State-Dependent Presynaptic Inhibition Regulates Central Pattern Generator Feedback to Descending Inputs

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Central pattern generators (CPGs) provide feedback to their projection neuron inputs. However, it is unknown whether this feedback is regulated and how that might shape CPG output. We are studying feedback from the pyloric CPG to identified projection neurons that regulate the gastric mill CPG, in the crab stomatogastric nervous system. Both CPGs are located in the stomatogastric ganglion (STG) and are influenced by projection neurons originating in the paired commissural ganglia (CoGs). Two extrinsic inputs [ventral cardiac neurons (VCNs) and postoesophageal commissure (POC) neurons] trigger distinct gastric mill rhythms despite acting via the same projection neurons [modulatory commissural neuron 1 (MCN1); commissural projection neuron 2 (CPN2)]. These projection neurons receive feedback inhibition from the pyloric CPG interneuron anterior burster (AB), resulting in their exhibiting pyloric-timed activity during the retraction phase of the VCN- and POC-triggered gastric mill rhythms. However, during the gastric mill protraction phase, MCN1/CPN2 exhibit pyloric-timed activity during the POC-triggered rhythm but fire tonically during the VCN-triggered rhythm. Here, we show that the latter, tonic activity pattern results from the elimination of AB inhibition of MCN1/CPN2, despite persistent AB actions within the STG and AB action potentials still propagating into each CoG. This loss of pyloric-timed AB input likely results from presynaptic inhibition of AB in each CoG because, when a secondary rhythmic AB burst initiation zone in the CoG is activated, the associated action potentials are selectively suppressed during the VCN protraction phase. Thus, rhythmic CPG feedback can be locally regulated, in a state-dependent manner, enabling the same projection neurons to drive multiple motor patterns from the same neuronal circuit.

Key words: stomatogastric; presynaptic regulation; neuromodulation; rhythm; projection; crustacea

Introduction

Central pattern generator (CPG) circuits generate the motor patterns underlying rhythmic behaviors (Bellingham, 1998; Gao et al., 2001; Marder and Bucher, 2001; Kristan et al., 2005; Kiehn, 2006; Grillner et al., 2008). As a result of input from different sets of projection neurons, sensory neurons, and circulating hormones, each CPG can generate a set of distinct but related motor patterns (Rosen et al., 1991; Frost and Katz, 1996; Blitz et al., 1999; Marder and Bucher, 2007; Büschges et al., 2008; Dubuc et al., 2008; Jordan et al., 2008). CPGs can in turn provide rhythmic feedback that causes their projection neuron inputs to fire bursts that are time-locked to the CPG-generated rhythm (Gillette et al., 1978; Nusbaum, 1986; Arshavsky et al., 1988; Dubuc and Grillner, 1989; Nagy et al., 1994; Frost and Katz, 1996; Norris et al., 1996; Ezure and Tanaka, 1997; Buchanan and Einum, 2008). Thus far, however, it is unknown whether this CPG feedback is differentially regulated by distinct inputs and, if so, what its consequences might be for motor pattern generation.

We are examining local regulation of CPG feedback using the

crab stomatogastric nervous system (STNS). The STNS contains four ganglia, including the paired commissural ganglia (CoGs) and the single oesophageal (OG) and stomatogastric (STG) ganglia. The STG contains the gastric mill (chewing) and pyloric (food filtering) CPGs, whereas the CoGs and OG contain the projection neurons that regulate the STG circuits (Coleman et al., 1992; Nusbaum et al., 2001; Marder and Bucher, 2007). Similar to other rhythmic motor systems, CoG projection neurons receive feedback from the STG circuits that they regulate (Coleman and Nusbaum, 1994; Norris et al., 1994, 1996).

Several pathways, including the mechanosensory ventral cardiac neurons (VCNs) and the postoesophageal commissure (POC) neurons, trigger different gastric mill rhythms despite activating the same two projection neurons, modulatory commissural neuron 1 (MCN1) and commissural projection neuron 2 (CPN2) (Beenhakker and Nusbaum, 2004; Blitz et al., 2008). One likely contribution to these different rhythms is the distinct MCN1 and CPN2 activity patterns that occur during each rhythm (Beenhakker and Nusbaum, 2004; Blitz et al., 2008). Specifically, during the protraction phase, MCN1 and CPN2 fire tonically in the VCN- rhythm but generate pyloric rhythm-timed bursts during the POC-rhythm. In contrast, both projection neurons exhibit pyloric-timed activity during the retraction phase of both gastric mill rhythms. This pyloric-timed activity results from feedback from the pyloric pacemaker anterior burster (AB) neuron.

Here, we examined whether the apparent lack of CPG feed-

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Figure 1. Schematic of the isolated stomatogastric nervous system, including identified projection neurons and extrinsic inputs. The projection neurons (MCN1, CPN2) occur as single neurons in each CoG, from where they project through either the *ion* (MCN1) or *son* (CPN2) to innervate the STG. The extrinsic inputs (VCNs, POCNs) are bilaterally symmetric neuronal populations that innervate each CoG via either the *dpon* (VCNs) or the *poc* (POCNs). The CoG arborization of the POC neurons is depicted as a blob to reflect its dense arborization within a neurohemal organ in the anterior CoG (Goldberg et al., 1988; Messinger et al., 2005; Blitz et al., 2008). The lines projecting posteriorly from the STG represent the axons of STG motor neurons projecting toward their muscle targets. Abbreviations: ganglia: TG, thoracic ganglion; nerves: *dgn*, dorsal gastric nerve; *lvn*, lateral ventricular nerve; *mvn*, medial ventricular nerve; extrinsic inputs: POCNs, postoesophageal commissure neurons.

back during VCN protraction resulted from its local regulation. We found that AB-mediated inhibition in MCN1 and CPN2 was selectively eliminated during VCN protraction, despite persisting rhythmic AB actions within the STG and AB activity entering each CoG. The loss of AB inhibition onto MCN1 and CPN2 during VCN protraction resulted from presynaptic inhibition of the AB axon terminals within each CoG during the VCN rhythm, but not during the POC rhythm. Thus, there is local, state-dependent regulation of CPG feedback, resulting in distinct projection neuron activity patterns that contribute to the generation of different motor patterns from a single CPG circuit.

Materials and Methods

Animals. Male *Cancer borealis* crabs were obtained from commercial suppliers (Commercial Lobster and Seafood Company; Marine Biological Laboratory). Crabs were maintained in commercial tanks containing recirculating, filtered, and aerated artificial seawater (10°C). Before dissection, crabs were cold anesthetized by packing in ice for at least 30 min. The STNS was dissected as described previously (Blitz et al., 2004). Briefly, the foregut was removed from the animal and pinned dorsal-side down in a Sylgard 170 (K. R. Anderson; World Precision)-coated glass bowl in chilled *C. borealis* saline. The postoesophageal commissure (*poc*) (Fig. 1) was visualized with a dissecting microscope and bisected, after which the stomach was bisected ventrally and pinned flat with the interior stomach wall against the Sylgard. The STNS, including all four ganglia plus their connecting and peripheral nerves (Fig. 1), was then freed from surrounding tissue, removed from the surface of the foregut, and pinned down in a Sylgard 184 (K. R. Anderson; World Precision)-coated Petri dish. The foregut and nervous system were maintained in chilled (9–12°C) saline throughout the dissection and subsequent experiment.

Solutions. *C. borealis* saline included the following (in mM): 440 NaCl, 26 MgCl₂, 13 CaCl₂, 11 KCl, 10 Trizma base, 5 maleic acid, and 5 dextrose, pH 7.4–7.6. High divalent cation saline (HiDi saline) consisted of the following (in mM): 440 NaCl, 52 MgCl₂, 65 CaCl₂, 11 KCl, 10 Trizma

base, 5 maleic acid, and 5 dextrose, pH 7.4–7.6. HiDi saline decreases the likelihood of activating polysynaptic pathways by raising action potential threshold, thereby also suppressing spontaneous neuronal activity (Nusbaum and Marder, 1989; Blitz and Nusbaum, 1999). When applied to the OG and CoGs, this saline decreased spontaneous activity, as was evident in extracellular recordings of the nerves [superior oesophageal nerve (*son*); inferior esophageal nerve (*ion*)] (Fig. 1) connecting these ganglia to the STG. In addition, during superfusion of HiDi saline to the anterior ganglia, spontaneous pyloric rhythm activity in the STG was weakened and resembled the activity in preparations in which the CoG output nerves were transected (Bartos and Nusbaum, 1997; Blitz and Nusbaum, 1999).

Electrophysiology. Extracellular recordings were made by isolating a small region of nerve with a petroleum jelly well (Vaseline; Medical Accessories and Supply Headquarters) and placing one of a pair of stainless-steel wires inside the well and the other wire inside the main bath compartment. Loose-patch recordings were made by placing glass electrodes near axons in the desheathed *son* (Fig. 1) and applying suction to the electrode. Electrode location was altered to obtain recordings from as few axons as possible. Extracellular nerve recordings and axonal loose-patch recordings were amplified using AM Systems Model 1700 AC amplifiers and Brownlee Precision model 410 amplifiers.

Intracellular microelectrodes were made from borosilicate glass filled with 0.6 M K₂SO₄ plus 10 mM KCl (current clamp, 20–25 MΩ; voltage clamp, 8–12 MΩ). Intracellular signals were amplified using Axoclamp 2B and 900A amplifiers (Molecular Devices) in bridge mode, discontinuous current-clamp mode (3–10 kHz sampling rate), or discontinuous single electrode voltage-clamp mode (4.5–15 kHz sampling rate), and digitized at ~5 kHz using a Micro 1401 data acquisition interface and Spike2 software (Cambridge Electronic Design). Voltage-clamp gain was 0.7–2.5 nA/mV.

To facilitate intracellular recordings, ganglia were desheathed and viewed with light transmitted through a dark-field condenser (Nikon). STG and CoG neurons were identified based on their activity patterns, synaptic connectivity, and axonal projection patterns (Weimann et al., 1991; Beenhakker and Nusbaum, 2004; Saideman et al., 2007). The VCN-gastric mill rhythm was triggered by extracellular stimulation of the dorsal protractor oesophageal nerve (*dpon*) (Figs. 1, 2) (intra-burst frequency, 15 Hz; interburst frequency, 0.06 Hz; burst duration, 6 s) (Beenhakker and Nusbaum, 2004; Beenhakker et al., 2004). The POC-gastric mill rhythm was triggered by extracellular stimulation of the *poc* (Figs. 1, 2) (tonic stimulation, 15 Hz; duration, 30 s) (Blitz et al., 2008). As noted above, the *poc* was bisected during the dissection and left and right portions were stimulated either separately or simultaneously (Blitz et al., 2008).

The pyloric rhythm was routinely monitored by an extracellular recording of the pyloric dilator nerve (*pdn*), which contains the axons of the pyloric dilator (PD) neurons (Figs. 1, 2). PD neurons are part of the pyloric pacemaker ensemble because of their electrical coupling to the conditional oscillator AB neuron (Eisen and Marder, 1982; Miller and Selverston, 1982a,b). The gastric mill rhythm was routinely monitored via intracellular recording of the lateral gastric (LG) protractor neuron, a gastric mill CPG neuron, and/or an extracellular recording of the LG axon in the lateral gastric nerve (*lgn*) (Figs. 1, 2).

Data analysis. Data analysis was performed using Spike2 software. Time-locked IPSPs were detected by overlapping multiple sweeps (≥20/ recording) of simultaneous recordings of AB and the indicated projection neuron. All recordings were aligned to the peak of AB action potentials. For Figure 3, we selected AB action potentials from the end of each AB burst, to minimize the differences in the projection neuron membrane potential at the onset of each IPSP. The number of AB action potentials per pyloric-timed burst was measured from loose-patch recordings to eliminate the possibility of a change in spike number caused by impalement injury. In some figures, a raw extracellular recording (*ion* or *son*) was duplicated with the activity of a unit digitally subtracted for clarity, as indicated in each such figure legend. A custom written script in Spike2 was used to digitally subtract the secondary unit after manually inspecting the trace to verify that only that particular unit was selected for subtraction.

Figures were made using Spike2 and Corel-Draw (Corel). Statistical significance was assessed with SigmaStat (Systat Software). The paired Student *t* test was used, and significance was considered to be $p < 0.05$. Data are expressed as mean $\pm$ SEM.

Results

The STG contains the pyloric (food filtering) and gastric mill (chewing) CPGs. The pyloric rhythm (cycle period, ~ 0.5 – 2 s) is a pacemaker-driven, three-phase motor pattern that is spontaneously active both *in vivo* and *in vitro* (Fig. 2, left) (Marder and Bucher, 2007). The gastric mill rhythm (cycle period, ~ 5 – 20 s) is a two-phase motor pattern (protraction, retraction) that is usually not spontaneously active *in vivo* or *in vitro* (Fig. 2, left) (Beenhakker et al., 2004; Marder and Bucher, 2007). Instead, its activity requires the activation of particular CoG projection neurons, such as MCN1 and CPN2 (Nusbaum et al., 2001; Beenhakker and Nusbaum, 2004; Blitz et al., 2004, 2008; Saideman et al., 2007). The gastric mill rhythm drives the alternating protraction and retraction of the paired lateral teeth and unpaired medial tooth within the gastric mill stomach compartment (Heinzel et al., 1993). In all preparations used in this study, the pyloric rhythm was spontaneously active, whereas the gastric mill rhythm was only active when triggered by VCN or POC stimulation (Fig. 2).

Stimulation of either the mechanosensory VCNs or the POC neurons triggers a lasting activation of MCN1 and CPN2, which in turn drive the gastric mill rhythm via their synaptic connections onto CPG neurons within the STG (Beenhakker and Nusbaum, 2004; Beenhakker et al., 2004; Blitz et al., 2008). However, VCN and POC stimulation trigger distinct gastric mill rhythms along with rhythm-specific activity patterns in MCN1 and CPN2. For example, MCN1 and CPN2 exhibit pyloric-timed activity during both phases of the POC-gastric mill rhythm, whereas their activity is also pyloric timed during VCN retraction but is tonic during VCN protraction (Fig. 2, right) (Beenhakker and Nusbaum, 2004; Beenhakker et al., 2004; Blitz et al., 2008). Insofar as these two projection neurons drive the activity of the protractor neuron LG (Coleman and Nusbaum, 1994; Norris et al., 1994), LG also generates pyloric-timed bursts during the POC rhythm and tonic bursts during the VCN rhythm (Fig. 2) (Beenhakker and Nusbaum, 2004; Beenhakker et al., 2004; Blitz et al., 2008).

MCN1 and CPN2 receive feedback inhibition from a pyloric CPG neuron

All four identified CoG projection neurons, including MCN1, CPN2, and modulatory commissural neurons 5 and 7 (MCN5, MCN7), exhibit pyloric-timed activity caused by feedback from the pyloric CPG (Coleman and Nusbaum, 1994; Norris et al., 1994, 1996; Beenhakker and Nusbaum, 2004; Beenhakker et al., 2004). The pyloric pacemaker neuron AB is the likely source of the pyloric-timed activity in the projection neurons, because the AB neuron is the only pyloric neuron that projects to the CoGs

Figure 2. The MCN1 and CPN2 activity patterns are distinct during the POC- and the VCN-triggered gastric mill rhythms. **A, B**, Left, Before POC or VCN stimulation, there was an ongoing pyloric rhythm (*pdn*) and no gastric mill rhythm (LG silent). At these times, MCN1 was silent (**A**) or weakly active (**B**), whereas CPN2 was weakly active. **A, B**, Right, After POC or VCN stimulation, there was a long-lasting excitation of MCN1 and CPN2 that resulted in a gastric mill rhythm being elicited from the gastric mill circuit in the STG. The gastric mill rhythm is represented by the rhythmic bursting of the LG neuron. During the POC-triggered gastric mill rhythm, MCN1 and CPN2 exhibited a pyloric rhythm-timed activity pattern, as did the LG neuron. In contrast, during the VCN-triggered gastric mill rhythm, MCN1 and CPN2 activity was pyloric-timed during retraction (black bar, Ret) but was tonic during protraction (white bar, Pro), despite the fact that the pyloric rhythm (PD) persisted during each protraction phase. Note that, in **A**, the bottom *ion* recording is the same as the top *ion* recording except that the oesophageal motor neuron (OMN) activity was digitally subtracted to more explicitly show the MCN1 activity pattern (see Materials and Methods).

(Fig. 3A) (Claiborne and Ayers, 1987). In turn, several of these projection neurons, including MCN1, excite the AB neuron (Norris et al., 1996; Bartos and Nusbaum, 1997).

Each of the four identified CoG projection neuron receives IPSPs that occur during the AB/PD neuron phase of the pyloric rhythm (Fig. 3B) (Coleman and Nusbaum, 1994; Norris et al., 1994, 1996; Beenhakker and Nusbaum, 2004; Beenhakker et al., 2004). We confirmed this synaptic relationship by pairing extracellular recordings of the AB axon in the stomatogastric nerve (*stn*) (Fig. 1) with intracellular recordings of the identified CoG projection neurons. For example, the overlay of multiple sweeps aligned to the peak of the AB action potential in Figure 3C illustrates the consistent presence of a discrete IPSP in these projection neurons after every AB action potential (MCN1, $n = 8$; CPN2, $n = 3$; MCN5, $n = 5$).

The reliability of these AB-elicited IPSPs in the identified projection neurons suggested that there was a direct synapse from AB to each of them. To further assess whether these were direct connections, we superfused the anterior region of the STNS, including the desheathed OG and CoGs, with HiDi saline to increase action potential threshold and decrease the likelihood of AB activating an intervening neuron (see Materials and Methods). In the presence of HiDi saline, rhythmic AB/PD-timed inhibition of the CoG projection neurons persisted (MCN1, $n = 2$ of 2; MCN5, $n = 2$ of 2), supporting the likelihood that the AB neuron directly inhibited these projection neurons.

Figure 3. The pyloric pacemaker neuron, AB, inhibits MCN1 and CPN2 in the CoGs. **A**, Schematic diagram indicating that the AB interneuron projects from the STG to the CoGs, where it inhibits MCN1 and CPN2. Note that AB and the PD motor neurons are electrically coupled, enabling them to fire in phase (Eisen and Marder, 1982; Miller and Selverston, 1982a,b). The PD neurons do not project to the CoGs. Filled circles, Synaptic inhibition; resistor, nonrectifying electrical coupling; arrows, projection path. **B**, The activity of the projection neurons CPN2 and MCN1 was inhibited during each AB/PD burst (gray bars). **C**, Reliably occurring IPSPs were recorded in MCN1 and CPN2 after each AB action potential (recorded extracellularly in the *stn*). Each collection of IPSPs in MCN1 and CPN2 represents 50 sweeps triggered by an AB action potential. The thick black lines are the average of each set of 50 sweeps. The vertical dashed lines indicate the latency from the peak of the AB action potential to the onset of the IPSPs. The AB recording site in the *stn* was ~ 1.5 cm from the CoG. The observed latencies are consistent with those reported for CoG projection neuron-initiated action potentials in comparably sized axons traveling this distance to the STG and eliciting PSPs in target neurons (Coleman et al., 1995; Norris et al., 1996). The recordings in **B** and the left and right recordings in **C** are from three different preparations.

Figure 4. The activity of the AB neuron proximal to the CoGs is not inhibited during the protraction phase of the VCN-triggered gastric mill rhythm. Left, Before VCN stimulation, there was no gastric mill rhythm (LG silent), but there was an ongoing pyloric rhythm (AB). Note that the AB recording was from a loose-patch recording of its axon in the *son* nerve (Fig. 1). Right, After VCN stimulation triggered the gastric mill rhythm, AB fired rhythmic bursts of action potentials throughout both retraction (Ret) and protraction (Pro), despite the tonic MCN1 activity during protraction. The bottom *ion* recording is a duplicate of the top *ion* recording except that the oesophageal motor neuron (OMN) was digitally subtracted to better illustrate the MCN1 activity pattern. Likewise, the bottom *son* recording is a duplicate of the top *son* recording with an additional, larger amplitude oesophageal-timed unit digitally subtracted for clarity (see Materials and Methods).

AB neuron activity persists proximal to the CoGs during VCN protraction

The loss of pyloric-timed activity in MCN1 and CPN2 during VCN protraction might have resulted from suppression of the pyloric rhythm during this phase of the gastric mill rhythm. However, this was not the case because the pyloric rhythm consistently persisted throughout both phases of the VCN-triggered gastric mill rhythm (Fig. 2B) ($n = 29$ of 29 preparations).

Given that MCN1 and CPN2 are inhibited by AB but fire tonically during VCN protraction despite an ongoing pyloric rhythm, it is possible that the AB action potentials were phasically suppressed at or near the STG during the protraction phase of the VCN-gastric mill rhythm. This was a possibility because, within the STG, AB synaptic transmission is mediated primarily by graded transmitter release (Raper, 1979; Graubard et al., 1980, 1983; Anderson and Barker, 1981). Thus, suppression of AB action potentials could occur without having much impact on the ability of AB to drive the pyloric rhythm in the STG. However, by recording AB activity extracellularly near the entrance to the CoG, we found that AB action potentials were not eliminated proximal to the CoG during VCN protraction (Fig. 4). Specifically, rhythmic AB neuron activity persisted at the level of the *son* nerves after the VCN-gastric mill rhythm was triggered, during both protraction and retraction (Fig. 4) ($n = 6$ of 6). Furthermore, there was no difference in the number of AB action potentials nor its firing frequency during the retractor and protractor phases of the VCN-gastric mill rhythm (AB spike number: protraction, 6.0 ± 0.2 ; retraction, 5.8 ± 0.1 , $p > 0.05$, $n = 4$; firing frequency: protraction, 28.0 ± 1.8 Hz; retraction, 29.6 ± 1.1 , $p > 0.05$, $n = 4$). This result suggested the possibility that the AB neuron activity was locally regulated within each CoG.

Local regulation of AB synapses onto CoG projection neurons

As an initial approach to determine the site at which the AB neuron synaptic actions onto CoG projection neurons was being regulated during VCN protraction, we took advantage of the bilaterally symmetric nature of the anterior STNS and stimulated the VCN and POC pathways on opposite sides (Fig. 5). The gastric mill rhythm that was triggered by this costimulation commonly included a mixture of VCN-like (tonic) and POC-like (pyloric-timed) LG neuron activity (Fig. 5). How-

Figure 5. The synaptic actions of the AB neuron are separately regulated in each CoG. Costimulation of VCNs_{right} and POCs_{left} triggered a gastric mill rhythm (LG) during which MCN1_R and MCN1_L exhibited distinct protraction-phase activity patterns. Left, The AB-mediated inhibition was apparent in both MCN1s during each retractor phase (Ret) because their activity was interrupted during each AB/PD burst (*pdn*). In contrast, during protraction (Pro) the MCN1_R (VCN-stimulated side) fired tonically, whereas MCN1_L (POC-stimulated side) continued to exhibit a pyloric-timed activity pattern. Right, Schematic illustration of the sites of stimulation for the POCs (*poc_L*) and VCNs (*dpon_R*). Note that the diagonal bars through the *poc* indicate that this commissure was transected before nerve stimulation (Blitz et al., 2008).

ever, the associated protraction phase activity pattern of each MCN1 reflected the pattern expected to occur based on the pathway used to activate it. Specifically, the MCN1 protraction phase activity pattern was tonic on the VCN-stimulated side, whereas it was pyloric-timed on the POC-stimulated side ($n = 5$) (Fig. 5). This result further supported the hypothesis that the tonic firing in MCN1 and CPN2 during VCN protraction was not attributable to a global change in AB activity but occurred locally within each CoG. It further suggested that the decreased efficacy of AB inhibition onto MCN1 and CPN2 occurred via a postsynaptic shunting of the AB inhibition in the projection neurons and/or a presynaptic inhibition of the AB axon terminals in the CoGs (AB_{CoG}).

The possibility that the decreased efficacy of AB inhibition in the CoG resulted from a postsynaptic shunting mechanism seemed unlikely because the same event would have to occur with a consistent effectiveness in all four identified CoG projection neurons (MCN1, CPN2, MCN5, MCN7). This is because these four projection neurons all lose their pyloric-timed inhibitory input during VCN protraction (Norris et al., 1994, 1996; Beenhakker et al., 2004). This occurs in MCN5 ($n = 8$ of 8) and MCN7 ($n = 1$ of 1) despite the fact that they do not contribute to driving the VCN-gastric mill rhythm and are either inactive or weakly active after VCN stimulation (Beenhakker and Nusbaum, 2004; Beenhakker et al., 2004). These observations suggested that a single event that would have a simultaneous and equivalent impact on all four projection neurons, such as presynaptic inhibition of AB_{CoG}, was more likely to mediate the loss of pyloric-timed activity during VCN protraction in these neurons.

Presynaptic inhibition of the AB axon terminals in each CoG

To determine whether the AB inhibition of CoG projection neurons was being suppressed presynaptically or postsynaptically, we recorded AB-mediated IPSCs with voltage-clamp recordings of MCN1 and CPN2. During these recordings, pyloric rhythm-timed barrages of AB-mediated IPSCs were consistently recorded in MCN1 ($n = 12$) and CPN2 ($n = 12$) (Fig. 6A). After VCN

stimulation, the AB-mediated IPSCs were selectively eliminated in both MCN1 ($n = 8$ of 8) and CPN2 ($n = 8$ of 8) during VCN protraction (Fig. 6B). When the VCN-gastric mill rhythm eventually terminated, these IPSCs again occurred during every cycle of the pyloric rhythm (MCN1, $n = 6$ of 6; CPN2, $n = 6$ of 6). During these experiments, we stimulated the left and right VCN (or POC, see below) pathways so that the contralateral MCN1 or CPN2 activity would continue to drive the gastric mill rhythm when we placed MCN1 or CPN2 into voltage clamp and eliminated its activity. During protraction, the current level in both MCN1 ($n = 7$ of 8) and CPN2 ($n = 8$ of 8) was consistently more negative than the current measured during the trough in between the bouts of AB IPSCs. We did not pursue the underlying cause of this distinction.

Consistent with the hypothesis that there was a single event underlying the loss of pyloric-timed activity during VCN protraction in all four identified CoG projection neurons, the AB-mediated IPSCs were also eliminated in MCN5 ($n = 8$ of 8) and MCN7 ($n = 1$ of 1) during VCN protraction. This result further implicated the single event as being a presynaptic inhibition of AB_{CoG} and not a postsynaptic shunting of the influence of the AB synapse.

The elimination of AB-mediated IPSCs in MCN1 and CPN2 was specific to the VCN-type of gastric mill rhythm. During POC-gastric mill rhythms, the rhythmic barrages of AB-mediated IPSCs occurred during both protraction and retraction phases in MCN1 ($n = 4$ of 4) and CPN2 ($n = 4$ of 4) (Fig. 7).

To assess further whether there was presynaptic inhibition of AB_{CoG}, we took advantage of the presence of a secondary burst initiation zone in AB_{CoG}. Multiple spike or burst initiation zones are found in many neurons, with a primary/dominant zone defined as the one with the fastest intrinsic firing rate (Calabrese and Kennedy, 1974; Moulins et al., 1979; Meyrand et al., 1992; Zecević, 1996; Bucher et al., 2003). In such cases, when the primary zone is hyperpolarized, other zones can become active (Calabrese, 1980). In rhythmically bursting neurons, the secondary zone can generate bursts of action potentials (Calabrese, 1980).

Figure 6. The AB neuron-mediated IPSCs in MCN1 and CPN2 are eliminated during the protraction phase of the VCN-triggered gastric mill rhythm. **A**, During an ongoing pyloric rhythm (PD), pyloric rhythm-timed barrages of AB-mediated IPSCs (gray bars) occurred in MCN1 (left) and CPN2 (right). The left and right panels are from different preparations. The holding potential was -60 mV for MCN1 and -65 mV for CPN2. **B**, During the VCN-triggered gastric mill rhythm (indicated by the tonic LG bursts), the AB-mediated IPSCs in both MCN1 (left) and CPN2 (right) were present during each retractor phase (Ret). However, these IPSCs were eliminated during each protractor phase (Pro), despite the continued presence of the pyloric rhythm in the STG (PD). The left and right panels are from different preparations. The holding potential was -65 mV for MCN1 and -70 mV for CPN2.

After VCN stimulation, hyperpolarizing the STG region of the AB neuron (AB_{STG}) consistently activated a secondary AB burst initiation zone in the CoGs ($n = 7$). We hyperpolarized AB_{STG} either directly or indirectly via the electrically coupled PD and/or LPG (lateral posterior gastric) neurons. The activation of a secondary AB burst initiation zone was evident by recording the AB neuron at multiple sites simultaneously, including an intra-axonal AB recording near the STG and extracellular *stn* and/or *son* recordings located more anteriorly (Fig. 8A). In some experiments, we instead obtained a loose-patch AB axon recording in the *son* (Fig. 8A). In the absence of hyperpolarizing current injection, the AB action potentials always initiated near the STG and occurred later in the *stn* and *son* ($n = 10$ of 10) (Fig. 8B). In contrast, when AB_{STG} was hyperpolarized, the AB action potentials occurred first in the *son* and propagated toward the STG ($n = 4$ of 4) (Fig. 8B). These antidromic AB action potentials occurred in rhythmic bursts with a longer cycle period than the orthodromic AB bursts (Fig. 8C). The CoG burst initiation zone consistently elicited rhythmic bursts with longer cycle periods than the STG burst initiation zone (control, 1.14 ± 0.1 s; AB_{STG} hyperpolarized, 1.73 ± 0.2 s; $p < 0.05$; $n = 6$). Although consistently longer than the control pyloric cycle periods, the AB antidromic burst cycle periods were similar to typical pyloric cycle periods (0.5–2 s), and distinct from gastric mill cycle periods (5–20 s) (Beenhakker et al., 2004; Marder and Bucher, 2007).

At the STG, the antidromic bursts of AB action potentials propagated into the region in which the AB membrane potential was held hyperpolarized by current injection, which prevented transmitter release from AB_{STG} . Consequently, at these times no pyloric rhythm occurred ($n = 6$ of 6) (Fig. 8C). These AB action potentials were, however, effective in the CoGs as is evident from

the associated disruptions in MCN1 activity ($n = 6$ MCN1s in five preparations) (Fig. 8C).

We used these CoG-initiated AB action potential bursts, recorded intra-axonally in AB near the STG, to assess the possibility that AB was presynaptically inhibited during VCN protraction. We anticipated that, if AB was indeed presynaptically inhibited, then the antidromic AB bursts would likely be selectively eliminated during VCN protraction. In each experiment, before hyperpolarizing AB_{STG} to activate its CoG burst initiation zone, AB exhibited pyloric-timed membrane potential oscillations with action potential bursts at the depolarized peak of each oscillation (Fig. 9A). These AB bursts were coactive with the PD neurons, as occurs consistently during the pyloric rhythm (Marder and Bucher, 2007). We then triggered the VCN-gastric mill rhythm and found that the pyloric-timed AB_{STG} activity persisted, as usual, during both protraction and retraction (Fig. 9B). In contrast, when AB_{STG} was hyperpolarized and its CoG burst initiation zone was activated, the CoG-initiated AB action potentials persisted during VCN retraction but were consistently eliminated during each protraction phase ($n = 6$ of 6) (Fig. 9C). This result supported the occurrence of presynaptic inhibition of AB_{CoG} during VCN protraction.

Discussion

In this study, we determined that CPG feedback to its descending inputs is locally regulated, in a state-dependent manner. Specifically, after stimulating the mechanosensory VCN pathway, there was a gastric mill rhythm-timed presynaptic inhibition of the AB_{CoG} axon terminals. This presynaptic inhibition occurred during each gastric mill protractor phase, eliminating the inhibitory synaptic actions of AB on the CoG projection neurons, including

MCN1 and CPN2, and changing their activity pattern from pyloric-timed to tonic. We found no evidence for comparable events during the POC-gastric mill rhythm (Fig. 10). The distinct activity patterns in MCN1 and CPN2 during these two gastric mill rhythms contributed to their ability to drive different versions of the gastric mill rhythm (Beenhakker and Nusbaum, 2004; Blitz et al., 2008) (Fig. 10). The presence of CPG feedback to its descending inputs is well documented in motor systems, as is its ability to impose a rhythmic activity pattern on these inputs (Gillette et al., 1978; Nusbaum, 1986; Arshavsky et al., 1988; Dubuc and Grillner, 1989; Nagy et al., 1994; Frost and Katz, 1996; Norris et al., 1996; Ezure and Tanaka, 1997; Buchanan and Einum, 2008). To our knowledge, the ability of such CPG feedback to be locally regulated at the site of its feedback synapses has not been previously demonstrated.

Local regulation of AB neuron activity in the CoGs enables it to continue to function in other regions of the nervous system. For example, it continues to drive the pyloric rhythm in the STG during times when its transmitter release is suppressed in the CoGs. Comparable presynaptic regulation of transmitter release is a common feature of all nervous systems (Chevalyere et al., 2006; Rossignol et al., 2006; Pelkey and McBain, 2007; El Manira et al., 2008; Pinheiro and Mülle, 2008). More specifically with respect to motor systems, presynaptic regulation of transmitter release not only influences CPG feedback to projection neurons as shown here for the AB feedback pathway, but it also focally regulates feedforward input onto CPGs (Coleman and Nusbaum, 1994; Lomeli et al., 1998; Perrins and Weiss, 1998; Westberg et al., 2000; Sasaki et al., 2007).

Aside from changing the activity pattern of its projection neuron targets, the function of the CPG feedback pathway remains to be determined in most systems. In the isolated STNS, one documented function for CPG feedback to projection neurons is to enable the pyloric circuit to regulate the gastric mill rhythm (Wood et al., 2004). For example, when MCN1 activity is pyloric-timed, the gastric mill protractor LG neuron also exhibits pyloric-timed bursts, as occurs during the POC-gastric mill rhythm. When MCN1 activity is tonic, the LG bursts are tonic, as occurs during the VCN-gastric mill rhythm. These distinct protractor phase activity patterns appear to have behavioral correlates insofar as, in intact crabs, the lateral teeth can protract either smoothly or in a pyloric-timed manner (Heinzel et al., 1993). Although the behavioral function of these two chewing modes is not known, their presence supports the need for the motor system to generate these distinct motor patterns.

The gastric mill-timed presynaptic inhibition of AB also provides a timing cue to CoG neurons regarding the ongoing gastric mill rhythm. Thus, during the VCN-gastric mill rhythm, the CoG neurons that are targets of AB exhibit activity patterns timed to both the pyloric and gastric mill rhythms. This information may be used to coordinate the gastric mill and pyloric rhythms with other motor systems. Coordination between different rhythmic behaviors is common in all animals, such as the coordination between locomotion and respiration (Bramble and Carrier, 1983; Kawahara et al., 1989; Syed and Winlow, 1991; Morin and Viala, 2002; Saunders et al., 2004). Such intercircuit coordination exhibits flexibility, including changes in coupling ratios as well as a complete loss of coordination under different behavioral conditions (Bernasconi and Kohl, 1993; Clemens et al., 1998; Saunders et al., 2004). Although little is known regarding the underlying cellular mechanisms, state-dependent local regulation of CPG feedback seems well suited to play a role in these events. In other systems, there are also neurons in higher centers that exhibit

Figure 7. The AB neuron-mediated IPSCs in MCN1 and CPN2 persist during the protraction phase of the POC-triggered gastric mill rhythm. **A**, During the POC-triggered gastric mill rhythm (evident in the pyloric-timed LG bursts), the AB-mediated IPSCs in MCN1 were present during both retraction (Ret) and protraction (Pro). The pyloric rhythm and the timing of AB activity are represented by the PD (pdn) recording. The holding potential of MCN1 was -65 mV. **B**, The AB-mediated IPSCs in CPN2 were also present throughout both retraction (Ret) and protraction (Pro) during the POC-triggered gastric mill rhythm. The holding potential of CPN2 was -65 mV. **A** and **B** are from separate preparations.

activity patterns that are time-locked to multiple rhythms. For instance, some neurons in the medullary lateral reticular nucleus exhibit activity patterns that are time-locked with both the respiratory and locomotor rhythms (Ezure and Tanaka, 1997). However, neither the pathways that mediate this coordinated activity nor the function of these activity patterns is identified in most systems.

Although the extent to which MCN1 and CPN2 are necessary and sufficient for driving the POC-gastric mill rhythm remains to be determined, they likely play significant roles in driving this version of the rhythm (Wood et al., 2004; Blitz et al., 2008). Previous work established that coactivation of MCN1 and CPN2 is necessary and sufficient for driving the VCN-gastric mill rhythm as well as the distinct gastric mill rhythm triggered by the gastropyloric receptor (GPR) neuron (Beenhakker and Nusbaum, 2004; Blitz et al., 2004). Unlike the distinct protraction phase activity patterns that occur during the VCN- and POC-gastric mill rhythms, the VCN and GPR rhythms differ in cycle period and the activity levels of the associated motor neurons but share an overall tonic activity pattern during protraction and pyloric-timed activity during retraction. The distinction between these latter two rhythms appears to result from the additional ability of GPR to presynaptically inhibit the MCN1 axon termi-

Figure 8. Hyperpolarizing the STG region of AB activates a secondary burst initiation zone in the CoGs. **A**, Schematic illustration of the AB recording sites, including a sharp electrode intra-axonal recording near the STG (AB_{STG}) and a loose-patch recording of the AB axon in the *son* nerve (AB_{son}). **B**, Multiple (20) overlaid traces were aligned to AB action potentials recorded intra-axonally. Left, When the AB_{STG} was not regulated by current injection (V_{mv} , -57 mV), its action potentials were recorded first at AB_{STG} and then at AB_{son} . Right, When AB_{STG} was hyperpolarized (-73 mV), the AB action potentials occurred first at AB_{son} and then at AB_{STG} , indicating that a secondary spike initiation zone was activated near or within a CoG. Both panels are from the same preparation. **C**, Left, Under control conditions, AB acted as the pacemaker for the pyloric rhythm (note the coactive AB and PD bursts) and each AB burst (bars) inhibited MCN1 activity. Right, When AB_{STG} was hyperpolarized, its secondary burst initiation zone was activated at a location anterior to the STG. This antidromic AB activity (bars) failed to effectively invade the STG, as evident from the lack of any associated PD neuron (*pdn*) activity. Note that these antidromic bursts occurred with a longer cycle period than the orthodromic bursts. Despite not driving the pyloric rhythm in the STG, this secondary burst generating zone effectively inhibited MCN1 activity in the CoGs. The two *ion* recordings are the same, except that the OMN action potentials were digitally subtracted for the bottom *ion* recording to improve the clarity of the MCN1 activity (see Materials and Methods).

nals in the STG (Beenhakker et al., 2005). In several other motor systems, population coding is proposed to be the mechanism underlying the ability of single networks to generate multiple motor patterns (Georgopoulos, 1995; Kristan and Shaw, 1997; Lewis and Kristan, 1998; Liu and Fetcho, 1999). Recent work at the level of identified projection neurons in the *Aplysia* feeding system has provided additional support for the role of population coding (Morgan et al., 2002). This concept centers on the ability of distinct but related stimuli to activate separate, albeit overlapping populations of projection neurons. The ability of different extrinsic inputs to trigger distinct gastric mill rhythms despite their convergent coactivation of MCN1 and CPN2 supports the hypothesis that motor pattern selection can be mediated either by recruiting distinct projection neuron populations or differentially modifying the activity level and/or pattern of the same population.

It has also been suggested that CPG feedback may restrict the influence of higher-order and sensory inputs onto projection neurons to a particular phase of a rhythmic behavior (Deliagina et al., 2000; Pflieger and Dubuc, 2004). Therefore state-dependent regulation of CPG feedback such as we demonstrate here may provide flexibility in the influence of sensory signals during different motor patterns. Recent work has shown that there is both phase- and state-dependent filtering

of sensory feedback to MCN1 and CPN2 during the VCN-gastric mill rhythm, albeit not obviously linked to the presynaptic inhibition of AB_{CoG} (Beenhakker et al., 2005, 2007).

We have not yet determined the cellular and synaptic mechanisms by which the AB neuron is presynaptically inhibited within the CoG. Presumably, this presynaptic inhibition is mediated by one or more unidentified CoG neurons. It appears likely, however, that this CoG pathway has its activity regulated by the gastric mill CPG neuron interneuron 1 (Int1). Int1 is the only gastric mill neuron that projects to and influences CoG neurons (Claiborne and Ayers, 1987; Norris et al., 1994). Further, Int1 and LG are reciprocally inhibitory and burst in alternation during the gastric mill rhythm (Coleman et al., 1995; Bartos et al., 1999). Therefore, Int1 could provide the appropriate timing to the neuron(s) that inhibits AB_{CoG} during VCN protraction by inhibiting that neuron during retraction. In fact, hyperpolarizing Int1 can elicit a VCN protraction-like response in MCN1 and CPN2 even without previous VCN stimulation (Norris et al., 1994; B. J. Norris, M. J. Coleman, and M. P. Nusbaum, unpublished data). Thus, identifying targets of Int1 within the CoGs may enable identification of the neuron(s) that inhibit the AB_{CoG} terminals. It is not clear why there is no comparable presynaptic inhibition of AB_{CoG} during the POC-gastric mill rhythm, although the neuron(s) responsible for this focal action may be selectively inhibited by POC stimulation.

In conclusion, this study demonstrates that phasic presynaptic inhibition of CPG feedback helps shape projection neuron activity patterns. Furthermore, this local regulation is state dependent and consequently enables the same descending inputs to exhibit distinct activity patterns and drive different versions of a motor pattern under different conditions. Whether this state-dependent regulation of feedback alters the impact of other incoming signals to projection neurons, such as phase-specific sensory inputs, remains to be determined.

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Figure 9. The secondary burst generating zone of the AB neuron is suppressed during the protraction phase of the VCN-triggered gastric mill rhythm. *A*, Before VCN stimulation, AB_{STG} exhibited pyloric-timed membrane potential oscillations and fired action potentials at the peak of each oscillation. There was an ongoing pyloric rhythm (*pdn*) and no gastric mill rhythm (LG). *B*, During the VCN-triggered gastric mill rhythm, pyloric-timed bursts of AB_{STG} action potentials persisted during both protraction (Pro) and retraction (Ret). *C*, When AB_{STG} was hyperpolarized, the pyloric rhythm ceased (note lack of AB_{STG} membrane potential oscillations and elimination of PD activity) and the secondary AB burst initiation zone was activated. These presumably CoG-initiated action potentials were eliminated during each protraction phase (Pro). The smaller amplitude events, most evident during each LG burst, are EPSPs from the projection neuron MCN5 (Norris et al., 1996). All panels are from the same preparation.

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Figure 10. State-dependent presynaptic inhibition of AB feedback onto MCN1 and CPN2 underlies the distinct POC- and VCN-triggered gastric mill protractor phase activity patterns. **A**, During the VCN-gastric mill rhythm, the CoG axon terminals of the AB neuron are presynaptically inhibited during the protractor phase (Pro). This eliminates AB inhibitory feedback onto MCN1 and CPN2 in the CoG, whereas AB activity persists in the STG. This presynaptic inhibition switches the projection neurons from a pyloric rhythm-timed activity pattern during retraction (Ret) to a tonic firing pattern during protraction. This tonic firing pattern in turn drives tonic firing of the gastric mill protractor neuron LG in the STG. **B**, During the POC-gastric mill rhythm, there is no presynaptic inhibition of AB in the CoGs. Therefore, MCN1 and CPN2 activity is pyloric-timed during both protraction (Pro) as well as retraction (Ret), which in turn drives the protractor LG neuron to fire in a pyloric rhythm-timed pattern.

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