Differential Modulation of Synaptic Strength and Timing Regulate Synaptic Efficacy in a Motor Network

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INTRODUCTION

Neural network output can be altered by changes in the electrophysiological properties of the network neurons and their synapses (Briggman and Kristan 2008; Dickinson 2006; Marder and Bucher 2007). Neuromodulators modify network output by altering neuronal firing properties and synaptic strength at multiple sites; however, the functional importance of each site is often unclear. We determined the importance of monoamine modulation of a single synapse for regulation of network cycle frequency in the oscillatory pyloric network of the lobster. The pacemaker kernel of the pyloric network receives only one chemical synaptic feedback, an inhibitory synapse from the lateral pyloric (LP) neuron to the pyloric dilator (PD) neurons, which can limit cycle frequency. We measured the effects of dopamine (DA), octopamine (Oct), and serotonin (5HT) on the strength of the LP→PD synapse and the ability of the modified synapse to regulate pyloric cycle frequency. DA and Oct strengthened, whereas 5HT weakened, LP→PD inhibition. Surprisingly, the DA-strengthened LP→PD synapse lost its ability to slow the pyloric oscillations, whereas the 5HT-weakened LP→PD synapse gained a greater influence on the oscillations. These results are explained by monoamine modulation of factors that determine the firing phase of the LP neuron in each cycle. DA acts via multiple mechanisms to phase-advance the LP neuron into the pacemaker’s refractory period, where the strengthened synapse has little effect. In contrast, 5HT phase-delays LP activity into a region of greater pacemaker sensitivity to LP synaptic input. Only Oct enhanced LP regulation of cycle period simply by enhancing LP→PD synaptic strength. These results show that modulation of the strength and timing of a synaptic input can differentially affect the synapse’s efficacy in the network.

METHODS

General procedures

California spiny lobsters (Panulirus interruptus) were supplied by Don Tomlinson Commercial Fishing (San Diego, CA) and maintained...
Synaptic connections are chemical inhibitory (●) and electrical (nonrectifying, resistor symbols; rectifying, diode symbols). The LP neuron activity by the following criteria: 1) matched extracellularly recorded action potentials with intracellularly recorded action potentials, 2) the characteristic shape and amplitude of membrane potential oscillations and action potentials of pyloric neurons, and 3) the pattern of synaptic connectivity (Johnson and Harris-Warrick 1997; Johnson et al. 1994).

Pyloric cells release transmitter as a continuous function of presynaptic voltage after release threshold is reached and before release saturates (graded synaptic transmission; Hartline and Graubard 1992). Because these graded synaptic interactions are thought to shape the pyloric pattern in the lobster (Hartline et al. 1988), we recorded PD graded inhibitory postsynaptic potentials (gIPSPs) evoked by the LP neuron, after adding 10–7 M TTX (control conditions) to block spiking activity. We examined graded LP→PD chemical transmission during two-electrode voltage clamp of the presynaptic LP neuron to drive the LP membrane potential, and two electrode current clamp to maintain the postsynaptic PD membrane potential at ~50 mV, using Axoclamp-2A and 2B amplifiers (Molecular Devices) as previously described (Johnson et al. 2005; Mamiya et al. 2003).

Dynamics of LP→PD graded synaptic transmission

For these experiments, we drove the presynaptic LP neuron in voltage clamp with realistic waveforms; these waveforms simulate the natural LP neuron membrane potential oscillations under control and amine conditions with descending modulatory input intact. To generate these waveforms, original LP recordings (Fig. 1B) were low-pass filtered at 30 Hz to remove spike transients but preserve the slope of LP rebound from pacemaker inhibition (Fig. 1C). Ten consecutive oscillation cycles in control and amine conditions were averaged from representative experiments. Oscillations were sampled with 1,000 points, the first and last points corresponding to the beginning and ending midpoint voltage values of a single LP oscillation. The original averaged period and amplitude were preserved as separate values. Control and amine waveforms were adjusted to 30 mV amplitude to drive the LP neuron from a holding value of ~55 mV (Johnson et al. 2005) to a peak of ~25 mV, which evokes the near maximum LP-elicited gIPSP in PD neurons in response to presynaptic square pulses (Johnson et al. 1994; Manor et al. 1997).

In all DA experiments and most of the Oct and 5HT experiments, the AB neuron was photoinactivated by intracellular iontophoresis of 5,6-carboxyfluorescein and illumination with bright blue light (Miller and Selverston 1979). This was done because DA activates rhythmic bursting in the AB neuron, which would rhythmically inhibit the LP unless it is removed. We waited ≥1 h after the AB lost its resting potential to allow recovery before starting the experiments. In control TTX conditions, a train of 10 linked voltage waveforms was injected as a voltage-clamp command into the presynaptic LP neuron. This was repeated at varying intervals after amine superfusion began. In some preparations, multiple amines were applied, but the order of amine application was shuffled between preparations. We measured the peak postsynaptic PD response to the first LP oscillation, and the mean steady-state PD response to repeated LP oscillations, as calculated from the average of the last five PD gIPSP amplitudes in the train. A steady-state synaptic depression index (DI) was calculated as the steady-state peak response divided by the initial peak response.

Amine effects on LP regulation of cycle period and pacemaker and LP firing properties during rhythmic pyloric activity

To examine the functional importance of the LP→PD synapse in the intact network (without TTX application or AB photoinactivation)
under control and amine conditions during normal network activity, we temporarily removed the LP neuron from the network by hyperpolarizing it. We used $-7$ to $-9\,\text{nA}$ current steps to block LP activity; we monitored LP spiking on its motor nerve (Ivn), and PD and AB membrane potential oscillations for any signs of LP synaptic input to ensure proper LP inhibition. The effects of LP removal were measured on the pyloric period from the average of the 10 consecutive cycles before and after LP hyperpolarization. The pyloric period was calculated from the average duration of the AB or PD slow wave oscillations. We also characterized the PD firing properties and AB oscillation amplitude before and during LP hyperpolarization and LP firing properties under control and amine conditions, including the LP firing delay relative to the peak AB or PD oscillation potential (see Fig. 3A), again all averaged over 10 consecutive pyloric cycles.

In a separate set of experiments, we examined the contribution of PY neurons, which inhibit the LP neuron (Fig. 1A), to the LP regulation of pyloric period. The experiments and analysis above were repeated after four to six PY neurons had been photoimactivated, as described above for AB inactivation.

Data acquisition and analysis

Electrophysiological recordings were digitized at 4 kHz using a PCI-6070-E board (National Instruments) and stored on a PC using custom-made recording software (Scope) written in LabWindows/CVI (National Instruments). The same software controlled the injection of artificial control and amine waveforms as voltage-clamp commands into the LP neuron. All data were analyzed using the related custom-made software program ReadScope also written in Lab Windows/CVI (software available at http://stg.rutgers.edu/software). For statistical comparisons, we used JMP and SAS software to run paired $t$-tests, regression analysis, one-way ANOVA, and multivariate analysis with mixed models, followed by post hoc multiple comparison tests when necessary to determine specific statistical differences between individual data groups. When the main effects of an experimental treatment were significant, we report the $P$ values from post hoc tests for specific data comparisons, unless stated otherwise. Significant differences between mean values were accepted with $P < 0.05$ (2-tailed probability) for $F$ and $t$ values. Mean measured data values and percentages are reported ± SD (Curran-Everett 2008). Estimated values from statistical models are expressed as means ± SE, as noted.

RESULTS

Amines modulate synaptic dynamics of the LP→PD graded chemical synapse

CONSTRUCTION AND USE OF REALISTIC WAVEFORMS FOR LP STIMULATION. The LP neuron control and amine-induced waveforms, which we constructed for our presynaptic voltage-clamp stimulation, reflect the shapes of the LP slow wave oscillations during LP activity under control and amine conditions with intact descending input to the STG (Fig. 1B) (Johnson et al. 2005). We previously described our construction of artificial waveforms reflecting the LP oscillations under control and DA conditions, with periods of 645 and 692 ms, respectively, and their use to examine DA modulation of synaptic dynamics at LP→PY synapses (Johnson et al. 2005). The control LP waveform is monophasic, whereas the DA LP waveform is biphasic (Fig. 1C) (Flamm and Harris-Warrick 1986a; Mamiya and Nadim 2004; see Johnson et al. 2005 for a more detailed description of the LP control and DA waveforms). The waveform shapes of the Oct- and 5HT-induced waveforms are monophasic and more closely resemble the control waveform (Fig. 1C). The biological Oct and 5HT LP waveforms had mean periods not significantly different from their control values (Oct, 678 ± 145 ms; paired $t$-test, $P = 0.39, n = 4$) and (5HT, 652 ± 127 ms; paired $t$-test, $P = 0.42, n = 3$). The LP artificial waveform periods used to drive the synaptic experiments are not significantly different from the mean periods in our physiological measurements of LP→PD synaptic strength changes during pyloric activity.

Our earlier work using square pulse stimulation of LP showed that DA and Oct enhanced, whereas 5HT reduced LP→PD graded synaptic strength (Johnson et al. 1995). We re-examined aminergic modulation of the graded LP→PD synapse using the constructed waveforms as trains of presynaptic voltage-clamp commands in the LP neuron. Control and amine waveforms were both presented as stimuli under both control conditions and during bath application of an amine; this allowed us to separate the direct effects of an amine on the LP→PD synapse from its indirect effects on synaptic strength caused by changes in the LP waveform. The PD neuron responded to a series of linked control or amine LP waveforms under control conditions with an initial large gIPSP that depressed to a steady-state amplitude of approximately one half of the first gIPSP (DI = 0.51 ± 0.08, $n = 11$ preparations, measured before any amine treatment; see also Mamiya and Nadim 2004). In different preparations, the initial PD gIPSP amplitudes under control conditions varied over more than an order of magnitude (0.33–5.79 mV). However, there was no dependence of the DI on initial gIPSP amplitude [slope of regression line for the DI/gIPSP relationship ($-0.014$) not significantly different from 0, regression analysis, $n = 11$; $P = 0.28$].

DOPAMINE AND PRESYNAPTIC WAVEFORM MODULATION OF LP→PD TRANSMISSION. DA enhanced the graded synaptic strength at the LP→PD synapse in five of five preparations (Fig. 2A, DA). DA increased the initial peak amplitude of the PD gIPSP elicited by the first control or DA LP waveforms by 59 ± 48 ($P = 0.04$) and 46 ± 35% ($P = 0.04$), respectively, when the gIPSPs from each waveform in DA were normalized to the control gIPSP amplitude from that experiment. The steady-state gIPSP amplitudes at the end of the waveform train were also significantly increased in DA by 41 ± 30% ($P = 0.005$) using the control LP waveform (Fig. 2B) and by 41 ± 30% ($P = 0.01$) using the DA LP waveform. Figure 2B compares the absolute mean sizes of the gIPSPs in control and DA conditions using the control LP waveforms. DA application had no significant effect on the DI using either the control (Fig. 2C) or DA waveform ($P > 0.4$).

Interestingly, the waveform shape (control vs. DA) did not significantly alter the amplitude of the initial gIPSP, under either control or DA conditions. The initial peak amplitudes of the PD gIPSPs elicited by the control and DA LP waveforms under control conditions were not significantly different from each other ($P = 0.16$); similarly, the initial gIPSP amplitude during DA application was not different when driven by the control or DA LP waveforms ($P = 0.81, n = 5$ for both comparisons). However, under both control and DA conditions, the amplitude of the steady-state PD response at the end of the train elicited by the DA LP waveform was significantly larger than that elicited by the control LP waveform (control conditions: 15 ± 11% larger with DA waveform, $P = 0.02$;
DA conditions: 32 ± 35% larger with DA waveform, $P = 0.04$). This reflected significantly less synaptic depression using the DA waveform under both control conditions (DI = 0.53 ± 0.14, $P = 0.03$) and during bath application of DA (DI = 0.58 ± 0.15, $P = 0.004$) than that seen using the control waveform under either control (DI = 0.45 ± 0.14) or DA conditions (DI = 0.45 ± 0.15; Fig. 2C; $n = 5$). This result is similar to our earlier work on the LP→PY graded synapse (Johnson et al. 2005), where a train of DA LP waveforms elicited less synaptic depression than the control LP waveform. This may be caused by the shorter duration of the initial major depolarizing phase of the presynaptic DA waveform (Fig. 1C), which caused less transmitter release; this would in turn reduce the accumulation of depression (Johnson et al. 2005). Thus DA directly enhances synaptic strength at the LP→PD synapse, and, by altering the LP oscillation waveform, indirectly reduces the degree of steady-state synaptic depression at the LP→PD synapse.

Oct modulation of LP→PD synaptic dynamics

Oct also enhanced graded synaptic strength at the LP→PD synapse (Fig. 2A, Oct; $n = 6$). The control and LP waveforms during Oct application are both monophasic (Fig. 1C), and the choice of waveform did not affect either the initial peak or the steady-state gIPSP amplitude, or the DI values of synaptic depression, under either control or Oct conditions ($P > 0.1$ for all comparisons between waveforms). Thus we show our results with Oct application using only the control LP waveform (Fig. 2, D and E). Oct significantly increased the mean initial PD gIPSP by 89 ± 102% ($P = 0.05$) and the steady-state PD response at the end of the train by 110 ± 104% ($P = 0.05$, $n = 6$; Oct gIPSPs normalized to control gIPSPs from the same experiment; mean absolute values shown in Fig. 2D). The proportionately greater increase in the PD steady-state response during Oct application resulted in a significant 13 ± 7% reduction in synaptic depression (increase in DI from 0.47 ± 0.06 in control to 0.53 ± 0.09 under Oct conditions; $P = 0.007$, $n = 6$; Fig. 2E). We conclude that Oct acts directly to enhance synaptic transmission and reduce synaptic depression at the LP→PD graded synapse and does not evoke any indirect effects on synaptic release by altering the LP waveform.

SEROTONIN MODULATION OF LP→PD SYNAPTIC DYNAMICS. In contrast to DA’s and Oct’s enhancement of LP→PD graded transmission, 5HT reduced graded synaptic strength at this synapse (Fig. 2A, 5HT). As in the Oct experiments, the 5HT and control LP waveforms did not significantly differ in their synaptic drive: the mean peak and steady-state amplitudes of the PD gIPSPs elicited by the control and 5HT LP waveforms in control or 5HT conditions were not significantly different.
(P > 0.2 for all, n = 6). 5HT significantly reduced the mean initial peak PD response to the first LP control waveform stimulation by 9 ± 14% (P = 0.03; Fig. 2F) and the steady-state PD gIPSP at the end of the train by 15 ± 14% (P = 0.03, n = 6; Fig. 2F). There was no significant difference in synaptic depression in control (DI = 0.52 ± 0.12) and 5HT conditions (DI = 0.48; P = 0.16, n = 6) in the PD response to the control LP waveform. Thus 5HT acts directly to weaken the PD response to LP stimulation and does not act indirectly by significantly changing the LP oscillation waveform.

**Amines change LP regulation of pyloric cycle period**

Based on these results, we hypothesized that DA and Oct, which strengthen the LP→PD synapse, would increase the ability of the LP neuron to regulate the pyloric period by increasing LP inhibition of the pacemaker group; in contrast, 5HT was hypothesized to mildly decrease the LP effect on pacemaker cycle frequency because of its weak reduction of the LP→PD synapse.

In these experiments, we determined the role of LP feedback inhibition in regulation of pyloric cycle period during intact network activity (no TTX application) under control and amine-modulated conditions. This was done by measuring the change in cycle period after the LP neuron was hyperpolarized to eliminate its graded transmitter release (Fig. 3A). For ease of reading, we will refer to this change in cycle period after LP hyperpolarization as the “LP effect.” Under control conditions, without prior amine exposure, the mean LP effect was to slightly but significantly shorten the mean pyloric period (763 ± 313 ms) by 66 ± 95 ms (8% reduction; P < 0.001, paired t-test, n = 30; range: 2% period increase to 24.8% period decrease with LP hyperpolarization); this shows that, on average, LP inhibition of the pacemaker kernel slows the pyloric rhythm under our control conditions. The strength of this LP effect varied positively and significantly with the duration of the control period over a wide range of periods (459–1,869 ms; Fig. 3B; slope of linear regression fit = 0.27; P < 0.001). A least squares fit model identified 600 ms as the duration of the pyloric period where the LP effect was significantly greater than zero [P = 0.02; estimated LP effect = 20.7 ± 8.5 (SE) ms at 600 ms period; Fig. 3B]. This relation shows that the LP neuron’s inhibition has a greater effect on cycle period when the period is relatively slow, whereas the LP→PD synapse plays virtually no role in frequency regulation at high cycle frequencies.

In the intact, actively cycling pyloric network, DA significantly accelerated the pyloric cycle frequency once the steady-state DA effect was reached (7–10 min after DA perfusion began). DA reduced the mean period by 17% (control period, 858 ± 383 ms; DA period, 711 ± 175 ms; P = 0.048, paired t-test, n = 13). Oct and 5HT did not significantly change the cycle period; the respective mean control and amine periods at steady state were as follows: Oct, 779 ± 220 and 767 ± 201 ms (P = 0.61, n = 11); 5HT, 660 ± 116 and 676 ± 133 ms (P = 0.37, n = 11; paired t-test).

All three amines significantly altered the LP control of pyloric cycle frequency. Although DA significantly increased LP→PD synaptic strength, this surprisingly did not result in greater LP control of the pyloric rhythm. Instead, DA significantly weakened the LP effect on cycle frequency. In 12 of 13 DA experiments, the LP regulation of pyloric period was greatly weakened or even abolished; in these, the mean LP effect during DA application was only 17 ± 11% of the control LP effect. In the 13th experiment, DA increased the LP effect on period; we consider this informative exception in more detail below. Considering all the DA experiments, the linear regression slope of the relationship between the initial pyloric period and the change in period after LP hyperpolarization was 8.5 (SE) ms at 600 ms period; Fig. 3A].

**FIG. 3.** Effect of pyloric period on the LP effect to decrease the pyloric period. A: examples of anterior burster (AB), PD, and LP activity before and after LP hyperpolarization (LP Hyp) under control conditions. Measurements of the cycle period (in ms) before and after LP hyperpolarization are given above the AB trace in each set. The measurements of period and delay are indicated. Vertical dashed lines on consecutive PD oscillations after the LP hyperpolarization indicate the expected timing of the 2nd burst, based on the period before LP hyperpolarization. Vertical markers, 10 mV. B: relationship between the initial pyloric period and the change in period after LP hyperpolarization. Slope of linear fit = 0.27; P < 0.001 for a significant positive correlation between control period and the LP effect under control conditions. Asterisk and downward arrow indicate the period value (600 ms) where the LP effect is significantly greater than 0 (P = 0.02).
little effect on period at high frequencies even under control conditions (Fig. 3B), we identified the duration of the pyloric period where the LP effect during DA became significantly smaller than the paired control LP effect. DA’s weakening of the LP effect was statistically significant at mean periods of 700 ms and longer (P = 0.04, differences of least square means; Fig. 4A). Thus DA greatly reduced or abolished the LP effect on cycle frequency, despite strong excitation of the LP neuron (Flamm and Harris-Warrick 1986b) and enhancement of the LP→PD graded synapse (Fig. 2).

Oct also increased the strength of the LP→PD synapse (Fig. 2), but unlike DA, this resulted in a significantly greater LP effect on the pyloric rhythm, leading to a greater acceleration in the cycle frequency after LP removal in 11 of 11 experiments. In the presence of Oct, the LP effect increased significantly with the cycle period (P = 0.007), and the slope of the Oct LP effect/period relationship (slope = 0.35) was significantly greater than the slope from the paired control values (slope = 0.04, regression analysis; P = 0.002, n = 11; Fig. 4B). The shallow slope of the paired control values was caused by a LP effect that increased the pyloric period under control conditions in two atypical experiments; Oct reestablished the typical LP effect to decrease period in these two experiments (see short arrows in Fig. 4B; control slope without these atypical values = 0.19) and enhanced the LP effect in all the other experiments. The Oct LP effect was significantly different from the control LP effect at periods of 625 ms (P = 0.01, differences of least square means) and above (Fig. 4B), well below the mean period of 752 ms measured during Oct application. These results suggest that Oct enhancement of the LP→PD graded synapse is at least in part responsible for the enhanced LP effect.

Finally, 5HT’s weak reduction of LP→PD synaptic strength did not correlate with 5HT’s action on the LP effect. Contrary to expectation, the average LP effect increased modestly but significantly during 5HT application by 34 ± 30.3 ms (P = 0.02; n = 11). The LP effect was small in these experiments, and there was no dependence of the LP effect on the initial period under control or 5HT conditions; neither the slope of the paired control (0.09) nor 5HT (0.1) LP effect/period relationship was significantly different from each other (P = 0.94, regression analysis) or from zero (P = 0.45; Fig. 4C). This lack of a correlation with pyloric period may have been influenced by the more limited range of rapid pyloric periods measured in these 5HT experiments. The y-intercept of the 5HT LP effect/period regression line was significantly greater than the y-intercept of the paired control regression line (regression analysis, P = 0.004, n = 11; Fig. 4C). In one atypical experiment, LP hyperpolarization increased the pyloric period under control conditions; 5HT restored the LP effect to decrease pyloric period (arrow in Fig. 4C). In 10 of 11 experiments, the LP regulation of pyloric period was strengthened in 5HT by 40 ± 25 ms over the control LP effect. In an 11th experiment, the LP effect decreased by 23 ms from control. Thus despite weakening the LP→PD synapse, the mean effect of 5HT was to weakly enhance the LP effect on cycle frequency.

Amine modulation of LP regulation of PD and AB excitability

We proceeded to explore possible reasons why strengthening or weakening the LP→PD synapse did not always enhance or decrease the LP effect as expected. One possibility is that the monoamines are simultaneously directly influencing the intrinsic electrophysiological properties of the pacemaker AB and PD neurons, and this changes their responses to LP inhibition. To test this, we monitored the firing properties of the PD neurons and the AB oscillation amplitude before and after LP hyperpolarization under control conditions and in the presence of the three amines.

Under control conditions, hyperpolarizing the LP neuron had relatively little effect on PD firing properties (Fig. 3A) (see
also Weaver and Hooper 2003). Under these conditions, there were no significant effects of LP hyperpolarization on the PD burst duration (control = 219 ± 53 ms, LP = 228 ± 66 ms; P = 0.19, n = 14) or the number of action potentials (APs)/burst (control and LP = 9 ± 3, P = 0.13). LP hyperpolarization weakly but significantly lengthened the PD duty cycle (control = 0.27 ± 0.06, LP = 0.30 ± 0.06; P = 0.02), but this was probably because of the shortening of the cycle period described above. LP hyperpolarization also significantly reduced the mean oscillation amplitudes of both the PD (control = 16.5 ± 4 mV, LP = 14.2 ± 3 mV; P = 0.004) and AB neurons (control = 16.5 ± 9 mV, LP = 15 ± 8 mV; P = 0.02, n = 5). This was caused by the loss of the hyperpolarization from the LP synaptic inhibition in PD (Fig. 3A) that is transferred through electrical synapses to the AB neuron.

As previously shown (Flamm and Harris-Warrick 1986b; Klopenburg et al. 1999), 10^{-4} M DA directly inhibited the PD neuron and excited the AB neuron. These opposing effects on the pacemaker neurons reduce the pacemaker oscillation frequency below that of the sympathetically isolated AB neuron (Ayali and Harris-Warrick 1999). Application of DA significantly reduced all parameters of PD activity, including the burst duration (73 ± 27% shorter; P < 0.001), duty cycle (−69 ± 33%; P < 0.001), APs/burst (−76 ± 18%; P < 0.001) and slow wave oscillation amplitude (−18 ± 15%; P = 0.002) compared with control values (n = 13). There was a near significant trend to increase the AB’s slow wave oscillation amplitude by 51.4 ± 40% (P = 0.058, n = 5).

Despite strengthening the LP→PD synapse, DA significantly reduced several of the effects of the LP synapse on the pacemaker neuron firing properties. In particular, during DA application, LP hyperpolarization had no effect on PD duty cycle (P = 0.48), and the LP effect on PD oscillation amplitude was significantly reduced by 20 ± 74% in DA (P = 0.04, n = 13). There was a trend for the LP effect on PD oscillation amplitude to be reduced in DA by 72 ± 29% (P = 0.06, n = 5). Thus the modest effects of LP inhibition on PD duty cycle and PD/AB oscillation properties were eliminated or weakened by direct effects of DA on the pacemaker neurons. Stronger AB oscillations in DA could contribute modestly to the weakening of the LP effect in DA.

Oct directly excites both isolated PD and AB neurons (Flamm and Harris-Warrick 1986b) but had no significant direct effect on any of the PD firing parameters or on PD oscillation amplitude (n = 11) or AB oscillation amplitude (n = 4; P > 0.7 for all). Oct superfusion did not create a significant LP effect on the PD burst duration or the number of APs/burst (P > 0.3 for both). LP removal reduced the PD and AB oscillation amplitude to the same extent in Oct as under control conditions (P > 0.3 for both). Octopamine did weakly but significantly increase the LP effect to increase PD duty cycle (duty cycle change − LP in control, 0.01 ± 0.03 units; duty cycle change − LP in Oct, 0.05 ± 0.06 units; P = 0.05). Thus direct effects of Oct on the pacemaker neurons did not contribute to Oct’s enhancement of the LP effect.

Finally, 5HT excites the AB neuron and has no effect on the PD neurons when these cells are isolated from other network neurons (Flamm and Harris-Warrick 1986b). This amine had no direct effect on PD or AB firing properties during network activity (n = 11; P > 0.7 for PD burst duration, APs/burst and duty cycle, and P = 0.44 for AB oscillation amplitude, n = 4). However, 5HT did significantly reduce the PD slow wave oscillation amplitude by 8.9 ± 5% (P = 0.002) because of removal of the LP-evoked hyperpolarization. The only effect of LP hyperpolarization during 5HT was to decrease the PD oscillation amplitude because of loss of the LP gIPSP, but this was not significantly different from that seen under control conditions (P = 0.74). Serotonin did not change the LP effect on PD burst duration, APs/burst, duty cycle, or AB oscillation amplitude (P > 0.2 for all). Thus Oct and 5HT had very little direct effect on pacemaker firing properties and did not alter the LP effect on PD and AB firing properties.

**Dopamine does not reduce transfer of LP inhibition to AB**

A second possible explanation for the loss of the LP effect on cycle frequency during DA application is that the LP neuron’s synaptic inhibition is not successfully reaching the primary pacemaker AB neuron. The LP neuron directly synapses on and inhibits only the PD neurons in the pacemaker kernel; transfer of LP→PD inhibition to the pacemaker AB neuron occurs through the PD→AB electrical synapse (Eisen and Marder 1982). This explanation is unlikely because this electrical synapse is strengthened by DA, when measured in isolated PD→AB neuron pairs with presynaptic square pulses (Johnson et al. 1993a), despite DA’s direct reduction of PD excitability. We tested the fidelity of the electrotonic transfer of LP inhibition through the PD to the AB neuron during DA application in two experiments, using realistic waveform stimulation of the LP neuron in the intact network. In both experiments, DA did not compromise signal transfer from LP to the AB neuron but rather enhanced it (data not shown). Clearly, during DA application, the LP neuron still communicates with and more strongly inhibits the AB neuron through the PD neuron.

**Dopamine may reduce LP regulation of pyloric period through changes in LP firing properties and phasing in the cycle**

An alternative hypothesis is that DA could change the phasing of LP firing, such that LP→PD inhibition occurs at a phase during the AB/PD oscillation cycle that is insensitive to entrainment (Ayali and Harris-Warrick 1999; Thirumalai et al. 2006). Under control conditions, the LP normally fires a single burst of action potentials per cycle (Fig. 3A), whereas during DA application, the LP neuron fires a major primary burst and has a second smaller oscillation near the end of the cycle, which may or may not initiate a few APs (Fig. 1B) (see also Johnson et al. 2005); this second oscillation usually does not contribute significantly to the synaptic drive to the PD neuron. DA significantly changed most of the LP firing parameters during the cycle (n = 12; Table 1). It decreased the delay from the peak of the pacemaker oscillation to the first spike of the LP burst (P < 0.001), advanced the firing onset phase (P < 0.001), decreased the burst duration (P = 0.04), advanced the LP offset phase (P < 0.001), and increased the AP frequency during the initial main LP burst (P < 0.001). The advance in LP firing onset is caused by an accelerated rate of LP rebound after pacemaker inhibition during DA (Harris-Warrick et al. 1995a). DA had a trend to decrease the LP duty cycle by 11 ±
TABLE 1.  Effect of monoamines on LP firing properties

<table>
<thead>
<tr>
<th>Firing Parameter</th>
<th>DA Control</th>
<th>DA</th>
<th>Oct Control</th>
<th>Oct</th>
<th>5HT Control</th>
<th>5HT</th>
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<tr>
<td>Firing delay, ms</td>
<td>278 ± 104</td>
<td>111 ± 41*</td>
<td>254 ± 89</td>
<td>222 ± 60*</td>
<td>222 ± 49</td>
<td>227 ± 46</td>
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<tr>
<td>Burst duration, ms</td>
<td>344 ± 231</td>
<td>203 ± 96*</td>
<td>245 ± 159</td>
<td>272 ± 186</td>
<td>191 ± 110</td>
<td>256 ± 121*</td>
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<tr>
<td>Firing offset phase</td>
<td>0.69 ± 0.13</td>
<td>0.47 ± 0.09*</td>
<td>0.61 ± 0.17</td>
<td>0.64 ± 0.19</td>
<td>0.62 ± 0.14</td>
<td>0.72 ± 0.18*</td>
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<tr>
<td>Duty cycle</td>
<td>0.37 ± 0.14</td>
<td>0.30 ± 0.11</td>
<td>0.28 ± 0.17</td>
<td>0.34 ± 0.18</td>
<td>0.28 ± 0.13</td>
<td>0.38 ± 0.14*</td>
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<tr>
<td>APs/burst</td>
<td>7 ± 2</td>
<td>9 ± 2</td>
<td>6 ± 4</td>
<td>8 ± 4*</td>
<td>7 ± 3</td>
<td>8 ± 3*</td>
</tr>
<tr>
<td>AP firing, Hz</td>
<td>28 ± 16.6</td>
<td>46 ± 11.3*</td>
<td>29 ± 17.1</td>
<td>37 ± 17.8</td>
<td>37 ± 18.1</td>
<td>32 ± 14.0</td>
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<tr>
<td>n =</td>
<td>12</td>
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Values are means ± SD. Mean control and amine firing properties are shown as matched pairs. *Significantly different from matched control value at \( P < 0.05 \).

36% (\( P = 0.076; \) Table 1). DA’s phase advance of the LP duty cycle could shift the LP pacemaker inhibition into a refractory zone where the pacemaker neurons are hyperpolarized with low input resistance and thus are insensitive to LP regulation or hyperpolarizing current injection (Ayali and Harris-Warrick 1999).

In support of this hypothesis, we noted three experiments in which the LP had an unusually prolonged firing period and the LP effect was not reduced by DA. In the one atypical DA experiment mentioned above, hyperpolarization of the LP had the same effect under control and DA conditions, decreasing the cycle period by 5 and 6%, respectively, despite a much stronger AB oscillation in DA (Fig. 5A). In this experiment, the LP neuron developed an unusually strong second burst during DA application, which combined with the first burst, prolonged the LP duty cycle (by 189%) and delayed the offset phase (by 82%; Fig. 5A) so that the LP offset firing phase was not advanced by DA. This might explain the failure of DA to weaken the LP effect in this experiment. In two other experiments, we delayed the offset-phase of the LP neuron during DA by photoinactivating a subset of the PY neurons. The termination of the initial burst of LP firing (its offset phase) is determined by the onset of bursting of a subset of the eight PY neurons, which synaptically inhibit the LP neuron. During DA superfusion, these PY neurons are greatly excited (Flamm and Harris-Warrick 1986b; Johnson et al. 2005), and the PY→LP inhibitory synapse is significantly strengthened, thus truncating the LP burst (Johnson et al. 1993b, 1995). To test whether this enhanced PY inhibition causes the phase advance of the LP firing offset, we photoinactivated all the PY neurons we could find (4–6) in six experiments. In two of these preparations, PY inhibition was sufficiently reduced and the LP neuron did not show its typical biphasic waveform during DA application but fired almost continuously between AB/PD bursts; as a consequence, the LP effect was not reduced by DA. This indicated that many of the PY neurons that terminate the LP burst and set the earlier offset phase of the LP in DA had been photoinactivated and were no longer participating in network activity. In the experiment shown in Fig. 5B, after six PY cells were photoinactivated, DA no longer weakened the LP effect: LP hyperpolarization reduced the pyloric period by 9% before DA superfusion, these PY neurons are greatly excited (Flamm and Harris-Warrick 1986b; Johnson et al. 2005), and the PY→LP inhibitory synapse is significantly strengthened, thus truncating the LP burst (Johnson et al. 1993b, 1995). To test whether this enhanced PY inhibition causes the phase advance of the LP firing offset, we photoinactivated all the PY neurons we could find (4–6) in six experiments. In two of these preparations, PY inhibition was sufficiently reduced and the LP neuron did not show its typical biphasic waveform during DA application but fired almost continuously between AB/PD bursts; as a consequence, the LP effect was not reduced by DA. This indicated that many of the PY neurons that terminate the LP burst and set the earlier offset phase of the LP in DA had been photoinactivated and were no longer participating in network activity. In the experiment shown in Fig. 5B, after six PY cells were photoinactivated, DA no longer weakened the LP effect: LP hyperpolarization reduced the pyloric period by 9% before DA and 8% during DA perfusion. Significantly, in this experiment the LP duty cycle and offset phase were prolonged by 60 and 8% during DA perfusion. Significantly, in this experiment the LP duty cycle and offset phase were prolonged by 60 and 8% during DA perfusion.

FIG. 5. Effects of removing the LP→PD synapse on pyloric period when the LP firing offset phase is prolonged during DA.  
A: unusual experiment where DA evokes a strong 2nd burst in each LP cycle, thus prolonging the LP firing phase. Period measurements are indicated above the AB trace under control (left traces) conditions and during DA perfusion (right traces) before and during LP hyperpolarization (LP Hyp). Vertical dashed lines on consecutive PD oscillations after the LP hyperpolarization indicate the expected timing of the 2nd burst, based on the period before LP hyperpolarization. B: a separate experiment where photoinactivation of 6 PY neurons prolongs LP firing. Left: control. Right: 10^{-4} M DA does not reduce the LP effect in this experiment. Vertical dashed lines on consecutive PD oscillations after the LP hyperpolarization indicate the expected timing of the 2nd burst, based on the period before LP hyperpolarization. Vertical markers, 10 mV.
offset phase) in the intact network normally moves LP inhibition of the AB/PD group into the pacemaker’s refractory period, reducing the LP effect.

We further explored the effect of the LP offset phase advance in reducing the functional role of the LP→PD synapse during DA application. Figure 6A shows the relationship between the LP offset phase and the LP effect on cycle frequency during application of the three amines. Here, the x-axis plots the LP offset phase during amine application minus the LP offset phase under control conditions, whereas the y-axis shows the LP effect during amine application minus the LP effect under control conditions, for all the experiments in this study. We see that, as the LP offset phase is advanced to earlier values during DA (red squares, more negative difference between amine and control offset phases), the difference between the LP effect in control and in DA increases (except for the atypical experiment described in Fig. 5A, red square above the 0 line in Fig. 6A). The linear regression fit of all the DA data points is significantly different from zero (P = 0.04; with removal of the most negative outlier, P = 0.01, linear regression analysis; Fig. 6A). Thus as the LP offset phase is advanced during DA, the LP effect is reduced.

Oct may enhance LP regulation of pyloric period by strengthening the LP→PD graded synapse

Oct (10^-5 M) weakly increased LP excitability (n = 9), a direct effect previously reported by Flamm and Harris-Warrick (1986b) (Table 1). In our experiments, this slightly enhanced LP excitability significantly shortened the pacemaker to LP delay (P = 0.04) and increased the number of APs/burst (P = 0.02). However, there was no significant effect of Oct on the LP firing onset phase, burst duration, offset phase, or AP burst frequency (P > 0.1 for all; Table 1). There is no significant interaction between any Oct-induced offset phase shift and the amplitude of the Oct-induced change in LP effect (linear regression analysis, P = 0.21; Fig. 6A, blue circles), because there is no significant change in the LP offset phase (x-axis values cluster around 0). Thus the enhancement of the LP effect during Oct is best explained by Oct’s direct action to strengthen the LP→PD synapse and its regulation of cycle period.

5HT may enhance the LP effect on pyloric period through changes in LP firing properties and phasing of activity

5HT significantly changed most of LP’s firing properties (Table 1; n = 9). It increased the LP burst duration (P = 0.05) and duty cycle (P = 0.01), significantly delayed the firing offset phase (P = 0.01), and increased the number of APs/burst (P = 0.03), but weakly decreased the average AP frequency within a burst (P = 0.03; Table 1). There was a trend for the LP onset phase to be weakly delayed during 5HT (P = 0.06). Thus during 5HT application, the LP neuron fired during a later phase than under control conditions. In the only experiment where 5HT reduced the LP effect, it only delayed the LP offset phase by 0.02 (0.79–0.81) compared with an average delay in the LP offset phase of 0.11 ± 0.09 in the eight preparations where 5HT increased the LP effect. We suggest that the later LP offset phase could prolong the duration of LP inhibition, thus holding the pacemaker neurons below threshold for the next burst for a longer time and slowing the cycle frequency. Thus despite 5HT’s weakening of the LP→PD synapse, hyperpolarization of the LP neuron would cause a significant increase in pyloric frequency by allowing the AB-PD neurons to rebound more rapidly to the next burst. This is reinforced by our analysis of Fig. 6A. As the LP offset phase is delayed to later values during 5HT (green diamonds, more positive dif-
ference between amine and control offset phases), the LP effect is enhanced, as seen by a more positive difference between the LP effect during 5HT and under control conditions (except for the single exception described above, green diamond below the 0 line; Fig. 6A). The linear regression fit of all the 5HT data points is significantly different from 0 (linear regression analysis, \( P = 0.006; \) Fig. 6A). Because 5HT only weakly affects LP excitability when this neuron is isolated from all synaptic input (Flamm and Harris-Warrick 1986b), it seems likely that the prolonged firing and offset phase of the LP neuron arises from 5HT’s direct effect to weaken PY→LP inhibition (Johnson et al. 1995). This could override the 5HT reduction in LP→PD synaptic strength to increase the LP effect.

Interaction of synaptic strength and LP phasing determines the amine modulation of LP effect on cycle frequency

Figure 6B summarizes the effects of amines on the LP firing phase during the pyloric cycle. DA significantly advances both onset and offset phases of the LP’s duty cycle, shifting its activity to a time when the AB-PD pacemaker kernel is fully hyperpolarized at the end of the previous burst. Oct does not change the LP’s firing phase. 5HT does not strongly affect the onset phase but significantly delays the offset phase of the LP duty cycle; thus the overall duty cycle of the LP is prolonged, and the LP fires later in the cycle than normal, when the AB-PD kernel is repolarizing toward threshold for the next burst. As described above, the LP offset phase in particular seems to play an important role in setting the functional consequences of the LP→PD synapse. Thus amine modulation of the LP effect in the pyloric network can be explained by a complex interaction between amine modulation of the LP→PD synaptic strength and amine modulation of LP firing phase, especially its offset phase (Ayali and Harris-Warrick 1999).

DISCUSSION

The LP→PD synapse provides the only inhibitory feedback to the AB-PD pacemaker kernel in the pyloric network. In the spiny lobster, LP→PD inhibition normally acts as a brake to limit the pyloric cycle frequency over much of its normal frequency range (Johnson et al. 2005; Selverston and Miller 1980; Weaver and Hooper 2003). Thus changes in this synapse’s strength could significantly change cycle frequency. Here we show that the steady-state amplitude of the LP→PD gIPSP was enhanced by DA and Oct and modestly weakened by 5HT. If the most important parameter for LP period control is simply the strength of the LP→PD synapse, our results would predict that the LP regulation of cycle frequency should increase with DA and Oct and decrease with 5HT.

The efficacy of a perturbing input on the firing pattern of a neuron is described by the neuron’s phase response curves (PRCs) (Smeal et al. 2010). PRCs for the pyloric oscillator show insensitivity to inhibitory input about midcycle, with earlier perturbations increasing cycle frequency and later ones decreasing cycle frequency (Ayali and Harris-Warrick 1999; Ayers and Selverston 1977; Prinz et al. 2003; Thirumalai et al. 2006). When we removed the LP from the network during amine application, our only accurate prediction was that Oct enhancement of the LP→PD synapse increased LP’s regulation of pyloric period. The failure of our predictions for DA and 5HT can be explained by other actions of these amines in the network that change the phasing of LP activity into or out of the sensitivity range of the pyloric pacemaker’s PRC and thus determine the functional effectiveness of the LP→PD synapse. LP regulation of pyloric period depends on both the strength and the timing of LP→PD inhibition (Ayali and Harris-Warrick 1999; Prinz et al. 2003; Thirumalai et al. 2006). Our results emphasize that analyses of changes in strength of a single synapse, taken out of context of other changes throughout the network that affect the neuron’s firing phase, can give misleading ideas of the synapse’s functional importance (Prinz et al. 2003).

In our experiments, Oct’s direct enhancement of LP→PD inhibition strengthened the LP regulation of pyloric frequency. Oct has a number of actions that regulate pyloric cycle frequency. Oct excites the oscillator kernel, initiating AB oscillations from a quiescent state and directly exciting the PD neurons (Ayali and Harris-Warrick 1999; Flamm and Harris-Warrick 1986a,b). These effects should increase the cycle frequency, unless there is compensatory LP inhibitory feedback. In fact, the pyloric cycle frequency did not change significantly during Oct (see also Ayali and Harris-Warrick 1999; Peck et al. 2001). This can be explained by the stronger LP regulation of the pyloric oscillator. In our Oct experiments, the LP→PD inhibition was strengthened without a change in LP firing phase (Fig. 6B). Our results contrast with the enhancement of the LP→PD synapse by red pigment concentrating hormone (RPCH) in the lobster Homarus, also without a change in LP firing phase, that failed to increase steady-state LP regulation of pyloric frequency (Thirumalai et al. 2006). In Homarus, LP inhibition arrives in the PD at a relatively insensitive range of the pacemaker PRC under both control and RPCH conditions and increased synaptic strength without a change in its phasing is not functionally important (Prinz et al. 2003; Thirumalai et al. 2006). In the spiny lobster, LP inhibition arrives in the PD at a sensitive phase of the pacemaker PRC, so increased strength of LP inhibition of the oscillator kernel by Oct has an effect on the cycle frequency. The balance between Oct enhancement of pacemaker cycle frequency and Oct enhancement of inhibitory LP→PD feedback can account for the cycle frequency constancy during Oct.

In most DA experiments, despite stronger LP→PD inhibition, the LP neuron lost its feedback regulation of the pyloric cycle frequency. The pyloric cycle frequency increased during DA, reflecting in part this loss of the LP effect. DA also weakened the modest effects of LP hyperpolarization on PD and AB firing properties. DA has opposite effects on the pacemaker neurons: PD excitability is reduced, whereas AB oscillations are accelerated and larger during ongoing network activity and can be initiated from a quiescent state (Flamm and Harris-Warrick 1986b; Kloppenburg et al. 1999). DA’s enhancement of AB oscillations combined with weaker LP feedback regulation of cycle frequency increases the cycle frequency by upsetting the balance between intrinsic oscillator frequency and LP feedback inhibition.

Our data suggest that DA-induced advance of the LP firing phase can explain its loss of pacemaker regulation (Ayali and Harris-Warrick 1999; Thirumalai et al. 2006). DA significantly advanced both the LP onset and offset phases and shortened the LP burst duration (Table 1; Fig. 6B). The advanced onset phase arises from faster postinhibitory rebound from AB/PD inhibi-
tion, caused in part by an increase in $I_h$ and a reduction in $I_A$ (Harris-Warrick et al. 1995a). The advanced offset phase is caused by stronger PY inhibition of LP, terminating LP activity when the PY neurons begin to fire. The PY neurons show faster rebound from AB/PD inhibition and increased spike frequency during DA, in part because of reduced $I_A$ (Harris-Warrick et al. 1995b). DA increases the PY→LP synaptic strength (Johnson et al. 1995), in part because of enhanced $I_{Ca(V)}$ (Johnson et al. 2003). These combined effects truncate the LP burst at an earlier phase during DA.

Further analysis showed that the advanced LP offset phase during DA can explain the loss of LP regulation of cycle frequency. Figure 6A shows that the degree of advance in LP offset phase during DA correlates strongly with the ability of the LP to slow the cycle frequency. In experiments where the LP offset phase was not advanced in DA, the LP effect was not reduced (Fig. 5). Thus despite DA’s enhancement of LP→PD inhibition, it simultaneously advances LP activity into a phase where the AB/PD neurons are just terminating their oscillation and are not sensitive to this inhibitory input. (Ayali and Harris-Warrick 1999; Thirumalai et al. 2006).

5HT weakly reduced the strength of LP→PD graded inhibition, but it increased the LP effect on cycle frequency. Despite this, the pyloric period was not significantly changed by 5HT (Ayali and Harris-Warrick 1999). 5HT excites the AB neuron and initiates AB oscillations from a quiescent state (Flamm and Harris-Warrick 1986b; Harris-Warrick and Flamm 1987), suggesting that increased oscillator excitability was balanced by the increased LP effect to maintain cycle frequency constancy during 5HT application. 5HT directly inhibits the LP (Flamm and Harris-Warrick 1986b; Marder and Eisen 1984), but in the intact network, the LP duty cycle and offset phase were prolonged (Fig. 6B). The offset phase again seems critical, because the extent of 5HT’s prolongation of the offset phase correlates with the extent of enhancement of the LP effect on cycle frequency (Fig. 6A). This prolongation of LP activity arises from 5HT weakening the PY→LP synapse (Johnson et al. 1994, 1995). Thus in contrast to DA, 5HT prolongs LP activity such that LP→PD inhibition, even though it is weaker, occurs in a more sensitive range of the oscillator’s PRC (Ayali and Harris-Warrick 1999; Ayer and Selverston 1977; Prinz et al. 2003; Thirumalai et al. 2006), and thus creates a stronger LP regulation of cycle frequency.

These results emphasize that the consequences of changes in strength of targeted synapses in a neural network depend on simultaneous changes in network neuron excitability and timing (Prinz et al. 2003), which may play a more important role in network reconfiguration. For example, we previously showed that even when DA inverted the sign of the mixed electrical/chemical inhibitory LP→PY synapse or activated silent LP→PY synapses, this did not alter the PY firing onset (Johnson et al. 2005). Instead, DA enhanced the PY postinhibitory rebound properties, partly by reducing $I_h$ (Harris-Warrick et al. 1995b) and enhancing $I_{Ca(V)}$ (Johnson et al. 2003); this played the major role in determining the PY onset phase (Harris-Warrick et al. 1995b; Johnson et al. 2005). Such plasticity in intrinsic firing properties, along with synaptic strength, is now recognized in many systems as an important contributor to network configuration (Benjamin et al. 2008; Debanne and Poo 2010). Even in the hippocampus, where research on memory formation has primarily focused on changes in synaptic strength, changes in intrinsic neuronal excitability can also shape learning networks (Mozzachiodi and Byrne 2010). Synaptic and intrinsic excitability mechanisms can work together to shape neuronal output (Weaver et al. 2010), often with multiple targets of action within the network (Harris-Warrick and Johnson 2010).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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