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Follower Neurons in Lobster (*Panulirus interruptus*) Pyloric Network Regulate Pacemaker Period in Complementary Ways

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Weaver, Adam L. and Scott L. Hooper. Follower neurons in lobster (*Panulirus interruptus*) pyloric network regulate pacemaker period in complementary ways. *J Neurophysiol* 89: 1327–1338, 2003. First published November 13, 2002; 10.1152/jn.00704.2002. Distributed neural networks (ones characterized by high levels of interconnectivity among network neurons) are not well understood. Increased insight into these systems can be obtained by perturbing network activity so as to study the functions of specific neurons not only in the network's "baseline" activity but across a range of network activities. We applied this technique to study cycle period control in the rhythmic pyloric network of the lobster, *Panulirus interruptus*. Pyloric rhythmicity is driven by an endogenous oscillator, the Anterior Burster (AB) neuron. Two network neurons feed back onto the pacemaker, the Lateral Pyloric (LP) neuron by inhibition and the Ventricular Dilator (VD) neuron by electrical coupling. LP and VD neuron effects on pyloric cycle period can be studied across a range of periods by altering period by injecting current into the AB neuron and functionally removing (by hyperpolarization) the LP and VD neurons from the network at each period. Within a range of pacemaker periods, the LP and VD neurons regulate period in complementary ways. LP neuron removal speeds the network and VD neuron removal slows it. Outside this range, network activity is disrupted because the LP neuron cannot follow slow periods, and the VD neuron cannot follow fast periods. These neurons thus also limit, in complementary ways, normal pyloric activity to a certain period range. These data show that follower neurons in pacemaker networks can play central roles in controlling pacemaker period and suggest that in some cases specific functions can be assigned to individual network neurons.

INTRODUCTION

Central pattern generator (CPG) networks underlie rhythmic motor pattern production (Delcomyn 1980; Marder and Calabrese 1996). The outputs of these networks show large variations in cycle period (e.g., fast vs. slow breathing) and pattern phasing (e.g., breathing vs. gasping) (Arbas and Calabrese 1984; Calabrese et al. 1995; Cohen et al. 1988; Combes et al. 1999a,b; Harris-Warrick and Marder 1991; Jing and Weiss 2001, 2002; Lieske et al. 2000; Nadim and Calabrese 1997; Ramirez 1998; Tegner et al. 1998). We focus here on cycle period control.

CPG rhythmicity arises from network-based or endogenous oscillator mechanisms (Selverston and Moulins 1985). Network-based rhythmicity arises from interactions among multiple neurons, and in these networks, modifying the cellular or

synaptic properties of any of several network neurons generally alters network cycle period (DiCaprio and Fournier 1984, 1988; Namba and Mulloney 1999; Pearson and Ramirez 1990; Ramirez 1998; Reye and Pearson 1987; Wolf and Pearson 1988).

Endogenous oscillator CPGs are driven by pacemaker neurons that fire rhythmic spike bursts. In these networks, one mechanism for cycle period control is alteration of pacemaker intrinsic period (Ayali and Harris-Warrick 1999; Hooper and Marder 1987; Thoby-Brisson and Ramirez 2000). In some networks, the pacemaker is electrically coupled to other network neurons to form a synchronously firing pacemaker ensemble, and in these networks, pacemaker ensemble period can also be altered by changing the cellular properties of the nonoscillatory neurons (Kepler et al. 1990; Marder et al. 1992). Pacemaker neurons or ensembles often also receive feedback from network "follower" neurons (Grillner et al. 1995; Selverston et al. 1976)—neurons whose rhythmic activity is elicited by the pacemaker and that generally fire out of phase with it. These neurons can alter pacemaker period, and in one case, it has been shown that increasing the activity of an identified follower neuron increases pacemaker period (Massabau and Meyrand 1996).

The work noted in the preceding text has significantly expanded our understanding of cycle period control in endogenous oscillator driven networks and has clearly shown that network cycle period is determined by the activity of both network pacemaker and follower neurons. However, a systematic experimental examination of the role follower neurons play in determining pacemaker cycle period, across a wide range of pacemaker activities but in a constant neuromodulatory regime, has to our knowledge not been performed. The lobster (*Panulirus interruptus*) pyloric network is normally driven by a pacemaker ensemble, and this ensemble receives feedback from two pyloric follower neurons, the Lateral Pyloric (LP) and Ventricular Dilator (VD) neurons. Current injection into the endogenous oscillator alters ensemble period (Hooper 1997), and hyperpolarizing individual follower neurons below their transmitter release threshold removes follower neuron chemical feedback onto the pacemaker ensemble (Ayali and Harris-Warrick 1999; Graubard 1978; Graubard et al. 1980, 1983).

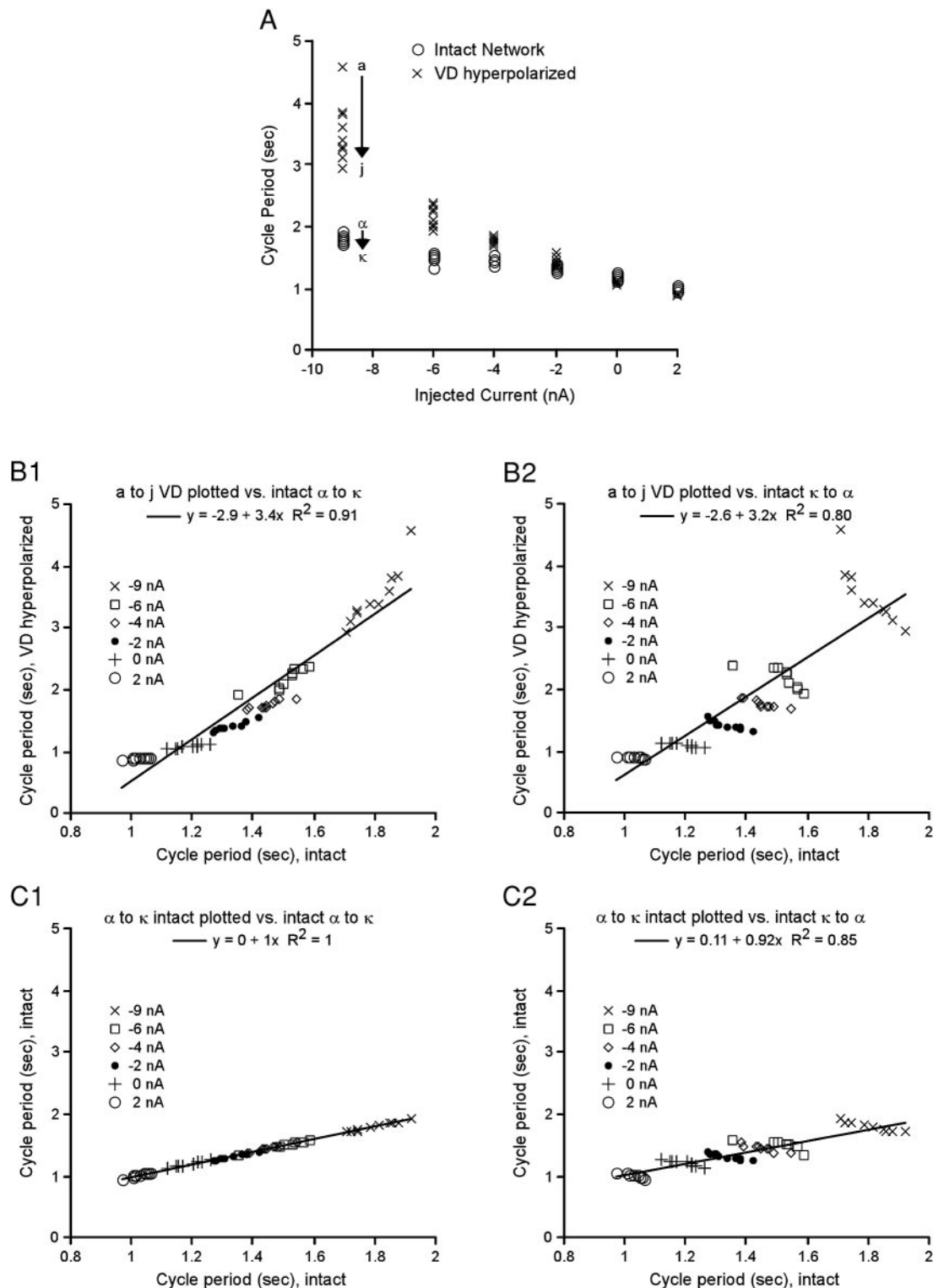
In this network, the role of the follower neurons in cycle

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period control can therefore be investigated across a range of pyloric activities by altering pacemaker period with current injection and, at each cycle period, by removing the LP or VD neuron by hyperpolarization to determine its effect on cycle period. Within a certain range of pacemaker periods, feedback from the VD neuron speeds the pacemaker, whereas feedback from the LP neuron slows it. For pacemaker periods outside this range, one or the other of the

follower neurons disrupts pyloric activity by failing to follow the pacemaker in a 1:1 manner. The VD neuron cannot follow short periods, whereas the LP neuron cannot follow long ones. Thus within a certain pacemaker period range, these neurons serve as complementary frequency governors in that they decrease the range of cycle frequency the network would produce in the absence of follower neuron feedback. Outside this range, they have complementary



effects in that one neuron disrupts slow pyloric rhythms, whereas the other disrupts fast ones.

METHODS

Pacific spiny lobsters (*P. interruptus*) of both sexes (0.5–1 kg) were obtained from Don and Laurice Tomlinson Commercial Fishing (San Diego, CA), and maintained in aquaria with chilled (10–15°C) circulating artificial seawater. *Panulirus* saline was composed of (in mM) 479 NaCl, 12.8 KCl, 13.7 CaCl₂, 3.9 Na₂SO₄, 10 MgSO₄, 10.9 glucose, 11.1 Tris base, and 5.1 maleic acid, pH 7.5–7.6. All salts were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Stomatogastric nervous systems were dissected and prepared for extracellular nerve recording and intracellular neuron recording using standard techniques (Selverston et al. 1976). In all experiments, the stomatogastric nerve, which carries input from the rest of the stomatogastric nervous system to the pyloric network, was left intact. The data presented here are from nine experiments. Nerve recordings were performed using stainless steel pin electrodes insulated with petroleum jelly and an A-M Systems (Everett, WA) differential amplifier. Intracellular recordings and stimulation were made with glass microelectrodes (filled with 0.55 M K₂SO₄, 0.02 M KCl, resistance: 10–20 MΩ) and an Axoclamp 2A or 2B (Foster City, CA). Signals were recorded on a Microdata (S. Plainfield, NJ) DT-800 digital tape recorder. Data were digitized with a Cambridge Electronic Design (CED, Cambridge, UK) 1401 plus interface and analyzed using the CED Spike2 software. Statistical tests (univariate general linear model/analysis of covariance, Student's *t*-test) were performed with SPSS (Chicago, IL) statistical software. Plots and 95% confidence interval lines were generated using Microcal Origin (Northampton, MA). Figures were prepared in Corel Draw (Ottawa, Ontario).

Cycle period was altered by constant current injection into the pacemaker Anterior Burster (AB) neuron. At each AB neuron current injection level, the LP and VD neurons were alternately removed from the network for 20–40 pyloric cycles by hyperpolarization to at least –100 mV, which blocked neuron firing and at least greatly reduced graded synaptic release (see DISCUSSION). In all cases, a single electrode was used for voltage recording and current injection. Hyperpolarized neurons were monitored for escape by examination of extracellular recordings, presence of inhibitory postsynaptic potentials in the neuron's synaptic target neurons, and, when possible, observation of the neuron's membrane potential via a bridge-balanced electrode. Cycle period was calculated from extracellular or intracellular recordings of Pyloric Dilator (PD) neuron activity.

Period was averaged over 6–10 pyloric cycles. Fewer than 10 cycles were used when interference from other stomatogastric nervous system networks (gastric mill, cardiac sac) perturbed pyloric activity (Bartos and Nusbaum 1997; Bartos et al. 1999; Marder et al. 1998; Mulloney 1977; Nadim et al. 1998, 1999; Thuma and Hooper 2002) or when the hyperpolarized neuron escaped from hyperpolarization. This latter problem occurred because originally we did not have a headstage capable of injecting sufficient current to keep the neuron hyperpolarized and only occurred in the first two experiments. In all

cases, no difference was seen between cases in which fewer than 10 cycles were used and those in which 10 were used.

At each level of AB current injection, with or without LP or VD neuron hyperpolarization, a range of cycle periods were observed (Fig. 1A shows an example for VD neuron hyperpolarization). This led to an ambiguity as to how to construct plots in which LP or VD neuron hyperpolarized data were plotted against intact network data because it was unclear which intact case cycle period should be associated with which LP or VD neuron hyperpolarized cycle period. Our association convention, and the logic behind it, are shown in Fig. 1, B1–C2.

Figure 1A shows network cycle periods from one experiment with 2, 0, –2, –4, –6, and –9 nA injected into the AB neuron with the network intact (○) and with the VD neuron hyperpolarized (×). The 10 cycle periods observed with –9 nA of injected current are labeled, in order of decreasing cycle period, from α to j for the VD neuron hyperpolarized case and from α to κ for the intact case (κ is the 10th letter of the Greek alphabet). Figure 1B, 1 and 2, shows the VD neuron hyperpolarized cycle periods plotted versus the intact case cycle periods in two ways. In the first, the periods from each case are plotted with both series in decreasing order (α vs. α , β vs. β , ..., j vs. κ ; Fig. 1B1). In this case, the VD neuron hyperpolarized data from each AB neuron current injection level increase with increased intact cycle period. In the second, the same data are plotted with one series in decreasing order and the other in increasing order (α vs. κ , β vs. ι , ..., j vs. α ; Fig. 1B2). In this case, the VD neuron hyperpolarized data from each AB neuron current injection level decrease with increased intact cycle period.

The trend of the data across AB neuron current injection levels is that the cycle period increase induced by VD neuron hyperpolarization becomes larger as intact cycle period increases. Figure 1B1's plotting convention therefore results in the data from each current injection level supporting the overall trend of the data and Fig. 1B2's plotting convention results in these data opposing the overall trend of the data. Consequently, Fig. 1B1's convention results in a linear fit to the data that has a higher slope and a larger R^2 value. Figure 1C, 1 and 2, shows the effect of the two plotting conventions when the intact network data are plotted against themselves. As expected, when α versus α , β versus β , ..., κ versus κ are plotted, a line with slope 1 and an R^2 of 1 results (Fig. 1C1). Alternatively, when α versus κ , β versus ι , ..., κ versus α are plotted, a line with a slope and $R^2 < 1$ results (Fig. 1C2).

The purpose of these plots is to decide how to use a univariate general linear model/analysis of covariance analysis to determine if the VD neuron hyperpolarized and intact network data differ. The plotting convention in Fig. 1, B1 and C1, makes it more likely that the univariate general linear model/analysis of covariance analysis would find a significant difference between the intact and hyperpolarized cases. In the example at hand, comparing Fig. 1, B1 versus C1 gives P values of 5×10^{-29} for intercept and 8×10^{-65} for slope whereas comparing Fig. 1, B2 and C2, gives P values of 2×10^{-14} for intercept and 3×10^{-41} for slope. The null hypothesis is that VD neuron hyperpolarization had no effect, and therefore the plotting convention in Fig. 1, B2 and C2, is most likely to result in the null hypothesis

FIG. 1. Convention for plotting Ventricular Dilator (VD) and Lateral Pyloric (LP) neuron hyperpolarized cycle period vs. intact cycle period. A: cycle periods in 1 experiment of intact network and with VD neuron hyperpolarized for Anterior Burster (AB) neuron current injections of +2, 0, –2, –4, –6, and –9 nA. At each AB neuron current injection level a range of cycle periods occurred. For the –9 nA case, the individual cycle periods have been labeled α – j (VD neuron hyperpolarized) and α – κ (intact network). B, 1 and 2: VD neuron hyperpolarized cycle periods vs. intact cycle periods are plotted with the data in both series in descending order (α with α , β with β , ..., j with κ ; B1) or with the data in one series in descending order and that data in the other in ascending order (α with κ , β with ι , ..., j with α ; B2). Because in both plots the overall trend of the data is to increase with cycle period, the plotting convention used in B1 gives a better R^2 value than that used in B2. C, 1 and 2: similar plots of the intact data vs. themselves. As expected, C1 gives a perfect fit, whereas C2 gives a much worse fit. Because the null hypothesis in this work was that VD (and LP) neuron hyperpolarization would not affect cycle period and because comparisons between the hyperpolarized and intact cases when plotted as in C2 were less likely to be significant, the conservative plotting convention shown in B2 and C2 was used in the work reported here. Symbol identification in B, 1 and 2, and C, 1 and 2, refer to level of AB neuron current injection.

being supported (i.e., that the data do not support this article's conclusions). We analyzed all our data using the convention shown in Fig. 1, B2 and C2, to ensure our analysis was as conservative as possible. One final point on this issue is that this plotting convention requires equal numbers of intact and LP or VD neuron hyperpolarized cycles at each level of AB neuron hyperpolarization. When different numbers of cycles were present in the intact or LP or VD neuron hyperpolarized cases, cycle numbers were equalized by discarding the most extreme cycle period cycles (e.g., if cycles had needed to be discarded in the VD neuron hyperpolarized case for -9 -nA AB neuron current injection in Fig. 1A, first point a, then j, then b, then i, etc., would have been discarded).

RESULTS

The pyloric network is a small, well-characterized network of 14 neurons consisting of six neuronal types (Eisen and Marder 1982; Johnson et al. 1993; Miller and Selverston 1982a,b; Selverston and Miller 1980; Selverston et al. 1976). Figure 2A shows the pyloric circuit diagram. Circles indicate inhibitory chemical synapses and resistors and diodes indicate electrical coupling. The AB neuron is an endogenous oscillator (pacemaker) neuron. The two PD neurons are electrically coupled to the AB neuron and burst with it. These three neurons form the pyloric pacemaker ensemble. The network has four follower neuron types, LP, VD, Inferior Cardiac (IC), and Pyloric (PY). We are primarily concerned here with the LP and VD neurons because they directly feed back onto the pacemaker ensemble. The LP neuron inhibits the PD neurons, and the VD neuron makes a rectifying electrical synapse onto both the PD and AB neurons.

Under the experimental conditions employed here (esophageal and commissural inputs intact), all pyloric neurons show postinhibitory rebound and plateau potentials (Russell and Hartline 1982). However, different pyloric neuron types, when isolated, have different responses to injected current protocols (Hartline 1979; Hartline and Gassie 1979; Hartline and Graubard 1992) and have different conductance complements (Baro et al. 1994, 2000, 2001). In addition to typical spike-mediated synaptic release, all pyloric neurons also release transmitter as a graded function of membrane potential, and

normally phased, rhythmic slow wave depolarizations continue when spiking is blocked with tetrodotoxin (Graubard 1978; Graubard et al. 1980, 1983; Raper et al. 1979).

The pyloric neural output is a triphasic rhythmic pattern in which first the AB/PD neuron pacemaker ensemble fires, then the LP and IC neurons fire and then the VD and PY neurons fire, after which the pattern repeats (Fig. 2B). In the work reported here, we investigated the effect of the LP and VD neurons on pacemaker ensemble cycle period. To this end, we injected varying levels of constant current into the AB neuron to alter network cycle period and then alternately hyperpolarized the LP and VD neurons to functionally remove them from the network. The LP and VD neurons were chosen because they are the only follower neurons that feed back onto the pacemaker ensemble and the differing nature of their feedback—the VD neuron makes rectifying electrical synapses onto the pacemaker ensemble, whereas the LP neuron inhibits the ensemble. VD and LP neuron removal from the network was carried out by hyperpolarization instead of photoinactivation (Miller and Selverston 1979) for two reasons. First, hyperpolarization is reversible and thus allows the effects of removing both follower neurons to be tested in the same preparation. Second, it takes several hours to perform the number of AB neuron current injections that were done here. Thus were these experiments to have been done with cell kills, we would have been comparing intact and neuron removed data taken at least 1–2 h apart. Pyloric baseline cycle frequency can slowly change over long periods, and we would have been unable to distinguish whether the cycle frequency changes were due to the neuron removal or to changes in pyloric period arising from other sources. With hyperpolarization, the intact and neuron removed cases are immediately alternating. Thus with hyperpolarization, even if pyloric baseline cycle period is slowly changing, the relative effect on that baseline of removing a neuron is preserved.

LP and VD neuron input affects pacemaker period in complementary ways

Figure 3 shows the effect of LP neuron removal on pacemaker cycle period. In each panel, *top* is an intracellular recording of the LP neuron and *middle* is an extracellular recording of PD neuron activity. *Bottom* shows PD neuron activity with the LP neuron hyperpolarized. The three panels show the effect of LP neuron removal at three AB neuron hyperpolarization levels (A, 0 nA; B, -5 nA; C, -10 nA). LP neuron removal consistently shortened average pacemaker cycle period (in A, from 0.67 to 0.57; in B, from 0.78 to 0.70; in C, from 0.94 to 0.85 s). Similar results were seen in five of five experiments.

Figure 4 shows the effect of VD neuron removal on pacemaker cycle period in the same preparation shown in Fig. 3. Figure layout and AB neuron current injection levels are the same as in Fig. 3. VD neuron removal consistently lengthened average pacemaker cycle period (in A, from 0.67 to 0.91; in B, from 0.78 to 1.18; in C, from 0.94 to 1.75 s). Similar results were seen in six of seven experiments.

Figures 3 and 4 show only AB neuron hyperpolarizing current injections. In almost all experiments, the control cycle periods of the preparations were near the minimum that the pyloric network produces in control saline (approximately

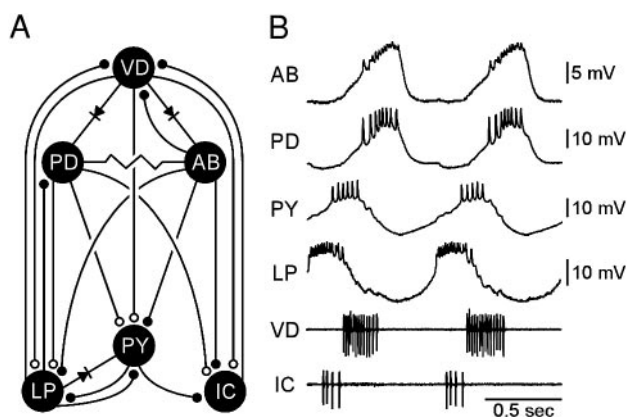


FIG. 2. The pyloric network synaptic connectivity diagram (A) and typical pyloric output pattern (B). The pyloric pattern is a triphasic rhythmic pattern in which the AB/Pyloric Dilator (PD) pacemaker ensemble fires, then the LP and Inferior Cardiac (IC) neurons fire, and then the VD and Pyloric (PY) neurons fire, after which the pattern repeats. ●, inhibitory glutamatergic synapses; ○, inhibitory cholinergic synapses; resistor, nonrectifying electrical coupling; diode, rectifying electrical coupling.

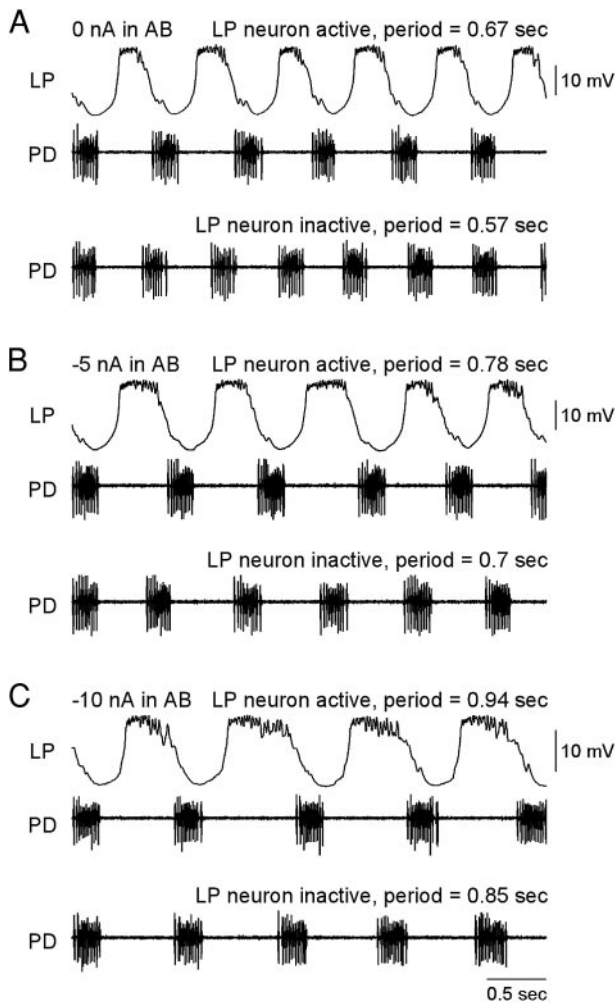


FIG. 3. LP neuron removal shortened pacemaker period. In each panel the top trace is an LP neuron intracellular recording and the second trace is a PD neuron extracellular recording. The third trace shows PD neuron activity with the LP neuron hyperpolarized. The three panels show the effect of LP neuron removal at three AB neuron hyperpolarization levels (0, -5, -10 nA).

0.5 s). We therefore were usually unable to significantly depolarize the AB neuron without the VD neuron disrupting pyloric activity (see *Pattern disruption*). Figure 5 shows data (from 1 of 2 LP neuron and 1 of 3 VD neuron removal experiments) in which we were able to depolarize the AB neuron somewhat without pattern disruption. In both panels of this figure, 0.5 nA has been injected into the AB neuron, decreasing cycle period from 0.73 to 0.67 s. In each panel, *top* and *middle* show follower and PD neuron activity and *bottom* shows PD neuron activity with the follower neuron hyperpolarized. LP neuron removal continued to cause a reduction in cycle period (from 0.68 to 0.56 s; Fig. 5A), whereas VD neuron removal had no effect (0.67 to 0.67 s; Fig. 5B).

It was important to compare the effects of LP and VD neuron removal across the entire range of AB neuron current injections and to test the significance of these effects. These tasks were difficult because at any level of AB neuron current injection, a range of cycle periods was observed in both the intact and LP or VD neuron removed cases. As a result of these ranges of cycle periods, it was unclear what x axis (intact cycle period) values to use for the LP and VD neuron removed data

in plots of LP and VD neuron removed cycle period versus intact network cycle period. As detailed in METHODS, we resolved this difficulty by discarding, if necessary, data points until, at each level of AB neuron current injection, the intact and LP or VD neuron removed cases had equal numbers of data points and, for each level of AB neuron current injection, by plotting the series of LP or VD neuron removed cycle periods in reverse order against the series of intact cycle periods. This method was the most conservative with respect to the univariate general linear model/analysis of covariance analysis in that it was the most likely to find that the LP or VD neuron removed data did not differ from the intact case.

Figure 6 compares VD neuron removed and LP neuron removed data to intact data from one typical experiment. At all intact cycle periods, VD neuron removal increased cycle period and LP neuron removal decreased cycle period. However, the effects of LP and VD neuron removal were not precisely complementary. LP neuron removal decreased average cycle period by approximately 20% at all AB neuron current injection levels. In contrast, the increase in cycle period induced by VD neuron removal increased with AB neuron hyperpolariza-

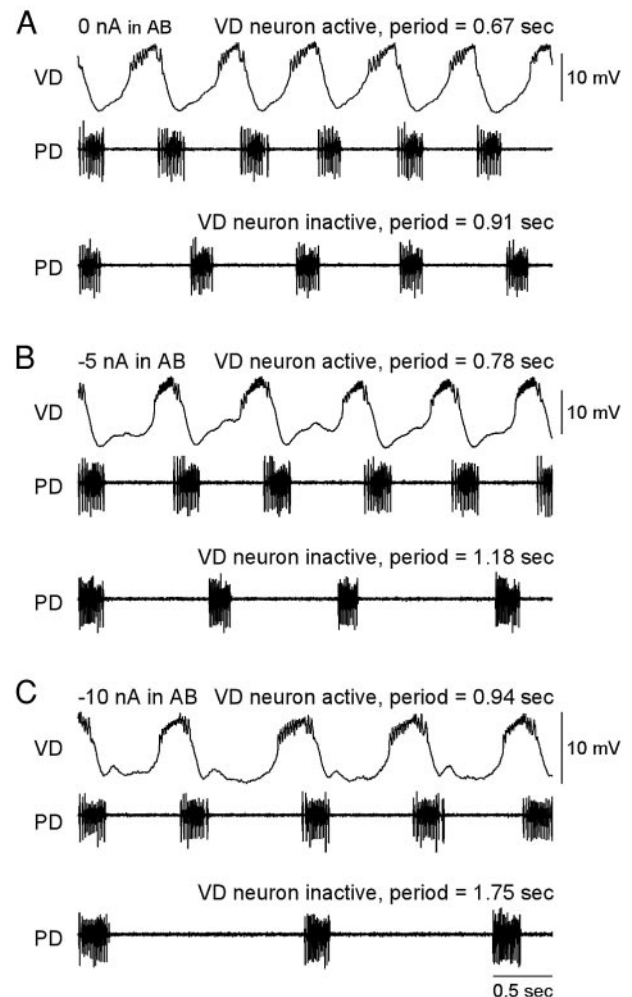


FIG. 4. VD neuron removal lengthened pacemaker period. In each panel, *top*: a VD neuron intracellular recording; *middle*: a PD neuron extracellular recording; *bottom*: PD neuron activity with the VD neuron hyperpolarized. A–C show the effect of VD neuron removal at 3 AB neuron hyperpolarization levels (0, -5, -10 nA).

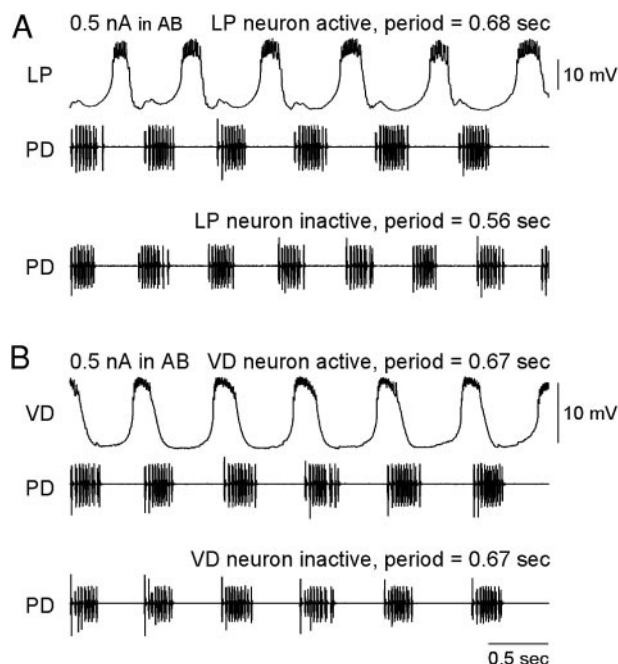


FIG. 5. When the AB neuron is depolarized, LP neuron removal continued to reduce cycle period (A), whereas VD neuron removal had little effect (B). In each panel, *top* and *middle*: follower and PD neuron activity; *bottom*: PD neuron activity with the follower neuron hyperpolarized. In each panel, 0.5 nA has been injected into the AB neuron.

tion (from 50% at 0 nA to 100% at -17 nA injection into the AB neuron). The 95% confidence lines in Fig. 6 do not overlap at any intact network cycle period, and these data sets differ significantly (these data are from the experiment shown in Figs. 3 and 4).

In five of five LP-neuron-removal experiments, LP neuron removal significantly altered pyloric cycle period, and in six of seven VD-neuron-removal experiments, VD neuron removal significantly altered pyloric cycle period. In the one VD-neuron-removal experiment in which VD neuron hyperpolarization did not alter pacemaker cycle period, VD neuron activity was unusual in that, instead of firing before the AB/PD neuron burst (see Fig. 2), the VD neuron fired with the pacemaker ensemble. It is likely that this preparation-specific difference in VD neuron activity is the reason that VD neuron removal did not alter cycle period in this experiment (see DISCUSSION).

To verify these experiment by experiment analyses, an analysis of covariance general linear model comparing the intercepts and slopes of the linear fits of the LP or VD neuron removed and intact data were performed across all experiments using the data from each experiment's plot similar to Fig. 6. The calculated P values were: LP neuron removed versus intact, intercept, 4.9×10^{-5} , slope, 0.039 ; VD neuron removed versus intact: intercept, 5.1×10^{-9} , slope, 8.5×10^{-9} . These analyses indicate that both VD and LP neuron removal significantly changed pyloric cycle period.

However, the analysis of covariance general linear model does not indicate whether the changes are consistent across preparations. We therefore also performed a post hoc comparison of the intercepts and slopes of the intact and the LP or VD neuron removed linear fits to resolve these questions. With respect to the nature of these changes, slope represents how

much neuron removal affects the changes in pacemaker period AB neuron current injection induced, and intercept represents how much neuron removal offsets the neuron removed line from the intact line. LP neuron removal decreased the average intercept from 0.14 to -0.2 ($P = 0.05$) and increased average slope by 0.95 (not significant, $P = 0.3$). VD neuron removal decreased the average intercept from 0.13 to -0.66 (not significant, $P = 0.06$) and increased average slope from 0.87 to 1.87 ($P = 0.007$; Student's t -test for all comparisons).

If the LP and VD neuron effects on cycle period were independent, it would be expected that if both neurons were simultaneously hyperpolarized, their effects would cancel, and thus the change in pyloric period would be decreased or reversed. Due to the length of these experiments, we only performed such dual hyperpolarizations in two of them. In these two experiments, this prediction was exactly borne out. In each experiment, hyperpolarizing the other of the LP or VD neuron when one of them was already hyperpolarized decreased the effect of the first hyperpolarization and resulted in cycle periods that were closer to those observed in the intact network for that level of AB neuron current injection (data not shown).

One explanation for the increased pacemaker period with VD neuron hyperpolarization could be leakage of hyperpolarizing current into the pacemaker ensemble through the VD to AB and PD neuron rectifying electrical synapses. Figure 7 shows that this explanation is unlikely to be correct. In each panel, the *top* is an intracellular recording of the VD neuron, the *middle* is an intracellular recording of the AB neuron, and the *bottom* is an extracellular recording of PD neuron activity. The two panels show the effect of VD neuron hyperpolarization at two AB neuron injection levels (A, 0 nA; B, -6 nA). In neither case did VD neuron hyperpolarization hyperpolarize the AB neuron. If VD neuron hyperpolarization had any effect in these examples, it was to slightly depolarize the AB neuron. These data suggest that the effects of VD neuron hyperpolarization were not due to the trivial explanation of simple AB neuron hyperpolarization through the VD to AB neuron rectifying synapse.

Pattern disruption

For sufficiently large AB neuron current injections both follower neurons disrupted pyloric cycling by not bursting 1:1 with the rest of the network. The LP neuron disrupted pyloric cycling when the AB neuron was extremely hyperpolarized (very slow cycle periods, Fig. 8). In both panels, -30 nA had been injected into the AB neuron, and the network was cycling extremely slowly (approximately 2-s period). In A, the network was intact. The *top*, *middle top*, and *middle bottom* are intracellular recordings of the LP, VD, and PY neurons, the *bottom* is extracellular recording of PD neuron activity. B shows network activity when the LP neuron was removed by hyperpolarization (LP neuron trace not shown). When the LP neuron was active (A), it intermittently fired two bursts per AB/PD neuron burst (boxes) and thus disrupted the pattern by lengthening VD neuron interburst interval, PY neuron burst duration, and network cycle period. LP neuron hyperpolarization below threshold restored regular pyloric cycling (B). Similar results were seen in six of six experiments.

The VD neuron disrupted pyloric cycling when the AB neuron was extremely depolarized (very fast cycle periods, Fig.

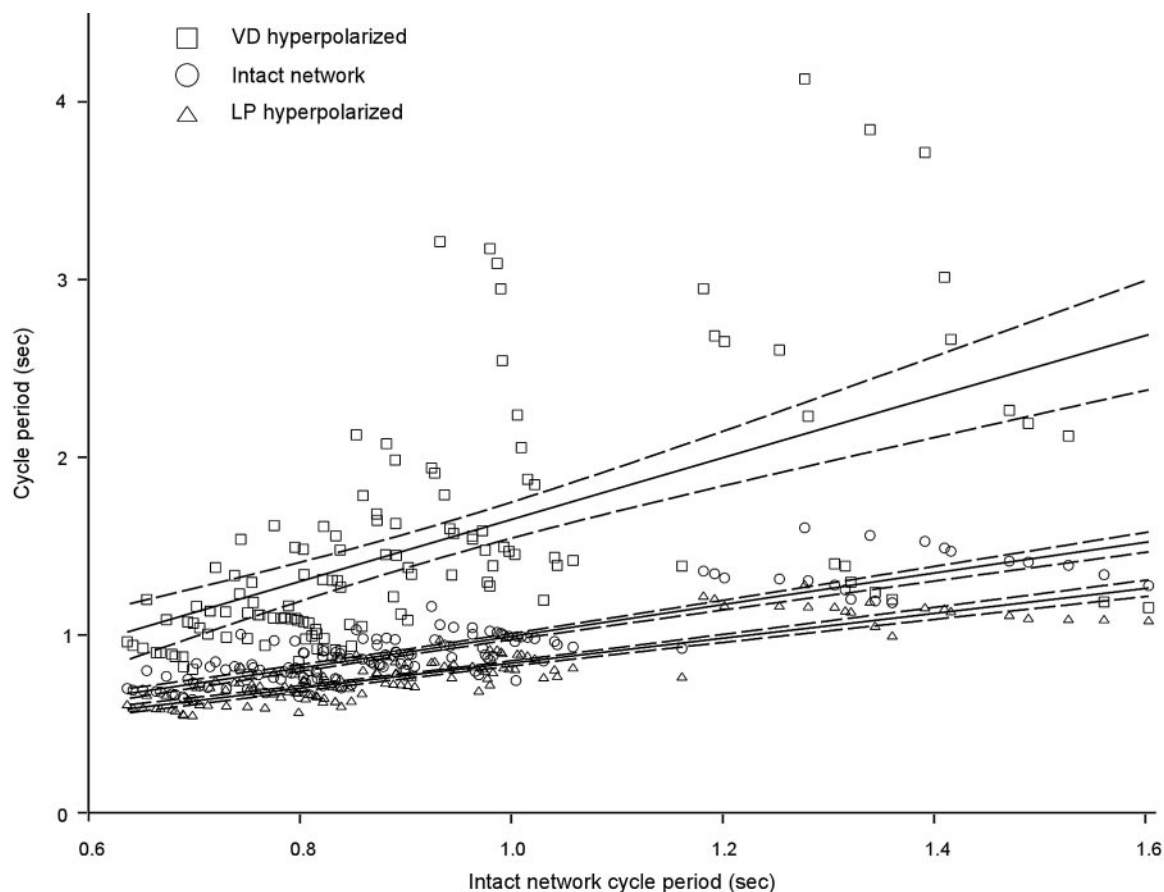


FIG. 6. Cycle period effects of LP or VD neuron removal. LP neuron removal reduces cycle period by a nearly constant amount compared with the intact network at all AB neuron current injection levels, whereas the slowing effect of VD neuron removal increases with increased intact network cycle period. The best-fit lines to all the data points of each condition are also plotted along with their 95% confidence interval lines.

9). In both panels, +20 nA had been injected into the AB neuron, and the network was cycling extremely quickly (approximately 0.5-s period, note the difference in time scale in

Figs. 8 and 9). In A, the network was intact. The *top*, *middle top*, and *middle bottom* are intracellular recordings of the VD, LP, and PY neurons, the *bottom* an extracellular recording of PD neuron activity. B shows the activity of these neurons when the VD neuron was removed by hyperpolarization (VD neuron trace not shown). When the VD neuron was active (A), it fired one burst every two to four AB/PD neuron bursts and thus disrupted the pattern by increasing PY and LP neuron interburst interval and decreasing PD neuron burst duration. VD neuron hyperpolarization below threshold restored regular pyloric cycling (B). Similar results were seen in four of five experiments in which depolarizing current was injected into the AB neuron.

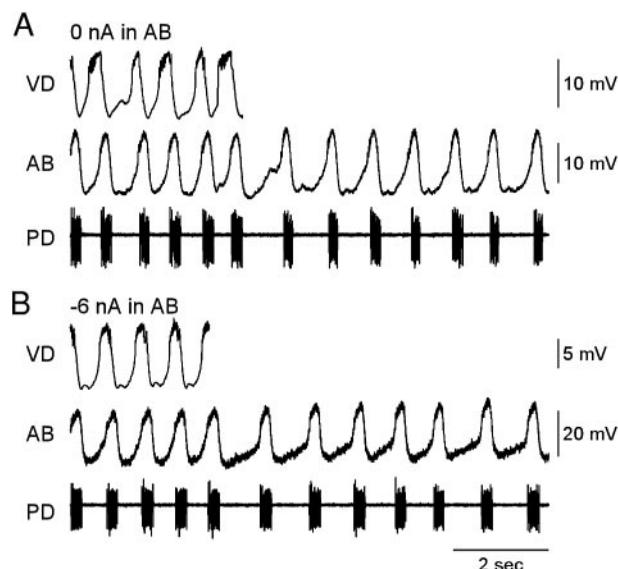


FIG. 7. VD neuron hyperpolarization does not hyperpolarize the AB neuron. In each panel, *top*: an intracellular VD neuron recording; *middle*: an intracellular AB neuron recording; *bottom*: an extracellular PD neuron recording. A and B show the effect of VD neuron hyperpolarization at 2 AB neuron injection levels.

DISCUSSION

Figure 10 is a schematic summarizing the effects of the LP and VD neurons on pyloric network activity. The *top* shows the effects of the LP neuron on the pacemaker, the *middle* the effects of the VD neuron, and the *bottom* the AB neuron current injection level. The triangle in the VD neuron trace symbolizes its increased effect on cycle period as AB neuron hyperpolarization level increases. The analogous portion of the LP neuron trace is a line because removal of this neuron affected the offset of the linear fits, not the slope, and thus the magnitude of the effect of LP neuron removal did not change with the period of the intact network. Each neuron can disrupt

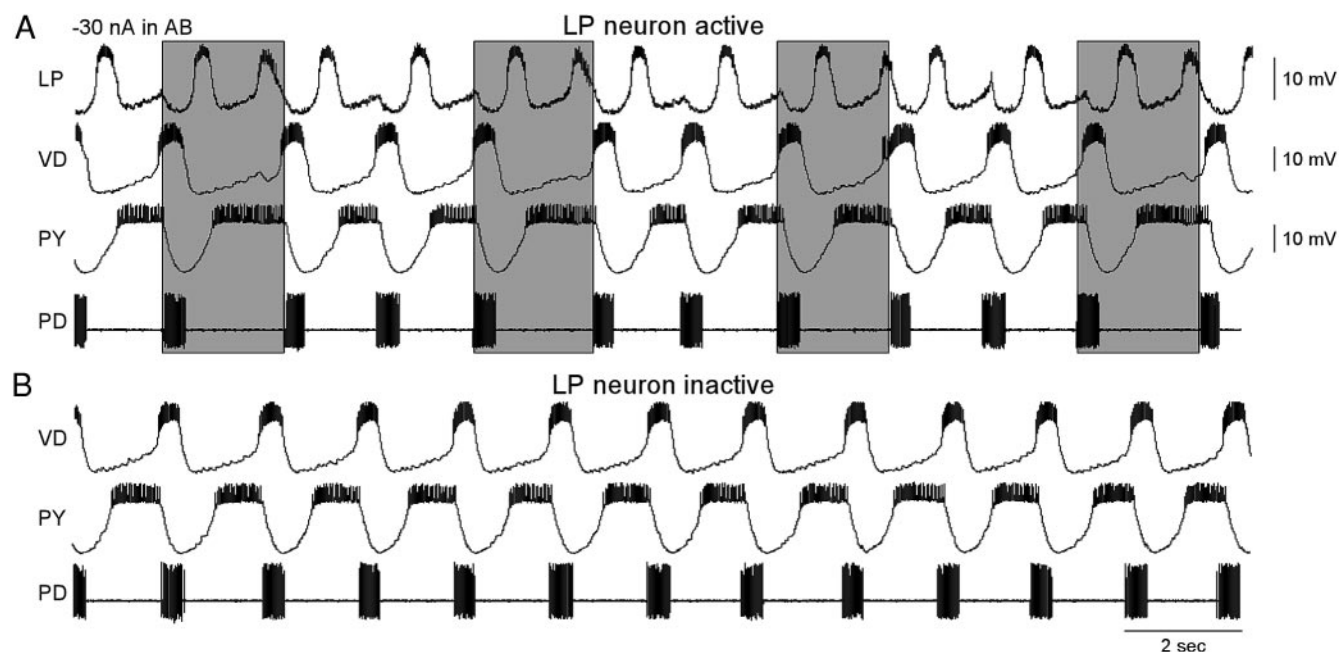


FIG. 8. The LP neuron disrupts pyloric activity when the AB neuron is strongly hyperpolarized (slow cycle periods). *A*, *top*, *middle top*, and *middle bottom*: intracellular recordings of the LP, VD, and PY neurons; *bottom*: an extracellular PD neuron recording. The LP neuron intermittently fired 2 bursts per AB/PD neuron burst (boxes). *B*: the activity of these neurons when the LP neuron was removed by hyperpolarization (LP neuron trace not shown). LP neuron hyperpolarization restored regular pyloric cycling.

the pattern, but on opposite ends of the network cycle period range. The LP neuron disrupts at slow cycle periods, while the VD neuron disrupts at fast cycle periods. These results suggest that each neuron can fire 1:1 with the rest of the network only within certain period ranges. The LP neuron can follow fast to moderately slow periods, whereas the VD neuron can follow slow to moderately fast ones. In the cycle period range in which neither neuron disrupts the pattern, each neuron alters

cycle period in complementary ways. Although the LP neuron can follow fast cycle periods 1:1, its presence slows the network, and it appears to do so by a nearly constant percentage at all network cycle periods. Similarly, although the VD neuron can follow slow cycle periods 1:1, its presence speeds the network. However, this effect lessens as network cycle period decreases and may be lost entirely when the AB neuron is depolarized.

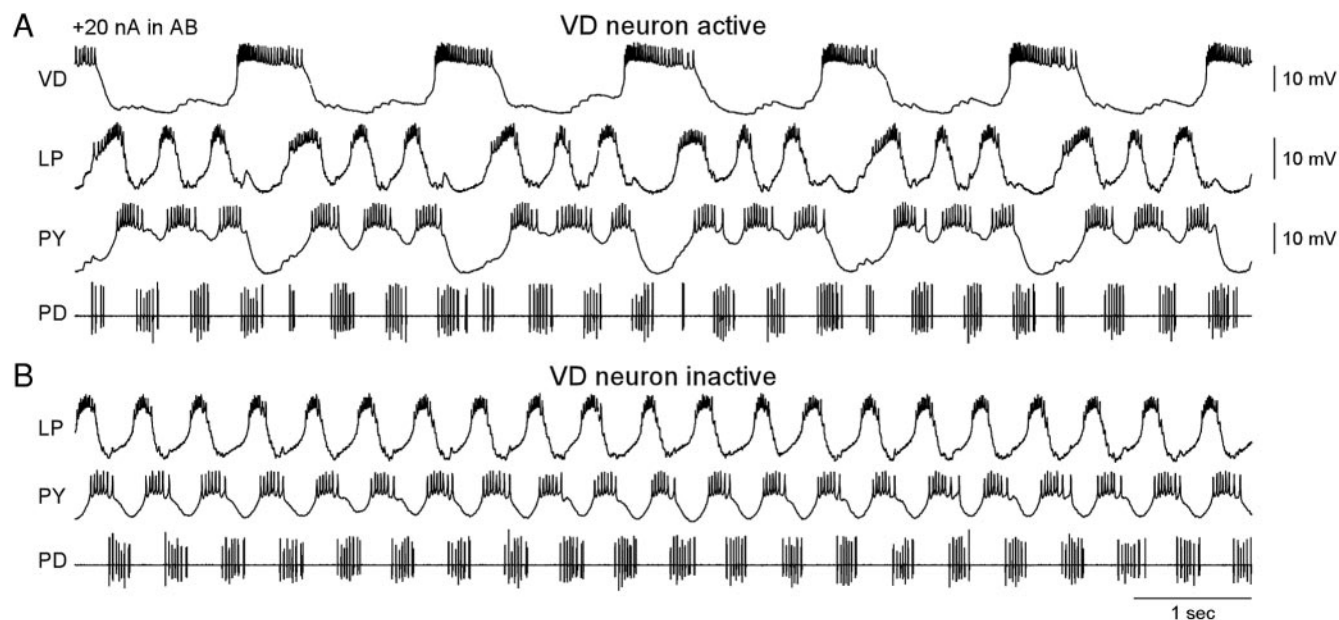


FIG. 9. The VD neuron disrupts pyloric activity when the AB neuron is strongly depolarized (fast cycle periods). In *A*, *top*, *middle top*, and *middle bottom*: intracellular recordings of the VD, LP, and PY neurons; *bottom*: an extracellular PD neuron recording. The VD neuron fired only once for every 2–4 AB/PD neuron bursts (boxes). *B*: the activity of these neurons when the VD neuron was removed by hyperpolarization (VD neuron trace not shown). VD neuron hyperpolarization restored regular pyloric cycling.

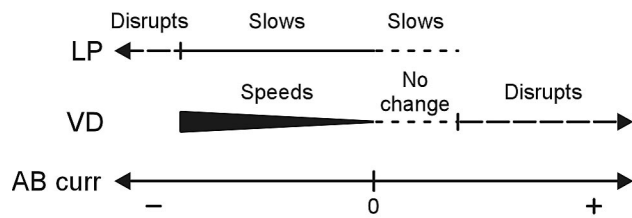


FIG. 10. Schematic diagram summarizing effects of LP and VD neurons on pyloric network activity. *Top*: the effects of the LP neuron; *middle*: those of the VD neuron; *bottom*: the AB neuron current injection level. The triangle for the VD neuron indicates its increasing effect as AB neuron hyperpolarization increases. The LP neuron disrupts pyloric activity at slow cycle periods, whereas the VD neuron speeds the network at fast periods. In the period range in which neither neuron disrupts the pattern, the LP neuron slows the network whereas the VD neuron speeds the network.

Experimental considerations

Pyloric neurons release transmitter as a graded function of membrane potential (nonspiking release), and hyperpolarization therefore may not remove all of the LP and VD neuron synaptic effects due to space clamp problems within the neuron (Hartline and Graubard 1992). However, the hyperpolarizations used (to at least -100 mV) were well below graded transmitter release threshold (Graubard 1978; Graubard et al. 1980, 1983) and have been successfully used in this system to reversibly remove neurons from the network (Ayali and Harris-Warrick 1999). Examination of the membrane voltages of the LP and VD neuron postsynaptic targets showed no signs the neurons were releasing transmitter, and in cases in which the membrane potential of the hyperpolarized neuron could be followed, no membrane potential oscillations were present. Even if transmitter release were occurring, this release is clearly less than in the intact network, and so our data would only underestimate the effects of LP and VD neuron removal on pacemaker period.

Comparison to earlier work

LP NEURON. Selverston and Miller (1980) showed that removal (by photoinactivation) (Miller and Selverston 1979) of the LP neuron in *Panulirus*, under experimental conditions similar to ours (descending input from the esophageal and commissural ganglia intact), decreased pyloric cycle period. Massabau and Meyrand (1996) showed in the lobster, *Homarus gammarus*, that increased LP neuron activity increased, and decreased (but not abolished) LP neuron activity decreased, pacemaker cycle period. These data are consistent with our 0-nA AB neuron current injection data points, which also showed that LP neuron input slows pyloric cycle period at this level of network activity. However, because the authors did not inject current into the AB neuron, they do not show that this effect is general across a wide cycle period range, as was done here.

In a study of proctolinergic modulation of the pyloric network, Hooper and Marder (1987) found that removal of the LP neuron by photoinactivation had no effect on pyloric cycle period. However, this work was performed with esophageal and commissural input to the network blocked. The cellular properties of the network were consequently very different from those in the preparations studied here, and in particular LP neuron activity was very weak (average, 1.5 spikes fired per burst). It is thus likely that the reason for the discrepancy

between their study and this one is that their LP neuron activity was so reduced that, even when in the network, the LP neuron had little effect on pacemaker activity.

Ayali and Harris-Warrick (1999) showed that the LP neuron played different roles in helping mediate the effects of the aminergic modulators, octopamine and dopamine, on pyloric cycle period in *Panulirus* (again, with esophageal and commissural inputs intact). In dopamine, the LP neuron played no role in the dopamine induced change in pyloric cycle period, whereas octopamine's effects on cycle period required the presence of the LP neuron. However, the role of the LP neuron in determining pyloric cycle period in control saline was not assessed in this work nor was it determined if the LP neuron in the modulators would continue to have the same effects on pyloric cycle period at multiple cycle periods (achieved, for instance, by independent current injection into the AB neuron). As such, although this work clearly shows that the LP neuron can have different effects on pyloric cycle period in different modulatory conditions, it is not directly relevant to the work reported here. Nonetheless, it is interesting that in the modulator (octopamine) in which the LP neuron did affect pyloric cycle period, the effect of the neuron, as in the work reported here, was to increase cycle period.

VD NEURON. We have been unable to find similar earlier work in which the role of the VD neuron on pyloric cycle period was investigated. Kepler et al. (1990) showed in a modeling study that electrically coupling a passive neuron to an endogenous oscillator could either increase or decrease oscillator cycle period, depending on the strength of the electrical coupling. This work is, unfortunately, not relevant to the experimental work reported here because the VD to AB neuron electrical coupling is rectifying (in Kepler et al. the coupling was bidirectional), the VD neuron does not normally fire in phase with the AB/PD neuron pacemaker (in Kepler et al. the electrically coupled neurons fired together), and Kepler et al. altered cycle period by changing coupling strength (as opposed to measuring the effect of coupling at different oscillator periods, as was done here).

Mechanisms of cycle period governance

One possible mechanism by which LP and VD neuron hyperpolarization could alter pyloric cycle period would be if these neurons were electrically coupled to descending modulatory projection neurons whose activity altered pacemaker pyloric period, and if LP or VD neuron hyperpolarization altered transmitter release from the projection neuron terminals (Coleman and Nusbaum 1994; Coleman et al. 1995). In this scenario, the LP neuron would be coupled to a projection neuron that slowed the network (because LP neuron hyperpolarization increases network cycle frequency) and the VD neuron would be coupled to a projection neuron that increased network cycle frequency (because VD neuron hyperpolarization slows the network). Although we cannot rule out this possibility, modulatory effects would likely occur relatively slowly, and thus the effects of LP and VD neuron hyperpolarization would also occur relatively slowly. This result was never observed. The cycle period changes induced by neuron hyperpolarization always occurred either within the pacemaker cycle in which the neu-

rons were hyperpolarized or in the next cycle, depending on whether the hyperpolarization began early or late in the pacemaker cycle.

Assuming that the cycle period changes are not due to changes in descending input activity, in distributed networks, changes in network activity can arise either via direct or network-mediated mechanisms (see Hooper and Marder 1987; Hooper and Moulins 1990 for pyloric network examples). For instance, LP neuron hyperpolarization could decrease pyloric cycle period either because of a direct effect of the lack of LP neuron input to the pacemaker ensemble or because the lack of LP neuron input alters PY neuron activity, which then alters VD neuron activity, which then alters pacemaker activity. We have examined the effects of LP and VD neuron hyperpolarization on all the pyloric network neurons (Weaver and Hooper 2000). When cycle period dependent changes are accounted for, LP neuron removal has no significant effect on any aspect of VD, PY, or IC neuron phase or spiking activity, and VD neuron removal has no significant effect on any aspect of LP, PY, or IC neuron phase or spiking activity. Direct inputs of the LP and VD neurons onto the pacemaker ensemble, not network mediated mechanisms, therefore most likely underlie the effects of LP and VD neuron removal.

LP NEURON. LP neuron presence increases period, and the LP neuron inhibits the PD neurons (Fig. 2). The most parsimonious explanation of the LP neuron effects is that, through the PD to AB neuron electrical coupling, LP to PD neuron inhibition increases AB neuron cycle period by increasing AB neuron interburst interval. Comparing the intact network to the LP neuron hyperpolarized traces in Figs. 4 and 6 shows precisely this effect. PD neuron interburst interval increases whereas PD neuron burst duration (which mirrors AB neuron burst duration) remains constant.

VD NEURON. The speeding effect of the VD neuron on pacemaker period can be explained by considering the mechanism underlying VD neuron bursting, the timing of these bursts, and the rectifying nature of the VD to AB neuron electrical coupling. Follower pyloric neurons fire because the inhibitions they receive induce postinhibitory rebound (Selverston et al. 1976) and plateau potentials (Russell and Hartline 1978, 1982). The LP and IC neurons inhibit the VD neuron, their bursts likely induce VD neuron firing, and the VD neuron therefore generally fires before the AB and PD neurons (Figs. 2, 4, and 5). The direction of the rectifying synapse is such that when the VD neuron is depolarized relative to the AB neuron, depolarizing current would flow from the VD to the AB neuron. The early VD neuron depolarization and firing therefore likely injects depolarizing current into the pacemaker ensemble and hence advances its firing and shortens pacemaker period.

This explanation is consistent with the increased speeding effect of the VD neuron with increased AB neuron hyperpolarization (Fig. 6). As the AB neuron is further hyperpolarized, the membrane potential difference between the VD and AB neuron would increase. More depolarizing current would flow from the VD to the AB neuron, and removal of this current by VD neuron hyperpolarization would more greatly alter pacemaker period. Further support for this explanation is provided by the one of seven experiments in

which VD neuron hyperpolarization did not alter pacemaker period (data not shown). In this experiment, the VD neuron fired in synchrony with, instead of before, the pacemaker. According to the above mechanism, in this phase relationship the VD neuron would not advance pacemaker activity.

Relevance to pyloric network function

The goal of this work was to investigate the effects of the LP and VD neurons in the pyloric network's typical in vitro state (connected to the esophageal and commissural ganglia but without exogenous neurotransmitter application or input stimulation) across a wide range of network activities. To achieve this goal, current injection into the AB neuron was used to alter pyloric cycle period. In vivo, pyloric cycle period is instead altered by neurotransmitter release from descending inputs. This release can alter the voltage dependence, kinetics, and expression of AB and other pyloric neuron membrane conductances and network synaptic strengths, which our current injections are presumably not inducing. It would therefore be incorrect to assume that, because the LP and VD neurons alter cycle period in complementary fashions when network period is altered by current injection into the AB neuron, these neurons continue to do so when network period is altered by neuromodulator application.

The data obtained with zero current injection into the AB neuron, however, are directly relevant to pyloric network activity in the esophageal/commissural connected preparation. Our data show that in these preparations decreases in VD neuron activity are unlikely to alter pyloric cycle period. In agreement with the results of Selverston and Miller (1980) in *Panulirus* and Massabau and Meyrand (1996) in *Homarus*, our data also show that in such preparations decreased LP neuron activity decreases pyloric cycle period. This observation suggests that pyloric cycle period could be altered by modulation of LP neuron activity, or LP to PD neuron synaptic strength (Harris-Warrick and Flamm 1986), without altering AB neuron properties.

The most valuable contribution of these data to understanding pyloric network function, however, is in providing a description of the LP and VD neuron effects on pyloric period across a wide range of pyloric periods as opposed to at a single period. These data provide a graded, continuous baseline of the effects of the LP and VD neurons in the typical in vitro pyloric network. Any model of the pyloric network in this state must also be able to reproduce this activity, and this requirement is presumably a more stringent test of model validity than measurements of the effects of LP and VD neuron removal at a single network period.

Similar experiments in the presence of neuromodulators or input stimulation, with and without esophageal and commissural input, can be easily performed. In these different neurotransmitter milieus, the cellular properties and synaptic strengths of the pyloric network are altered. Whether the LP and VD neurons continue to function as complementary period regulators in these different network states should provide both increased insight into the mechanisms underlying network activity in them and the mechanisms underlying the ability of the LP and VD neurons to do so.

Relevance to small distributed systems in general

This work has two points of general relevance to small systems neuroscience. The first is the importance, where possible, of examining the role a given neuron or synapse plays in generating network activity across a wide range of network activities because of the more complete description this procedure provides. It is essential to stress that this does not mean examining network mechanism under different neuromodulator milieus because, although such work is essential to understand network multifunctionality, by its very nature it can tell us relatively little about how networks function in any one neuromodulator milieu. Rather, it is necessary to use methods of altering network activity that do not, or as little as possible, alter conductance phosphorylation state, synaptic strength, or other fundamental network characteristics. Presumably in general, as was the case here, the most obvious first choice is current injection into key network neurons to provide a series of network activities in which to perform the tests of interest.

An immediate objection that can be raised is that such manipulations are not physiological. However, small systems are generally not studied because of their scientific interest per se, but because of the belief that by studying them, general principles applicable to other systems of greater import, particularly human, can be discerned. The situation is analogous to the voltage-clamp studies that defined the fast sodium current. These protocols were completely nonphysiological, but they resulted in a quantitative description of the conductance's "operating principles" (the differential equations describing its activation and inactivation) and qualitative concepts (absolute and relative refractory periods, one form of rebound firing) that are generally applicable and would have been extremely difficult to achieve otherwise.

This observation leads to the second issue of general relevance—the question of whether in distributed networks specific functions can be associated with individual neurons and synapses. Some theoretical and general discussions have argued that it is unlikely that such association will, in general, be possible (Rumelhart et al. 1988; Selverston 1980), but experimental work in several systems has often ascribed specific functions to specific neurons and synapses (Dickinson et al. 1990; Hooper and Marder 1987; Hooper and Moulins 1989, 1990; Katz 1995; Katz et al. 1994; Kepler et al. 1990; Marder et al. 1992). The data presented here suggest that two types of follower neuron synaptic feedback—chemical inhibition, rectifying electrical coupling—applied out of phase onto an endogenous oscillator can endow the followers with specific functional roles (respectively, slowing and speeding) with respect to oscillator frequency. The ability of the follower neurons to continue to alter oscillator period in the same manner across a wide range of cycle periods suggests that these synaptic connectivity patterns onto the oscillator may be robustly appropriate for these functions. Modeling will be required to determine the range of oscillator and follower neuron cellular properties over which these connectivity patterns continue to regulate cycle period. If this range is broad, these synaptic connectivity patterns could be added to the rather small collection of patterns known to serve specific functional roles in generating network activity.

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