Hormonal Modulation of Two Coordinated Rhythmic Motor Patterns

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Wood DE, Varrecchia M, Papernov M, Cook D, Crawford DC. Hormonal modulation of two coordinated rhythmic motor patterns. J Neurophysiol 104: 654–664, 2010. First published June 2, 2010; doi:10.1152/jn.00846.2009. Neuromodulation is well known to provide plasticity in pattern generating circuits, but few details are available concerning modulation of motor pattern coordination. We are using the crustacean stomatogastric nervous system to examine how co-expressed rhythms are modulated to regulate frequency and maintain coordination. The system produces two related motor patterns, the gastric mill rhythm that regulates protraction and retraction of the teeth and the pyloric rhythm that filters food. These rhythms have different frequencies and are controlled by distinct mechanisms, but each circuit influences the frequency of the other via identified synaptic pathways. A projection neuron, MCN1, activates distinct versions of the rhythms, and we show that hormonal dopamine concentrations modulate the MCN1 elicited rhythm frequencies. Gastric mill circuit interactions with the pyloric circuit lead to changes in pyloric rhythm frequency that depend on gastric mill rhythm phase. Dopamine increases pyloric frequency during the gastric mill rhythm retraction phase. Higher gastric mill rhythm frequencies are associated with higher pyloric rhythm frequencies during retraction. However, dopamine slows the gastric mill rhythm frequency despite the increase in pyloric frequency. Dopamine reduces pyloric circuit influences on the gastric mill rhythm and upregulates activity in a gastric mill neuron, DG. Strengthened DG activity slows the gastric mill rhythm frequency and effectively reduces pyloric circuit influences, thus changing the frequency relationship between the rhythms. Overall dopamine shifts dependence of frequency regulation from intercircuit interactions to increased reliance on intracircuit mechanisms.

INTRODUCTION

Some neuronal networks co-express multiple rhythms that must be coordinated for adaptive behavior (Briggman and Kristan 2008; Dickinson 1995). Coordination of distinct rhythmic motor patterns occurs in many animals, including breathing and locomotion in mammals (Kawahara et al. 1989; Morin and Viala 2002), and swimmeret beating and locomotion in crustaceans (Cattaert and Clarac 1983; Copp and Hodes 2001). Coordination can be influenced by afferent sensory and central descending inputs to circuits (Blitz and Nusbaum 2008; Blitz et al. 2008; Faumont et al. 2005; Giraudin et al. 2008; Morin and Viala 2002; Wood et al. 2004), by independent intracircuit mechanisms, and by local interactions between circuits (Bartos et al. 1999; Clements et al. 1998; Faumont et al. 2005; Rauscent et al. 2009). Presumably, any of these mechanisms can be modulated to flexibly coordinate motor patterns, but details of this are few (Dickinson 1995; Nusbaum and Beenakker 2002).

Using the crustacean stomatogastric nervous system (STNS), we are examining how modulatory influences alter rhythm frequencies of two coordinated motor patterns. The STNS produces distinct rhythms underlying the behaviors of chewing (gastric mill rhythm) and filtering of food (pyloric rhythm). In the crab, the overlapping circuits underlying these rhythms have distinct mechanisms that regulate their frequencies. Pyloric rhythm frequency is regulated by a subcircuit of neurons with pacemaking properties (Marder and Bucher 2007). In the crab, gastric mill rhythm frequency is driven by reciprocal inhibition between two core neurons (Bartos et al. 1999). Identified pathways for interactions of between the pyloric and gastric mill core circuits allow straightforward analysis of rhythm coordination (Bartos and Nusbaum 1997; Bartos et al. 1999).

Multiple modulatory inputs are delivered to these circuits as neurohormones and locally from projection neurons (Marder and Bucher 2007). Distinct rhythms are elicited from the circuits when specific modulatory inputs are available (Nusbaum and Beenakker 2002). Dopamine (DA) is a neurohormone in crustaceans (Fort et al. 2004; Siwicki et al. 1987; Sullivan et al. 1977). Hormonal concentrations of DA modulate axonal activity in peripheral nerves (Ballo and Bucher 2009; Bucher et al. 2003). Effects of hormonal concentrations on circuits have not been demonstrated.

We have found that DA applied as a hormone can modulate the rhythms elicited by an identified projection neuron, commissural neuron 1 (MCN1). The pyloric pacemaker circuit regulates gastric mill rhythm frequency using an identified pathway that also mediates the tendency for the frequencies of these rhythms to increase together (Bartos et al. 1999). With DA present, the gastric mill rhythm frequency decreases, and the typical relationship between pyloric and gastric mill rhythm frequencies is altered. We show that the influence of pyloric circuit regulation of gastric mill rhythm frequency is reduced, but phase-locking between the rhythms tightens. DA also affects intracircuit frequency regulation: activity in the dorsal gastric neuron (DG) is upregulated, and this contributes to decrease gastric mill rhythm frequency. Thus when DA is available as a hormone, the gastric mill rhythm frequency depends more on intracircuit mechanisms and less on intercircuit regulation without loss of coordination between the rhythms.

METHODS

Animals

Adult male Cancer borealis were obtained from Commercial Lobster (Boston, MA) and kept in an aerated, artificial seawater tank. All animals were anesthetized at −20°C for 20–30 min prior to dissection. The stomach was removed, and the STNS was dissected in chilled saline following the standard protocol for this preparation (Beenakker and Nusbaum 2004). The STNS was pinned out in a silicone elastomer-lined Petri dish (Sylgard 184, KR Anderson, Santa
Clara, CA), and for some experiments it was kept overnight at 4°C. We used a total of 92 crabs for these experiments.

Solutions

_C. borealis_ physiological saline had a composition of (in mM) 440 NaCl, 26 MgCl₂, 13 CaCl₂, 11 KCl, 10 Trisma base, and 5 maleic acid; pH 7.4–7.6. Dopamine hydrochloride was obtained from Sigma Aldrich (St. Louis, MO). The DA was dissolved initially in deionized water and stored as a 10⁻² M stock solution at −20°C. Immediately before use DA was diluted to working concentrations (10⁻⁹ to 10⁻⁶ M) in physiological saline. Low calcium saline was used to eliminate transmitter release (Blitz and Nusbaum 1997) and had the following composition (mM): 439 NaCl, 26 MgCl₂, 13 CaCl₂, 11.7 MnCl₂, 11 KCl, 10 Trisma base, and 5 maleic acid (pH 7.4–7.6).

Electrophysiology

Electrophysiology was performed using standard methods (Beenhakker and Nusbaum 2004; Saideman et al. 2007). The stomatogastric ganglion (STG) was desheathed and illuminated using a darkfield condenser to facilitate intracellular recording and to provide access to the circuits for applied DA. The isolated STNS was superfused continuously with physiological _C. borealis_ saline (7–12 ml/min) and maintained at a temperature of 10–13°C. The inferior esophageal nerves (ions) and superior esophageal nerves (sons) were transected to reduce modulatory input from the commissural ganglia (CoGs) to the circuits and to allow for selective activation of modulatory commis- sional neuron 1 (MCN1, Fig. 1A) (Bartos and Nusbaum 1997; Bartos et al. 1999). Under these conditions, the pyloric rhythm either stops or slows from a cycle frequency that is typically near 1 Hz. We only used preparations where the pyloric rhythm frequency was spontaneously active (0.7–1.2 Hz). When the gastric mill rhythm was active before the ions and sons were transected, the rhythm terminated as soon as these nerves were cut and did not activate spontaneously during experiments (Coleman and Nusbaum 1994; Coleman et al. 1995).

Microelectrodes (18–25 MΩ) were filled with 0.6 M K₂SO₄ with 20 mM KCl. Circuit neurons in the STG were identified by their activity patterns, interactions with other neurons, and axonal branching patterns (Blitz and Nusbaum 1997; Blitz et al. 1999; Norris et al. 1994; Weimann et al. 1991). DA was perfused using a peristaltic pump (Rabbit Minipuls 2, Rainin Instrument, Oakland, CA) for 30 min. A petroleum jelly (Vaseline) well was placed around the STG to confine activation of DA receptors to those located within the STG. The preparation was then washed in saline for 1 h after DA application.

Focal applications of DA were performed using picospritzer III (General Value, Fairfield NJ). A pipette (~1 MΩ) filled with DA 10⁻⁴ M was placed over the STG neuropil during the application (~0.5–1 s, 5–8 psi); otherwise it was maintained some distance away to prevent DA leakage. Concentrations of ≥10⁻⁴ M are typically required to elicit responses during focal applications.

The selective activation of MCN1 (10–15 Hz) was accomplished by extracellular stimulation of its axonal projection in the ions (Grass Model S-88, Astro-Med, West Warwick, RI; Fig. 1A) (Bartos and Nusbaum 1997; Coleman and Nusbaum 1994; Wood et al. 2000). Effective MCN1 activation was monitored by intracellular recording of the lateral gastric (LG) neuron and by its characteristic activation of neurons in the STG circuits (Blitz et al. 1999; Wood et al. 2000).

Electrical excitatory postsynaptic potentials (EPSPs) from MCN1 to LG were used to monitor MCN1 action potential frequency (Fig. 1C) (Coleman et al. 1995; Wood et al. 2000). Kirby et al. (2007) found that during the course of several MCN1 activations (10 Hz, ~1.5 h), the gastric mill rhythm frequency decreases over time. We found a similar result and strictly maintained our experiment durations to <1.5 h. Previous reports indicate that the spontaneous pyloric rhythm frequency remains stable for prolonged time periods (Hooper and Marder 1987; Miller and Selverston 1982).

**FIG. 1.** Activation of the modulatory commissural neuron 1 (MCN1) initiates a gastric mill rhythm and modulates the spontaneous pyloric rhythm. 
_A:_ a schematic preparation shows MCN1 in the commissural ganglia (CoG) and its projections to the stomatogastric ganglion (STG). _B:_ key circuit neurons that regulate rhythm frequencies. _C:_ left: the pyloric rhythm is spontaneously active (PD, intracellular) and the gastric mill rhythm is not. _Right:_ with MCN1 activated, the pyloric rhythm is strengthened and the gastric mill rhythm activates (LG, intracellular), DG (extracellular). Bar above AB indicate decreased rhythm frequency during protraction. _ions_ stomatogastric nerve; _sons_ superior esophageal; _Stn_ stomatogastric nerve; _s_ lateral esophageal; _lvn_ lateral ventricular; _dgn_ dorsal gastric; _lgn_ lateral gastric; _Prot_ protraction; _Ret_ retretaction.

Data analysis and acquisition

The data were acquired by computer using Axoscope, Pclamp 9.0 software and a Digidata 1300A (Molecular Devices), or Spike II software with a CED 1401 A/D board with interface, including custom scripts for Spike II (v 4.0) software (CED). Data were analyzed using custom scripts (courtesy of M. Beenhakker and W. Stein). Statistics and analysis were performed using Sigma Plot 9.0 and Sigma Stat 3.1 (SPSS, Chicago, IL). Data are expressed as means ± SD. All tests of two means are paired t-test except where nonparametric tests are noted. For multiple sample testing, ANOVA, Friedman repeated measures ANOVA, and Kruskal-Wallis ANOVA on ranks are noted where used. Post hoc comparison tests for ANOVA were performed using custom scripts for Spike II software (courtesy of M. Beenhakker and W. Stein). Methods that are explicitly for data with measurement variability (Warton et al. 2006). Data points acquired for this analysis were means of pyloric and gastric mill rhythm frequencies from individual preparations, computed as indicated in the following text. Computing software for these methods was obtained from: //www.bio.mq.edu.au/ecology/SMATR/.

The frequency of the pyloric rhythm was monitored via recordings of neurons that are a part of the pyloric pacemaker ensemble (Nusbaum and Beenhakker 2002) (Fig. 1, B and C). To compute the pyloric rhythm frequency, recordings of either a pyloric dilator neuron (PD) or the anterior burster (AB) neuron were used to measure the time from the first spike within a burst to the first in the next burst (20 consecutive cycles were averaged). During MCN1 elicited rhythms
the LG neuron is necessary for the MCN1 elicited gastric mill rhythm and its activity pattern critically shapes the frequency and form of this rhythm (Coleman and Nusbaum 1994). The frequency of the gastric mill rhythm was determined by measuring the LG cycle period from the first impulse within a LG burst until the start of the next burst (10 consecutive cycles were averaged). Burst duration for each neuron was determined by start and end of spiking in each cycle. Each LG burst is preceded by an AB or PD burst. The latency between the pyloric and gastric mill rhythms was computed as the time between the first spike in an AB or PD burst and the first spike in the next LG burst.

Because alternating bursting of the DG and LG motor neurons underlie fictive retraction and protraction of the teeth (Clemens et al. 1998; Coleman and Nusbaum 1994; Heinzel et al. 1993), we used changes in the activity patterns of these neurons to assess alteration of the form of the gastric mill rhythm (Fig. 1C). The activity of these neurons was measured by their average firing frequency (number of spikes minus 1 divided by burst duration), burst duration, and phase relationship (start time of DG bursting as a percentage of normalized LG cycle duration).

RESULTS

Applied dopamine actions on key neurons in the pyloric and gastric mill circuits

Much detail is known about DA actions on spontaneously active pyloric rhythms in lobsters (Harris-Warrick et al. 1998; Marder and Bucher 2007) but less is known for crabs. We began by examining DA actions on rhythm frequency in crab preparations with spontaneously active pyloric rhythms. Using DA concentrations reported as circulating hormone levels in crustaceans (Livingstone et al. 1980), we found that its application had no effect on spontaneous pyloric rhythm frequency and that the frequency was unchanged after DA wash (control, 0.65 ± 2.8 Hz; DA 10⁻⁹ M, 0.63 ± 2.74 Hz; 10⁻⁹ M, 0.64 ± 2.72 Hz; 10⁻⁷ M, 0.72 ± 2.29 Hz; 10⁻⁶ M, 0.76 ± 2.12 Hz; Kruskal-Wallis ANOVA on Ranks, P > 0.05, n = 26–87).

In conditions where modulatory inputs to the STG circuits are reduced, the gastric mill rhythm is not spontaneously active in the crab (Bartos and Nusbaum 1997; Coleman and Nusbaum 1994). Gastric mill rhythms are characterized by alternating bursting between the LG and DG motor neurons (Nusbaum and Beenhakker 2002) (Fig. 1C). The rhythm was not activated after bath application of DA (Fig. 2B). Applied DA increased spontaneous action potentials in the DG neuron and depolarized LG without initiating spiking (Fig. 2B). Average control LG Vm, −66 ± 1.2 mV; DA 10⁻⁹ M, −61.5 ± 1.0 mV; repeated measures on ranks, n = 15, P < 0.05; after wash Vm is similar to control, 67.1 ± 1.4 mV, P > 0.05).

Interneuron 1 (Int1) and LG are the core central pattern generating (CPG) neurons necessary for gastric mill rhythm generation in the crab (Fig. 1B) (Coleman and Nusbaum 1994; Nusbaum and Beenhakker 2002). Membrane potential of Int1 did not change significantly, even with DA concentrations above the thresholds for DG and LG (control Vm range, −48.7 ± 0.05 to −39.9 ± 0.06 mV; DA 10⁻⁷ M, −47.9 ± 0.04 to −38.7 ± 0.06 mV; n = 4, P > 0.05; DA 10⁻⁶ M, −48.1 ± 0.08 to 37.9 ± 0.09 mV, n = 3, P > 0.05). Firing frequency of Int1 was unaffected by DA (control firing frequency 14.9 ± 2.9 Hz; DA 10⁻⁷ M 15.1 ± 3.1 Hz, n = 4, P > 0.05; DA 10⁻⁶ M, n = 5, P > 0.05).

Dopamine actions on MCN1 elicited pyloric and gastric mill rhythms

Activation of MCN1 initiates a gastric mill rhythm and enhances the pyloric rhythm (Fig. 1C). Pyloric rhythms have much higher frequencies than gastric mill rhythms, completing several cycles during each gastric mill cycle (Fig. 1C). The average pyloric rhythm frequency is increased by MCN1 activation (Fig. 1C) (Blitz et al. 1999; Wood et al. 2000). The application of DA did not alter the average rhythm frequency (control, 1.1 ± 0.13 Hz, DA 10⁻⁹ M, 1.18 ± 0.12 Hz; not different across all concentrations 10⁻⁸ to 10⁻⁷ M; Kruskal-Wallis ANOVA on ranks n = 19–24, P > 0.05, Dunn’s test).

With DA present, the gastric mill rhythm is altered in both its frequency and form (Fig. 2). Applied DA decreases the average gastric mill rhythm frequency (Fig. 2, B and D) and the LG burst duration increases (Fig. 2, A and D, n = 15, * P < 0.025, repeated measures ANOVA). The average burst duration of DG is unchanged by DA (control, 3.2 ± 1.1 s; 10⁻⁹ M DA, 3.3 ± 0.8 s; n = 8, repeated measure ANOVA, P > 0.05). The phase relationship between DG and LG bursting represents the fraction of the normalized LG cycle period where DG fires bursts of action potentials. The onset phase of DG bursting was...
altered after DA application (control, 51.3 ± 4.1; DA 10^-9 M, 62.2 ± 3.8, n = 8, P < 0.05; Mann-Whitney rank sum test) but DG’s offset was unchanged (n = 8, P > 0.05; Mann-Whitney rank sum test). The delayed onset of DG’s burst is consistent with LG’s increased burst duration and an extended protractor phase of the rhythm. The firing frequencies of both LG and DG are increased in the presence of DA (LG, 7.88 ± 0.62 Hz control; DA 10^-9 M, 10.12 ± 0.81 Hz, n = 15, P < 0.01, ANOVA) with DG, 12.6 ± 1.25 Hz control; 10^-6 M DA, 14.2 ± 1.35 Hz, n = 8, P < 0.05; wash similar to control, P > 0.05; repeated measure ANOVA).

During MCN1 activation bursting in the LG neuron results from slow excitation that builds during the gastric mill retractor phase, allowing LG to escape from Int1’s inhibition (Fig. 1, B and C) (Bartos et al. 1999; Beenakker et al. 2005; Coleman et al. 1995). Because LG presynaptically inhibits MCN1, this slow MCN1 excitation wanes during the LG burst, contributing to LG burst termination. Bursting activity in the DG neuron is not necessary to maintain the rhythm and DG is a follower of LG activity (Coleman and Nusbaum 1994; Coleman et al. 1995). During an ongoing gastric mill rhythm, the Int1 neuron displays bursting activity during the retraction phase in time with the DG bursting (Bartos et al. 1999; Coleman et al. 1995). Int1 bursting displays gastric mill timing due to inhibition from LG and pyloric timing due to direct synaptic inhibition from the pyloric pacemaker (Fig. 1B) (Bartos et al. 1999; Nusbaum and Beenakker 2002). With DA present, Int1’s gastric-timed burst durations decrease (control average, 3.3 ± 0.09 s; DA 10^-7 M, 2.8 ± 0.11 s, P < 0.05, n = 4). This is consistent with the increase in LG burst duration and firing frequency. Int1’s firing frequency is unaltered in this condition (average, 16.8 ± 1.15 Hz control, 16.1 ± 1.23 Hz with DA, n = 4, P > 0.05) and the duration of its pyloric-timed bursts do not differ from control (average, 0.56 ± 0.08 s control, 0.52 ± 0.06 s with DA, n = 4, P < 0.05).

Concentration dependent effects of DA on the gastric mill rhythm

In determining the threshold concentration for DA effects on these rhythms, we found DA actions were very different for concentrations >10^-7 M. The pyloric rhythm frequency averaged across both gastric mill rhythm phases was unchanged with 10^-6 M DA (n = x, P > 0.05; repeated measures ANOVA) but the effects on the gastric mill rhythm opposed those produced by lower DA concentrations (Fig. 3). In this case, LG bursting was weakened: its burst duration was decreased (Fig. 3, B and D; n = 10, P < 0.03; repeated measure ANOVA), and its firing frequency was lowered (average firing frequency control, 8.1 ± 0.8 Hz; with DA, 6.2 ± 1.1 Hz, n = 10, P < 0.05). Most strikingly, the gastric mill rhythm frequency increased over control (Fig. 3, B and C, P < 0.05, n = 10, repeated measures ANOVA). In some preparations, bursting activity in the LG neuron was completely suppressed after 10^-4 M DA was applied (n = 8/26; 100% LG suppression with 10^-4 M DA, not shown) (Wood et al. 2006). In preparations with active LG bursting, the DG burst duration is slightly but significantly increased with 10^-6 M DA (n = 12, P < 0.05, 3.2 ± 1.1 s; with 10^-4 M DA 4.1 ± 0.7 s; repeated measures ANOVA, burst duration returned to control after wash) and its firing frequency was increased (12.3 ± 1.1 Hz control, 15.1 ± 1.4 with DA; n = 12, P < 0.01, repeated measures ANOVA; firing frequency returned to control after wash).

**Direct effects of DA on key gastric mill neurons**

Our results suggest that DA has direct actions on LG and DG but not on Int1. Changes in Int1 activity such as the reduced duration of its gastric timed bursts may be explained in part by changes in LG activity (see DISCUSSION). However, unexpected changes in synaptic interactions can arise during modulation (Ayali and Harris-Warrick 1999; Hooper and Marder 1987; Thirumalai et al. 2006). To investigate modulation of intracircuit frequency regulation, we began by elucidating whether DA acts directly on these key neurons and then compared this to responses during the DA modulated MCN1 rhythm.

Using low Ca^2+ saline to reduce chemical synaptic influences, we found that focal application of DA (10^-4 M) depolarized LG and DG but had no influence on Int1 (n = 4/4; Fig. 3E). Both LG and DG responded with action potentials after DA application (n = 4/4; Fig. 3E). DA is known to enhance voltage dependent Ca^2+ currents in some pyloric neurons and reduce it in others (Johnson et al. 2003). This suggests that use of low Ca^2+ saline could alter the responses of gastric mill neurons to DA. DA is also known to modulate several different conductances in neurons where I_{Ca} is also modulated (Marder and Bucher 2007). Our tests showed that some DA actions on LG and DG are detectable in these conditions and that Int1...
consistently displayed no net change in its response to DA with or without low Ca²⁺ saline present. The LG neuron is electrically coupled to the inferior cardiac neuron (IC), medial gastric neuron (MG), and the gastric mill neurons (GM) (Nusbaum and Beenhakker 2002). Both IC and MG are activated by MCN1 (Coleman and Nusbaum 1994), but neither demonstrates activity changes during MCN1 activation with bath application of DA (firing frequency IC control, 14.9 ± 2.1 Hz; DA, 14.7 ± 1.8 after; n = 25, P > 0.05; MG control, 9.9 ± 3.1; DA, 11.8 ± 4.1 Hz; n = 16, P > 0.05). This indicates that IC and MG likely do not contribute to excitation of LG by bath applied DA. The GM neurons are only weakly activated by MCN1 (Stein et al. 2007), and applied DA did not alter GM activity during MCN1 activation (control firing frequency, 2.9 ± 1.2 Hz; DA, 3.1 ± 1.4 Hz; n = 10, P > 0.05). It appears unlikely that these neurons contribute to excitation of LG by DA. The DG neuron is not electrically coupled with other circuit neurons (Coleman and Nusbaum 1994; Nusbaum and Beenhakker 2002; Saideman et al. 2007). These results indicate that DG and LG have receptors for DA.

The Int1 neuron is electrically coupled to one neuron, the ventricular dilator (VD) (Nusbaum and Beenhakker 2002; Saideman et al. 2007). We observed increased excitation of VD with DA present during MCN1 activation (control, 15.1 ± 2.9 Hz; n = 10, P > 0.05). If VD activity influenced Int1 in these conditions, we would expect Int1 activity to be enhanced, and as described in the preceding text, during focal DA application and it was not. Overall Int1 exhibited no change in response to DA, and this suggests that it likely has no receptors for DA.

**Dopamine alters gastric mill circuit regulation of pyloric rhythm frequency**

Presynaptic inhibition of MCN1 by LG diminishes MCN1 actions during each LG burst (Fig. 1, B and C) (Bartos and Nusbaum 1997; Bartos et al. 1999). This feedback decreases the pyloric rhythm frequency during LG bursting, as compared with rhythm frequency during the LG interburst (Fig. 1C) (Bartos and Nusbaum 1997). Potentially, DA may modulate both the pyloric circuit and this feedback inhibition to affect rhythm frequency. We showed that average pyloric rhythm frequency is unchanged with DA present. However, pyloric rhythm frequency averaged over both gastric mill phases may not demonstrate DA influences; therefore, we removed this gastric mill circuit interaction to examine DA actions directly on pyloric rhythm frequency (Fig. 4). When we suppressed LG bursting activity the average pyloric rhythm frequency increased (MCN1 15 Hz activation, Fig. 4, C and D; n = 17, repeated measures ANOVA, *P < 0.05). In the presence of DA, LG suppression led to a significantly greater increase in pyloric rhythm frequency (Fig. 4, C and D; n = 17, repeated measures ANOVA, **P < 0.05).

Because the pyloric rhythm frequency increased with LG suppression, we expected that with DA present the pyloric rhythm frequency may be increased during the LG interburst. As previously shown, pyloric rhythm frequency averaged during the LG interburst was greater than during its burst (Bartos and Nusbaum 1997) (Fig. 5, A and C; repeated measures ANOVA, n = 17, *P < 0.05). With DA applied, the rhythm frequency during the LG interburst was significantly increased over control (Fig. 5, B and C; repeated measures ANOVA, n = 17, **P < 0.05). In this condition, the rhythm frequency averaged during LG bursting increased for some preparations (n = 9/17), and this average was nearly significantly different from control (Fig. 5C, n = 17, P = 0.06). We found that the first pyloric cycle within each LG burst is prolonged with DA present, suggesting that the effect of LG feedback to MCN1 is altered (n = 16/17 preparations; P = 0.02; average cycle duration control, 1.02 ± 0.15 s; DA 10⁻⁷ M, 1.2 ± 0.12 s). Overall DA influences on pyloric rhythm frequency depend on the phase of the gastric mill rhythm, demonstrating a dependence on intercircuit interactions.

**Pyloric circuit influences on gastric mill rhythm frequency are reduced with DA present**

The pyloric pacemaking circuit regulates the gastric mill rhythm frequency via an inhibitory synapse from the pacemaking neurons (AB/PD) to the core gastric mill neurons (Int1/LG; Fig. 1B) (Bartos et al. 1999). It was previously shown that increasing

![Diagram](http://jn.physiology.org/)

FIG. 4. With gastric mill rhythm influences removed, the average pyloric rhythm frequency increases with DA present. A: the pyloric rhythm cycle frequency slows during each LG burst (* AB). Hyperpolarization of LG (△, -2 mV) decreases pyloric rhythm frequency (bar 1). B: with DA present, LG suppression (▲, -2 nA) increases the pyloric rhythm frequency (bar 2). C: during LG suppression, the pyloric rhythm frequency increases with DA (△, bar 1; ▲, bar 2). D: pyloric rhythm frequency increases after LG suppression (*) but is increased significantly more with DA (**).
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FIG. 5. Applied DA influences gastric mill rhythm phase-dependent changes in pyloric rhythm frequency. A: the pyloric rhythm frequency decreases during each LG burst and increases during the interburst. B: in the presence of DA, the pyloric rhythm frequency decreases during LG's burst and increases during LG's interburst. C: in control rhythms, average pyloric rhythm frequency during LG's interburst (*) is greater than during the LG burst and greater than rhythm frequency with both gastric mill phases averaged (Avg. black bar). With DA applied, the average pyloric rhythm frequency during LG's interburst decreases significantly (**) as compared with all other conditions.

The MCN1 elicited gastric mill and pyloric rhythms are phase-locked via the same frequency regulating pathway described in the preceding text (Bartos et al. 1999). Prior results demonstrated a constant latency between each AB/PD burst and the next LG burst and that this latency remains stable.

Depolarizations of LG during its interburst are time-locked to the pyloric rhythm and occur when the pyloric pacemaker subcircuit inhibits Int1 to remove Int1 inhibition of LG (Bartos et al. 1999) (Fig. 7A, dotted line). With DA applied, these depolarizations remain evident, and this indicates the continued presence of both pyloric pacemaker and Int1 input to LG (Fig. 7B). To test whether pyloric pacemaker regulation of the gastric mill rhythm is altered by DA, we suppressed pacemaker activity during an ongoing gastric mill rhythm (Fig. 7). Effective suppression of the pyloric rhythm removes these depolarizations during the LG interburst (Fig. 7A, right) (Bartos et al. 1999). Consistent with prior results, removal of pyloric pacemaker input greatly reduced the gastric mill rhythm frequency (Bartos et al. 1999; Wood et al. 2004) (Fig. 7, B and C, *P < 0.001, n = 15–17, repeated measures ANOVA). When we inhibited the pyloric pacemaker with DA present, the gastric mill rhythm frequency decreased (Fig. 7, B and C; **P = 0.009, n = 15–17; repeated measure ANOVA; with 10−7 M DA applied LG spikes during the interburst). But under this condition, frequency was decreased significantly less than control without DA (Fig. 7C; n = 15–17, ΔP < 0.05; repeated measure ANOVA). In the presence of DA, pyloric circuit input regulates gastric mill rhythm frequency but has less impact.

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mill rhythm elicited by a peptide hormone, inhibition by DG (Saideman et al. 2007; Weimann and Marder 1994). In a gastric other modulatory conditions (Nusbaum and Beenhakker 2002; Coleman and Nusbaum 1994; Saideman et al. 2007). However, gastric mill rhythm frequency (Bartos and Nusbaum 1997; Nusbaum and Beenhakker 2002). The LG burst duration across a wide range of pyloric rhythm frequencies (Bartos et al. 1999). We found that the average latency was reduced in the presence of DA (latency without DA, 315 ± 28 ms; latency with 10−7 M DA, 248 ± 19, n = 15, P < 0.05). The variability of latency is decreased (Levene’s test, P > 0.05), and this indicates tighter phase-locking between the rhythms.

**DG neuron regulates gastric mill rhythm frequency with DA present**

As mentioned in the preceding text, DG is a follower of LG activity during MCN1 activation, and it does not influence gastric mill rhythm frequency (Bartos and Nusbaum 1997; Coleman and Nusbaum 1994; Saideman et al. 2007). However, an inhibitory synapse from DG to LG has been reported in other modulatory conditions (Nusbaum and Beenhakker 2002; Saideman et al. 2007; Weimann and Marder 1994). In a gastric mill rhythm elicited by a peptide hormone, inhibition by DG suppresses LG interburst membrane potentials and regulates the timing of LG bursting (Saideman et al. 2007). We noted a similar depression of LG interburst membrane potentials after DA application (n = 14/16; Fig. 2B, †), and this motivated our test of whether DG actions are altered by DA.

Consistent with prior results, removal of DG during the MCN1 elicited rhythm had no effect on rhythm frequency (Fig. 8, A and C; n = 8, P > 0.05, repeated measures ANOVA) (Coleman and Nusbaum 1994; Saideman et al. 2007). After DA application, the average rhythm frequency decreased (Figs. 2D and 8C). With DA applied, the rhythm frequency increased when DG was removed (Fig. 8, B and C; repeated measures ANOVA, n = 8, *P < 0.01), and the suppression of LG membrane potential during the interburst often disappeared (n = 13/17). The continued activation of a rhythm after DG removal confirms that DG does not become essential for rhythm generation as are Int1 and LG (Coleman and Nusbaum 1994; Nusbaum and Beenhakker 2002). The LG burst duration did not change after DG removal when DA is present (n = 8, 4.67 ± 1.10, DG removed 4.70 ± 1.23; P > 0.05), indicating that DG does not contribute to LG burst termination. The longer LG cycle period that occurs in the presence of DA when DG is active is due to the increased retraction phase. This demonstrates that DG slowed the onset of LG bursting.

It is possible that the activity of other circuit neurons contribute to alter DG actions in the DA modulated rhythm. However, as mentioned in the preceding text, during MCN1

![FIG. 7](image-url) Pyloric pacemaker influence on the gastric mill rhythm frequency is decreased with DA. A, left: MCN1 activates LG bursting that is time-locked with AB (dotted line). Right: suppression of AB by current injection (†, −2.5 nA) slows LG’s bursting frequency. B, left: applied DA slows the rhythm and increases LG’s burst duration (LG spikes during interburst with 10−7 M) and strengthens AB bursting. Right: suppression of AB slows LG bursting (†, −3.6 nA). C: average LG cycle frequency decreases when AB is hyperpolarized (** AB Hyp.). With DA present, the rhythm frequency decreases (Fig. 2D). Suppressing AB significantly decreased the frequency (**), and it differs from control with AB removed (Δ).

![FIG. 8](image-url) The removal of DG neuron activity increases gastric mill rhythm frequency with DA present. A: in control, DG hyperpolarization does not alter rhythm frequency (bar = 1 LG cycle, arrow −1.5 nA current injection DG). B: with DA applied and DG suppressed, the rhythm frequency increases (arrow −1.7 nA, bars indicate 1 LG cycle). C: with DA present, the average gastric mill rhythm frequency decreases (Fig. 2D). Frequency increases significantly when DG is suppressed (asterisk).
activation, DG does not receive direct synaptic input from other circuit neurons (Kirby and Nusbaum 2007; Nusbaum and Beenhakker 2002). The only other known DG synapse inhibits the GM neurons (Nusbaum and Beenhakker 2002), but again as mentioned in the preceding text, these neurons are not activated during the DA modulated rhythm. We cannot rule out an unexpected change in the circuit but direct DG inhibition of LG appears likely. Regardless of the participation of other neurons, these results show additional evidence for modulation of rhythm frequency that may contribute to the reduced impact of the intercircuit frequency regulation.

Because the DA modulated rhythm is slowed by DG activity, it was unclear whether experiments where we suppressed the pyloric pacemaker can clearly show the relative contribution of the pyloric circuit to regulate the gastric mill frequency. This led us to test the possibility that with DA and both DG and pyloric pacemaker inputs suppressed, the gastric mill CPG can generate rhythms with frequencies similar to typical rhythm frequencies.

During the control rhythm with the pyloric pacemaker input suppressed, we expected that the added removal of DG influences would have no effect on rhythm frequency (n = 15–17; average with pyloric pacemaker removed, 0.052 ± 0.011 Hz; average with DG and pyloric input removed, 0.056 ± 0.018 Hz, P > 0.05; repeated measures ANOVA). In both of these conditions, the rhythm frequencies are well below control (as in Figs. 7C and 9B). During DA modulation with the pyloric input suppressed, the added removal of DG significantly increased the rhythm frequency (Fig. 9, A and B; n = 15–17, repeated measure ANOVA, *P < 0.05). However, the rhythm frequency remained slower than the control frequency with both pyloric and DG inputs intact (Fig. 9B, 15 Hz MCN1 activation, n = 15–17, ΔP < 0.05, repeated measure ANOVA).

Notably, in conditions with both DG and pyloric pacemaker inputs removed, the DA modulated rhythm is much faster than control with these inputs removed (Fig. 9B; n = 15–17, **P < 0.008, repeated measures ANOVA). Activation of MCN1 at a lower frequency (10 Hz, with DG and pyloric inputs intact) elicited a rhythm that was significantly slower than the 15 Hz control rhythm (Fig. 9B, n = 17, ***P < 0.05). When compared with the rhythms generated with 10 Hz MCN1 activation frequency, DA modulated rhythms (15 Hz MCN1) without DG and pyloric inputs were similar (right white bar versus black stripe, Fig. 9B, n = 15–17, P > 0.05, repeated measures ANOVA). Thus with DA modulation present the gastric mill CPG can generate rhythm frequencies nearer to typical ranges without input from the pyloric pacemaker.

Overall these results show a contribution for pyloric pacemaker input to maintain typical gastric mill rhythm frequency but its impact is less with DA present. The increased influence of DG in the DA modulated rhythm demonstrates a shift of frequency control from intercircuit mechanisms to greater reliance on intracircuit mechanisms.

DISCUSSION

We have shown that modulation can change the relative frequencies of co-expressed motor patterns by acting on the discrete underlying circuits and by altering the influence of circuit interactions. Specifically, we show that with DA applied as a hormone, the frequencies of MCN1 elicited pyloric and gastric mill rhythms change in opposing directions. The frequency coupling changes between the rhythms, but the variability in latency is decreased, indicating tighter phase-locking. DA influences the activity patterns of key gastric mill neurons, and in the case of the DG neuron, it can participate in frequency regulation. With DA present the relative influence of pyloric pacemaker input is reduced, making gastric mill rhythm frequency control less dependent on intercircuit regulation and more on intracircuit control.

The effects of DA on this rhythm are similar to those of the peptide hormone CCAP (Kirby and Nusbaum 2007). Like CCAP, applied DA likely provides tonic excitation that enhances the phasic MCN1 excitation of LG (Delong et al. 2009b; Kirby and Nusbaum 2007). Similar to CCAP actions, DA likely has no influence on Int1 during MCN1 activation (Kirby and Nusbaum 2007), and this unbalanced LG excitation allows it to more effectively escape from Int1 inhibition. This prolongs the LG burst and the rhythm protraction phase. Unlike the CCAP modulated rhythm, DA upregulates DG activity to delay LG bursting and extend the retraction phase.
In all documented MCN1 elicited gastric mill rhythms, only Int1 and LG are essential for rhythm generation and other gastric mill neurons are followers (Beenhakker et al. 2005; Coleman and Nusbaum 1994; Kirby and Nusbaum 2007; Nusbaum and Beenhakker 2002; Saideman et al. 2007). We demonstrate that DA likely has direct actions on LG and DG but not on Int1. However, as we mentioned, it is possible that DA influences unexpected changes in the circuit (Ayali and Harris-Warrick 1999; Hooper and Marder 1987; Thirumalai et al. 2006). For example, it is difficult to test whether electrical coupling is modulated. Testing DA actions more fully on other STG circuit neurons may reveal other interesting circuit modifications.

It will be important to determine whether DA acts directly on MCN1. MCN1 axonal terminals in the STG are considered part of the gastric mill circuit (Bartos et al. 1999; Coleman et al. 1995). If DA enhances MCN1 transmitter release, we would expect rhythm frequency to increase as it does with increased MCN1 activation frequency (Bartos et al. 1999) (Fig. 9). When pyloric pacemaker activity and DG activity were removed in the presence of DA (Fig. 9), the resulting rhythm frequency resembled slower control rhythms with lowered MCN1 activation frequency. This suggests that DA may contribute to reduce MCN1 transmitter release. Application of $10^{-6}$ M DA can suppress LG bursting, and this also reflects possible DA inhibition of MCN1. In this condition, increasing MCN1 firing frequency can restore LG bursting, suggesting that DA suppresses MCN1 actions by increasing the threshold firing frequency for target neuron activation. However, pyloric rhythm activation is undiminished during LG suppression (Wood et al. 2006). Recent evidence supports that serotonin can selectively inhibit MCN1 actions on LG (Delong et al. 2009a), leaving the possibility that DA exerts a similar effect.

As previously shown, we found that higher gastric mill rhythm frequencies are associated with higher pyloric rhythm frequencies (Fig. 9A) (Bartos et al. 1999). These rhythms are latency locked but their coupling ratios vary between preparations (Bartos et al. 1999). Therefore we were not surprised that the correlation between rhythm frequencies is low but significant. This correlation remains significant with DA present; however, gastric mill rhythm frequencies tended to be slower relative to control with pyloric rhythm frequencies greater than the median (Fig. 9B). Gastric mill rhythm frequency depends also on the strength of the synaptic pathway from the pyloric pacemaker to Int1/LG (Fig. 1B) (Bartos et al. 1999). Whether reduced synaptic strength contributes to decrease gastric mill rhythm frequency remains to be tested. It was clear that pyloric pacemaker input to the gastric mill CPG remained important for latency coordination between DA modulated rhythms regardless of the degree of impact on frequency.

In all described gastric mill rhythms, depolarizations in LG during the interburst are mediated by pyloric pacemaker input (Figs. 1C and 7A) and by rhythmic excitations of LG by descending inputs. These depolarizations are necessary to maintain rhythm frequency (Bartos et al. 1999; Beenhakker et al. 2005; Blitz et al. 2008; Kirby and Nusbaum 2007; Saideman et al. 2007; Wood et al. 2004): absence of LG interburst depolarizations either eliminates the rhythm or greatly reduces its frequency ($\geq50\%$ reduction). As we show, with DG and pyloric inputs removed, the DA modulated rhythm frequency is much closer to a typical MCN1 rhythm frequency (Fig. 9). This differs from the CCAP modulated gastric mill rhythm where the rhythm without pyloric pacemaker input is further slowed by CCAP application (Kirby and Nusbaum 2007).

During MCN1 activation with DA present, the pyloric rhythm frequency is increased when gastric mill circuit influences are removed (Fig. 4). Because the pyloric rhythm frequency depends on gastric mill rhythm phase, DA influences were unapparent on the rhythm frequency averaged over both phases. With DA present, many preparations displayed a greater decrease in the first pyloric rhythm cycle duration during LG bursting (Fig. 5). This suggests that LG inhibition of MCN1 transmitter release is stronger than in control, and this could be directly tested. More importantly, we show that DA can influence MCN1 elicited rhythm frequency independently of gastric mill circuit interactions. In lobsters, applied DA has distinct effects on pyloric neuron subtypes but has no net effect on spontaneous pyloric network frequency (Ayali and Harris-Warrick 1999). Threshold DA concentrations for these effects ($10^{-5}$ to $10^{-4}$ M ) are much higher than we used, but in other preliminary work, we found that higher DA concentrations increased spontaneous rhythm frequency in crabs ($10^{-5}$ to $10^{-4}$ M ) (Wood et al. 2006). These results suggest underlying differences in DA actions between these species, making comparisons unclear.

In this system, DA actions on currents in gastric mill neurons are undescribed, but DA affects different combinations of several identified currents on pyloric neuron subtypes (Harris-Warrick et al. 1998; Marder and Bucher 2007). Despite complex effects for DA on multiple currents (Harris-Warrick et al. 1998; Marder and Bucher 2007), circuit level changes can become apparent from DA actions on an individual current such as $I_h$ (Peck et al. 2006). These issues can be investigated in the crab, but given that responses to similar DA concentrations are different between these species, it is unclear whether $I_h$ modulation will have a similar result.

MCN1’s peptide cotransmitters are known to activate a nonselective cationic current in pyloric and gastric mill neurons (Delong et al. 2009b; Golowasch and Marder 1992; Swensen and Marder 2000). This current appears distinct from those modulated by DA. Our preliminary studies show that MCN1 does not contain DA (Wood, unpublished data), suggesting that MCN1 actions and DA actions on currents are distinct. In the CCAP modulated MCN1 elicited rhythm, enhanced protraction with decreased retraction occurs because CCAP tonically increases the peptide activated current in the LG neuron (Delong et al. 2009b). Whether the actions of MCN1 cotransmitters are entirely parallel or overlap with DA actions remains to be determined.

The notable DA concentration dependent ($10^{-6}$ M) effects that weaken LG bursting are not likely due to altered interactions with DG and Int1 (Fig. 3). It appears unlikely that DA enhances Int1 inhibition of LG. Similarly, the modest concentration dependent increases in DG activity likely do not contribute to reduce LG burst duration. The DG burst onset is not phase advanced by $10^{-6}$ M DA and remains similar to that with lower concentrations applied (Fig. 3B) (Wood, unpublished observations). It remains to be seen if DA actions on MCN1 and directly on LG can account for the concentration dependent weakening of LG bursting.

There is good evidence in several decapod crustaceans that DA is available as a hormone (Cooke and Goldstone 1970;
Mechanosensory activation of a motor

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