Dopamine Modulation of Phasing of Activity in a Rhythmic Motor Network: Contribution of Synaptic and Intrinsic Modulatory Actions

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Abstract

The phasing of neuronal activity in a rhythmic motor network is determined by a neuron's intrinsic firing properties and synaptic inputs; these could vary in their relative importance under different modulatory conditions. In the lobster pyloric network, the firing of eight follower PY neurons is shaped by their intrinsic rebound after pacemaker inhibition and by synaptic input from the LP neuron, which inhibits all PY neurons and is electrically coupled to a subset of them. Under control conditions, LP inhibition is weak and has little influence on PY firing. We examined modulation that could theoretically enhance the LP's synaptic contribution to PY firing. We measured the effects of dopamine (DA) on LP→PY synapses, driving the LP neuron with trains of realistic waveforms constructed from prerecorded control and DA LP oscillations, which differed in shape and duration. Under control conditions, chemical inhibition underwent severe depression and disappeared; in the mixed synapses, electrical coupling dominated. Switching between control and DA LP waveforms (with or without DA present) caused only subtle changes in synaptic transmission. DA markedly enhanced synaptic inhibition, reduced synaptic depression and weakened electrical coupling, reversing the sign of the mixed synapses. Despite this, removal of the LP from the intact network still had only weak effects on PY firing. DA also enhances PY intrinsic rebound properties, which still control the onset of PY firing. Thus, in a rhythmic network, the functional importance of synaptic modulation can only be understood in the context of parallel modulation of intrinsic properties.

Key Words: lobster, pyloric network, central pattern generator, mixed synapse

Introduction

The phasing of rhythmic activity of neurons and muscles shapes rhythmic motor behaviors, for example, distinguishing walking from skipping or galloping. This timing of neuronal activity during a rhythmic motor cycle is generally considered to be determined by the interactions of neurons in the central pattern generator (CPG) network that organizes the behavior (Harris-Warrick et al. 1992; Getting 1989; Orlovsky et al. 1999), as well as the pattern of sensory feedback (Pearson and Ramirez 1997; Pearson 2004). In many CPGs, the timing of onset and offset of neuronal firing in the networks is determined by a dynamic interaction between the intrinsic firing properties of the neurons (such as post-inhibitory rebound, bistability and bursting) and the pattern of synaptic interactions within the network (Cymbalyuk et al. 2002; Getting 1989; Harris-Warrick et al. 1992; Hooper 1997; Katz 1999; Ramirez et al. 2004; Stein et al. 1997). The phasing of neurons in the cycle is not fixed, but can vary, generating new motor "gaits", due to sensory input or the actions of neuromodulators such as amines and peptides. Plasticity in phasing can arise from the recruitment of new neurons that provide new synaptic inhibition or excitation (Selverston et al. 1997; Jing and Weiss 2001; Jing et al. 2003), modulation of existing synapses (Johnson et al. 1995; Sillar et al. 1998; Marder and Thirumalai 2002), and modulation of the intrinsic properties of the CPG neurons (Harris-Warrick et al. 1995ab, 1998; Kloppenburg et al. 1999; Matsushima et al. 1993; Marder and Thirumalai 2002). While the involvement of these parameters is widely accepted, their relative contributions in determining changes in phasing are not well understood in any system.

In this paper, we look at a specific example of modulation of phasing, how dopamine (DA) modifies the phasing of the 8 Pyloric (PY) neurons in the pyloric network of the crustacean stomatogastric ganglion (STG). The pyloric network contains 14 neurons, and generates a rhythmic motor pattern driven by a 3-neuron pacemaker kernel (Johnson and Hooper 1992; Ayali and Harris- Warrick 1999). Under control conditions, the PY neurons fire with a delay after being inhibited by the network pacemaker kernel. This delay is thought to be established by the combined actions of intrinsic post-inhibitory rebound (PIR) after pacemaker inhibition and by synaptic input

from the Lateral Pyloric (LP) neuron, which fires earlier in the triphasic rhythm (Hartline and Gassie 1979; Selverston et al. 1998). The LP chemically inhibits the PY neurons via graded transmission, and is additionally electrically coupled to a subset of them (Fig. 1A; Johnson et al. 1994). Under control conditions, the chemical inhibition is weak and undergoes significant synaptic depression during normal LP oscillations, leaving the electrotonic coupling between LP and PY as the dominant synaptic interaction (Mamiya et al. 2003). Removal of the LP neuron has only slight effects on the phasing of PY firing in the intact network (Mamiya et al. 2003; Weaver and Hooper 2003).

Dopamine causes a significant PY phase advance in the pyloric rhythm. DA enhances PY post-inhibitory rebound after pacemaker inhibition primarily by reducing I_A (Harris-Warrick et al. 1995a). At the same time, it enhances LP→PY chemical inhibition and weakens LP→PY electrical coupling, reversing the sign of the LP→PY interaction from depolarizing to hyperpolarizing (Johnson et al. 1993b, 1994). In theory, these changes should cause the LP→PY synapse to oppose and constrain the advance in PY onset phase caused by PY PIR. However, two factors might lessen its impact. First, the LP neuron appears to oscillate with a significantly narrower waveform in DA compared to control (Flamm and Harris-Warrick 1986), potentially reducing synaptic transmission. Second, the degree of synaptic depression of the DA-modified LP→PY synapse is unknown, as previous studies were done with single square pulse depolarizations of the LP neuron (Johnson et al. 1994). Here, we address these two factors and determine the relative role of the LP→PY synapse in determining the onset phase of PY firing in the DA-modified pyloric rhythm. Using pre-recorded realistic waveforms to drive synaptic transmission, we show that the LP waveform shape plays a surprisingly small role in determining the strength of the LP→PY synapse. Even though the synapse undergoes synaptic depression, it still becomes strongly inhibitory in the presence of DA. Despite this sign reversal, the LP→PY synapse continues to play only a minor role in determining PY onset phase, which is dominated by DA's enhancement of the PY neurons' intrinsic PIR properties.

Materials and Methods

California spiny lobsters (*Panulirus interruptus*) were supplied by Don Tomlinson Commercial Fishing (San Diego, CA) and maintained in marine aquaria at 16° C. Lobsters were cooled in ice until immobile. The stomatogastric nervous system (STNS) was removed as previously described (Selverston et al. 1976), and pinned in a Sylgard–coated petri dish in chilled *Panulirus* saline of the following composition (mM): 479 NaCl, 12.8 KCl, 13.7 $CaCl_2$, 3.9 Na_2SO_4, 10.0 $MgSO_4$, 2 glucose, 11.1 Tris base, pH 7.35 (Mulloney and Selverston 1974). The STG was desheathed, enclosed in a 1ml pool walled with Vaseline and superfused at 5 ml/min with oxygenated *Panulirus* saline (18-19°C). In all experiments, DA was prepared in the appropriate control solution to a final concentration of 10^{-4}M just before application (DA conditions). All measurements were taken after 5 min perfusion with DA. Results were discarded if the effects of DA did not show reversal after a 30 min wash. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Electrophysiological recording, cell identification and synaptic isolation. We recorded pyloric neuron activity using extracellular pin electrodes and standard intracellular recording techniques. We examined the LP → PY synaptic interaction using two electrode voltage-clamp of the pre-synaptic LP neuron and current injection to maintain the post-synaptic PY membrane potential at the desired level (3 M KCl filled electrodes, 10 to 15 MΩ resistance) using Axoclamp-2A and 2B amplifiers (Axon Instruments) as previously described (Johnson et al. 1994; Mamiya et al. 2003). Our methods for identifying the six major classes of pyloric neurons, for photoinactivation to kill specific network neurons, and for pharmacological block of chemical transmission at mixed pyloric synapses have also been previously described in detail (Johnson and Harris-Warrick 1997; Johnson et al. 1994).

LP waveform construction and LP → PY synaptic transmission. We constructed artificial, realistic LP waveforms from pre-recorded LP activity in control and DA conditions to use as pre-synaptic voltage clamp commands. LP recordings were low pass filtered at 30 Hz to preserve the slope of LP rebound from pacemaker inhibition and filter

out spike transients. An averaged, normalized LP waveform, sampled at 1000 points with the first and last points corresponding to the beginning and ending midpoint voltage values of a single oscillation, was constructed from the average of 10 consecutive oscillation cycles in a preparation. The original average period and amplitude of these cycles were preserved as separate values. The waveforms from 6 different preparations were then averaged and adjusted for the appropriate averaged period. Since LP waveform amplitudes were not significantly different in control and 10^{-4} DA conditions (see Results), both control and DA waveforms were scaled to 30 mV amplitude. This waveform amplitude drove the LP neuron from a holding value of -55 mV, near the resting potential of silent LP neurons (Johnson et al. 1992), to a peak of approximately -25 mV, a value that evokes the largest chemical synaptic response in PY neurons (Johnson et al. 1994).

Pyloric cells release transmitter as a continuous function of pre-synaptic voltage, by a process called graded synaptic transmission (Hartline and Graubard 1992). These graded synaptic interactions shape the pyloric pattern in the lobster (Hartline et al. 1988). To record PY graded inhibitory post-synaptic potentials (IPSPs), we added 10^{-7} M TTX to the saline to block spiking activity. In these experiments the AB neuron was killed by photoinactivation using 5,6-carboxyflourescein (Miller and Selverston 1979). In control-TTX conditions, the PY neuron was held at -55 mV with current injection in current clamp, while 10 linked control or DA waveforms were injected as voltage clamp commands into LP. This was repeated in the presence of 10^{-4} M DA; in separate runs, the PY was either held at – 55 mV or allowed to depolarize to its DA-induced value. We measured the PY peak response to the first LP oscillation, and the mean steady state response to repeated LP oscillations, as calculated from the average amplitudes of the last 5 PY IPSPs. A synaptic depression index (DI) was calculated as the steady state peak response divided by the initial peak response. We also examined the voltage dependence of the PY electrotonic response alone in control-TTX conditions after adding 5 X 10^{-6} M picrotoxin (PTX) to block LP glutamatergic chemical transmission (Bidaut 1980; Eisner and Marder 1982) and 20 mM TEA to block voltage-gated K^+ conductances that might shunt electrical coupling during PY depolarization.

Firing properties of PY neurons during rhythmic activity. To examine the functional importance of DA modulation of LP → PY synaptic dynamics on PY onset phasing during rhythmic pyloric activity, we measured the delay between the onset of LP and PY spiking in control and DA conditions; these experiments were done with intact descending modulatory inputs activating the pyloric network. In addition, we hyperpolarized the LP neuron to temporarily remove it from network activity, and measured the timing of PY firing onset relative to the AB pacemaker. In these experiments we also characterized the following PY parameters in control and 10^{-4} M DA conditions: number of spikes/burst, burst duration, duty cycle, and PY onset phase relative to AB onset. For each PY neuron, we averaged burst and firing measurements from 5 to 10 oscillation cycles in control and DA conditions.

Data acquisition and analysis. Electrophysiological recordings were digitized at 4KHz using a PCI-6070-E board (National Instruments), and stored on a PC using custom-made recording software written in Lab Windows/CVI (National Instruments). The same software was also used to inject artificial control and DA waveforms as voltage clamp commands into the LP neuron. All data were analyzed using another custom-made software program also written in Lab Windows/CVI (software available upon request). We determined the significant differences between individual data groups using an analysis of variance (ANOVA), followed by protected t-tests. Statistical significance was accepted with $P< 0.05$ for F or t values. Mean measured values and percentages are reported ± SD.

Results

Construction of realistic waveforms to drive the LP neuron

The LP neuron displays quite different waveform shapes during control and DA-modulated pyloric rhythms (Fig. 1B). The control LP waveform is monophasic: it rebounds from AB/PD pacemaker inhibition to fire a burst of spikes, which is terminated by synaptic inhibition from the PY and VD neurons (Fig. 1A) before the next round of pacemaker inhibition (Fig. 1B top left trace). During modulation by 10^{-4} M DA, the LP waveform is biphasic (Fig. 1B top right trace; Flamm and Harris-Warrick 1986). Its

rebound from pacemaker inhibition is accelerated by DA (Flamm and Harris-Warrick 1986; Harris-Warrick et al. 1995b), but its firing is quickly terminated by DA-enhanced PY inhibition (Fig.1b, PY right traces; Johnson et al. 1995). The second, brief depolarizing phase in DA is caused by its release from tonic PY inhibition as the AB/PD pacemaker group inhibits the PY cells (Johnson and Harris–Warrick 1997). This second depolarizing phase is terminated by pacemaker inhibition, which is also enhanced by DA (Johnson et al. 1995).

We generated realistic waveforms for the LP neuron in control and 10^{-4} M DA (DA4) conditions. As synaptic transmission between pyloric neurons is primarily graded, we filtered (30 Hz) and averaged recordings from 6 LP neurons in control and in DA; these averaged waveforms (Fig. 2B, top traces) reflected the shapes of the LP slow wave oscillations in Figure 1B. The control and DA4 waveforms did not differ significantly in amplitude (12.2 ± 1.84 mV and 13.7 ± 1.79 mV, respectively) or period (645 ± 40.62 ms and 692 ± 57.9 ms, respectively). The trend to a longer mean period of the DA waveform reflects the tendency of DA to slightly slow down the rhythm (Ayali and Harris-Warrick 1998). However, the half-durations of the first depolarizing phase of the control and DA4 waveforms were significantly different (389.6 ± 57.45 ms vs. 211.3 ± 17.54 ms). These two waveforms were applied periodically in trains of 10 as pre-synaptic voltage commands in the LP neuron to examine DA modulation of LP → PY graded synaptic dynamics. We drove the LP neuron with both the control and the DA waveforms under both control conditions and in the presence of 10^{-4}M DA; this allowed us to discriminate between the direct effects of DA on the LP→PY synapse and its indirect effects due to changes in the LP waveform.

Dopamine reverses the sign of mixed LP → PY synapses driven by realistic LP waveforms

In the pyloric network, a subset of PY neurons is both chemically inhibited by and electrically coupled to the LP neuron. The remaining PY neurons only receive chemical inhibition from the LP neuron. We first looked at DA's effects on the mixed chemical-electrical LP→ PY synapses with TTX added to the saline to block spontaneous activity and spike-evoked transmitter release (Fig. 2). In response to both control and DA pre-synaptic LP waveforms, the steady-state PY response in three preparations under control

conditions was weakly depolarizing. However, in 10^{-4}M DA, the synaptic response to both LP waveforms reversed in sign to become strongly hyperpolarizing. In the example of Figure 2, which is the same cell pair shown in Figure 1B, the first LP waveform in the series elicited weak, biphasic PY responses, consisting of an electrotonic depolarization that outweighed the weak chemical inhibition (Fig. 2A, middle traces). This response occurred with both the control and the DA LP waveforms. By the second or third waveform in the series, only depolarizing electrotonic responses remained, due to marked synaptic depression of the chemical component (Fig. 2A, middle traces, note PY responses above dashed line marking the resting potential; see also Mamiya et al. 2003). Application of DA depolarized this PY by 15 mV. Both control and DA LP waveforms elicited large, hyperpolarizing graded chemical synaptic potentials, which depressed to a steady state hyperpolarized value by the forth or fifth repeated LP waveform (Fig. 2A, bottom traces, note PY IPSPs below dashed line marking the resting potential, and the ten-fold reduced voltage scale). In between IPSPs in DA, the PY neuron depolarized above the initial resting potential, reflecting the marked enhancement of post-inhibitory rebound that DA evokes in these neurons (Harris-Warrick et al. 1995a).

In theory, the DA-induced depolarization of the post-synaptic PY neuron could by itself explain the enhanced chemical IPSP in Figure 2A by increasing the driving force on the inhibitory synapse. At the 3 mixed synapses where synaptic sign was reversed, DA depolarized the PY neurons an average of 14 ± 1.7 mV. However, when the PY neuron was depolarized to the same extent under control conditions without DA, only a small, initial hyperpolarization was seen, and this initial response depressed into the noise level with repeated LP oscillations (Fig. 2B, same synapse as shown in 2A, note the dashed line marking the resting potential, and the expanded voltage scale). We could not analyze PY IPSPs at –55 mV in the presence of DA because these PY neurons generated slow rhythmic membrane potential oscillations when hyperpolarized by current injection.

Figure 3 shows the mean peak PY responses to the first LP waveform and the steady state PY responses at the end of the LP train at these 3 mixed synapses, using both control and DA waveforms under both control and DA conditions. Surprisingly, the different LP waveform shapes (control and DA) did not significantly affect the peak amplitudes of either the initial or steady state PY responses in either control or DA

conditions. Notably, in DA, the second depolarizing phase of the DA waveform has no detectable effect on the PY neuron. These experiments suggest that the rather dramatic change in LP waveform evoked by DA has only subtle effects on its synaptic output (see below). A minority of the 8 PY neurons do not respond to DA (Johnson, Schneider and Harris-Warrick, unpublished observations), and at a fourth mixed LP → PY synapse, DA did not depolarize the PY neuron. The chemical synapse onto this PY was undetectable under control conditions, and became apparent only during the first LP oscillation in DA. Like the other mixed synapses we studied in control conditions, the chemical component of this synapse depressed with repeated LP oscillations to leave a predominantly electrotonic, steady state component (not shown). Thus, at all of these synapses, DA enhanced chemical inhibition, and in the majority of the mixed synapses, this reversed the synaptic sign from depolarizing to hyperpolarizing. This predominant hyperpolarization was maintained during repeated LP oscillations despite relatively strong chemical synaptic depression.

Dopamine is known to weaken the electrical coupling between LP and PY neurons when measured at constant pre- and post-synaptic membrane potentials (Johnson et al. 1993a). This is a rectifying electrical junction where depolarization of the LP is transferred to the PY neuron as a function of the difference between their membrane potentials. Thus, DA-induced PY depolarization, by lessening the differential voltage between the LP and PY neurons, could further contribute to weakening the electrotonic component of the mixed synapse. This is demonstrated in Figure 4, which shows the isolated electrotonic component of the LP→PY synapse after PTX was added to block the chemical component, and 20 mM TEA was added to partially block voltage-gated K^+ conductances and enhance control of the electronic component. Under control conditions (with no DA), LP depolarization drove a relatively large electrotonic potential (1.9 mV) in the PY neuron (Fig. 4, left traces). Depolarization of the PY neuron by 15 mV (to mimic the effect of DA) reduced the electrotonic potential amplitude to 0.62 mV (Fig. 4, middle PY trace). This diminished PY response could be partially restored when the LP waveform amplitude was in turn increased by 10 mV (Fig. 4a, right PY trace). This shows that LP → PY rectifying electrical coupling is dependent upon the voltage

difference between the two neurons. Similar results were seen in one other experiment with chemical transmission blocked by PTX.

*Dopamine activates silent synapses at purely chemical LP → PY synapses during realistic network activity*A subset of the PY neurons are not electrically connected to the LP and show only chemical inhibition upon LP stimulation (Johnson et al. 1994). At these purely chemical LP → PY synapses, the PY neuron displayed a hyperpolarizing response to the first LP waveform in control conditions, but this depressed to little or no postsynaptic response with repeated LP oscillations using either the control or the DA LP waveforms (Fig. 5A, middle traces; note dashed line marking the resting potential). Addition of DA only weakly depolarized the PY neuron at these synapses (n = 3; mean= 3.7 ± 3.2 mV), and enhanced synaptic inhibition by 313 ± 213% for the first control waveform and 378 ± 301% for the first DA waveform. Although these synapses depressed strongly, there remained a small hyperpolarizing response at steady state during the LP train using both pulse types (Fig. 5A, bottom traces; note PY responses below dashed line marking the resting potential). We could eliminate the DA-evoked PY depolarization with current injection; the IPSP was still significantly enhanced (Fig. 5B, same synapse as in Fig. 5A). Figure 6 shows the mean peak PY responses to the first LP waveform and the steady state PY responses at the end of the LP train at these chemical synapses, using both control and DA waveforms under both control and DA conditions. In 2 out of 3 experiments, under control conditions the PY steady state response to LP stimulation was completely eliminated. Again, switching between control and DA LP waveform shapes had no significant effect on the initial PY responses or the steady state PY responses under control conditions or during DA application (Fig 6). Thus, during realistic network activity, DA enhanced the purely chemical LP → PY synapses strongly enough to maintain chemical inhibition that depressed to silence in most cases under control conditions.

Control and DA pre-synaptic LP waveforms cause subtle differences in the shape and depression of DA-enhanced PY inhibitory responses

We were surprised that changing the pre-synaptic LP waveform from the control to the DA shape did not cause significant differences in the amplitudes of the initial and steady state PY IPSPs (Figs. 3 and 6). We looked for more subtle effects of waveform shape and found two small, but statistically significant, differences in the duration and depression of PY inhibitory responses to the two types of waveforms during DA application. Here, we combined experiments on the DA-enhanced IPSPs from both mixed and purely chemical LP → PY synapses because they were large enough to measure accurately, and because DA-enhanced steady state IPSPs did not depress completely, allowing calculation of the depression index (DI). First, in the presence of DA, the mean half-duration of the first PY IPSP in response to the control LP waveform was significantly longer (216.5 ± 33 ms) than the response to the DA waveform (192.7 ± 29 ms), correlating with the different durations of the two LP waveforms (Fig. 7A, middle traces). Depolarizing PY electrotonic responses in control conditions, measured after pharmacological blockade of the chemical inhibition, were, not surprisingly, an attenuated version of the LP waveform and were significantly different with control and DA waveforms (Fig. 7A, bottom traces). Second, we calculated the DI as the average steady state peak IPSP divided by the initial peak IPSP for the LP responses during bath application of DA. The DI was slightly, though significantly, smaller for the control LP waveform than the DA waveform, indicating slightly greater synaptic depression in DA with the longer control waveform than with the shorter DA waveform (Fig. 7B). This appears to contradict our results that initial and steady state PY responses were not significantly different between the two waveforms (Figs. 3 and 6). Despite variability in the initial and steady state amplitude of the PY IPSPs, the ratio of steady state to initial amplitude was consistently lower with the control waveform (see bottom traces in Figs. 2 and 5); the effect is, however, subtle. These results show that fairly large differences in LP waveform shape cause only small changes in PY inhibitory response. The changes that we did detect are probably due to differences in the two LP waveform durations (see Discussion).

LP → PY firing delay in control and DA conditions

Under control conditions, the LP → PY synapse appears to have little effect on the onset of PY firing in the rhythmic pyloric motor pattern, in that LP hyperpolarization does not strongly advance the onset of PY spiking (Weaver and Hooper 2003; Mamiya et al. 2003). We hypothesized that application of DA, by weakening electrical coupling and very significantly strengthening the functional LP → PY inhibition, may increase the LP control of PY firing onset. We first examined the onset time delay between LP and PY (first LP spike to first PY spike) in experiments where the cycling network activity of both neuron types was recorded simultaneously in the absence and presence of DA. Apparently consistent with our hypothesis, the mean LP-PY delay across our population of cell pairs was 42% greater in DA than in control conditions (Fig. 8A). This effect was not statistically significant because of significant variability in firing delay of individual PY neurons. Figure 8C shows the range of PY firing delays in control and DA conditions. This figure plots the LP to PY firing delay for each of 21 PY neurons under control and DA conditions; it divides these PY neurons by their firing onset relative to LP and by the effects of DA on their firing times. Most (17 out of 21) PY neurons began firing after the LP neuron under control conditions; 11 of these increased, while 6 decreased, their firing delay in DA (Fig. 8C; see also example in Fig. 1B). Interestingly, the PY neurons that increased their firing delay in DA all had shorter control delays than those that decreased their firing delay in DA. Thus, the major effect of DA was to regularize the LP-PY delay by significantly decreasing the delay variability in these PY cells (control delay= 120.7 ± 82.86 ms; DA delay= 132.8 ± 41.07 ms). Thus, these cells fired together to a much greater extent in DA. Two PYs were silent under control conditions and fired only after a long delay during application of DA. The remaining two PY neurons fired before the LP neuron in control and switched to fire after the LP neuron in DA (Fig. 8C). Although we did not test the LP → PY synaptic connection for every cell pair, the PY firing delay did not seem to correlate with the type of synaptic connection with the LP (purely chemical vs. mixed chemical/electrical). Thus, for most PY neurons, DA appeared to delay and regularize the onset of their firing relative to the LP neuron.

At first sight, this appears to be consistent with stronger LP control of PY firing. However, during DA application the pyloric cycle period also became slightly longer, as

described above (Ayali and Harris-Warrick 1999). When the LP-PY delay is converted into a fraction of the period to take this into account, the phasing between LP and PY is unchanged by DA (Fig. 8B).

LP influence on the AB→PY firing delay and PY burst properties

To further examine the LP's influence on PY firing onset, we examined the effect of removing its synaptic input by hyperpolarizing the LP neuron and measuring the change in the delay of PY firing relative to the AB pacemaker during network activity in the presence and absence of DA. If the LP neuron exerted more control over PY firing during DA application due to its stronger synaptic inhibition, its temporary removal from the network should advance PY firing relative to the pacemaker AB to a greater extent in DA than under control conditions. Figure 9A shows a cycling preparation where the LP neuron was hyperpolarized under control conditions and during application of DA. Block of LP activity immediately accelerated the cycle frequency in both control and DA conditions, due to the removal of LP inhibitory feedback to the pacemaker kernel (Fig. 9A, Fig. 1A). When the LP neuron was released from hyperpolarization, it fired a strong post-inhibitory rebound burst which inhibited AB firing in control (Fig. 9A, left traces), and delayed AB firing and slowed PY firing frequency in DA (Fig. 9a, right traces). This showed that LP chemical synapses were active, and their steady state depression was removed by the hyperpolarization; this change in cycle frequency returned to normal within a cycle or two (Fig. 9A).

LP hyperpolarization had only a small and insignificant effect to decrease the AB → PY delay under control conditions (PY delay decrease: 30.5 ± 15.9 ms, n = 6, from 4 preparations; Fig. 9B, control). Surprisingly, this effect of removing LP did not change significantly when LP → PY synaptic inhibition was enhanced by DA (17.5 ± 18.1 ms; Fig. 9B, DA). Due to the acceleration of the pyloric rhythm during LP hyperpolarization, there were no significant changes in the phase of PY onset firing relative to AB during LP hyperpolarization in either control or DA conditions (Fig. 9C). These results suggest that, contrary to our earlier hypothesis, the addition of DA did not increase LP control of PY firing onset.

These small changes in AB → PY delay during LP hyperpolarization cannot account for the tendency for the LP → PY time delay to increase during DA application (Fig. 8A); if this increase were due to stronger LP inhibition of the PY neurons, it would also delay PY onset relative to the AB neuron. We further compared the AB-LP and AB-PY time delays during control and DA network activity. These experiments showed that in fact, DA decreases the AB-LP firing delay more than the AB-PY firing delay; that is, the increased LP-PY delay (Fig. 8A) is caused by the LP advancing more, relative to the AB, than the PY, rather than the LP delaying the PY (Fig. 9D). The slightly longer cycle period during DA application neutralized the slightly longer time delay between LP and PY onset; indeed, in these experiments, the DA-evoked phase advances for LP (0.25 ± 0.02) and PY (0.26 ± 0.09) relative to AB were similar (Fig. 9E), consistent with the constancy of LP-PY phase under control and DA conditions (Fig. 8B).

LP hyperpolarization also had no significant effects on other aspects of PY activity in the presence or absence of DA, including the burst duration (Fig. 10A), the number of APs per burst (Fig. 10B), or the duty cycle (Fig. 10C). Note, however, that DA did significantly enhance excitability in these PY neurons, as indicated by the increases in burst duration, number of action potentials per burst and duty cycle (Figs. 10A, B and C). We have previously documented DA's significant enhancement of post-inhibitory rebound in the PY neurons (Harris-Warrick et al. 1995a).

In essence, the timing of LP and PY firing onset is primarily determined by their rates of intrinsic post-inhibitory rebound from the pacemaker AB/PD inhibition. During DA application, both the LP and most of the PY neurons are highly excited (Figs. 1B, 9), but the LP shows a more rapid post-inhibitory rebound after pacemaker inhibition than the PY neurons. Thus the LP neuron fires earlier and increases the time delay (though not the phase delay) between the LP and PY neurons. Despite DA's enhancement of LP → PY inhibition (and even its sign reversal in isolated conditions), this synapse is still not strong enough to significantly delay PY firing onset in an intact, rhythmic network. Thus, intrinsic PY firing properties and their enhancement by DA determine PY firing under both control and DA conditions.

Discussion

Mechanisms of DA modulation of LP→PY synaptic transmission

Graded synaptic transmission is the predominant form of interaction between neurons in the pyloric network (Hartline et al. 1988). These graded chemical synapses show marked depression during normal cycling membrane potential oscillations (Manor et al. 1997; Mamiya et al. 2003; Mamiya and Nadim 2004); the relative importance of each synapse under these partially depressed conditions is not known. DA can dramatically strengthen the inhibitory component and weaken the electrotonic component of the mixed LP → PY synapse in the pyloric network under conditions of realistic network activity. Despite short term depression, DA reversed the sign of most LP → PY mixed chemical-electrical synapses from depolarizing to strongly hyperpolarizing, and it activated purely chemical LP → PY synapses that are silenced by depression under control conditions. The enhancement of chemical inhibition was caused at least in part by DA's pre-synaptic action to enhance transmitter release from LP terminals (Johnson and Harris-Warrick 1997), possibly by increasing I_{Ca} (Johnson et al. 2003). In addition, DA acts post-synaptically to depolarize and increase the input resistance of PY neurons (Johnson et al. 1993a; Harris-Warrick et al. 1995a), and to enhance their response to iontophoresed glutamate, the LP neuron's transmitter (Johnson and Harris-Warrick 1997). These post-synaptic actions are partially caused by a decrease in I_A (Harris-Warrick et al. 1995a), and perhaps to a small extent by an increase in I_h (Peck and Harris-Warrick, unpublished observations). Furthermore, electrical coupling at mixed LP → PY synapses is weakened directly by DA (Johnson et al. 1993a), and as we show here, also indirectly as a result of postsynaptic depolarization which decreases the voltage difference between the two neurons at this rectifying synapse.

In addition, DA might reduce the steady state level of synaptic depression at the LP→PY synapse. Dopamine has been shown to prevent the development of synaptic depression in other systems (Baimoukhametova et al. 2004). Unfortunately, we were not able to accurately quantitate the effects of DA on LP→PY depression dynamics, as the chemical component of the mixed synapses under control conditions was completely masked by electrical coupling, and most purely chemical synapses depressed almost completely to undetectable levels during a train of 10 pre-synaptic oscillations. However,

these depressing effects seem larger than we saw in DA, where none of the synapses depressed completely. In control conditions similar to ours, the chemical component of LP → PY mixed synapses depresses by approximately 90% (Mamiya and Nadim, unpublished observations). This is significantly greater than the 65% depression we observed in DA, suggesting that DA indeed reduces the magnitude of depression at this synapse.

Effect of control and DA LP waveform shape on LP→PY dynamics

Our ability to drive the LP cell with either control or DA waveforms in the absence or presence of DA allows us to distinguish direct DA modulation of the pre-synaptic release process itself from indirect modulation caused by DA-induced changes in the LP pre-synaptic waveform. The amplitude and time courses of graded IPSPs have been shown to depend on the shape of the pre-synaptic waveform (Olsen and Calabrese 1996; Manor et al 1997; Simmons 2002: Mamiya and Nadim 2004). Our control and DA LP waveforms differed significantly in their shape; the DA waveform was biphasic, with a shorter main depolarizing phase than the control waveform, which was a single broader wave. This difference arises from DA-enhanced PY→LP reciprocal inhibition, which prematurely terminates the LP firing plateau in DA until the PY neurons are inhibited by the pacemaker group (Fig. 1B; Johnson et al. 1995). Despite these marked differences in waveform shape, we saw no significant differences between control and DA LP waveforms in the amplitudes of the initial or steady state PY IPSPs, when tested under the same conditions (control or during application of DA). Thus, DA enhancement of the peak LP→PY inhibition arises from direct actions of DA at the synapse and not from indirect changes in pre-synaptic waveform shape.

We did observe two significant, though subtle, effects of using control vs. DA waveforms in the presence of DA. First, the control waveform evoked a slightly longer IPSP duration than the DA waveform; this correlated with the relatively longer duration of the control waveform. Second, we observed a slightly greater synaptic depression of the PY response to the control waveform. Since higher output synapses characteristically display greater depression than lower output synapses (Atwood and Karunanithi 2002), the longer duration of the control waveform may have allowed transmitter release from LP for a slightly longer period, leading to more depression. Longer artificial LP

waveforms do lead to greater depression at LP→PY mixed synapse under control conditions (Mamiya et al. 2003). These subtle effects of pre-synaptic waveform shape are probably too small to have significant functional consequences for the network, especially since the enhanced LP→PY inhibition did not contribute much to the onset time of PY firing (see below).

Intrinsic rebound properties outweigh LP synaptic inhibition in determining the onset of PY firing

The control of patterned firing in motor networks is normally thought to be achieved through a balance between synaptic interactions and the intrinsic firing properties of the network neurons. Under control conditions of ongoing rhythmic network activity, we found, as others had before (Weaver and Hopper 2003; Mamiya et al. 2003) that hyperpolarization of the LP neuron (to eliminate the effect of the LP→PY synapse) had only a small effect to advance the onset of PY firing. It appears that the PY neurons are primarily responding to the very strong periodic inhibition from the pacemaker kernel (AB-PD), and their intrinsic postinhibitory rebound from this pacemaker inhibition plays the dominant role in setting their onset time under control conditions. However, we expected that after DA strongly enhanced LP→PY inhibition, this synapse would assume a more important role in delaying PY onset. Surprisingly, despite the dramatic dopaminergic enhancement in LP→PY chemical inhibition, this synapse did not exert any greater control over PY firing than under control conditions; removal of LP had equally small effects on PY onset times in the presence and absence of DA (Fig. 9A,B) and had no effect on PY phasing relative to the pacemaker kernel (Fig. 9C). Instead, it appears that the intrinsic post-inhibitory rebound of the PY neurons after pacemaker inhibition, which is strongly enhanced in most PY neurons by DA (Harris-Warrick et al. 1995a), still outweighs the effects of the LP→PY synapse in setting PY onset times. Thus, during DA application the PY neurons show a shortened delay to firing onset relative to AB pacemaker, an increased number of APs per burst and increased burst duration (Fig. 10). These intrinsic excitatory effects appear to offset the enhanced inhibitory input from the LP neuron to maintain the same balance of intrinsic and synaptic control of PY rebound firing as before DA application. While the overall effect of DA was to phase advance the PY neurons relative to the pacemaker kernel (AB/PD;

Fig. 9C), DA's effect on LP-PY timing was more variable and complex (Fig. 8). On average, DA slightly delayed the time from LP onset to PY onset (Fig. 8A), thus maintaining constant LP-PY phasing in the slightly slower pyloric rhythm (Fig. 8B). While this would suggest that DA enhances the effective LP inhibition of PY onset, our further studies showed that this increased delay reflects the intrinsic rebound of both LP and PY neurons after pacemaker inhibition. In DA, the LP neuron was time-advanced even more than the PY, relative to the pacemaker AB neuron (Fig. 9D); this then led to the apparent timing delay of PY relative to LP. Again, due to the slightly slower pyloric period in DA, the relative phasing of LP and PY remains constant (Fig. 9E).

Interestingly, when all the individual experiments are compared (Fig. 8B), it is clear that another net effect of DA is to regularize the delay between LP and PY onset. PY neurons that had short delays relative to LP had these delays prolonged by DA, while PY neurons with long delays had them shortened by DA, so the variance of the LP to PY delay is significantly smaller in DA. Thus, the PY neurons fire more synchronously as a group in DA than under control conditions. Since the PY neurons poly-innervate non-spiking muscles, this more synchronized and strengthened PY activity should evoke stronger PY contractions in DA than under control conditions. It is likely that this enhanced regularity of PY firing onset arises primarily from intrinsic changes in their rebound from AB/PD inhibition, with LP→PY inhibition playing a minor role.

These reset experiments were performed with an actively cycling pyloric preparation, driven by mixed modulatory inputs from higher ganglia. It is thus possible that DA failed to enhance the effect of hyperpolarizating the LP on PY onset because these synapses are already fully modulated by the inputs from higher centers. DA is a natural neuromodulator in crustaceans (Nusbaum and Beenhakker 2002; Tierney et al. 2003), and DA and other modulators may already be affecting the LP→PY synapses during pyloric activity, occluding the effects of additional DA. However, we think this unlikely for several reasons. First, under the same conditions, bath-applied DA still strongly excites PY firing. In addition, hyperpolarization of the LP also removes its inhibition of the PD neuron in the pacemaker group (Fig. 1), accelerating the pyloric rhythm (Fig. 9A). DA strongly enhances LP→PD inhibition (Johnson et al. 1995; Ayali et al. 1998), and LP removal in the presence of DA causes a larger acceleration of

network cycle period than under control conditions (Fig. 9A; Johnson, Schneider and Harris-Warrick, unpublished observations). Thus, several other effects of DA on the pyloric network are easily detectable in the cycling preparation, suggesting that its effects on the LP→PY synapse are not occluded by other modulators. Finally, in preliminary experiments, we have monitored the effect of LP hyperpolarization on AB-PY timing in an isolated pyloric network, with no neuromodulators present except DA. Even in this simplified preparation, LP removal does not appear to significantly advance the onset of PY firing relative to the pacemaker group.

We still do not understand why DA's enhancement of the LP→PY synapse fails to increase the efficacy of this synapse in determining the PY onset time. The competing increase in PY excitability appears to counterbalance the strengthening of this synaptic inhibition, yet when the inhibition is removed (by hyperpolarizing the LP) we do not see a dramatic advance in PY onset relative to control conditions. Further work will be needed to test how this arises from complex network interactions that are not yet fully understood.

Separating the complementary contributions of intrinsic and synaptic mechanisms that shape the patterns of neuronal output in a functioning neural network is a difficult challenge in most systems. This is especially true for complex vertebrate networks, though progress has been made in studies of contrast adaptation in the cat visual cortex (Nowak et al 2005). New models of the mechanisms underlying memory consolidation are also beginning to consider both synaptic and intrinsic mechanisms (Xu et al. 2005; Zhang and Linden 2003). As we have shown here, modulatory actions that dramatically alter network synaptic interactions may in fact not be quantitatively very important for follower cell activity, when compared to intrinsic changes that are happening simultaneously. This emphasizes our finding in the pyloric network that the functional importance of synaptic modulation can only be understood in the context of parallel modulation of intrinsic properties in a rhythmic network.

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Figure Legends

Figure 1. Schematic diagram of the pyloric network of the spiny lobster *Panulirus interruptus* (A) and typical LP and PY neuron activity under control and dopamine (DA, 10^{-4}M) conditions (B). In the pyloric network, the synaptic connections are either electrical (nonrectifying: resistor symbols, and rectifying (diode symbols) or chemical inhibitory (filled circles). The LP → PY synapses examined in this study are marked in bold lines.

Figure 2. Dopamine modulation of synaptic transmission at LP → PY mixed synapses. A: Pre-synaptic Control and dopamine (DA) LP waveforms (top traces) and PY responses to these waveforms in Control (middle traces) and DA (10^{-4}M) conditions (bottom traces). Note 10-fold difference in voltage scales in control and DA traces, and DA-evoked depolarization. B: PY response to the control LP waveform under control conditions with the PY neuron artificially depolarized to the membrane potential (-40 mV) seen in the presence of DA. Dashed line in PY traces indicates resting potential. Note different PY voltage scales in A and B.

Figure 3. Summary of DA effects on LP → PY mixed synapses. Mean PY peak synaptic responses are shown to LP control (Ctl, left bars) and dopamine (DA, right bars) waveforms in control (Ctl) and dopamine (DA) conditions. The PY responses to the first waveform in each train are shown by the open bars, while the steady state PY responses, averaged from the last 5 waveforms in the train, are shown by the closed bars. PY responses were depolarizing in control conditions and hyperpolarizing in DA conditions.

Figure 4. Effects of LP and PY voltage differences on LP → PY electrotonic coupling. To isolate the electrotonic coupling between these neurons, chemical inhibition is eliminated with 5 x 10^{-6} M pictrotoxin, and 20 mM TEA is added to reduce the shunting effect of $I_{K(V)}$. An LP depolarization of 25 mV from -55 to -30 mV, using the DA waveform, produces a PY electrotonic response at -55 mV (left traces) which is reduced when the PY neuron is depolarized to -40 mV with current injection (middle traces).

Subsequently increasing the LP depolarization to -20 mV while the PY is depolarized to -40 mV restores most of the PY electrotonic response (right traces).

Figure 5. Dopamine modulation of synaptic transmission at LP → PY purely chemical synapses. A: Pre-synaptic Control and dopamine (DA) LP waveforms (top traces) and PY responses to these LP waveforms under Control (middle traces) and DA (10^{-4}M) conditions (bottom traces). Note PY depolarization from -55 mV to -50 mV in DA. B: PY response to the DA LP waveform in the presence of DA, while the PY is hyperpolarized back to the control membrane potential (-55 mV). Dashed lines in PY traces indicate resting potentials; values shown to the left of each trace.

Figure 6. Summary of DA effects on LP → PY purely chemical synapses. Mean PY peak synaptic responses are shown to LP control (Ctl, left bars) and dopamine (DA, right bars) waveforms in control and DA conditions. The PY responses to the first waveform in each train are shown by the open bars, while the steady state PY responses, averaged from the last 5 waveforms in the train, are shown by the closed bars.

Figure 7. Effects of LP waveform shape on PY IPSP duration and synaptic depression. A: Control (Ctl) and dopamine (DA) LP waveforms (top traces) elicit different duration IPSPs in PY neurons in DA conditions (middle traces). Isolated PY electronic responses in control conditions (with chemical response blocked by PTX) are an attenuated version of the pre-synaptic waveform (bottom traces). B: Depression indices for PY responses are significantly smaller to control (Ctl; open bar) than to DA waveforms (DA, closed bar).

Figure 8. Dopamine (DA) effects on time delay between LP and PY onset, and LP to PY onset phase difference during normal pyloric network activity. A: Mean effect of DA on LP to PY time delay. B: Mean effect of DA on LP to PY onset phase difference. Neither difference is statistically significant. C: Time delay between LP and PY onset for each of 21 PY neurons under control (white bars) and DA (black bars) conditions. PY neurons are grouped by their firing in control conditions: silent, firing before LP, and firing after

LP. The last group is subdivided by whether DA causes a phase advance or a phase delay.

Figure 9. Effect of removing the LP→PY synapse on PY onset time and phase, and comparison of LP and PY firing phases in control and dopamine conditions. A: Example showing the effects of hyperpolarizing the LP to remove its synaptic effects on pyloric network activity. The AB neuron traces show that the cycle accelerates, but the PY phase does not change significantly. B-C: Comparisons of the effects of LP hyperpolarization on time delay between AB and PY firing onset (B), and AB to PY firing onset phase (C) under control and dopamine (DA) conditions. None of these differences are significant. D-E: Comparison of LP and PY time delay to onset (D), and phase delay to onset under control (white bars) and dopamine (black bars) conditions. DA causes a significant time and phase advance of both LP and PY relative to AB.

Figure 10. DA enhances PY excitability, and this is unaffected by removal of LP. Comparisons of the effects of LP hyperpolarization (to remove the LP→PY synaptic interaction) on PY burst duration (A), the number of action potentials per PY bursts (B), and the PY duty cycle (C), under control and dopamine (DA) conditions. DA significantly excites the PY neurons by all three measures, but removal of LP has no effect.

Figure 1

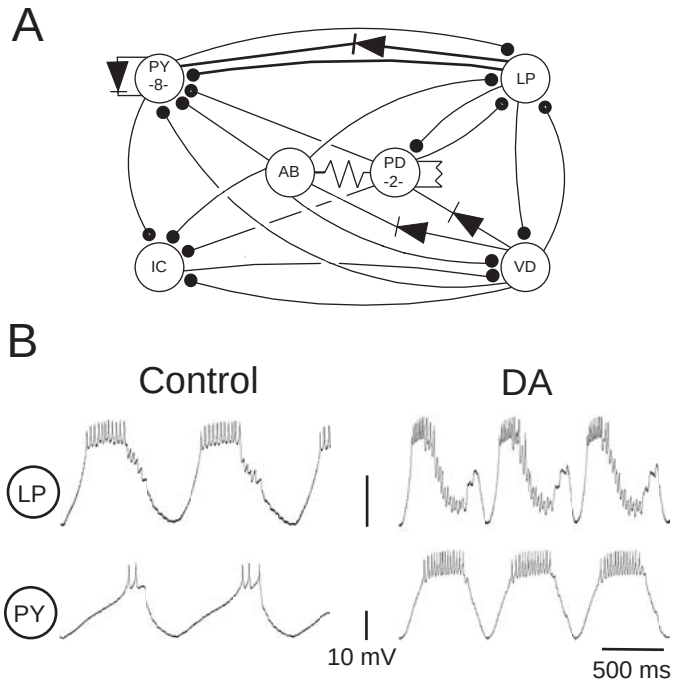


Figure 2

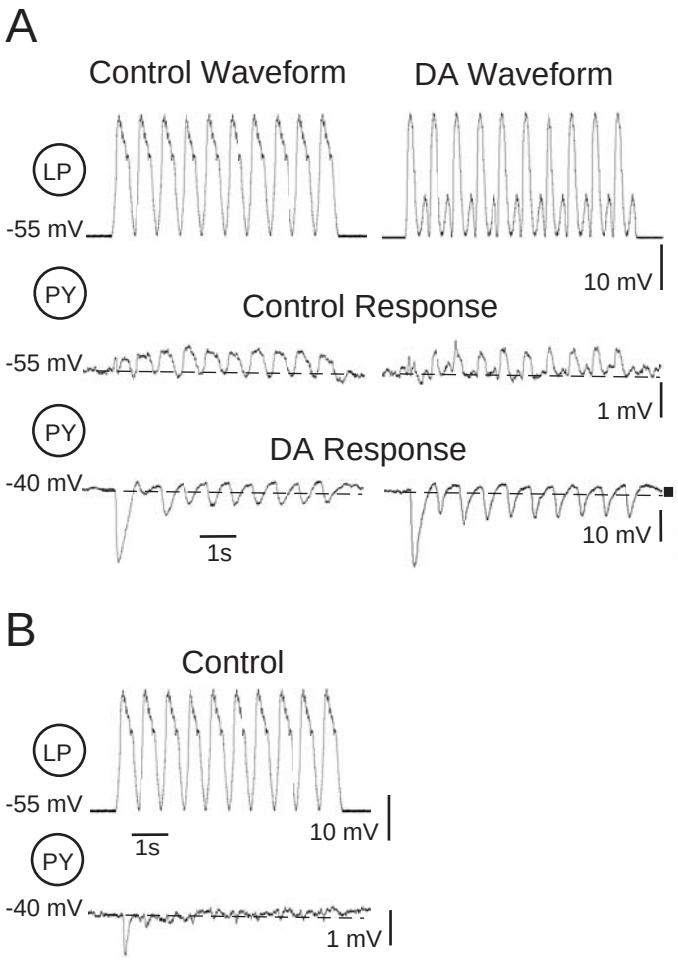

A
Control Waveform
DA Waveform
LP
-55 mV
10 mV
PY
Control Response
-55 mV
1 mV
PY
DA Response
-40 mV
1s
10 mV
B
Control
LP
-55 mV
10 mV
1s
PY
-40 mV
1 mV

Figure 3

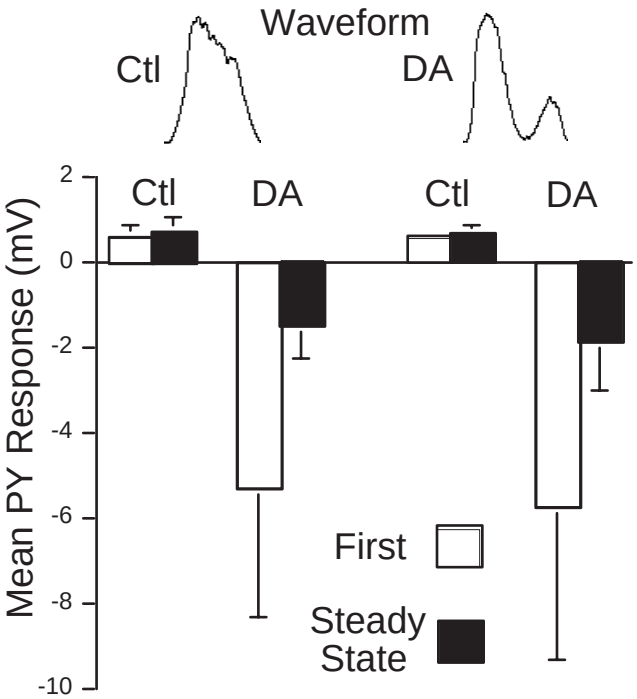

Waveform
Ctl
DA
Ctl
DA
Ctl
DA
Mean PY Response (mV)
2
0
-2
-4
-6
-8
-10
First
Steady
State

Figure 4

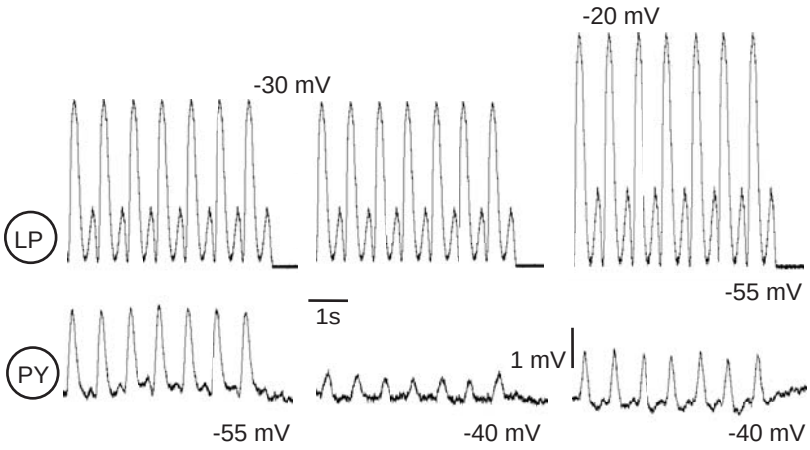


Figure 5

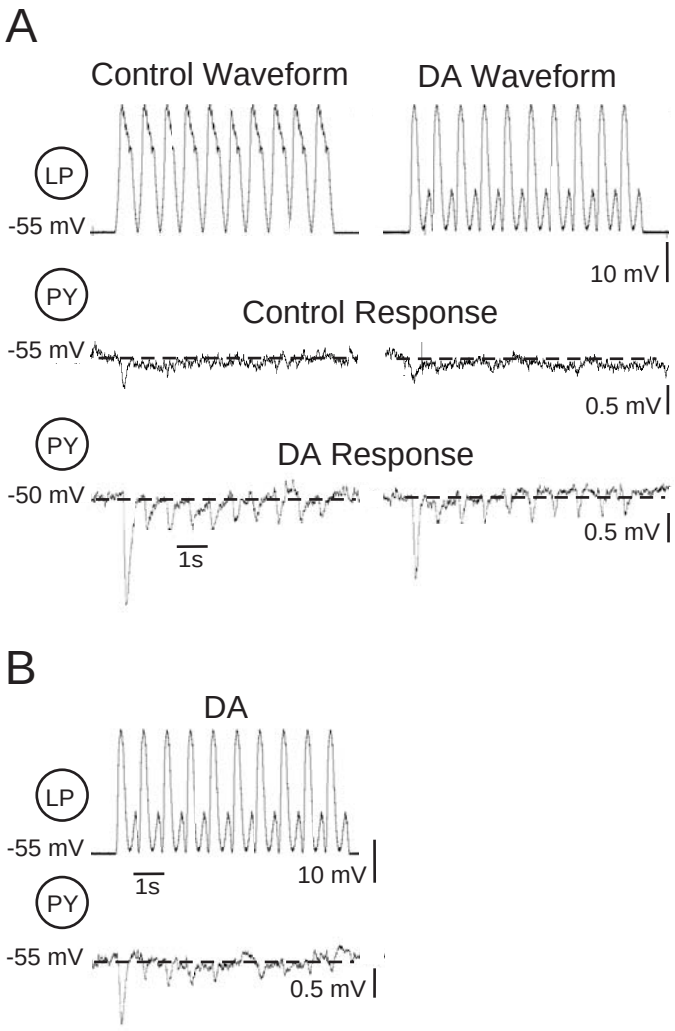

A
Control Waveform
DA Waveform
LP
-55 mV
10 mV
PY
Control Response
-55 mV
0.5 mV
PY
DA Response
-50 mV
0.5 mV
1s
B
DA
LP
-55 mV
1s
10 mV
PY
-55 mV
0.5 mV

Figure 6

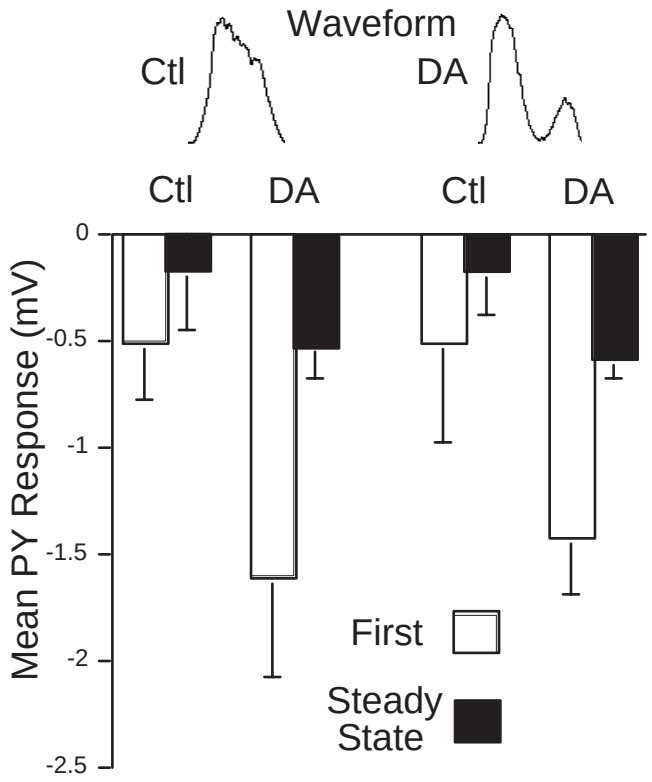

Waveform
Ctl
DA
Ctl
DA
Ctl
DA
0
-0.5
-1
-1.5
-2
-2.5
Mean PY Response (mV)
First
Steady State

Figure 7

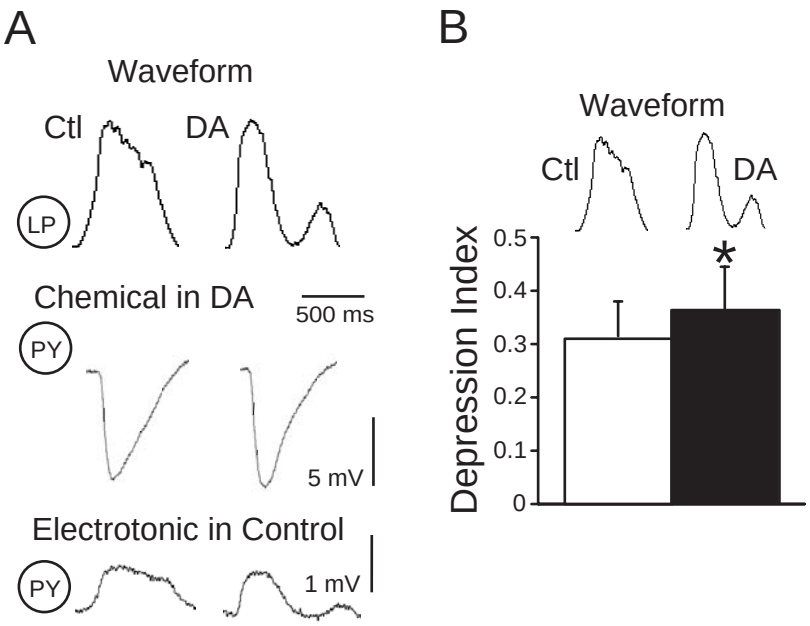

A
Waveform
Ctl
DA
LP
Chemical in DA
500 ms
PY
5 mV
Electrotonic in Control
PY
1 mV
B
Waveform
Ctl
DA
Depression Index
0.5
0.4
0.3
0.2
0.1
0
*

Figure 8

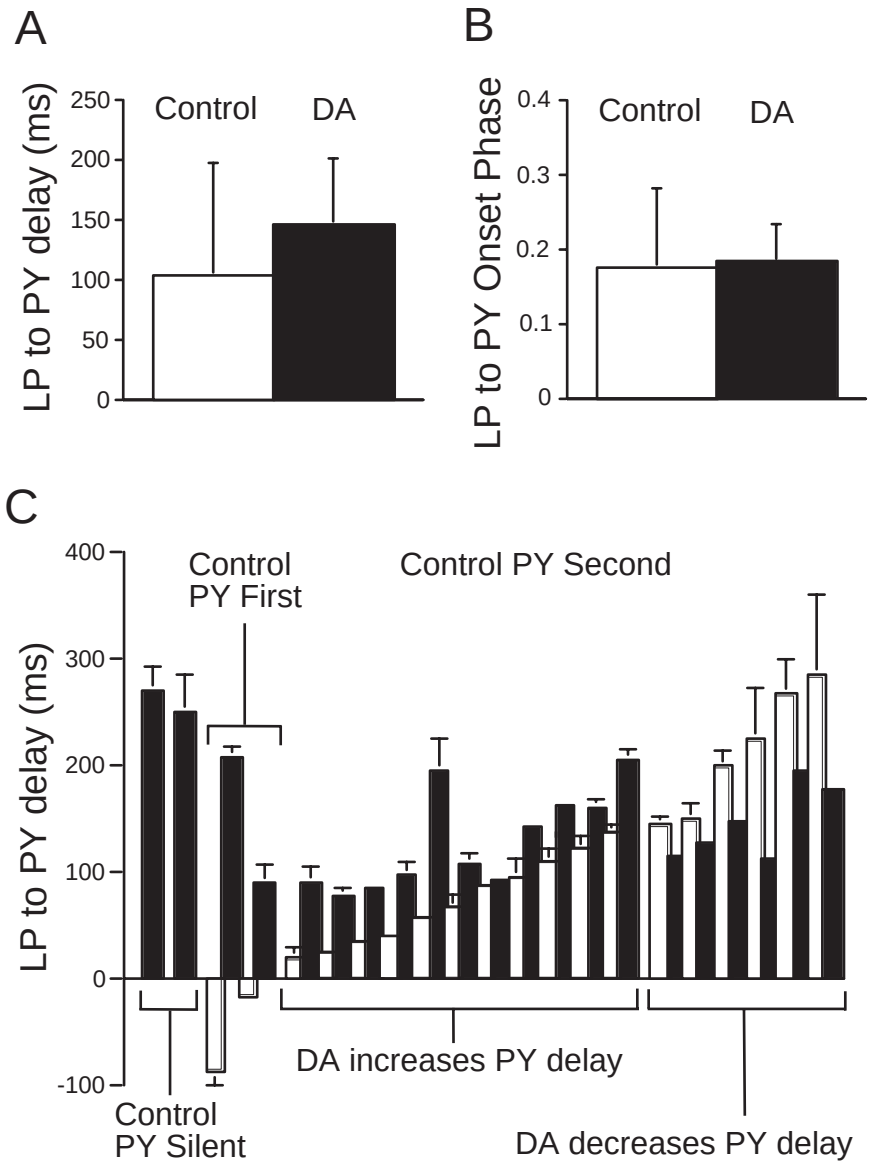

A
LP to PY delay (ms)
250
200
150
100
50
0
Control
DA
B
LP to PY Onset Phase
0.4
0.3
0.2
0.1
0
Control
DA
C
LP to PY delay (ms)
400
300
200
100
0
-100
Control
PY First
Control PY Second
DA increases PY delay
Control
PY Silent
DA decreases PY delay

Figure 9

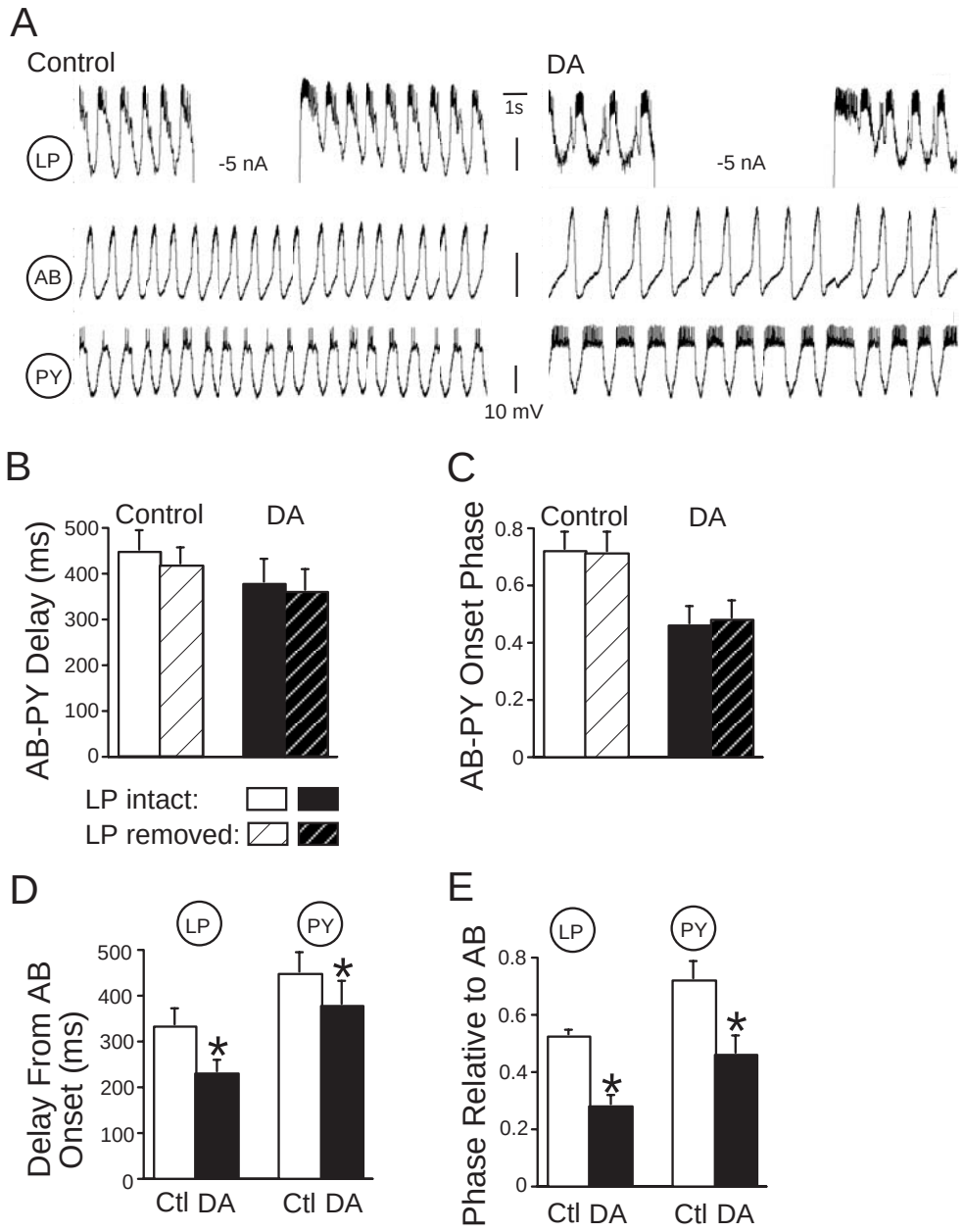
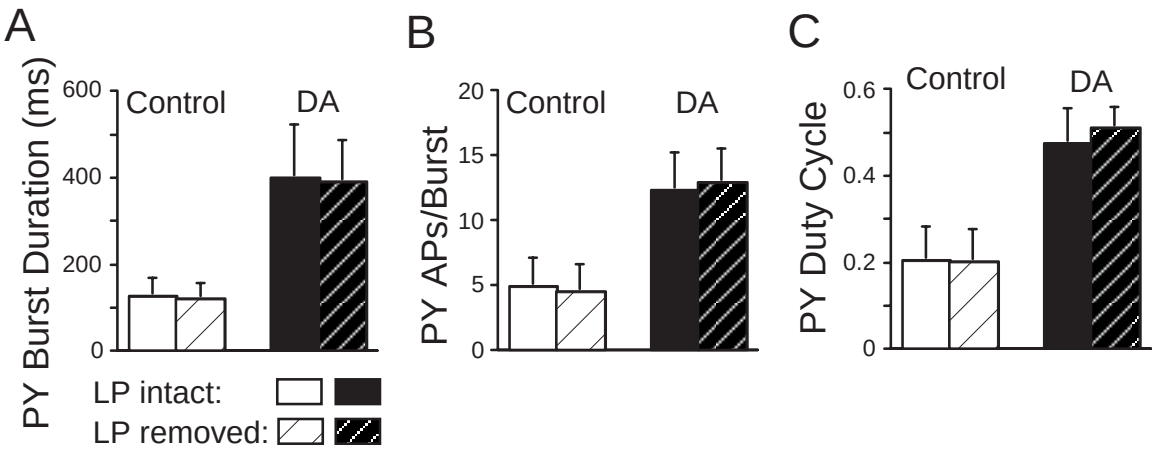

A
Control
DA
1s
LP
-5 nA
-5 nA
AB
PY
10 mV
B
Control
DA
AB-PY Delay (ms)
500
400
300
200
100
0
LP intact:
LP removed:
C
Control
DA
AB-PY Onset Phase
0.8
0.6
0.4
0.2
0
D
LP
PY
Delay From AB Onset (ms)
500
400
300
200
100
0
Ctl DA
Ctl DA
E
LP
PY
Phase Relative to AB
0.8
0.6
0.4
0.2
0
Ctl DA
Ctl DA

Figure 10


A
PY Burst Duration (ms)
600
400
200
0
Control
DA
LP intact:
LP removed:
B
PY APs/Burst
20
15
10
5
0
Control
DA
C
PY Duty Cycle
0.6
0.4
0.2
0
Control
DA