Peptide Hormone Modulation of a Neuronally Modulated Motor Circuit

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Kirby MS, Nusbaum MP. Peptide hormone modulation of a neuronally modulated motor circuit. J Neurophysiol 98: 3206–3220, 2007. First published October 3, 2007; doi:10.1152/jn.00795.2006. Rhythmically active motor circuits are influenced by neuronally released and circulating hormone modulators, but there are few systems in which the influence of a peptide hormone modulator on a neuronally modulated motor circuit has been determined. We performed such an analysis in the isolated crab stomatogastric system by assessing the influence of the hormone crustacean cardioactive peptide (CCAP) on the gastric mill (chewing) rhythm elicited by identified modulatory projection neurons. The gastric mill circuit is located in the stomatogastric ganglion. In situ, this ganglion is located within the ophthalmic artery and thus is in the path of circulating hormones such as CCAP. Focally-applied CCAP directly excited some gastric mill neurons, including the gastric mill central pattern generator neurons LG and Int1, but it did not elicit a sustained gastric mill rhythm. At concentrations as low as $10^{-10}$ M, however, CCAP did influence gastric mill rhythms elicited by coactivating the projection neurons MCN1 and CPN2 and by selectively stimulating MCN1. In both cases, CCAP slowed this rhythm by selectively prolonging the protraction phase, although its influence on the MCN1-elicited rhythm was limited to those with relatively brief cycle periods. Interestingly, CCAP also reduced the threshold MCN1 firing frequency for activating the gastric mill rhythm. Last, the gastric mill neurons that exhibited altered activity during these CCAP-influenced rhythms did not correspond completely to the set of CCAP-responsive neurons. These results highlight the ability of hormonal modulation to enhance the flexibility provided by the neuronal modulation of rhythmically active motor circuits.

INTRODUCTION

Individual neuronal networks are influenced by many different neuromodulators, which enable each network to generate multiple activity patterns (Alford et al. 2003; Marder and Bucher 2001; Marder et al. 2005; Ramirez et al. 2004). Network modulation results from the local neuronal release of modulatory transmitters and from circulating hormones. Distinct modulatory inputs to a neuronal network can work independently, but there are likely times when their influences overlap. In such cases, one neuromodulator can alter the influence of the co-active one, a condition termed metamodulation (Katz and Edwards 1999). There are, however, few examples documenting the consequences of metamodulation for network activity. Thus far, changes resulting from metamodulation include the generation of distinct motor patterns and/or behaviors and limits on the maximal effect of each co-released modulator on the assayed motor pattern relative to when they act separately (Crisp and Mesce 2004; Edwards et al. 2002; McLean and Sillar 2004; Svensson et al. 2001; Wood et al. 2000). Mechanistically, a metamodulator can act both in series and in parallel with its target modulator (Edwards et al. 2002; McLean and Sillar 2004; Svensson et al. 2001; Wood et al. 2000).

We are addressing the consequences of metamodulation at the circuit level, using the stomatogastric nervous system (STNS) of the crab Cancer borealis (Marder and Bucher 2007; Nusbaum and Beenhakker 2002). The STNS contains two well-defined central-pattern-generating (CPG) circuits in the unpaired stomatogastric ganglion (STG), including the gastric mill (chewing) and pyloric (filtering of chewed food) circuits. These circuits are modulated by a set of projection neurons that innervate the STG from the paired commissural ganglia (CoGs) and unpaired esophageal ganglion (OG). There are also identified peptide hormones that influence these two circuits (Marder and Bucher 2007). Many of these hormones are released into the cardiac chamber from the pericardial organs (POs), from where they are pumped through the ophthalmic artery to influence the STG, which is located within this artery (Li et al. 2003; Skiebe 2001; Turrigiano and Selverston 1990). One of these neuromodulators, crustacean cardioactive peptide (CCAP), influences the STG only as a circulating hormone (Billimoria et al. 2005; Marder and Bucher 2007). CCAP modulates the pyloric rhythm in Cancer borealis by activating a voltage-dependent, depolarizing current in several pyloric circuit neurons (Swensen and Marder 2000, 2001; Weimann et al. 1997).

In this study, we assessed the CCAP influence on gastric mill rhythms in the isolated STNS (Beenhakker and Nusbaum 2004; Coleman and Nusbaum 1994). CCAP application to the STG did not activate a sustained gastric mill rhythm, but it directly excited several gastric mill neurons including the gastric mill CPG neurons lateral gastric (LG) and interneuron 1 (Int1). CCAP slowed the gastric mill rhythms elicited by modulatory commissural neuron 1 (MCN1) and by coactivation of MCN1 and commissural projection neuron 2 (CPN2), even when applied at low concentrations ($10^{-10}$ M). It also reduced the threshold firing frequency at which MCN1 activated the gastric mill rhythm. Further, only some gastric mill targets of CCAP exhibited altered activity during these CCAP-modulated rhythms, and the activity of one nontarget was also altered. These results indicate that a peptide hormone can facilitate and alter neuronal modulation of motor circuit activity, thereby expanding the available output patterns of rhythmically active motor circuits.

Some of these data were published in abstract form (Kirby and Nusbaum 2003, 2004).
METHODS

Animals

Jonah crabs (C. borealis) were obtained from commercial suppliers (Commercial Lobster and Seafood, Boston, MA; Marine Biological Laboratory, Woods Hole, MA). The crabs were housed in commercial tanks containing recirculating, aerated and filtered artificial seawater (10–12°C). Before dissection, the crabs were cold-anesthetized by packing them in ice for ≥30 min. The foregut was then removed and maintained in chilled physiological saline while the STNS was dissected from it.

Solutions

Under most experimental conditions, the isolated STNS was maintained in physiological saline (5–10 ml) containing (in mM) 439 NaCl, 26 MgCl$_2$, 13 CaCl$_2$, 11 KCl, 10 Trizma base, and 5 maleic acid (pH 7.4–7.6). In some experiments, transmitter release was eliminated using saline that contained a reduced (0.1× normal) concentration of Ca$^{2+}$ plus a compensatory addition of Mn$^{2+}$ (“low-Ca$^{2+}$ saline”) (Blitz and Nusbaum 1997). Low-Ca$^{2+}$ saline contained (in mM) 439 NaCl, 26 MgCl$_2$, 13 CaCl$_2$, 11.7 MnCl$_2$, 11 KCl, 10 Trizma base, and 5 maleic acid (pH 7.4–7.6).

Electrophysiology

All experiments were conducted using the completely isolated STNS (Fig. 1). The preparation was pinned down in a saline-filled silicone elastomer (Sylgard)-lined petri dish (Sylgard 184, KR Anderson, Santa Clara, CA) and superfused continuously (7–12 ml/min) with physiological saline and/or low-Ca$^{2+}$ saline (10–12°C). Intracellular recordings of STNS neurons were made using routine methods for this system (Beenhakker and Nusbaum 2004). Glass microelectrodes (15–30 MΩ) filled with 4 M K-acetate plus 20 mM KCl or 0.6 M K$_2$SO$_4$ plus 10 mM KCl were used for intracellular recording. Intracellular recordings were made with Axoclamp 2 amplifiers (Molecular Devices, Sunnyvale, CA). Intracellular current injections were performed in single-electrode discontinuous current clamp (DCC) mode with sampling rates of 2–5 kHz. To facilitate intracellular recordings, the STNS ganglia were desheathed and visualized with light transmitted through a dark-field condenser (Nikon, Tokyo, Japan).

Each extracellular nerve recording was made using a pair of stainless steel wire electrodes (reference and recording), the ends of which were pressed into the Sylgard-coated dish. A differential AC amplifier (Model No. 1700: AM Systems, Carlsborg, WA) amplified the voltage difference between the reference wire, placed in the bath, and the recording wire, placed near an individual nerve and isolated from the bath by petroleum jelly (Vaseline: Lab Safety Supply, Janesville, WI). This signal was then further amplified and filtered (Model No. 410 Amplifier: Brownlee Precision, Santa Clara, CA). Extracellular nerve stimulation was accomplished by placing the pair of wires used to record nerve activity into a stimulus isolation unit (SIU 5: Astromed/Grass Instruments, West Warwick, RI) that was connected to a stimulator (Model No. S88: Astromed/Grass Instruments).

The influence of the neuropeptide CCAP on individual neurons within the STG was tested by direct application of the peptide onto the desheathed STG neuropil during superfusion of low-Ca$^{2+}$ saline. For these experiments, a recording electrode was filled with a CCAP solution (10$^{-4}$ or 10$^{-5}$ M) in low-Ca$^{2+}$ saline to be consistent with the bath condition. The tip of the electrode was then broken to create an electrode resistance of 1–1.5 MΩ. The CCAP solution was forced from the pipette using a Picospritzer II pressure ejection device (General Valve, Fairfield, NJ). CCAP was applied at 4–6 psi for a duration of 0.5–1.0 s during continual superfusion of low-Ca$^{2+}$ saline. In between CCAP puffer applications, the CCAP-containing pipette was maintained at a distance away from the STG to prevent the possibility of leak-mediated CCAP actions on STG neurons.

To determine the effects of CCAP on the gastric mill rhythm, a CCAP solution (10$^{-6}$–10$^{-11}$ M) was superfused across the STG. The CCAP solution flowed for 10–15 min before any experimental manipulations, ensuring that the CCAP solution had completely displaced the normal saline solution. This time period was verified by determining the time taken for the effects of CCAP on the pyloric circuit to reach steady state (Weimann et al. 1997).

Gastric mill rhythms were elicited using one of two methods. First, we used extracellular stimulation of one or both dorsal posterior esophageal nerves (dpns), in preparations with the CoGs still connected with the STG, to activate the mechanosensory ventral cardiac neurons (VCNs) (Beenhakker et al. 2004). Second, we selectively activated MCN1 and thereby evoked MCN1-elicited gastric mill rhythms by extracellular stimulation of one or both inferior esophageal nerves (ions), after their transaction to separate them from the CoGs (Fig. 2C) (Barts and Nusbaum 1997; Barts et al. 1999). Each ion stimulation (1-ms stimulus duration) elicits a single action potential in MCN1 (Barts and Nusbaum 1997). Activation of MCN1 action potentials was confirmed by maintaining an intracellular recording of the LG neuron, which receives an electrical EPSP from each MCN1 action potential (Coleman et al. 1995).
A: VCN-triggered gastric mill rhythm (see METHODS) results from the persistent activation of the projection neurons MCN1 and CPN2 by the mechanosensory VCN neurons (Beenhacker and Nusbaum 2004). This version of the gastric mill rhythm includes coordinated bursting of the lateral gastric (LG) and gastric mill (GM) protractor neurons that alternates with the retractor neurons dorsal gastric (DG) and ventricular dilator (VD). Note also that the inferior cardiac (IC) neuron fires primarily during retraction. The protractor neuron MG and retractor neurons interneuron 1 (Int1) and anterior median (AM) also participate in this rhythm (not shown). A gastric mill cycle period extends from the burst duration. The cycle period of gastric mill and pyloric rhythms was determined by calculating the duration between the onset of consecutive LG neuron bursts and two successive pyloric dilator (PD) neuron bursts, respectively (e.g., see Fig. 2 C). The intraburst firing frequency based on their individual pyloric-timed bursts) during a gastric mill cycle. In contrast, for these neurons we analyzed their number of action potentials per burst as well as their intraburst firing frequency based on their individual pyloric-timed bursts.

Data analysis

Individual STNS neurons were identified by their axonal pathways, activity patterns and interactions with other neurons (Beenhacker and Nusbaum 2004; Blitz et al. 1999; Weimann et al. 1991). Data were collected onto a chart recorder (Models No. MT 95000 and Everest: Astromed) and, in parallel, digitized (∼5 kHz) and collected onto a PC computer using data-acquisition/analysis tools (Spike2, Cambridge Electronic Design, Cambridge, UK). Figures were made from Spike2 files incorporated into Adobe Photoshop (Adobe, San Jose, CA) and Powerpoint graphics programs (Microsoft, Seattle, WA).

Data analysis was facilitated with a custom-written program for Spike2 that determines the activity levels and burst relationships of individual neurons (freely available at http://cuniculina.biologie.uni-ulm.de/wsstein/spike2/The_Crab_Analyzer.s2s). Unless otherwise stated, each datum in a data set was derived by determining the average of 10 consecutive gastric mill- or pyloric rhythm-time impulse bursts. Briefly, burst duration was defined as the duration (s) between the onset of the first and last action potential in an impulse burst (e.g., see LG burst in Fig. 2C). The intraburst firing rate was determined by dividing the number of action potentials minus one by the burst duration. The cycle period of gastric mill and pyloric rhythms was determined by calculating the duration between the onset of two successive LG neuron bursts and two successive pyloric dilator (PD) neuron bursts, respectively (e.g., see Fig. 2C). The burst relationship among gastric mill neurons (phase relationships) was determined for normalized gastric mill cycles in which the onset and offset of a cycle was determined by the onset of successive LG neuron bursts. Specifically, we determined the mean point in a normalized gastric mill cycle at which the burst onset and offset occurred for each gastric mill neuron. To identify these points, we divided the duration from each cycle onset to burst onset (and offset) by its associated cycle period.

In the case of STG neurons (Int1, VD, IC, MG) whose gastric mill rhythm-related activity was subdivided into pyloric-timed bursts, we determined their gastric mill-related burst duration as the duration of their entire period of activity (i.e., the cumulative duration of all of their pyloric-timed bursts) during a gastric mill cycle. In contrast, for these neurons we analyzed their number of action potentials per burst as well as their intraburst firing frequency based on their individual pyloric-timed bursts.

We determined the MCN1 firing frequency threshold for eliciting the gastric mill rhythm by tonically, and simultaneously, stimulating both ions (60- to 300-s train duration) at a series of constant instantaneous stimulus frequencies in the range of threshold for rhythm activation (2–8 Hz). There was a minimum lag of 1 min between successive stimulus trains. It is noteworthy that the MCN1-elicted gastric mill rhythm consistently stops immediately when MCN1 stimulation is terminated (Bartos and Nusbaum 1997).

Over the course of any single experiment, repeated activation of the gastric mill rhythm generally resulted in a progressively slower rhythm (see RESULTS). This occurred even when the sequentially activated gastric mill rhythms were each elicited under the same condition, such as during normal saline superfusion. This progressive slowing contrasted with the pyloric rhythm, which is often continu-
ously active for hours in the isolated preparation at a relatively constant cycle period, presumably due to the presence of an endogenously oscillatory pacemaker neuron (Hooper and Marder 1987; Miller and Selverston 1982). There is no such pacemaker neuron within the gastric mill CPG (Bartos et al. 1999; Coleman et al. 1995).

Due to the progressive slowing of the gastric mill rhythm with repeated activation, it was difficult to generate matched control rhythms before and after CCAP application. It was, however, routinely possible to elicit two equivalent gastric mill rhythms (saline 1:saline 2 or S1:S2) with intervals that matched those of the pre-CCAP application and during-CCAP application rhythms (supplementary Tables 1–3; see RESULTS). To ensure that any changes that occurred in the gastric mill rhythm during CCAP superfusion were due to the presence of the peptide and not to the progressive slowing of the rhythm within each preparation, we compared two successive gastric mill rhythms during saline superfusion (S1, S2) with intervals comparable to our experimental interval (S, CCAP). We performed these controls in separate preparations from those in which CCAP was applied, so that the rhythms could be elicited at comparable times after the start of each experiment.

To most appropriately represent the results of our experiments, we used a two-stage process for determining which analyzed parameters were significantly different between the control and experimental conditions. In stage 1, we used a paired Student’s t-test to obtain each within-group comparison (S1 vs. S2; S vs. CCAP). If, for any particular parameter (i.e., cycle period) a significant change was found in the S:CCAP data set, we progressed to stage 2.

The stage 2 test was used to determine whether a change in activity induced by the addition of CCAP was significantly different from the condition when saline was applied during consecutively elicited gastric mill rhythms. We determined the difference between S1 and S2 for each experiment (S2 minus S1) to measure the change in a particular gastric mill parameter induced by no intervention. For each experiment where saline was followed by an application of CCAP, we again determined the difference between values (CCAP minus saline). We then performed an unpaired Student’s t-test to determine whether the change induced by CCAP across preparations was significantly different from the change, if any, induced in the experiments when saline was applied during both gastric mill rhythms. In some cases, where noted, we instead used the Mann-Whitney rank sum test in our stage 2 analysis. We used this alternative test when the variance between the data sets was unequal. In the text, any result for which a significant difference is noted during CCAP application represents the results of our stage 2 analysis.

To determine whether CCAP consistently increased the gastric mill cycle period during MCN1 stimulation, regardless of the value of the control cycle period, we used a sliding window analysis of the complete data set. Specifically, we compared the control (S1, S2) and experimental (S, CCAP) data within successive 4-s-duration windows. After each set of comparisons, these 4-s-duration windows were shifted 0.5 s to the right, and the analysis was performed again, until all possible windows were analyzed.

For each window, we used our stage 2 analysis (see preceding text) to compare the change induced in the gastric mill cycle period by the presence of CCAP with the change induced in the S1:S2 condition. The result (significant change or no change) was then assigned to the value at the center of each window (i.e., a significant change was assigned to 6 s for the 4-s-duration window that spanned cycle periods of 4–8 s). We note the distinction between significant and nonsignificant changes at the center of the 4-s-duration window encompassing the largest values of cycle period that showed a significant difference between S:CCAP and S1:S2.

We did not see any change in the results using time increments <0.5 s (<0.25 s; not shown). We also obtained the same results with 4- and 5-s-duration windows (not shown). We did not use a 3-s-duration window because it often resulted in too few data points to enable an accurate determination of significant changes.

Statistical analyses were performed with SigmaStat 3.0 and SigmaPlot 8.0 (SPSS, Chicago, IL). Data are expressed as means ± SE except where explicitly noted to be expressed as means ± SD.

**Gastric mill model**

We implemented a computational model modified as indicated in the following text from an existing conductance-based model of the gastric mill circuit (Beenhakker et al. 2005; Nadim et al. 1998). We retained all aspects of the model implemented by Beenhakker et al. (2005), including modeled versions of the LG, Int1, and MCN1 neurons having multiple compartments separated by an axial resistance, with each compartment possessing intrinsic and/or synaptic conductances, as documented originally by Nadim et al. (1998). The voltage-dependent trajectory of the MCN1 input to LG was based on the neuropeptide-activated current in pyloric neurons in *C. borealis* (Golowasch and Marder 1992; Swensen and Marder 2000). The only parameters that were altered from the model version presented in Beenhakker et al. (2005) were an increase in the maximal conductance value for the inhibitory synapse from Int1 to LG, from the previously used value of 1.4 to 2.1 nS, and elimination of the gastro-pyloric receptor (GPR) synapses onto MCN1 and Int1 in the STG.

**RESULTS**

**Gastric mill system in *C. borealis***

The gastric mill rhythm (cycle period: ~5–20 s) controls the rhythmic protraction and retraction chewing movements of the teeth in the gastric mill stomach compartment (Heinzel et al. 1993). Thus this rhythm is composed of alternating impulse bursts of protractor and retractor motor neurons, plus a single retractor phase interneuron (Int1; Fig. 2) (Marder and Bucher 2007). Several gastric mill neurons also exhibit rhythmic impulse bursts that are time-locked to the pyloric rhythm, which is a faster rhythm (cycle period: ~0.5–2 s) that controls the filtering of chewed food in the pylorus, immediately posterior to the gastric mill (Weimann et al. 1991). The neurons exhibiting this dual rhythmic pattern during the gastric mill rhythms studied in this paper include the inferior cardiac (IC), medial gastric (MG), and ventricular dilator (VD) neurons as well as Int1 (e.g., Fig. 2, A and C). The LG neuron also exhibits pyloric-timed activity during a distinct version of the gastric mill rhythm (Wood et al. 2004). All gastric mill neurons are present as single neurons in the *C. borealis* STG except the protractor gastric mill (GM) neuron, which is present as four apparently equivalent neurons.

There are several distinct versions of the gastric mill rhythm in *C. borealis*, including one driven by selective activation of the projection neuron MCN1 and one triggered by the mechanosensory VCNs, which activate the rhythm via their excitation of MCN1 and CPN2 (Fig. 2) (Beenhakker and Nusbaum 2004; Beenhakker et al. 2004; Coleman and Nusbaum 1994). These two rhythms share several features, including the presence of alternating protractor and retractor phases (Fig. 2, A and C). Both rhythms also include rhythmic alternating bursting of the CPG neurons LG and Int1 and, in both cases, the retractor neurons VG and DG are coactive with Int1. Distinctions between these rhythms include the relative timing of the impulse bursts in the IC and MG neurons as well as the level of participation of the GM and anterior median (AM) neurons. For example, as shown in Fig. 2, IC neuron activity is distinct...
during the MCN1- and VCN-elicited gastric mill rhythms while GM neuron bursting occurs only during the VCN-elicited rhythm.

The projection neuron MCN1 excites all of the gastric mill neurons (Fig. 2B) (Bartos and Nusbaum 1997; Coleman and Nusbaum 1994; Stein et al. 2007). However, its influence on the GM and AM neurons is modest, particularly when they are inactive prior to MCN1 stimulation. Consequently, GM and AM are generally silent or only weakly active during the MCN1-elicited gastric mill rhythm. During this version of the gastric mill rhythm, the core CPG includes the gastric mill neurons LG and Int1 plus the STG terminals of MCN1 (Bartos et al. 1999; Coleman et al. 1995).

During the VCN-triggered gastric mill rhythm, MCN1 is active during both phases but it fires tonically during protrac-

tion and is pyloric-timed during retraction (Beenhakker and Nusbaum 2004). The projection neuron CPN2 is also tonically active during the protractor phase but exhibits reduced or no activity during retraction (Beenhakker and Nusbaum 2004; Norris et al. 1994). CPN2 excites the LG and GM neurons but inhibits the IC and MG neurons, thereby shifting the activity of the latter two neurons during the VCN-triggered rhythm (Beenhakker and Nusbaum 2004; Norris et al. 1994) (Fig. 2, A and B). CPN2 also inhibits the retractor neuron DG (Norris et al. 1994) (Fig. 2B).

**CCAP directly excites a subset of gastric mill neurons**

Swensen and Marder (2000, 2001) showed previously that three of the seven pyloric circuit neurons, including the anti-

erior burster (AB), lateral pyloric (LP) and IC neurons, are direct targets of CCAP in *C. borealis*. We determined whether CCAP had any direct actions on the gastric mill circuit neu-

rons. To this end, we pressure ejected CCAP (10−4 or 10−5 M) onto the desheathed STG neuropil under conditions where neurotransmitter release was suppressed (low-Ca2+ saline, see METHODS) while recording intracellularly from each gastric mill neuron.

In low-Ca2+ saline, CCAP excited a subset of the gastric mill neurons. For example, as shown in Fig. 3, brief (1 s) pressure application of CCAP in low-Ca2+ saline caused a depolarization and action potential burst in the LG neuron (n = 19/19) and Int1 (n = 9/11). For both of these neurons, their response to CCAP outlasted its application by many seconds (Fig. 3, A and B). There was also an excitatory response in the MG (n = 11/11), IC (n = 13/13), and AM (n = 7/7) neurons, although the MG neuron response tended to be weaker than that of the other neurons. In contrast, the DG (n = 13/13; Fig. 3C) and GM (n = 20/20) neurons never responded to CCAP application in low-Ca2+ saline.

To assess the possibility that the DG and GM neurons had a voltage-dependent response to CCAP application, this peptide was also applied at times when these neurons were depolarized by intracellular current injection (<1.0 nA) so that they fired continuously at a modest frequency (e.g., Fig. 3C). At these times, neither neuron responded to peptide application [DG: pre-CCAP, 1.2 ± 0.9 (SD) Hz; CCAP, 1.4 ± 1.1 Hz; n = 12, P = 0.34; GM: pre-CCAP, 0.8 ± 0.7 Hz; CCAP, 0.6 ± 0.9 Hz; n = 16, P = 0.71]. Moreover, in every case in which these gastric mill neurons did not respond to CCAP application, the other gastric mill neurons were responsive. We also deter-

mined, via intra-axonal recordings near the entrance to the STG (Beenhakker and Nusbaum 2004; Coleman and Nusbaum 1994), that there was no change in the membrane potential at the STG terminals of the MCN1 and CPN2 projection neurons in response to CCAP application (MCN1: pre-CCAP, −45.7 ± 7.8 (SD) mV; CCAP, −46.7 ± 7.4 mV; n = 6, P = 0.20; CPN2: pre-CCAP, −56.0 ± 8.8 mV; CCAP, −54.0 ± 7.4 mV; n = 4, P = 0.18). Additionally, CCAP never elicited action potentials in either projection neuron (MCN1: n = 7; CPN2, n = 6).

The LG, IC, and MG neurons are electrically coupled in *C. borealis* (M. P. Beenhakker, M. S. Kirby, M. P. Nusbaum, unpublished data). Therefore to obtain a more accurate assessment of whether each of them was a direct target of CCAP, we performed additional experiments in which we hyperpolarized two of them during any particular CCAP application. These current injections consistently suppressed activation of spiking in the hyperpolarized neurons in response to CCAP but never caused a hyperpolarization of >5 mV in the un.injected neuron (n = 12/12). When the LG and IC neurons were hyperpolarized, the MG neuron failed to respond to pressure applied CCAP (n = 9/9). Moreover, under these conditions MG remained unresponsive even when it was depolarized to spike threshold via intracellular current injection (n = 9). In contrast, when LG and MG were hyperpolarized, the IC neuron consistently responded as vigorously as when no neurons were hyperpolarized (n = 10/10). Similarly, the LG neuron exhibited an unchanged excitatory response when both the IC and MG neurons were hyperpolarized (n = 6/6). Thus four of the eight types of gastric mill neurons (LG, IC, Int1, AM) appeared to be direct targets of CCAP. The remaining neurons (DG, VD, MG, GM) as well as the STG terminals of the projection neurons MCN1 and CPN2 were not directly responsive to this
peptide. Last, no gastric mill neurons ever responded to CCAP application in low-Ca\(^{2+}\) saline with an inhibitory response.

It remains possible that we obtained a false negative result regarding the neurons that were unresponsive to CCAP in low-Ca\(^{2+}\) saline because CCAP might also influence a Ca\(^{2+}\)-sensitive current in these neurons. Such a current would have been minimal or absent due to the 10-fold reduced Ca\(^{2+}\) concentration in the low-Ca\(^{2+}\) saline. Although the presence of an additional, Ca\(^{2+}\)-sensitive current remains a possibility, previous work indicated that CCAP influenced only a single ionic current in C. borealis pyloric neurons, and this current persisted in low-Ca\(^{2+}\) saline (Golowasch and Marder 1992; Swensen and Marder 2000).

**CCAP elicits a transient, incomplete gastric mill rhythm**

The reciprocally inhibitory neurons LG and Int1 are key gastric mill CPG neurons (Bartos et al. 1999; Coleman et al. 1995). Because both of these neurons were directly excited by CCAP application, we determined whether application of this peptide in normal saline would activate the gastric mill rhythm. CCAP superfusion (10\(^{-6}\) or 10\(^{-7}\) M) to the isolated STG often elicited a gastric mill rhythm (n = 30/38), but this rhythm never included the DG retractor neuron nor did it persist for the duration of the application. This rhythm did, however, include coordinated bursting of several gastric mill neurons, including LG and Int1 (Fig. 4, A and B). These CCAP elicited rhythms persisted for 7.4 ± 3.5 (SD) min (n = 19). Generally this rhythm gave way to intermittent spiking or inactivity in LG and the return to exclusively pyloric-timed activity in the other participating neurons before the end of each CCAP application (Fig. 4C). During these CCAP-elicited rhythms, not every gastric mill neuron participated. As mentioned in the preceding text, the DG neuron was never activated under these conditions (Fig. 4, B and C). The lack of DG participation in the CCAP-elicited rhythm was not a consequence of damage during dissection, because DG was effectively activated subsequently during the MCN1-elicited gastric mill rhythm (Fig. 4D).

**MCN1-elicited gastric mill rhythm slows with repeated activation**

As noted in METHODS, we found that repeated activation of the gastric mill rhythm with sufficiently long inter-stimulus intervals resulted in increased gastric mill cycle periods even without CCAP application. For example, in a sampling of 15 preparations where we superfused CCAP (10\(^{-7}\) M), the initial MCN1-elicited gastric mill cycle period during saline superfusion was 9.6 ± 0.6 s. After a 1-h washout of CCAP, the gastric mill rhythm had slowed (cycle period: 10.7 ± 0.9 s, P < 0.001, paired Student’s t-test). Similarly, when we elicited consecutive gastric mill rhythms in normal saline with intervals (~1.5 h) that mimicked the duration of the application and washout of CCAP, the gastric mill cycle period of the postinterval rhythm was prolonged relative to the preinterval rhythm (saline, pre-interval: 9.5 ± 1.3 s; saline, post-interval: 11.9 ± 1.9 s; n = 8; P < 0.05, paired Student’s t-test).

In contrast to the gradual slowing of gastric mill rhythms during saline superfusion that occurred across intervals of 1 h or more, the MCN1-elicited rhythms in saline were equivalent when they had an interval comparable to the one occurring between a control rhythm and the rhythm elicited in CCAP. Comparison of these matched controls revealed that none of the studied parameters differed between consecutively elicited gastric mill rhythms in saline (supplementary Table 1, Table 2). For the VCN-triggered gastric mill rhythms, the only parameter that was distinct between the consecutively elicited rhythms in saline was the burst onset phase of the GM neuron (n = 10, P < 0.05; Fig. 5, supplementary Table 3). For example, there was no change in the VCN-triggered gastric mill cycle period (S1: 9.8 ± 0.6 s; S2: 9.8 ± 0.7 s; n = 11, P = 0.98, paired Student’s t-test).

**CCAP modifies the MCN1/CPN2-elicited gastric mill rhythm**

We analyzed the response of the gastric mill rhythm triggered by mechano sensory (VCN neurons)-mediated coactivation of MCN1 and CPN2 to CCAP application (Fig. 2A) (Beenhakker and Nusbaum 2004; Beenhakker et al. 2004). The VCN-triggered rhythm requires the CoGs to remain connected to the STG because this rhythm requires properly timed synaptic feedback from STG neurons onto MCN1 and CPN2 (Beenhakker and Nusbaum 2004). Consequently, in these experiments, we used a petroleum jelly wall to separate the STG from the anterior ganglia and selectively superfused CCAP onto the STG while the anterior compartment was continually superfused with normal saline.

CCAP slowed the MCN1/CPN2-elicited gastric mill rhythm by prolonging the protractor (LG burst) phase (Fig. 5; supplementary Table 3). This was a consistent effect across preparations, regardless of the control cycle period which ranged from ~7 to 14 s in different preparations (Fig. 5B). During saline superfusion, the mean gastric mill cycle period was 10.4 ± 0.5 s, whereas during superfusion of CCAP (10\(^{-7}\) M), the cycle period increased to 11.9 ± 0.6 s (n = 15; P < 0.001; Fig. 5C). In parallel, the LG burst duration was consistently increased by the presence of CCAP (10\(^{-7}\) M; Fig. 5C; supple-
FIG. 5. CCAP slows the VCN-triggered gastric mill rhythm. A, top left: during normal saline before VCN stimulation, there was no gastric mill rhythm. The LG and DG neurons were silent while the IC and VD neurons exhibited pyloric-timed activity. Top right: after VCN stimulation, the gastric mill rhythm was triggered. Bottom left: during CCAP superfusion but before VCN stimulation, there was no gastric mill rhythm. Bottom right: VCN-triggered gastric mill rhythm was slowed by the presence of CCAP. Note that, for the same duration, there were 4 complete LG neuron bursts during saline superfusion but only 3 complete LG bursts, and a fraction of a fourth, during CCAP application. These recordings were made with the CoGs connected with the STG. Both panels are from the same preparation. B: scatter plot showing the mean (±SD) value for the gastric mill cycle period during CCAP (10⁻⁷ M) application relative to its saline control for each preparation (n = 15), and the mean (±SD) value for the same parameter from 2 successive gastric mill rhythms elicited during saline superfusion (S1, S2: ⋅), obtained from a separate set of preparations. —, equivalent points on the y and x axes. C: CCAP (10⁻⁷ M) application altered several parameters of the VCN-triggered gastric mill rhythm across preparations. Specifically, relative to the same parameter during saline superfusion (S), CCAP increased the gastric mill cycle period (Ci, n = 15), LG burst duration (Cii, n = 15), LG intraburst firing frequency (Ciii, n = 15), and number of LG action potentials per burst (Cvi, n = 15). There was no change in any of these parameters during consecutive rhythms triggered during saline superfusion (S1, S2: P > 0.05, n = 11). **P < 0.01.

mentary Table 3). This increase in LG neuron burst duration was accompanied by increases in the number of LG neuron action potentials per burst and LG intraburst firing frequency (Fig. 5C; supplementary Table 3).

There were additional CCAP (10⁻⁷ M)-mediated changes in the activity of gastric mill neurons during the VCN-triggered rhythm. These included increased activity in the DG and AM neurons (supplementary Table 3). The DG neuron, despite not being a direct target of CCAP, exhibited an increase in its burst duration (P < 0.05, n = 13). The AM neuron, which was directly excited by CCAP, also showed an increased burst duration (P < 0.05, n = 7) and number of action potentials per burst (P < 0.01, n = 7). The activity of both DG and AM were unchanged in the sequentially triggered, saline control rhythms (supplementary Table 3). The activity (burst duration, number of spikes per burst, and intraburst firing frequency) of the other gastric mill neurons was unchanged by the presence of CCAP during the VCN-triggered gastric mill rhythm (supplementary Table 3).

Finally, CCAP (10⁻⁷ M) superfusion changed several aspects of the phase relationships among the gastric mill neurons during the VCN-triggered rhythm (supplementary Table 3). Phase relationships represent the fraction of a normalized cycle during which each neuron fires its burst of action potentials (see METHODS). Because the normalized gastric mill cycle is designated as extending from the onset of successive LG neuron bursts, the protractor neurons are generally active during approximately the first half of each cycle while the retractor neurons fire during the latter half of the cycle (i.e., Fig. 2). During the VCN-triggered rhythm, CCAP (10⁻⁷ M) superfusion altered the active phase of one protractor neuron (MG) and one retractor neuron (DG). Specifically, the onset phase of MG neuron activity was significantly delayed by CCAP superfusion (P < 0.05, n = 8) with no corresponding change in MG offset phase (P = 0.11, n = 8). Further, CCAP delayed both the onset (P < 0.05, n = 13) and offset phase (P < 0.05, n = 13) of DG activity.

**CCAP reduces the MCN1 firing frequency threshold for gastric mill rhythm activation**

Because CCAP effectively influenced the VCN-triggered gastric mill rhythm when applied selectively to the STG, we aimed to study these influences in more detail in a further reduced preparation. To this end, we removed the CoGs by transecting the ions and superior esophageal nerves (sons) and studied the CCAP actions on the MCN1-elicited gastric mill rhythm by selectively stimulating MCN1 extracellularly (see METHODS).

CCAP reduced the threshold MCN1 firing frequency for eliciting the gastric mill rhythm (Fig. 6, A and B). With the isolated STG superfused with normal saline, the mean thresh-
old MCN1 firing frequency that elicited the gastric mill rhythm was ~5 Hz (4.7 ± 0.4 Hz; n = 10). During CCAP superfusion (10^{-7} M), this threshold firing rate was reduced to ~3 Hz (2.9 ± 0.2 Hz; n = 10; P < 0.01; Fig. 6B). This effect of CCAP on the threshold MCN1 firing frequency for activating the gastric mill rhythm was effective even at CCAP concentrations as low as 10^{-10} M [saline: 5.4 ± 0.3 Hz; CCAP (10^{-10} M): 4.3 ± 0.3 Hz; P < 0.05, n = 10; Fig. 6B]. At lower concentrations (10^{-11} M), CCAP had no effect on this parameter (P = 0.60, n = 7). In control experiments in which CCAP was not present during the second activation of the gastric mill rhythm, there was no change in the threshold firing frequency at which MCN1 elicited the gastric mill rhythm (saline 1: 6.6 ± 0.6 Hz; saline 2: 6.2 ± 0.4 Hz; P = 0.20, paired Student’s t-test, n = 9; Fig. 6B).

**CCAP directly alters the MCN1-elicited gastric mill rhythm**

Previous work showed that CCAP also influences the pyloric rhythm (Weimann et al. 1997), and the pyloric rhythm regulates the MCN1-elicited gastric mill rhythm (Bartos et al. 1999). Therefore the possibility existed that the CCAP influence on the gastric mill rhythm was a secondary consequence of the peptide action on the pyloric rhythm. We tested this possibility by superfusing the STG with CCAP (10^{-7} M) while suppressing the pyloric rhythm via intracellular hyperpolarizing current injection into the two PD neurons which, along with the AB neuron, are members of the electrically coupled pyloric pacemaker group (Fig. 7). As shown previously, the MCN1-elicited gastric mill rhythm was slower when the pyloric rhythm was suppressed (Bartos et al. 1999).

First, as in the preceding text, we determined whether the successively elicited gastric mill rhythms during saline superfusion were equivalent when the pyloric rhythm was suppressed. Using inter-rhythm intervals matched to those in the CCAP experiments, we found these consecutively elicited rhythms in normal saline to be indistinguishable (cycle period: saline 1: 17.2 ± 0.9 s; saline 2: 17.3 ± 1.2 s; n = 10, P = 0.96; LG burst duration: saline 1: 8.7 ± 0.8 s; saline 2: 9.2 ± 0.8 s; n = 10, P = 0.11; Fig. 7B).

With the pyloric rhythm suppressed, CCAP (10^{-7} M) still altered the MCN1-elicited gastric mill rhythm (Fig. 7). Under this condition, the CCAP-elicited changes included prolonging the cycle period of the rhythm (saline: 18.8 ± 1.2 s; CCAP: 22.6 ± 1.4 s; n = 10, P < 0.01) and the LG neuron burst duration (saline: 9.7 ± 0.8 s; CCAP: 11.4 ± 1.0 s; n = 10, P < 0.05), and increasing the number of LG spikes per burst (saline: 88.9 ± 10.1 spike; CCAP: 111.1 ± 12.5 spike; n = 10, P < 0.01). There were also increases in the DG neuron burst duration (saline: 9.3 ± 1.0 s; CCAP: 12.0 ± 1.0 s; n = 8, P < 0.01) and the number of DG spikes per burst (saline: 113.9 ± 13.0 spike; CCAP: 136.9 ± 12.9 spike; n = 8, P < 0.05).

**CCAP modifies the MCN1-elicited gastric mill rhythm**

We also examined the MCN1-elicited gastric mill rhythm response to CCAP application when the pyloric rhythm remained active. In these experiments, we delayed MCN1 stimulation until after any CCAP-elicited gastric mill rhythmicity had ended. At these times, CCAP influenced the MCN1-elicited gastric mill rhythm in a manner comparable to its effects on the VCN-triggered rhythm, including prolonging the...
gastric mill cycle period (Fig. 8). Across preparations, the gastric mill cycle period increased from 9.6 ± 0.6 s during saline superfusion to 10.5 ± 0.5 s in the presence of CCAP (10⁻⁷ M; n = 27, P < 0.05). However, there was no significant change in the duration of either the protractor (saline: 6.4 ± 0.5 s; CCAP: 7.1 ± 0.4 s; n = 27, P = 0.07) or retractor phase (saline: 3.2 ± 0.2 s; CCAP: 3.4 ± 0.2 s; n = 27, P = 0.15) of this rhythm.

It was surprising to obtain a significant increase in the gastric mill cycle period without concomitant changes in the duration of one or both phases of this rhythm. However, based on previous studies, we realized that this outcome might have resulted from an inconsistent data set in which CCAP had a stronger impact on MCN1-elicited gastric mill rhythms with shorter cycle periods. We considered this possibility because it seemed likely that at a given concentration, CCAP would activate a steady-state level of its target current in CCAP-responsive gastric mill neurons, whereas for a constant MCN1 firing frequency, the MCN1 peptide-activated current amplitude likely increased with the duration of the retractor phase (LG interburst) (Beenhakker et al. 2005).

Our current understanding of the mechanisms underlying the generation of the MCN1-elicited gastric mill rhythm include the periodic buildup and decay of a slowly developing excita-

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**FIG. 7.** The CCAP influence on the gastric mill rhythm is not an indirect consequence of CCAP modulation of the pyloric rhythm. A: CCAP continued to slow the gastric mill rhythm and increase LG neuron burst duration when the pyloric rhythm was suppressed by hyperpolarizing current injection into both PD neurons. The electrical coupling of the PD neurons with the pyloric pacemaker neuron AB ensured that AB also was maintained at a hyperpolarized level (~80 mV) before, during, and after CCAP application. Note that, as expected (Bartos et al. 1999), suppressing the pyloric rhythm caused an increase in the gastric mill cycle period during saline superfusion (compare with Figs. 2 and 8). B: MCN1-elicited gastric mill rhythm was consistently altered by CCAP (10⁻⁷ M) application in the absence of the pyloric rhythm. Under this condition there were increases in the gastric mill cycle period (left, n = 10), LG burst duration (middle, n = 10), and number of LG action potentials per burst (right, n = 10). None of these parameters were altered by successive rhythms (S1, S2) elicited during saline superfusion (n = 10). **•** P < 0.01.
The amount of MCN1-mediated excitation necessary to enable LG to reach burst threshold is a function of the level of inhibition received by LG from Int1 (Beenhakker et al. 2005). Thus for a constant MCN1 firing frequency, increasing the Int1 inhibition of LG prolongs the time needed for the MCN1-activated current to enable LG to reach burst threshold (Fig. 9B). Prolonging the retractor phase in this manner prolongs the subsequent protractor phase due to the apparently unchanged decay rate of the MCN1 excitation of LG (Beenhakker et al. 2005).

In the pyloric circuit neurons of *C. borealis*, several neuropeptides including CCAP and CabTRP Ia selectively activate the same, voltage-dependent inward current (Swensen and Marder 2000, 2001). Because CCAP and CabTRP Ia converge on the same ionic current in pyloric neurons, we tested the hypothesis that there was a similar convergence in the gastric mill neurons and this convergence limited CCAP, at the concentrations used (∼10⁻⁷ M), to only influencing relatively fast gastric mill rhythms. As suggested in the preceding text, we anticipated that this limitation resulted from a given CCAP concentration activating a relatively constant amount of the aforementioned peptide-activated current whereas the amount of this current contributed by MCN1 would be larger when the retractor phase was prolonged, as occurs during slower gastric mill rhythms (i.e., Fig. 9). Thus the impact of the CCAP action would be proportionally larger during the faster rhythms.

There was indeed a cycle period-dependent influence of CCAP on the MCN1-elicited gastric mill rhythm (Fig. 10). As shown in Fig. 10Ai, CCAP (10⁻⁷ M) appeared to more consistently increase the cycle period during relatively fast gastric mill rhythms. We confirmed this qualitative observation by using a sliding window analysis to analyze 4-s-duration windows of the data from these 27 preparations relative to controls (see METHODS). We thereby determined that CCAP consistently increased the gastric mill cycle period when the control cycle period was ∼9.0 s (0.001 < P < 0.05 for each window between 4 and 8 and 7 and 11 s). Overall, when the control gastric mill cycle period was ∼9.0 s, CCAP (10⁻⁷ M) increased the cycle period from 6.9 ± 0.3 s in saline to 8.5 ± 0.4 s in CCAP (n = 12, P < 0.001; Fig. 10Aii). At the level of individual experiments, the presence of CCAP increased the gastric mill cycle period in 10/12 preparations with control cycle periods of ∼9 s, whereas the cycle period increase occurred in only 5/15 preparations with longer-duration control cycle periods (Fig. 10Ai).

**FIG. 10.** Quantitative analysis of the CCAP-altered gastric mill rhythm elicited by MCN1 stimulation. Ai: data set includes the full range of MCN1-gastric mill cycle periods; Aii and B–D: only gastric mill rhythms the cycle period of which was ∼9.0 s during saline superfusion. Each scatter plot shows the mean (±SD) value for the indicated gastric mill rhythm parameter during CCAP (10⁻⁷ M) application relative to its saline control for each preparation (●). Also plotted are the mean (±SD) value for the same parameter from 2 successive gastric mill rhythms elicited during saline superfusion (S1, S2: ○) from a separate set of preparations. - - - , equivalent points on the y and x axis. A: CCAP increased the MCN1-elicited gastric mill cycle period relative to the rhythm that occurred during saline superfusion, when the pre-CCAP cycle period was ∼9.0 s. Ai: in most preparations (10/12) where the pre-CCAP cycle period was ∼9.0 s, the gastric mill cycle period was longer in CCAP than during its corresponding saline control, whereas this CCAP effect occurred in only 5/15 preparations when the pre-CCAP cycle period was >9.0 s. For all preparations, there was little difference in the cycle period between the S1 and S2 rhythms. Aii: mean gastric mill cycle period during S1 and S2 was equivalent across preparations (P = 0.20, n = 10), whereas the mean cycle period during CCAP application was longer than during the preceding saline superfusion (n = 12). B: CCAP increased the LG burst duration. Bi: in most preparations (9/12), CCAP increased the LG burst duration relative to its saline control, whereas there was little difference in this parameter during S1 and S2 rhythms in each preparation. Bii: across preparations, the mean LG burst duration was equivalent during S1 and S2 (P = 0.17), but it was longer during CCAP application relative to its saline control. C: CCAP increased the number of LG spikes per burst. Ci: in all preparations (12/12), CCAP caused an increased number of LG spikes per burst relative to their saline controls. In contrast, there was no difference in this parameter between the S1 and S2 rhythms in each preparation. Cii: across preparations, the mean number of LG spikes per burst was equivalent during S1 and S2 (P = 0.72) but was larger during CCAP application relative to its saline control. D: CCAP increased the intraburst firing frequency of the LG neuron. Di: in most preparations (9/12), CCAP caused an increased intraburst firing frequency in the LG neuron relative to their saline controls. In contrast, there was no difference in this parameter between the S1 and S2 rhythms in each preparation. Dii: across preparations, the mean LG intraburst firing frequency was equivalent during S1 and S2 (P = 0.07) but was higher during CCAP application relative to its saline control. **P < 0.01; ***P < 0.001.
Our sliding window analysis also determined that CCAP (10^{-7} M) did not consistently alter the cycle period for rhythms in which the control cycle period was >9.0 s (0.07 < P < 0.77 for each 4-s-duration window between 7.5 and 11.5 and 12 and 16 s; P = 0.20 for the 4-s window centered at 9.5 s). When the data from this range were analyzed collectively, the gastric mill cycle period was unchanged by CCAP (saline: 11.7 ± 0.6 s; CCAP: 12.1 ± 0.5 s; n = 15, P = 0.18). We therefore separated the remaining data into two sets, including fast and slow gastric mill rhythms, for further analysis.

Because we compared the results of S:CCAP to the results of a set of control experiments (S1, S2), we separated the control data sets into those with fast (≤9.0 s) and slow (>9.0 s) cycle periods to match them to the cycle-period-dependent actions of CCAP. All of the analyzed parameters were equivalent for the slow group, and nearly all parameters were equivalent for the fast group (supplementary Table 1, Table 2). For example, the gastric mill cycle period was unchanged in both control groups (fast group: S1, 6.5 ± 0.5 s; S2, 6.7 ± 0.4 s; n = 10, P = 0.20, paired Student’s t-test; slow group: S1, 11.2 ± 0.6 s; S2, 11.3 ± 0.5 s; n = 11, P = 0.77, paired Student’s t-test) as were the LG burst duration, intraburst firing frequency, and number of spikes per burst (Fig. 10; supplementary Tables 1, Tables 2). There were two parameters that were distinct for the S1 versus S2 controls in the fast group, including MG neuron burst duration (P < 0.01, n = 4) and the phase off of the MG neuron burst (P < 0.01, n = 4; supplementary Table 1).

The CCAP-mediated slowing of these relatively fast rhythms resulted largely from its causing a prolongation of the protractor (LG neuron active) phase (Figs. 8 and 10 and supplementary Table 1). For example, the LG neuron burst duration increased during CCAP (10^{-7} M) application to these fast rhythms (saline: 4.4 ± 0.3 s; CCAP: 5.6 ± 0.4 s; n = 12, P < 0.001; Fig. 10B). There was no change in LG burst duration during the slower rhythms (saline: 8.0 ± 0.5 s; CCAP: 8.3 ± 0.4 s; n = 15, P = 0.30). In contrast to the cycle-period-dependent actions of CCAP on the gastric mill cycle period and LG burst duration, CCAP caused an increase in the number of LG neuron action potentials per gastric mill cycle and in its intraburst firing frequency during both fast and slow rhythms (Fig. 10, C and D; supplementary Table 1, Table 2).

Based on the preceding information, we reasoned that if CCAP was providing a smaller fraction of the total excitation to LG when the rhythm was slow, then providing a higher CCAP concentration to slow rhythms would increase its influence on LG relative to the contribution from MCN1 and therefore effectively influence these rhythms in a manner comparable to the lower CCAP concentrations on faster rhythms. Consistent with this hypothesis, application of CCAP (10^{-6} M) to slow rhythms did cause an increased cycle period (saline: 10.8 ± 0.3 s; CCAP: 13.6 ± 1.1 s; n = 8, P < 0.05) and increased LG burst duration (saline: 7.6 ± 0.5 s; CCAP: 10.4 ± 1.2 s; n = 8, P < 0.05; Fig. 11).

CCAP (10^{-7} M) superfusion also caused an increase in the pyloric-timed activity of the protractor phase neuron IC during the MCN1-elicited gastric mill rhythm. We observed an increased number of action potentials per pyloric-timed burst in IC during peptide application for the fast rhythms only (P < 0.05, n = 9), although its intraburst firing frequency was unchanged during the fast rhythms (P = 0.12, n = 9; supplementary Table 1, Table 2). CCAP did not activate the protractor neuron GM (n = 19) nor did it alter the gastric mill- or pyloric-timed activity of the protractor neuron MG (n = 7).

CCAP also did not alter the activity of any of the retractor neurons during these fast rhythms, including Int1, DG, and VD (supplementary Table 1). A review of the data set for these neurons indicated that their mean burst duration (across 10 consecutive gastric mill cycles) was in fact prolonged in many but not all individual preparations. For example, the mean DG burst duration was increased during CCAP (10^{-7} M) superfusion (n = 7) despite the fact that AM was directly excited by focally applied CCAP (supplementary Table 1). Aside from the LG neuron, none of the recorded gastric mill neurons exhibited altered activity when CCAP was applied during slow gastric mill rhythms (supplementary Table 2).

Unlike the effects of CCAP on the VCN-triggered rhythm, CCAP application did not alter the phase relationships of any gastric mill neurons during either the fast or slow MCN1-elicited gastric mill rhythms (supplementary Table 1, Table 2). The lack of a change in its active phase was not surprising for the LG neuron because its burst duration was increased along with the cycle period during the fast rhythms (Fig. 10, A and B; supplementary Table 1). However, the unchanged phase relationships of the retractor neurons Int1 (n = 2; phase on, P = 0.20; phase off, P = 0.20), VD (n = 5; phase on, P = 0.77; phase off, P = 0.93), and DG (n = 6; phase on, P = 0.49; phase off, P = 0.73) as well as the protractor neurons IC (n = 5; phase on, P = 0.40; phase off, P = 0.82) and MG (n = 6; phase on, P = 0.93; phase off, P = 0.97) were surprising because their burst durations were not altered during fast rhythms, whereas the cycle period was prolonged (supplementary Table 1). A review of the data set for these neurons indicated that their mean burst duration (across 10 consecutive gastric mill cycles) was in fact prolonged in many but not all individual preparations. For example, the mean DG burst duration was increased during CCAP (10^{-7} M) superfusion relative to each saline control in four of six preparations.

**CCAP influence on the gastric mill rhythm is dose-dependent**

We also determined the threshold concentration at which CCAP influenced the MCN1-elicited gastric mill rhythm. For
CCAP modifies the gastric mill rhythm regulation of the pyloric rhythm

The MCN1-elicted gastric mill rhythm also regulates the pyloric rhythm. Specifically, the pyloric rhythm is slowed and weakened during each protractor (LG burst) phase (Bartos and Nusbaum 1997). This effect results from the presynaptic inhibition of the STG terminals of MCN1 by the LG neuron, which reduces the MCN1 influence on the pyloric rhythm during the protractor phase (Bartos and Nusbaum 1997; Coleman and Nusbaum 1994). Therefore we determined whether the influence of the gastric mill rhythm on the pyloric rhythm was altered in the presence of CCAP. Relative to saline superfusion, CCAP application (10⁻⁷ M) did not alter the pyloric cycle period during either the LG neuron burst (saline: 1.2 ± 0.0 s; CCAP: 1.2 ± 0.1 s; n = 11, P = 0.20) or interburst (saline: 1.0 ± 0.0 s; CCAP: 1.1 ± 0.0 s; n = 11, P = 0.22). Additionally, as shown originally by Bartos and Nusbaum (1997), in our experiments, the pyloric cycle period retained its longer duration during the LG burst, relative to the LG interburst, during saline superfusion (LG burst: 1.2 ± 0.0 s; LG interburst: 1.0 ± 0.0 s, n = 11, P < 0.001). This relationship persisted in the presence of CCAP (10⁻⁷ M; LG burst: 1.2 ± 0.1 s; LG interburst: 1.1 ± 0.1 s, n = 11, P < 0.001).

In contrast to its lack of influence on the pyloric cycle period, CCAP (10⁻⁷ M) did alter the activity of the pyloric neuron LP in a gastric mill phase-dependent manner. In saline, the LP neuron produced more action potentials per pyloric-timed burst during the retractor phase (LG interburst) than during the protractor phase (LG burst: 7.5 ± 0.4 spike; LG interburst: 7.9 ± 0.4 spike; n = 13, P < 0.05, paired Student’s t-test). As reported originally by Weimann et al. (1997), the presence of CCAP (10⁻⁵ M) increased the number of LP action potentials per burst. Moreover, during the MCN1-elicted gastric mill rhythm, the number of LP action potentials per burst became larger during the LG burst phase of the gastric mill rhythm relative to the LG interburst phase (LG burst: 11.1 ± 0.5 spike; LG interburst: 10.2 ± 0.4 spike; n = 13, P < 0.05, paired Student’s t-test).

CCAP application increases the spontaneous activity of Int1. A. top: during saline superfusion, Int1 exhibited spontaneous pyloric rhythm-timed bursts. Bottom: CCAP application enhanced spontaneous Int1 activity. Note the increase in the Int1 firing frequency and its more depolarized membrane potential at the trough of each oscillation. B: across preparations, the intraburst firing frequency of Int1 was higher during CCAP application than during saline superfusion (saline: 6.4 ± 1.1 Hz; CCAP: 8.8 ± 1.3 Hz; n = 7, P < 0.05).

The threshold concentration at which CCAP influences several gastric mill rhythm parameters is within the range of circulating hormone levels. Data were normalized for each individual preparation (CCAP/saline) and then averaged across preparations. Symbols represent statistical significance when tested on the raw data for each parameter. A–C: at concentrations of 10⁻⁷ M, CCAP increased the gastric mill cycle period (A), LG neuron burst duration (B), and LG intraburst firing frequency (C).
DISCUSSION

In this paper, we show that the peptide hormone CCAP alters two different versions of the gastric mill rhythm elicited by identified modulatory projection neurons in the crab STNS. In both cases, CCAP slowed the gastric mill rhythm by prolonging its protractor phase. This prolongation was concomitant with an increased burst duration of the LG neuron, which is directly excited by CCAP and is the protractor half of the reciprocally inhibitory pair of neurons at the core of the gastric mill CPG (Bartos et al. 1999; Coleman et al. 1995). CCAP similarly prolongs motor neuron burst durations in the crab pyloric system and cardiac ganglion as well as in the crayfish swimmeret system (Fort et al. 2007; Mulloney et al. 1997; Weimann et al. 1997). In contrast, although the retractor half of this gastric mill CPG pair (Int1) was also directly excited by CCAP, this peptide application did not alter Int1 activity during these gastric mill rhythms (Fig. 14). These results suggest that, despite its multiple targets, the primary site of action by which CCAP modulates the gastric mill rhythm is the LG neuron (see following text).

The modulation of a modulatory action has been termed metamodulation (Edwards et al. 2002; Katz and Edwards 1999; McLean and Sillar 2004; Mesce 2002; Parker and Grillner 1999; Svensson et al. 2001). Although metamodulation is likely to be a common phenomenon, there are currently few examples of its consequences for motor circuit activity, and, in most cases, it has not been possible to assess the studied metamodulatory actions on identified circuit neurons. For example, application of either substance P or serotonin to the isolated lamprey spinal cord has long-lasting actions on spinal interneurons involved in the lamprey swim motor pattern, including modifying NMDA-mediated synaptic actions (Parker and Grillner 1999). Further, either serotonin or dopamine application alters these substance P-mediated actions (Svensson et al. 2001). None of the previous studies on metamodulation involved the release of a neuromodulator from an identified neuron. However, recent work by McLean and Sillar (2004) showed that pharmacological manipulation of the nitric oxide (NO) system influences a neuronal source of NO in a manner comparable to that of exogenously applied NO donors on noradrenergic modulation of the amphibian spinal locomotor system.

The phase-specific influence of CCAP on the MCN1-elicited gastric mill rhythm was surprising given that CCAP had a balanced, direct excitatory influence on protractor (LG, IC) and retractor (Int1, AM) neurons. The unbalanced influence of CCAP on these neurons during this rhythm might have resulted from this peptide having distinct dose-response effects on these different neurons, perhaps with Int1 and AM being more weakly affected at lower CCAP concentrations than LG and IC. Alternatively, the CCAP modulation of these rhythms might have resulted from its enabling increased transmitter release from MCN1. This latter possibility remains to be directly examined, but it appears to be an unlikely explanation. Specifically, when MCN1 transmitter release is enhanced by directly increasing MCN1 firing frequency, the gastric mill cycle period is decreased instead of increased, and it is associated with a decreased retractor phase duration instead of an increased protractor phase duration (Bartos et al. 1999). CCAP also produces phase specific modulation in the crayfish swimmeret system by increasing the activity of power stroke motor neurons without changing the activity of the return stroke motor neurons (Mulloney et al. 1997).

Despite the apparent lack of direct CCAP action on the STG terminals of MCN1, what are likely to be hormonally relevant concentrations of CCAP (see following text) did facilitate the ability of MCN1 to activate the gastric mill rhythm by reducing its threshold firing frequency for rhythm activation. This CCAP action was likely to result from the same convergent activation of the aforementioned voltage-dependent inward current by this peptide and MCN1-released CabTRP Ia in the LG neuron that likely underlies the CCAP influence on the gastric mill cycle period. Although a direct excitatory action of CCAP onto MCN1 could have also facilitated MCN1 activation of the gastric mill rhythm, the aforementioned previous studies suggest that the resulting cycle period would have been reduced instead of increased, due to a reduced retractor phase duration instead of an increased protractor phase duration (Bartos et al. 1999; Beenhakker et al. 2005).

It remains to be determined why Int1 activity was not altered during the gastric mill rhythm despite its continued responsiveness to CCAP at least when applied at $10^{-7}$ M. The lack of change in Int1 activity when CCAP was applied during MCN1 stimulation was not likely to be an occlusion effect resulting from the known convergent activation by different peptide transmitters of the same ionic current in STG neurons (Swensen and Marder 2000, 2001). This possible mechanism is unlikely because MCN1 influences Int1 exclusively by a picrotoxin-sensitive, ionotropic GABAergic excitation (Stein et al. 2007; Swensen et al. 2000).

Interestingly, the proprioceptor neuron GPR also effectively excites Int1 in the absence of the MCN1-elicited gastric mill rhythm, yet Int1 activity is not altered when GPR is stimulated during this rhythm (Beenhakker et al. 2005). The GPR excitation of Int1 also appears to be ionotropic, but unlike the MCN1 action, it is not a GABAergic action but is likely cholinergic (Beenhakker 2004, 2005; Katz et al. 1989; Swensen et al. 2000). The fact that the Int1 response to both CCAP application and GPR stimulation is gated out during the gastric mill rhythm suggests a common underlying mechanism. One possible mechanism is a ceiling effect wherein the Int1 firing...
frequency is already maximal during the gastric mill rhythm. Testing this hypothesis awaits further experiments.

Although the most obvious and pronounced effects of CCAP were its influence on LG neuron activity and the resulting slowing of the gastric mill rhythms, CCAP did also influence additional gastric mill neurons during these rhythms (Fig. 14). However, in neither of the two studied gastric mill rhythms did CCAP alter the activity of all four gastric mill neurons that responded directly to its application. Additionally, DG neuron activity was altered by CCAP during the VCN-triggered gastric mill rhythm despite its not being directly influenced by this peptide. The lack of congruence between direct targets of CCAP and those neurons the activity of which was altered during the gastric mill rhythm reinforces previous work documenting that, due to unanticipated circuit interactions, it is not sufficient to simply identify the individual targets of neuromodulation to understand neuronal circuit modulation (Ayali and Harris-Warrick 1999; Hooper and Marder 1987; Thirumalai et al. 2006).

Surprisingly, the cycle-period dependent action of CCAP on the MCN1-elicited gastric mill rhythm was eliminated when the pyloric rhythm was suppressed as CCAP effectively prolonged the cycle period of these slow rhythms. The mechanistic explanation for this discrepancy awaits further experiments. However, it is noteworthy that the pyloric-time depolarizations in LG during each retractor phase are a pivotal mechanism underlying the natural cycle period of this gastric mill rhythm (Bartos et al. 1999). Thus when the pyloric rhythm is absent, the relative influence of each remaining circuit synapse on gastric mill rhythm generation may be altered, enabling CCAP to have an enhanced influence on these long cycle periods.

There is no information available regarding the circulating levels of CCAP during feeding. However, CCAP release in behaving crabs (Carcinus maenas L., Orconectes limosus) occurs during ec dysis (molting) behavior, a time when crabs are explicitly not feeding. During ec dysis, CCAP in the hemolymph rises to an average value of $10^{-9}$ M, whereas the average CCAP concentration during the intermolt period is nearly 100-fold lower ($\sim 3 \times 10^{-11}$ M) (Philippen et al. 2000). In the study by Philippen et al. (2000), hemolymph samples were collected from the ventral abdominal sinus (O. limosus) and hypobranchial sinus (C. maenas). These sites are downstream from the ophthalmic artery, which is a major artery exiting the STG also contain a larger volume of hemolymph than the ophthalmic artery. Therefore the concentration of CCAP at the STG was likely to be higher than the molt- and intermolt-related values reported by Philippen et al. (2000; see also Fort et al. 2007). Thus even the steady-state CCAP concentration of the intermolt period was likely to be at or above threshold for both our reported CCAP actions on the gastric mill circuit ($> 10^{-11}$ M) and for the previously reported CCAP actions on the pyloric circuit (Weimann et al. 1997). Additionally, a rise in hormone levels is not unexpected in relation to feeding. For example, Turrigiano and Selverston (1990) showed that levels of a cholecystokinin-like peptide, again assayed downstream from the ophthalmic artery, rise to $> 10^{-9}$ M in the hour after feeding.

In conclusion, the ability of the peptide hormone CCAP to modulate the MCN1-elicited gastric mill rhythms indicates that hormonal modulation can expand the flexibility of rhythmically active motor circuits to levels beyond those provided by neuronal modulation. Additionally, despite having multiple targets within the gastric mill circuit, CCAP may well be altering the gastric mill rhythm largely via its influence on a single, pivotal CPG neuron.

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