Recruitment of a Projection Neuron Determines Gastric Mill Motor Pattern Selection in the Stomatogastric Nervous System of the Crab, *Cancer borealis*

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SUMMARY AND CONCLUSIONS

1. In the isolated stomatogastric nervous system of the crab *Cancer borealis* (Fig. 1), the muscarinic agonist oxotremorine elicits several distinct gastric mill motor patterns from neurons in the stomatogastric ganglion (STG; Fig. 2). Selection of a particular gastric mill rhythm is determined by activation of distinct projection neurons that influence gastric mill neurons within the STG. In this paper we identify one such neuron, called commissural projection neuron 2 (CPN2), whose rhythmic activity is integral in producing one form of the gastric mill rhythm.

2. There is a CPN2 soma and neuropilar arborization in each commissural ganglion (CoG). The CPN2 axon projects through the superior esophageal nerve (son) and the stomatogastric nerve (stn) to influence neurons in the STG (Figs. 3 and 4A).

3. CPN2 activity influences most of the gastric mill neurons in the STG. Specifically, CPN2 excites gastric mill neurons GM and LG (gastric mill and lateral gastric, respectively) and inhibits the dorsal gastric (DG), anterior median (AM), medial gastric (MG), and inferior cardiac (IC) neurons (Figs. 5 and 6). CPN2 also indirectly inhibits gastric mill neurons Int1 and VD (interneuron 1 and ventricular dilator neuron, respectively) through its activation of LG. The CPN2 excitatory effects are mediated at least partly via discrete excitatory postsynaptic potentials (EPSPs; Fig. 4B), whereas its inhibitory effects are produced via smooth hyperpolarizations.

4. Within the CoG, CPN2 receives excitatory synaptic input from the anterior gastric receptor neuron (AGR), a gastric mill proprioceptive sensory neuron (Fig. 7) and inhibitory synaptic input from the gastric mill interneuron, Int1 (Fig. 8).

5. During one form of the gastric mill rhythm, CPN2 fires rhythmically in time with the gastric mill motor pattern, whereas it is silent or fires weakly during other gastric mill rhythms (Fig. 9).

6. When CPN2 rhythmic activity is suppressed during a CPN2-influenced gastric mill rhythm, the gastric mill rhythm continues, but the pattern is altered (Fig. 10). Moreover, transiently stimulating CPN2 during any ongoing gastric mill motor pattern can reset the timing of that rhythm (Fig. 11).

7. Tonic activity in CPN2 is insufficient to elicit a gastric mill rhythm (Fig. 12). Phasic activity in CPN2 can elicit a gastric mill rhythm only in preparations in which gastric mill neurons are already in an excited state (Figs. 12 and 13).

8. CPN2 recruitment plays a pivotal role in determining the final form of the gastric mill rhythm. Moreover, its patterned influence on this rhythm, as well as its ability to reset ongoing gastric mill rhythms, shows it to be a member of the underlying neural network that generates this motor pattern (Fig. 14).

INTRODUCTION

The output of rhythmically active neural networks is modified by neurons projecting from other regions of the nervous system (Grillner and Matsushima 1991; Harris-Warrick and Marder 1991; Steriade et al. 1993). This modification of rhythmic neural activity is often accomplished through modulation of the membrane properties of the network neurons and/or modification of the synaptic strengths between these neurons (Dekin et al. 1985; Dickinson and Moulins 1992; Harris-Warrick and Marder 1991; Harris-Warrick et al. 1992; Marder and Weimann 1992; Ramirez and Pearson 1991; Sigvardt et al. 1986; Steriade et al. 1993).

The rhythmic output of these neural networks can also be modified by recruitment of previously silent neurons with synaptic connections to the active network neurons. This recruitment changes one or more aspects of the ongoing motor pattern. For example, in the marine mollusk *Clione limacina*, activation of a projection neuron in the pleural ganglion alters the ongoing swimming motor pattern in the pedal ganglion from a weak to a more vigorous rhythm (Arshavsky et al. 1985a,b, 1989). In *Xenopus* embryos, swimming frequency is related to the number of premotor interneurons that fire action potentials during an episode of swimming (Sillar and Roberts 1993). Sensory stimulation increases the rate of swimming by recruiting additional interneurons to participate in generating the swim rhythm. For both of these systems, neuronal recruitment produces a faster and more vigorous rhythm, but with little change in the phase relationships of the rhythmically active neurons.

The stomatogastric nervous system (STNS) is an ideal system for studying the modification of patterned motor outputs. The STNS includes the paired commissural ganglia (CoGs), the esophageal ganglion (OG), and the stomatogastric ganglion (STG), plus their connecting and motor nerves. These ganglia contain a set of well-characterized neural networks that produce the rhythmic motor patterns controlling the movements of the striated muscles of the crustacean foregut. The two best characterized motor patterns in this system are the gastric mill and pyloric rhythms. The neuronal components that generate these two rhythms include most of the 30 neurons in the STG (Harris-Warrick et al. 1992; Selverston and Moulins 1987). In the crab STG there is considered to be a single neural network underlying both the pyloric and gastric mill rhythms because there is...
The STG neural network is strongly influenced by modulatory transmitters that are released from neurons that project to the STG from the CoGs and OG, as well as from the periphery (Coleman et al. 1992; Harris-Warrick et al. 1992; Marder and Weimann 1992). These modulatory inputs influence many aspects of the STG motor patterns, including cycle frequency, phase relationships, and the intensity of activity in individual pyloric and gastric mill components. Additionally, some modulatory inputs cause STG network neurons to functionally switch between different motor patterns (Dickinson and Moulin 1992; Hooper and Moulin 1989, 1990; Weimann et al. 1993), or cause a fusion of previously separate neural networks to produce novel motor patterns (Dickinson et al. 1990; Meyrand et al. 1991, 1994).

The gastric mill rhythm is particularly reliant on modulatory influences. In the isolated crab STNS the gastric mill rhythm is rarely spontaneously active and, when it is active, the rhythm stops when inputs from the CoGs and OG are removed. Exogenous application of any one of several modulatory transmitters, in vitro and in vivo, can elicit a gastric mill rhythm or modify an ongoing rhythm in distinct ways (Dickinson et al. 1990; Elson and Selverston 1992; Heinzel 1988b; Heinzel and Selverston 1988; Heinzel et al. 1993b; Marder et al. 1986; Turrigiano and Selverston 1989; Weimann et al. 1993). Several projection neurons that influence the gastric mill rhythm have also been identified in different species (Coleman et al. 1993; Harris-Warrick et al. 1992; Norris et al. 1994; Nusbaum et al. 1992).

In this paper, we show that the gastric mill rhythm can be changed not only by modifying the membrane properties and/or synaptic interactions of STG neurons, but also by recruitment of a newly identified projection neuron, called commissural projection neuron 2 (CPN2; previously called MCN2) (Norsin et al. 1993), which synthetically influences many of the gastric mill neurons in the STG. Because the gastric mill rhythm is rarely spontaneously active in the isolated crab STNS, we elicited gastric mill rhythms by bath applying the muscarinic agonist oxotremorine. The modulatory effects of muscarinic agonists on the STG have been reported previously (Bal et al. 1994; Elson and Selverston 1992; Marder and Paupardin-Tritsch 1978). We found that, in the isolated STNS of the crab Cancer borealis, oxotremorine elicits three distinct gastric mill rhythms. The generation of one of these rhythms required the rhythmic activation of CPN2. CPN2 activity was necessary, but not sufficient, to elicit this rhythm. In addition to synthetically influencing many gastric mill neurons, CPN2 also fulfilled the classic criteria for inclusion in the neural network underlying generation of the gastric mill rhythm. Some of this work was published previously in abstract form (Norris et al. 1993).

**METHODS**

**Animals**

*C. borealis* were obtained commercially from Neptune Seafood and Lobster (Boston, MA) and the Marine Biological Laboratory (Woods Hole, MA). Crabs were maintained in aerated, filtered, artificial sea water aquaria at 10–13°C. Data were obtained from 78 male crabs weighing between 300 and 800 g. Animals were cold anesthetized by packing in ice for 20–40 min before dissection. The stomach was then removed and kept in chilled physiological saline (~4°C) while the STNS was dissected away from the rest of the stomach.

**Solutions**

*C. borealis* physiological saline contained (in mM) 440 NaCl, 26 MgCl₂, 13 CaCl₂, 11 KCl, 10 Trizma base, and 5 maleic acid, pH 7.4–7.5. All experiments involving superfusion of oxotremorine saline were done with the use of 10⁻⁴ M oxotremorine sesquifumarate (Sigma) dissolved in standard saline. Oxotremorine saline was prepared immediately before it was used.

**Anatomy**

Intracellular Lucifer yellow (LY: Lucifer yellow-CH, Sigma) dye injections were performed as described in Nusbaum et al. (1992). Electrode tips contained 5% LY in distilled water while the shaft was backfilled with 0.1 M LiCl, with an air bubble separating the two solutions. Electrode resistances were 20–50 MΩ. LY dye-fills were amplified with the use of anti-LY antiserum provided by J. Kuwada (Dept. of Biology, Univ. Michigan, Ann Arbor). Anti-LY was used at a final dilution of 1:2,000. Antibody staining was processed as described in Coleman et al. (1992). Immunolabeling was visualized with the use of a fluorescein-conjugated goat anti-rabbit secondary antiserum (Calbiochem). The preparation was mounted on a microscope slide in 80% glycerol and 20% Na₂CO₃ (20 mM). Preparations were viewed and images collected with the use of a Biorad MRC 600 laser scanning confocal microscope equipped with a krypton/argon mixed gas laser. Micrographs were printed with the use of a Sony Mavigraph color video printer.

**Electrophysiology**

All electrophysiological experiments were performed on the isolated STNS (Fig. 1). Each preparation was continuously superfused (7–12 ml/min) with physiological saline (10–13°C). Intracellular and extracellular recordings were made with the use of standard techniques for this system as described in Nusbaum et al. (1992). The CoGs and STG were desheathed and viewed with light transmitted through a darkfield condenser (Nikon) to facilitate intracellular recordings. Intracellular recordings were made with the use of microelectrodes containing 4 M potassium acetate plus 20 mM KCl (15–40 MΩ). All intracellular current injections were made via explicitly unbalanced bridge circuits. Data were collected by chart recorder (Astromed MT-95000) and videotape (Vetter Instruments). Figures were made by scanning data into a graphics program (CorelDraw, version 3.0) with the use of a Scan- jet IIE (Hewlett Packard). Data were acquired from 45 intracellular recordings of CPN2.

Individual STG neurons were identified by their axonal pathways, activity patterns, and interactions with other neurons (Coleman and Nusbaum 1994; Nusbaum et al. 1992; Weimann et al. 1991, 1993). In extracellular recordings of the inferior cardiac (IC) and ventricular dilator (VD) neurons within the medial ventricular nerve (mvn), the IC action potentials were often, but not always, smaller than those of VD. Thus, in all experiments, IC and VD were identified in mvn recordings on the basis of their pyloric-timed impulse bursts. The pyloric-timed activity of VD occurs during the bursts in the pyloric (PY) constrictor neurons and the pyloric-timed bursts in interneuron 1 (Int1) (Heinzel et al. 1993b; Weimann et al. 1991). The pyloric-timed IC neuron bursts overlap with those of the lateral pyloric (LP) constrictor neurons as...
GASTRIC MILL MOTOR PATTERN SELECTION

FIG. 1. Schematic illustration of the stomatogastric nervous system (STNS), including the CPN2 projection pattern. Note that CPN2 arborizes in both the CoG and the STG. Stippling indicates the neuropil region of each ganglion. CoG, commissural ganglion; CPN2, commissural projection neuron 2; dgn, dorsal gastric nerve; dvn, dorsal ventricular nerve; ion, inferior esophageal nerve; lvn, lateral ventricular nerve; mvm, medial ventricular nerve; OG, esophageal ganglion; son, superior esophageal nerve; STG, stomatogastric ganglion; stn, stomatogastric nerve.

well as the pyloric-timed bursts in the lateral gastric (LG) and medial gastric (MG) neurons (Heinzel et al. 1993b; Weimann et al. 1991). During every experiment there was an ongoing pyloric rhythm, and the activity in the LP and PY neurons was recorded extracellularly in the dorsal ventricular nerve (dvn) or lateral ventricular nerve (lvn), where their activity is readily distinguishable (not shown) (Heinzel et al. 1993b; Nusbaum et al. 1992; Weimann et al. 1993).

Data analysis

Gastric mill cycle periods were defined as extending from the onset of an impulse burst in the LG neuron to the onset of the subsequent LG burst. Cycle frequency was calculated as the reciprocal of the cycle period. Individual data points of all gastric mill rhythm parameters involved determination of means from 5–10 consecutive cycles of activity during ongoing gastric mill rhythms. Data are presented as means ± SD. Statistical analyses consisted of unpaired and paired Student's t tests that were performed with the use of SigmaPlot for Windows, version 1.0 (Jandel Scientific).

RESULTS

Oxotremorine elicits three distinct gastric mill rhythms

In our preparations, oxotremorine elicited three distinct gastric mill motor patterns (Fig. 2). In 26% of these preparations (10 of 38), the gastric mill rhythm shown in Fig. 2A was produced. This rhythm was characterized by alternating bursts in the LG and dorsal gastric (DG) neurons. In some preparations, the DG bursts partly overlapped with the onset of each LG burst. In addition, the IC neuron fired strongly and was coactive with LG, whereas the gastric mill (GM) neuron was at best only weakly active. The MG neuron was also rhythmically active with the LG and IC neurons (not shown; see Fig. 9A). In another 47% of these preparations (18 of 38), the gastric mill rhythm shown in Fig. 2B was elicited. During this form of the rhythm, LG and DG activity was similar to that in Fig. 2A, although there was generally less overlap between their bursts. IC and MG fired vigorously at the onset of each LG burst, but their activity would then abruptly stop while the LG burst continued (MG recording not shown; see Fig. 9B). GM neuron activity was inhibited at the start of each LG burst by IC activity (Weimann 1992) (see also Fig. 2), but GM became coactive with LG once IC activity ceased. Both of these gastric mill rhythms were also sometimes expressed spontaneously in the STNS during saline superfusion.

In four additional preparations (11%), oxotremorine elicited a gastric mill rhythm that was quite different from the previous two rhythms. This rhythm was characterized by the coactivity of the IC, LG, and DG neurons, and their alternation with GM neuron bursts (Fig. 2C). This rhythm was strongly influenced by the vigorous activity in the IC neuron (Coleman et al. 1993). GM neuron activity was restricted to firing primarily in alternation with IC, LG, and DG because GM was inhibited by the enhanced activity in IC. In this third rhythm, DG generally exhibited less intense bursts. In the final 16% of the preparations (6 of 38) in which oxotremorine elicited a gastric mill rhythm, the preparation first produced one of the patterns described above and then switched to another of these patterns.

In addition to differences in IC, MG, and GM activity, there were other differences that distinguished the gastric mill rhythms in Fig. 2, A and B. For example, the rhythm exemplified by Fig. 2H tended to include more vigorous impulse bursts in the LG neuron. Thus the LG firing frequency within each burst was significantly faster during these rhythms (17.1 ± 4.8 Hz, mean ± SD) than during those exemplified by Fig. 2A (11.5 ± 6.8 Hz; P < 0.05). The rhythm shown in Fig. 2B also tended to have a longer cycle period (10.0 ± 2.7 s; n = 15) than the rhythm shown in Fig. 2A (8.7 ± 2.7 s; n = 10), although the difference was not statistically significant (P > 0.05). The lack of difference between the cycle period in these two types of gastric mill rhythm appeared to be due to considerable variability between different preparations. To test this possibility, we compared the cycle period in seven preparations where the gastric mill rhythm switched between these two patterns, either spontaneously or through experimental manipulations (c.g., Fig. 2, A and B; see also Fig. 10). In all seven of these preparations, the gastric mill cycle period was consistently, and significantly, longer during the rhythm exemplified by Fig. 2B (9.8 ± 2.6 s vs. 7.0 ± 2.4 s; P < 0.01).

As described below, expression of the gastric mill rhythm shown in Fig. 2B resulted primarily from the recruitment of a CoG projection neuron that we have recently identified, called CPN2.

Identification and localization of CPN2

The CPN2 soma was commonly located on the dorsal surface of the CoG, close to the anterior or lateral side of the L neuron. The L neuron is a previously identified neuron with the largest soma in the CoG (Robertson and Moulins 1981). As shown schematically in Fig. 1, CPN2 arborizes in
Oxotremorine (10^{-3} M) elicits several distinct gastric mill rhythms in the isolated STNS. A: during this form of the gastric mill rhythm, the lateral gastric neuron (LG) and dorsal gastric neuron (DG) fire in alternation, although there is some overlap between the end of each DG burst and the onset of each LG burst. The inferior cardiac neuron (IC; mvn: smaller unit) is coactive with LG, and the gastric mill neuron (GM) is inactive. B: in this gastric mill rhythm, LG and DG again fire in alternation, but with less overlap than in (A). Additionally, IC is inhibited during most of each LG burst, and GM is coactive with LG. C: in this gastric mill rhythm, IC, LG, and DG are coactive and fire in alternation with GM. The small-amplitude, depolarizing events in the GM recordings in A and B are excitatory postsynaptic potentials (EPSPs). Recordings in A and B are from the same preparation.

the CoG and projects an axon to the STG via the superior esophageal nerve (son) and the stomatogastric nerve (stn). LY dye-fills of CPN2 within the CoG showed that the CPN2 soma was connected by a fine process to a larger diameter neurite within the CoG neuropil (Fig. 3). Its arborization within the CoG originated entirely from this enlarged neurite. The CPN2 soma diameter was \(~40\) \(\mu\)m. We confirmed, electrophysiologically, that the CPN2 axon projected through the son and stn to innervate the STG by eliciting action potentials in the CPN2 soma and recording their time-locked presence first in the son, and then in the stn (Fig. 4 A). We never found more than one CPN2 in any CoG.

**Synaptic effects of CPN2 in the STG**

The gastric mill rhythm controls the movements of the three teeth in the crustacean stomach, including a single pair of lateral teeth and an unpaired medial tooth. During gastric mill rhythms, the lateral teeth commonly move together to the center of the stomach to hold a piece of food, and the medial tooth swings down from its position in the dorsal stomach to rasp the food particle (Hartline and Maynard 1975; Heinzel 1988a,b). The neurons that control the movements of the teeth are functionally subdivided into the medial and lateral tooth subsystems.

In the STG, CPN2 activity influenced nearly all of the gastric mill neurons. The medial tooth subsystem includes four functionally equivalent GM neurons whose activity moves the medial tooth forward and down (protraction) (Heinzel et al. 1993b). There is also a single DG neuron whose activity retracts the medial tooth. Intracellular stimulation of CPN2 excited the GM neurons and inhibited DG (Figs. 4 B and 5). The excitation of GM was accomplished at least partly via discrete excitatory postsynaptic potentials (EPSPs; Fig. 4 B). No PSPs were recorded during the CPN2 inhibition of DG. Instead, DG exhibited a smooth hyperpolarization (Fig. 5 A). We performed no tests to determine whether the CPN2-mediated inhibition of DG was direct, but we recorded a strong inhibitory response in every preparation in which we stimulated CPN2 while recording from...
the DG neuron \((n = 21)\). The CPN2 inhibition of DG was not mediated through activation of either the GM neurons or any other STG neuron, because none of these neurons synaptically inhibit DG (Coleman and Nusbaum 1994; Weimann 1992). The GM neurons have no neurotransmitter-mediated effects within the STG, although they are weakly electrically coupled to the lateral teeth protractor neurons (Weimann 1992; unpublished observations). The only documented synaptic output of DG within the crab STG is its inhibitory effect on the GM neurons (Fig. \(5 \, B\)) (Coleman and Nusbaum 1994; Weimann 1992; unpublished observations). The anterior median (AM) motor neuron, although it does not innervate any medial tooth-related muscles, commonly bursts in time with \(\text{DG}\) during gastric mill rhythms. We found that CPN2 activity also caused a smooth inhibition of AM \((n = 4\); data not shown). The effects of CPN2 on the neurons controlling movements of the lateral teeth are shown in Fig. 6. Activity in the LG and MG neurons cause the lateral teeth to move together (protraction), whereas separation of these teeth (retraction) occurs either passively or is facilitated by the two lateral posterior gastric (LPG) neurons (Heinzel et al. 1993b). Additionally, the IC neuron is coactive with the lateral teeth protractor neurons, and vigorous IC neuron activity causes a forward thrusting of the lateral teeth and protracts the small, lateral accessory teeth. As shown in Fig. 6, CPN2 had opposite effects on the lateral teeth protractor neurons because it excited LG \((n > 40)\) and inhibited MG \((n = 24 \, \text{of} \, 26)\). CPN2 activity also inhibited the IC neuron \((n = 40 \, \text{of} \, 43)\). As was the case in the medial tooth subsystem, the CPN2 excitation of LG was mediated at least partly via discrete EPSPs (Fig. 4 \(B\)), whereas its inhibitory effects on MG and IC showed no evidence of discrete PSPs (Fig. 6 \(A\)). The CPN2 inhibition of MG and IC was not mediated via its excitation of LG, because the LG neuron does not inhibit either of these neurons (Weimann 1992). Moreover, CPN2 stimulation still inhibited MG and IC during times when LG was not activated. CPN2 had no observable effect on the LPG neurons. However, the LPG neurons never participated in the gastric mill rhythms that occurred in these experiments (see also Weimann et al. 1991).

Although the MG and IC neurons were commonly inhibited by CPN2 activity, in a few preparations CPN2 did not inhibit one or the other of these neurons \((n = 5)\). Instead, that neuron was either excited or else displayed no change in activity. In an additional three preparations, CPN2 stimulation initially inhibited IC, but during subsequent CPN2 stimulations IC was excited. The cause of this lack of inhibition was unclear, because in these cases CPN2 still inhibited both DG and either IC or MG. However, the CPN2 excitation of MG and IC may have resulted from the lack of inhibition enabling LG to enhance MG and/or IC activity, because these three neurons are electrically coupled (Fig. 6 \(B\)) (Weimann 1992; unpublished observations). MG appears to have no neurotransmitter-mediated effects within the crab STG (Weimann 1992; unpublished observations).

The sole STG interneuron in the gastric mill system, Int1, fires in alternation with the lateral teeth protractor neurons during gastric mill rhythms. Int1 is reciprocally inhibitory with LG, and it also inhibits the MG and IC neurons (Meyrand et al. 1992; Weimann 1992; unpub-
lished observations). CPN2 had no direct effect on Int1, but it indirectly inhibited Int1 via its excitation of LG. Thus we found that when LGi was hyperpolarized by current injection, CPN2 activity had no effect on Int1. Finally, the VD neuron was also inhibited by CPN2 activity, as a result of the CPN2 activation of LG (Weimann 1992; unpublished observations), and when we hyperpolarized LG, CPN2 no longer influenced VD neuron activity. VD is coactive with Int1, and its gastric mill–timed activity retracts the small accessory teeth (Heinzel et al. 1993b). CPN2 activity only influenced STG neurons that participated in gastric mill rhythms. Thus we found no response to CPN2 activity in either the pyloric pacemaker neurons AB and PD (anterior burster neuron and pyloric dilator neuron, respectively) or the pyloric constrictor neurons LP and PY.

Gastric mill input to CPN2 in the CoG

CPN2 received excitatory input in the CoG from the anterior gastric receptor (AGR) sensory neuron. As was originally documented in the European lobster, Homarus gammarus (Simmers and Moulins 1988a,b) and in the California spiny lobster, Panulirus interruptus (Elson et al. 1994), the AGR soma is commonly located either at the posterior end of the STG or in the dvn. Also as in these lobsters, AGR has neither a neuropilar arborization nor direct synaptic effects within the crab STG. Instead, AGR projects via the stn and sons (or, in the lobsters, the stn and inferior esophageal nerve (ions)) to the CoGs, where it excites neurons that in turn project to the STG and influence STG neurons (Coleman et al. 1992; Combes et al. 1993; Elson et al. 1994; Simmers and Moulins 1988a,b). Figure 7A shows that AGR stimulation in the crab STNS produced a vigorous excitatory response in CPN2. This led to an excitation of LG and GM, and an inhibition of IC and VD (Fig. 7A). These responses in the STG appeared to be mediated entirely by the AGR excitation of CPN2, because when CPN2 was hyperpolarized by current injection, AGR stimulation had little effect on the STG neurons (n = 4; Fig. 7B). In these experiments the contralateral son was transected to eliminate the participation of the contralateral CPN2. We saw discrete EPSPs in CPN2 that were time locked to every AGR action potential in only some preparations. This variability may have been due to the filtering characteristics of the thin neurite connecting the CPN2 soma to its neuropilar processes (Fig. 3).

In addition to receiving excitatory input in the CoG from AGR, CPN2 is inhibited in the CoG by Int1 (n = 8). For example, at times when oxotremorine superfusion did not elicit a gastric mill rhythm but did enhance STG neuron activity, suppression of Int1 activity by hyperpolarizing current injection increased tonic CPN2 activity and activated LG (Fig. 8A). LG activation resulted both from its release from Int1-mediated inhibition and from the in-
FIG. 8. Gastric mill interneuron (Int1) inhibits CPN2 within the CoG. A: during oxotremorine (10^{-5} M) superfusion, Int1 exhibited vigorous, pyloric rhythm-timed activity. Suppressing Int1 activity (-3.5 nA; arrowheads) increased activity in both CPN2 and LG, whereas releasing Int1 from hyperpolarization again reduced CPN2 and LG activity. B: during oxotremorine (10^{-7} M) superfusion, CPN2 exhibits postinhibitory rebound (PIR) after inhibition from Int1. Intracellular stimulation of Int1 (+5 nA; arrowheads) inhibited CPN2 and LG. Termination of Int1 stimulation caused CPN2 and LG to rebound and fire long-duration burst of action potentials. C: schematic representation of the synapses between CPN2, LG, and Int1. The Int1 synapse onto CPN2 occurs within the CoG, whereas all other illustrated synapses occur within the STG. Synapses labeled as in Fig. 5. Membrane potentials: A: Int1, -52 mV (most hyperpolarized potential); CPN2, -44 mV; LG, -80 mV (most hyperpolarized potential). B: most hyperpolarized potentials: Int1, -50 mV; CPN2, -69 mV; LG, -69 mV. Recordings in A and B are from different preparations.

creased excitatory input that LG received from CPN2 (Fig. 8C). This enhanced activity in both CPN2 and LG was again diminished when Int1 was released from hyperpolarization. In addition, the Int1 inhibition of CPN2 could prime CPN2 to fire a long-duration burst via postinhibitory rebound (PIR; n = 4), as shown in Fig. 8B. When Int1 was stimulated to fire strongly, during oxotremorine superfusion, it inhibited both LG and CPN2. When Int1 stimulation was terminated, CPN2 rebounded from inhibition to fire an intense, long-duration burst. LG also rebounded from inhibition to fire a burst of action potentials, which was enhanced by the synaptic excitation it received from CPN2. The LG inhibition of Int1 caused the hyperpolarization that occurred in Int1. In normal saline, CPN2 only occasionally exhibited a strong rebound burst when released from Int1 inhibition. We also found that CPN2 routinely exhibited strong rebound bursts after hyperpolarizing current injection in the presence of oxotremorine, but not during saline superfusion (data not shown). Figure 8C shows, schematically, the connectivity of Int1, LG, and CPN2.

As is also evident in Fig. 8B, in some preparations CPN2 received rhythmic inhibition that was time locked to the impulse bursts of the pyloric rhythm pacemaker neurons. This pyloric-timed input was particularly pronounced during oxotremorine superfusion, although in some recordings it was also evident during saline superfusion.

CPN2 influence on the gastric mill rhythm

The synaptic effects of CPN2 made it an ideal candidate to participate in generating the gastric mill rhythm shown in Fig. 2B. Therefore we studied the activity of CPN2 during the production of gastric mill rhythms. Figure 9, A and B, shows intracellular recordings of CPN2 activity during gastric mill patterns similar to those shown in Fig. 2, A and B, respectively. During all eight preparations in which we recorded CPN2 activity during the rhythm illustrated in Figs. 2A and 9A, CPN2 was at best only weakly active and had little if any influence on the gastric mill rhythm. As a result, the MG and IC neurons were inactive with LG. Figure 9B shows the rhythm we expected would result from the participation of CPN2. As predicted, MG and IC were inhibited throughout most of the LG burst, and CPN2 was rhythmically active with the LG neuron (n = 13). CPN2 did not participate in the rhythm shown in Fig. 2C (data not shown; n = 2).

To show that CPN2 was participating in the generation of the gastric mill rhythms exemplified by Fig. 9B, and was not simply being driven by gastric mill neurons, we suppressed CPN2 activity by hyperpolarizing current injection (n = 6; Fig. 10). In this experiment bath-applied oxotremorine elicited a gastric mill rhythm in which CPN2 was firing gastric mill-timed bursts (Fig. 10A). When CPN2 activity was suppressed, the gastric mill pattern switched from the CPN2-influenced rhythm to a rhythm like that shown in Figs. 2A and 9A (Fig. 10B). This changed the timing and intensity of activity in the MG, IC, and GM neurons. Thus hyperpolarization of CPN2 eliminated its inhibition of IC and MG, enabling their activity to persist during the LG neuron impulse bursts (MG recording not shown). The CPN2 excitation of GM was also removed, which resulted in a substantial decrease in GM neuron activity (not shown). As is evident in Fig. 10, hyperpolarization of CPN2 also increased the gastric mill cycle frequency and decreased both the LG burst duration and its firing frequency within each burst.

We considered the possibility that CPN2 was a member
of the gastric mill pattern-generating network because it exhibited synaptic interactions with gastric mill neurons, it showed rhythmic activity that was time locked to one form of the gastric mill rhythm, and it significantly altered the speed and pattern of this rhythm. To test this possibility, we determined whether manipulating the CPN2 membrane potential could reset the time of onset of each subsequent cycle during ongoing gastric mill rhythms (n = 6). Thus, during stable CPN2-influenced gastric mill rhythms, we either depolarized CPN2 to fire before its next expected burst or hyperpolarized it to suppress its next burst. In the example shown in Fig. 11, CPN2 stimulation prematurely activated LG and GM, prematurely terminated Int1 activity, and eliminated the expected DG neuron burst. This CPN2 stimulation also reset the time of onset of subsequent cycles, as is indicated in Fig. 11 by the filled circles. The filled circles during and after CPN2 stimulation indicate the expected time of LG burst onset in the absence of any perturbation. These times were determined by averaging the time between the onset of consecutive LG bursts for 10 cycles immediately before CPN2 stimulation. Although the onset of each gastric mill cycle occurred earlier then expected after CPN2 stimulation, the gastric mill cycle frequency was unchanged. Altering CPN2 activity reset the gastric mill rhythm in all six preparations tested. We also found that CPN2 stimulation could reset ongoing gastric mill rhythms in which CPN2 was not participating (data not shown).

Given the importance of CPN2 in determining the form and frequency of the gastric mill rhythm in which it participates, we wanted to test whether activating CPN2 was sufficient to elicit this rhythm. Furthermore, we wanted to test whether rhythmic activity in CPN2 was important for generating a CPN2-influenced rhythm. We found that, in the isolated STNS superfused with normal saline, tonic stimulation of CPN2 never elicited a gastric mill rhythm (n = 43). In similar situations, other COG projection neurons do elicit gastric mill rhythms (Coleman and Nusbaum 1994; Coleman et al. 1993; Norris et al. 1994; Nusbaum et al. 1992). We next examined the ability of CPN2 to elicit a gastric mill rhythm during oxotremorine superfusion when gastric mill neuron activity was enhanced but no gastric mill rhythm was in progress. Because oxotremorine also increased tonic activity in CPN2, we
Neither tonic nor rhythmic activity in CPN2 is sufficient to elicit a gastric mill rhythm. The preparation was superfused with oxotremorine ($10^{-5}$ M), which failed to elicit a gastric mill rhythm. Because oxotremorine excited CPN2, its activity was suppressed by hyperpolarizing current injection (1.5 nA: black line). A: when CPN2 was released from hyperpolarization and fired tonically, LG produced gastric mill-like bursts, but DG (dgn) was tonically inhibited. B: when CPN2 was released from hyperpolarization to fire in gastric mill-like bursts, alternation in activity occurred between LG and DG. DG, however, did not burst as strongly as it normally does during CPN2-influenced gastric mill rhythms. Membrane potentials: CPN2, -57 mV; LG, -69 mV (most hyperpolarized potential).

Under the appropriate conditions, it was not necessary to directly stimulate CPN2 to evoke a CPN2-influenced gastric mill rhythm. For example, in some preparations where gastric mill neuron activity was enhanced, gastric mill-time pulses in either AGR (not shown) or Int1 were sufficient to elicit a CPN2-influenced gastric mill rhythm ($n = 5$). In Fig. 13B, rhythmic activation of Int1 caused rhythmic PIR bursts in CPN2, enabling CPN2 to rhythmically influence its gastric mill targets. Although vigorous activity in Int1 was able to drive CPN2 bursting via PIR, Int1 activity was not necessary for CPN2 to burst in time with the gastric mill rhythm. Thus, in two preparations superfused with oxotremorine, rhythmic bursting persisted in CPN2 even though Int1 was either weakly active or was hyperpolarized.
AGR. AGR is activated by contraction of a medial tooth protractor muscle innervated by the GM neurons (Heinzel et al. 1993b). In intact animals CPN2 would be recruited to participate in gastric mill rhythms via the gastric mill-timed excitatory input that it receives from these times or instead is synaptically driven in a rhythmic fashion by an unidentified input remains unresolved. It may be that CPN2 is activated to participate in gastric mill rhythms as a result of input it receives from other CoG neurons, some of which are known to activate gastric mill rhythms in the crab (Coleman and Nusbaum 1994; Coleman et al. 1993; Nusbaum et al. 1992).

In the isolated STNS, AGR activity is both weak and tonic. As a result, there must be another mechanism(s) underlaying the rhythmic activation of CPN2 during gastric mill rhythms in vitro. One candidate for this role is the rhythmic inhibition that CPN2 receives from Int1, which can cause postinhibitory rebound bursts in CPN2. Whether CPN2 also exhibits endogenous oscillatory activity during these times or instead is synaptically driven in a rhythmic fashion by an unidentified input remains unresolved. The rhythmic activation of CPN2 during gastric mill rhythms in vitro means that CPN2 would normally be rhythmically excited during the protraction phase of the gastric mill rhythm, which is also its gastric mill-timed pattern in the isolated STNS. This timing would enable CPN2 activity to facilitate protraction of the teeth by its synaptic excitation of the lateral and medial teeth protractor neurons (LG and GM, respectively) and inhibition of the medial tooth retractor neuron (DG; Fig. 14B). As proposed previously for the AGR pathway in the lobster STNS (Elson et al. 1994; Simmers and Moulins 1988a), during chewing in the intact animal the positive feedback provided by the AGR pathway to the protractors might participate in strengthening the grasp by the lateral teeth and the rasp by the medial tooth of particularly large or dense pieces of food.

It appears that CPN2 plays a similar role in vivo. In vivo recordings from the gastric mill system, as well as endoscope recordings of movements of the gastric mill teeth in intact crabs and lobsters, have shown that the teeth move in several different coordination patterns (Heinzel 1988a,b; Heinzel et al. 1993a,b). One of these in vivo patterns described in the crab Cancer pagurus corresponds to the in vitro CPN2-influenced gastric mill pattern in our preparations (Heinzel et al. 1993b). In intact animals CPN2 would be recruited to participate in gastric mill rhythms via the gastric mill-timed excitatory input that it receives from AGR. AGR is activated by contraction of a medial tooth protractor muscle innervated by the GM neurons (Heinzel 1990; Heinzel et al. 1993a; Simmers 1988a,b). This excitatory input from AGR in vivo means that CPN2 would normally be rhythmically excited during the protraction phase of the gastric mill rhythm. Hence CPN2 synapses are indicated as broken lines. B: modulatory input (arrow) elicits a gastric mill rhythm that includes rhythmic bursting in CPN2, enabling CPN2 to influence the resulting motor pattern. Hence CPN2 synapses indicated as solid lines. This produces a different gastric mill motor pattern.

The role of CPN2 in generating a specific gastric mill rhythm. The top row of gastric mill neurons in A and B includes all STG neurons active during the protraction phase of the gastric mill rhythm. The bottom row includes all STG neurons active during the retraction phase of this rhythm.

A : modulatory input (arrow) elicits a specific gastric mill motor pattern without activating CPN2. Although CPN2 has synaptic connections to the gastric mill neurons in the STG, it is silent and does not participate. Hence CPN2 synapses are indicated as broken lines. B: modulatory input (arrow) elicits a gastric mill rhythm that includes rhythmic bursting in CPN2, enabling CPN2 to influence the resulting motor pattern. Hence CPN2 synapses indicated as solid lines. This produces a different gastric mill motor pattern.

DISCUSSION

Function of CPN2

We have identified and characterized CPN2, a projection neuron whose soma is located in the CoG of the crab, C. borealis. Our results indicate that the recruitment of CPN2 plays a pivotal role in determining which gastric mill motor pattern will be produced in the isolated STNS. CPN2 influences the gastric mill rhythm in three ways (Fig. 14). First, its synaptic effects separate the activity patterns of the three lateral teeth subsystem neurons that would otherwise be coactive. Thus CPN2 causes MG and IC to be inactive during most of each LG burst. Second, CPN2 activity ensures that the LG and DG neurons exhibit an alternating activity pattern, which they commonly display during CPN2-influenced gastric mill rhythms. There appears to be no mechanism intrinsic to the crab STG neurons that would otherwise enable LG and DG bursts to alternate (Coleman and Nusbaum 1994). Finally, CPN2 strongly activates the GM neurons, enabling them to participate in the gastric mill rhythm and to be coactive with LG.

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In the isolated STNS, AGR activity is both weak and tonic. As a result, there must be another mechanism(s) underlaying the rhythmic activation of CPN2 during gastric mill rhythms in vitro. One candidate for this role is the rhythmic inhibition that CPN2 receives from Int1, which can cause postinhibitory rebound bursts in CPN2. Whether CPN2 also exhibits endogenous oscillatory activity during these times or instead is synaptically driven in a rhythmic fashion by an unidentified input remains unresolved. It may be that CPN2 is activated to participate in gastric mill rhythms as a result of input it receives from other CoG neurons, some of which are known to activate gastric mill rhythms in the crab (Coleman and Nusbaum 1994; Coleman et al. 1993; Nusbaum et al. 1992).

We found that CPN2 activity was necessary, although not always sufficient, to elicit a specific gastric mill motor pattern. Rather, CPN2 is incorporated into gastric mill rhythms to produce a distinct motor pattern (Fig. 14). Thus, rather than acting as a "trigger" or "gating" neuron for the gastric mill rhythm, CPN2 is best defined as a member of the gastric mill network that is only recruited to participate in the generation of a specific gastric mill pattern (Fig. 14). Its membership in the gastric mill pattern-generating network is based on the fact that it satisfies all of the conditions generally ascribed to members of rhythmi-
cally active neural networks. This includes exhibiting rhythmic activity that is time locked to that of the network, and having synaptic access to the network such that it can perturb and reset the ongoing rhythm (Peterson and Calabrese 1982). A major distinction between CPN2 and the gastric mill neurons in the STG is that only CPN2 synaptically influences all of the other gastric mill neurons, either directly or indirectly. CPN2 might also provide modulatory influences to this system, because, in the presence of oxotremorine, tonic CPN2 activity selectively elicited rhythmic bursting in LG. However, it remains uncertain whether or not this represents a modulatory influence because, in the presence of oxotremorine, rhythmic LG activity could also sometimes be elicited by depolarizing LG via depolarizing current injection. Recent work, both in the stomatogastric system (Coleman and Nusbaum 1994; Nusbaum et al. 1992) and in the escape system of the mollusk Tritonia diomedea (Katz et al. 1994), has shown that individual neurons can provide modulatory influences to a rhythmically active neural network and also be an integral component of that network.

Other projection neurons that influence the gastric mill rhythm

Several other projection neurons that influence the gastric mill rhythm have been identified. In Homarus gammarus this includes the commissural gastric (CG) neuron (Robertson and Moulins 1984) and the gastric inhibitor (GI) neuron (Combes et al. 1993). In the red lobster, Palinurus vulgaris, the anterior pyloric modulator (APM) neuron can either trigger or entrain a specific gastric mill rhythm (Dickinson et al. 1988; Nagy et al. 1988). In the spiny lobster, Panulirus interruptus, the excitatory (E) neuron excites at least some gastric mill neurons (Elson et al. 1994; Russell 1976; Selverston et al. 1976).

CPN2 may be the species equivalent of the CG neuron in H. gammarus and the E neuron in P. interruptus. All three of these neurons are located within the CoG, project to the STG via the son and stn, and have the same synaptic connectivity to a subset of the gastric mill STG neurons. For example, all three of these projection neurons are excited by the sensory neuron AGR, and they all excite the GM and LG neurons. However, only the CG and E neurons excite LPG. Interestingly, although LPG commonly participates in gastric mill rhythms in the lobster in vitro STNS (Dickinson et al. 1988; Elson and Selverston 1992), this is an uncommon occurrence in the crab in vitro system (Weimann et al. 1991; this paper). The remaining synaptic targets of the E neuron have not been described.

There are several additional differences between CPN2 and CG. For example, CG differs from CPN2 in that CG has the unusual property of firing action potentials only within a narrow window of membrane potentials (Robertson and Moulins 1984). When the CG membrane potential is either more depolarized than -30 mV or more hyperpolarized than -60 mV, no action potentials are produced. Perhaps the most dramatic difference between CPN2 and CG is that CG has no known inhibitory effects on gastric mill neurons. Recent work in Homarus has shown that a separate projection neuron, GI, does have extensive inhibitory effects on the gastric mill system (Combes et al. 1993). Additionally, AGR excites GI, although GI has a higher response threshold to AGR activity than does CG (Combes et al. 1993). No GI equivalent has yet been recorded in Cancer. However, CPN2 may play the role of both CG and GI, because suppression of CPN2 eliminated all of the AGR effects in the crab STG.

In C. borealis, modulatory commissural neuron 1 (MCN1) provides modulatory drive to many gastric mill neurons and initiates a gastric mill rhythm that is similar to the rhythm elicited by oxotremorine in Fig. 2A (Coleman and Nusbaum 1994; Nusbaum et al. 1992). Additionally, MCN7 elicits a gastric mill rhythm in C. borealis that is very similar to the one shown in Fig. 2C (Coleman et al. 1993). The gastropyloric receptor (GPR) sensory neuron also influences several gastric mill neurons in C. borealis (Katz and Harris-Warrick 1991). The membership of MCN1 and CPN2 in the gastric mill network in C. borealis may explain why gastric mill rhythms occur so infrequently when the STG is isolated from the CoGs. Activity in both MCN1 and CPN2 commonly originates in the CoG, so that their activity level within the STG is greatly reduced when the STG is separated from the CoGs. This removes an important source of both modulatory and patterned input to the gastric mill system.

Motor pattern selection

In the C. borealis STNS, exogenous application of different modulatory transmitters produce distinct pyloric rhythms by their direct effects on STG neurons (Marder and Weimann 1992). Although these modulatory transmitters also directly influence the membrane properties of the gastric mill neurons, the different gastric mill rhythms elicited by oxotremorine in this system appear to result partly from its excitatory effects on distinct CoG projection neurons. These particular gastric mill rhythms are not exclusively activated by oxotremorine, because they can also be elicited by other bath-applied modulators, including two FMRFamide-related peptides (Heinzel et al. 1993b; Weimann et al. 1993). This suggests that the FMRFamide-related peptides probably also influence the gastric mill system in part by activating these same CoG projection neurons.

Nagy et al. (1988) reported that distinct gastric mill rhythms also occur in vitro in P. vulgaris. Although they differ in both detail and underlying mechanism from the gastric mill patterns produced in C. borealis, these different rhythms in P. vulgaris also involve changes in the activity levels and phase relationships of the LG, MG, GM, and DG neurons. Moreover, Nagy et al. (1988) suggested that one of the P. vulgaris patterns results at least partly from enhanced activity in the CG neuron.

In the swimming systems of both Clione and Xenopus, neuronal recruitment causes a faster and more vigorous rhythm without modifying the phasing of the motor pattern (Arshavsky et al. 1985b, 1989; Sillar and Roberts 1993). In contrast, we found that CPN2 recruitment not only alters the frequency of the gastric mill rhythm, but it also causes substantial changes in the motor pattern (Fig. 14). A similar mechanism could well account for switches that occur in
other sets of related behaviors where different forms of the behavior are believed to be generated by the same or overlapping subsets of neural network components (Pershson 1993).

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