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J Neurophysiol 90:2378-2386, 2003. First published Jun 11, 2003; doi:10.1152/jn.00705.2002

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Submitted 19 August 2002; accepted in final form 3 June 2003

Weaver, Adam L. and Scott L. Hooper. Relating network synaptic connectivity and network activity in the lobster (*Panulirus interruptus*) pyloric network. *J Neurophysiol* 90: 2378–2386, 2003. First published June 11, 2003; 10.1152/jn.00705.2002. The lobster pyloric network has a densely interconnected synaptic connectivity pattern, and the role individual synapses play in generating network activity is consequently difficult to discern. We examined this issue by quantifying the effect on pyloric network phasing and spiking activity of removing the Lateral Pyloric (LP) and Ventricular Dilator (VD) neurons, which synapse onto almost all pyloric neurons. A confounding factor in this work is that LP and VD neuron removal alters pyloric cycle period. To determine the effects of LP and VD neuron removal on pyloric activity independent of these period alterations, we altered network period by current injection into a pyloric pacemaker neuron, hyperpolarized the LP or VD neuron to functionally remove each from the network, and plotted various measures of pyloric neuron activity against period with and without the LP or VD neuron. In normal physiological saline, in many (or most) cases removing either neuron had surprisingly little effect on the activity of its postsynaptic partners, which suggests that under these conditions these neurons play a relatively small role in determining pyloric activity. In the cases in which removal did alter postsynaptic activity, the effects were inconsistent across preparations, which suggests that either despite producing very similar neural outputs, pyloric networks from different animals have different cellular and synaptic properties, or some synapses contribute to network activity only under certain modulatory conditions, and the "baseline" level of modulatory influence the network receives from higher centers varies from animal to animal.

INTRODUCTION

Central pattern generator networks underlie rhythmic motor pattern production (Delcomyn 1980; Marder and Calabrese 1996). These networks often produce complicated neural outputs with stereotyped neuron firing order, phase relationships, and spiking activity. Modulatory or sensory input induces many such networks to produce single patterns at multiple cycle periods (fast vs. slow walking) or multiple patterns with differing neuron phasing and spiking activity (skipping vs. walking) (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). Many central pattern generator networks also have highly distributed synaptic connectivities in which each neuron synapses onto, and receives synapses from, a large percentage of the network's neurons. One consequence of such complex connectivity patterns is that these networks can produce multiple outputs because network synapse function changes in different network conditions (Dickinson et al. 1990). An extreme example of changed synaptic function would be synapses that were functionally important in only certain network conditions.

The lobster (*Panulirus interruptus*) pyloric network is a highly interconnected network whose six neuron types make 20 intra-network synapses (Eisen and Marder 1982; Selverston et al. 1976); a completely interconnected six-neuron network would make 30. The network also receives a variety of inputs that alter network output (Harris-Warrick and Marder 1991). This network is thus highly suitable for investigating the function of specific synapses under different network conditions. We examined this issue using the Lateral Pyloric (LP) and Ventricular Dilator (VD) neurons in the network ground (physiological saline without exogenously applied modulation) condition. The network contains only one LP and one VD neuron, and the effect of their synapses on network activity can be assessed by individually hyperpolarizing them.

Interpreting these data is difficult because removing either neuron alters pyloric cycle period (Weaver and Hooper 2003), and any effects of neuron removal could thus arise merely from the cycle period changes. To control for this confounding factor, we altered pyloric cycle period by injecting varying levels of current into the network's pacemaker Anterior Burster (AB) neuron and then sequentially hyperpolarized the LP and VD neurons (see DISCUSSION). We could thus observe network activity with and without the LP or VD neuron across a wide cycle period range and so determine any changes their absence induces independent of cycle period.

We measured beginning and ending delay/phase, burst duration/duty cycle, burst spike number, intraburst spike frequency, and overall spike frequency (burst spike number/cycle period) for all pyloric neurons. Under the experimental conditions used here, some LP and VD neuron synapses appeared to play no role in determining postsynaptic target spiking activity or phasing. Other synapses did have significant effects, but the nature of these effects varied from animal to animal, and the significance depended on only a few, strongly affected preparations. These data suggest two hypotheses. The first is that pyloric cellular and synaptic properties vary from animal to animal, and in only some animals were these properties such

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that, in the "ground" state, LP and/or VD neuron input altered the activity of the postsynaptic neuron in question. The second is that in different experiments the network was receiving different modulatory input from higher centers, under the influence of only some of which did LP or VD neuron output alter postsynaptic target activity.

A preliminary account of some of these data has appeared in abstract form (Weaver and Hooper 2000).

METHODS

Lobsters (0.5–1 kg) of both sexes were obtained from Don and Laurice Tomlinson Commercial Fishing (San Diego, CA) and maintained in aquaria with 10–15°C circulating artificial seawater. Stomatogastric nervous systems were dissected using standard techniques (Selverston et al. 1976). The stomatogastric nerve, which carries input from the rest of the stomatogastric nervous system to the pyloric network, was intact in all experiments. *Panulirus* saline (pH 7.5–7.6) was composed of (in mM) 479 NaCl, 12.8 KCl, 13.7 $CaCl_2$, 3.9 Na_2SO_4, 10.0 $MgSO_4$, 10.9 glucose, 11.1 Tris base, and 5.1 maleic acid, obtained from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Nerve recordings were made with stainless steel pin electrodes insulated with petroleum jelly and an A-M Systems (Everett, WA) differential amplifier. Intracellular recordings and stimulations were made with an Axoclamp 2A or 2B (Foster City, CA) using 10- to 20-MΩ glass microelectrodes filled with 0.55 M K_2SO_4, 0.02 M KCl. Data were recorded on a Microdata (S. Plainfield, NJ) DT-800 digital tape recorder, digitized with a Cambridge Electronic Design (CED, Cambridge, UK) 1401*plus* interface, and analyzed using CED Spike2 software. Statistical tests (multivariate general linear model/analysis of covariance with Dunn-Šidák α-level compensation for multiple comparisons, nominal α-level 0.05) were performed with SPSS (Chicago, IL). Plots and 95% confidence lines were generated with Microcal Origin (Northampton, MA) and figures 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 blocked or at least greatly reduced graded synaptic release (see DISCUSSION). The same electrode was used for voltage recording and current injection. Hyperpolarized neurons were monitored for escape by examination of extracellular recordings, absence of inhibitory postsynaptic potentials in the neuron's postsynaptic partners, and when possible, observing neuron activity with a bridge-balanced electrode. Pyloric output was measured for 6–10 cycles with the LP and VD neurons both present, with the LP neuron absent, and with the VD neuron absent. Fewer than 10 cycles were used when pyloric activity was perturbed by escapes from hyperpolarization or interference from other stomatogastric nervous system networks (gastric mill, cardiac sac) (Bartos and Nusbaum 1997; Bartos et al. 1999; Marder et al. 1998; Mulloney 1977; Nadim et al. 1998; Thuma and Hooper 2002, 2003). In cases in which different cycle period ranges were obtained from the intact and LP or VD neuron removed cases, only data from overlapping cycle period ranges were used. The data presented here are from 10 experiments, with 3–6 experiments for each parameter and neuron.

RESULTS

The pyloric network consists of 14 neurons, divided into 6 neuron types, interconnected by inhibitory chemical synapses (○ and ●) and electrical coupling (resistors and diodes) (Fig. 1*A*). The AB neuron is an endogenous oscillator (pacemaker) neuron. The PD and AB neurons are electrically coupled and

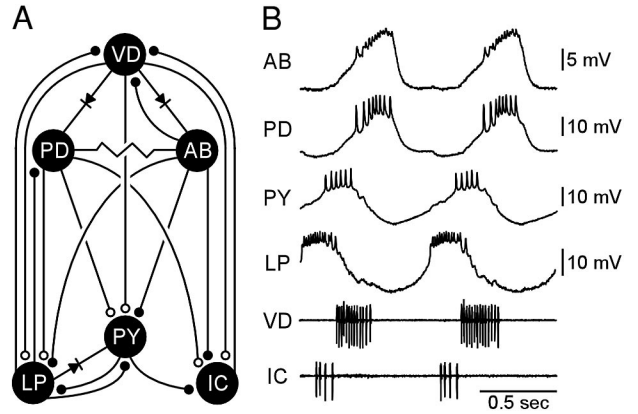

FIG. 1. Pyloric network synaptic connectivity (*A*) and typical output (*B*). The pyloric pattern is a triphasic rhythm in which the Anterior Burster (AB)/Pyloric Dilator (PD) neuron pacemaker ensemble fires, then the Lateral Pyloric (LP) and Inferior Cardiac (IC) neurons fire, and then the Ventricular Dilator (VD) and Pyloric (PY) neurons fire, after which the pattern repeats. Synaptic connectivity symbols: ○, inhibitory cholinergic synapse; ●, inhibitory glutamatergic synapse; resistor, electrical coupling; diode, rectifying electrical synapse.

form the 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 inhibits the LP, IC, and PY neurons and makes a rectifying electrical synapse onto the PD and AB neurons (Eisen and Marder 1982; Johnson et al. 1993; Selverston et al. 1976). LP and VD neuron synapses could thus directly alter the activity of most (LP neuron) or all (VD neuron) pyloric neurons. The pyloric output pattern (Fig. 1*B*) is a rhythmic (0.5- to 2.0-s 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.

We investigated the effect of the LP and VD neurons on pyloric phasing and spiking activity by alternately hyperpolarizing the LP and VD neurons to functionally remove them from the network. Hyperpolarization was used instead of photoinactivation (Miller and Selverston 1979) because hyperpolarization is reversible, and the effects of removing both follower neurons can thus be tested in each preparation. A difficulty in this work was that LP or VD neuron removal changes pyloric cycle period (Weaver and Hooper 2003), which itself alters pyloric neuron activity (Hooper 1997). Figure 2 shows an example of how changing cycle period alone can alter network activity. The *top panel* shows PY (*1st trace*, intracellular recording) and PD (*2nd trace*, extracellular recording) neuron activities with no current injected into the AB neuron; ↔ shows PY neuron firing delay relative to PD neuron burst beginning. When cycle period was increased by AB neuron hyperpolarization, PY neuron burst beginning and ending delay (relative to PD neuron burst beginning), burst duration, and burst spike number increased (*bottom*).

A variety of measures of pyloric neuron activity were made (Fig. 3). Burst beginning and ending delay were measured from PD neuron burst beginning; phase was obtained by dividing these delays by PD neuron cycle period. Burst duration is the time between a burst's first and last spike, duty cycle is burst duration divided by cycle period, burst spike frequency is burst

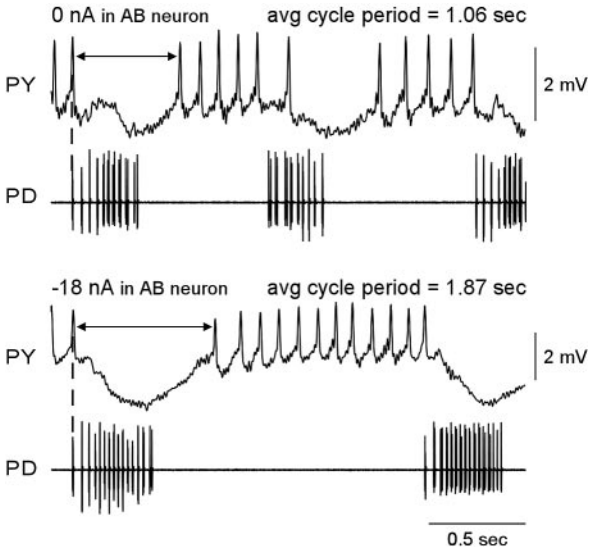

FIG. 2. Changing network cycle period (by current injection into the AB neuron) alters pyloric neuron activity. *Top*: intracellular PY (*top trace*) and extracellular PD (*bottom trace*) neuron recordings with no AB neuron current injection. ↔, PY neuron firing delay relative to PD neuron burst beginning. *Bottom*: the activity of the same neurons when the network was slowed by AB neuron hyperpolarization; PY neuron burst beginning and ending delay, burst duration, and burst spike number increased.

spike number minus 1 divided by burst duration, and overall spike frequency is burst spike number divided by cycle period. Because PD neuron activity defined cycle period, for PD neurons, burst beginning delay and phase were always zero and ending delay and phase equaled 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 1997). 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 4*A* shows a typical experiment. The *top two traces* are VD and LP neuron intracellular recordings, the *third trace*

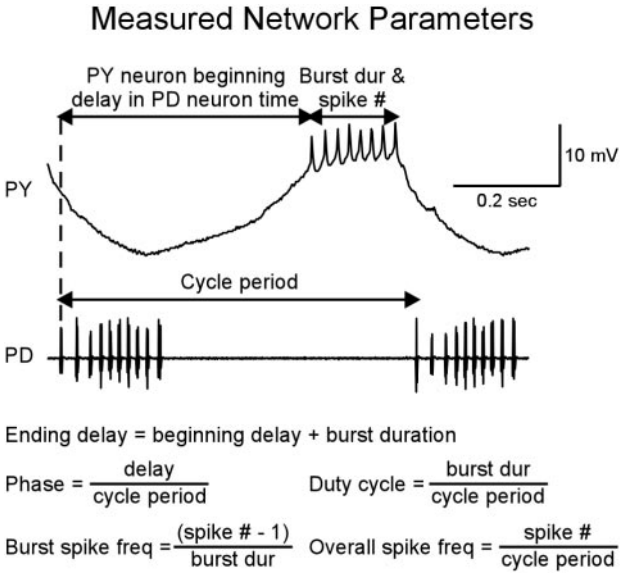

FIG. 3. Computed measures of pyloric neuron phasing and spiking activity. See text for explanation.

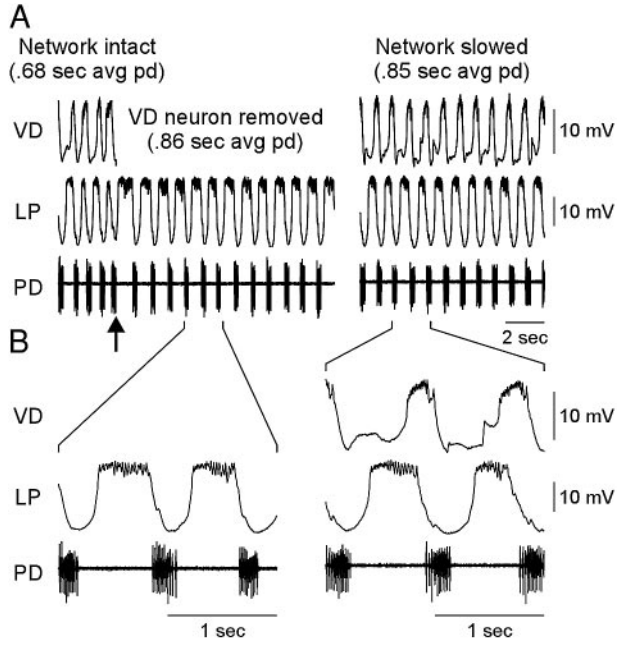

FIG. 4. Typical VD neuron removal data. *A*: intracellular VD and LP (*top 2 traces*) and extracellular PD (*3rd trace*) neuron recordings. *Left*, ↑: the VD neuron was hyperpolarized well below rest. After a brief transient, the network produced a new pattern in which cycle period and LP neuron burst duration increased. *Right*: activity of the same neurons with the VD neuron active and cycle period being made (by AB neuron hyperpolarization) to match the period observed after VD neuron removal. *B*: time expansions of recordings in *A*. LP neuron activity in the slowed, intact condition was similar to that after VD neuron removal.

is a PD neuron extracellular recording. At ↑, the VD neuron was hyperpolarized well below rest. After a brief transient, the network adopted a new pattern in which cycle period and LP neuron burst duration increased. It was thus unclear if the increased LP neuron activity was a direct effect of VD neuron removal or an indirect effect of the period change. The *right panel* shows the activity of the same neurons with the VD neuron active and cycle period being made (by AB neuron hyperpolarization) to match the period observed after VD neuron removal. Figure 4*B* shows time expansions of the recordings from *A*. LP neuron activity when the VD neuron was removed (Fig. 4*B*, *left*), and in the intact, slowed network (Fig. 4*B*, *right*), was very similar, suggesting that the changes in LP neuron activity were primarily due to the cycle period change induced by VD neuron removal.

To remove the confounding effects of the cycle period variation induced by LP and VD neuron removal, current was injected into the AB neuron to vary cycle period, and at each injection level, the LP and VD neurons were alternately hyperpolarized. Pyloric phasing and spiking parameters were plotted against cycle period or cycle frequency to observe the effects of LP or VD neuron removal at matched cycle periods. Figure 5 shows the effect of VD neuron removal on LP neuron beginning delay (*A*) and burst duration (*B*) in one experiment. ○ are data from intact network conditions, □ are with the VD neuron hyperpolarized. Best fit (—) and 95% confidence interval (- - -) lines are 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. A general

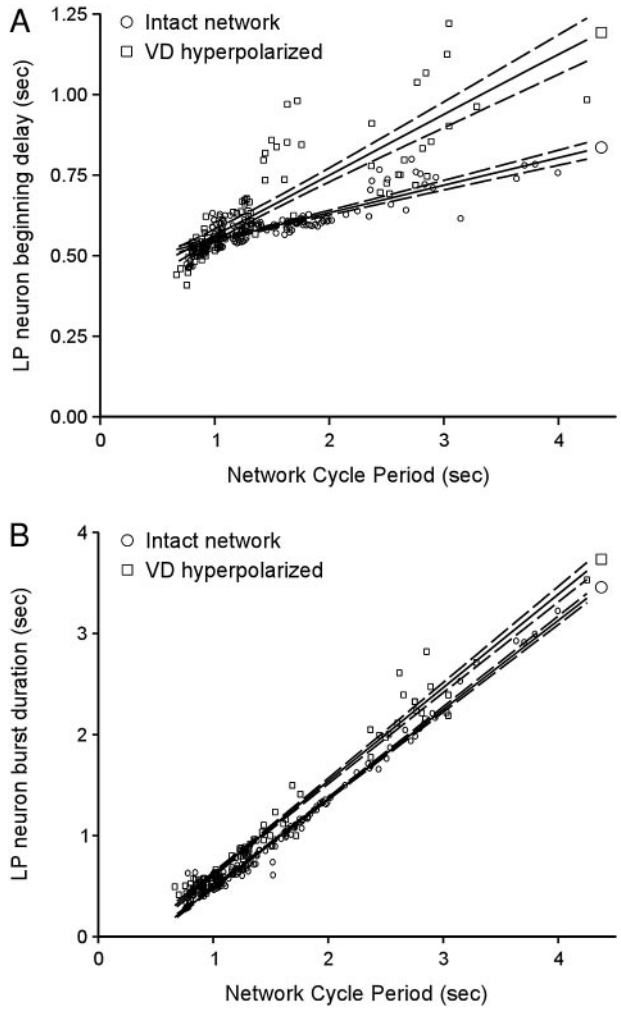

FIG. 5. Effect of VD neuron removal on LP neuron beginning delay (*A*) and burst duration (*B*) in 1 experiment (○ are data from intact network; □ with the VD neuron hyperpolarized). Best fit (—) and 95% confidence interval lines (- - -) are plotted for each data set. In this experiment, the confidence interval lines do not overlap over most of the cycle period range, suggesting these data differ in the intact and VD neuron hyperpolarized conditions.

linear model statistical comparison of the data confirms this suggestion.

Similar experiment by experiment analyses were performed for all measured parameters (Fig. 6). These analyses showed that in 16 (bold) of the 44 measured parameters LP neuron removal and in 21 of the parameters VD neuron removal, had significant (α-level 0.5) effects on half or more of the experiments. Figure 7 shows the pronounced experiment by experiment variation in the effectiveness of LP neuron removal on VD neuron overall spike frequency (this parameter was most consistently—4 of 6 experiments—affected by LP neuron removal). Comparison of the data and best fit and 95% confidence lines shows that in only *experiments B–D* did LP neuron removal result in large changes in activity. The intercept, but not slope, of *experiment F* is also statistically significant, but the change is clearly nonetheless very small.

This observation raised the disturbing possibility that the variation in the effects of neuron removal we observed was due to variation in pyloric network "ground state" among our preparations (because networks from different animals do not have identical synaptic strengths and membrane properties even in the absence of modulation, because in different preparations different descending input to the networks were active, or because of variable damage to the networks during dissection). Figure 8 shows additional evidence for this possibility, which shows best fit lines for LP neuron beginning delay versus cycle period for five experiments. Each — (intact) and - - - (VD neuron removed) line of similar horizontal length are data from one experiment. Lines a and a′ are the data shown in Fig. 5; lines b and b′ are from a different experiment. In experiment a/a′, VD neuron removal increased LP neuron beginning delay, and in experiment b/b′, VD neuron removal decreased LP neuron beginning delay. In the other experiments, VD neuron removal had intermediate effects on LP neuron beginning delay. Nonetheless, in four of the five experiments, the changes were significant. These data could thus be interpreted as indicating that VD neuron removal does alter LP neuron beginning delay, but that that effect ranges, depending on experimental preparation, all the way from increasing to decreasing beginning delay.

State-dependent effects of modulator application have been observed at least once in the pyloric system in which proctolin's effect on AB neuron cycle period depended on AB neuron preapplication cycle period (Nusbaum and Marder 1989). We attempted to associate the variation in the effects of neuron removal we observed with differences in control activity such as network cycle period and various measure of pyloric neuron spiking activity but were unsuccessful. From this data set, we are thus unable to resolve the question of whether the variation in effects we observe is true state dependence or statistical variation.

However, we can use an across experiment multivariate general linear model (GLM) to identify at least in which parameters neuron removal significantly changes best fit line slope and intercept. An intercept difference without a change in slope indicates that neuron removal changed the measured parameter by a constant amount across the entire independent variable (cycle period or frequency, as appropriate) range (e.g., the line was just raised or lowered relative to control). A slope difference without a change in intercept indicates neuron removal increased or decreased the rate of change of the parameter in question as cycle period changes, but the value of the parameter at a cycle period of zero is unchanged. Changes in both intercept and slope indicate a combination of these two effects was occurring. The GLM model is independent of the direction of the induced changes and thus would be unaffected by the variation shown in Fig. 8. A liberal initial α-level of 0.05 was chosen. However, due to the multiple (44) comparisons for each neuron removed condition, the Dunn-Šidák α-level compensation method had to be employed; the critical α-level for 44 comparisons was $1.17 \cdot 10^{-3}$.

Figure 9 shows the 17 cases (13 LP neuron and 4 VD neuron) in which significant differences (bold) between control and LP or VD neuron removed cases were found. LP removal significantly affected various aspects of PD, VD, and PY neuron activity (the lack of effect on the IC neuron is not surprising as the LP neuron does not synapse onto this neuron). VD neuron removal significantly affected various aspects of PD, LP, and IC neuron activity but not, despite the VD to PY neuron inhibitory synapse, PY neuron activity. However, it is critical to note that comparison to Fig. 6 shows that in many of

LP Down vs Normal			N	# Signif. Slope	# Signif. Intercept
PD	Burst Dur / Duty Cycle	Per	6	2	2
		Freq	6	2	2
	Spike Number	Per	5	1	1
		Freq	5	1	1
	Burst Spike Freq	Per	5	1	1
		Freq	5	1	2
	Overall Spike Freq	Per	5	1	1
		Freq	5	1	1
VD	Begin Delay/Phase	Per	6	2	1
		Freq	6	1	2
	End Delay/Phase	Per	6	**3**	**3**
		Freq	6	**3**	**3**
	Burst Dur / Duty Cycle	Per	6	2	**3**
		Freq	6	**3**	2
	Spike Number	Per	6	**3**	**3**
		Freq	6	**3**	2
	Burst Spike Freq	Per	6	2	1
		Freq	6	2	**4**
	Overall Spike Freq	Per	6	**3**	**4**
		Freq	6	**3**	**3**
IC	Begin Delay/Phase	Per	6	2	2
		Freq	6	**3**	**3**
	End Delay/Phase	Per	6	1	1
		Freq	6	1	1
	Burst Dur / Duty Cycle	Per	6	1	1
		Freq	6	1	1
	Spike Number	Per	6	2	1
		Freq	6	0	0
	Burst Spike Freq	Per	6	1	1
		Freq	6	1	1
	Overall Spike Freq	Per	6	1	1
		Freq	6	1	1
PY	Begin Delay/Phase	Per	3	0	0
		Freq	3	1	0
	End Delay/Phase	Per	3	0	1
		Freq	3	1	1
	Burst Dur / Duty Cycle	Per	3	0	0
		Freq	3	0	0
	Spike Number	Per	3	0	0
		Freq	3	0	0
	Burst Spike Freq	Per	3	1	1
		Freq	3	1	1
	Overall Spike Freq	Per	3	0	0
		Freq	3	0	0

VD Down vs Normal			N	# Signif. Slope	# Signif. Intercept
PD	Burst Dur / Duty Cycle	Per	6	**4**	2
		Freq	6	2	**4**
	Spike Number	Per	5	0	0
		Freq	5	1	2
	Burst Spike Freq	Per	5	2	1
		Freq	5	2	**3**
	Overall Spike Freq	Per	5	0	0
		Freq	5	0	1
LP	Begin Delay/Phase	Per	5	**4**	**4**
		Freq	5	**3**	**4**
	End Delay/Phase	Per	5	**3**	1
		Freq	5	1	**3**
	Burst Dur / Duty Cycle	Per	5	**5**	1
		Freq	5	1	**4**
	Spike Number	Per	5	1	1
		Freq	5	2	2
	Burst Spike Freq	Per	5	**3**	**4**
		Freq	5	**4**	1
	Overall Spike Freq	Per	5	2	2
		Freq	5	2	2
IC	Begin Delay/Phase	Per	6	**3**	1
		Freq	6	2	**3**
	End Delay/Phase	Per	6	2	1
		Freq	6	1	2
	Burst Dur / Duty Cycle	Per	6	1	0
		Freq	6	0	1
	Spike Number	Per	6	0	0
		Freq	6	2	2
	Burst Spike Freq	Per	6	0	0
		Freq	6	1	2
	Overall Spike Freq	Per	6	0	0
		Freq	6	1	1
PY	Begin Delay/Phase	Per	4	1	0
		Freq	4	0	0
	End Delay/Phase	Per	4	**3**	**2**
		Freq	4	**2**	**3**
	Burst Dur / Duty Cycle	Per	4	0	0
		Freq	4	0	0
	Spike Number	Per	4	1	0
		Freq	4	**2**	**2**
	Burst Spike Freq	Per	4	1	1
		Freq	4	**2**	1
	Overall Spike Freq	Per	4	0	0
		Freq	4	1	1

FIG. 6. For most measured parameters, in only a minority of experiments did LP (*left*) or VD (*right*) neuron removal alter pyloric activity. General linear model statistical comparisons of intact and LP or VD neuron removed activity were performed on an experiment by experiment basis for all measure parameters. The numbers in the "# Signif. Slope" and "# Signif. Intercept" columns are the numbers of experiments in which removal affected the parameter in question, and column "*N*" is the number of experiments in which the parameter was measured. Burst duration, beginning delay, and ending delay were compared relative to cycle period. Duty cycle, beginning phase, and ending phase were compared relative to cycle frequency. All other parameters were compared relative to both cycle period and frequency. Per., period; Freq., frequency; Dur., duration; Signif., significance. Bold text indicates instances in which neuron removal significantly altered half or more experiments.

these cases, it is the presence of only a few strongly affected experiments that results in a significant difference being found. LP neuron removal affected PD neuron burst duration/duty cycle, and PY neuron ending delay, in only two of six and one of three experiments, respectively, when the experiments were analyzed individually. VD neuron removal affected PD neuron burst spike frequency, and IC neuron burst spike frequency, in only two of five and zero of six experiments, respectively, when the experiments were analyzed individually.

Taken together, these data suggest that the effects of the LP neuron on PD and PY neuron spiking and phasing activity, and of the VD neuron on PD and IC neuron activity, if they are real, either occur in only a minority of our experiments (LP neuron to PD and PY neurons, VD neuron to PD neuron), or are too weak to be observed in individual experiments (VD to IC neuron). The most common and strongest effects of the LP and VD neurons are thus on each other, with the LP neuron affecting a wide range of VD neuron activity parameters and the VD neuron primarily affecting only LP neuron burst spike frequency (Fig. 9).

Examination of the data in these cases, however, suggested that these significances resulted from data at cycle periods far

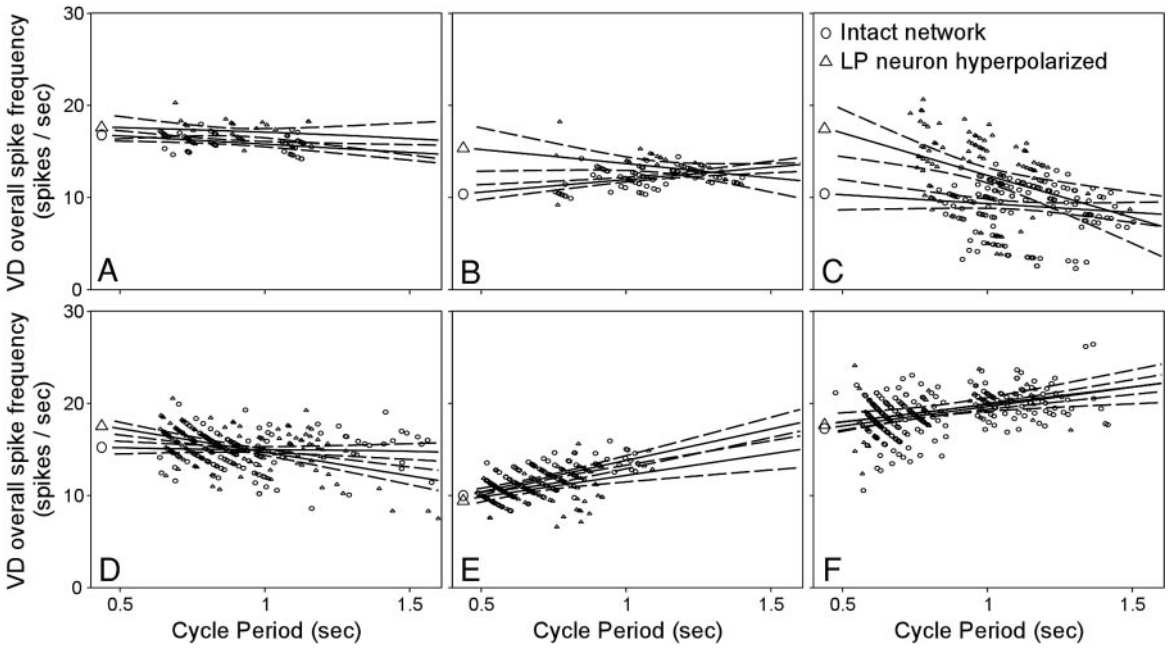

FIG. 7. VD neuron overall spike frequency plots with (○) and without (△) the LP neuron for 6 experiments (*A–F*). In only *experiments B–D* does LP neuron removal have visually apparent effects on data points or best fit and 95% confidence lines.

from the intact network rest cycle period. Although these cycle periods are within the network's physiological cycle period range, in the animal, they are unlikely to be achieved by conductance changes analogous to current injection into the AB neuron. The comparison of the greatest physiological interest is therefore between intact and neuron removed data near rest cycle period. We consequently examined the effects of LP and VD neuron removal in a data set restricted to points with cycle periods within ±25% of rest period for all parameters for which the GLM showed a significant effect (Fig. 9) and in which half or more of the individual experiments were significantly affected (Fig. 6; for the LP-neuron-removed case, VD neuron end delay/phase, burst duration/duty cycle, spike number, and overall spike frequency; for the VD-neuron-removed case, LP neuron burst spike frequency). Because this restriction removed cycle period variation as a confounding variable, the intact and neuron removed data could be compared by a simple repeated measure *t*-test (except that, for the LP-neuron-removed case, Dunn-Šidák multiple measures α-level compensation again had to be used; the critical α-level for 6 comparisons was 0.085 for a nominal α-level 0.05). Comparison of these data showed that LP or VD neuron removal had no significant effect on any parameter near rest cycle periods. Strikingly, this lack of significance remained even when data were used only from experiments in which, when the entire cycle period range was examined, a significant change was observed in the individual GLM tests (e.g., for VD neuron overall spike frequency, only data from *experiments B–D* and *F*, Fig. 7).

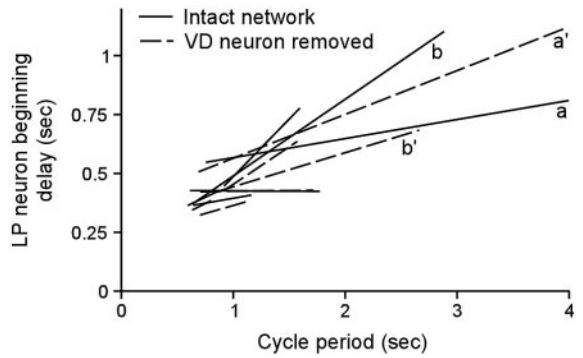

FIG. 8. VD removal did not consistently change LP neuron activity. LP neuron beginning delay vs. cycle period linear best fits for 5 experiments. Each — (intact) and - - - (VD neuron removed) line of similar horizontal length represents data from 1 experiment. Lines a and a′ are the same data shown in Fig. 5; b and b′ are from a different experiment.

DISCUSSION

This work was motivated by the hypothesis that some pyloric synaptic connections have functional relevance only in certain network states (as induced by modulatory or sensory inputs). To this end, we analyzed the effects of LP or VD neuron removal on all other pyloric neurons. The most powerful of these analyses was a multivariate general linear model (Fig. 9). This analysis unambiguously supports the hypothesis for at least one pyloric network connection, the VD to PY neuron synapse, for which VD neuron removal altered no parameter.

However, detailed comparison of the cases in which the grouped GLM did find significance suggests that the hypothesis is likely also true for all LP and VD neuron synapses, at least near rest cycle period. First, in one case (the effect of VD neuron removal on IC neuron burst spike frequency), no individual experiment showed significant change. Thus although this effect may be real, it is extremely small. It is possible that this synapse has been maintained throughout evolution to produce effects too small to be detected in individual preparations. An attractive alternative explanation, however, is that it exists instead to alter IC neuron activity during the activity of another stomatogastric network, the cardiac sac network. Cardiac sac network bursts strongly excite the VD neuron and strongly

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
VD	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
	Overall Spike Freq	Per	6	**7.85E-8**	**1.24E-10**
		Freq	6	**1.13E-6**	**4.02E-4**
PY	End Delay/Phase	Per	3	0.143	0.016
		Freq	3	**5.7E-4**	0.027

VD Down vs Normal			N	Slope Signif.	Intercept Signif.
PD	Burst Spike Freq	Per	5	0.500	0.620
		Freq	5	**3.5E-4**	0.029
LP	Burst Spike Freq	Per	5	**1.14E-4**	**1.43E-8**
		Freq	5	0.015	0.654
IC	Burst Spike Freq	Per	6	0.353	**1.61E-4**
		Freq	6	0.019	0.553

FIG. 9. Summary of ANCOVA results. Across experiment multivariate general linear model (GLM) on best-fit line slopes and intercepts; only parameters in which a significant difference was found are included. Numbers in bold are significant (uncompensated α-level, 0.05; Dunn-Šidák compensated level for multiple comparisons α-level, 1.17×10^{-3}).

inhibit the IC neuron (Russell and Hartline 1981; Sigvardt and Mulloney 1982b; Thuma and Hooper 2003). Certain cardiac sac network neurons excite the VD neuron, but none are known to inhibit the IC neuron (Claiborne and Selverston 1984; Russell and Hartline 1981; Sigvardt and Mulloney 1982a). As such, under the experimental conditions used here (with the stomatogastric nerve intact but without exogenous modulator application) the VD to IC neuron synapse may not exist to fulfill any functional role in generating pyloric network activity but instead to (at a minimum) transmit inhibition to the IC neuron during cardiac sac network bursts.

Second, in many parameters in which the GLM identified a significant change, in only a minority of preparations did significant change occur or different effects occurred in different preparations. These data can be interpreted in two ways. The first is that not all pyloric networks, even in identical modulatory milieus, are identical. How much animal to animal variation in pyloric network "ground state" conductance makeup and synaptic strength exists is unknown. Regardless, this interpretation would support our hypothesis because, in at least some of these networks, the LP and VD synapses would play no significant role in determining any aspect of pyloric activity (except for cycle period, which is always affected) (Weaver and Hooper 2003).

The second interpretation is that all pyloric networks are identical, and the preparation specific variation we observed was because our preparations were receiving varying modulatory input (in all experiments the stomatogastric nerve, which carries modulatory input from the rest of the stomatogastric nervous system, was intact). All animals were housed in identical facilities, and all preparations were removed from the animal several hours before experiments began. However, decapod crustacea form behavioral hierarchies in captivity (Edwards and Kravitz 1997), and hierarchical or other experience dependent effects could have induced long-lasting changes in pyloric modulatory input activity. Regardless, this interpretation again supports our hypothesis as it argues that in only some of these modulatory milieus did the LP and VD neuron synapses alter pyloric neuron phasing and spiking activity.

The third argument in support of our hypothesis is comparison of the intact and neuron-removed cases when the analysis was restricted to cycle periods near the intact network rest period. This analysis removes the confounding period changes LP or VD neuron removal induces, allows use of simple statistical tests, and is more likely to be physiologically relevant. In this analysis, LP or VD neuron removal altered no aspect of pyloric phasing or spiking activity.

It is important to stress that a very liberal α-level of 0.05 was chosen in all analyses and that for the near rest cycle period analysis, the analysis was even re-run using only data from experiments that, when analyzed across the entire data range, showed significant change when GLM analyzed as individuals (an obviously dubious data selection). Our failure to observe consistent, statistically significant changes even under conditions designed to maximize their detection further supports the contention that LP and VD neuron synapses play little or no role in pyloric phasing and spiking activity in at least some network states. These synapses presumably do not exist for no reason, and thus our observations support the hypothesis that some pyloric synapses are functionally relevant in only in certain network states. It is also important to contrast the present work with the effects of the LP and VD neuron on pyloric cycle period (Weaver and Hooper 2003) in which the effects were large, consistent across preparations, and no "special" efforts to obtain statistical significance were necessary. As such, at least some LP and VD neuron synapses (those to the pyloric pacemaker ensemble) have the same effects in the multiple pyloric networks, or multiple modulatory states that could be hypothesized to explain our results.

LP and VD neuron activity was not increased in this work and increasing their activity may thus alter pyloric phasing and spiking activity. We often observed alterations in pyloric neuron activity during the short lasting high-frequency LP and VD neuron firing that occurred on release from hyperpolarization, although it is difficult to separate these effects from the simultaneous cycle period changes that occur.

A possible criticism of this work is that because pyloric neurons release transmitter as a graded function of membrane potential, hyperpolarization may not remove all LP and VD neuron output. This concern is unlikely to be significant for several reasons. First, the hyperpolarizations were to membrane voltages (below –100 mV) at which graded release does not occur (Graubard 1978; Graubard et al. 1980, 1983), and this technique has been used before to reversibly remove pyloric neurons from the network (Ayali and Harris-Warrick 1999). Second, the membrane potential of the hyperpolarized neuron was almost always observed with a bridge-balanced electrode. Although at these current injection levels the elec-

trode was unlikely to be still balanced, relative membrane potential changes could be observed. These recordings showed that, in general, the neuron remained at fairly constant hyperpolarized membrane potentials without subthreshold "escapes;" in instances when subthreshold escapes occurred, data near them were not analyzed. Third, even if some graded release did occur, it was certainly very much reduced.

Comparison to earlier work

LP NEURON. Selverston and Miller (1980) reported that LP neuron removal by photoinactivation increased cycle frequency and slightly altered PY and IC neuron activity. However, due to the lack of computerized data analysis techniques at the time, the changes in PY and IC neuron activity were not quantified, and so whether they were significant is unknown. Furthermore, the confounding effect of the concurrent cycle period changes was not removed by altering pyloric cycle period. It is thus unclear if their data disagree with those reported here. Massabuau and Meyrand (1996) showed in *Homarus gammarus* that increased PO_2 levels in superfusion saline increased LP neuron activity, pyloric cycle period, and PY neuron burst beginning phase and duty cycle. Blocking changes in LP neuron activity blocked all these changes, indicating they were secondary consequences of the changes in LP neuron activity. However, because cycle period was again not independently controlled, it is impossible from these data to determine if the change in PY neuron activity was an indirect effect of an LP-neuron-induced change in pyloric cycle period or a direct effect of increased LP neuron activity. Changes in PY neuron activity similar to those reported by Massabuau and Meyrand are seen in *Panulirus* when pyloric cycle period is increased by current injection into the AB neuron (Hooper 1997), which suggests that the observed changes could result solely from the LP neuron induced changes in cycle period.

VD NEURON. Stimulating a sensory input in *Palinurus vulgaris* induces the VD neuron to become silent and causes a variety of other changes in pyloric phasing and spiking activity without inducing large cycle period changes (Hooper and Moulins 1989, 1990). The IC neuron was particularly strongly affected, and these changes in IC neuron activity resulted solely from the VD neuron ceasing to fire. These data differ from those reported here in which VD neuron removal induced insignificant changes in IC neuron activity. These experiments were also performed without experimentally applied modulators and with the stomatogastric nerve intact, and thus differences in network modulatory state in these two works are unlikely to be an explanation. However, although the pyloric networks of different species produce similar outputs, their synaptic connectivities and the cellular properties of their neurons are not identical (Katz and Tazaki 1992). The difference between these works may thus be due to species-specific variation in pyloric network structure and function.

Relevance to pyloric network phase maintenance

The pyloric network maintains phase when its period is altered by current injection into the AB neuron (Hooper 1997). The cellular basis of this phase maintenance has been investigated in isolated PY neurons (Hooper 1998). That work shows that PY neuron postinhibitory rebound slows as PY neuron inhibition period and duration increase, which would help maintain phase as cycle period changes. However, the observed changes are only approximately half those required to explain the PY neuron phase maintenance observed in the intact network.

We began this work in part to investigate whether changes in LP and VD neuron activity as pyloric cycle period changes could, through their synapses onto the PY neurons, provide a mechanism for the increased PY neuron phase maintenance observed in the intact network. The data reported here strongly suggest this is not the case as LP neuron removal did not consistently alter, and group GLM analysis indicated VD neuron removal had no effect on, PY neuron activity. The basis of the increased phase maintenance in the intact network is thus still unknown, but an attractive possibility is that it arises from period and duration-dependent changes in the synaptic transfer function from the AB/PD neuron pacemaker ensemble (Nadim et al. 1999). The relative importance of endogenous and synaptic mechanisms in phase maintenance for the other pyloric neurons is unknown. However, our data showing that LP or VD neuron removal does not consistently alter pyloric phasing or spiking activity suggest that, at least in control saline, LP and VD neuron synaptic input is unlikely to be critical for phase maintenance for any neuron.

Relevance to small distributed systems in general

These data raise three issues of general importance. First, VD or LP neuron removal alters pyloric period (Weaver and Hooper 2003), and altering pyloric period alters pyloric spiking and phasing activity (Hooper 1997; Hooper and Thuma 1996; Nadim et al. 1999). As a result of these period altering effects, the effect of LP and VD removal on pyloric spiking and phasing activity could not have been determined without using AB neuron current injection to match cycle period in the intact and neuron removed conditions. It would not be surprising if similar period alterations occur in other highly distributed systems when neurons are removed from the network. In these systems, as well, independent alteration of period may thus be required to investigate neuron function in generating network output.

Second, the LP and VD neurons synapse onto both the pacemaker ensemble and most or all other pyloric neurons. In normal saline, however, LP or VD neuron removal consistently alters only pyloric period. Activity changes in neurons with widespread synaptic contacts may thus nonetheless consistently alter only one aspect of network activity. The observation that LP or VD neuron activity increases can alter pyloric activity independent of period changes (Hooper and Marder 1987) also suggests that modulatory input could have qualitatively different effects depending on its sign. In the case at hand, modulation that decreases LP or VD neuron activity would consistently directly alter only pyloric cycle period whereas input that increases LP or VD neuron activity might directly alter both pyloric period and pyloric phasing and spiking activity.

Third, the inconsistency of the observed effects suggests that a much touted advantage of small invertebrate neural networks—that they are essentially identical in all individuals of a species—may need to be re-examined. The data presented here showing only a preparation-specific variation in statistical

significance are too weak to prove that pyloric networks are not essentially identical in all individuals of the same developmental stage and habitat. However, pyloric neurons show long-lasting alterations in response to varying input (Turrigiano et al. 1994, 1995) and can produce very similar activity with different conductance compositions (Golowasch et al. 2002). Given the importance of this issue for correct data interpretation across experiments not only in the pyloric system, but in small network work in general, it may be time to re-examine this issue in detail.

We thank R. DiCaprio and J. Thuma for comments on the manuscript.

DISCLOSURES

This work was supported by an Ohio University Doctoral Fellowship to A. L. Weaver and a Human Frontier Science Project grant, National Science Foundation Grant 9309986, and National Institute of Mental Health Grant MH-57832 to S. L. Hooper.

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