THE FUNCTIONAL ROLES OF THE LATERAL PYLORIC AND VENTRICULAR DILATOR NEURONS IN THE PYLORIC NETWORK OF THE LOBSTER, *Panulirus interruptus*.

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The functional roles of the Lateral Pyloric and Ventricular Dilator neurons in the pyloric network of the lobster, *Panulirus interruptus* (114pp.)

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Recent work has suggested that particular functions can be ascribed to specific neurons in distributed networks. To investigate this issue, we carried out hyperpolarization "removal" studies of the Lateral Pyloric (LP) and Ventricular Dilator (VD) neurons in the lobster (*Panulirus interruptus*) pyloric network. We found that these neurons regulate network cycle period; the LP neuron slows the network when present and the VD neuron speeds it. When sufficient current is injected into the pacemaker Anterior Burster (AB) neuron to force the network to cycle outside of its physiological range, the LP neuron disrupts normal patterning when the network is driven too slow, and the VD neuron disrupts when the network is driven too fast.

If larger AB current depolarizations are employed, the VD neuron role switches from network disruption to network pacemaker and entrains the other network neurons to follow its slow cycling. Hyperpolarization studies have shown that the other network neurons can cycle faster in the absence of the VD neuron and that the AB neuron is entrained to the slow VD neuron rhythm through the LP neuron's synapse. Thus, this anomalously slowed rhythm is VD neuron driven and mediated onto the network pacemaker by the LP neuron.

Finally, hyperpolarization studies of the LP or VD neuron have shown significant differences in pyloric neuron spiking and phasing activity compared with that of the intact network. However, there are confounding changes in network cycle period in the

absence of either neuron which must be accounted for to assess the direct effects of each neuron removal. When this compensation is applied, consistent effects of removal are not observed.

In the unmodulated "ground" state, these neurons seem to play little role in network activity. However, these neurons do play a significant role in pyloric network cycle period regulation. That is, the LP neuron slows the network when present and the VD neuron speeds the network when present. Additionally, the VD neuron can serve as network pacemaker and the LP neuron mediates this pacemaker effect onto the AB neuron. Thus, we have suggested two roles for these neurons in the absence of modulation.

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Chapter 1. Introduction

Background

A wide range of important behaviors are driven by rhythmic motor patterns. In such widely varying modalities as scratching, walking, swimming, breathing and digestion, networks of neurons called central pattern generators (CPGs) provide the spontaneous cyclic activity to sustain these behaviors without the need for external rhythmic input (Delcomyn, 1980). These oscillatory networks can be modulated (via sensory and higher center feedback) so as to modify the behavioral output over a range of speeds (period) and relative timings of the pattern elements (phase). This modulation allows the motor pattern to adapt to changing conditions, thus ensuring the generation of functionally relevant behaviors (Harris-Warrick and Marder, 1991; Hooper and Marder, 1987; Katz, 1995; Kristan, Jr. and Calabrese, 1976). Additionally, since CPGs are themselves simple examples of neural networks, they can be used to further understand the organization and function of more complex neural networks that may or may not have a rhythmic component. The study of CPGs has shown that there is a significant complexity at all major levels of organization within CPGs: 1. The networks are composed of highly-connected, non-hierarchical synaptic connectivity patterns (Calabrese and Peterson, 1983; Eisen and Marder, 1982; Kristan, Jr. et al., 1988; Roberston, 1986; Selverston et al., 1976). 2. The constituent neurons of the network possess active, non-linear cellular properties (Arshavsky et al., 1986, 1988, 1989; Bal et al., 1988; DiCaprio, 1997; Elson and Selverston, 1992; Merickel and Gray, 1980;

Ramirez and Pearson, 1991; Russell and Hartline, 1982; Wallen and Grillner, 1987). 3. Synapses can be graded, single, and/or multi-transmitter mediated, and membrane conductances can be voltage, calcium, and/or second-messenger dependent (Angstadt and Calabrese, 1989, 1991; Arbas and Calabrese, 1987; Eisen and Marder, 1982; Golowasch et al., 1992; Graubard, 1978; Graubard et al., 1980, 1983; Graubard and Hartline, 1991; Katz et al., 1994; Olsen and Calabrese, 1996; Raper, 1979; Tierney and Harris-Warrick, 1992). As a result, it is difficult to ascertain completely the functional role of an individual neuron or synapse within a CPG (Lockery and Sejnowski, 1993). However, relatively small CPGs can provide an effective preparation for the study of the neural basis of behavior with modern techniques of neurophysiology, due to their measurable biological function and motor pattern, known network components, complex properties (as described above), and tractability for mathematical modeling.

In this regard, the pyloric network of crustaceans provides an excellent model system for this study. The pyloric CPG is a subset of the larger stomatogastric (STG) network and controls the muscular contractions of the pylorus, a box-shaped structure that pumps and filters food particles passing between the gastric mill and hindgut (Maynard and Dando, 1974). The pyloric network possesses the following advantages: 1. The network is small in cell type and number (6 and 14, respectively) and all neurons are accessible and identifiable (Hartenstein, 1997). 2. All of the neuron types, the synaptic connections and many of the intrinsic properties are known (Abbott et al., 1991; Eisen and Marder, 1982; Golowasch and Marder, 1992; Harris-Warrick et al., 1995; Miller and Selverston, 1982a, 1982b; Russell and Hartline, 1978; Selverston et al., 1976; Tierney

and Harris-Warrick, 1992). 3. The network produces a stereotyped tri-phasic rhythm that can be experimentally manipulated over a wide range of physiological cycle periods (0.4 to 2.0 sec) (Hooper, 1997a, 1997b, 1998) and network configurations (Golowasch and Marder, 1992; Harris-Warrick et al., 1995; Harris-Warrick and Flamm, 1987; Hooper and Moulins, 1989, 1990; Johnson and Harris-Warrick, 1990; Kiehn and Harris-Warrick, 1992a, 1992b; Marder and Eisen, 1984; Simmers et al., 1995; Simon et al., 1992; Thompson and Calabrese, 1992; Zhang and Harris-Warrick, 1994).

Figure 1A shows a schematic diagram of the network's synapses (adapted from Hooper, 1998). Table 1 shows that out of 32 possible synaptic connections, 21 (66%) are present, illustrating the high level of connectivity within the network. These synaptic connections include fast onset and recovery glutamatergic, slow onset and recovery cholinergic, electrotonic (electrical coupling), and rectifying (electrotonic synapses that primarily flow in one direction) synapses. Each of the synapses in the network are subject to modulatory control – the efficacy of chemical synapses (Dickinson et al., 1990; Marder and Calabrese, 1996) and the direction of current flow in electrotonic synapses (Johnson et al., 1993a, 1993b, 1994) are both subject to the neuromodulators that interact with the network. All chemical synapses are inhibitory; therefore, neurons of the pyloric network burst primarily as a result of post-inhibitory rebound (PIR), an endogenous property of these neurons causing them to depolarize above rest after being inhibited. Additionally, these neurons possess the ability to express plateau potentials, prolonged regenerative depolarizations resulting from active membrane properties (Russell and Hartline, 1978).

Figure 1B shows the pyloric rhythm, which consists of a tri-phasic recurring pattern in which the network's pacemaker ensemble (Anterior Burster (AB) and 2 Pyloric Dilator (PD) neurons) burst first, followed by the Lateral Pyloric (LP) and Inferior Cardiac (IC) neurons, and finally the Ventricular Dilator (VD) and 6-8 Pyloric (PY) neurons. Important terms for motor pattern descriptions include burst duration and cycle period, the length of the rise above threshold and the time between bursts, respectively; interburst interval, which is the difference between cycle period and burst duration; rebound delay, which is the time from the end of inhibition to the beginning of the burst, and duty cycle (DC), which is the ratio of burst duration over cycle period. These terms are essential for descriptions of phasing within the motor pattern.

Cycle Period Changes

An important characteristic of CPGs is their ability to produce a functionally relevant rhythmic output over a wide cycle period range. Clearly, the behaviors under the command of CPGs can be expressed over a variety of speeds (e.g., slow vs. fast walking), and CPGs must be able to alter the duration of its pattern elements so as to maintain function over a wide cycle period range.

Phase maintenance is not a trivial issue. In the hypothetical network of Figure 2A, Neuron 1 is an endogenous oscillator with a 3 sec cycle period and 1 sec burst duration. Neurons 2 & 3 are plateauing neurons with PIR. Neuron 3 recovers from inhibition 1 sec slower than Neuron 2. As illustrated in Figure 2B (middle panel), Neuron 1 would start the cycle, followed immediately by Neuron 2. Neuron 3 would then inhibit Neuron 2, and Neuron 1 would then inhibit Neuron 3 at the beginning of the next cycle. Thus, each

neuron would fire for 1 sec (33% DC) in the cycle. However, if the endogenous period for Neuron 1 was instead 1.5 sec (left panel) while maintaining a constant burst duration (as in the isolated AB neuron (Abbott et al., 1991)), then Neuron 1 would still burst for 1 sec (67% DC), leaving Neuron 2 to only burst for 0.5 sec (33% DC), before Neuron 1 would burst again in the next cycle; in this case, Neuron 3 would not burst at all. If instead Neuron 1's endogenous period were increased to 6 sec (right panel) while maintaining burst duration, Neuron 1 would still burst for 1 sec (17% DC). Neuron 2 would also fire for 1 sec (17% DC) until Neuron 3 began to burst; however, Neuron 3 would burst for the remainder of the cycle, 4 sec (67% DC). Clearly, without phase-maintaining properties (endogenous to the network) that can properly shift the synaptic delays, rebound delays, or burst durations, the network is unable to maintain phase over a wide range of cycle periods. Proper phase maintenance is illustrated in Figure 2C. In this example network, Neuron 1 would have to increase its burst duration and Neuron 3 would have to increase its rebound delay to properly maintain phase as the cycle period of Neuron 1 is increased, and both neurons would have to shorten these parameters as cycle period is decreased to maintain phase.

Real neural networks can be divided into two groups based on response patterns to changes in cycle period. Motor patterns that interact with a solid substrate (e.g., walking) primarily change the burst duration of the motor pattern that elicits the power stroke (Grillner, 1981) when cycle period changes; thus, they do not maintain phase. For motor patterns with more equal force strokes that do not interact with a solid substrate (e.g., air stepping), phase for all elements is maintained over a wide range of cycle

periods (Cohen et al., 1992; DiCaprio, 1997; Eisen and Marder, 1984; Grillner et al., 1988; Johnston and Bekoff, 1992). Previous work (Eisen and Marder, 1984; Hooper, 1997a) has shown that the lobster, *Panulirus interruptus*, pyloric network approximately maintains phase as cycle period is altered by tonic current injection into the AB neuron. This ability to maintain phase is endogenous to the network and may arise from either cellular- or network-based mechanisms.

Cellular-based Mechanisms

A recent study (Hooper, 1998) has shown that isolated PY neurons can change their rebound delays in response to changes in the protocol of rhythmic hyperpolarizing current injections, with shorter delays for shorter current injection cycle periods and/or duty cycles. This delay shift helps the neuron to remain duty cycle constant over a four-fold cycle period range (0.5 to 2.0 sec) and happens relatively quickly (i.e., within 3-4 cycles). However, the delay shifts of the isolated neurons were half those observed when the network was intact.

Network-based Mechanisms

Thus, it seems likely that network mechanisms also play an important role in phase maintenance. However, it should be noted that there is no clear distinction between endogenous and network-based phase maintaining properties. Previous work (Hooper and Moulins, 1989, 1990) has shown that sensory input can induce changes in the *cellular* properties of the VD neuron that cause further "downstream" changes in IC neuron activity via *network-based* mechanisms. Thus, both mechanisms can play a role simultaneously within the network. Nonetheless, a significant body of work has been

compiled that demonstrates the further importance of network mechanisms and these results will be described below.

Electrical Coupling

Of the 21 synaptic connections, 6 (29%) are electrotonic in nature. Clearly, these electrical synapses must be important to network function. In fact, the weakly coupled electrotonic (20-30%) and rectifying (5-10%) synapses are the only source of excitatory potentials intrinsic to the network (Johnson et al., 1993a). Additionally, Eisen and Marder (1982) have shown that an inhibitory post-synaptic potential (IPSP) seen in the AB neuron results from an inhibitory post-synaptic current (IPSC) conducted from the PD neurons that was induced by the LP to PD neuron chemical synapse. Thus, neurons that are not directly connected can still be affected by an indirect connection via an electrical synapse. Further, Johnson et al. (1993a) have shown that the strength and direction of electrical coupling in the pyloric network is under modulatory control, providing another means to functionally "rewire" this network and fix the neuron phases within the network.

Other work has shown the importance of electrical coupling in maintaining phase and determining cycle period. 1) Tonic current injection used to control the cycle period of an isolated AB neuron leads to a constant burst duration and varying interburst interval (Abbott et al., 1990, 1991). Under identical current injection conditions, an isolated PD neuron bursts irregularly with a significantly lengthened cycle period compared with the coupled condition (Bal et al., 1988). In contrast, the isolated electrically coupled PD-AB neuron ensemble maintains an approximately constant duty cycle over a wide range of

cycle period. Modeling studies suggest that the ensemble maintains phase because current flow from the PD neuron to the AB neuron during their burst reduces PD neuron burst duration and lengthens AB neuron burst duration as cycle period increases. 2) To further illustrate the importance of network properties to rhythm generation, Hooper and Marder (1987) have shown that (in the presence of proctolin) the isolated AB neuron fires at 2 Hz, while the intact network only fires at 1 Hz. Proctolin induces increased activity in the isolated LP neuron. A priori, one possible explanation for this slowing of the intact proctolin-bathed network is the increased activity from the LP neuron. However, deletion of the LP neuron showed that the slowing of the AB neuron arises from its electrical coupling with the PD and VD neurons, not the proctolin-induced increased LP neuron activity. Thus, electrical coupling can play an important role in determining cycle period. Models by Kepler et al. (1990) further explain the effect of electrical coupling on cycle period as a function of the intrinsic currents of the driving pacemaker neuron. The amount of time that the neuron experiences a net inward versus a net outward current is important in determining the effect on cycle period of increased coupling to a passive follower neuron. Oscillators that spend a majority of the cycle with a net inward current will slow down when coupled to a passive neuron that is more hyperpolarized and speed up with a more depolarized neuron. A predominantly outward current neuron has the opposite effects.

Finally, Bal et al. (1994) have shown that under certain reduced conditions, the electrically coupled AB, PD, and VD neuron ensemble fire a distinct rhythm from each of the neurons alone or the AB-PD neuron pair. This new pacemaker ensemble is sensitive

to both the modulatory inputs it receives and the membrane potential of each of the neurons, suggesting that the AB neuron is not solely responsible for maintaining the network oscillations nor controlling cycle period and pattern phasing.

Entrainment

Previous work has argued that a neuron can be considered part of a CPG if altering that neuron's activity disrupts or resets the ongoing pattern (Friesen and Stent, 1978). Thus, it follows that the functional role of a neuron in a network can be deduced (in part) by the response of the CPG to experimental manipulations of that neuron, specifically through rhythmic current injections into the neuron and removal of the neuron from the network by hyperpolarization or photoinactivation (discussed in the following section). A change in network activity in response to a change in an individual neuron's activity is defined as "access" for that neuron to the network pacemaker. Specifically, rhythmic current injections can be used to determine if a neuron has the access to entrain the network.

In the case of rhythmic current injections, there are two separate types of entrainment. First, there is entrainment of the neuron that is being injected to the period of the stimulating current. The second type is the entrainment of the network (as measured by the pacemaker ensemble) to the entraining (stimulated) neuron. Ayers and Selverston (1977) found that the pacemaker (PD) neurons could be entrained by both rhythmic excitatory post-synaptic potentials (EPSP; via IVN stimulation) or inhibitory post-synaptic potentials (IPSP; via LP neuron depolarization). To be capable of entrainment, the follower must have a periodically varying sensitivity to its stimulus,

which the pyloric network does. The entrainment seen in the pyloric network occurred at a different phase in the excitatory and inhibitory conditions. In another study (Ayers and Selverston, 1979), rhythmic current injections were performed over a range of cycle periods. In these experiments, phase locking was more pronounced near the endogenous period of the pyloric network. Additionally, IPSPs were better able to entrain with slightly slower rhythms than the endogenous period, while EPSPs produced stronger entrainment at faster cycle periods. Thus, synaptic input to the PD neurons can successfully entrain the pyloric network.

Neuron Removal

The final technique for ascertaining neuronal function in a network is to eliminate its ability to influence its post-synaptic neurons by removing it from the network. In the pyloric network, there are three techniques for the effective removal of a neuron. First, bath application of picrotoxin (PTX) will block all neurons that use glutamatergic synapses (Johnson and Hooper, 1992), leaving only the VD and PD neurons' cholinergic chemical synapses and the network's electrical synapses active. Second, injection of hyperpolarizing current to bring an identified neuron well below threshold provides a reversible means of removal. Third, an identified neuron can be injected with a dye (e.g., Lucifer Yellow) and irradiated with an intense blue light to photoinactivate the neuron (Miller and Selverston, 1979). This latter technique has been applied in a number of experiments and their results are summarized below. However, it has limited applicability in isolating neurons that are electrically coupled due to the permeability of the dye through gap junctions and the possibility of damage to its electrically coupled partners.

First, Selverston and Miller (1980) have shown that, when the anterior inputs are left intact, pattern generation continues when the pacemaker ensemble (AB and the two PD neurons), or any other neuron or pair of neurons are removed. However, when the pacemaker ensemble *and* the VD neuron are removed, pattern generation ceases. LP neuron inactivation has only modest effects on the pattern, and PY or IC neuron inactivation has no significant effect on the pattern. Some argue that the pyloric synaptic connectivity is sufficient for pattern generation, but the pacemaker ensemble serves to "tune" the activity to a behaviorally relevant pattern. Finally, they argue that despite inactivation of "integral" component neurons, the motor pattern continued suggesting the network has a built-in robustness and redundancy. However, cycle period was not controlled and changes in phasing were not analyzed.

In another study using photoinactivation (Miller and Selverston, 1982b), the network was reduced to the minimal subset of neurons that through network interactions could generate rhythmic activity. The minimum was any two neurons which were synaptically coupled through mutually inhibitory synapses; these neurons fired alternately in a manner similar to a classical "half-center" oscillator (i.e., mutually inhibitory) pair. Motor pattern genesis was explained as the result of oscillatory membrane properties of individual neurons and the multiple reciprocally inhibitory ("half-center") interactions within the network. The AB neuron controls overall pattern cycle period via its endogenous bursting characteristics and its strong synapses on all of the pyloric neurons except the PD neurons. Phase relationships are derived from synaptic connectivity, relative synaptic strengths, PIR, rebound delay, and the kinetics of the

endogenous burst generation mechanisms. These results again suggest that both cellular and network mechanisms play a role in phase maintenance within the pyloric network.

In the intact pyloric network, the PD and PY neurons burst alternately. Eisen and Marder (1984) studied the mechanisms that underlie the phase relations between these neurons and found that the PY neurons remain phase constant relative to the PD neurons over a wide range of cycle frequencies. Deletion of the PD neurons or reduction of their activity by application of dopamine leaves only the effects of the AB-evoked IPSPs on the PY neurons, leading to a phase advance and (in some cases) an increased burst duration for the PY neurons at all cycle periods. Excitation of the PD neurons (via IVN stimulation) leads to the opposite effect. Based on these results, the authors suggest that pyloric cycle period and phasing within the pyloric cycle can be regulated independently. However, to properly maintain phase, rebound delay and burst duration must change to compensate for cycle period changes (see Figure 2). In this case, the PD neurons may change their bursting pattern (in response to AB neuron cycle period changes), so as to keep the PY neurons phase-constant.

Specific Aims

We have investigated network-based mechanisms in phase maintenance through the use of several experimental techniques. Removal of constituent neurons through hyperpolarization (while network cycle period was varied through tonic AB neuron current injection) was used to determine their role in cycle period regulation, phase maintenance and pacemaker switching. More recently, rhythmic current pulses were injected into neurons, other than the pacemaker ensemble, to investigate entrainment of

the intact network and to determine these neuron's synaptic "access" onto the pacemaker and the rest of the network, and computer modeling techniques were employed using knowledge of individual neuronal properties to attempt to further understand the role of these neurons in entraining network cycle period.

Chapter 2. Relating Network Structure to Network Output: "Follower" Neurons Can Govern Pacemaker Ensemble Cycle Period

Abstract

Many neural networks that have been described on the cellular level are driven by endogenous oscillator neurons or by rhythmic neuron assemblies (half-center oscillators). These pacemaker units often receive strong feedback from "follower" neurons in the rest of the network. This distributed arrangement between pacemaker and follower suggest that, although rhythmicity arises with the pacemaker, input from the rest of the network may play a role in pacemaker activity.

This issue was examined in a well-investigated distributed neural network, the rhythmic pyloric network of the lobster, *Panulirus interruptus*. A pacemaker ensemble of three electrically coupled neurons, the endogenous oscillator Anterior Burster (AB) neuron and two Pyloric Dilator (PD) neurons, drives the network. This ensemble inhibits two other pyloric neurons, the Lateral Pyloric (LP) and Ventricular Dilator (VD) neurons. Both the LP and VD neurons feed back onto the ensemble, the LP neuron by inhibiting the ensemble and the VD neuron via electrical coupling to it. The effect of these follower neurons on pacemaker ensemble period was examined by 1) altering pacemaker period by current injection into the AB neuron, and then 2) hyperpolarizing the LP or VD neurons to functionally remove each from the network.

Within a certain range of pacemaker periods, LP neuron removal speeds the network, and VD neuron removal slows it. In this period range, these neurons thus play complementary roles as pacemaker frequency governors. Outside this range, network

activity is disrupted because the LP neuron cannot follow slow cycle periods, and the VD neuron cannot follow fast ones. These two neurons thus also limit normal pyloric activity to a certain period range, again in complementary ways. These data show that follower neurons in pacemaker networks can play central roles in pacemaker cycle period control, and suggest that in at least some cases specific functional roles in generating network activity can be associated with 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 (fast vs. slow breathing) and pattern phasing and neuron spiking activity (breathing vs. gasping) (Arbas and Calabrese, 1984; Calabrese et al., 1995; Cohen et al., 1988; Harris-Warrick and Marder, 1991; Lieske et al., 2000; Nadim and Calabrese, 1997; Ramirez, 1998; Ramirez and Richter, 1996; 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 as such, modifying the cellular or synaptic properties of any of several network neurons generally alters network cycle period (DiCaprio and Fourtner, 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 pacemaker ensemble, and these electrically coupled neurons can also affect pacemaker period (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 which generally fire out of phase with it. These neurons could alter pacemaker period, and in one case it has been shown that increasing the activity of an identified follower neuron increases the pacemaker period (Massabuau and Meyrand, 1996). However, except for this study, this process is relatively little investigated.

The lobster (*Panulirus interruptus*) pyloric network is normally driven by a pacemaker ensemble, and this ensemble receives feedback from two pyloric follower neurons. Tonic current injection into the endogenous oscillator alters ensemble period, and hyperpolarizing individual follower neurons below their transmitter release threshold removes follower neuron feedback to the pacemaker ensemble. Therefore, the role of the follower neurons in cycle period control was investigated by 1) altering pacemaker period with current injection, and 2) at each period, assessing the effect of removing each follower neuron. Follower neuron feedback alters pacemaker activity in two ways, and in each case the neurons have complementary effects. First, within a certain range of pacemaker periods, feedback from one follower neuron speeds the pacemaker while feedback from the other slows it. Second, 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; one follower cannot follow short periods whereas the other cannot follow long ones. Thus, within a certain pacemaker period range, the follower neurons serve as complementary frequency governors; outside this range they have complementary effects in that one neuron disrupts slow pyloric rhythms whereas the other disrupts fast ones.

Materials and Methods

Pacific spiny lobsters (*Panulirus 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): NaCl 479, KCl 12.8, $CaCl_2$ 13.7, Na_2SO_4 3.9, $MgSO_4$ 10.0, glucose 10.9, tris base 11.1, maleic acid 5.1, pH 7.5–7.6. All salts were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Stomatogastric neuromuscular systems were dissected and prepared for extracellular nerve recording and intracellular neuron recording using standard techniques (Selverston et al., 1976). 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 0.55M K_2SO_4, 0.02M KCl, resistance 10 to 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

were performed (univariate general linear model/analysis of covariance) 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 tonic current injection into the 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 presumably greatly reduced graded synaptic release (see Discussion). In all cases the same electrode was used for voltage recording and current injection. Hyperpolarized neurons were monitored for escape by examination of extracellular recordings, presence of inhibitory post-synaptic potentials in the neuron's synaptic partners, 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 neuron activity. Period was averaged over 6-10 pyloric cycles; less than ten cycles were used when the removed neuron escaped from its hyperpolarization, or interference from other stomatogastric nervous system networks (gastric mill, cardiac sac), perturbed pyloric activity (Bartos et al., 1999; Bartos and Nusbaum, 1997; Marder et al., 1998; Mulloney, 1977; Nadim et al., 1998; Thuma and Hooper, 1999). In all experiments the stomatogastric nerve, which carries input from the rest of the stomatogastric nervous system to the pyloric network, was intact. The data presented here are from 10 experiments.

Results

The pyloric network is a small, well-characterized network of 14 neurons consisting of 6 neuronal types. All synapses within the network are known and are either inhibitory chemical synapses or electrical coupling. Figure 1A shows the pyloric circuit diagram; circles indicate inhibitory chemical synapses and resistors and diodes indicate electrical coupling. The Anterior Burster (AB) neuron is an endogenous oscillator (pacemaker) neuron. The Pyloric Dilator (PD) neurons are electrically coupled to the AB neuron, and these three neurons form the pyloric AB/PD pacemaker ensemble. The network has four follower neuron types: Lateral Pyloric (LP), Ventricular Dilator (VD), Inferior Cardiac (IC), and Pyloric (PY). The LP and VD neurons inhibit each other, the LP neuron inhibits the PD neuron, and the VD neuron makes a rectifying electrical synapse onto both the PD and AB neurons (Johnson et al., 1993a). The pyloric output pattern (Fig. 1B) is a triphasic rhythmic pattern in which first the AB/PD pacemaker ensemble fires, then the LP and IC neurons fire, and then the VD and PY neurons fire, after which the pattern repeats; this pattern underlies food filtering in the pyloric chamber of the lobster stomach.

In the work reported here the effect of the LP and VD neurons on pacemaker ensemble cycle period was investigated. To this end, varying levels of tonic current into the AB neuron were injected to alter network cycle period, and then the LP and VD neurons were alternately hyperpolarized to functionally remove them from the network. The LP and VD neurons were chosen because 1) they are the only follower neurons that feed back onto the pacemaker ensemble and 2) 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) because hyperpolarization is reversible, and thus allows the effects of removing both follower neurons to be tested in each preparation.

The LP and VD neurons are cycle period governors

Figure 3 shows the effect of LP neuron removal on pacemaker cycle period. In each panel the top trace is an intracellular recording of the LP neuron and the second trace is an extracellular recording of PD neuron activity. 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 (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 sec). Similar results were seen in 4 of 4 experiments.

Figure 4 shows the effect of VD neuron removal on pacemaker cycle period in the same preparation shown in Fig. 3. The figure layout and AB neuron current injection levels are the same as in Fig. 3. VD neuron removal consistently increased 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 sec). Similar results were seen in 5 of 7 experiments.

In Figs. 3 and 4, only AB neuron hyperpolarizing current injection conditions are shown. In almost all experiments the control cycle periods of the preparations were near the minimum that the pyloric network produces (~0.5 sec). Because of the fast endogenous periods, we were therefore usually unable to inject significant depolarizing

current into the AB neuron without the VD neuron disrupting pyloric activity (see Pattern Disruption below). Figure 5 shows data (from one of two LP neuron and one of three VD neuron removal experiments) in which we were able to depolarize the AB neuron without pattern disruption; these experiments were possible due to the large variation in AB neuron sensitivity to injected current. 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 sec. In each panel the first two traces show follower and PD neuron activity and the third trace 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 sec; Fig. 5A), whereas VD neuron removal had no effect (0.67 to 0.67 sec; Fig. 5B).

Figure 6 summarizes the effects of LP or VD neuron removal on average network cycle period as a function of the intact network cycle period for the experiment shown in Figs. 3 and 4. LP neuron removal reduces cycle period 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. In order to properly plot the three cycle period conditions against the intact cycle period, criteria for matching each y-value with a given x-value was needed. To this end, the intact network x-axis values were isolated by AB neuron current injection condition and within each condition the order was reversed. These reversed points were matched up with sorted increasing values from each of the three neuron removal conditions. This procedure maximized the scatter around the best-fit line and gave us a worst-case

scenario for line overlap. Despite using a procedure which would maximize any chances for overlap, there was no significant overlap of the 95% confidence interval lines.

These data show that when the network was slowed by current injection into the AB neuron, VD neuron feedback speeded the network and LP neuron feedback slowed the network. However, the effects of LP and VD neuron removal were not precisely complimentary. LP neuron removal decreased average cycle period by approximately 20% at all AB neuron current injection levels. Alternatively, the increase in cycle period induced by VD neuron removal increased with AB neuron hyperpolarization from 50% at 0 nA to 100% at –17 nA injection into the AB neuron (data not shown). Similar results were observed in all experiments (4 of 4, LP neuron; 5 of 7, VD neuron) in which follower neuron hyperpolarization altered pacemaker cycle period.

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 synapse. Figure 7 shows that this explanation is unlikely to be the case. In each panel the first trace is an intracellular recording of the VD neuron, the second is an intracellular recording of the AB neuron, and the third 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 does VD neuron hyperpolarization hyperpolarize the AB neuron; if VD neuron hyperpolarization has any effect, it is to slightly depolarize the AB neuron. These data suggest that the effects of VD neuron hyperpolarization are 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 the 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 has been injected into the AB neuron, and the network is cycling at the slowest edge (~2 sec period) of the physiological range. In A, the top three traces are intracellular recordings of the LP, VD, and PY neurons and the fourth trace is an extracellular recording of PD neuron activity. Panel B shows the activity of these neurons 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 (gray 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 6 of 6 experiments.

The VD neuron disrupted pyloric cycling when the AB neuron was extremely depolarized (very fast cycle periods, Fig. 9). In both panels +20 nA has been injected into the AB neuron, and the network is cycling at the fastest end (~0.5 sec period) of the physiological range (note the difference in time scale in this figure and Fig. 8). In A, the top three traces are intracellular recordings of the VD, LP, and PY neurons and the fourth trace is an extracellular recording of PD neuron activity. Panel 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 per two to four AB/PD neuron bursts, and thus disrupted the pattern by increasing PY and LP neuron interburst intervals and decreasing PD neuron burst duration. VD neuron hyperpolarization below threshold restored regular pyloric cycling (B). Similar results were seen in 4 of 4 experiments.

Discussion

Figure 10 is a schematic diagram summarizing the effects of the LP and VD neurons on pyloric network activity. The first line of this figure shows the effects of the LP neuron on the pacemaker, the second the effects of the VD neuron, and the third 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. Each neuron can disrupt 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; the VD neuron slow to moderately fast ones. In the cycle period range in which neither neuron disrupts the pattern, each neuron limits (governs) cycle period. Although the LP neuron can follow fast cycle periods 1:1, its presence slows the network, and it appears to do so by a 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 cycled period decreases, and may be lost entirely when the AB neuron is depolarized.

Experimental considerations

Two experimental issues affect interpretation of these data. First, pyloric neurons release transmitter as a graded function of membrane potential (non-spiking 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 were well below rest (to at least –100 mV), examination of the membrane voltages of the LP and VD neuron postsynaptic partners 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 these data would only underestimate the effects of LP and VD neuron removal on pacemaker period.

Second, in these preparations the pyloric network was almost always rapidly cycling at rest. Thus, although we were able to test the effects of LP and VD neuron removal across the entire physiological cycle period range (0.5 to 2 sec), in most cases we did so by slowing rapidly cycling preparations. It is possible that in slowly cycling preparations, in which we would depolarize the AB neuron to span the physiological period range, the frequency governor effects of the LP and VD neurons reported here would not occur. However, it is important to note that in the 4 experiments in which we were able to

depolarize the AB neuron without disrupting the pattern, data similar to those shown here were observed.

Comparison to earlier work

In a study of proctolinergic modulation of the pyloric network Hooper and Marder (Hooper and Marder, 1987) found that removal of the LP neuron by photoinactivation (Miller and Selverston, 1979) had no effect on pyloric cycle period. However, this work was performed with activity in the stomatogastric nerve blocked by sucrose, and consequently 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. Massabuau and Meyrand (1996) showed in the lobster, *Homarus gammarus*, that increased LP neuron activity increased pacemaker cycle period. These data are consistent with ours, but since the authors did not inject current into the AB neuron, they correspond only to our 0 nA AB neuron current injection data points.

Mechanisms of cycle period governance

In distributed networks changes in network activity can arise either via direct or network mediated mechanisms (Hooper and Marder, 1987; Hooper and Moulins, 1990). 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 (see Chapter 3). LP neuron removal has no effect on any aspect of VD, PY, or IC phasing or spiking activity, and VD neuron removal has no effect on any aspect of LP, PY, or IC phasing or spiking activity. Instead of network mediated mechanisms, direct inputs of the LP and VD neurons onto the pacemaker ensemble most likely underlie the cycle period effects of LP and VD neuron removal.

LP neuron presence increases cycle period, and the LP neuron inhibits the PD neurons (Fig. 1). 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 the AB neuron interburst interval. Comparing the intact network to the LP neuron hyperpolarized traces in Figs. 3 and 5 shows precisely this effect; PD neuron interburst interval increases whereas PD neuron burst duration (which mirrors AB neuron burst duration) remains constant.

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 inhibition they receive induces postinhibitory rebound (Selverston et al., 1976) which triggers 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. 1, 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 injects depolarizing current into the pacemaker ensemble and hence advances its firing.

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 two experiments in which VD neuron hyperpolarization did not alter pacemaker period (data not shown). In these experiments (and only these), 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 ground (unmodulated) state 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 neuromodulator release. This release can alter the voltage dependence, kinetics, and expression of AB and other pyloric neuron membrane conductances and network synaptic strengths. AB neuron current injection presumably changes cycle period without inducing these effects. It would therefore be incorrect to assume that, because the LP and VD neurons serve as cycle period governors when the network is slowed by current injection into the AB neuron,

these neurons continue to do so when the network is slowed by neuromodulator application.

The data obtained with zero current injection into the AB neuron, however, are directly relevant to pyloric network function. Our data show that, in preparations with rapid rest periods, decreases in VD neuron activity are unlikely to alter pyloric cycle period. However, in agreement with the results of Massabuau and Meyrand (1996) in *Homarus*, our data show that pyloric cycle period is decreased by decreased LP neuron activity. 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 a single period. These data provide a graded, continuous baseline of the effects of the LP and VD neurons in the unmodulated pyloric network. Similar experiments in the presence of neuromodulators in which pyloric cycle period is also altered by current injection into the AB neuron can be easily performed. These descriptions of the varied effects of LP and VD neuron presence across a wide range of pyloric cycle periods will provide a more complete understanding of their role in shaping the dynamics of this system.

Relevance to small distributed systems in general

The data presented here suggest that two types of follower neuron synaptic feedback (chemical inhibition, rectifying electrical coupling) onto an endogenous oscillator can

endow the followers with specific functional roles with respect to oscillator period (slowing and speeding, respectively). These data are relevant to the question of whether specific functions can be associated with individual neurons and synapses in distributed networks. 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, 1990; Katz et al., 1994; Kepler et al., 1990; Marder et al., 1992). Our demonstration that the LP and VD neurons act as complementary cycle period governors in the pyloric network adds to this latter body of work, and provides additional hope that understanding the relationship between structure and function, at least in small neural networks, may be an achievable goal.

Chapter 3. Relating Network Structure to Network Output: Synapses That Appear, in Network "Ground" State, to Have No Function

Abstract

Many neural networks that have been described on the cellular level have highly distributed synaptic connectivity patterns in which many of the network's neurons make synapses onto, and receive synapses from, a large percentage of the other neurons in the network. In such networks it is difficult to discern from the synaptic connectivity pattern what role individual neurons play in generating network activity. It has been suggested that such networks can only be understood as a collective, and that in general assigning specific functional roles to individual neurons may be impossible (Selverston, 1980).

This issue was examined using the Lateral Pyloric (LP) and Ventricular Dilator (VD) neurons of the well-investigated rhythmic pyloric network of the lobster, *Panulirus interruptus*. The pyloric network is highly distributed, and the LP and VD neurons synapse onto, and receive synaptic input from, the large majority of pyloric neurons, including the pacemaker ensemble. The effects of the removal of LP and VD neuron activity on the phasing and spiking activity of the pyloric network are described here.

A confounding factor in this work is that the absence of LP and VD neuron activity alters pyloric cycle period (see Chapter 2). To control for these period changes, we 1) altered pacemaker period by current injection into a pyloric pacemaker neuron, 2) hyperpolarized the LP or VD neurons to functionally remove each from the network, and 3) plotted a wide variety of measures of the activity of all the pyloric neurons against period with and without the LP or VD neuron. We were thus able to determine the effects

of LP and VD neuron functional removal on pyloric network phasing and spiking activity independent of the period-altering effects of LP and VD neuron removal.

We report here that, in the unmodulated ground condition, removal of LP and VD neuron input to the network does not significantly alter phasing or spiking activity of any pyloric network neuron. Previous work has shown that, in the presence of a modulator that increases LP neuron activity, LP neuron removal does alter phasing and spiking activity of other network neurons compared with the intact case (Hooper and Marder, 1987). Taken together, these data and those reported here thus suggest that, in complex distributed systems such as the pyloric network, some synaptic connections may contribute to network activity in only certain network states.

Introduction

Central pattern generator (CPG) networks underlie rhythmic motor pattern production (Delcomyn, 1980; Marder and Calabrese, 1996). Many central pattern generator networks described on the cellular level have highly complicated, distributed synaptic connectivity patterns in which each network neuron both makes and receives synaptic contact from a large percentage of the rest of the network's neurons. Many such networks also produce complicated neural output patterns in which the various network neurons fire in fixed phase relationships with stereotyped spiking activities (hence their ability to produce multi-phasic rhythmic motor outputs such as walking). The outputs of these networks can show large variations in cycle period (fast vs. slow breathing). Furthermore, many such networks can also, in response to modulatory or sensory input, produce multiple output patterns in which the phasing and spiking activities of the network's neurons change to

produce different behaviors (e.g., running vs. walking; breathing vs. gasping) (Arbas and Calabrese, 1984; Calabrese et al., 1995; Cohen et al., 1988; Harris-Warrick and Marder, 1991; Lieske et al., 2000; Nadim and Calabrese, 1997; Ramirez, 1998; Tegner et al., 1998). *A priori*, it is possible that these different activities arise because, in different network conditions, specific network synapses play different functional roles in helping determine network activity. An extreme example of changes in synaptic function would be that some network synapses are functionally important only in certain network conditions.

We show here that, in the unmodulated ground state of the lobster (*Panulirus interruptus*) pyloric network, certain synaptic connections appear to play no role in determining the spiking and phasing activity of their postsynaptic neurons. The pyloric network is a highly interconnected network whose six neuron types make a total of 20 intranetwork synaptic connections; a completely interconnected 6 neuron network would have 30 connections. The network also receives a variety of modulatory and sensory inputs that alter network condition and output. As such, the pyloric network is highly suitable for investigating the functional roles of different synaptic connections in different network conditions.

This issue was examined using two neurons, the Lateral Pyloric (LP) and Ventricular Dilator (VD) neurons, in the network ground (normal saline) condition. There is only one LP and one VD neuron in the network, and thus their input to the rest of the network can be assessed by individually hyperpolarizing them. Interpretation of the results so obtained, however, is complicated because removal of either neuron alters pyloric

network cycle period (see Chapter 2), and thus the effects of removal could arise as a consequence of network cycle period changes. To control for this factor, pyloric cycle period was altered by injecting varying levels of current into the network's pacemaker Anterior Burster (AB) neuron, and sequentially the LP and VD neuron were hyperpolarized. This procedure allowed us to observe network phasing and spiking activity with and without the LP or VD neuron across a wide cycle period range, and thus measure the changes induced by their absence independent of cycle period. This procedure also allowed us to explore the effects of LP and VD neuron removal throughout the entire state space of the network's ground condition (as opposed to the single "snapshot" that would be provided at only one level of AB neuron activity), and thus to fully characterize the role these neurons play in network activity in this condition.

The phasing and spiking activities of all pyloric neurons were measured including beginning and ending delay/phase relative to the pacemaker, burst duration/duty cycle, burst spike number, intraburst spike frequency, and overall spike frequency (burst spike number/cycle period). We report here that the removal of the LP or VD neuron affected none of these parameters in a statistically significant fashion for any pyloric neuron. These data thus suggest that, at least with respect to decreases in LP and VD neuron activity, the LP and VD neuron synapses serve no functional role in pyloric network phasing or spiking activity in the network's ground (i.e., normal saline and no added modulators) condition.

Materials and Methods

Pacific spiny lobsters (*Panulirus 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): NaCl 479, KCl 12.8, $CaCl_2$ 13.7, Na_2SO_4 3.9, $MgSO_4$ 10.0, glucose 10.9, tris base 11.1, maleic acid 5.1, pH 7.5–7.6. All salts were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Stomatogastric neuromuscular systems were dissected and prepared for extracellular nerve recording and intracellular neuron recording using standard techniques (Selverston et al., 1976). 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 0.55M K_2SO_4, 0.02M KCl, resistance 10 to 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 were performed (multivariate general linear model/analysis of covariance with Dunn-Šidák α-level compensation for multiple comparison, nominal α-level 0.05) 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). The data presented here are from 10 experiments.

Cycle period was altered by tonic current injection into the 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 greatly reduced or blocked graded synaptic release (see Discussion). In all cases the same electrode was used for voltage recording and current injection. Hyperpolarized neurons were monitored for escape by examination of extracellular recordings, presence of inhibitory post-synaptic potentials in the neuron's synaptic partners, and when possible, observation of the neuron's membrane potential via a bridge-balanced electrode. In cases in which different cycle period ranges could be obtained in control or without the LP or VD neurons, only data from overlapping cycle period ranges were used in statistical comparisons.

In each experimental condition (3 cases for each level of AB neuron current injection—with the LP and VD neurons present, with the LP neuron absent, with the VD neuron absent) pyloric activity was measured over 6-10 pyloric cycles; less than ten cycles were used when escapes from hyperpolarization, or interference from other stomatogastric nervous system networks (gastric mill, cardiac sac), perturbed pyloric activity (Bartos et al., 1999; Bartos and Nusbaum, 1997; Marder et al., 1998; Mulloney, 1977; Nadim et al., 1998; Thuma and Hooper, 1999). The pyloric period was calculated from intracellular or extracellular recordings of PD neuron activity. Phase and delay were measured with the beginning of the PD neuron burst defined as cycle beginning; as such the PD neurons always had a beginning delay and phase of 0. Burst duration and delay were plotted vs. cycle period; duty cycle and phase were plotted vs. cycle frequency

(Hooper, 1997a). All other variables were plotted vs. both cycle period and cycle frequency. In all experiments the stomatogastric nerve, which carries input from the rest of the stomatogastric nervous system to the pyloric network, was intact. The data reported here are drawn from 3 to 6 experiments for each parameter and neuron.

Results

The pyloric network is a small, well-characterized network of 14 neurons consisting of 6 neuronal types. All synapses within the network are known and consist of either inhibitory chemical synapses or electrical coupling. Figure 1A shows the pyloric circuit diagram; circles indicate inhibitory chemical synapses and resistors and diodes indicate electrical coupling. The Anterior Burster (AB) neuron is an endogenous oscillator (pacemaker) neuron. The two Pyloric Dilator (PD) neurons are electrically coupled to the AB neuron, and these three neurons form the pyloric AB/PD pacemaker ensemble. The network has four "follower" neuron types: Lateral Pyloric (LP), Ventricular Dilator (VD), Inferior Cardiac (IC), and Pyloric (PY). The LP neuron inhibits the PD, VD, and PY neurons and has a rectifying electrical synapse to the PY neurons. The VD neuron makes a rectifying electrical synapse onto the PD and AB neurons, and inhibits the LP, IC, and PY neurons (Johnson et al., 1993a; Selverston et al., 1976). As such, the LP and VD neurons have synaptic connections appropriate for altering the phase and firing activity of most or all of the other neurons of the pyloric network. The pyloric output pattern (Fig. 1B) is a rhythmic (0.5-2.0 sec cycle period), triphasic pattern in which first the AB/PD pacemaker ensemble fires, then the LP and IC neurons fire, and then the VD and PY neurons fire, after which the pattern repeats.

In the work reported here we investigated the effect of the LP and VD neurons on pyloric phasing and spiking activity. To this end, we alternately hyperpolarized the LP and VD neurons to functionally remove them from the network. VD and LP neuron removal from the network was carried out by hyperpolarization instead of photoinactivation (Miller and Selverston, 1979) because hyperpolarization is reversible, and thus allows the effects of removing both follower neurons to be tested in each preparation.

A difficulty in this work was that LP or VD neuron removal also changes pyloric cycle period (see Chapter 2), and changing cycle period alters delay and spiking activity of network neurons (Hooper, 1997a). Figure 11 shows an example of this phenomenon for the PY neuron when pyloric cycle period is changed by current injection into the AB neuron. The first trace in the top panel is an intracellular recording from a PY neuron and the second trace is an extracellular recording from a PD neuron when no current is injected into the AB neuron. The double-headed arrow shows the PY neuron delay to firing relative to the beginning of the PD neuron burst. The bottom panel shows the activity of the same neurons when hyperpolarizing current was injected into the AB neuron to slow the network. As cycle period slows, PY neuron burst beginning and ending delay (after PD neuron burst beginning), burst duration, and burst spike number increase.

A variety of pyloric neuron phasing and spiking activity measures were computed (Fig. 12). Burst beginning and ending delay were measured from the beginning of the PD neuron burst, burst beginning and ending phases were determined by dividing these

delays by PD neuron cycle period. Burst duration is the duration between the first and last spike of the burst, duty cycle is this duration divided by cycle period, burst spike frequency is burst spike number minus 1 divided by burst duration ($(spike\#-1)/burst\ duration$), and overall spike frequency is burst spike number divided by cycle period. Since the PD neurons were used to define cycle period, their burst beginning delay and phase were always zero, and their ending delay and phase equal their burst duration and duty cycle. Pyloric neuron delay and burst duration vary linearly with cycle period, and phase and duty cycle vary linearly with cycle frequency (Hooper, 1997a). Delay and burst duration were therefore plotted against cycle period and phase and duty cycle against cycle frequency. The other parameters were plotted against both cycle period and frequency.

Figure 13 shows a typical experiment in which the VD neuron was removed by hyperpolarization. The top two traces are intracellular recordings of a VD and an LP neuron; the third trace is an extracellular recording of PD neuron activity. At the arrow the VD neuron was hyperpolarized well below rest. After a brief transient effect, the network assumed a new pattern in which both cycle period and LP neuron burst duration were increased. As such, it is unclear if the increase in LP neuron activity was a direct effect of VD neuron removal or an indirect effect of the change in cycle period. The right panel shows the activity of the same neurons with the VD neuron active and pyloric cycle period being made (by AB neuron current injection) to match the cycle period observed when the VD neuron was removed (right panel). LP neuron activity under these conditions was similar to that when the VD neuron was removed, suggesting that the

changes in LP neuron activity in the right panel were an indirect effect of the cycle period changes induced by VD neuron removal.

AB neuron current injection was used to vary cycle period, and at each injection level, the LP and VD neurons were alternately hyperpolarized. Each of the previously mentioned phasing and spiking parameters were measured and plotted against either cycle period or cycle frequency, as appropriate. Typical results for LP neuron beginning delay (A) and burst duration (B) in one VD neuron removal experiment are shown in Figure 14; circles are data from intact network conditions while squares are with the VD neuron hyperpolarized. A best fit line (solid) and 95% confidence interval lines (dashed) were plotted for each data set. In this experiment the confidence interval lines do not overlap over most of the cycle period range, suggesting that these data differ in the intact and VD neuron hyperpolarized conditions.

Comparison across experiments shows, however, that, although LP or VD removal often induced significant changes in pyloric activity in individual experiments, these changes were not consistent across experiments. Figure 15A shows best fit lines for LP neuron beginning delay versus cycle period for 5 experiments. Each solid (intact) and dashed (VD neuron removed) line of similar horizontal length represents the results from one experiment. Lines a and a' are the same data shown in Fig. 14; b and b' represent a different experiment. Note that while in experiment a VD neuron removal increased LP neuron beginning delay, in experiment b VD neuron removal decreased LP neuron beginning delay. In the other experiments VD neuron removal had smaller and similarly inconsistent effects in LP neuron beginning delay. Figure 15B shows LP neuron burst

duration versus cycle period best fits for 4 experiments. Unlike the changes in beginning delay, VD neuron removal induced small, but consistent changes in LP neuron burst duration.

A similar range of responses including large and small inconsistent changes as well as small and consistent changes was observed in comparing the effects of LP and VD neuron removal on the other measured parameters. To determine if these changes were significant, we utilized a multivariate general linear model (GLM) on the slopes and intercepts of the best-fit lines across experiments. A liberal initial α-level of 0.05 was chosen to increase the chances that significant differences would be seen. However, due to the multiple (88) comparisons, the Dunn-Šidák α-level compensation method had to be employed; the critical α-level for 88 comparisons is 5.8×10^{-4}. Figure 16 summarizes the probabilities obtained from these GLM tests for all measured parameters.

Perusal of this table shows that in 17 instances (i.e., numbers shown in green), significant differences between control and LP or VD neuron removed cases existed. However, the general linear model does not measure whether the observed changes are consistent across experiments. We therefore performed post-hoc paired sample *t*-tests on the slopes and intercepts of the best-fit lines for each of these instances to determine if the observed changes were consistent, again using Dunn-Šidák to compensate for multiple comparisons (nominal α-level of 0.05, compensated α-level: 3×10^{-3}). By this comparison, none of these *t*-tests achieved significance. To ensure that we were not being too stringent in our statistical analyses, we also examined the raw data from the cases which were significant by the general linear model and had paired *t*-test *p* values near or

less than 0.05. This examination supported the statistical analyses in that, although there were occasionally experiments, or more accurately, narrow period domains within experiments, in which LP or VD removal appeared to induce changes in the phasing or spiking parameter in question, these changes were not consistent across experiments.

An example of this is shown in Fig. 17, which shows VD neuron overall spike frequency with (circles) and without (triangles) the LP neuron for six experiments (panels A-F). Fig. 16 shows that this parameter had extremely low general linear model *p* values for both best-fit slope and intercept when plotted against either cycle period or cycle frequency. Student *t*-test analysis of this data showed that, in the cycle period case, the data with and without the VD neuron differed with a p values of 0.031 (slope) and 0.035 (intercept) (this was the lowest Student *t*-test *p* value of all comparisons). In panel C it appears that at short cycle periods LP neuron removal increases VD neuron overall spike frequency. However, in the other panels it is less clear that LP neuron removal has any consistent effect. Examination of the data on an experiment-by-experiment basis thus supports the statistical analyses.

Discussion

We have shown that in the lobster pyloric network that removal of two neurons that make extensive synaptic contact within the network appears to induce no consistent changes in the network spiking or phasing activity of any pyloric neuron. As such, it appears that in the ground state of normal saline and no added modulators the only functional role these neurons play is regulation of pyloric cycle period (see Chapter 2). It is essential to stress that LP and VD neuron synaptic activity was not increased in this

work, and thus it is possible that increasing their activity would alter the activity of other pyloric neurons. In support of this, we often observed alterations in pyloric neuron activity during the short-term high frequency LP and VD neuron firing that occurred when they were released from hyperpolarization. It is difficult to separate these effects from the simultaneous changes that also occurred in cycle period. Nonetheless, these data provide evidence that, when firing at their normal ground state activity level, the LP and VD neuron synapses onto other pyloric neurons play no role in pyloric activity (except for cycle period regulation).

A possible criticism of this work is that, since pyloric neurons release transmitter as a graded function of membrane potential, hyperpolarization may not have removed all LP and VD neuron input onto their synaptic targets. This concern is unlikely to be significant for several reasons. First, the hyperpolarizations were to membrane voltages (below –100 mV) at which graded release is not believed to occur (Graubard, 1978; Graubard et al., 1983). Second, in almost all cases the membrane potential trajectory of the hyperpolarized neuron was observed with a bridge-balanced electrode. At these levels of current injection the electrode was unlikely to be still in balance, but relative changes of neuron membrane potential could still be observed. These recordings showed, in general, that the hyperpolarized neuron remained at fairly constant hyperpolarized membrane potentials without subthreshold "escapes"; in those instances in which subthreshold escapes occurred, data near these escapes were not analyzed. Third, even if some graded release was occurring at these very hyperpolarized membrane potentials, release was nonetheless certainly dramatically reduced from physiological levels. The lack of

significant change in pyloric neuron phasing or spiking activity under these conditions clearly shows that much of the LP and VD neuron input can be removed without altering pyloric phasing or spiking activity.

Comparison to earlier work

The role of the LP and VD neurons in pyloric activity has been investigated in several different ways. The first such work was by Miller and Selverston (Miller and Selverston, 1982b), in which photoinactivation was used to sequentially reduce pyloric network neural complement. This work showed that the LP and VD neurons can form a half-center oscillator pair, but did not investigate what effect LP or VD neuron removal had on phasing and spiking activity of their followers. Massabuau and Meyrand (1996) showed that decreased O_2 levels increased LP neuron burst duration and induced an increased cycle period in the pyloric network. Blocking changes in LP neuron activity prevented the changes in network activity, which indicates that this change was a secondary consequence of the changes in LP neuron activity. However, the effects of decreasing or removing LP neuron activity were not investigated.

More directly relevant is work on VD (Hooper and Moulins, 1989, 1990) and LP (Hooper and Marder, 1987) neuron removal. A defined sensory input in the lobster, *Palinurus vulgaris*, results in the VD neuron becoming silent, and a variety of other changes in pyloric phasing and spiking activity, without inducing large changes in pyloric cycle period. A particularly strongly affected neuron is the Inferior Cardiac (IC) neuron. Work in which the VD neuron was hyperpolarized without stimulation of the sensory input, and in which the VD neuron was forced to fire in a normal manner after the

sensory input had been stimulated, showed that the changes in IC neuron activity after sensory stimulation resulted solely from the lack of VD neuron activity at this time. These data are in stark contrast to those reported here, in which VD neuron removal induced insignificant changes in IC neuron activity. The experiments with VD neuron hyperpolarization were performed under identical modulatory conditions (stomatogastric nerve, which attaches the ganglion that contains the pyloric network to anterior stomatogastric system ganglia, intact) as the experiments here, and thus differences in network condition in the earlier and this work are unlikely to be an explanation. However, although the pyloric networks of different species produce remarkably similar outputs, their synaptic wiring diagrams, and the cellular properties of their constituent neurons, show considerable variation (Meyrand et al., 2000). A possible explanation for the difference between these works is likely species-specific variation in pyloric network structure and function.

Work in *Panulirus* on the role of the LP neuron in the presence of the peptide modulator, proctolin, is more directly relevant. These data showed that several changes in pyloric neuron spiking and phasing activity in the presence of proctolin were indirect effects of proctolin-induced changes in LP neuron activity. This work was done with stomatogastric nerve input from anterior ganglia blocked, and so is not directly comparable to the present data. Furthermore, these authors did not investigate the role of the LP neuron in pyloric activity in control saline, only in the presence of proctolin (when stomatogastric nerve input is blocked, LP neuron activity in normal saline is very weak or absent entirely). Nonetheless, their demonstration that LP neuron removal does alter

pyloric spiking and phasing activity under at least one condition (proctolin modulation without stomatogastric nerve input) shows that LP neuron input alters the phasing and spiking activity of its postsynaptic targets in at least one network condition.

Relevance to pyloric network modulation

The pyloric network is subject to a wide variety of modulatory inputs that decrease and increase pyloric neuron activity. The data presented here showing that removal of at least much of VD or LP neuron input to the rest of the network has very little effect on pyloric spiking and phasing activity suggests that reduction of VD or LP neuron activity by modulatory input would have very little effect on spiking or phasing activity of other pyloric neurons (when the data is frequency matched).

Relevance to pyloric network phase maintenance

The pyloric network shows strong phase maintenance when pyloric period is altered by current injection into the AB neuron (Hooper, 1997a). The cellular basis of this phase maintenance has been investigated for the PY neurons (Hooper, 1998). This work shows that the postinhibitory rebound properties of the PY neurons are altered as the duration and period of inhibition they receive changes. These alterations result in PY neuron beginning delay (Fig. 11) increasing as inhibition period and duration increase. These endogenous changes would tend to maintain phase as cycle period changes. However, these endogenous changes are only approximately half as large as are required to explain the PY neuron phase maintenance observed in the intact network. This work was began in part to investigate whether phase-maintaining changes in LP and VD neuron activity observed when pyloric cycle period is altered could, through their synapses onto the PY

neurons, provide the mechanism for the increased PY neuron phase maintenance in the intact network. The data reported here showing that the removal of LP and VD neuron input does not alter PY neuron phasing shows that these neurons are not responsible for the increased PY neuron phase maintenance in the intact network. The basis of the increased phase maintenance in the intact network is thus still unknown, but an attractive possibility is that this arises from period and duration dependent changes in the synaptic transfer function from the AB/PD neuron pacemaker ensemble (Nadim et al., 1999). With respect to phase maintenance of the other pyloric neurons, the relative importance of endogenous and synaptic mechanisms is unknown. However, the data showing that LP or VD neuron removal does not alter phase maintenance for any pyloric neuron shows that, at least in the control saline condition, LP and VD neuron synaptic input is not important for phase maintenance for any pyloric neuron.

Relevance to small distributed systems in general

These data highlight two issues of general importance. First, VD or LP neuron removal alters pyloric period (see Chapter 2), and altering pyloric period alters pyloric spiking and phasing activity (Hooper, 1997a; Hooper and Thuma, 1996; Nadim et al., 1999). As a result of these period altering effects, whether LP and VD removal affected pyloric spiking and phasing activity could not have been determined without using AB neuron current injection to match the network period in the intact and neuron removed conditions. It would not be surprising if similar period alterations occurred in other highly distributed systems when neurons are removed from the network, and thus, in these as well, independent alteration of period is required to investigate neuron function in

generating network output. As such, these data underscore the necessity of examining the system over a wide activity range (Hooper and Weaver, in preparation).

Second, the LP and VD neurons make synaptic input both to the pacemaker ensemble and the other neurons of the pyloric network. LP or VD neuron removal, however, appears (in normal saline) to alter only pyloric period. As such, in complex networks, changes in the activity of neurons that make widespread synaptic contact may nonetheless alter only one aspect of network activity (in this case, only cycle period). The possibility that increases in LP or VD neuron activity, alternatively, may alter pyloric phasing or spiking activity independent of changes in pyloric period (Hooper and Marder, 1987; Massabuau and Meyrand, 1996), also suggests that modulatory input, depending on its sign, could have qualitatively different effects. For instance, in the case at hand, modulatory input that decreases LP or VD neuron activity would only affect pyloric cycle period directly; input that increases LP or VD neuron activity may directly affect both pyloric cycle period and pyloric phasing and spiking activity.

Chapter 4. A Possible Mechanism for Pacemaker Switching in the Lobster (*Panulirus interruptus*) Pyloric Network

Abstract

Central pattern generator (CPG) networks underlie rhythmic motor pattern production. These networks can produce multiple outputs by modulating the strength of network synapses, intrinsic neuronal properties, and changing network neuronal complement. All neurons of the pyloric network of the stomatogastric system are endogenous oscillators, but the network is generally driven by the fastest of these, the Anterior Burster (AB) neuron. Depolarizing current injection into the AB neuron initially decreases network cycle period, but large currents slow network cycling. This anomalous slowing is due to the activity of two other pyloric neurons, the Lateral Pyloric (LP) and Ventricular Dilator (VD). As AB neuron cycle period decreases, eventually the VD neuron can no longer follow and so it begins to oscillate at a slow cycle period. It then entrains the rest of the network to its slow cycle period, and thus limits the network's cycle period. Further experiments showed that the VD neuron entrains all network neurons but the AB neuron. AB neuron entrainment is mediated by the LP neuron. This work demonstrates that the neuron serving as the network pacemaker is not fixed, but can shift under certain conditions.

Introduction

Central pattern generator (CPG) networks underlie rhythmic motor pattern production (Delcomyn, 1980; Marder and Calabrese, 1996), and these rhythmic motor patterns are often driven by endogenous oscillator neurons. Endogenous oscillator neurons contain

active conductances that lead to spontaneous rhythmic spiking activity. These oscillator neurons are often synaptically coupled to other neurons within the same network, some of which are also oscillator neurons, and this coupling leads to a stereotyped rhythmic motor pattern. An open question is how these synaptic interactions bring about properly patterned behavior as network activity (e.g., cycle period) changes.

The pyloric network of the stomatogastric (STG) system is a good model network to study this issue for two reasons. First, as has been shown to also occur in several other well-defined neuronal networks, modulatory influences that alter the pyloric network's synaptic strengths and the conductance properties of its neurons induce the network to produce multiple outputs (Marder, 1991; Marder and Calabrese, 1996). Additional work has shown that this modulation can also act to switch neurons between different CPG networks by altering synaptic strengths (Hooper and Moulins, 1989, 1990; Weimann and Marder, 1994). A key observation in this work was that, due to the pyloric network's dense synaptic interconnectivity pattern, understanding the mechanisms underlying the network response required considering both the neurons directly affected by modulation, and feedback effects of other network neurons not directly affected by the modulatory input.

Second, the pyloric network is driven by an endogenous oscillator, the Anterior Burster (AB) neuron. The AB neuron is electrically coupled to a pair of Pyloric Dilator (PD) neurons, and these three neurons generally form the network's pacemaker ensemble. However, when synaptically isolated from the rest of the network, all pyloric network neurons are conditional endogenous oscillators (Bal et al., 1988). These neurons

endogenously cycle more slowly than the AB neuron, and thus presumably the AB/PD neuron ensemble generally serves as network pacemaker because it entrains the other oscillators to its relatively rapid cycle period. Nonetheless, the Lateral Pyloric (LP) and Ventricular Dilator (VD) neurons both synapse onto the pacemaker ensemble, and thus these neurons have the necessary synaptic connectivity to alter AB/PD neuron ensemble activity.

We have been investigating the role of the LP and VD neuron synapses within the pyloric network by alternately hyperpolarizing the LP and VD neurons well below rest to functionally remove each of these neurons' synaptic activity from the network and comparing network activity in the intact network and each removal case. In order to investigate the effects of these neurons across a wide range of pyloric network activity, we performed these experiments across a wide range of pyloric cycle periods by injecting tonic current into the AB neuron. We have shown previously (see Chapter 2) that, in the physiological range of pyloric cycle periods (0.5-2 sec), the LP neuron slows the network while the VD neuron speeds it. We also showed that, when sufficient current is injected into the AB neuron, these neurons can disrupt pyloric cycling; the LP neuron disrupts the network when the network is driven too slowly and the VD neuron disrupts when it is driven too fast. These data suggest that, in addition to altering period in the physiological cycle period range, the LP and VD neurons also serve to determine the boundaries of this range.

In the VD neuron disrupted case (high levels of depolarizing current injected into the AB neuron), the disruption occurred because the VD neuron would frequently escape

from AB/PD neuron entrainment, cycle independently at a slower period, and alter the activity of the rest of the pyloric network through the VD neuron's extensive synapses within the network. This raised the question of whether the VD neuron could perhaps, if even more current were injected into the AB neuron, entrain the entire network and thus become the network pacemaker. We show here that this occurs, that the VD neuron becomes the functional pacemaker of the network and causes the entire network to cycle slowly despite the ability of the other neurons, in the absence of VD neuron input, to cycle at faster periods. LP neuron hyperpolarization shows that the VD neuron input entrains all pyloric neurons (including the LP neuron) except the AB/PD pacemaker ensemble; pacemaker ensemble entrainment is mediated indirectly as a result of LP neuron input onto the ensemble. By showing that pacemaker identity can switch among network neurons, this work significantly extends prior work by showing that not only can neuron phasing, burst duration, and spiking activity be altered, but the neurons that form the functional core of a rhythmic network can change.

Materials and Methods

Pacific spiny lobsters (*Panulirus 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): NaCl 479, KCl 12.8, $CaCl_2$ 13.7, Na_2SO_4 3.9, $MgSO_4$ 10.0, glucose 10.9, tris base 11.1, maleic acid 5.1, pH 7.5–7.6. All salts were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Stomatogastric neuromuscular systems were dissected and prepared for extracellular nerve recording and intracellular neuron recording using standard techniques (Selverston et al., 1976). 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 0.55M K_2SO_4, 0.02M KCl, resistance 10 to 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. 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 tonic current injection into the 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 Chapter 3). In all cases the same electrode was used for voltage recording and current injection. Hyperpolarized neurons were monitored for escape by examination of extracellular recordings, presence of inhibitory post-synaptic potentials in the neuron's synaptic partners, 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 neuron activity. Period was averaged over 6-10 pyloric cycles; less than ten cycles were used when escapes from hyperpolarization, or

interference from other stomatogastric nervous system networks (gastric mill, cardiac sac), perturbed pyloric activity (Bartos et al., 1999; Bartos and Nusbaum, 1997; Marder et al., 1998; Mulloney, 1977; Nadim et al., 1998; Thuma and Hooper, 1999). In all experiments the stomatogastric nerve, which carries input from the rest of the stomatogastric nervous system to the pyloric network, was intact. The data presented here are from 5 experiments.

Results

The pyloric network is a small, well-characterized network of 14 neurons consisting of 6 neuron types. All synapses in the network are known. Figure 1A shows the pyloric circuit diagram; circles indicate inhibitory chemical synapses and resistors and diodes indicate electrical coupling. The AB neuron is an endogenous oscillator neuron. The PD neurons are electrically coupled to the AB neuron, and these three neurons form the pyloric AB/PD pacemaker ensemble. The network has four "follower" neuron types: LP, VD, Inferior Cardiac (IC), and Pyloric (PY). The LP neuron inhibits the PD, VD, and PY neurons and makes a rectifying electrical synapse onto the PY neurons. The VD neuron makes a rectifying electrical synapse onto the PD and AB neurons, and inhibits the LP, IC, and PY neurons (Johnson et al., 1993a; Selverston et al., 1976). As such, the LP and VD neurons have synaptic connections appropriate for altering the phase and firing activity of most or all of the other neurons of the pyloric network. The pyloric output pattern (Fig. 1B) is a rhythmic, triphasic pattern in which the AB/PD pacemaker ensemble fires, then the LP and IC neurons fire, and then the VD and PY neurons fire, after which the pattern repeats.

We were interested in investigating the response of the network to changes in pacemaker ensemble cycle period, and therefore injected tonic current into the AB neuron to alter pyloric network period; we report here on the effects of depolarizing current injections. Figure 18 shows typical effects from one experiment on PD neuron cycle period as AB neuron current injection is increased. The three traces (top to bottom) of panel A show PD neuron extracellular activity for 0, +4, and +8 nA AB neuron current injection. Cycle period clearly decreases as injected current is increased from 0 to +4 nA with little change in PD neuron burst duration. When the level of injected current was increased to +8 nA, PD neuron spiking activity had a much lower interspike interval, and was much less regular than in the +4 nA case (for intermediate +6 nA data, see discussion). However, despite this decreased regularity, very long PD neuron bursts with a greatly increased cycle period ("anomalous slowing") can be identified.

The irregular nature of the PD neuron bursts in the +8nA case raised the question of whether we had correctly identified the PD neuron burst and interburst intervals, or if perhaps the short silent intervals inside the long bursts should also be counted as interburst intervals. Several lines of evidence suggest that using the larger interval to measure cycle period is correct. First, these longer interburst intervals are similar in magnitude to the interburst intervals present at other depolarization levels (+4 nA, 0.58 ± 0.03 sec; +8 nA, 0.48 ± 0.05 sec). Second, when binned, the long interburst intervals stand out as a discrete peak well separated (by a 100-150 msec gap) from the short interval group. Panel B of Figure 18 is an interspike interval histogram for the data from the +8 nA condition. The inset shows the entire range of data and the larger plot is an

expansion of the non-grayed data. Third, when the long interburst intervals were averaged and this average minus three standard deviations was taken as the minimum interburst interval (this criterion corresponds to a 99.87% confidence interval), none of the short interspike intervals met this definition.

The most compelling evidence that the long interburst intervals properly define cycle period, however, was obtained by intracellular and extracellular recordings from other pyloric neurons. Figure 19 shows the same experimental conditions as in Fig. 18 with the addition of all pyloric neurons except the AB neuron (whose activity, due to their electrical coupling, will likely be similar to that of the PD neuron). Starting from the top, each panel has 5 traces: a PD neuron extracellular trace, PY and LP neuron intracellular traces, an IC neuron extracellular trace, and a VD neuron intracellular trace. For the cases in which 0 and +4 nA were injected into the AB neuron, all the pyloric neurons burst in a normal pattern once per PD neuron cycle period. In the anomalous slowed condition (+8 nA), the network continues to produce a regular motor pattern with clear one to one firing of the PY, LP, IC, and VD neurons.

To ascertain if the LP or VD neurons played a role in the anomalous slowing each neuron was alternately hyperpolarized well below rest while recording the activity of the remaining neurons. In the 0 and +4 nA conditions, removal of the LP neuron speeded the pattern and removal of the VD neuron had little effect (data not shown; see Chapter 2 for more complete description). Figure 20 shows 3 cases, in each of which +8 nA was injected into the AB neuron; the five neuron recordings are shown in the same order as in Fig. 19. The first panel is with the intact network, and is the same as the third panel of

Fig. 19. The second panel is with the VD neuron hyperpolarized. In this case, the network cycled much faster than the +8 nA intact condition, and also faster than the +4 nA intact condition. In this case the PY neurons show only small decreases in firing with each PD neuron burst, the LP neuron shows large variations in the amplitude of its slow wave oscillation, and the IC neuron burst duration and spike number also show considerable variation. However, all the neurons still show a one to one bursting pattern tied to the PD neuron cycle period, and thus the network is clearly capable, in the absence of the VD neuron, of expressing this very rapidly cycling pattern. In the third panel, the LP neuron was hyperpolarized. Without the LP neuron, the PD neuron becomes uncoupled from the rest of the network's neurons (i.e., IC and PY neurons), which continue to cycle slowly while the PD neuron fires with an even shorter (although less consistent) period than in the previous cases. These data thus show two results. First, without the VD neuron, anomalous slowing does not occur, since, as expected, the network cycles faster when +8 nA was injected into the AB neuron than when +4 nA was injected. Second, without the LP neuron's synaptic influence onto the PD neuron, the PD (and presumably the AB) neurons can cycle very quickly, but the other network neurons continue to cycle slowly with the VD neuron.

Figure 21 shows the average cycle period and standard deviation of the PD neuron for the intact network (dark gray), VD neuron removed (red), and LP neuron removed (blue) cases at AB neuron current levels of 0, +2, +4, +6, and +8 nA. Considering first the intact network case, we see no change in cycle period as AB neuron current injection was increased from 0 to +2 nA, a decrease in period from +2 to +4 nA, no change in period

but a large increase in standard deviation from +4 to +6 nA, and a significant increase in period but no increase in standard deviation from +6 to +8 nA. Removing the VD neuron has little effect except in the +6 nA injection case, in which the standard deviation is reduced (compared with the intact case) and the +8 nA case, in which the anomalous slowing no longer occurs. Removing the LP neuron results in a nearly linear decline in PD cycle period as AB neuron current injection is increased. Similar results were seen in four of the five experiments carried out; in the fifth experiment anomalous slowing was not observed.

Discussion

We have shown that, in the lobster pyloric network, increasing AB neuron depolarization first speeds and then anomalously slows network cycle period. VD neuron hyperpolarization showed that the rest of the network is capable of responding to increased AB neuron current injection with decreased cycle periods across the entire range of current injections used here. LP neuron hyperpolarization freed the PD (and presumably the AB) neuron to respond to increased AB neuron current injection with continuously decreased cycle period. However, in this case at high levels of AB neuron current injection, the other pyloric neurons cycled one for one with the more slowly cycling VD neuron.

At an intermediate condition (+6 nA) in the intact network, the previously reported VD neuron pattern disruption (see Chapter 2) is evidenced by large cycle period standard deviations. This disruption is likely due to the VD and AB neurons vying for pacemaker control of the network. In this case, the VD neuron incompletely controls PD neuron

cycling and both short (AB neuron driven) and long (VD neuron driven) PD neuron cycle periods are present (data not shown, but see Fig. 9 of Chapter 2). It thus appears that VD neuron pattern disruption is an incomplete form of pacemaker switching. The LP neuron removal data indicate that PD/AB neuron ensemble entrainment to the VD neuron depends on LP neuron synaptic input to the ensemble. As such, it appears that the direct electrical rectifying synapses the VD neuron makes onto the pacemaker ensemble do not play a significant role in the pacemaker switching described here.

A possible criticism of this work is that the tonic current injections used here do not mimic the physiological mechanisms used to alter AB neuron activity. Two arguments against this criticism can be raised. First, our primary goal was to examine the functional capabilities inherent to the pyloric network, and altering network cycle period by AB neuron current injection allowed examining network function over a wide activity range without the widespread changes that modulator application would induce. Second, nicotine application induces a strong, ionotropically-mediated, AB neuron depolarization that can cause a large decrease in AB neuron oscillation period (Marder and Meyrand, 1989). Although ionotropic channel opening and current injection differ due to ionotropic current's dependence on driving force, the two are nonetheless much more comparable than the effects on voltage dependent conductances induced by many modulatory substances. The presence of muscarinic and nicotinic receptors on pyloric neurons suggests that acetylcholine may be used to modulate pyloric activity (Marder and Hooper, 1985; Marder and Meyrand, 1989). Given the extremely strong effects of nicotine on AB neuron activity, it is thus possible that ACh release onto the AB neuron

could decrease its cycle period sufficiently for the pacemaker switching described here to occur.

Another criticism of this work is that pacemaker switching did not occur in one of the five experiments. One explanation is that in this preparation the intrinsic properties of the VD neuron were such that it could cycle as quickly as the AB neuron throughout the full range of current injections used in this experiment, or that the strength of the VD to LP neuron synapses, or the LP to PD/AB neuron ensemble synapses, was insufficient to result in PD/AB neuron ensemble entrainment. Another explanation is that, in this experiment, insufficient current was injected into the AB neuron to make its cycle period decrease sufficiently that the VD neuron could not continue to follow it.

Comparison to earlier work

Alternative mechanisms of pyloric rhythm generation were examined by Miller and Selverston (1982b) in which the AB neuron was photoinactivated. In this work the PD and LP neurons formed a half-center oscillator that could support pyloric network activity in the absence of the AB neuron. That a similar PD/LP neuron half center is not responsible for rhythm generation in our case is clearly shown by the LP neuron hyperpolarization experiments, in which both the AB/PD neuron ensemble and the rest of the network continue to cycle, although with different cycle periods.

Weimann et al. (1997) have shown in *Cancer borealis* that crustacean cardioactive peptide activates slow intrinsic oscillatory properties in the LP neuron, and can induce a network configuration in which the LP neuron fires one burst for every 2 to 4 cycles of the rest of the pyloric network. Although these data support ours and the work of Bal et.

al. (1988) in that they show that all the pyloric neurons can be endogenous oscillators and that these oscillators can have different inherent cycle periods, pacemaker switching in which the entire pyloric network cycled one for one with the slow LP neuron oscillations was not observed. Given that it is the LP neuron that mediates the VD neuron entrainment of the PD/AB neuron ensemble in *Panulirus*, the data of Weimann et al. (1997) and ours may seem in contradiction. However, the experimental conditions in these two studies are different. First, there may be species dependent effects. Second, since Weimann et al. were not injecting current into the AB neuron, in their work the PD/AB neuron ensemble was cycling at a slower period than in ours. It is thus possible that the ensemble was less susceptible to LP neuron entraining influence at this cycle period. Third, crustacean cardioactive peptide likely affects the membrane properties, synaptic strengths, or both of a variety of pyloric neurons and synapses, and it is possible that these changes prevented LP neuron entrainment of the ensemble.

Relevance to other rhythmic systems in general

These data underscore the central importance that indirect connections can play in determining network activity. Although the VD neuron makes direct electrical connections onto the PD/AB neuron ensemble, this input is insufficient for the VD neuron to entrain the ensemble. This entrainment instead occurs via an indirect route in which the VD neuron entrains the LP neuron, which in turn entrains the ensemble. On a more general level, considerable previous work has shown that intrinsic synaptic and conductance properties are subject to modulation that can result in single neural networks producing multiple output patterns (Marder, 1991; Marder and Calabrese, 1996). The

data presented here expand on this work by showing that, in pacemaker driven networks, as fundamental an aspect of network structure as which neuron serves as the pacemaker can also change. Another network that is believed to be pacemaker driven is vertebrate respiration, which is known to function in at least two modes (normal breathing, gasping) characterized by different cycle periods (Ramirez, 1998). This network is not known well enough on the cellular level to say if this activity change arises due to a switch in pacemaker neuron identity, but our data suggest that such a mechanism might underlie this behavioral switch.

Chapter 5. Conclusions

This work was originally begun to investigate the network mechanisms that underlie phase maintenance in the intact pyloric network. However, it became clear over time that we were investigating several important roles of the LP and VD neurons in this network. In this chapter, we would like to briefly describe three general conclusions from this work, provide some more recent results, and outline future directions for this project.

Summary of presented work

Functional roles of individual neurons in a distributed network

Previous work in neural networks has been pessimistic with regard to ascribing function to individual neurons and synapses in distributed networks (Rumelhart et al., 1988; Selverston, 1980). However, some experimental work has begun to assign specific functions to certain neurons and synapses in small neural systems (Dickinson et al., 1990; Hooper and Marder, 1987; Hooper and Moulins, 1990; Katz et al., 1994; Kepler et al., 1990; Marder et al., 1992) We have added to these results with the work in Chapter 2.

In this work, we have found that the LP neuron serves to slow the network while the VD neuron speeds it. Additionally, outside of the physiological range of cycle periods, the LP neuron disrupts the proper patterning of the network by escaping from inhibition periodically and intermittently cycling 2 to 1 with the rest of the network. In an analogous but opposite manner, the VD neuron also disrupts the pyloric network when the network is speeded by oscillating slowly compared with the rest of the network (i.e., one VD neuron cycle for every two to four cycles of the rest of the network). This work suggests that the synapses onto the pacemaker ensemble from the LP and VD neurons serve as one

means to regulate cycle frequency within the pyloric network. Outside of the physiological cycle period range, disruption of the network occurs due to the inability of the LP and VD neurons to cycle slow or fast enough, respectively. These two neurons serve as both cycle frequency governors in the pyloric network and define the extent of the physiological range of cycle periods.

Pacemaker switching in central pattern generator networks

An important issue in central pattern generator research is whether the fundamental pacemaker of a CPG network is coupled half-center oscillator neurons or an endogenous oscillator neuron. Previous work by Miller and Selverston (1982b) has shown through photoinactivation of the endogenous oscillator AB neuron that the rest of the network can continue to cycle due to the half-center oscillator PD-LP neuron pair. Thus, pyloric network rhythmicity, while pacemaker-driven, is reinforced by half-center oscillator connections. More recent work by Bal et. al. (1988) has shown that all of the pyloric neurons are conditional neuronal oscillators, although they cycle with a period slower than the AB neuron. As such, each neuron may have the ability to entrain the network to its rhythm. However, the pyloric neurons are typically entrained by the faster AB neuron.

Our work in Chapter 4 provides an example of the VD neuron serving as the entraining influence of the entire network. As previously mentioned, the VD neuron intrinsic properties limit how fast it can cycle compared with the rest of the network, leading to network pattern disruption when the network is speeded. If an attempt to speed it further is made, the VD neuron asserts control of its chemical synapse connected partners and then becomes the network pacemaker. This entrainment effect is mediated

further onto the pacemaker ensemble through the LP neuron's synapse onto the PD neuron. Under these conditions, the network cycles slowly despite the ability of each of the other pyloric neurons to cycle faster in the absence of the VD neuron.

Why have so many synaptic connections in the pyloric network?

An unresolved question in the field of small neural systems is why networks are often so highly distributed. Table 1 shows that out of 32 possible unique synaptic connections, 21 (66%) are present, illustrating the high level of connectivity within the pyloric network. This multitude of connections is much more than is necessary to produce the network's triphasic rhythm. One explanation that has been given for this phenomenon is that it will allow a fixed network to produce multiple outputs (Harris-Warrick and Marder, 1991; Marder and Calabrese, 1996). Implicit in this idea is that synaptic strengths are subject to modulation and this is borne out by prior work (Johnson et al., 1995). An additional assumption is that some synapses play little role in maintaining the network pattern in certain modulatory conditions, including the unmodulated ground state.

Our work in Chapter 3 seems to have proven this assumption true. Again, hyperpolarization studies were carried out across a wide range of cycle periods and at each condition, the LP and VD neuron were alternately removed from the network. The phasing and spiking activity of the other neurons were compared with the intact network condition. We found no significant difference in any remaining neuron as a result of either neuron's removal.

Recent entrainment results

It is known that both the LP and VD neurons make strong synapses onto the pacemaker ensemble. We have shown here that these synapses are involved in cycle period regulation and pacemaker switching in the network. However, it is unclear under what conditions these synapses have functional access to the pacemaker and to what extent this access allows for network entrainment. In more recent work, we began an entrainment study to better understand this issue where we injected alternating pulses of rest and hyperpolarizing current levels into either the LP or VD neuron and measured network entrainment to these rhythmic pulses. We unexpectedly found 1:1 entrainment occurred over a relatively narrow range of cycle periods (±10%) relative to the endogenous network period. Typical forms of 1:n or n:1 ("integer") entrainment outside this range were observed at the expected relative periods (e.g., 33%, 200% of endogenous), but these forms of entrainment occurred over an even narrower range of periods. Surprisingly, we found a wide variety of n:m ("non-integer") coupling between these ranges of integer entrainment and these forms of entrainment occurred over a wider range of relative cycle periods than did the integer forms of coupling. To our knowledge, this is the first example of n:m coupling seen in neuronal networks.

In collaboration with Kevin Hobbs, we have attempted to explain this phenomenon through a single oscillator and two oscillator network models (relaxed Van der Pol oscillator adapted from Rowat and Selverston, 1993, 1997). We biased the two cell network models to match the neuronal dynamics of the LP neuron plateau potential and pacemaker endogenous oscillator properties in order to better recreate the experimental

setup. We found that the single oscillator model could generate n:m entrainment with the stimulus if the stimulation current were sufficiently weak. This n:m entrainment occurred at the expected stimulation periods along with other forms of 1:n entrainment. Each form of entrainment occurred across a nearly equal cycle period range. However as stimulation current increases, first the non-integer forms of entrainment were overtaken by the other integer forms of entrainment, then the 1:1 entrainment would force out the remaining forms of integer entrainment. In order to properly emulate the experimental setup, in the network model we injected strong levels of current into the "LP" model to force it to cycle 1:1 with the stimulus and measured the entrainment response of its inhibitory coupled "pacemaker ensemble" model cell. The network model gave similar results to the single neuron model with n:m coupling being squeezed out by 1:n coupling which was in turn forced out by 1:1 coupling, as the "LP" to "PD/AB" synaptic strength was increased. Investigations of the phase response curves (PRC) of these models suggested that a flat PRC at zero indicates no entrainment, and a linearly decreasing PRC was necessary for 1:1 entrainment to occur over the entire range of stimulation periods. Entrainment with n:m and 1:n coupling required a PRC somewhere between these two. We are now investigating how non-integer entrainment is preferentially lost to integer forms of entrainment. Thus, we have a relatively simple model that emulates much of the previous entrainment experimental results. This model suggests that weak synaptic coupling is a necessary ingredient for non-integer entrainment in a network and predicts the form of PRC we should find experimentally.

Future directions

There are several lines of future research that could prove fruitful to continue with this work. While each of these future directions have some limitations, they help to more fully explore the system and to explain the results described here

These experiments were carried out through comparisons of hyperpolarized and intact network conditions. Although important for future work, we avoided performing cell kills through photoinactivation primarily because it is not reversible. This lack of reversibility is a problem because the endogenous cycle period often changes over the time it takes to run en entire range of AB neuron current injections in the intact network condition. So, direct comparisons with and without the LP or VD neuron at a particular AB neuron current injection would not be possible. Additionally, only one neuron could be removed from each study so comparisons of the differential effects of these two neurons within the same preparation could not be achieved. However in the hyperpolarization studies, we have no way of independently knowing whether all synaptic activity was removed by simple hyperpolarization, especially since the space clamp in pyloric neurons is believed to be inadequate in their neuropil. We do feel though that the synaptic effects of these neurons were greatly limited by this hyperpolarization and if anything, this work provides a lower bound for the results seen.

We chose the LP and VD neurons for this study because they exist as single cells and have strong synapses onto most of the network's neurons. Investigations of the varied effects of the remaining motor neurons of the network should also be carried out. Table 2 shows the possible techniques for removal of each of the network's neurons. The next

obvious candidate for study would be the single IC neuron, but it is believed to be less important in *Panulirus* because it only synapses onto the VD neuron and its synaptic strength is lower than many of the other neurons. The next candidate would be the two PD neurons that make up part of the pacemaker ensemble. It is unclear what effect their removal might have as the AB neuron synapses onto all of the other neurons in the network. Finally, the 6-8 PY neurons would provide a technically challenging removal since in most preparations not all the PY neurons can be found. Like the IC neuron, it is also unclear what effect removal of these neurons might have as they synapse only onto the LP and PY neurons.

After the removal studies have been carried out for each of the network's motor neurons, then these removal studies should be carried out on reduced networks. Without the influence of a single neuron (through photoinactivation), the network would be reduced in complexity and hyperpolarization studies would further allow us to understand the mechanisms that underlie pattern generation in this distributed network. Additionally, dynamic clamp (Sharp et al., 1993) studies could be carried out to functionally replace a missing neuron, modify synaptic strengths, or alter conductance properties of a neuron.

Like the entrainment work, we are now at a point where modeling could be fruitful for understanding the mechanisms behind our results. Simple models that incorporate the synaptic connections of the network may alone be enough to explain why we see some of our results. If not, conductance-based models exist for several pyloric neurons and could be incorporated into a more detailed model of the network.

Finally, we have stated that the LP and VD neuron synapses play no significant role in pattern phasing or spiking, butthat these connections may be important under certain modulatory conditions. Thus, investigations into the modulatory conditions that bring about this change and what effect these newly important synapses might have on the network should be carried out. Unfortunately, few of the known modulators have single effects within the network. Thus, these modulation studies will have to deal with the confounding factors of this myriad of effects. Work by Brezina and Weiss (1997) provides a method for dealing with this complexity by mapping multiple modulator inputs to multiple network activity outputs. Then, the network of interactions is charted through experiments using different combinations of multiple transmitters and building a model that reproduces these experimental results.

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Table 1. Pyloric network synaptic connections: electrical (E), rectifying electrical (R), cholinergic chemical (S), glutamatergic chemical (S). Rows list the originating neuron; columns list the receiving neuron. Gray squares indicate single neurons to themselves.

		Receiving					
		AB	PD	VD	LP	IC	PY
Originating	AB		E	S	S	S	S
	PD		R		S	S	S
	VD	R	R		S	S	S
	LP		S	S			R S
	IC			S			
	PY				S	S	R

Table 2. Possible pyloric neuron removal techniques.

Neurons	Possible Removal Treatments
AB	Hyperpolarize
	Photoinactivate
	PTX Block
PD	Hyperpolarize both
	Photoinactivate one and hyperpolarize the other.
	Kill both
VD	Hyperpolarize
	Photoinactivate
LP	Hyperpolarize
	Photoinactivate
	PTX Block
IC	Hyperpolarize
	Photoinactivate
	PTX Block
PY	In a preparation with many PY neurons, photoinactivate a majority of them and hyperpolarize the rest to reduce the effect of the remaining neurons.
	PTX Block

Table 3. List of Abbreviations

AB	Anterior Burster
ANCOVA	Analysis of Covariance
CPG	Central Pattern Generator
DA	Dopamine
DC	Duty Cycle
EPSC	Excitatory Post-Synaptic Current
EPSP	Excitatory Post-Synaptic Potential
IC	Inferior Cardiac
IPSC	Inhibitory Post-Synaptic Current
IPSP	Inhibitory Post-Synaptic Potential
IVN	Inferior Ventricular Nerve
LP	Lateral Pyloric
PD	Pyloric Dilator
PIR	Post-Inhibitory Rebound
PRC	Phase-Response Curve
PTX	Picrotoxin
PY	Pyloric
SEC	Seconds
STG	Stomatogastric Ganglion
STN	Stomatogastric Nerve
VD	Ventricular Dilator

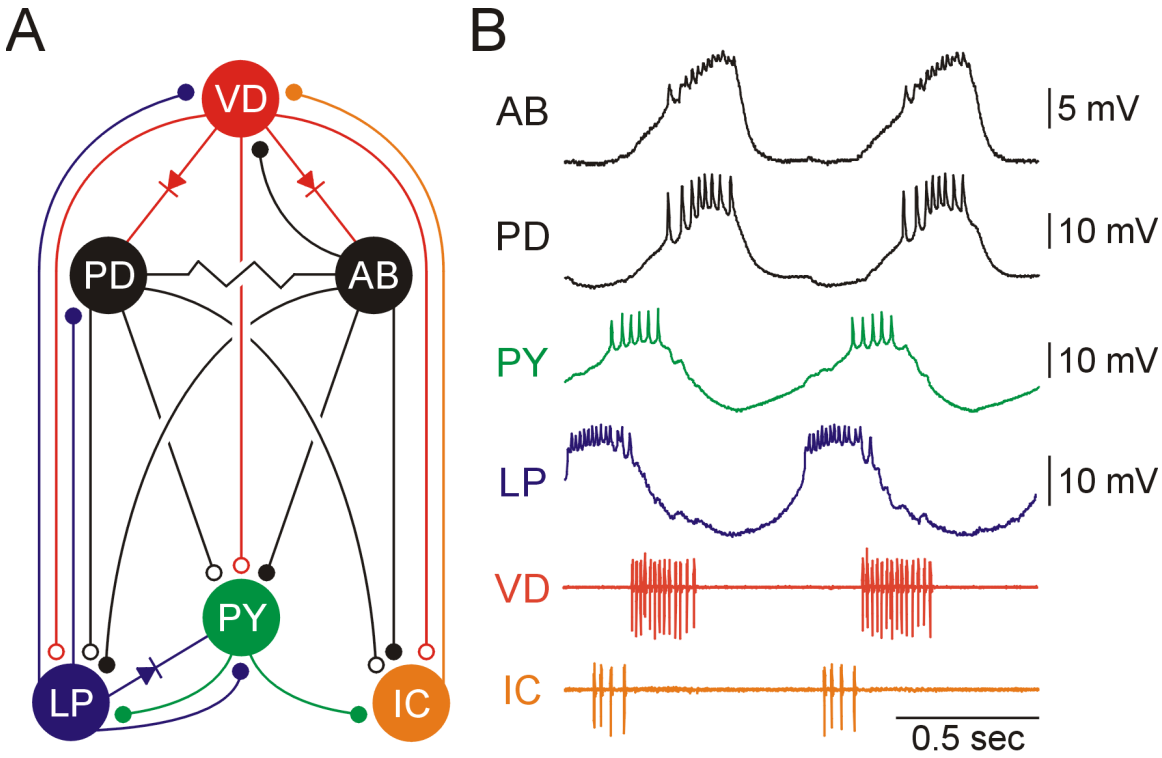

Fig. 1. The pyloric network synaptic connectivity diagram (A) and typical pyloric output pattern (B). See text for explanation of pyloric rhythmicity. The pyloric pattern is a triphasic rhythmic pattern in which the AB/PD pacemaker ensemble fires, then the LP and IC neurons fire, and then the VD and PY neurons fire, after which the pattern repeats. Synaptic connectivity symbols: closed circle, inhibitory cholinergic synapses; open circle, inhibitory glutamatergic synapses; resistor, electrical coupling; diode, rectifying electrical synapse.

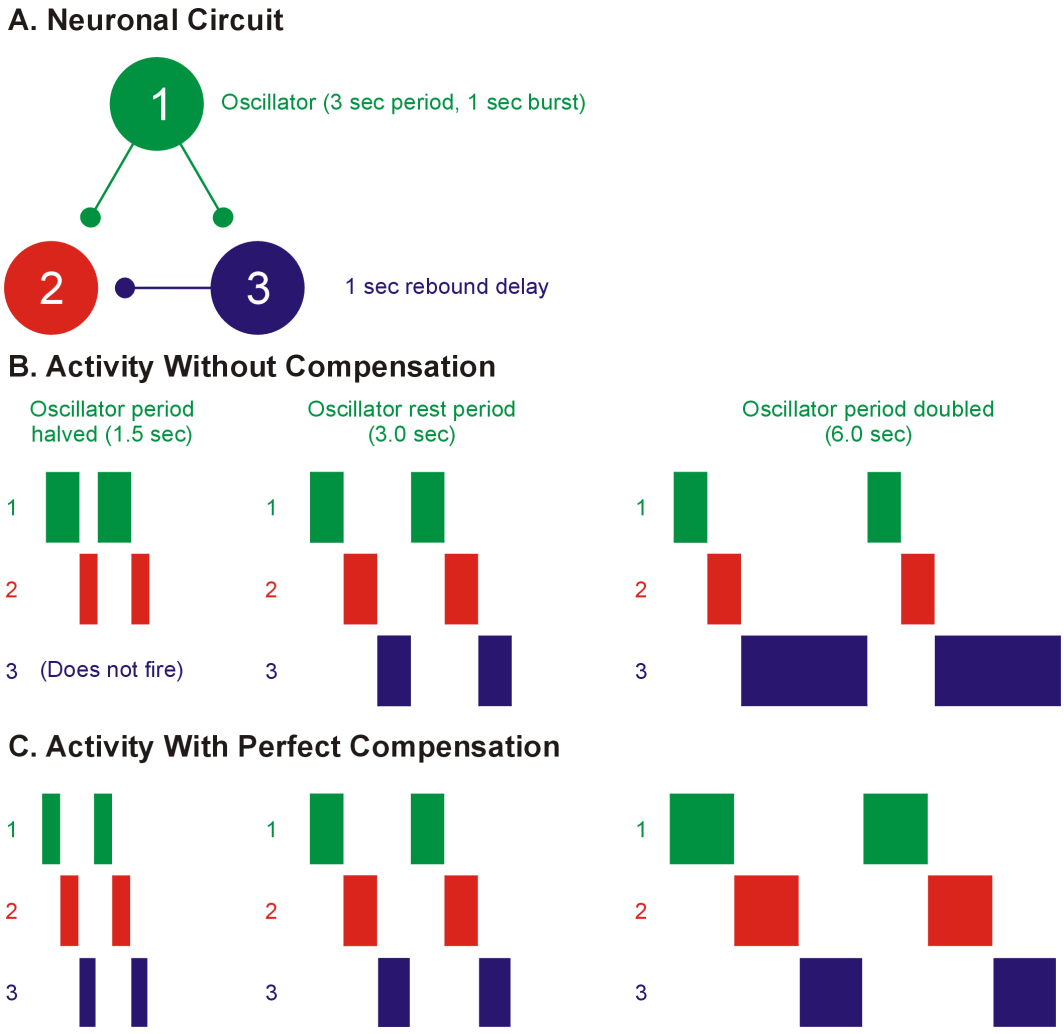

Fig. 2. Phase maintenance is not a trivial issue. In the hypothetical network of the top panel, Neuron 1 is an endogenous oscillator with a 3 sec cycle period and 1 sec burst duration. Neurons 2 & 3 are plateauing neurons with PIR. Neuron 3 recovers from inhibition 1 sec slower than Neuron 2. Without phase-maintaining properties that can properly shift the synaptic delays, rebound delays, or burst durations, the network is unable to maintain phase over a wide range of cycle periods (middle panel). Proper phase maintenance is illustrated in the bottom panel.

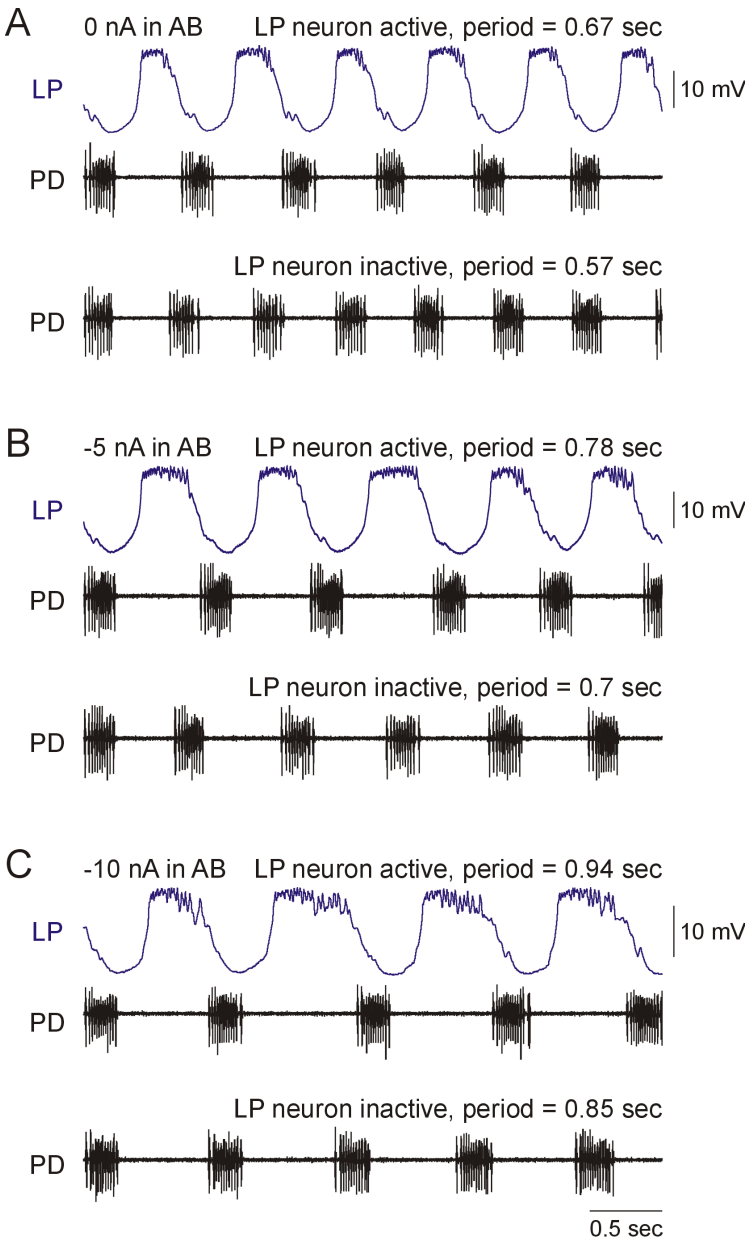

Fig. 3. LP neuron removal consistently shortened pacemaker period. In each panel the top trace is an LP neuron intracellular recording and the second trace is a PD neuron extracellular recording. The third trace shows PD neuron activity with the LP neuron hyperpolarized. The three panels show the effect of LP neuron removal at three AB neuron hyperpolarization levels.

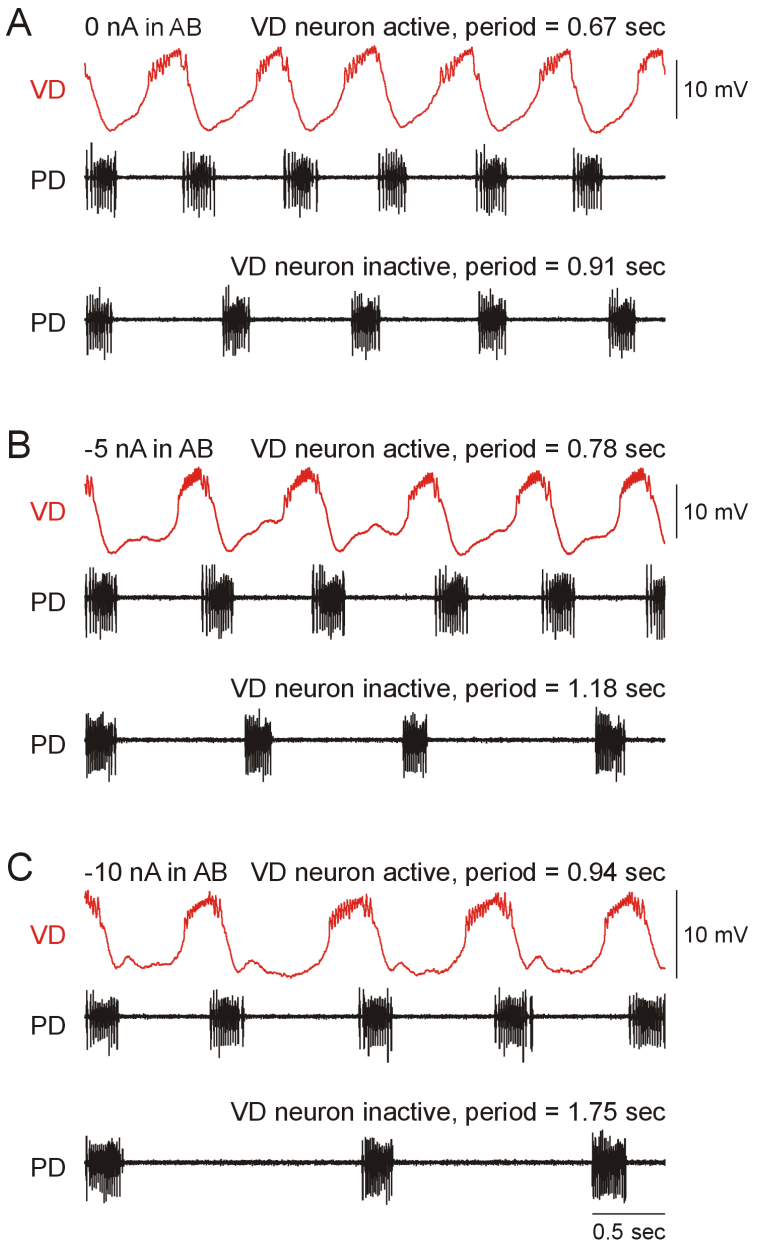

Fig. 4. VD neuron removal consistently lengthened pacemaker period. In each panel the top trace is a VD neuron intracellular recording and the second trace is a PD neuron extracellular recording. The third trace shows PD neuron activity with the VD neuron hyperpolarized. The three panels show the effect of VD neuron removal at three AB neuron hyperpolarization levels.

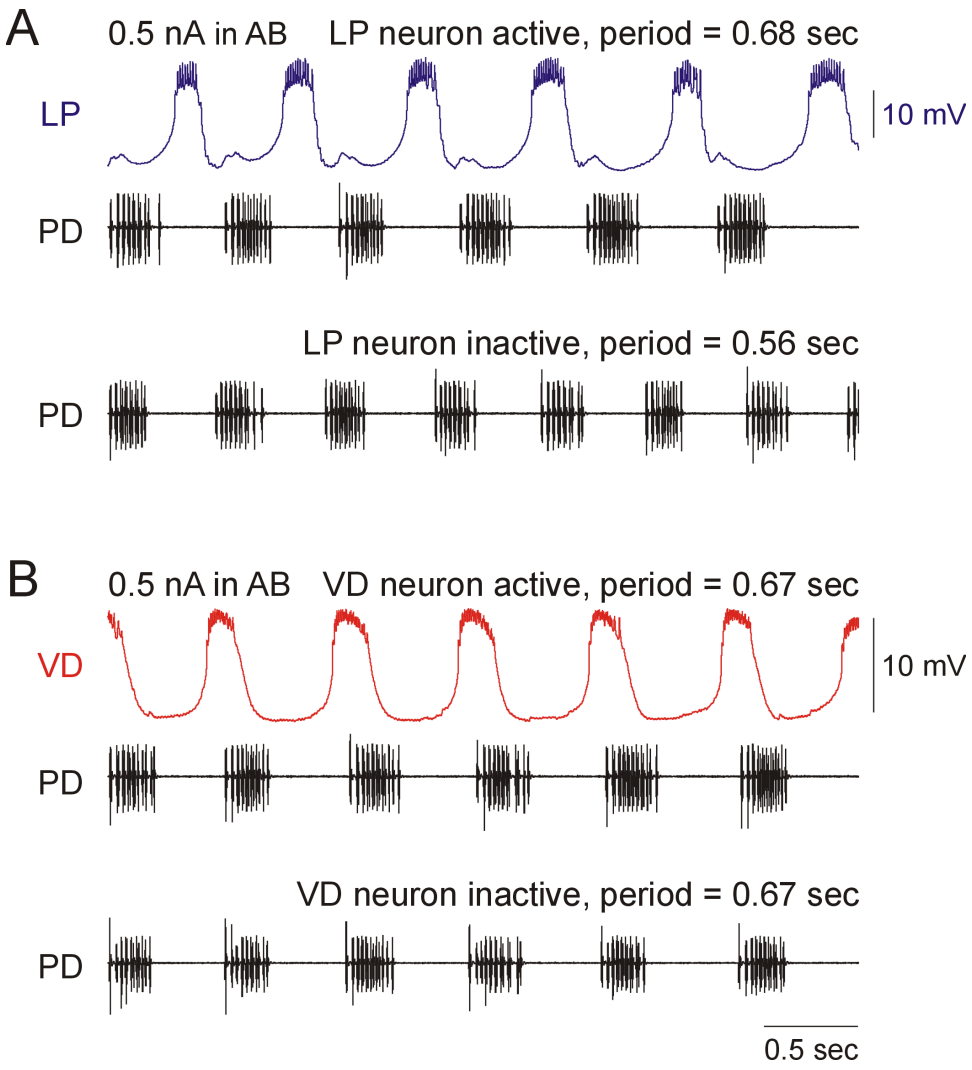

Fig. 5. When the AB neuron is depolarized, LP neuron removal continued to reduce cycle period (A), whereas VD neuron removal had little effect (B). In each panel the first two traces show follower and PD neuron activity and the third trace shows PD neuron activity with the follower neuron hyperpolarized; in each panel 0.5 nA has been injected into the AB neuron.

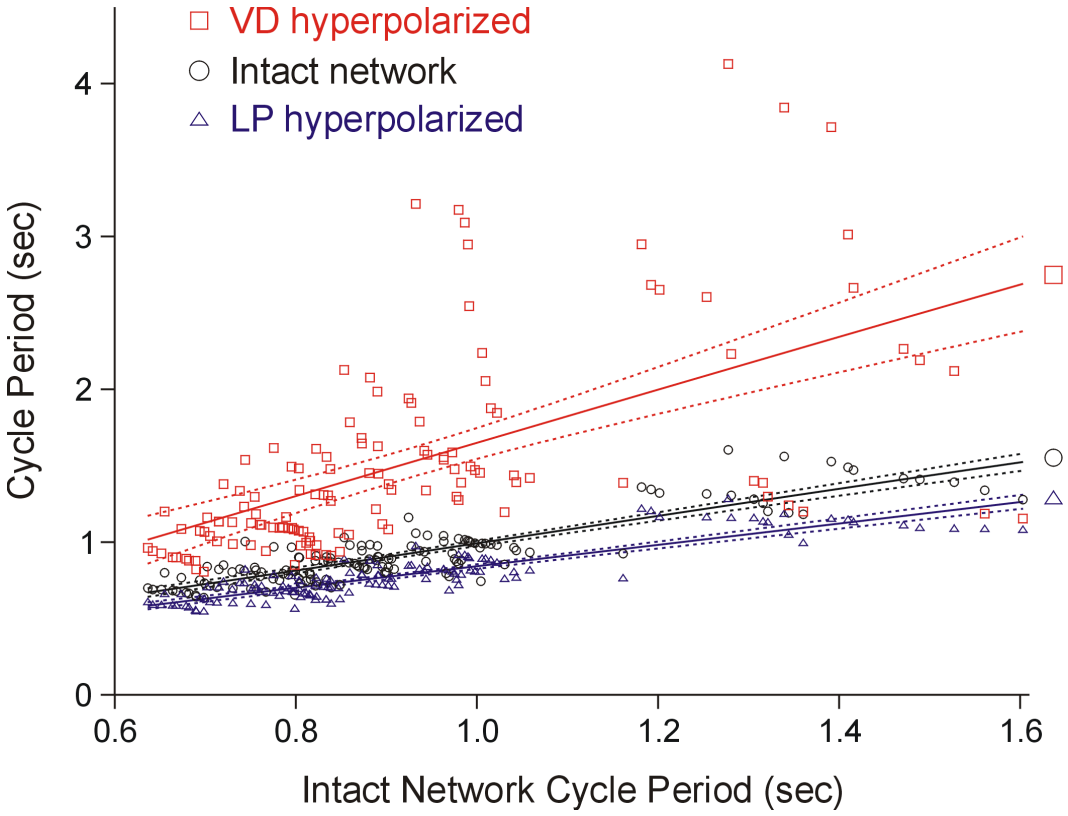

Fig. 6. Cycle period effects of LP or VD neuron removal. LP neuron removal reduces cycle period by a constant amount compared with the intact network at all AB neuron current injection levels, whereas the slowing effect of VD neuron removal increases with increased intact network cycle period. The best-fit lines to all the data points of each condition are also plotted, along with their 95% confidence interval lines. Please see results text for more discussion on how the data scatter was generated.

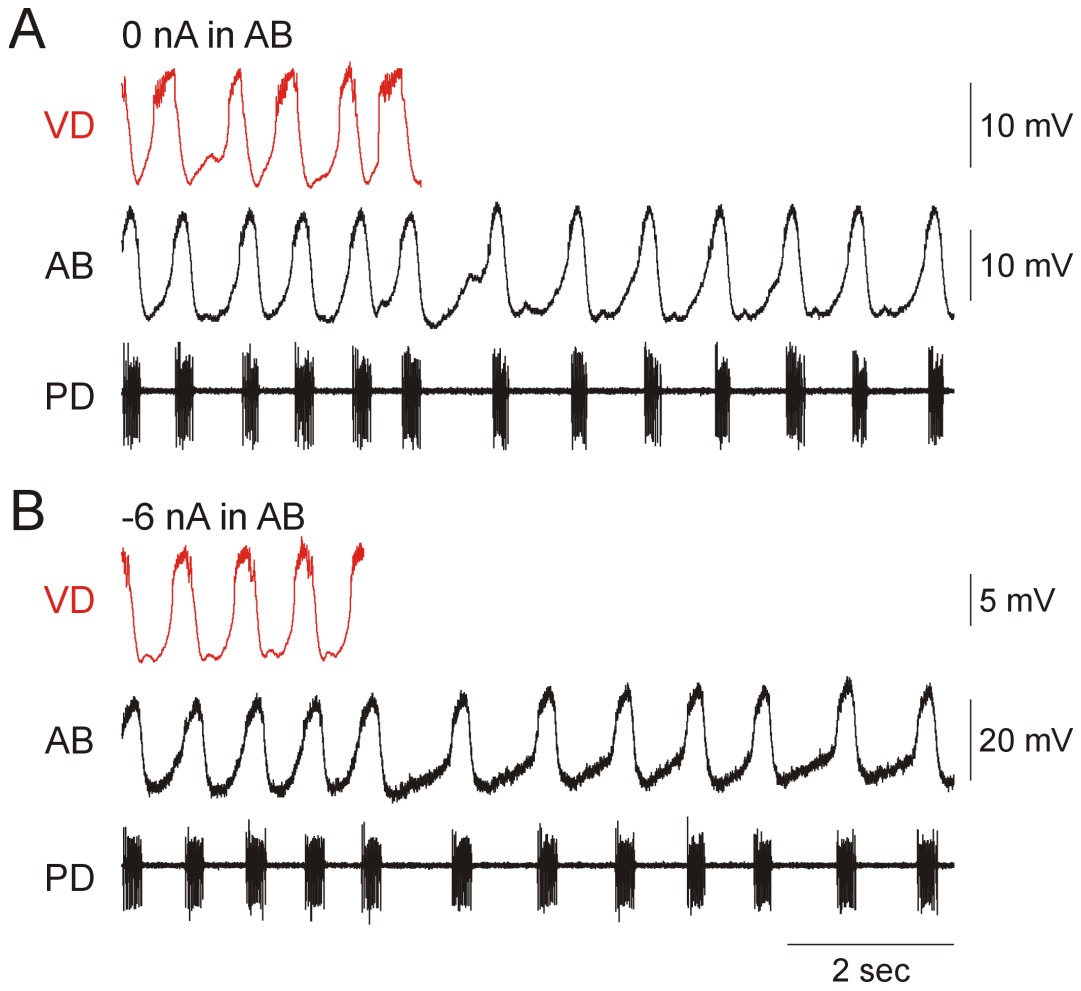

Fig. 7. VD neuron hyperpolarization does not hyperpolarize the AB neuron. In each panel the first trace is an intracellular VD neuron recording, the second is an intracellular AB neuron recording, and the third is an extracellular PD neuron recording. The two panels show the effect of VD neuron hyperpolarization at two AB neuron injection levels.

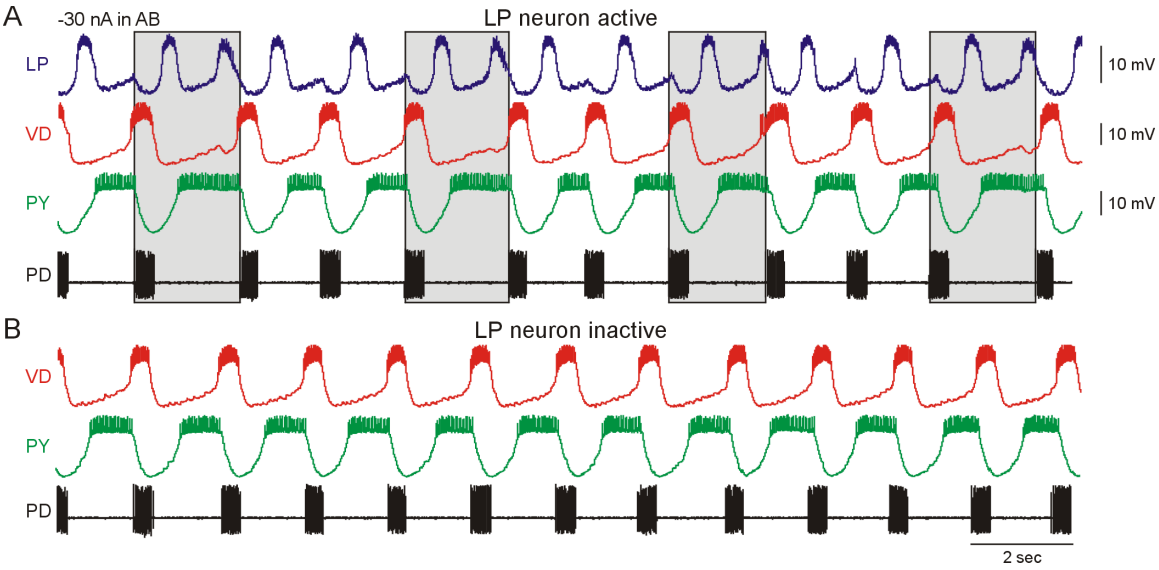

Fig. 8. The LP neuron disrupts pyloric activity when the AB neuron is strongly hyperpolarized (slow cycle periods). In A the top three traces are intracellular recordings of the LP, VD, and PY neurons and the fourth trace is an extracellular PD neuron recording; the LP neuron intermittently fired two bursts per AB/PD neuron burst (gray boxes). Panel B shows 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.

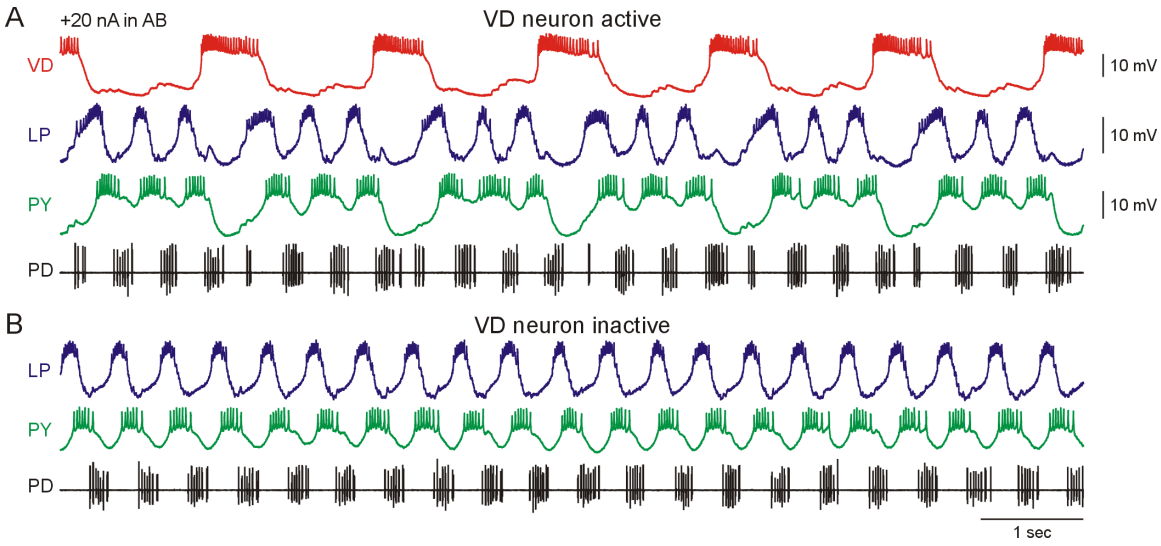

Fig. 9. The VD neuron disrupts pyloric activity when the AB neuron is strongly depolarized (fast cycle periods). In A the top three traces are intracellular recordings of the VD, LP, and PY neurons and the fourth trace is an extracellular PD neuron recording; the VD neuron fired only once for every two to four AB/PD neuron bursts. Panel B shows 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.

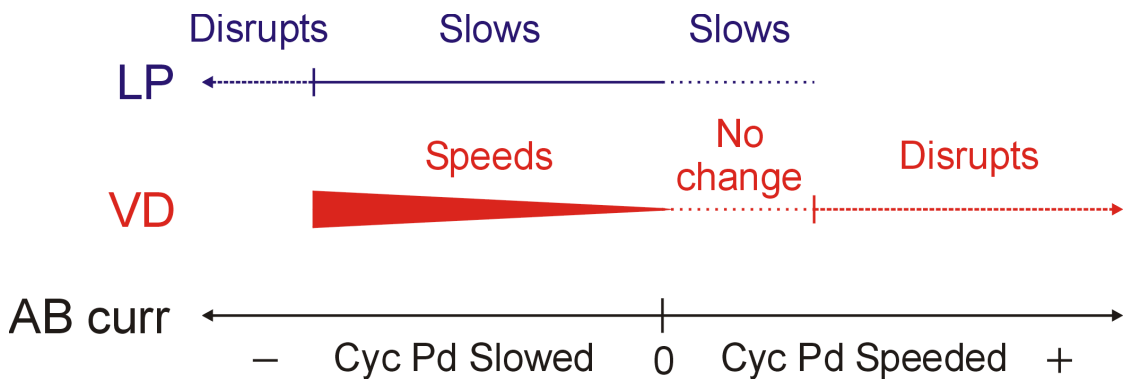

Fig. 10. Schematic diagram summarizing effects of LP and VD neurons on pyloric network activity. The first line shows the effects of the LP neuron, the second those of the VD neuron, and the third the AB neuron current injection level. The triangle for the VD neuron indicates its increasing effect as AB neuron hyperpolarization increases. The LP neuron disrupts pyloric activity at slow cycle periods whereas the VD neuron disrupts 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.

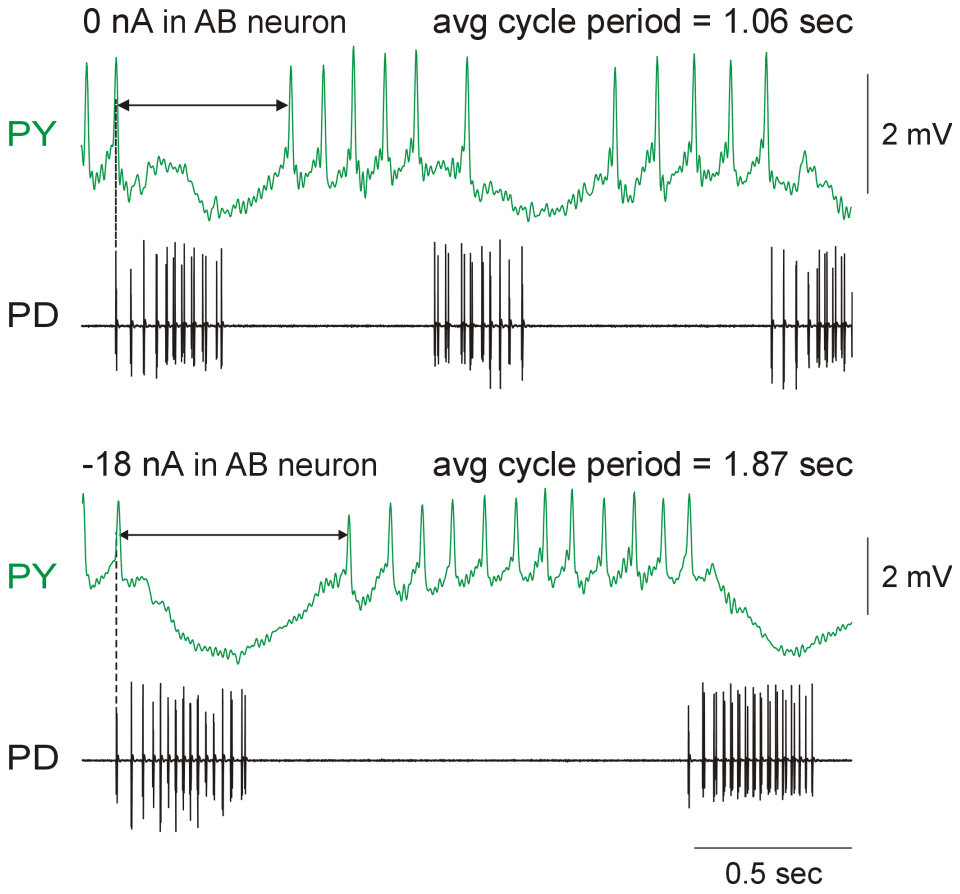

Fig. 11. Changing network cycle period alters delay and spiking activity of pyloric network neurons. This figure shows an example of this phenomenon for the PY neuron when the pyloric cycle period is changed by current injection into the AB neuron. The first trace in the top panel is an intracellular recording from a PY neuron and the second trace is an extracellular recording from a PD neuron when no current is injected into the AB neuron. The double-headed arrow shows the PY neuron delay to firing relative to the beginning of the PD neuron burst. The bottom panel shows the activity of the same neurons when hyperpolarizing current was injected into the AB neuron to slow the network. As cycle period slows, PY neuron burst beginning and ending delay (after PD neuron burst beginning), burst duration, and burst spike number increase.

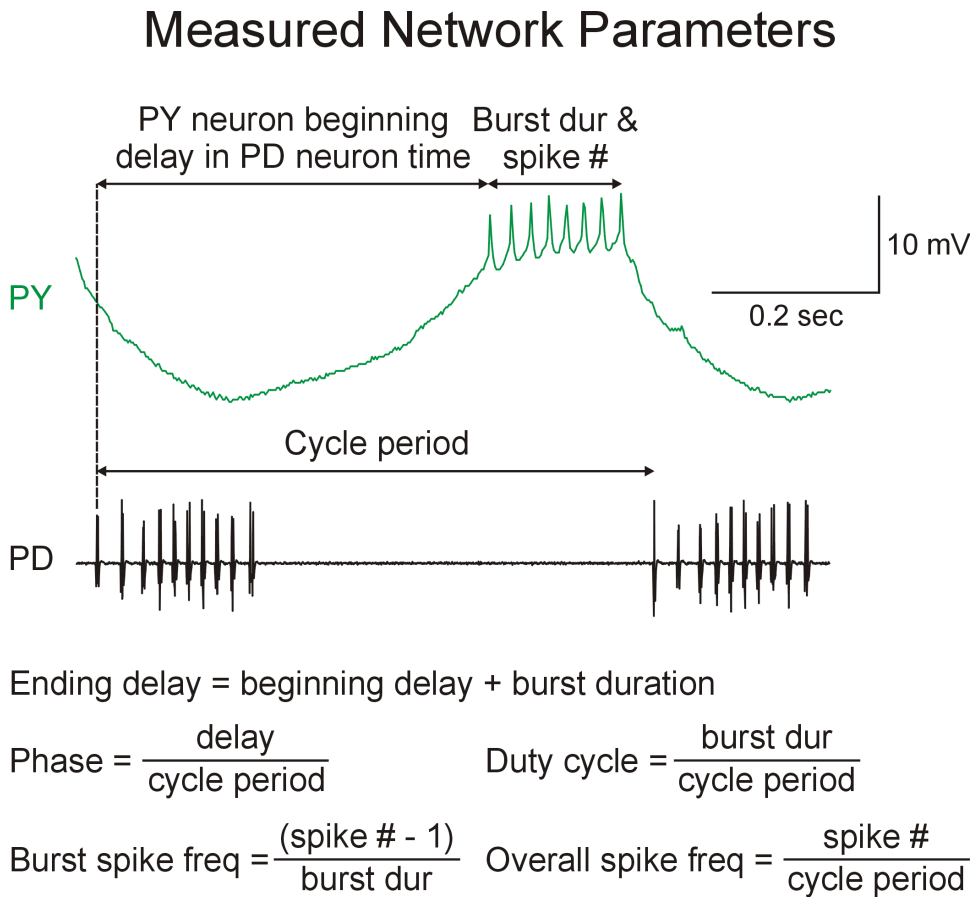

Fig. 12. Computed measures of pyloric neuron phasing and spiking activity Burst beginning and ending delay were measured from the beginning of the PD neuron burst, burst beginning and ending phases were determined by dividing these delays by PD neuron cycle period. Burst duration is the duration between the first and last spike of the burst, duty cycle is burst duration divided by cycle period, burst spike frequency is burst spike number minus 1 divided by burst duration (($spike\#-1$) / *burst duration*), and overall spike frequency is burst spike number divided by cycle period. Since the PD neurons were used to define cycle period, their burst beginning delay and phase were always zero, and their ending delay and phase equal their burst duration and duty cycle.

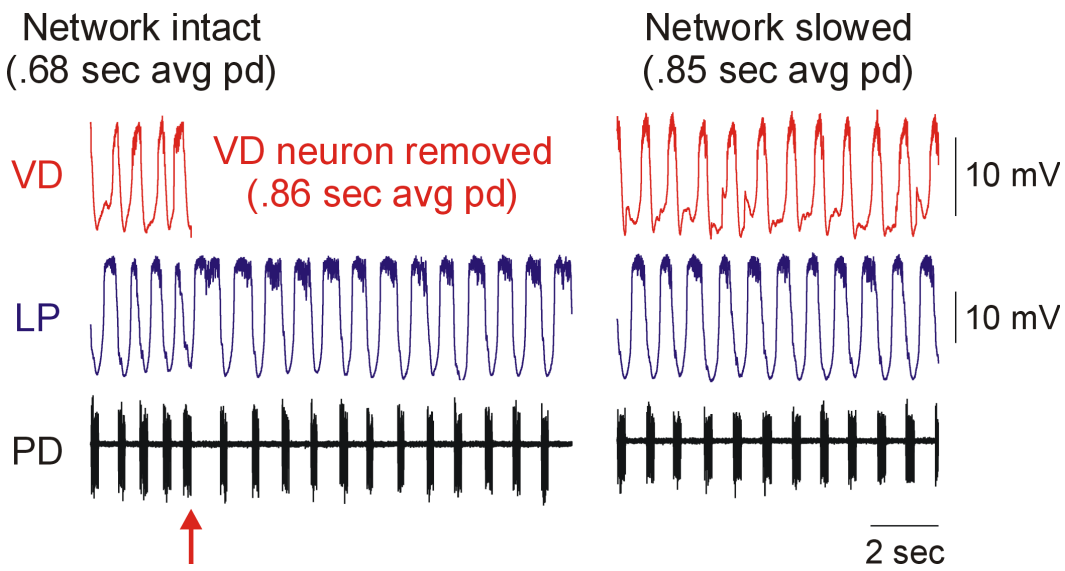

Fig. 13. Typical VD neuron hyperpolarization experimental results. The top two traces are intracellular recordings of a VD and an LP neuron; the third trace is an extracellular recording of PD neuron activity. At the arrow the VD neuron was hyperpolarized well below rest. After a brief transient effect, the network assumed a new pattern in which both cycle period and LP neuron burst duration were increased. As such, it is unclear if the increase in LP neuron activity was a direct effect of VD neuron removal or an indirect effect of the change in cycle period. The left panel shows the activity of the same neurons with the VD neuron active and pyloric cycle period being made (by AB neuron current injection) to match the cycle period observed when the VD neuron was removed (right panel). LP neuron activity under these conditions was similar to that when the VD neuron was removed, suggesting that the changes in LP neuron activity in the right panel were an indirect effect of the cycle period changes induced by VD neuron removal.

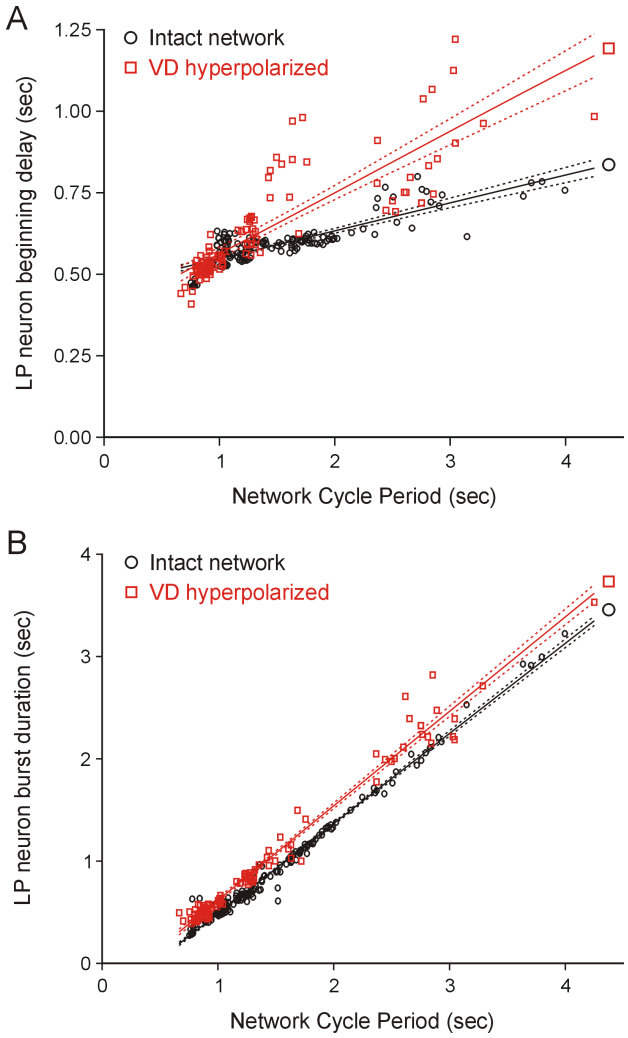

Fig. 14. Variation in LP neuron phasing as cycle period is varied with and without the VD neuron. Typical results for LP neuron beginning delay (A) and burst duration (B) in one VD neuron removal experiment are shown; circles are data from intact network conditions while squares are with the VD neuron hyperpolarized. A best fit line (solid) and 95% confidence interval lines (dashed) were plotted for each data set. In this experiment the confidence interval lines do not overlap over a majority of the cycle period range, suggesting that these data differ in the intact and VD neuron hyperpolarized conditions.

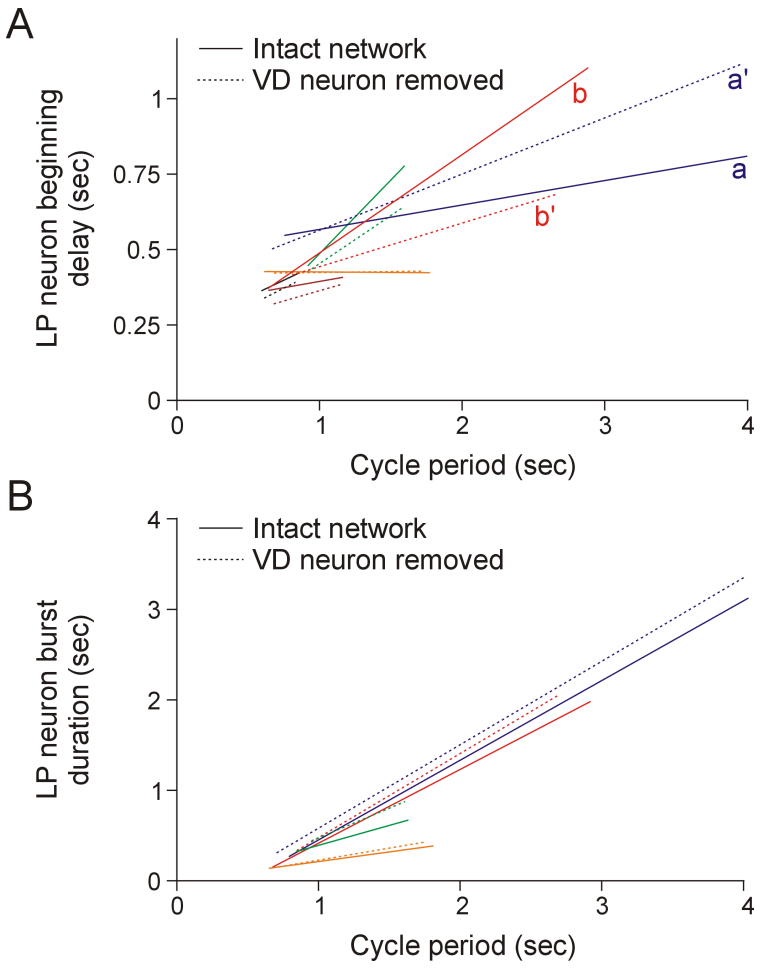

Fig. 15. Multiple comparisons across experiments. Although LP or VD removal often induced significant changes in pyloric activity in individual experiments, these changes were not consistent across experiments. Panel A shows LP neuron beginning delay versus cycle period linear best fits for 5 experiments. Each solid (intact) and dashed (VD neuron removed) line of similar horizontal length represents the results from one experiment. Lines a and a' are the same data shown in Fig. 14; b and b' represent a different experiment. Panel B shows LP neuron burst duration versus cycle period best fits for 4 experiments. Unlike the changes in beginning delay, VD neuron removal induced small, but consistent changes in LP neuron burst duration.

LP Down vs Normal			N	Slope Signif.	Intercept Signif.
PD	Burst Dur / Duty Cycle	Per	6	0.015	1.11E-5
		Freq	6	1.21E-5	0.016
	Spike Number	Per	5	0.991	0.593
		Freq	5	0.357	0.048
	Burst Spike Freq	Per	5	0.401	0.905
		Freq	5	0.166	0.262
	Overall Spike Freq	Per	5	0.313	0.135
		Freq	5	0.257	0.546
VD	Begin Delay/Phase	Per	6	0.470	0.300
		Freq	6	0.909	0.636
	End Delay/Phase	Per	6	0.074	1.04E-5
		Freq	6	3.34E-7	0.027
	Burst Dur / Duty Cycle	Per	6	0.031	3.79E-6
		Freq	6	3.65E-6	0.109
	Spike Number	Per	6	7.19E-7	6.78E-5
		Freq	6	0.072	0.975
	Burst Spike Freq	Per	6	0.086	0.005
		Freq	6	0.220	0.945
	Overall Spike Freq	Per	6	7.85E-8	1.24E-10
		Freq	6	1.13E-6	4.02E-4
IC	Begin Delay/Phase	Per	6	0.113	0.672
		Freq	6	0.477	0.793
	End Delay/Phase	Per	6	0.691	0.503
		Freq	6	0.206	0.294
	Burst Dur / Duty Cycle	Per	6	0.183	0.370
		Freq	6	0.462	0.237
	Spike Number	Per	6	0.073	0.123
		Freq	6	0.169	0.202
	Burst Spike Freq	Per	6	0.423	0.091
		Freq	6	0.627	0.609
	Overall Spike Freq	Per	6	0.152	0.270
		Freq	6	0.267	0.151
PY	Begin Delay/Phase	Per	3	0.184	0.136
		Freq	3	0.043	0.074
	End Delay/Phase	Per	3	0.143	0.016
		Freq	3	5.7E-4	0.027
	Burst Dur / Duty Cycle	Per	3	0.684	0.959
		Freq	3	0.952	0.634
	Spike Number	Per	3	0.288	0.634
		Freq	3	0.635	0.347
	Burst Spike Freq	Per	3	0.312	0.320
		Freq	3	0.232	0.227
	Overall Spike Freq	Per	3	0.468	0.859
		Freq	3	0.547	0.259

VD Down vs Normal			N	Slope Signif.	Intercept Signif.
PD	Burst Dur / Duty Cycle	Per	6	0.232	0.583
		Freq	6	0.849	0.395
	Spike Number	Per	5	0.384	0.567
		Freq	5	0.121	0.015
	Burst Spike Freq	Per	5	0.500	0.620
		Freq	5	3.5E-4	0.029
	Overall Spike Freq	Per	5	0.197	0.520
		Freq	5	0.166	0.102
LP	Begin Delay/Phase	Per	5	0.021	0.059
		Freq	5	0.459	0.136
	End Delay/Phase	Per	5	0.465	0.316
		Freq	5	0.176	0.356
	Burst Dur / Duty Cycle	Per	5	0.005	0.404
		Freq	5	0.575	0.016
	Spike Number	Per	5	0.344	0.709
		Freq	5	0.020	0.016
	Burst Spike Freq	Per	5	1.14E-4	1.43E-8
		Freq	5	0.015	0.654
	Overall Spike Freq	Per	5	0.036	0.006
		Freq	5	0.226	0.638
IC	Begin Delay/Phase	Per	6	0.409	0.051
		Freq	6	0.025	0.393
	End Delay/Phase	Per	6	0.107	0.861
		Freq	6	0.436	0.265
	Burst Dur / Duty Cycle	Per	6	0.272	0.243
		Freq	6	0.021	0.605
	Spike Number	Per	6	0.409	0.370
		Freq	6	0.022	0.020
	Burst Spike Freq	Per	6	0.353	1.61E-4
		Freq	6	0.019	0.553
	Overall Spike Freq	Per	6	0.159	0.105
		Freq	6	0.118	0.202
PY	Begin Delay/Phase	Per	4	0.742	0.301
		Freq	4	0.120	0.538
	End Delay/Phase	Per	4	0.321	0.539
		Freq	4	0.004	0.007
	Burst Dur / Duty Cycle	Per	4	0.738	0.622
		Freq	4	0.772	0.331
	Spike Number	Per	4	0.877	0.849
		Freq	4	0.517	0.170
	Burst Spike Freq	Per	4	0.021	0.012
		Freq	4	0.086	0.129
	Overall Spike Freq	Per	4	0.177	0.427
		Freq	4	0.934	0.821

Fig. 16. Summary table of ANCOVA results. To determine if activity changes were significant across experiments, we utilized a multivariate general linear model (GLM) on the slopes and intercepts of the best-fit lines across experiments. A liberal initial α-level of 0.05 was chosen to increase the chances that significant differences would be seen. However, due to the multiple (88) comparisons, the Dunn-Šidák α-level compensation method had to be employed; the critical α-level for 88 comparisons is 5.8×10^{-4}. Figure 16 summarizes the probabilities obtained from these GLM tests for all measured parameters. The numbers shown in green indicate significance.

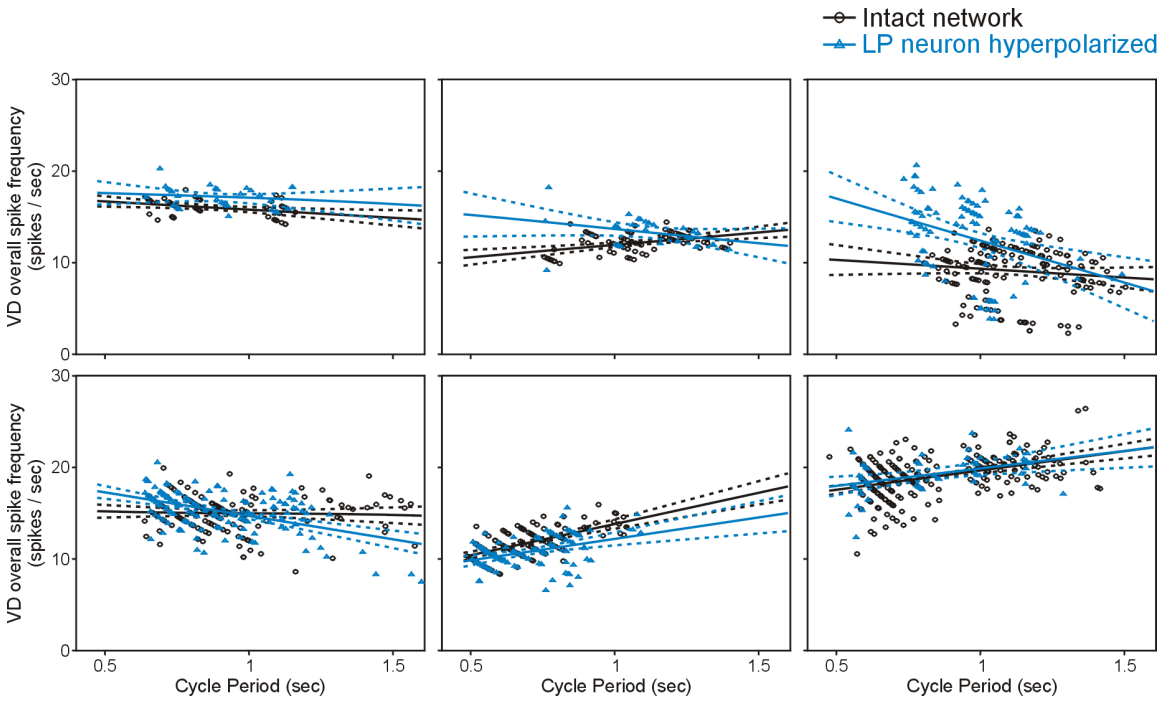

Fig. 17. VD neuron overall spike frequency plots with (circles) and without (triangles) the LP neuron for six experiments (panels A-F). Fig. 16 shows that this parameter had extremely low general linear model *p* values for both best-fit slope and intercept when plotted against either cycle period or cycle frequency. Student *t*-test analysis of this data showed that, in the cycle period case, the data with and without the VD neuron differed with a p values of 0.031 (slope) and 0.035 (intercept) (this was the lowest Student *t*-test *p* value of all comparisons). In panel C it appears that at short cycle periods LP neuron removal increases VD neuron overall spike frequency. However, in the other panels it is less clear that LP neuron removal has any consistent effect. Examination of the data on an experiment-by-experiment basis thus supports the statistical analyses.

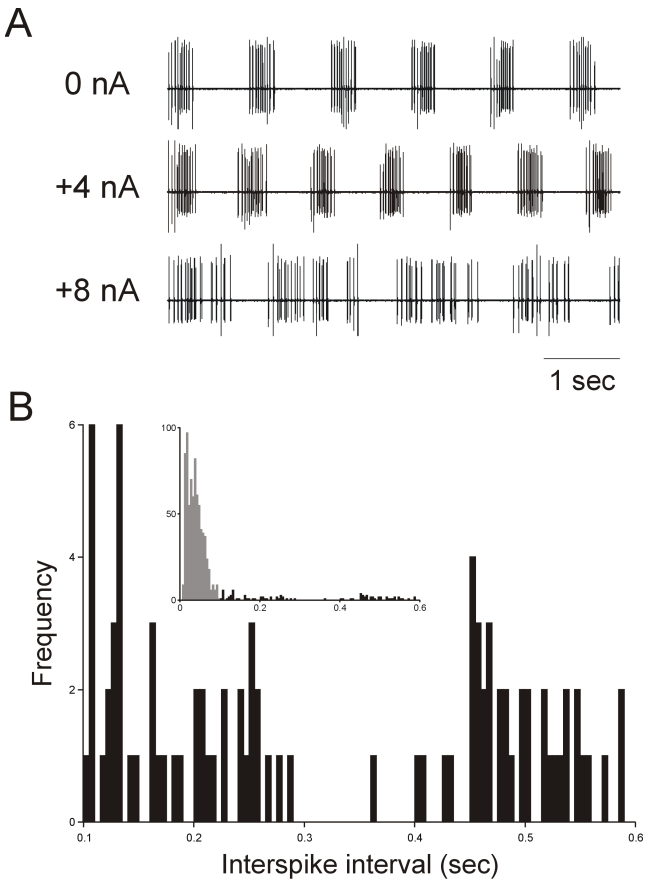

Fig. 18. Response of the PD neuron to changes in AB neuron current injection. Figure 18 shows typical effects from one experiment on PD neuron cycle period as AB neuron current injection is increased. The three traces (top to bottom) of panel A show PD neuron extracellular activity for 0, +4, and +8 nA AB neuron current injection. Cycle period clearly decreases as injected current is increased from 0 to +4 nA with little change in PD neuron burst duration. When the level of injected current was increased to +8 nA, PD neuron spiking activity had a much lower interspike interval, and was much less regular than in the +4 nA case. Panel B is an interspike interval histogram for the data from the +8 nA condition. The inset shows the entire range of data and the larger plot is an expansion of the non-grayed data.

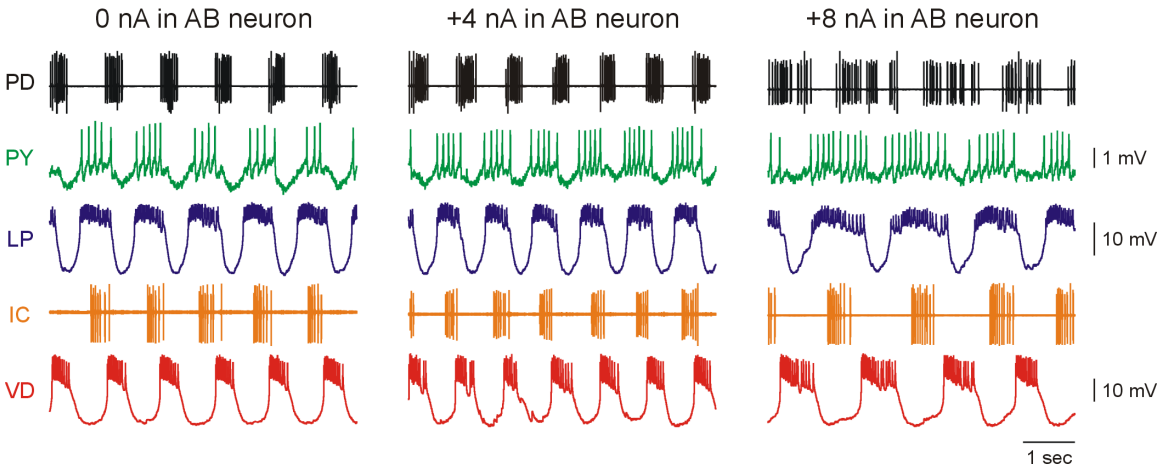

Fig. 19. Response of the entire network to changes in AB neuron current injection. Figure 19 shows the same experimental conditions as in Fig. 18 with the addition of all pyloric neurons except the AB neuron (whose activity, due to their electrical coupling, will likely be similar to that of the PD neuron). Starting from the top, each panel has 5 traces: a PD neuron extracellular trace, PY and LP neuron intracellular traces, an IC neuron extracellular trace, and a VD neuron intracellular trace. For the cases in which 0 and +4 nA were injected into the AB neuron, all the pyloric neurons burst in a normal pattern once per PD neuron cycle period. In the anomalous slowed condition (+8 nA), the network continues to produce a regular motor pattern with clear one to one firing of the PY, LP, IC, and VD neurons. Comparison of this pattern with the PD neuron bursts shows that the long interburst intervals have the same cycle period as the cycling of the other pyloric neurons, supporting our contention that these intervals mark PD neuron cycles.

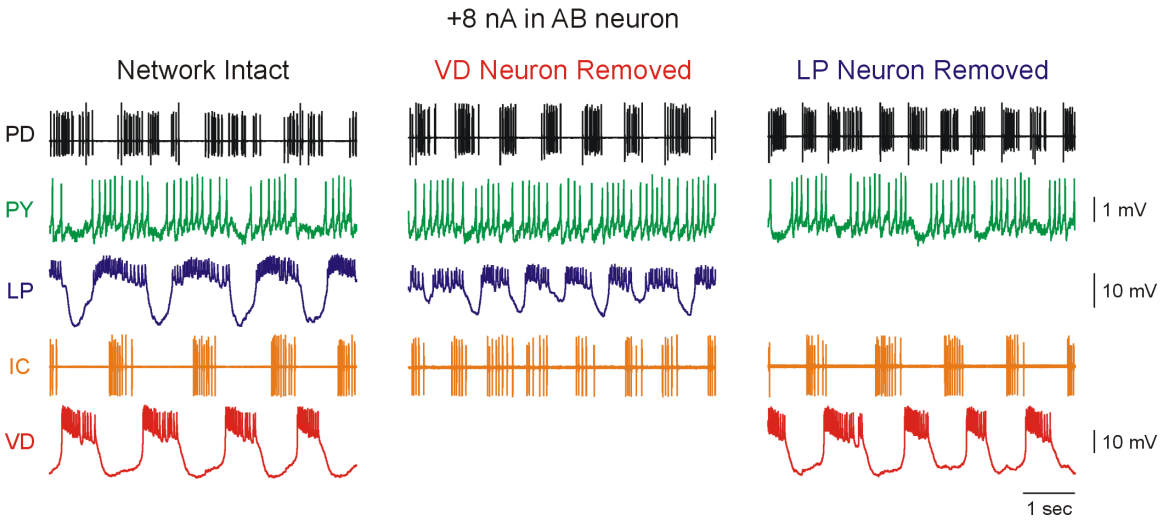

Fig. 20. The role of the LP and VD neurons in this anomalous slowing. Figure 20 shows 3 cases, in each of which +8 nA was injected into the AB neuron; the five neuron recordings are shown in the same order as in Fig. 19. The first panel is with the intact network, and is the same as the third panel of Fig. 19. The second panel is with the VD neuron hyperpolarized. In this case, the network cycled much faster than the +8 nA intact condition, and also faster than the +4 nA intact condition. In the third panel, the LP neuron was hyperpolarized. Without the LP neuron, the PD neuron becomes uncoupled from the rest of the network's neurons, which continue to cycle slowly while the PD neuron fires with an even shorter (although less consistent) period than in the previous cases.

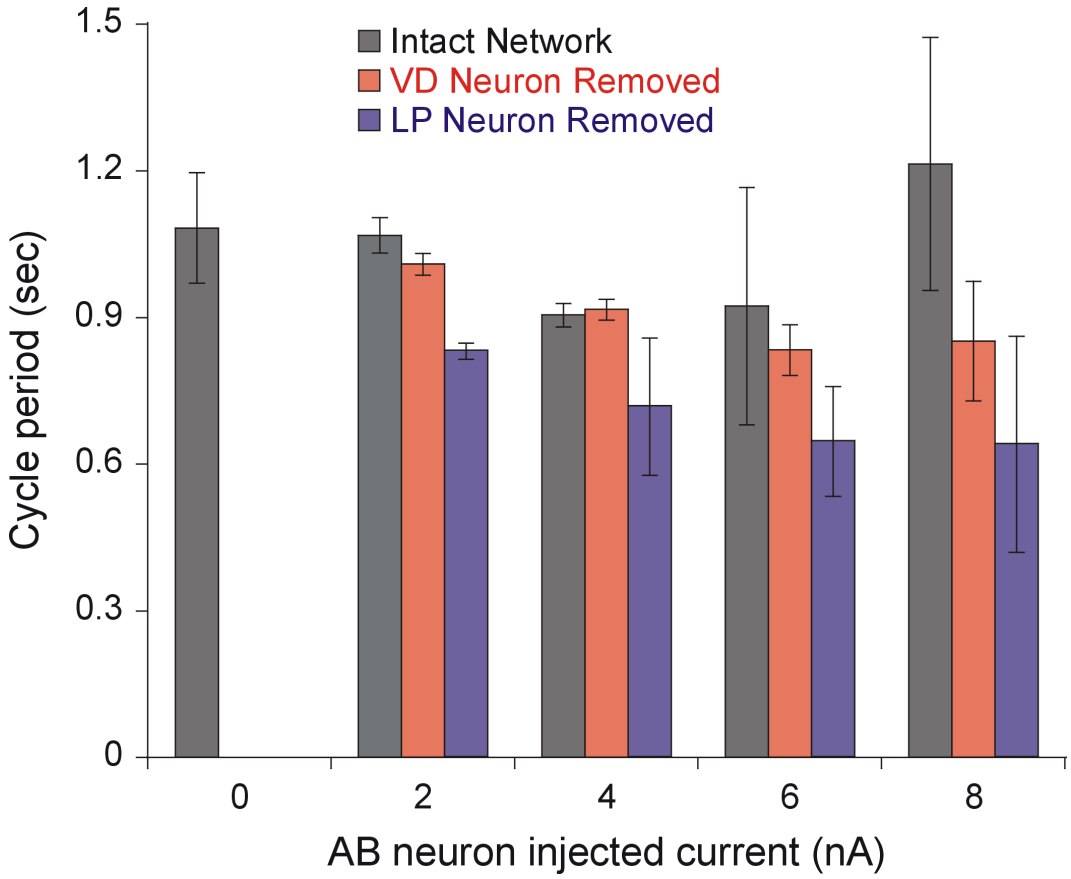

Fig. 21. Summary histogram of cycle period effects of LP and VD neuron removal under different AB neuron current injection conditions. Figure 21 shows the average cycle period and standard deviation of the PD neuron for the intact network (dark gray), VD neuron removed (red), and LP neuron removed (blue) cases at AB neuron current levels of 0, +2, +4, +6, and +8 nA. Similar results were seen in four of the five experiments carried out; in the fifth experiment anomalous slowing was not observed.