by the amplitudes chosen (20, 40, 60 mV) to be injected in the PD neuron.

Comparison of waveforms. In this study, the LP neuron was injected with different waveforms for variety of reasons. First, to study the effects of neuromodulators on graded transmission, the LP neuron was stimulated with single square pulses at a fixed duration (2000 msec) with increasing amplitudes (10-50 mV). This stimulation enabled us to create input/output curve to characterize the effects of neuromodulators on the synaptic strength. Second, a train of 500 msec, 40 mV multiple pulses were injected into the LP neuron and varied the duration between interpulse intervals (250-2000 msec) to study the short-term dynamics (depression, recovery from depression, recovery time from depression). Third, the LP neuron was injected with 40 mV sinusoidal waveforms to study the effects of neuromodulators on frequency and steady state gIPSP amplitude. A previous study revealed that the amplitude of the PSP depends on the slope of the rising phase of the presynaptic depolarization [2]. Finally, the realistic LP waveforms that are closest to the ongoing rhythm were used. Cable properties may not enable a presynaptic neuron that is voltage clamped at the soma to be fully space-clamped, and square pulses would make this effect more significant. Realistic waveforms with a smooth shape and slow rise time of the envelope minimize these presynaptic cable properties. The postsynaptic response to a realistic LP waveform might be different from the response to a square pulse. Square pulses are widely used, but not the best to underlie the short-term dynamics. Moreover, it has been indicated that it was important to use realistic waveforms when studying graded transmission [2, 21]. In an ongoing rhythm, the pyloric neurons show smooth envelopes of slow-wave depolarizations with action potentials on them. Since our interest is the graded transmission, the LP neuron realistic waveforms to mimic realistic oscillations in the LP neuron were used. Also, the response to filtered LP waveforms injected into the LP neuron with the response to non-filtered waveforms were compared in order to study the additional effect of presynaptic action potentials on the IPSP.

Measuring gIPSPs in the PD neuron. In this study, only graded synaptic transmission was focused on, which has been shown to be the main component of synaptic release and thus key in controlling network dynamics [2]. It is also involved in the production of the tri-phasic pyloric rhythm. For this reason, after identification of the neurons, the preparation was super-fused with 10^{-7} M TTX (Biotium, Hayward, CA) in order to block sodium inward currents, action potentials and action potential mediated synaptic transmission in the ganglion and to block release of modulators from projection axons, thereby abolishing pyloric activity and giving us a better control of the neurons' membrane potentials. In order to activate the LP to PD synapse, the LP neuron was voltage clamped with two electrodes at –60 mV (approximate resting membrane potential of the LP neuron) in TTX and stimulated with multiple square pulses, sine waves, and LP waveforms at different amplitudes (20, 30, 40, 60 mV) and interpulse intervals (100-4000 ms). Different frequencies (0.5-10 Hz) of sinusoidal and realistic waveforms were used. The resulting graded inhibitory post-synaptic potentials (gIPSPs) were recorded from the PD neuron in current clamp mode in control and in the presence of RPCH. This protocol was repeated two times for each type of injection and the resulting gIPSPs were averaged over the two repetitions. A 20 second interval was allowed between the two injections to allow the synapse to completely recover from any form of short-term synaptic plasticity. The PD neuron resting membrane potential were in the range of –55 mV± 5 mV in all preparations.

Effects of RPCH on the ability of the neuron in controlling the cycle period. The LP neuron was impaled with one electrode and injected with hyperpolarizing and depolarizing currents (-5nA, 5nA) with increasing periods to drive the pyloric rhythm, and the LP neuron activity was monitored extracellularly from the lateral ventricular nerve (*lvn*) and the PD neuron activity was monitored from the pyloric dilator neuron (*pdn*) and *lvn*. Fifteen cycles of continuous injections of these hyperpolarizing and depolarizing currents were used. With each cycle, the duration of the depolarization injection was kept constant (300 msec), but the duration of the hyperpolarization injection was increased until the PD neurons stopped entraining. Experiments were repeated in three conditions: intact control (IC) saline, front-end cut stn control (FECC) saline, and RPCH.

Analysis and Statistics of Data. The acquired data were saved as individual binary files and detection of the peak time, amplitude of the postsynaptic response, averaging the synaptic response and all other analysis were done by custom made programs written in C in Linux. Statistical tests were done using Statistica (Stat Soft, Tulsa, OK) and Origin (Northampton, MA).

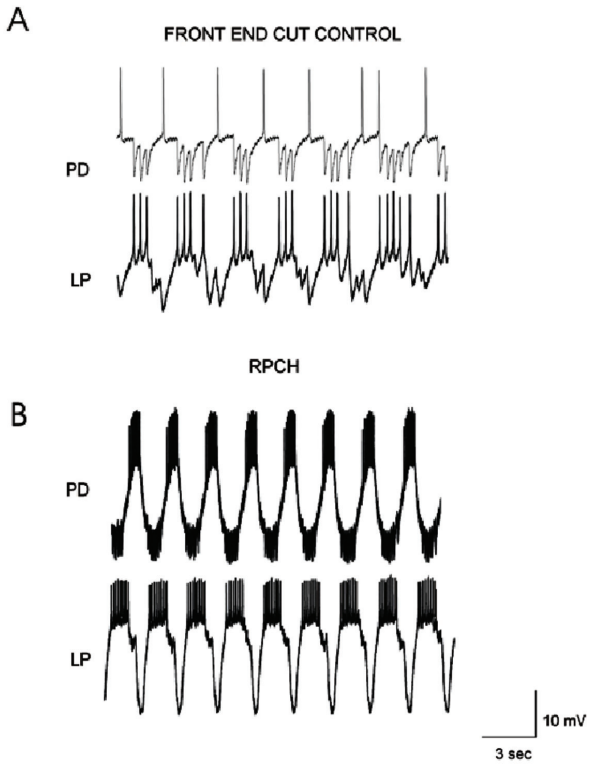

Figure 2. RPCH activates rhythmic pyloric activity in a quiescent preparation. A. Intracellular recordings of the LP and the PD neurons in control saline after stn is cut (top panel). B. Bath application of 10-6 M RPCH restored the LP and the PD neuron activity (bottom panel).

RESULTS

The graded LP to PD postsynaptic potential (gIPSP) was strengthened in a dose-dependent manner in RPCH

Modulation of the intrinsic membrane properties of the STG neurons can result in a change of the functional strengths of the synaptic connections between these neurons. Neurons of the STG release their neurotransmitter via graded synaptic transmission, which depends on membrane potential. Alterations in the membrane potential of the presynaptic neuron may change the amount of neurotransmitter released from that neuron and hence subsequently affect the magnitudes of the graded inhibitory postsynaptic potentials (gIPSPs) evoked in the postsynaptic neuron. Therefore, to study the effects of RPCH on the graded transmission of the LP to PD synapse, the LP neuron was two-electrode voltage clamped and stimulated with various types of injection protocols. The gIPSPs in the PD neuron were recorded in current clamp mode in control and in the presence of RPCH (10^{-9} M to 10^{-5} M). Different concentrations of RPCH was studied because of the possibility that different metabotropic receptors show sensitivity to different concentrations [32].

Figure 2A shows intracellular recordings both the LP and the PD neurons in front-end-cut control. Figure 2B shows intracellular recordings both the LP and the PD neurons in RPCH.

Figure 3A shows the traces of gIPSPs in the PD neuron in response to a 40 mV depolarization step in control and in different doses of RPCH (10^{-9} M to 10^{-5} M). RPCH caused an increase in the gIPSP amplitude in a dose-dependent manner thereby strengthening the LP to PD synapse. This was also true for other depolarization steps. In order to quantify the strength of the LP to PD synapse in RPCH, the peak amplitudes of the gIPSPs versus the depolarization steps were plotted in the LP neuron (10-60 mV) in increasing RPCH doses (Fig. 3B, two-way ANOVA, $p<0.05$, N=12). All the curves exhibited a sigmoidal dependence on the presynaptic depolarization and RPCH was shown to strengthen the LP to PD gIPSPs by shifting the synaptic input-output curve by 6 mV and increasing the slope of the I/O curves in every RPCH dose.

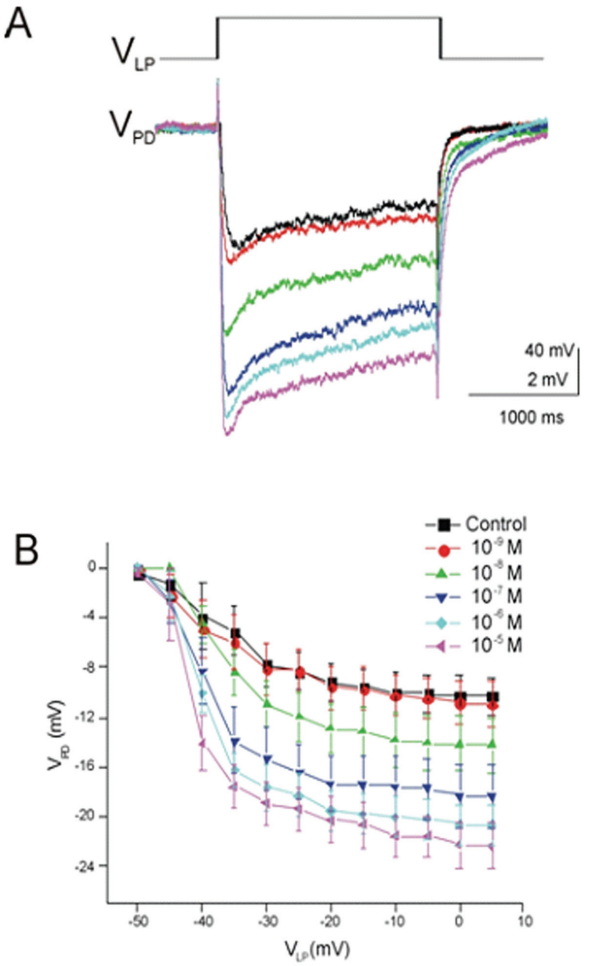

Figure 3. The LP to PD synapse was strengthened in a dose-dependent manner in RPCH. A. The LP neuron membrane potential was voltage clamped at –60 mV in TTX. The LP neuron was stimulated with single square pulses of fixed duration (2000 msec) and increasing depolarization steps (10-65 mV) and the resulting gIPSP was recorded in current clamp mode (only response to 40 mV shown) in control and in RPCH (10^{-9} M to 10^{-5} M). B. The amplitudes of the gIPSPs at the peak were measured and plotted against the presynaptic potential in control and in different doses of RPCH to obtain the input/output curves (mean± SEM; N=12). The gIPSPs increased in a dose-dependent manner indicating the strengthening of the LP to PD synapse.

Short-term dynamics of the LP to PD synapse in RPCH

To test what occurs to synaptic strength *in vitro* both in control and in the presence of RPCH, the LP neuron was voltage clamped to a holding potential of –60mV in TTX and stimulated with trains of five voltage pulses with fixed amplitude (40mV) and duration (500 msec) and increasing interpulse intervals (IPI; 250-2000 msec) while the synaptic potentials in the PD neuron were simultaneously monitored. Figure 4A shows an example trace of voltage pulses with 250 msec IPI applied to the LP neuron and the resulting gIPSPs in the PD neuron elicited both in control (black trace) and in 10^{-6} M RPCH (red trace). Because the first peak (A1) of the gIPSP was greater in amplitude compared to the steady state peak (A5) in both traces, the synapse was a depressing synapse. To compare the extent of depression and the rate of recovery of the LP to PD synapse in control and in different doses of RPCH, the ratio of the A5 to A1 versus IPI was calculated. This ratio was between 0 and 1. Any value close to or equal to 1 represents complete recovery of the synapse. Figure 4B shows the extent of depression of the LP to PD synapse in control and in 10^{-6} M RPCH (mean±SEM; N=10). Depression was greater in RPCH at every IPI as compared to control. At 2000 msec IPI, recovery of >95% in control and ~88% in 10^{-6} M RPCH was achieved, indicating that at even high IPIs, RPCH can elicit depression in the PD neuron. Also the rate of recovery from depression was calculated to fully characterize the short-term dynamics of the LP to PD

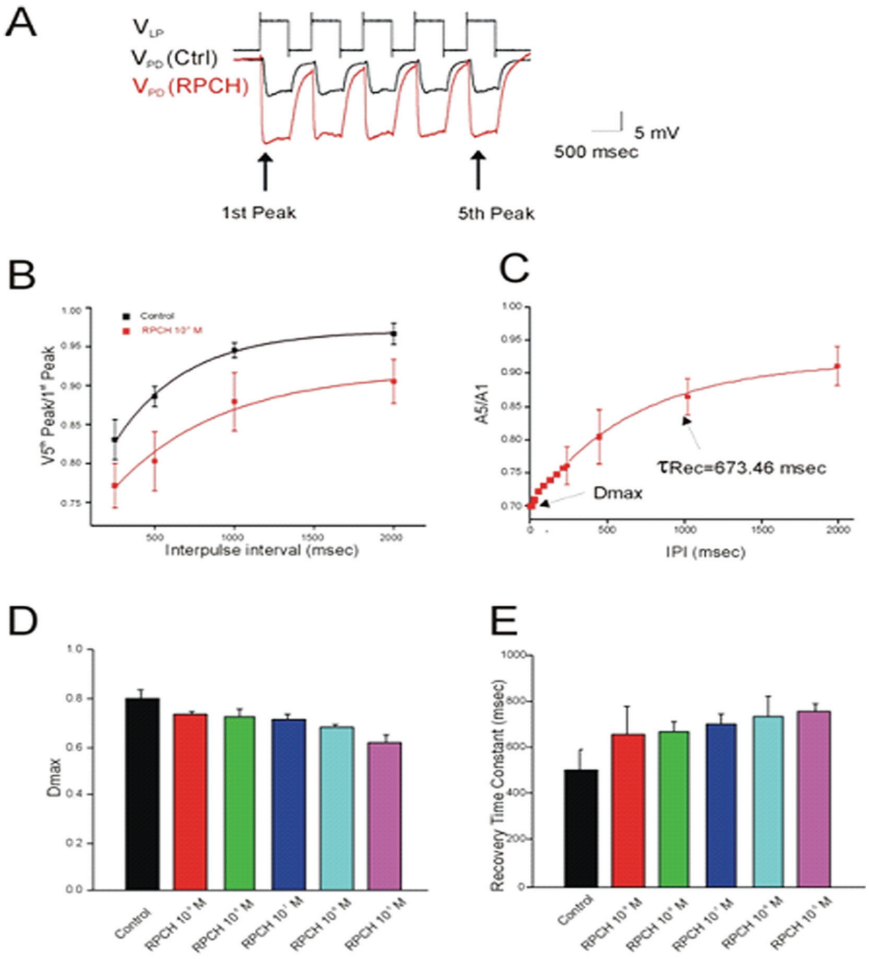

Figure 4. Short-term dynamics of the LP to PD synapse changed in response to trains of pulses in RPCH. A. An example of the gIPSPs in the PD neuron in control (black trace) and in 10-6 M RPCH (red trace) in response to stimulation of the LP neuron with a train of five 40 mV, 500 msec voltage pulses separated by 250 msec IPI. IPI was changed between 250-2000 msec each time to measure the synaptic dynamics. B. The amplitudes of the 1st and the steady state gIPSPs were measured and the ratio of A5/A1 plotted against IPI then fitted with a first order exponential decay curve with recovery time constant (τ_{rec}) In control and in RPCH (only 10-6 M shown). The LP to PD synapse depressed more in RPCH. C. Maximum depression (Dmax) was calculated for each experiment, when the IPI tends to 0 in control and in 10-6 M RPCH D. Dmax values were plotted for control and different doses of RPCH (Only 250 msec IPI was shown as representative). The synapse depressed more as the doses of RPCH increased. E. τ_{rec} were plotted against the doses of RPCH (10^{-9} M-10^{-5}M). The synapse recovered more slowly as the concentration of RPCH was increased (mean± SEM; N=10).

synapse. In each experiment, for control and increasing doses of RPCH, the ratio of A5/A1 versus IPI was graphed and fit the resulting trace. Such an example is shown in Fig. 5.3C with a recovery time constant τ_{rec} = 673 msec in 10^{-6} M RPCH. The y-intercept of the exponential fit shows the depression when the IPI tends to zero. This intercept represents the maximum depression (Dmax) in response to trains of voltage pulses. In Fig. 4D, the Dmax values of the LP to PD synapse was plotted in control and in different concentrations of RPCH (10^{-9} M to 10^{-5} M). Maximum depression was achieved as the concentration of RPCH was increased (two-way ANOVA, $p<0.05$, N=10). This implies that the LP to PD synapse regulates its efficacy in RPCH in a dose-dependent manner. Also, the recovery time constant (τ_{rec}) for different doses of RPCH was plotted (Fig. 4E). Increases in recovery time occurred at higher concentrations of RPCH, whereas the lower levels of neuromodulation led to a decrease in recovery time. Synapse recovered the fastest in 10^{-5} M RPCH, whereas synapse recovered the slowest in 10^{-9} M RPCH.

Frequency- dependency of the LP to PD synapse in RPCH

It was previously shown that the amplitude of a depressing synapse changes in proportion to the changes in the firing frequency of the presynaptic neuron and the steady state amplitude of a depressing synapse is inversely proportional to the firing frequency [33]. In another study, researchers showed that the LP to PD synapse was sensitive to changes in frequency of the presynaptic neuron in lobster *Panulirus interruptus* [2]. Therefore to study the effects of frequency on the gIPSP amplitudes, the LP neuron was voltage clamped (to -60mV) and injected sinusoidal waveforms at a fixed amplitude (40mV) and different frequencies (0.5-10 Hz). Then, the peak amplitudes of gIPSPs from both the first pulse and the steady state pulse (5^{th} pulse) in control and in RPCH (10^{-9} M to 10^{-5} M) were measured. Figure 5A shows an example of voltage traces of the LP and the PD neurons during the activation of the synapse with five repetitions of the sinusoidal waveform at fixed amplitude (40 mV) and 4 Hz frequency in control and in RPCH (only 10^{-6} M shown). The top trace shows the voltage clamped LP neuron and the bottom traces shows the PD neuron responses in control (black trace) and in 10^{-6} M RPCH (red trace). The amplitudes of all peaks increased in RPCH as compared to control. In order to quantify the changes in the peak gIPSP amplitudes, the 1^{st} pulse (Fig. 5B) and the steady pulse amplitudes (Fig. 5.C) versus frequency (0.5-10 Hz) were plotted in control and in RPCH. The amplitudes of both the first peak and the steady state gIPSPs showed a dose-dependent response at different frequencies, and the gIPSPs in response to the fifth stimulation was smaller than the first gIPSP for all

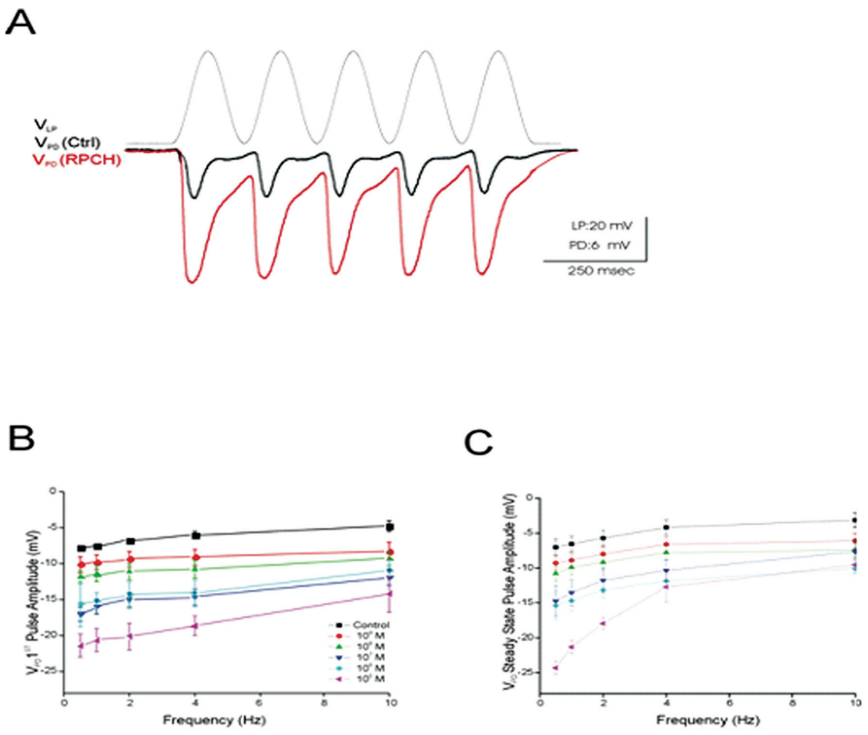

Figure 5.Effects of frequency on the gIPSP amplitude in RPCH. A. Traces of the LP neuron two-electrode voltage clamped with a sinusoidal waveform (40mV) and the subsequent gIPSPs were measured in the PD neuron in control (black trace) and in RPCH (red trace, only response to 10^{-6} M RPCH was shown). B., C. The amplitudes of the first pulse and the steady state gIPSPs were measured and graphed versus frequency in control and in different doses of proctolin (mean± SEM, N=8). Both the 1st pulse and the steady state pulse amplitudes enhanced in RPCH in a dose-dependent manner.

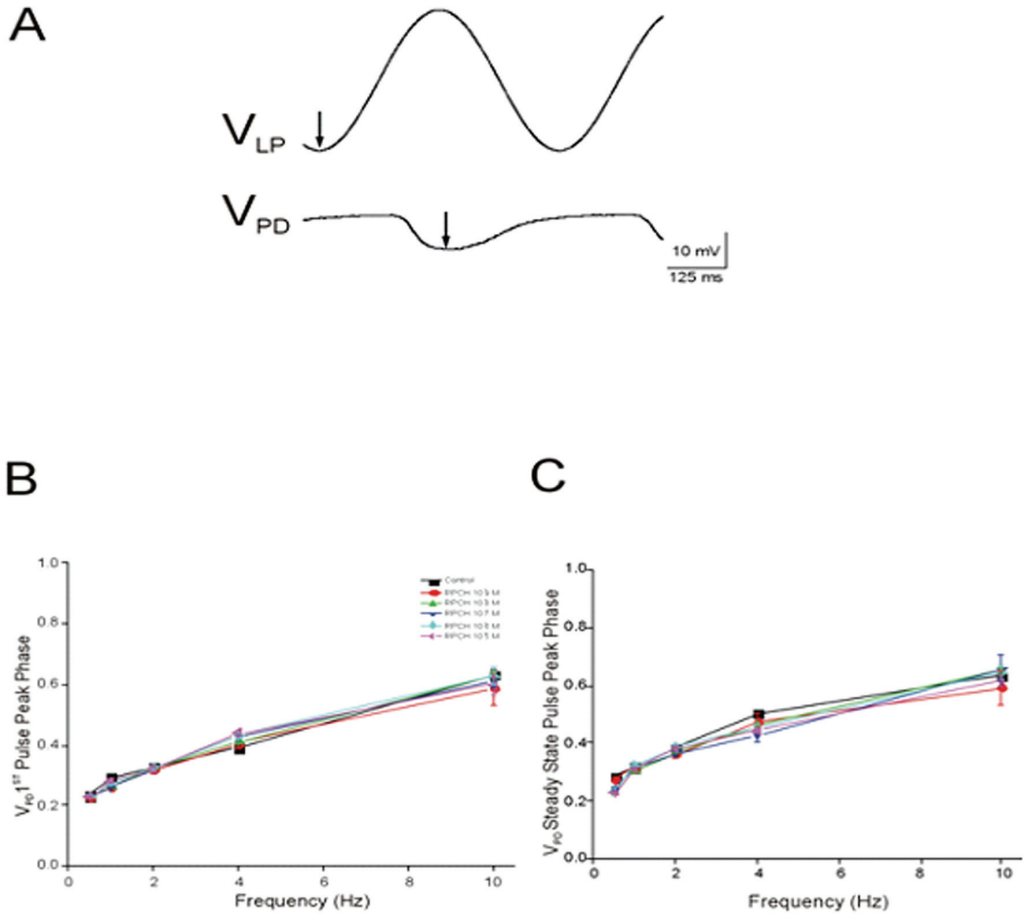

Figure 6. Peak phase of the gIPSPs was frequency dependent but not dose-dependent. A. Sinusoidal waveform was injected in the voltage clamped LP neuron and gIPSP was measured from the PD neuron in control and in RPCH. The trace shown is an example of gIPSP in response to 4 Hz sinusoidal waveform. Δt was calculated as the difference between the beginning of the LP waveform (arrow) and the peak of the gIPSP of the PD neuron (arrow) for the 1st peak and the steady state peak. B., C. The peak phase of the gIPSP was calculated as the ratio of Δt/Period (Φ=Δt/Period) then plotted versus frequency in control and in RPCH. Peak phases of both the 1st pulse and the steady state pulse gIPSPs showed frequency dependency, but different doses of RPCH had no effect.

conditions, indicating that this synapse shows short-term depression.

Peak phase of the gIPSPs was frequency dependent but not dose-dependent

Synaptic strength can be modulated by neuromodulators as well as the efficiency and the timing of the presynaptic activity [34]. In fact, the frequency of presynaptic activity may affect not only the amplitude (as seen in Fig. 4) but also the time to peak of the gIPSPs. In order to study the effects of frequency on the peak phase of the LP to PD synapse in different concentrations of RPCH, the LP neuron was voltage clamped and stimulated with sinusoidal waveforms (40mV) at different frequencies. The changes in peak phase of the first and steady state gIPSP were analyzed(mean± SEM) at frequencies ranging between 0.5 and 10 Hz. Figure 6A shows a sample response to a sinusoidal waveform at 4 Hz. For the purpose of calculating peak phase (Φ), the beginning trough of the LP sinusoidal waveform as a reference point was chosen and defined Δt as the difference between the beginning of the LP sinusoidal waveform and the peak of the gIPSP in the PD neuron. Then, Φ (defined as Δt/Period) versus frequency was plotted for control and for different doses of RPCH (Fig. 6B). The peak phase increased as the frequency increased in control and in each dose of RPCH (10^{-9} M-10^{-5} M) for both the 1st pulse and steady state pulse gIPSPs (two-way ANOVA, $p<0.05$). However, two-way ANOVA did not reveal a significant interaction between control and RPCH or between the different doses of RPCH ($p>0.05$). Therefore, the dependence of the phase on frequency was not affected by the neuromodulator RPCH.

Short-term dynamics of the LP to PD synapse changed in RPCH in response to realistic waveforms

In order to obtain direct measures of the different components contributing to the postsynaptic responses, such as stimulation with different amplitudes and periods, the LP neuron was stimulated with non-filtered and filtered waveforms with increasing amplitudes and periods in control and in RPCH. Figure 7A shows the traces of the gIPSPs elicited by the 47 mV non-filtered and 40 mV filtered LP waveforms at 500 msec period in control and in 10^{-6} M RPCH. RPCH caused an increase in gIPSP amplitudes as compared to control for both types of LP waveforms. However, the response to the non-filtered waveform was larger than to the filtered

waveform for all presynaptic amplitude stimulations in control and in RPCH (two-way ANOVA, $p<0.05$; data not shown).

Then, the effects of the neuromodulator RPCH on the short-term dynamics of both the non-filtered and the filtered waveforms at different amplitudes were quantified. The ratio of the steady state pulse amplitude to the 1st pulse in control and in 10^{-6} M RPCH was caluclated. Figures 7B and 7C show these ratios as a function of periods (500, 1000, 1500, 2000 msec) for 24, 47, 71 mV (non-filtered waveform) and 20, 40, 60 mV (filtered waveform) in control (Fig. 7B) and in RPCH (Fig. 7C). In control conditions, synaptic depression in response to both the filtered and non-filtered stimulations was similar at any given waveform amplitude or period. Also in the control condition, the LP to PD synapse achieved near full recovery as the waveform period was increased to 2000 msec for both types of waveforms. The extent of recovery for the 47 mV and 71 mV presynaptic amplitudes was significantly smaller at 500 msec period for non-filtered waveform (two-way ANOVA, $p<0.05$, N=12) and filtered waveform (two-way ANOVA, $p<0.05$, N=12). In RPCH (Fig. 7C), two-way ANOVA measurements also revealed a significant difference for 40 mV at 2000 msec for filtered waveform. The extent of recovery was the biggest for 24 mV (non-filtered) and 20 mV (filtered) amplitudes. However, 71 mV (non-filtered) and 60 mV (filtered) amplitudes showed more recovery than 47 mV (non-filtered) and 40 mV (filtered) amplitudes.

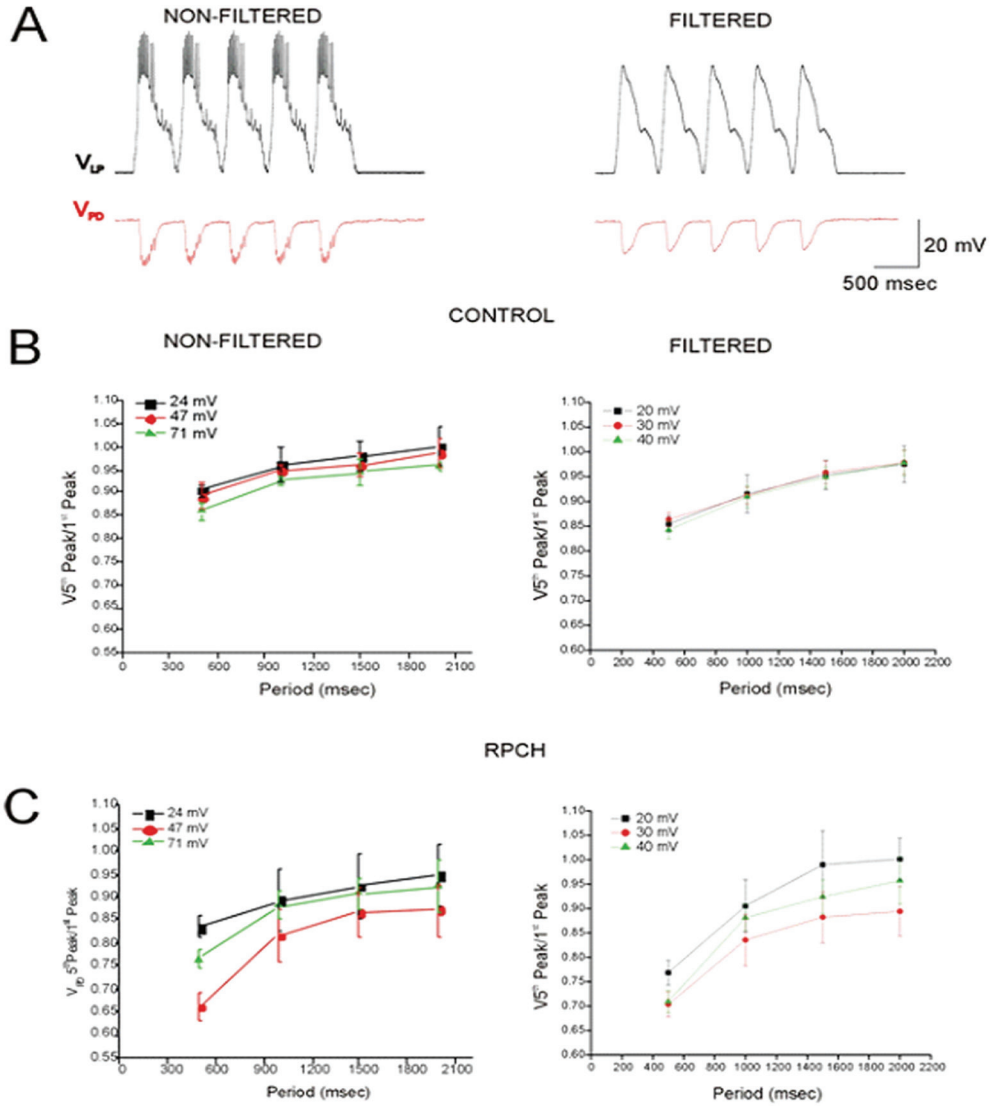

Figure 7. Postsynaptic responses to realistic waveform stimulation of the LP neuron at different amplitudes in RPCH. A. Sample traces of the gIPSPs in response to non-filtered and filtered realistic waveforms (Only 47 mV for non-filtered and 40 mV for filtered waveform were shown) in 10-6 M RPCH. B. Average effects of different realistic waveform amplitudes on the extent of depression as a function of period in control for non-filtered and filtered waveforms. Depression rate was calculated as the ratio of the steady state gIPSP peak amplitude versus the 1st gIPSP peak amplitude. As the period increased the LP to PD synapse recovered more and nearly showed fully recovery at 2000 msec period. C. The extent of depression was calculated at all amplitudes in 10-6 M RPCH for non-filtered (left panel) and filtered waveforms (right panel). The extent of depression showed period dependency and as the period increased the synapse recovered more in all amplitudes.

DISCUSSION

In this study, the effects of the RPCH on the short-term dynamics of a key synapse (extent of depression, recovery from depression and the time dependent properties) of the LP to PD synapse were investigated for the first time in crab *Cancer borealis*. The results show that RPCH can modulate both the strength of the graded chemical interaction and the short-term plasticity between the LP neuron and the PD neurons in a dose-dependent manner.

Strengthening of the LP to PD synapse in RPCH

Graded synaptic transmission, where transmitter is released as a continuous function of presynaptic voltage, has been observed in many systems, and it is useful for neuronal interactions allowing precise and continuous control of postsynaptic membrane potential [17,18]. Despite the importance of graded transmission, little is known about the effects of neuromodulators on the strength of these synapses.

Several previous studies examined the effects of amines on the pyloric synapses in spiny lobster *Panulirus interruptus* by using long, single square presynaptic current pulses [16]. These studies showed that octopamine, 5-HT, dopamine were capable of modulating the strength of graded chemical synaptic interactions within the pyloric motor circuit. The purpose of these sets of experiments was to study the effects of neuromodulators on graded synaptic transmission in the pyloric network, where graded synaptic transmission is important for network function and where small changes in synaptic strength have functional consequences for the network output. I therefore began by investigating the effects of RPCH in a dose-dependent manner on the strength of the LP to PD graded chemical synapse. Upon injection of single square pulses with increasing depolarizations steps (10-50 mV) into the LP neuron, the gIPSP amplitudes evoked in the PD neuron were bigger in RPCH as compared to those evoked in control conditions. This increase in gIPSP amplitude implied that the LP to PD synapse was strengthened in a dose-dependent manner in RPCH. This concentration dependence of on the LP to PD synapse suggests that the LP and the PD neurons might have a different threshold and dose-response relationship for RPCH. It has also previously been suggested that networks made up of different neurons with different dose-response relationships can be induced to produce different motor outputs by the different concentration of the same neuromodulator [35]. It should also be noted that the LP to PD synapse did not saturate at high depolarization steps for every dose of RPCH. This might be due to several factors: 1. Desensitization of the RPCH receptors might prevent the continuous depolarization and saturation of the receptors. 2. The concentrations used (10^{-9} M- 10^{-5} M) may reduce the dissociation rate of the neuromodulator from the receptor. Higher concentrations of the neuromodulator may prevent this phenomenon. 3. Or, these concentrations increase the number of receptors in an active conformation or retain the receptors in an active confirmation for a longer period. It should be noted that in this study, the mechanisms or sites of RPCH action in modulating the synaptic transmission were not investigated. Although results suggest that RPCH acts directly at the LP to PD synaptic site, it is unclear whether its actions are pre- (enhanced transmitter release) and/or postsynaptic (enhanced postsynaptic response).

Effects of trains of square voltage pulses

Up-to-date, studies that examined the effects of monoamines at a single dose on the pyloric synapses did so in a static context in lobster *Panulirus interruptus* by using the long, single, square presynaptic current pulses [11, 16]. The few studies that examined the dose-dependent effects of amines, octopamine, dopamine [29] and proctolin [30] also did so in a static context. These studies showed that the pyloric activity pattern changed in a dose-dependent manner in octopamine and dopamine [36] and the follower LP neuron was excited in different dose of proctolin [30] The efficacy of the graded synapses is use-dependent and transmission at these synapses depends on the timing of the action potentials that arrive at the presynaptic terminal and on the type of the waveform and the duration of its action potential. Previous studies revealed the importance of these properties on graded synapses. They showed that the amplitudes of the gIPSP responses are dependent on the shape, the slope of the rising phase of the presynaptic depolarizations and the frequency of the presynaptic stimulations [2, 21]. Therefore, since synapses in the pyloric circuit work in a dynamic context, a simple way to quantify the short-term dynamics of the LP to PD synapse is to stimulate the LP neuron with trains of multiple pulses and compare the gIPSPs evoked by the first pulse to those evoked by the steady-state pulse. The intervals between the pulses were varied in order to quantify the effect of the frequency of activity on the short-term dynamics. In this manner, I was able to measure synaptic depression, and recovery from depression. The LP to PD synapse showed more depression in RPCH in response to trains of pulses and as the duration of inter-pulse intervals was extended more recovery from depression was achieved. These results give support to the hypothesis that RPCH play important role in affecting the short-term dynamics of the LP to PD synapse. The fact that the recovery from depression slowed down in a dose-dependent manner at different periods and increased its extent may suggest a functional role of RPCH in shaping the pyloric rhythm by acting on the oscillation period. This effect would be due to the effects of RPCH on the presynaptic or postsynaptic neurons. The passive flow of current between input and output sites within a cell might affect the graded synaptic transmission and RPCH could act presynaptically to alter this graded transmission by two distinct mechanisms. First, it could modify the transmitter release machinery or terminal ionic conductances by directly acting

presynaptically. Second, it could act nonspecifically on ionic conductances at other parts of the LP neuron. Alternatively, RPCH could act postsynaptically to affect graded transmission via either a direct modification on the metabotropic glutamate receptors on the PD neuron or an indirect action on ionic conductances that might in turn affect current spread from the synapse to the other regions of the PD neuron.

Effects of frequency

Although neuromodulators do modify synaptic activity, the frequency with which the presynaptic neuron is stimulated plays an important role in how big of an effect the neuromodulators will have at the network level. It was found that the PD neuron gIPSPs' peak amplitudes changed in response to the LP neuron stimulations with sinusoidal waveforms of different frequencies. The amplitudes of the 1st and the steady state gIPSPs decreased as the stimulation frequency increased for control and every dose of RPCH. This may be explained by a decrease in the recovery time from depression (as the frequency increased). When the recovery from depression was measured, it was observed that the synapse was still depressed around 2 sec range. The duration of recovery from depression being longer than the normal period of the pyloric rhythm indicates that the strength of the LP to PD synapse might be altered by the frequency changes. It was also observed that RPCH enhanced the gIPSP amplitudes in a dose-dependent manner. I then looked at the time course of gIPSPs (peak phase), and found that the peak phases of the gIPSPs were frequency dependent. The LP to PD synapse peaked earlier in time as the frequency decreased in control, in RPCH. This phase advance of the onset of the PD burst might be due to a reduction in IA and entrainment of Ih. The RPCH effect on the LP to PD synapse is to slow down a fast rhythm and speed up a slow [21].

Taken together these data show that the effect of RPCH on the LP to PD synapse stabilizes the rhythm frequency. This stabilizing effect is consistent with state-dependent effects of RPCH.

Effects of stimulation with different amplitudes in the neuromodulator

In this study, recordings from a key synapse in the pyloric network of crab *Cancer borealis* were used to correlate the effects of neuromodulation on the dynamical properties of an individual synapse by using various presynaptic waveforms (see Methods) with different depolarization amplitudes. One waveform that was used is the realistic LP waveform. This was used because several studies have shown that the slope of the presynaptic membrane potential plays an important role in determining the amplitude of the postsynaptic response [2, 18].

Directions for future studies

Determining the sites and mechanisms of regulation by neuromodulators

Although a great number of studies have investigated the effects of neuromodulators on the pyloric network, no detailed study has been done to look at the their sites of actions, whether they are postsynaptic or presynaptic and mechanisms of graded synaptic modulation. In principle, a neuromodulator could change the network output at multiple sites [37]. Addressing the cellular sites and mechanisms of the neuromodulator action underlying short-term plasticity in the pyloric circuit should help us to better understand the dynamic reconfigurations giving the depression and facilitation. Therefore, the next step would be to define the pre- and postsynaptic contributions to neuropeptide-induced changes in graded synaptic strength.

Several mechanisms could underlie the action of neuromodulators on the presynaptic and postsynaptic sites of the synapse. They might act modify the terminal ionic conductances or the release machinery at the synapse or on ionic conductances at other sites of the cell when they act presynaptically. This would change the depolarization and release from the synaptic terminal due to modification of neuron's conductances. Similarly neuromodulators might act postsynaptically via direct and non-direct actions. This action might directly be on the transmitter receptors or indirectly by modification of ionic conductances, which may change the current spread from the neuron. One way to distinguish between these two possible synaptic sites of neuromodulator action would be to study the postsynaptic effects of neuromodulators, such as proctolin and RPCH on short-term synaptic dynamics to iontophoretic applications of Glutamate (Glu) along with the neuromodulators. Glutamate and acetylcholine are the inhibitory transmitters in STG. One possible mechanism for short-term synaptic depression is the desensitization of the postsynaptic receptors. If the application of Glutamate (or acetylcholine) and neuromodulator on the postsynaptic site reveals desensitization it would support postsynaptic actions of the neuromodulator. Desensitization of postsynaptic receptors has been shown to play a role in synaptic transmission in different systems. The calyceal synapse between the auditory nerve and the nucleus magnocellularis of the chick [38], the cones and bipolar cells in the retina [39], the giant cochlear nucleus synapses [40], the retinogeniculate synapses [41] are couple of these systems and all of these studies have shown desensitization of AMPA receptors. In addition to desensitization of postsynaptic receptors, decreased sensitivity of the exocytosis [42] and inhibition of presynaptic calcium channels through the action of G proteins [43] were shown to be the mechanisms for short-term synaptic depression. It is possible that all of these mechanisms might contribute to short-term synaptic plasticity in the pyloric circuitry by acting either presynaptically or postsynaptically or both.

Studying the effects of other neuromodulators on the short-term synaptic dynamics

Despite the success of current findings of neuromodulatory affects on short-term synaptic

dynamics, much remains to be done. The current findings could be stepping stones for investigating the actions of all neuromodulators present in the pyloric circuit, either individually or in an orchestrated way. The findings of this study place a strong emphasis on the roles of proctolin and RPCH on the short-term dynamics of a key synapse and subsequent shaping of the pyloric network output.

It is still puzzling that there are 15 different neuromodulators for 26 neurons in the STG that produces simple motor patterns. Understanding the effects of each neuromodulator at the network level would help us to better understand the circuit function and the final network output. In this manner dynamic clamp technique [44] would provide a tool to apply artificial neuromodulatory currents to the neurons and study the effects on the synapses between them.

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