Intercircuit Control via Rhythmic Regulation of Projection Neuron Activity

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Synaptic feedback from rhythmically active neuronal circuits commonly causes their descending inputs to exhibit the rhythmic activity pattern generated by that circuit. In most cases, however, the function of this rhythmic feedback is unknown. In fact, generally these inputs can still activate the target circuit when driven in a tonic activity pattern. We are using the crab stomatogastric nervous system (STNS) to test the hypothesis that the neuronal circuit-mediated rhythmic activity pattern in projection neurons contributes to inter-circuit regulation. The crab STNS contains an identified projection neuron, modulatory commissural neuron 1 (MCN1), whose tonic stimulation activates and modulates the gastric mill (chewing) and pyloric (filtering of chewed food) motor circuits in the stomatogastric ganglion (STG). During tonic stimulation of MCN1, the pyloric circuit regulates both gastric mill cycle frequency and gastro-pyloric coordination via a direct synapse onto a gastric mill neuron in the STG. However, when MCN1 is spontaneously active, it has a pyloric-timed activity pattern attributable to synaptic input from the pyloric circuit. This pyloric-timed activity in MCN1 provides the pyloric circuit with a second pathway for regulating the gastric mill rhythm. At these times, the direct STG synapse from the pyloric circuit to the gastric mill circuit is not necessary for pyloric regulation of the gastric mill rhythm. However, in the intact system, these two pathways play complementary roles in this inter-circuit regulation. Thus, one role for rhythmicity in modulatory projection neurons is to enable them to mediate the interactions between distinct but related neuronal circuits.

Key words: central pattern generator; neuromodulation; stomatogastric ganglion; pyloric circuit; gastric mill circuit; modulatory projection neuron

Introduction

Projection neurons that activate rhythmically active neuronal circuits commonly switch from a tonic to rhythmic activity pattern once their target circuit is activated as a result of feedback they receive from that circuit (Weeks, 1981; Dubuc and Grillner, 1989; Kasicki et al., 1989; Frost and Katz, 1996; Perrins and Weiss, 1996; Deliagina et al., 2000). Despite this rhythmic activity pattern, tonic stimulation of these projection neurons is often sufficient to elicit rhythmic activity from the target circuit, suggesting that these inputs provide no timing cues to the activated circuit. It has been suggested that the rhythmic activity exhibited by these projection neurons helps maintain their activity and thereby contributes to maintaining circuit activity (Weeks, 1981). However, the function(s) of the rhythmic activity pattern in such projection neurons remains to be determined.

To examine this issue, we are studying the interactions among

an identified modulatory projection neuron and two rhythmically active motor circuits in the stomatogastric nervous system (STNS) of the crab *Cancer borealis* (Nusbaum and Beenhakker, 2002). The STNS contains four ganglia, including the paired commissural ganglia (CoGs), the unpaired oesophageal ganglion (OG) and stomatogastric ganglion (STG). Overlapping sets of STG neurons comprise the rhythmically active gastric mill (chewing) and pyloric (filtering of chewed food) circuits (Weimann et al., 1991; Weimann and Marder, 1994). Projection neurons originating in the CoGs and OG innervate the STG and modulate the gastric mill and pyloric rhythms (Nusbaum et al., 2001). Each of these projection neurons effectively influences the STG circuits when stimulated tonically, but most of them normally exhibit rhythmic activity that is time-locked to these circuits (Robertson and Moulins, 1984; Coleman and Nusbaum, 1994; Nagy et al., 1994; Norris et al., 1994, 1996).

One well described CoG projection neuron is modulatory commissural neuron 1 (MCN1) (Blitz et al., 1999). Tonic stimulation of MCN1 elicits the gastric mill rhythm and modulates the pyloric rhythm. However, spontaneous MCN1 activity is pyloric rhythm-timed as a result of inhibitory input that it receives within the CoG from the anterior burster (AB) neuron, a pyloric pacemaker neuron (Coleman and Nusbaum, 1994). When this feedback pathway is eliminated and MCN1 is stimulated tonically

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to activate the gastric mill rhythm, the pyloric circuit continues to regulate gastric mill rhythm activity via a direct synapse from the AB neuron onto a gastric mill circuit neuron (Nadim et al., 1998; Bartos et al., 1999).

In this paper, we tested the hypothesis that the pyloric-timed activity of MCN1 provides a second pathway by which the pyloric circuit regulates and coordinates the gastric mill rhythm. We demonstrate that this indeed is the case. Moreover, this pathway alone is sufficient to enable pyloric regulation of the gastric mill rhythm, although both pyloric circuit pathways to the gastric mill circuit are necessary to replicate the naturally occurring rhythm. Thus, one role for rhythmic activity in a modulatory projection neuron is to enable one neuronal circuit to regulate the activity of a distinct but related neuronal circuit.

Parts of this paper have been published previously in abstract form (Wood and Nusbaum, 2001).

Materials and Methods

Animals. Adult male *C. borealis* were obtained from Commercial Lobster and Seafood Co. (Boston, MA) and the Marine Biological Laboratory (Woods Hole, MA). Crabs were maintained in artificial, aerated seawater (10–12°C) until used. Animals were anesthetized with ice for 20–40 min before dissection. The dissection of the STNS was done in *C. borealis* physiological saline, containing (in mM): 440 NaCl, 26 MgCl₂, 13 CaCl₂, 11 KCl, 10 Tris base, and 5 maleic acid, pH 7.4–7.6. Dissections were performed in chilled saline as described previously (Blitz and Nusbaum, 1997). Experiments were performed using the isolated STNS (see Fig. 1A).

Electrophysiology. Electrophysiology was performed using standard methods as described previously (Beenhakker et al., 2004). The STNS was pinned down in a silicone elastomer-lined (Sylgard 184; K. R. Anderson, Santa Clara, CA) Petri dish. The isolated nervous system was superfused continuously (7–12 ml/min) with chilled physiological saline (10–12°C). To facilitate intracellular recordings, the STG was desheathed and visualized with white light transmitted through a dark-field condenser (Nikon, Tokyo, Japan). Microelectrodes (15–30 MΩ) were filled with 4 M potassium acetate plus 20 mM KCl. Intracellular current injections were accomplished using Axoclamp 2B amplifiers in single-electrode discontinuous current clamp (DCC) mode (Axon Instruments, Foster City, CA). Sample rates in DCC mode ranged from 2.5 to 3 kHz.

Identification of STG neurons was done by documenting their activity patterns, synaptic interactions, and axonal projection paths as described previously (Weimann et al., 1991; Bartos and Nusbaum, 1997; Blitz and Nusbaum, 1997). Identification of MCN1 was accomplished via its axon location (Coleman and Nusbaum, 1994; Bartos and Nusbaum, 1997) and interactions in the STG (Blitz et al., 1999; Beenhakker and Nusbaum, 2004). Selective activation of MCN1 was achieved by stimulating the inferior oesophageal nerves (*ions*) (each *ion*, 10–25 Hz) (Bartos and Nusbaum, 1997). The selective activation of MCN1 via *ion* stimulation was assured by the fact that the axon of only one other STG-projecting CoG neuron, modulatory commissural neuron 5 (MCN5), is present in the *ion*, and MCN1 has a lower activation threshold than MCN5 during *ion* stimulation (Coleman et al., 1992; Norris et al., 1996; Bartos and Nusbaum, 1997). In some preparations, modulatory influence in the STG from projection neurons in the CoG was eliminated by transecting both *ions* and superior oesophageal nerves (*sons*) (see Fig. 1A). Under these conditions, the pyloric rhythm either stops or slows (0.2–0.8 Hz) from a cycle frequency that is typically >1 Hz. When the gastric mill rhythm was active before the *ions* and *sons* were transected, the rhythm terminated as soon as these nerves were cut.

Experimental control of a pyloric rhythm-timed activity pattern in MCN1 was accomplished by extracellular stimulation of the *ion(s)* between each set of successive bursts of the paired pyloric dilator (PD) neurons. This protocol involved triggering each *ion* stimulation from the start of a PD neuron burst with an added delay that included the entire PD burst and a duration that included the period between PD bursts. The PD neurons, along with the AB neuron to which they are electrically coupled, are members of the pyloric pacemaker group (see Fig. 1B). The

cycle frequency of the pyloric rhythm is determined primarily by this set of neurons (Hooper and Marder, 1987). Intracellular recording of the PD neuron was used to monitor the relevant parameters of the pyloric rhythm.

Detection of PD neuron activity and control of MCN1 stimulation was done using either a National Instruments (Austin, TX) analog-to-digital (A/D) board (AT-MI0–16E2) and interface with Lab Windows CVI (F.N. and Y.M.) or a Cambridge Electronic Design (Cambridge, UK) 1401 A/D board with interface, including custom scripts (D.E.W.) for Spike 2 data acquisition software (version 4.0; Cambridge Electronic Design). In all stimulation experiments, MCN1 activity was controlled directly by the stimulation protocol because each extracellular stimulation pulse (duration, 1 msec) elicits a single action potential from MCN1 (Bartos and Nusbaum, 1997). For experiments in which the pyloric rhythm was suppressed, the timing of the rhythmic activation of MCN1 was controlled directly from a stimulator (Grass model S-88; Astromed, West Warwick, RI).

Data analysis. Data were recorded using a chart recorder (Astromed MT-95000) and digitized via Spike 2 data acquisition software to a computer. Data analyses were conducted either manually or on digitized data using a custom-written Spike 2 program called “The Crab Analyzer” (freely available at <http://www.neurobiologie.de/>) to determine the activity levels and phase relationships of neurons. The pyloric cycle period was defined as the interval between the onset of an impulse burst in the PD neuron and the onset of the subsequent PD neuron burst. Pyloric cycle frequency was determined as the reciprocal of the pyloric cycle period. In each experiment, a sequence of 20 pyloric cycles was used to determine the mean pyloric cycle frequency.

There are eight types of gastric mill circuit neurons, most of which occur as single copies (Harris-Warrick et al., 1992; Blitz and Nusbaum, 1997). The core of the gastric mill rhythm generator includes reciprocal inhibition between interneuron 1 (Int1) and the lateral gastric (LG) neuron (see Fig. 1B) (Coleman et al., 1995; Bartos et al., 1999). In this paper, gastric mill rhythms were monitored by recording the activity of the LG neuron and the dorsal gastric (DG) neuron. The gastric mill rhythm-timed DG neuron bursts are coactive with those of Int1, and they alternate with the LG neuron bursts (see Fig. 2). The LG neuron was recorded intracellularly, whereas the DG neuron was recorded extracellularly from the dorsal gastric nerve (*dgn*) (see Figs. 1A, 2).

The gastric mill cycle period was defined as the interval between the onset of consecutive LG neuron bursts. Ten consecutive gastric mill cycles were used for computing the mean number of action potentials per pyloric-timed LG neuron burst, the mean LG neuron duty cycle (fraction of a gastric mill cycle during which the LG neuron was active), and the mean value for the gastric mill cycle period. When the LG neuron bursts were subdivided by pyloric-timed input during the gastric mill rhythm, its gastric mill-timed burst was defined as the duration from the onset of LG neuron activity until the next episode of Int1-mediated inhibition. This typically spanned several pyloric-timed LG neuron bursts. The hyperpolarization resulting from the Int1-mediated inhibition was typically discernable (more hyperpolarized) from that resulting from the pyloric-timed removal of the MCN1-mediated excitation that subdivided each LG neuron burst (see Fig. 2A). Barrages of unitary IPSPs from Int1 were also evident during Int1-mediated inhibition. The occasional action potential that occurred during the LG neuron interburst was not included as part of its burst. Coordination between the gastric mill and pyloric rhythms was computed as the latency from the onset of the PD neuron burst that immediately preceded each LG neuron burst to the start of that LG neuron burst (Nadim et al., 1998; Bartos et al., 1999). In some cases, the pyloric–gastric mill relationship was measured as the latency from the onset of an MCN1 burst that immediately preceded an LG neuron burst to the onset of that LG neuron burst.

Figures were prepared by scanning sequences of recordings using a Hewlett-Packard (Palo Alto, CA) Scanjet IIC with Deskscan II (version 2.0a) software or from digitized records. Final figures were prepared with CorelDraw (version 9.0 for Windows) or Microsoft Powerpoint 2002 (Microsoft, Seattle, WA). Graphics and statistics were generated using Sigma Plot 4.0 and Sigma Stat 2.03 (SPSS, Chicago, IL). Statistical tests used to analyze data were one-way ANOVA, repeated-measures

There were several qualitative differences between the spontaneously active gastric mill rhythms and those elicited by tonic stimulation of MCN1. One such difference was that the LG neuron burst pattern was divided into pyloric-timed bursts in the former condition, whereas it was tonic in the latter condition (Figs. 2, 3). A second qualitative difference between the gastric mill rhythms elicited by pyloric-timed and tonic MCN1 activity was in the pattern of the LG neuron membrane potential oscillations that occurred during each LG neuron interburst. In both cases, these subthreshold oscillations were pyloric-timed, and in both instances, the depolarizing aspect of these oscillations initiated during the AB–PD neuron burst (Figs. 2, 3). This timing resulted from the fact that the onset of each rhythmic depolarization represented the periodic removal of Int1 inhibition of the LG neuron. This disinhibition is a consequence of AB neuron inhibition of Int1 (Fig. 1*B*), and one of these rhythmic events triggers the LG neuron burst onset during each cycle of the gastric mill

rhythm elicited by tonic MCN1 stimulation (Bartos et al., 1999). At such times, these disinhibitions help regulate both the gastric mill cycle frequency and the coordination between the gastric mill and pyloric rhythms (Bartos et al., 1999). When MCN1 was stimulated tonically, these rhythmic depolarizations were smooth, single-step events because under this condition, the AB-mediated inhibition of Int1 was the only pyloric-timed depolarizing event occurring in the LG neuron (Bartos et al., 1999). However, when MCN1 activity was pyloric-timed, its excitation of the LG neuron was comparably rhythmic, thereby providing a second pyloric-timed depolarizing event to the LG neuron (Fig. 3B). MCN1 provides both electrical and modulatory excitation to the LG neuron (Coleman et al., 1995; Wood et al., 2000). The relative timing of the rhythmic MCN1-mediated excitation of the LG neuron was such that during the LG neuron interburst, each excitatory episode initiated during or just after the peak of the associated disinhibition (Fig. 3, arrows). Note that the duration of the rhythmic MCN1-mediated excitation was abbreviated by the Int1-mediated inhibition of the LG neuron that began soon after each MCN1 burst onset.

There was also a qualitative difference in the event that triggered LG neuron burst initiation when MCN1 activity was tonic and pyloric-timed. When MCN1 was tonically active, the start of every LG neuron burst occurred during a disinhibition event (Nadim et al., 1998; Bartos et al., 1999). In contrast, during rhythmic MCN1 activity, the onset of most LG neuron bursts occurred during an episode of MCN1-mediated excitation in LG (Fig. 3). In fact, during spontaneous gastric mill rhythms, the LG neuron burst initiated during an episode of MCN1-mediated excitation in 75% of the gastric mill cycles (113 of 150 cycles; $n = 5$).

Comparable gastric mill rhythms resulted from pyloric-timed MCN1 stimulation and spontaneous MCN1 activity

There were additional, quantitative changes in the gastric mill rhythm when MCN1 activity was pyloric-timed instead of tonic. However, it was not practical to use spontaneously occurring gastric mill rhythms to document these changes because these rhythms occurred too infrequently. Thus, to more reliably obtain gastric mill rhythms driven by pyloric-timed MCN1 activity and to have better experimental control, we developed a protocol in which we replaced the AB neuron inhibition of MCN1 with a computer-controlled, pyloric-timed rhythmic stimulation of MCN1 (see Materials and Methods) (Fig. 3A). As shown in Figure 3, in which this manipulation was performed in preparations where the CoGs remained connected to the STG, the gastric mill rhythm generated by this stimulation protocol was comparable with the one that occurred during spontaneous gastric mill

Figure 2. Pyloric-timed and tonic activity in the modulatory projection neuron MCN1 elicit distinct gastric mill rhythms in the isolated stomatogastric nervous system. *A*, Left, When MCN1 (arrow, *ion*) was not active, there was no gastric mill rhythm (*LG*, *dgn*) and a relatively slow pyloric rhythm (*PD*). Right, Spontaneous, pyloric-timed bursting of MCN1 elicited the gastric mill rhythm (*LG*, *DG*) and modulated the pyloric rhythm (*PD*). Each *ion* contains the axon of the ipsilateral MCN1 (see Fig. 1A). Note that MCN1 was inactive during each *PD* neuron burst and that each gastric mill-timed *LG* neuron burst was composed of a set of pyloric-timed bursts. Most hyperpolarized membrane potential: *LG*, -66 mV; *PD*, -52 mV. *B*, Left, As in *A*, without MCN1 activity there was no gastric mill rhythm (*LG*, *dgn*) and a weak pyloric rhythm (*PD*). Right, Selective, tonic stimulation (Stim.) of MCN1 activated the gastric mill rhythm and strengthened the pyloric rhythm. During tonic MCN1 stimulation, each *LG* neuron burst was tonic instead of pyloric-timed. Most hyperpolarized membrane potential: *LG*, -70 mV; *PD*, -54 mV.

rhythms. This included the presence of pyloric-timed *LG* neuron bursts and two-step, pyloric-timed depolarizations during each *LG* neuron interburst (Fig. 3). Note that, as described above for the spontaneous condition, the MCN1-mediated depolarizations during the *LG* neuron interburst were abbreviated by the presence of the rhythmic inhibitory input arriving in the *LG* neuron from Int1 soon after the start of each MCN1 burst (Fig. 3C).

There were two quantitative differences between the gastric mill rhythms that occurred when MCN1 activity was pyloric-timed and tonic. The first difference was that the gastric mill rhythm was significantly faster when MCN1 activity was tonic (tonic, 0.14 ± 0.04 Hz; spontaneous pyloric-timed, 0.12 ± 0.02 Hz; $n = 12$; $p < 0.02$). In contrast, there was no difference in the speed of this gastric mill rhythm between times when the rhythm was spontaneous and when it was evoked by pyloric-timed MCN1 stimulation (pyloric-timed stimulation, 0.12 ± 0.03 Hz; $n = 12$; $p > 0.05$).

We also found that the temporal relationship between the pyloric and gastric mill rhythms was changed when MCN1 activ-

Figure 3. Spontaneous and experimentally elicited rhythmic (pyloric-timed) activity in MCN1 elicits comparable gastric mill rhythms. *A*, Schematic representation of experiments in which MCN1 was rhythmically activated during the interburst of the pyloric pacemaker neurons (AB–PD). The onset of each pyloric cycle was used with an added delay to trigger the output of a stimulator to stimulate the *ion*(s) and thereby evoke a pyloric-timed activation of MCN1 that alternated with that of the AB and PD neurons (see Materials and Methods). *B*, Spontaneous MCN1 activity elicited the gastric mill rhythm. Note that MCN1 was inactive during each PD neuron burst and that the LG neuron bursts were pyloric-timed (coactive with MCN1). Also note that during each LG neuron interburst there were two consecutive subthreshold depolarizations in LG during each pyloric cycle (arrows). Most hyperpolarized membrane potential: LG, -62 mV; PD, -55 mV. *C*, Pyloric-timed stimulation (Stim.) of MCN1 (intra-burst frequency, 25 Hz) elicited a gastric mill rhythm comparable with the rhythm elicited by spontaneous MCN1 activity in *B*. Note the pyloric-timed bursting of the LG neuron and the two consecutive depolarizations per pyloric cycle in LG during each LG neuron interburst (arrows). Most hyperpolarized membrane potential: LG, -68 mV; PD, -57 mV.

ity was changed from tonic to rhythmic. Our previous work showed that during tonic MCN1 stimulation at 10 Hz, there is a constant latency relationship between the onset of each LG neuron burst and the immediately preceding PD neuron burst (Nadim et al., 1998; Bartos et al., 1999). This latency value was unchanged when MCN1 was activated tonically at 25 instead of 10 Hz ($p > 0.05$; $n = 12$) (data not shown). A consistent latency relationship was also maintained when MCN1 was rhythmically activated at an intra-burst frequency of 25 Hz with an 80% duty cycle (PD to LG latency, 323 ± 40 msec; $n = 12$) and during spontaneous MCN1-elicited gastric mill rhythms (362 ± 62.9 msec; $n = 13$). The latency value was not different for these latter two conditions when MCN1 activity was pyloric-timed ($p > 0.05$). However, both the mean latency and its variability were

decreased when MCN1 was tonically active relative to times when MCN1 activity was pyloric-timed, whether attributable to spontaneous activity or rhythmic MCN1 stimulation (312 ± 10 msec; $p < 0.01$; $n = 13$). There was also a comparable latency from the onset of the immediately preceding MCN1 burst to LG neuron burst onset during spontaneous and rhythmically stimulated MCN1 activity ($n = 11$; $p > 0.05$). It was not surprising that the mean latency value was lower when determined from MCN1 burst onset (141.7 ± 53.7 msec; $n = 12$; $p < 0.01$) than from PD neuron burst onset because, as indicated above for the spontaneous rhythms, when MCN1 activity was pyloric-timed, the LG neuron burst was more likely to initiate during an episode of MCN1-mediated excitation. The same was true during rhythmic MCN1 stimulations (139 of 150 cycles; 93%; $n = 5$ preparations).

After determining that the spontaneous MCN1-elicited gastric mill rhythm was well mimicked by pyloric-timed MCN1 stimulation, we used this stimulation protocol to determine whether the pyloric timing of the MCN1 activity pattern contributed to the previously documented pyloric regulation of the speed of the MCN1-elicited gastric mill rhythm (Bartos et al., 1999). As indicated above, Bartos et al. (1999) showed that when MCN1 was stimulated tonically in the isolated STG, the pyloric rhythm regulated the speed of the gastric mill rhythm via AB neuron inhibition of Int1. Thus, under these conditions, the gastric mill rhythm was slowed significantly whenever the pyloric rhythm was experimentally slowed or terminated (Fig. 4*A,C*) (Bartos et al., 1999). The speed of the pyloric rhythm is readily controlled by injecting current into the PD and/or AB neurons. In contrast to the slowing of the gastric mill rhythm that occurred when the pyloric rhythm was suppressed during tonic MCN1 activity, suppressing the pyloric rhythm while MCN1 was stimulated in a pyloric pattern resulted in no change in the gastric mill cycle frequency (Fig. 4*B,C*). As is evident from the intracellular recording of the LG neuron in Figure 4*B*, under this condition the rhythmic excitation of the LG neuron by MCN1 enabled LG to still exhibit pyloric-timed membrane potential oscillations when the pyloric rhythm was suppressed. At times when the pyloric rhythm was suppressed but MCN1 activity retained a pyloric-like pattern, the onset of every LG burst occurred during an episode of MCN1 activity (Fig. 4*B*). There was, however, an increase in the mean latency from MCN1 burst onset to LG burst onset from when the pyloric rhythm was present (141.7 ± 53.7 msec; $n = 12$) to when it was suppressed (240 ± 196 msec; $n = 12$; $p < 0.001$). Nonetheless, the pyloric-timed excitation from MCN1 to the LG neuron was sufficient to maintain the speed of the gastric mill rhythm.

The presence of pyloric timing in MCN1 also altered the pattern of the gastric mill rhythm. We assessed this influence on the gastric mill pattern by comparing the LG neuron duty cycle during rhythms elicited by both tonic and rhythmic MCN1 activity. We also determined whether the pyloric-timed activity of MCN1 alone was sufficient to maintain the normal LG neuron duty cycle by rhythmically stimulating MCN1 while suppressing the pyloric rhythm.

Neither source of pyloric input to the gastric mill circuit, when present by itself, was sufficient to mimic the natural condition of having both pyloric pathways present (Fig. 5). Thus, relative to having both pyloric pathways present, the LG neuron burst occupied a smaller fraction of each cycle when the only source of pyloric influence was the indirect pathway (AB neuron inhibition of MCN1, $p < 0.01$; $n = 12$), whereas it occupied a larger fraction of each gastric mill cycle when the only available pyloric pathway was the direct one (AB neuron inhibition of Int1, $p < 0.05$; $n =$

12) (Fig. 5). LG neuron duty cycle was most reduced when there was no source of pyloric input to the gastric mill circuit (MCN1 tonically active, pyloric rhythm suppressed, $p < 0.01$; $n = 12$) (Fig. 5).

Consequences of the coordination between the pyloric and gastric mill rhythms

We next aimed to determine whether the differences between gastric mill rhythms elicited by tonic and pyloric-timed MCN1 activity required the presence of the latency locking between the activity of MCN1 and the pyloric pacemaker neurons. Therefore, we compared the gastric mill rhythm elicited by pyloric-related activity patterns in MCN1 that were latency locked to the preceding PD neuron burst (the natural condition) with those in which the rhythmic MCN1 activity was free-running with respect to the pyloric rhythm (Fig. 6). In the free-running condition, we unlinked the pyloric-related activity of MCN1 from PD neuron activity but retained all other stimulation parameters, including those for MCN1 burst cycle frequency (1 Hz), intraburst firing frequency (25 Hz), and burst duration (800 msec). We focused our comparison on the gastric mill cycle frequency, the pyloric to gastric mill latency relationship, LG neuron duty cycle, and the waveform pattern in the LG neuron during its interburst. As elaborated below, unlinking the MCN1 activity pattern from the pyloric rhythm changed some of these parameters.

Some aspects of the gastric mill rhythm were unaffected by unlinking rhythmic MCN1 activity from the pyloric rhythm. For example, the speed of the gastric mill rhythm was the same whether rhythmic MCN1 activity was linked to the pyloric rhythm ($p > 0.05$; $n = 12$) (Fig. 6B1). The LG neuron duty cycle was also unaffected by changing the relationship between MCN1 activity and the pyloric rhythm (Fig. 6B2).

Several other aspects of the gastric mill rhythm were altered by unlinking rhythmic MCN1 activity from the pyloric rhythm. One example of such a change was in the LG neuron waveform during its interburst. As described above, when MCN1 activity was pyloric-linked, the LG neuron interburst usually consisted of a repeating set of three pyloric-timed events, including two consecutive depolarizing steps followed by an IPSP-associated hyperpolarization (Fig. 3). The first depolarizing step (AB-mediated removal of Int1 inhibition of LG) and the hyperpolarization (pyloric-timed Int1 inhibition of LG) are direct pyloric circuit-mediated synaptic events (Bartos et al., 1999). The second of the two depolarizing steps resulted from MCN1 excitation of the LG neuron. When MCN1 activity was unlinked from the pyloric rhythm, the occurrence of the MCN1 excitation of the LG neuron was similarly unlinked from the first depolarizing step and the subsequent hyperpolarization. Consequently, in the unlinked condition, the excitatory input from MCN1 to the LG neuron had a continually changing relationship with the direct pyloric circuit inputs to the LG neuron, resulting in an altered LG neuron waveform during each interburst (Fig. 6A).

Figure 4. Direct and indirect influence of the pyloric circuit on the gastric mill cycle frequency. *A*, Left, The gastric mill rhythm (LG, DG) resulting from tonic stimulation (Stim.) of MCN1 in a preparation in which the STG was isolated from the CoGs. Note the presence of the pyloric rhythm (PD). Right, Suppression of the pyloric rhythm by PD neuron hyperpolarization slowed the gastric mill rhythm. Suppression of the pyloric rhythm is evident both in the maintained PD neuron hyperpolarization and in the lack of pyloric-timed depolarizations during the LG interburst. Most hyperpolarized membrane potential: LG, -73 mV; PD, -59 mV. *B*, Left, The gastric mill rhythm resulting from pyloric-timed stimulation of MCN1. Right, The gastric mill rhythm resulting from pyloric-related stimulation of MCN1 while the pyloric rhythm was suppressed by PD hyperpolarization. Note that there are three gastric mill cycles during the same duration with and without the pyloric rhythm present, indicating that the gastric mill rhythm speed was unchanged by suppressing the pyloric rhythm. During the latter condition, each LG neuron burst initiated during an episode of MCN1 stimulation (dotted lines). The bar under each set of recordings represents the tonic (solid bar) or pyloric-timed (broken bar) MCN1 activity pattern. Most hyperpolarized membrane potential: LG, -71 mV; PD, -60 mV. *C*, Comparison of gastric mill rhythm (Rhyth.) speed during the above four experimental conditions. The absence of the pyloric rhythm did not alter the speed of the gastric mill rhythm as long as MCN1 retained a pyloric-like activity pattern ($p > 0.05$; $n = 12$). ^ΔSignificantly different from all other conditions ($p < 0.05$; $n = 12$). Freq., Frequency.

Figure 5. Direct and indirect influence of the pyloric circuit on LG neuron duty cycle during the MCN1-elicited gastric mill rhythm. *A*, The LG neuron duty cycle (i.e., the fraction of a gastric mill cycle during which the LG neuron burst occurred) was different in all four experimental conditions. *a*, Pyloric-timed MCN1 stimulation (intraburst firing rate, 25 Hz) during an ongoing pyloric rhythm; *b*, Pyloric-timed MCN1 stimulation while the pyloric rhythm was suppressed (intraburst firing rate, 25 Hz); *c*, Tonic MCN1 stimulation (25 Hz) during an ongoing pyloric rhythm; *d*, Tonic MCN1 stimulation (25 Hz) while the pyloric rhythm was suppressed. All four traces are from the same experiment. Note that each gastric mill rhythm-timed LG neuron burst in *a* and *b* is composed of a set of shorter-duration, (pyloric-timed) rhythmic bursts that are separated by smaller amplitude hyperpolarizations than those separating the gastric mill-timed bursts. *B*, Quantification of the LG neuron duty cycle when MCN1 activity was pyloric-timed and tonic, both in the presence and absence of the pyloric rhythm (Rhyth.). Significant difference from all other conditions: ^Δ $p < 0.01$, $n = 12$; * $p < 0.05$, $n = 12$.

Unlinking MCN1 activity from the pyloric rhythm also altered the regularity (mean duration) of the individual pyloric-timed LG neuron bursts (Fig. 6A,B3). Specifically, the mean pyloric-timed LG neuron burst duration was reduced in the un-

Figure 6. Pyloric rhythm regulation of MCN1 activity is necessary for normal LG neuron burst structure and gastric mill–pyloric coordination. *A*, Left, MCN1 stimulation was pyloric-timed (stimulus bars) (see Materials and Methods), eliciting the standard gastric mill rhythm as indicated by the regular duration of the pyloric-timed LG neuron bursts that combine to constitute each gastric mill-timed burst of LG. Note that the pyloric-timed LG bursts were separated by smaller-amplitude hyperpolarizations than were each gastric mill-timed LG neuron burst. The latter bursts were also separated by several pyloric-timed subthreshold depolarizations. Right, MCN1 was stimulated using the same pyloric-related parameters except that MCN1 activity was free-running relative to the pyloric rhythm (stimulus bars). Note the variability in the duration of the pyloric-timed LG neuron bursts. *B*, Consequences of unlinking MCN1 activity from the pyloric rhythm. *B1*, Gastric mill cycle frequency and the gastric mill-related duty cycle (*B2*) of LG were unchanged when MCN1 activity was switched from pyloric-timed to free-running ($p > 0.05$; $n = 12$). *B3*, There was a reduction in the mean duration of each pyloric-timed LG neuron burst when MCN1 activity was switched from pyloric-timed to free-running ($p < 0.05$; $n = 12$). *C*, There was an increase in the mean latency from the onset of the pyloric cycle immediately preceding the LG neuron burst to the onset of that LG (gastric mill) burst when MCN1 activity was free-running relative to the pyloric rhythm. This was the case whether the latency relationship was measured relative to the burst onset of the PD neuron or MCN1. * $p < 0.05$ relative to all other conditions except those with the same symbol; $^{\Delta}p < 0.01$ relative to all other conditions; $n = 20$ for all conditions.

linked condition ($p < 0.05$; $n = 11$) (Fig. 6*B3*), despite the fact that neither the mean gastric mill-related duty cycle of the LG neuron (Fig. 6*B2*) nor the mean gastric mill cycle period (Fig. 6*B1*) was altered. Moreover, these pyloric-timed LG neuron burst durations were more variable in the unlinked condition relative to gastric mill rhythms elicited with pyloric-timed activation of MCN1 ($p < 0.05$; $n = 11$) (Fig. 6*B3*).

Another consequence of unlinking MCN1 activity from the pyloric rhythm was an increase in the mean latency of the PD neuron burst onset relative to the onset of the LG neuron burst ($n = 12$; $p < 0.01$) (Fig. 6*C*). There was a concomitant increase in the variability of this latency relationship (Fig. 6*C*). This result suggested that the coordination of the two pyloric-timed inputs to the LG neuron was necessary for maintaining the normal coordination of the pyloric and gastric mill rhythms.

During gastric mill rhythms elicited by either spontaneous activity or pyloric-timed stimulation of MCN1, there was a shorter latency between MCN1 and LG neuron burst onsets than between PD neuron and LG neuron burst onsets (Fig. 6*C*). This relationship persisted in the free-running condition, but both of these latency measurements were larger than their counterpart measurements during pyloric-timed MCN1 activity ($n = 12$; $p < 0.01$) (Fig. 6*C*). Finally, as we showed previously, most LG neuron bursts initiated during an MCN1 burst when the gastric mill rhythm was elicited by either spontaneous MCN1 activity or pyloric-timed stimulations of MCN1. This relationship was not as strict when the pyloric and MCN1 activities were uncoordinated. During the free-running gastric mill rhythms, the LG neu-

ron burst initiated during an episode of MCN1-mediated excitation in only 42% of the gastric mill cycles (63 of 150 cycles; $n = 5$).

Discussion

We have shown that rhythmic synaptic feedback from a central pattern-generating circuit to a modulatory projection neuron can enable that circuit to regulate the activity of a distinct but related neuronal circuit. Specifically, we have demonstrated that the pyloric rhythm-timed activity of MCN1 enables the pyloric circuit to regulate several aspects of the gastric mill rhythm, including its speed, pattern, and coordination with the pyloric rhythm. The pyloric circuit also influences the gastric mill rhythm by a second pathway, within the STG, via an inhibitory synapse from the pyloric pacemaker AB neuron to the gastric mill pattern generator neuron Int1 (Bartos et al., 1999). As discussed below, these two pathways provide both complementary and distinct contributions to the pyloric regulation of the gastric mill rhythm.

Patterned synaptic feedback from central pattern-generating circuits to their modulatory inputs is well documented in many motor systems (Weeks, 1981; Robertson and Moulins, 1984; Nusbaum and Kristan, 1986; Dubuc and Grillner, 1989; Kasicki et al., 1989; Nagy et al., 1994; Frost and Katz, 1996; Perrins and Weiss, 1996; Deliagina et al., 2000). In the few previous studies that aimed to understand the function of this rhythmic feedback, the focus was generally restricted to potential consequences for the circuit responsible for this feedback (Weeks, 1981; Kasicki et al., 1989; Deliagina et al., 2000). The results of these studies were

inconclusive. In the case of the leech swim system, several possible roles were eliminated because the rhythmicity of the swim-initiating cell 204 did not contribute to swim initiation, maintenance, or cycle phasing (Weeks, 1981). In the lamprey swim system, there are correlative data supporting the possibility that the swim-related rhythmic activity in reticulospinal neurons serves a gating function (Deliagina et al., 2000). In the present study, we did not examine whether the pyloric-timed activity in MCN1 had a different influence on the pyloric rhythm than did tonic MCN1 activity. However, this rhythmic MCN1 activity pattern clearly participated in the pyloric regulation of the gastric mill rhythm.

Intercircuit interactions underlie many complex behaviors. There are, for example, well documented interactions between the respiratory and locomotory systems (Cattaert and Clarac, 1983; Kawahara et al., 1989; Morin and Viala, 2002), between respiration and vocalization (Larson et al., 1994; Suthers, 1997), and between different aspects of feeding (Perrins and Weiss, 1996; Clemens et al., 1998). However, there is little information available regarding the cellular basis of how such interactions coordinate the relevant neuronal circuits. In the stomatogastric system, previous studies showed that the gastric mill circuit regulates the pyloric rhythm via LG neuron presynaptic inhibition of the transmitter-mediated actions of MCN1, but not its electrical synapses, within the STG (Coleman et al., 1995; Bartos and Nusbaum, 1997). Moreover, as mentioned above, the pyloric circuit regulates the gastric mill rhythm via AB neuron inhibition of Int1 (Nadim et al., 1998; Bartos et al., 1999). The ability of one circuit to regulate and coordinate the activity of a related circuit by way of its synaptic influence on the activity pattern of a modulatory projection neuron provides a novel mechanism for how such regulation can occur.

Either of the two pyloric-influenced pathways alone was sufficient to enable the pyloric rhythm to regulate gastric mill cycle frequency and gastric mill–pyloric coordination. However, in the normal condition, when both pathways were present, some aspects of the gastric mill rhythm were regulated conjointly by both pathways whereas other aspects of this rhythm were patterned primarily by the pyloric-timed activity in MCN1. One example of the combined action of both pyloric-influenced pathways on the gastric mill rhythm was their influence on the LG neuron duty cycle. Either of these pathways alone was sufficient to maintain a steady LG neuron duty cycle, but neither one alone was sufficient to produce the same duty cycle that occurred when both pathways were present. Thus, because a given LG neuron duty cycle also defines the Int1 duty cycle for the gastric mill rhythm and these two neurons constitute the core of the gastric mill central pattern generator (Coleman et al., 1995; Bartos et al., 1999), these effects on LG neuron duty cycle indicate that neither pyloric pathway alone elicited the same gastric mill rhythm as did their combined influence. Changes in the LG neuron duty cycle represent a change in the fraction of each chewing cycle during which the teeth protract (LG neuron active) and retract (LG neuron inactive) (Heinzel et al., 1993). The behavioral relevance of this change in the chewing pattern, such as whether it underlies the chewing of different types of food, remains to be determined.

The combined influence of both pyloric-regulated pathways was also evident in the latency relationship that underlies the coordination of the pyloric and gastric mill rhythms. Although most LG neuron bursts were initiated during an episode of the rhythmic, pyloric-timed depolarizations it received during its interburst from MCN1, this MCN1 input consistently occurred at or near the peak of the immediately preceding pyloric-mediated

disinhibition. It was therefore not surprising that separating these two pyloric-timed depolarizing events decreased the effectiveness of each one and thereby increased the latency to LG neuron burst onset, relative to both AB–PD and MCN1 activity, and increased its variability.

An example of the selective influence of the pyloric-timed MCN1 activity on the gastric mill rhythm was the generation of pyloric-timed bursts in the LG neuron. Each pyloric-timed burst in the LG neuron occurred during the corresponding pyloric-timed burst in MCN1 and resulted from MCN1-mediated excitation of the LG neuron. In contrast, when MCN1 was tonically active but the pyloric rhythm was still influencing the gastric mill rhythm via AB inhibition onto Int1, each LG neuron burst was tonic. This change from a tonic to rhythmic LG burst pattern should change the protraction movements of the lateral teeth from a single smooth movement to fast rhythmic ones (Heinzel et al., 1993). When MCN1 is tonically active, each LG neuron burst is a consequence of both electrical and peptidergic input from MCN1 (Coleman et al., 1995; Wood et al., 2000). It will be interesting to determine whether there is a change in the balance of influence of the electrical and peptidergic actions when MCN1 activity is pyloric rhythm-timed.

The specific timing of MCN1 activity relative to the pyloric rhythm was not important to its influence on gastric mill cycle frequency. The MCN1 influence on gastric mill cycle frequency appeared to be determined primarily by the overall MCN1 firing frequency, insofar as tonic MCN1 activity at 25 Hz consistently elicited faster gastric mill rhythms than did pyloric-timed MCN1 activity with an intraburst firing rate of 25 Hz but an overall firing rate of ~20 Hz. Previous work showed that when MCN1 is tonically active, the speed of the gastric mill rhythm is directly correlated with MCN1 firing frequency (Bartos et al., 1999). These experiments also reinforced previous work documenting that the presence of a pyloric rhythm input always results in a faster gastric mill rhythm (Bartos et al., 1999). Here, the loss of AB neuron inhibition of Int1 did not slow the gastric mill rhythm as long as MCN1 was pyloric-timed instead of being tonically active.

Our work revealing that the pyloric circuit has two pathways by which it regulates the gastric mill rhythm suggests that feedback processes might include redundant pathways. However, closer analysis has revealed that these multiple feedback loops act synergistically to maintain coordination between these circuits. Additionally, there is a shift in the relative influence of the two pyloric-related inputs during different gastric mill rhythms. One such distinct gastric mill rhythm results from the activation of either an identified mechanosensory pathway or a projection neuron located in the crab brain. Both of these inputs elicit a gastric mill rhythm driven primarily or entirely by coactivation of MCN1 and a second projection neuron, CPN2 (Beenhakker and Nusbaum, 2004; Beenhakker et al., 2004; Christie et al., 2004). During the MCN1–CPN2-elicited gastric mill rhythm, MCN1 activity remains pyloric-timed during the LG neuron interburst but it is tonically active during the LG neuron burst. Consequently, during this latter rhythm, the LG neuron burst is tonic instead of pyloric-timed. The presence of multiple feedback pathways also provides the possibility that there might be state-dependent shifts in the balance of influence of the different feedback pathways caused by neuromodulation. For example, there are many different neuromodulators present in the STNS that alter the cellular and synaptic properties of the resident neurons (Nusbaum et al., 2001; Marder and Thirumalai, 2002).

Bidirectional communication between different levels is common in all types of neuronal circuits, but the role(s) of the feed-

back pathways in most circuits remains to be determined (Sillito and Jones, 2002; Alitto and Usrey, 2003). Additional analysis of the interactions between different functional levels of the feeding-related motor circuits in the STNS will therefore inform investigators aiming to unravel the function of circuit feedback not only in other rhythmic motor systems but also in those involved in processing other types of information.

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