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Thirumalai, Vatsala, Astrid A. Prinz, Christian D. Johnson, and Eve Marder. Red pigment concentrating hormone strongly enhances the strength of the feedback to the pyloric rhythm oscillator but has little effect on pyloric rhythm period. *J Neurophysiol* 95: 1762–1770, 2006. First published November 30, 2005; doi:10.1152/jn.00764.2005. The neuropeptide, red pigment concentrating hormone (RPCH), strengthened the inhibitory synapse from the lateral pyloric (LP) neuron to the pyloric dilator (PD) neurons in the pyloric network of the stomatogastric ganglion (STG) of the lobster, *Homarus americanus*. RPCH produced several-fold increases in the amplitude of both action potential–mediated and non–impulse-mediated transmission that persisted for as long as the peptide remained present. Because the LP to PD synapse is the only feedback to the pacemaker kernel of the pyloric network, which consists of the electrically coupled two PD neurons and the anterior burster (AB) neuron, it might have been expected that strengthening the LP to PD synapse would increase the period of the pyloric rhythm. However, the period of the pyloric rhythm increased only transiently in RPCH, and a transient increase in cycle period was observed even when the LP neuron was hyperpolarized. Phase response curves were measured using the dynamic clamp to create artificial inhibitory inputs of variable strength and duration to the PD neurons. Synaptic conductance values seen in normal saline were ineffective at changing the pyloric period throughout the pyloric cycle. Conductances similar to those seen in 10^{-6} M RPCH also did not evoke phase resets at phases when the LP neuron is typically active. Thus the dramatic effects of RPCH on synaptic strength have little role in modulation of the period of the pyloric rhythm under normal operating conditions but may help to stabilize the rhythm when the cycle period is too slow or too fast.

INTRODUCTION

Although synaptic plasticity at central and peripheral synapses has been the subject of intense study (Abbott and Nelson 2000; Kandel 2001; Zucker and Regehr 2002), there are relatively few cases in which the circuit consequences of changes in synaptic strength have been systematically established. This is partly because those experimental model systems that are best suited for studying synaptic physiology are not optimal for analyses of network function and vice versa. In many cases, evidence for the functional significance of synaptic plasticity is correlation-based (McKernan and Shinnick-Gallagher 1997; Oda et al. 1998; Rogan et al. 1997), and it is often assumed that all changes in synaptic strength are functionally significant. Here we show an example in which a several-fold increase in the strength of an input synapse to an oscillator caused by a peptide produces little effect on the period of the oscillator and the rhythm it drives.

Synapses between identified neurons of the pyloric network of decapod crustaceans are modulated by previous activity (Mamiya et al. 2003; Manor and Nadim 2001; Manor et al. 1997; Nadim et al. 1999) and by neuromodulators (Ayali and Harris-Warrick 1999; Ayali et al. 1998; Marder et al. 1997) present in the hemolymph and in the terminals of projection neurons from anterior ganglia. The pyloric network of decapod crustaceans consists of six different types of cells. The single anterior burster (AB) neuron is electrically coupled to the two pyloric dilator (PD) neurons, and together, these neurons act as the pacemaker kernel for the triphasic pyloric rhythm. The ventricular dilator (VD), the inferior cardiac (IC), the lateral pyloric (LP), and the pyloric (PY) neurons are inhibited by the pacemaker group. Inhibition from the LP neuron to the PD neurons provides the major feedback signal from the follower cells to the pacemaker group (Harris-Warrick et al. 1992; Selverston and Moulins 1987).

Red pigment concentrating hormone (RPCH) was one of the first neuropeptides discovered to have strong and robust actions on the crustacean stomatogastric nervous system (Dickinson and Marder 1989; Dickinson et al. 1990, 1993, 2001; Nusbaum and Marder 1988). RPCH is present in the pericardial organs (Christie et al. 1995; Pulver and Marder 2002) and in the neuropil of the stomatogastric ganglion (STG) within the terminals of neurons projecting from anterior ganglia (Fénelon et al. 1999; Nusbaum and Marder 1988; Thirumalai and Marder 2002). In the lobster, *Panulirus interruptus*, RPCH strongly potentiates the synapses from the inferior ventricular (IV) neurons to a number of the STG neurons, accounting for a massive circuit reconfiguration as RPCH produces a single conjoint rhythm from neurons that ordinarily form part of the cardiac sac and gastric mill circuits (Dickinson et al. 1990). In crabs and lobsters, RPCH strongly excites the LP neuron (Dickinson et al. 2001; Nusbaum and Marder 1988; Thirumalai and Marder 2002), increasing its burst duration and number of spikes per burst and altering its duty cycle in the pyloric rhythm. Nonetheless, the reported effects of RPCH on pyloric rhythm frequency are complex, and in some cases depend on the initial frequency of the rhythm (Dickinson et al. 2001; Nusbaum and Marder 1988; Thirumalai and Marder 2002).

In this paper, we studied the effects of RPCH on the pyloric rhythm in the lobster *Homarus americanus*. We show that

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RPCH elicits a strong and long-lasting potentiation of the LP to PD neuron synapse but has only transient effects on network behavior. Using the dynamic clamp (Sharp et al. 1993a,b) to replace the biological LP to PD synapse, we construct phase response curves (PRCs) of the oscillator (Pinsker 1977a,b; Winfree 1980) and ask how enhancing the strength and duration of the input synapse affects the oscillator period.

METHODS

The stomachs of adult *H. americanus* were dissected and pinned on to a Sylgard-coated dish. The stomatogastric nervous system, consisting of the paired commissural ganglia (CoG), the esophageal ganglion (OG), the stomatogastric ganglion (STG), the nerves connecting these ganglia with each other, and the pyloric motor nerves, was dissected away from the stomach and pinned onto a transparent Sylgard-coated dish containing chilled (9–14°C) *H. americanus* saline (Fig. 1*A*).

Saline composition was as follows (in mM): 479.12 NaCl, 12.74 KCl, 13.67 $CaCl_2$, 20 $MgSO_4$, 3.91 Na_2SO_4, and 2.4 HEPES (pH 7.45). Vaseline wells were constructed on the motor nerves for extracellular recording of action potentials. The STG was desheathed, and the preparation was continuously superfused with *H. americanus* saline at 9–14°C. STG neurons were impaled with sharp electrodes filled with 0.6 M K_2SO_4 and 20 mM KCl (resistance, 13–50 MΩ). The PD and LP neurons were identified by the constant latency of their spikes on the pyloric dilator nerve (pdn), the lateral pyloric-pyloric nerve (lp-pyn), and/or the lateral ventricular nerve (lvn) (Maynard and Dando 1974). Intracellular voltages were recorded using AxoClamp 2A and AxoClamp 2B (Axon Instruments, Foster City, CA) amplifiers and digitized using either a Digidata 1200 or Digidata 1322A data acquisition board and pClamp 8.0 or pClamp 9.0 software (Axon Instruments, Foster City, CA). Data were analyzed off-line using either in-house software or Spike2 (version 5, CED, Cambridge, UK). Burst durations were defined as the time from the first to last spike in a burst. Statistical tests were performed using Microcal Origin (Microcal Software, Northampton, MA) and SigmaStat (SPSS, San Rafael, CA). All error bars are SE.

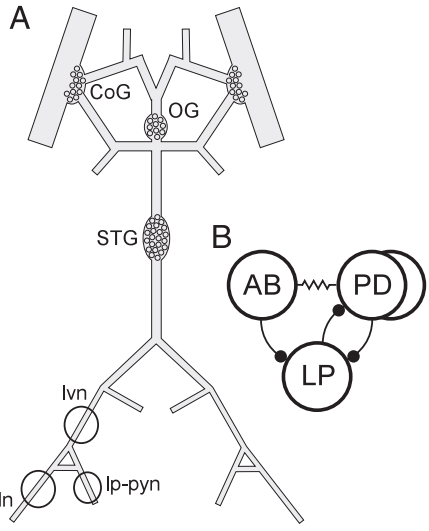

FIG. 1. Stomatogastric nervous system and circuit diagram. *A*: schematic of stomatogastric preparation with stomatogastric ganglion (STG), paired commissural ganglia (CoG), esophageal ganglion (OG), and lateral ventricular nerve (lvn). Typical positions of Vaseline wells for extracellular recording from the lower lvn, pdn, and lp-pyn are indicated. *B*: simplified schematic of the circuit studied here. A single anterior burster (AB) neuron electrically coupled to 2 pyloric dilator (PD) neurons forms the pyloric pacemaker kernel. Both kernel components inhibit the single lateral pyloric (LP) neuron, which in turn inhibits the PD neurons.

Saline containing 10^{-6} M RPCH (American Peptide Co., Sunnyvale, CA) was introduced in the bath using a switching manifold. When required, the STG was isolated from modulatory influences from the anterior ganglia by placing a conduction block on the stomatogastric nerve (stn; 750 mM sucrose and 10^{-6} M TTX from Alomone Laboratories, Jerusalem, Israel).

Measuring LP to PD neuron graded and spike-mediated inhibitory postsynaptic potentials

The LP and PD neurons were impaled with two electrodes each. The LP neuron was hyperpolarized to −80 mV. The membrane potential of the PD neuron was varied from −20 to −120 mV by injecting current. Brief depolarizations were triggered in the LP neuron to make it spike. The amplitude of the resulting spike-evoked inhibitory postsynaptic potential (sIPSP) in the PD neuron in response to the first spike in the LP neuron was measured at different membrane potentials of the PD neuron, which was measured immediately before the start of the IPSP. For the most part, sIPSPs were triggered during the trough of the slow oscillation in the PD neuron so that membrane potentials could be measured with little error. This experiment was performed both in normal saline and in 10^{-6} M RPCH. The amplitude of the sIPSP was plotted against the PD membrane potential at which it was evoked and was fit with a straight line. Using the fit, the amplitude of the sIPSP at −40 mV was calculated.

To measure the effect of RPCH on graded IPSPs (gIPSPs), all action potentials were blocked by superfusing the preparation with saline containing 10^{-6} M TTX. The LP neuron was voltage clamped at −80 mV and the PD neuron was brought to a given starting membrane potential by injecting current using two-electrode current clamp. The LP neuron was stepped to −30 mV for 5 s to release neurotransmitter. The amplitude of the gIPSP in the PD neuron was measured 500 ms before the end of the voltage pulse in the LP neuron to let the IPSP reach steady state so as to rule out any effects of RPCH on the kinetics of depression. This protocol was repeated for PD neuron membrane potentials ranging from −30 to −110 mV.

Measuring the effect of RPCH on pyloric rhythm properties

We recorded the pyloric rhythm extracellularly from the lvn, pdn, and lp-pyn. We impaled the LP neuron with a single electrode and the PD neuron with two electrodes. To assay the output of the intact pyloric circuit and the isolated pacemaker group, we followed the following protocol: we injected current to hyperpolarize the LP neuron for 29 s every 1.9 min. We injected between −8 and −12 nA and this abolished the LP spikes on the lvn and lp-pyn (Fig. 5*A*). We measured the PD neuron cycle period and burst duration in the presence and absence of the LP neuron. We repeated these measurements after bath-applying saline containing 10^{-6} M RPCH. We also compared the LP neuron burst duration in control saline with that in RPCH.

Phase response curves

Experiments were done by either placing two electrodes, one for injecting current and the other for recording the membrane potential V_m, or by recording in discontinuous current clamp (DCC) mode, using a single electrode for recording voltage and for injecting current. To remove the influence of the LP neuron on the PD neurons, one of three methods was used *1*) hyperpolarization by current injection, *2*) bath application of 10^{-5} M picrotoxin (PTX; Sigma Chemical Co., St. Louis, MO), which blocks the LP to PD neuron synapse, or *3*) photoablation of the LP neuron (Miller and Selverston 1979). An artificial inhibitory synapse was created using dynamic clamp (Sharp et al. 1993a,b) to inject pulses of synaptic conductance into the PD neuron. We used two versions of the dynamic clamp: one developed by Dr. R. D. Pinto (Physics Institute, University of Säo Paolo, Säo

Paolo, Brazil) (Pinto et al. 2001) and modified by us and a second version developed by Dr. F. Nadim (Department of Biology, Rutgers University, Rutgers, NJ). The LP to PD neuron synapse was modeled with a reversal potential E_r of −90 mV, which was within the range of experimentally measured reversal potentials for this synapse. The synaptic conductance g_{syn} was varied over a range from 20 to 1,000 nS. These conductances were chosen based on preliminary experiments in which the size of the PSP caused by the biological LP to PD synapse was compared with the size of the PSP caused by the dynamic clamp synapse of a certain conductance. From these estimates, dynamic clamp PSPs with a 20-nS conductance resembled closely the biological PSP in normal saline. The synaptic conductance had instantaneous activation and no inactivation and was modeled to replicate the envelope of the graded and spike-mediated synaptic inhibition from the LP neuron. For plotting the phase response curve (PRC) of the PD neuron, the artificial synaptic conductance was injected at different phases of the PD neuron oscillation (Prinz et al. 2003). The first spike of the PD neuron burst was designated as phase 0 and the conductance was injected at phases 0 through 1 in increments of 0.05. The average unperturbed cycle period P of the PD neuron was measured and used to calculate the time of the pulse. The injected synaptic current in the PD neuron, I_{syn}, was calculated from the measured V_m using the equation $I_{syn} = g_{syn} \times (V_m - E_r)$.

The duration of the synaptic conductance was varied over a range from 20 to 750 ms. The altered PD neuron cycle period P' caused by the pulse was measured. The phase reset in PD oscillation was calculated as $\Phi = \Delta P/P = (P' - P)/P$.

The phase of arrival of the stimulus pulse (θ) was calculated from its latency (L) after the PD burst onset as $\theta = L/P$.

The PRC was plotted with the phase reset Φ on the y-axis and the stimulus phase θ on the x-axis for each g_{syn} and duration used (Canavier et al. 1999; Pinsker 1977a,b).

RESULTS

Figure 1*B* depicts a simplified connectivity diagram for the neurons that are the subject of this study. The pacemaker kernel consists of the two PD neurons and a single AB neuron, which are electrically coupled. The LP neuron makes an inhibitory chemical synapse onto the two PD neurons, which in turn inhibit the LP neuron, as does the AB neuron (Eisen and Marder 1982).

RPCH alters the strength of the LP to PD neuron inhibitory synapse

STG neurons release neurotransmitter as a graded function of presynaptic depolarization (Graubard 1978; Manor et al. 1997) as well as in response to presynaptic action potentials. Consequently, we separately studied the effects of RPCH on the amplitude of the sIPSP from the LP neuron to the PD neuron and the gIPSP between the same neurons. In these first sets of experiments, RPCH was applied to preparations in which the effects of anterior descending neuromodulatory inputs were removed by blocking the stn.

RPCH increased the amplitude of the unitary sIPSPs evoked by the LP neuron in the PD neuron. Figure 2*A* shows recordings from a spontaneously active preparation in normal saline and in the presence of RPCH. In control saline, the LP neuron action potentials produced no obvious IPSPs in the PD neuron. However, after RPCH application, LP neuron action potentials evoked large, unitary sIPSPs. Note that the membrane potentials of the two recordings were approximately the same, so that the failure to record the IPSP in control saline was not caused by the membrane potential being close to the reversal potential for the IPSP.

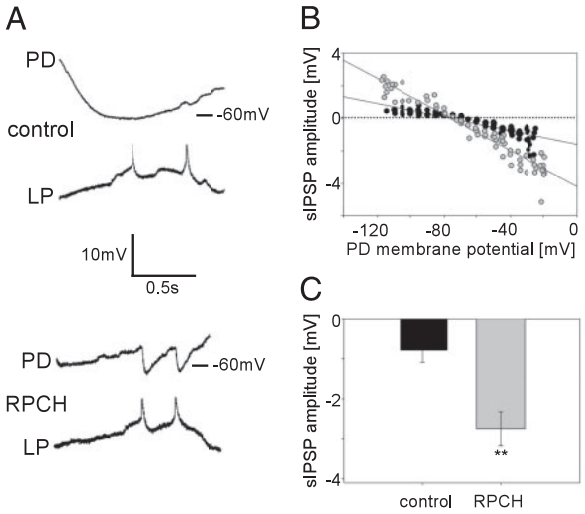

FIG. 2. Red pigment concentrating hormone (RPCH) potentiates spike-mediated synaptic transmission at the LP to PD synapse. *A*: in normal saline (control), LP spikes produce little or no spike-mediated inhibitory postsynaptic potentials (IPSPs) in PD. In the same preparation in RPCH, LP spikes cause large IPSPs in PD at similar membrane potentials of the PD neuron. In both conditions, the LP neuron was at −45 mV when spiking, and the trough of the PD neuron was close to −60 mV (indicated by the bars). *B*: amplitude of the spike-mediated IPSP (sIPSP) is larger in RPCH than in control at all PD membrane potentials, except around synaptic reversal. Black circles, control; gray circles, 10^{-6} M RPCH. Data in *B* are not from the same experimental preparation as in *A*. *C*: pooled data from 6 preparations shows that RPCH increases size of sIPSP. Mean sIPSP amplitude and SE at a PD neuron membrane potential of −40 mV in control and in RPCH are plotted. $^{**}P < 0.01$.

Figure 2*B* is a plot of the amplitude of the unitary IPSP evoked in a PD neuron by single LP neuron action potentials as a function of the PD neuron's membrane potential. Note that the amplitude of the IPSP was increased by RPCH at all postsynaptic membrane potentials but the reversal potential, which was unchanged. Figure 2*C* shows summary data from six different preparations in which RPCH increased the size of the sIPSP measured at −40 mV from −0.8 ± 0.3 mV in normal saline to −2.75 ± 0.43 mV ($P < 0.01$, Student's t-test).

Figure 3*A* shows that in normal saline with TTX the LP neuron to PD neuron gIPSP was very weak. There was only a small transient gIPSP seen in the PD neuron in response to the LP neuron depolarization from −80 to −30 mV. In contrast, in the presence of 10^{-6} M RPCH plus TTX the gIPSP increased in size several-fold. These gIPSPs show significant depression during the sustained presynaptic pulse (Manor et al. 1997), and Fig. 3*A* shows that both the early peak and the sustained level of the IPSP were enhanced in RPCH. The increase in gIPSP amplitude was seen at all membrane potentials of the PD neuron except at the reversal potential, which was unchanged (Fig. 3*B*). No observable changes in the PD neuron input impedance were seen (data not shown). Figure 3*C* shows that the size of the gIPSP measured at −40 mV increased from −2.2 ± 0.6 mV in normal saline to −11.2 ± 1.8 mV in 10^{-6} M RPCH ($n = 4$; $P < 0.01$, Student's t-test).

The previous experiments were done in preparations in which the modulatory inputs were removed to allow us to study the effects of RPCH on the LP to PD neuron synapse under conditions in which we could effectively control both the presynaptic and postsynaptic neurons' firing. Nonetheless,

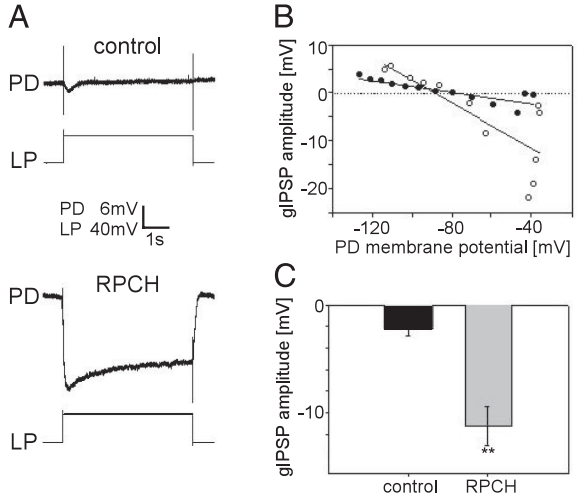

FIG. 3. Modulation of graded synaptic transmission at the LP to PD synapse by RPCH. *A*: graded IPSPs (gIPSPs) recorded from PD in response to a voltage jump from −80 to −30 mV in LP in normal saline and TTX (control) and in RPCH and TTX. PD holding potential was −38 mV in both conditions. The gIPSP is transient and small in control and persistent and larger in RPCH. *B*: increase in size of the persistent gIPSP is seen at all membrane potentials of the PD neuron except near synaptic reversal. Black circles, control; gray circles, 10^{-6} M RPCH. *C*: pooled data from 4 preparations shows that RPCH increases the amplitude of the persistent gIPSP. Mean persistent gIPSP amplitude and SE when the PD neuron was held at −40 mV are plotted. $^{**}P < 0.01$.

whereas these paradigms are useful in showing a modulatory action on an "isolated synapse," they do not necessarily show that RPCH applications would as strongly modulate the same synapses in preparations with ongoing pyloric rhythms seen in the presence of descending modulatory inputs. To address this question, during ongoing rhythms, we depolarized the PD neuron for 29 s every 1.9 min such that during the depolarization, the trough of the oscillation was close to about −40 mV and recorded the LP to PD neuron IPSPs in control saline and in the presence of RPCH.

Figure 4*A* shows simultaneous intracellular recordings from PD and LP neurons during one LP neuron burst in control saline and after 40 min in RPCH. The amplitude of the individual IPSPs is significantly larger in RPCH, as is the envelope composed of the graded and spike-mediated components. Figure 4*B* plots the average PSP amplitude during each bout of PD depolarization against time, in the same experiment as shown in Fig. 4*A*. Under these experimental conditions, the increase in average PSP amplitude took some time to occur but persisted as long as the RPCH was present. Figure 4*C* is a summary plot of the amplitude of the fifth IPSP in each LP neuron's burst ($n = 5$ experiments) in control saline, at the time of the peak of the RPCH effects on the pyloric rhythm frequency (~5–10 min after RPCH application), at 40 min, and after washing with normal saline. The enhanced IPSP amplitude was significantly different from the control, and the peak and 40 min values were not significantly different (repeated-measures ANOVA, $P < 0.01$; Tukey test, $P < 0.01$ for control vs. RPCH peak and control vs. RPCH 40 min, $P = 0.936$ for RPCH peak vs. RPCH 40 min).

Effects of RPCH on the pyloric rhythm

Given the sustained and strong effect of RPCH on the LP to PD synapse strength, we expected that the peptide might also have strong effects on the pyloric rhythm. To assay the effects of RPCH on the pyloric network, we bath-applied RPCH during an on-going pyloric rhythm and compared the cycle period of the rhythm in control saline and at different times during RPCH application. To assay the effects of RPCH on the pyloric pacemaker alone, we hyperpolarized the LP neuron for 29 s every 1.9 min and compared the cycle period in control saline and at different times during RPCH application. Each panel in Fig. 5*A* displays an extracellular recording from the lvn and an intracellular PD neuron recording. The lvn recordings show alternations of the LP neuron (largest spike) and the PD neurons when the LP neuron was not hyperpolarized and show that LP neuron action potentials were abolished by the somatic hyperpolarization. With the LP neuron active in the network (Fig. 5*A*, top 2 traces), RPCH application produced an increase in period, which decayed over time. At 40 min, in the continued presence of RPCH, the period had returned close to its baseline values. When the LP neuron was hyperpolarized (Fig. 5*A*, *bottom 2 traces*), RPCH application once again produced an increase in the PD neuron period, which again recovered to baseline levels during extended periods of time in the presence of RPCH. The time-course of the RPCH actions are shown in Fig. 5*B*, for the PD cycle period and for the durations of the PD and LP neuron bursts. These plots show clearly that the peak effects on the firing of the neurons were seen early in RPCH applications but did not persist after 40 min.

Pooling the data from five experiments, both RPCH application and the presence or absence of LP activity had a significant effect on the PD neuron cycle period (2-way repeated-measures ANOVA main effect of RPCH application, $P < 0.001$; main effect of LP activity, $P < 0.05$), with a significant interaction present between the two factors (2-way repeated-measures ANOVA, $P < 0.05$). A post hoc Tukey test showed that with the LP neuron in the circuit, RPCH increased the PD neuron cycle period from 1.13 ± 0.05 s in control saline to 1.56 ± 0.09 s at the peak of the RPCH effect ($P < 0.001$). By minute 40 of RPCH superfusion, the PD neuron cycle period dropped to 1.10 ± 0.06 s — a level not significantly different

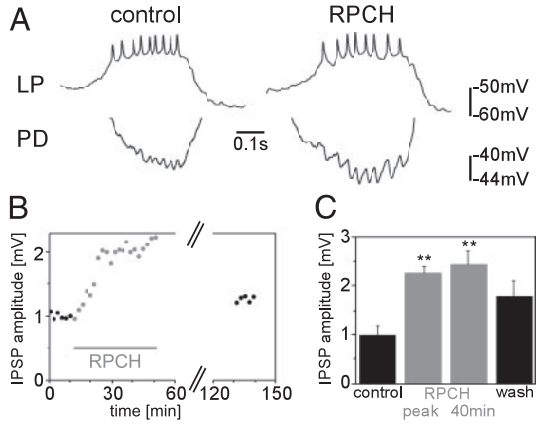

FIG. 4. RPCH enhances LP to PD synaptic transmission in the intact preparation. *A*: intracellular recordings from LP and PD in control saline and after 40 min in RPCH. Spike-mediated IPSPs are larger in RPCH. *B*: time-course of IPSP amplitude in PD before, during, and after RPCH application. IPSP amplitude increases slowly in RPCH and reaches a plateau after ~30 min of RPCH application. *C*: averages over 5 preparations and SE of IPSP amplitude in control, at the peak of RPCH effects on the rhythm frequency, after 40 min in RPCH, and after washing. $^{**}P < 0.01$ compared with control.

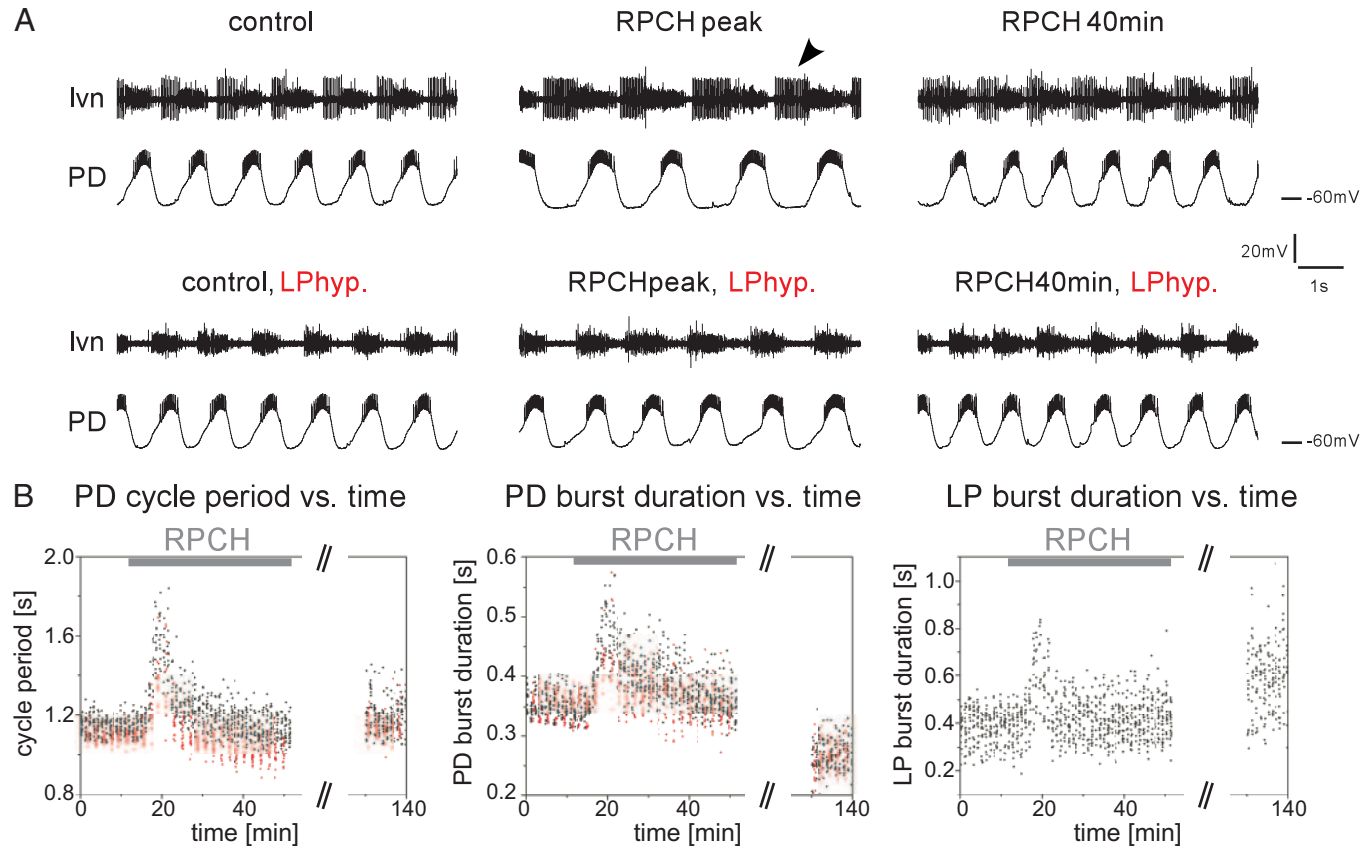

FIG. 5. Time-course of RPCH effects on the pyloric rhythm. *A*: nerve recordings from the lvn and intracellular recordings from PD in control saline (*left*), at peak of RPCH effect on the rhythm (*middle*), and after 40 min in RPCH (*right*). *Top rows* show recordings from the intact pyloric circuit, whereas LP was removed from the circuit by hyperpolarization in the *bottom rows*. Arrowhead points to the LP spikes, which are absent when LP is hyperpolarized. *B*: time-course of the pyloric cycle period (*left*), the PD burst duration (*middle*), and the LP burst duration (*right*) before, during, and after RPCH application. Dots show data from the same preparation with intact circuit (black) and with LP hyperpolarized (red).

from that seen in control saline ($P = 0.942$) or after RPCH washout (1.12 ± 0.05 s, $P = 0.983$). Although RPCH also increased the PD neuron cycle period when the LP neuron was hyperpolarized, raising it from 1.09 ± 0.05 s in saline to 1.39 ± 0.04 s at the peak of the RPCH effect ($P < 0.001$), the cycle period at peak effect was significantly shorter with LP hyperpolarized than when the LP neuron was active ($P < 0.001$). As before, the cycle period at minute 40 of RPCH superfusion (1.05 ± 0.05 s) did not differ significantly from either the cycle period in control saline ($P = 0.894$) or that after a saline wash (1.09 ± 0.04 s, $P = 0.924$).

RPCH application also had a significant effect on the PD neuron burst duration (2-way repeated-measures ANOVA main effect of RPCH application, $P < 0.01$), and RPCH's effect again differed depending on the presence or absence of LP activity (2-way repeated-measures ANOVA interaction between RPCH application and LP activity, $P < 0.01$). A post hoc Tukey's test showed that with the LP neuron in the circuit, RPCH increased the PD neuron burst duration from 0.34 ± 0.01 s in control saline to 0.55 ± 0.07 s at the peak of the RPCH effect ($P < 0.01$). The PD neuron burst duration followed the same pattern as the cycle period, dropping to a level at minute 40 of RPCH superfusion (0.35 ± 0.02 s) not significantly different from that seen in either control saline ($P = 0.998$) or after RPCH washout (0.33 ± 0.03 s, $P = 0.960$). Although RPCH did tend to increase the PD burst duration when the LP neuron was hyperpolarized (from 0.35 ± 0.01 s in saline to 0.47 ± 0.04 s at the peak of the RPCH effect, then dropping back down to 0.36 ± 0.01 s at RPCH minute 40), the increase was not statistically significant ($P = 0.119$), and the duration at peak effect was significantly smaller than when the LP neuron was active ($P < 0.01$).

RPCH had a significant effect on the LP neuron burst duration as well (repeated-measures ANOVA, $P < 0.01$). A post hoc Tukey's test showed that RPCH increased the LP neuron burst duration from 0.33 ± 0.03 s in saline to 0.53 ± 0.05 s at the peak of the RPCH effect ($P < 0.01$). The burst duration at minute 40 of RPCH superfusion (0.37 ± 0.05 s) did not differ significantly from either the duration seen in control saline ($P = 0.742$) or that seen after a saline wash (0.40 ± 0.05 s, $P = 0.915$).

Dissecting RPCH actions using PRCs

Together, the data on the effects of RPCH on the ongoing pyloric rhythm show that early after RPCH is applied to an ongoing rhythm it increases the cycle period, most likely caused by a direct action on one or more neurons of the pacemaker kernel, but that these effects do not persist during longer RPCH applications. In contrast, the effects of RPCH on the strength of the synapse from the LP neuron to the PD neuron did persist for as long as RPCH was present. Why is the period of the pyloric rhythm seen late in RPCH applications

apparently so insensitive to the strength of the LP to PD neuron synapse?

The answer to this question lies in the PRC of the PD neuron in response to inhibitory inputs. In these experiments, we used the dynamic clamp (Prinz et al. 2004; Sharp et al. 1993b) to construct an artificial IPSP of varying conductance and duration and measured families of PRCs resulting from moving these artificial IPSPs throughout the cycle of the PD neurons. In these experiments, the values of the LP to PD synaptic conductance were varied from 20 to 1,000 nS. The biological LP neuron was removed from the pyloric circuit (see METHODS).

Figure 6 shows an experiment in which the dynamic clamp was used to inject 500-ms inhibitory synaptic conductances into the PD neuron at different phases of its oscillation. The 500-ms duration was chosen because it is typical of the LP neuron burst duration under control conditions. The PRC of the PD neuron was plotted using the fractional change in cycle period caused by a pulse (phase reset) and the phase in the PD neuron oscillation at which the pulse occurred (stimulus phase). Figure 6*A* shows the membrane potential recording of a PD neuron when the dynamic clamp artificial synaptic input was applied during the PD neuron burst. The 20-nS pulse did not prematurely terminate the PD neuron firing, but the 50- and 200-nS conductance pulses did so. Figure 6*B* shows the response of the PD neuron for the same set of synaptic conductances delivered at a late phase of the PD neuron oscillation. The *bottom traces* in Fig. 6, *A* and *B*, are recordings of the dynamic clamp current injected into the PD neuron.

Figure 6*C* shows the PRCs plotted for the full data set at four different synaptic conductances. Weak inhibitory inputs produced modest phase advances when delivered early in the cycle and produced relatively little effect when delivered late in the cycle. Larger conductances caused phase advances when delivered at stimulus phase 0–0.4 and phase delays when delivered after 0.6. The PRCs are close to zero at phases 0.4–0.6 for all amplitude IPSPs. As the LP neuron is routinely active during ongoing rhythms at around phase 0.5, this means that the onset of the natural LP burst occurs at the time when the oscillator is least sensitive to the LP-evoked IPSP ($n = 10$ experiments for synaptic conductance from 20 to 1,000 nS).

Because one of our aims was to ask whether RPCH-mediated synapse strengthening affects the pyloric cycle period, we needed to determine whether the PD neuron PRC itself was altered in RPCH. In three experiments, we directly compared the PRCs of the PD neuron in normal saline and in 10^{-6} M RPCH, using 100-nS, 500-ms stimuli to obtain the PRCs. Figure 6*D* shows example PRCs obtained from a PD neuron in normal saline (black dots) and in 10^{-6} M RPCH (open dots). The PRCs were similar regardless of the presence or absence of RPCH (2-way ANOVA, $P < 0.001$ for stimulus phase; $P = 0.393$ for presence/absence of RPCH).

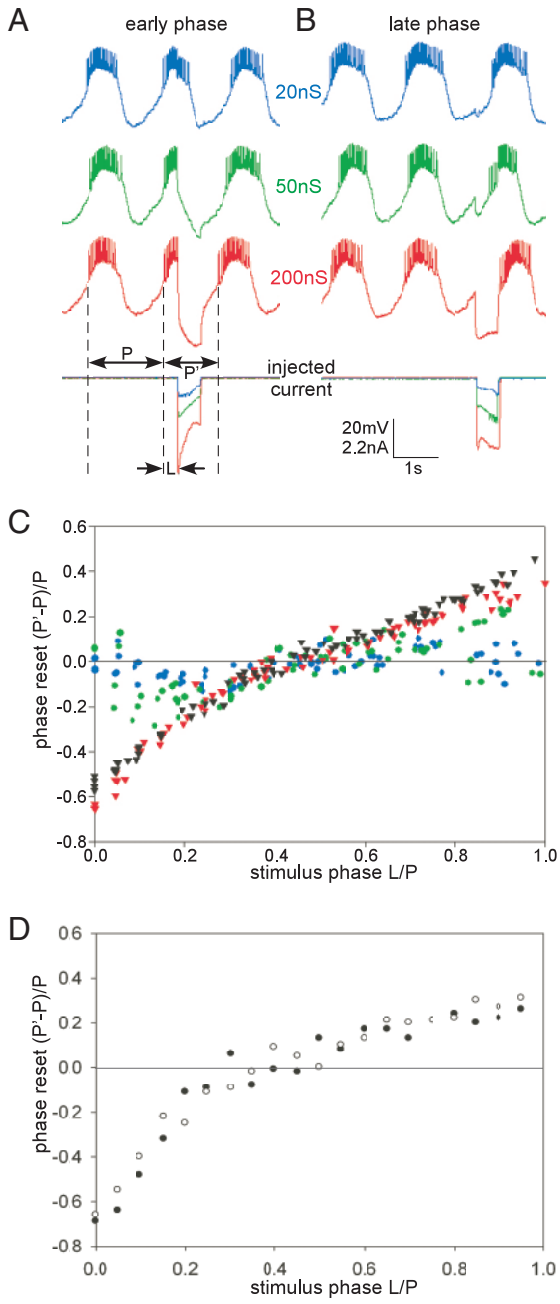

FIG. 6. Phase resetting of the pyloric pacemaker in response to stimuli of different amplitudes. *A*: recordings from a PD neuron during dynamic clamp injection of 500-ms inhibitory synaptic conductance pulses of 20- (blue), 50- (green), and 200-nS (red) amplitude at an early phase of the oscillation. Unperturbed period *P*, perturbed period *P*', and stimulus latency *L* are indicated. *B*: injection of the same conductance pulses at a late phase of the oscillation. *C*: phase resetting curves of a pyloric pacemaker for different amplitude conductance pulses. Color coding is the same as in *A* and *B*. Phase resetting in response to stimuli of 1,000-nS amplitude is shown in black. *D*: phase resetting curves of a pyloric pacemaker in normal saline and in 10^{-6} M RPCH. Resets in normal saline are shown with black dots and those in RPCH are shown in unfilled dots. Stimuli were of conductance 100 nS and duration 500 ms.

Changing the duration of the synaptic input can affect PD neuron cycle period

RPCH transiently increased the burst duration of the LP neuron (Fig. 5*B*). We previously showed that increasing the duration of the input to an oscillator influences its period (Prinz et al. 2003). Therefore we wished to determine the effects of altering the duration of the LP neuron activity. To do this, we measured PRCs in the PD neuron with a synaptic conductance of 500 nS while varying the duration of the stimulus to cover a range from 20 to 750 ms.

Figure 7*A* shows voltage traces from a PD neuron in response to pulses of 20 (blue trace), 500 (green trace), and 750 ms (red trace) injected during the early phase of its oscillation.

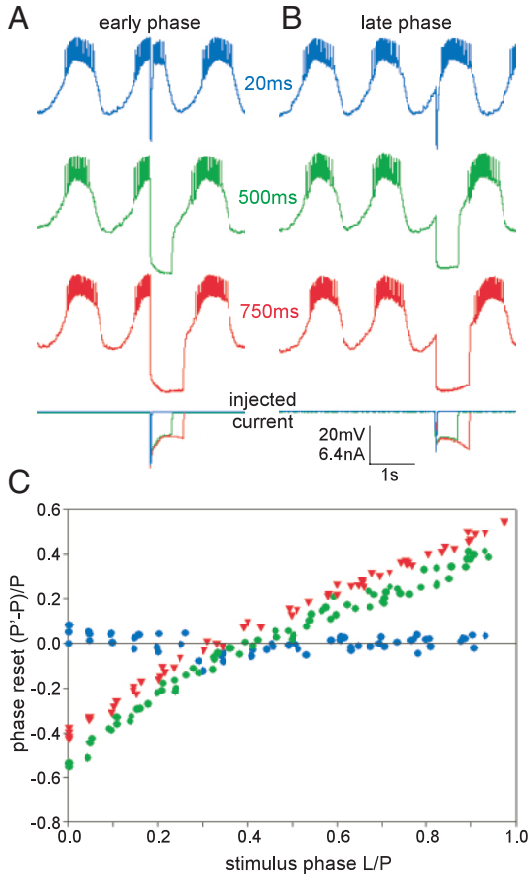

FIG. 7. Phase resetting of the pyloric pacemaker in response to stimuli of different durations. *A*: recordings from a PD neuron during dynamic clamp injection of 500-nS inhibitory synaptic conductance pulses of 20- (blue), 500- (green), and 750-ms (red) duration at an early phase of the oscillation. *B*: injection of the same conductance pulses at a late phase of the oscillation. *C*: phase resetting curves of a pyloric pacemaker for pulses of different durations. Color coding is the same as in *A* and *B*.

Figure 7*B* shows voltage traces from the same PD neuron injected with pulses of these same durations but at a late phase of the PD oscillation. The 20-ms pulse was too short to cause significant resets in the PD oscillation at any phase (Fig. 7, *A* and *B*, blue traces; Fig. 7*C*, blue circles). When the duration was set to 500 ms, pulses arriving early aborted the PD burst and caused an advance (Fig. 7*A*, green trace), whereas pulses arriving late caused a small delay (Fig. 7*B*, green trace). The PRC for the 500-ms pulse (Fig. 7*C*, green circles) crossed the *x*-axis at around phase 0.4–0.5. As the duration of the pulse was increased to 750 ms, the PRC shifted to greater delays (Fig. 7*C*, red triangles). The PRC for the 750-ms pulse crossed the *x*-axis at around phase 0.3–0.35. We constructed PRCs of the PD neuron for varying durations in 12 different preparations. In all cases, the PRCs shifted toward greater delays at all phases when the pulse duration was increased.

Figure 7*C* shows that a 500-ms pulse arriving at phase 0.5 causes a phase delay of ~0.05. At the same stimulus phase, a 750-ms-long pulse causes a phase delay of ~0.2. This is to say that when the LP burst duration is 500 ms, this would produce an increase in the PD cycle period by 5% and when the burst duration is 750 ms, this would contribute to an increase in the PD cycle period by 20%. This implies that when the LP burst duration increases from 500 to 750 ms, the PD oscillation period would increase by 15%. Therefore increases in the LP neuron burst duration caused by modulators such as RPCH are likely to contribute modestly to the increase in the pyloric cycle period.

DISCUSSION

It is usually assumed that large changes in synaptic strength will have important functional consequences for the circuit in which they are found, whether these changes are evoked by application of a neuromodulator or result from experience-dependent plasticity. In contrast, in this study we show that several-fold changes in the strength of a synapse evoked by a neuromodulator may have virtually no effect under many physiological conditions. Instead, the neuromodulatory control of synaptic strength may tend to stabilize ongoing network performance.

The synapse of interest in this study is an input to an oscillator. In earlier work, we showed that the PRCs of bursting and spiking oscillators saturate for increasing input strength and even a 10-fold increase in synaptic strength may not change the oscillator period if the synapse was strong to begin with (Oprisan et al. 2003; Prinz et al. 2003). In this study, the initial values of the LP to PD neuron synapses were often quite low in control conditions, but nonetheless the large potentiation seen in RPCH was without much effect because the LP neuron usually fires at the point of the cycle at which synaptic inputs have relatively little effect. Consequently, any change in the LP neuron duty cycle that expands the time at which the LP neuron is active will also have the effect of pushing the LP neuron's firing into the region of the cycle at which its synapse is more effective and therefore at which its strength is more relevant to the period of the oscillator.

In *H. americanus* it is not uncommon for the LP neuron evoked IPSPs in the PD neurons to be virtually invisible during ongoing pyloric rhythms, whereas in other crustacean stomatogastric systems such as *Cancer borealis* and *Panulirus interruptus*, the LP to PD IPSPs are often far more easily seen in intracellular recordings of the PD neuron (Eisen and Marder 1982; Manor and Nadim 2001; Manor et al. 1997; Maynard 1972; Nadim et al. 1999). Nonetheless, regardless of how large the IPSPs are in these different species, under control conditions the LP neuron's firing phase is relatively constant across species (Bucher et al. 2005; Hooper 1997a,b).

Several studies in the spiny lobster *P. interruptus* show that the LP neuron can regulate the pyloric cycle period through the LP to PD synapse (Mamiya and Nadim 2004, 2005; Nadim et al. 1999; Weaver and Hooper 2003). The PRCs shown here suggest that depending on where in the pyloric cycle they occur, LP bursts can either slow down or speed up the rhythm (Fig. 6*C* and 7*C*) and that these effects on the rhythm are enhanced with increasing LP to PD synaptic strength (Fig. 6). This characterization of LP's influence on the pyloric rhythm is consistent with those of Mamiya and Nadim (Mamiya and Nadim 2004, 2005), which suggest that rhythmic LP activity can lengthen the pyloric cycle period when the rhythm is fast and sometimes shorten the cycle period when the rhythm is slow. Mamiya and Nadim (2004) showed that slowing down the pyloric rhythm by hyperpolarizing the AB/PD pacemaker kernel would change the amplitude and phase of the IPSP produced in PD by LP. The IPSP peaked earlier in phase than

it had before, and, because the increased pyloric cycle period allowed for a greater recovery from synaptic depression, the IPSP had a larger amplitude. Our PRCs would predict that a strong IPSP firing with an early phase would speed up the pyloric rhythm (Fig. 6*C*), and this is what Mamiya and Nadim (2004) found.

Our work, in conjunction with the previous studies, argues that the LP to PD synapse functions to stabilize the pyloric rhythm. As our results show, the LP to PD synapse will have little to no effect on the pyloric rhythm when LP fires within a certain phase range (Figs. 6*C* and 7*C*). However, if the pyloric rhythm speeds up or slows down enough to shift the phase of the LP burst out of this range, the resulting IPSP in PD will have the effect of pushing the pyloric rhythm back toward its original frequency.

We hypothesize that it is because of the need for such a stabilizer that the potentiation of the LP to PD synapse by RPCH in *H. americanus* may be important. RPCH application not only strengthened the LP to PD synapse, it also enhanced bursting in the PD neurons. It is possible that to retain its ability to protect against drastic changes in pyloric frequency, the LP to PD synapse must become stronger to compensate for the increased strength in pacemaker function. If RPCH enhanced the strength of the pacemaker oscillator without also enhancing the LP to PD synapse, the synapse could become less effective as a period governor.

It is also possible that the potentiation of the LP to PD synapse may be attributed to a global increase in the strength of all synapses onto the PD neuron because of RPCH, which serves to better coordinate the pyloric rhythm with the gastric mill and cardiac sac rhythms. In fact, in some preparations, application of RPCH causes interruptions in the PD neuron oscillation that are timed with the cardiac sac or gastric mill rhythms (data not shown).

Whereas the effect of RPCH on the strength of the LP to PD synapse persisted for as long as the peptide was present, the effect on the pyloric cycle period was short-lived. Furthermore, RPCH increased the durations of the LP neuron and PD neuron bursts transiently (Fig. 5*B*). RPCH is both a hormone in the circulating hemolymph (Christie et al. 1995; Pulver and Marder 2002) and a neuromodulator released from the terminals of projection neurons (Fénelon et al. 1999; Nusbaum and Marder 1988; Thirumalai and Marder 2002). RPCH released hormonally may be present for longer durations while neurally released RPCH may be available for much shorter durations. By this token, hormonal RPCH might lead to a sustained increase in the LP to PD synaptic strength while neural RPCH might affect the cycle period of the rhythm. Thus the temporal dynamics of RPCH release will ultimately determine its network effects.

Finally, it is important to remember that in the animal RPCH is found as a cotransmitter with other modulators (Thirumalai and Marder 2002) and RPCH containing neurons, as has been shown in studies of the *C. borealis* modulatory projections, are likely to be part of a network of neuromodulatory projection neurons that work in concert (Blitz et al. 1999, 2004; Nusbaum and Beenhakker 2002). Therefore understanding the role of RPCH's increase in synaptic strength will ultimately require knowing the modulatory context in which RPCH is released.

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