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A newly identified extrinsic input triggers a distinct gastric mill rhythm *via* activation of modulatory projection neurons

Dawn M. Blitz[1], Rachel S. White[1], Shari R. Saideman[1,*], Aaron Cook[1], Andrew E. Christie[2,3], Farzan Nadim[4,5] and Michael P. Nusbaum[1,†]

[1]Department of Neuroscience, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA, [2]Department of Biology, University of Washington, Seattle, WA 98195-1800, USA, [3]Mount Desert Island Biological Laboratory, Salisbury Cove, ME 04672, USA, [4]Department of Mathematical Sciences, New Jersey Institute of Technology, Newark, NJ 07102, USA and [5]Department of Biological Sciences, Rutgers University, Newark, NJ 07102, USA

*Present address: Department of Biological Sciences, Columbia University, New York, NY 10027, USA
†Author for correspondence (e-mail: nusbaum@mail.med.upenn.edu)

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SUMMARY

Neuronal network flexibility enables animals to respond appropriately to changes in their internal and external states. We are using the isolated crab stomatogastric nervous system to determine how extrinsic inputs contribute to network flexibility. The stomatogastric system includes the well-characterized gastric mill (chewing) and pyloric (filtering of chewed food) motor circuits in the stomatogastric ganglion. Projection neurons with somata in the commissural ganglia (CoGs) regulate these rhythms. Previous work characterized a unique gastric mill rhythm that occurred spontaneously in some preparations, but whose origin remained undetermined. This rhythm includes a distinct protractor phase activity pattern, during which a key gastric mill circuit neuron (LG neuron) and the projection neurons MCN1 and CPN2 fire in a pyloric rhythm-timed activity pattern instead of the tonic firing pattern exhibited by these neurons during previously studied gastric mill rhythms. Here we identify a new extrinsic input, the post-oesophageal commissure (POC) neurons, relatively brief stimulation (30 s) of which triggers a long-lasting (tens of minutes) activation of this novel gastric mill rhythm at least in part *via* its lasting activation of MCN1 and CPN2. Immunocytochemical and electrophysiological data suggest that the POC neurons excite MCN1 and CPN2 by release of the neuropeptide *Cancer borealis* tachykinin-related peptide Ia (CabTRP Ia). These data further suggest that the CoG arborization of the POC neurons comprises the previously identified anterior commissural organ (ACO), a CabTRP Ia-containing neurohemal organ. This endocrine organ thus appears to also have paracrine actions, including activation of a novel and lasting gastric mill rhythm.

Key words: neuromodulation, central pattern generator, projection neurons, neuropeptide, *Cancer borealis*.

INTRODUCTION

Neuromodulation enables single motor circuits to generate multiple distinct activity patterns by changing the intrinsic and synaptic properties of circuit neurons (Marder et al., 2005; LeBeau et al., 2005; Kiehn, 2006; Gordon and Whelan, 2006; Tryba et al., 2006). Further flexibility in the output of these motor circuits is afforded by modulatory actions at the level of the projection neurons that drive circuit activity (Di Prisco et al., 2000; Beenhakker and Nusbaum, 2004; Blitz et al., 2004; McLean and Sillar, 2004; Brocard et al., 2005; Smetana et al., 2007). However, the extrinsic inputs that provide these modulatory influences on projection neurons are not well-documented in most systems.

We are using the stomatogastric nervous system (STNS) of the crab *Cancer borealis* to identify the extrinsic input responsible for the activation of a previously identified version of the gastric mill (chewing) rhythm (Wood et al., 2004). The stomatogastric nervous system is an extension of the decapod crustacean CNS that includes the unpaired stomatogastric (STG) and oesophageal (OG) ganglia plus the paired commissural ganglia (CoGs) (Nusbaum and Beenhakker, 2002; Marder and Bucher, 2007). Overlapping sets of the 26 neurons in the *C. borealis* STG contribute to the gastric mill and pyloric (filtering of chewed food) rhythms (Marder and Bucher, 2007). In *C. borealis*, these rhythms are regulated by input from no more than 20 projection neurons, most of which are present as single neurons within each CoG (Coleman et al., 1992; Nusbaum et al., 2001). In addition, extrinsic inputs that convey sensory and other information modify these rhythms by influencing circuit neurons and/or projection neurons (Meyrand et al., 1994; Combes et al., 1999; Christie et al., 2004; Beenhakker and Nusbaum, 2004; Blitz et al., 2004).

We have identified a novel extrinsic input to the STNS of *C. borealis*. This input, called the post-oesophageal commissure (POC) neurons, consists of bilateral peptidergic fiber bundles that project through the post-oesophageal commissure (*poc*) and circumoesophageal connectives (*cocs*) to innervate the CoGs.

Extracellular *poc* stimulation drives the POC neurons to trigger a long-lasting activation of CoG projection neurons, which in turn drive the gastric mill rhythm. Two of these projection neurons are modulatory commissural neuron 1 (MCN1) and commissural projection neuron 2 (CPN2) (Coleman and Nusbaum, 1994; Norris et al., 1994). Interestingly, despite the likely participation of MCN1 and CPN2 in the POC-triggered gastric mill rhythm, the POC-triggered activity pattern of these projection neurons and the associated gastric mill rhythm are distinct from previous versions

of this rhythm that are activated by these same two projection neurons (Beenhakker and Nusbaum, 2004; Blitz et al., 2004). Our data further suggest that the POC excitation of MCN1 and CPN2 is mediated by the neuropeptide transmitter *Cancer borealis* tachykinin-related peptide Ia (CabTRP Ia). The POC neurons also appear to be the source of the CabTRP Ia-containing anterior commissural organ (ACO), a dense neurohemal structure in the CoG neuropil (Messinger et al., 2005).

Some of this work was published previously in abstract form (White et al., 2005).

MATERIALS AND METHODS

Animals

Male Jonah crabs (*Cancer borealis* Stimpson) were obtained from Commercial Lobster and Seafood Co., Boston, MA, USA and the Marine Biological Laboratory, Woods Hole, MA, USA. Before experimentation, crabs were housed in commercial tanks containing recirculating, filtered and aerated artificial seawater (10°C). Crabs were cold anesthetized by packing in ice for at least 30 min prior to dissection. The STNS was dissected as described previously (Blitz et al., 2004). Briefly, the foregut was first removed and pinned down in a Sylgard 170 (KR Anderson, Morgan Hill, CA, USA, or World Precision Inc., Sarasota, FL, USA)-coated glass bowl in chilled *C. borealis* saline. The *poc* was bisected under a dissecting microscope, after which the stomach was bisected ventrally and pinned flat with the interior stomach wall against the Sylgard. The STNS, including all four ganglia (two CoGs, OG, STG) plus their connecting and peripheral nerves (Fig. 1), was next dissected from the surface of the foregut and pinned in a Sylgard 184 (KR Anderson)-coated Petri dish. The foregut and nervous system were maintained in chilled (10–13°C) saline throughout the dissection and subsequent experiment.

All *C. borealis* used for fiber counting, tracing the POC axons to the thoracic ganglion (TG) and axon diameter measurement were collected by hand at Mount Desert Island Biological Laboratory (Salisbury Cove, ME, USA) and maintained in flow-through natural seawater tanks at ambient water temperature (10–14°C). For ease of dissection and immunoprocessing, these animals were smaller than those used for electrophysiological experiments. For tissue collection, as above, these crabs were first anesthetized by packing in ice for at least 30 min. The dorsal carapace was then removed and the thoracic ganglion, with the *cocs* and CoGs attached, were isolated by microdissection in chilled (approximately 10°C) *C. borealis* saline.

Solutions

Cancer borealis saline for dissections had the following composition (in mmol l^{-1}): 440 NaCl, 26 $MgCl_2$, 13 $CaCl_2$, 11 KCl, 10 Trizma base, and 5 maleic acid (pH 7.4–7.6). During recording, 5 mmol l^{-1} dextrose was added to the saline. In high-divalent cation saline (Hi-Di), $MgCl_2$ and $CaCl_2$ were raised to 130 mmol l^{-1} and 65 mmol l^{-1}, respectively. Phosphoramidon (Sigma, St Louis, MO, USA) and CabTRP Ia (Biotechnology Center, University of Wisconsin, Madison, WI, USA) were stored as frozen aliquots and diluted in *C. borealis* saline immediately prior to use.

Electrophysiology

Extracellular recordings were made by isolating a section of nerve with petroleum jelly (Vaseline: Medical Accessories and Supply Headquarters, Alabaster, AL, USA) and placing one stainless steel wire of a pair inside the Vaseline compartment and the other wire in the main bath compartment. These recordings were amplified in a 2-stage process (stage 1: AM Systems Model 1700 AC Amplifier, Carlsborg, WA, USA; stage 2: Brownlee Precision Model 410 Amplifier, Santa Clara, CA, USA). To facilitate intracellular recordings, ganglia were desheathed and viewed with light transmitted through a darkfield condenser (Nikon, Tokyo, Japan). Intracellular recordings were accomplished using borosilicate microelectrodes filled with 0.6 mol l^{-1} K_2SO_4 plus 10 mmol l^{-1} KCl (20-25 MΩ). Intracellular signals were amplified using Axoclamp 2B amplifiers (Molecular Devices, Sunnyvale, CA, USA) and digitized at ~5 kHz using a Micro 1401 data acquisition interface and Spike2 software (Cambridge Electronic Design, Cambridge, England).

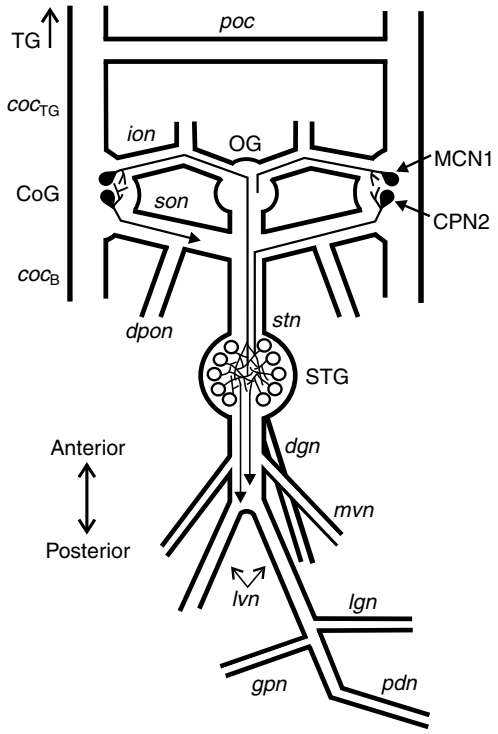

Fig. 1. Schematic of the isolated stomatogastric nervous system, including the axon projections of MCN1 and CPN2 to the STG. The two lines with arrowheads projecting posteriorly from the STG neuropil represent the projection pattern of most STG motor neurons. Ganglia: CoG, commissural ganglion; OG, oesophageal ganglion; STG, stomatogastric ganglion; TG, thoracic ganglion. Nerves: coc_{TG}, circumoesophageal connective from the CoG to the TG; coc_B, circumoesophageal connective from the CoG to the brain; *dgn*, dorsal gastric nerve; *dpon*, dorsal posterior oesophageal nerve; *ion*, inferior oesophageal nerve; *lgn*, lateral gastric nerve; *lvn*, lateral ventricular nerve; *mvn*, medial ventricular nerve; *pdn*, pyloric dilator nerve; *poc*, post-oesophageal commissure; *son*, superior oesophageal nerve. Neurons: CPN2, commissural projection neuron 2; MCN1, modulatory commissural neuron 1.

Network and projection neurons were identified based on their activity patterns, synaptic connectivity and axonal projection patterns (Weimann et al., 1991; Norris et al., 1994; Coleman and Nusbaum, 1994; Beenhakker and Nusbaum, 2004; Saideman et al., 2007a; Saideman et al., 2007b). In some experiments, the activity of the projection neuron CPN2 was monitored indirectly, *via* the presence of excitatory postsynaptic potentials in the gastric mill (GM) protractor motor neuron (Norris et al., 1994).

Each half of the bisected *poc* was surrounded by a Vaseline well. Axons in the *poc* were stimulated extracellularly using a Grass S88 stimulator (AstroMed, West Warwick, RI, USA) and stimulus

isolation unit (SIU5, AstroMed). The *poc* was stimulated tonically, using a range of voltages (4–15 V), at 15–30 Hz for 15–30 s. All *poc* stimulations were unilateral. To activate the gastro-pyloric receptor 2 neuron (GPR2) (Katz et al., 1989), the gastro-pyloric nerve was stimulated tonically at 10 Hz for 4 s. The ventral cardiac neurons (VCNs) (Beenhakker et al., 2004) were activated by stimulating the dorsal posterior oesophageal nerve in a rhythmic pattern (burst duration: 6 s, interburst freq.: 0.06 Hz, intraburst freq.: 15 Hz) (Beenhakker et al., 2004; Beenhakker and Nusbaum, 2004). CabTRP Ia was pressure ejected (10^{-4} mol l^{-1}, 41–69 kPa, 0.5–10 s) from a Picospritzer II device (General Valve Corporation, Fairfield, NJ, USA) into the desheathed CoG neuropil. The dorsal aspect of the CoG is covered with neuronal somata, and the neuropil is underneath these somata. Therefore, to focally apply CabTRP Ia into the CoG neuropil, we inserted the peptide-containing pipette through the soma layer and into the depth of the anterior neuropil (Blitz and Nusbaum, 1999). The endopeptidase inhibitor phosphoramidon (10^{-5} mol l^{-1}) was superfused to the anterior portion of the STNS, which was isolated from the STG by a Vaseline wall built across the recording dish. No data collection was made until phosphoramidon superfusion had occurred for at least 25 min.

Immunocytochemistry

Whole mounts of the isolated STNS and the thoracic ganglion (TG) with attached *cocs* and CoGs were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 12–24 h, rinsed at least five times, at 1-h intervals, in phosphate (*P*) buffer (0.1 mol l^{-1}) with 0.3% Triton X-100 (*P*-Triton) and then incubated for 24–72 h with a monoclonal rat anti-substance P antibody (1:300; Accurate Chemical and Scientific Corporation, Westbury, NY, USA; Abcam Incorporated, Cambridge, MA, USA) that has been used previously on this system (Goldberg et al., 1988; Christie et al., 1997; Blitz et al., 1999; Messinger et al., 2005). The nervous system was then again rinsed in *P*-Triton, five times at 1 h intervals, after which the STNS preparations were incubated with goat anti-rat Alexa Fluor 488 or 647 (1:300; Invitrogen Corporation, Carlsbad, CA, USA) for 12–16 h. In preparations where the TG was studied, the nervous system was incubated with donkey anti-rat IgG conjugated with either FITC or Rhodamine Red-X (Jackson ImmunoResearch, West Grove, PA, USA). In both cases, the preparations were then rinsed at least five times at 1-h intervals with *P* buffer and then mounted in 80% glycerol/20% 20 mmol l^{-1} sodium carbonate and a cover slip was place on top. For the STNS preparations, fluorescence was visualized and photographed with a Leica DMRB microscope, a Leica DC 350 FS camera, and Image-Pro Express software (Leica, version 4.5.1.3) using a L4 or Y5 (Leica) filter set (Leica Microsystems Inc., Bannockburn, IL, USA). The thoracic-CoG preparations were imaged using a Zeiss LSM 510 Meta confocal system (Carl Zeiss MicroImaging Inc., Thornwood, NY, USA), equipped with a Zeiss Observer.Z1 inverted microscope and argon and helium-neon lasers. Imaging was done using Zeiss EC plan-NEOFLUAR 10×/0.3 dry, Plan-Apochromat 20×/0.8 dry, EC plan-NEOFLUAR 40×/1.30 oil and Plan-Apochromat 63×/1.4 oil objective lenses, standard FITC and Rhodamine filter sets, and manufacturer-supplied software.

Data analysis

Data analysis was performed with custom written macros using Spike2 ('The Crab Analyzer', freely available at http://www.uni-ulm.de/~wstein/spike2/index.html). Gastric mill cycle period was measured as the duration from the onset of a lateral gastric (LG) neuron burst to the onset of the subsequent LG burst. An average of 10 consecutive cycles was obtained in each condition. Control MCN1 and CPN2 firing frequencies were measured continuously for 30 seconds prior to stimulation. MCN1 and CPN2 firing frequencies after stimulation were quantified during 10 consecutive protraction and retraction phases of the gastric mill rhythm in each preparation, as the average frequency across the entire protraction or retraction phase. MCN1 pyloric-timed activity was measured as the percentage of time it was active during each pyloric cycle, defined as the duration from the onset of a pyloric dilator (PD) neuron burst until the onset of the subsequent PD burst, for the pyloric cycles occurring during 10 consecutive protraction and retraction phases in each preparation.

The *coc* is a bilateral fiber bundle that connects the TG to the supra-oesophageal ganglion (brain), with the CoG being an outpocketing of the *coc* between its two termination points (Fig. 1). The *poc* connects the *cocs* on the TG side of the CoG. To refer specifically to a region of the *coc* relative to the CoG, we label the region of the *coc* projecting from the CoG towards the TG as the coc_{TG}, and the region of the *coc* projecting from the CoG towards the brain as coc_{B} (Fig. 1).

Figures were made using Spike2, CorelDraw (Corel Corporation, Ottawa, ON, Canada) and Igor Pro (Wavemetrics, Portland, OR, USA). Statistical analysis was performed with SigmaStat (Systat Software, San Jose, CA, USA). The Paired Student's *t*-test or repeated-measures (RM) one-way ANOVA followed by multiple comparisons using the Student–Newman–Keuls method were used as indicated. Significance was considered to be $P<0.05$. Data are expressed as mean ± s.e.m.

RESULTS

The gastric mill rhythm is a two-phase motor pattern driven by descending input

The gastric mill rhythm (cycle period: 5–20 s) drives the rhythmic protraction and retraction movements of the teeth in the gastric mill stomach compartment, thereby enabling the chewing of food (Heinzel, 1988; Heinzel et al., 1993). In *C. borealis* there are eight different types of gastric mill neurons, seven of which are motor neurons (Weimann et al., 1991; Saideman et al., 2007b; Stein et al., 2007). Four of these gastric mill neurons are protractor motor neurons, including the LG, GM, medial gastric (MG) and inferior cardiac (IC) neurons, although the IC and MG neurons can also exhibit retractor phase activity during some versions of the gastric mill rhythm (Beenhakker and Nusbaum, 2004; Blitz et al., 2004; Wood et al., 2004; Saideman et al., 2007b). There are also three retractor motor neurons, the dorsal gastric (DG), anterior median (AM) and ventricular dilator (VD) motor neurons, plus interneuron 1 (Int1), which is also active during the retractor phase and is the sole interneuron in this circuit. There is a single neuron of each type per STG, except the GM neurons of which there are four functionally equivalent copies.

In the isolated STNS of *C. borealis*, some of the gastric mill neurons (Int1, MG, IC, VD) are spontaneously active in pyloric rhythm-time, even in the absence of the gastric mill rhythm (e.g. VD and IC in Fig. 2, left panel) (Weimann et al., 1991; Blitz and Nusbaum, 1997). The pyloric rhythm (cycle period 0.5–2 s), which controls the filtering of chewed food in the posterior (pyloric) stomach compartment, is generated by a second motor circuit in the STG and is continuously active both *in vitro* and *in vivo* (Marder and Bucher, 2007).

The gastric mill rhythm is usually silent, in the isolated STNS as well as *in vivo*, unless the projection neurons that drive it are activated (Fleischer, 1981; Heinzel et al., 1993; Nusbaum et al.,

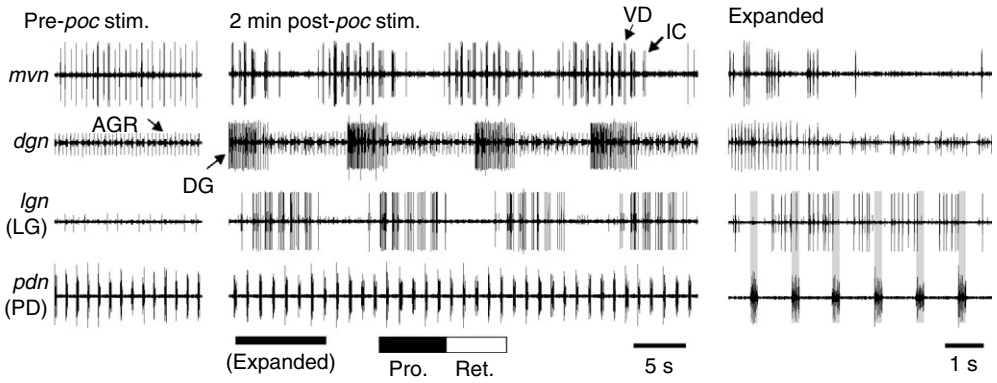

Fig. 2. The gastric mill rhythm is triggered by *poc* nerve stimulation. (Left) Prior to *poc* stimulation, there was an ongoing pyloric rhythm [medial ventricular nerve (*mvn*) and *pdn*], but no gastric mill rhythm (*dgn*, *lgn*). The large, tonically active unit in the *dgn* corresponds to the activity of the anterior gastric receptor (AGR) neuron. AGR is a muscle tendon proprioceptor neuron that is spontaneously active in the isolated STNS (Combes et al., 1995; Smarandache and Stein, 2007). (Middle) 2 min after tonic *poc* stimulation (15 Hz, 30 s), the gastric mill rhythm was triggered, as is evident from the rhythmic bursting in the protractor LG neuron that alternated with the retractor phase activity of the DG, VD and IC neurons. Note the pyloric-timed bursting in the LG neuron. (Right) This expanded section of the middle panel shows more explicitly that each protractor LG burst is time-locked to the pyloric rhythm. Each period of inactivity in LG starts with a pyloric dilator (PD) neuron burst (grey bars). Pro., protraction, Ret., retraction.

2001; Beenhakker and Nusbaum, 2004; Blitz et al., 2004). However, Wood et al. (Wood et al., 2004) characterized a version of the gastric mill rhythm that occurred in some preparations without any experimental manipulation of projection neuron activity. This gastric mill rhythm was unusual in that the gastric mill LG neuron and projection neuron MCN1 exhibited a pyloric rhythm-timed activity pattern during the protraction phase, instead of the tonic firing pattern that they exhibit during protraction in all other characterized gastric mill rhythms in *C. borealis* (Coleman and Nusbaum, 1994; Beenhakker et al., 2004; Blitz et al., 2004; Christie et al., 2004; Saideman et al., 2007b). This spontaneously active gastric mill rhythm was driven largely by an unusually high level of spontaneous activity in the projection neuron MCN1. This MCN1 activity was not only pyloric rhythm-timed, but the resulting gastric mill rhythm was largely replicated by pyloric rhythm-timed extracellular stimulation of MCN1 (Wood et al., 2004).

POC stimulation triggers a long-lasting gastric mill rhythm

Stimulating the *poc* nerve (15 Hz tonic stimulation, 30 s duration) consistently triggered the gastric mill rhythm, beginning soon after the stimulation was terminated (*N*=39). In the example shown in Fig. 2, this rhythm started approximately 2 min after the end of *poc* stimulation and, as is typical for gastric mill rhythms, there was rhythmic alternating bursting of the protractor (LG) and retractor (DG, VD) neurons. It is also noteworthy that, during these rhythms, the IC neuron was mostly active during the retractor phase instead of the protractor phase (Fig. 2). Across preparations, the *poc*-triggered gastric mill rhythm started approximately 1 min after the end of *poc* stimulation (mean latency post-stimulation: 0.91±0.05 min, *N*=39). These rhythms exhibited a cycle period of 13.1±0.9 s (*N*=20).

During each protractor phase, the LG neuron exhibited pyloric rhythm-timed bursts (Fig. 2). Within each pyloric-timed burst, LG activity alternated with activity in the pyloric pacemaker neurons (e.g. PD neuron in Fig. 2), as also occurred in the spontaneously active rhythm characterized by Wood et al. (Wood et al., 2004). The protractor GM motor neuron also exhibited pyloric-timed bursts in many preparations (not shown). In all other previously studied gastric mill rhythms, the protractor neuron bursts instead exhibited a tonic firing pattern (Beenhakker and Nusbaum, 2004; Blitz et al., 2004; Christie et al., 2004; Saideman et al., 2007b).

The *poc*-triggered gastric mill rhythm was also long-lasting. After a 30 s *poc* stimulation, the gastric mill rhythm tended to persist for many minutes, and sometimes for more than 1 h (*N*=39). Specifically, in a few preparations this rhythm lasted for less than 5 min (*N*=4/39), but it often persisted for 5–20 min (*N*=22/39) or longer (*N*=13/39). The pattern was consistent for the duration of the triggered gastric mill rhythm. For example, there was stable, alternating bursting between the retractor (e.g. DG) and protractor

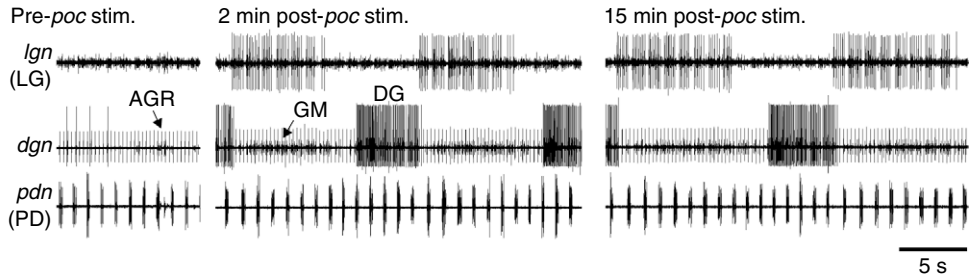

Fig. 3. The *poc*-triggered gastric mill rhythm is long-lasting. (Left) Before *poc* stimulation, there was an ongoing pyloric rhythm (*pdn*) but no gastric mill rhythm (*lgn*, *dgn*). (Middle) 2 min after tonic *poc* stimulation (15 Hz, 30 s), the gastric mill rhythm had been triggered and was ongoing. Note the pyloric-timed LG bursts. (Right) This rhythm persisted for more than 15 min after *poc* stimulation.

(e.g. LG) neurons, with consistent pyloric-timed interruptions in each LG burst (Fig. 3).

POC stimulation indirectly activates the gastric mill rhythm

Extrinsic inputs can alter STG circuit activity *via* synaptic actions on circuit neurons and/or descending projection neurons (Hooper and Moulins, 1990; Katz and Harris-Warrick, 1990; Meyrand et al., 1994; Combes et al., 1999; Beenhakker and Nusbaum, 2004; Blitz et al., 2004). To determine whether the input(s) activated by *poc* stimulation influenced the gastric mill circuit directly or indirectly, we selectively superfused the CoGs with high divalent cation (Hi-Di: 5×Ca^{2+}/5×Mg^{2+}) saline while continuing to supply normal *C. borealis* saline to the STG. The Hi-Di saline raises action potential threshold and reduces the likelihood of polysynaptic transmission (Blitz and Nusbaum, 1999). This allowed us to reversibly reduce the ability of any *poc*-stimulated synaptic actions to activate CoG projection neurons and thereby determine whether this input activated the gastric mill rhythm *via* direct actions on STG neurons.

After determining that *poc* stimulation triggered a gastric mill rhythm in control conditions (Fig. 4A), Hi-Di saline was superfused selectively to the CoGs to suppress *poc* activation of CoG projection neurons. Under these conditions, stimulating the *poc* did not activate the gastric mill rhythm (*N*=6), even when the stimulation voltage was increased by 2 V (Fig. 4B). To ensure that the inability of *poc* stimulation to activate the gastric mill rhythm was not a consequence of a dysfunctional gastric mill circuit, we used extracellular stimulation of the inferior oesophageal nerve to drive this rhythm *via* selective activation of the projection neuron MCN1 (Bartos et al., 1999). Tonic MCN1 stimulation elicits a distinct gastric mill rhythm from the one triggered by *poc* stimulation, but both rhythms involve the same gastric mill circuit neurons (Coleman et al., 1995; Bartos et al., 1999; Wood et al., 2004; Saideman et al., 2007b). Extracellular MCN1 stimulation consistently elicited the gastric mill rhythm despite the presence of high-divalent cation saline to the CoGs (*N*=3, data not shown). Furthermore, after washing out the Hi-Di saline, *poc* stimulation again triggered the gastric mill rhythm (Fig. 4C; *N*=5/6). Thus, axons in the *poc* appear to project into the CoGs to activate projection neurons and thereby indirectly activate the gastric mill rhythm. We have designated the *poc* input that triggers the gastric mill rhythm as the POC neurons (see below).

The POC neurons excite the projection neurons MCN1 and CPN2

Two previously identified CoG projection neurons in *C. borealis*, MCN1 and CPN2, are necessary and sufficient for driving two previously characterized gastric mill rhythms that are elicited by stimulation of a mechanosensory (VCN neurons) or proprioceptor (GPR neuron) input (Beenhakker and Nusbaum, 2004; Blitz et al., 2004). Furthermore, the spontaneously active gastric mill rhythm studied by Wood et al. (Wood et al., 2004) was largely mimicked by direct stimulation of MCN1. We therefore examined the activity of MCN1 and CPN2 before and after *poc* stimulation, and found that this stimulation consistently triggered a long lasting excitatory response in both projection neurons (*N*=39). This excitatory response included an increased firing rate and pyloric-timed activity (Fig. 5).

The POC-triggered excitation of MCN1 and CPN2 always coincided with the triggering of the gastric mill rhythm (*N*=39). After *poc* stimulation, the firing frequency of MCN1 was consistently higher than pre-stimulation (pre-*poc* stim.: 4.0±0.5 Hz; post-*poc* stim.: protraction phase (LG burst), 14.5±1.2 Hz, retraction phase (LG inter-burst), 14.7±1.2 Hz, *N*=10; protraction and retraction significantly different from control, $P<0.05$, protraction not significantly different from retraction, $P>0.05$, RM one-way ANOVA and Student–Newman–Keuls test of multiple comparisons). Similarly, CPN2 firing frequency was consistently increased after POC stimulation (pre-POC: 2.8±1.1 Hz; post-POC: protraction, 18.2±3.3 Hz; retraction, 15.6±3.3 Hz, *N*=4; protraction and retraction significantly different from control, $P<0.05$,

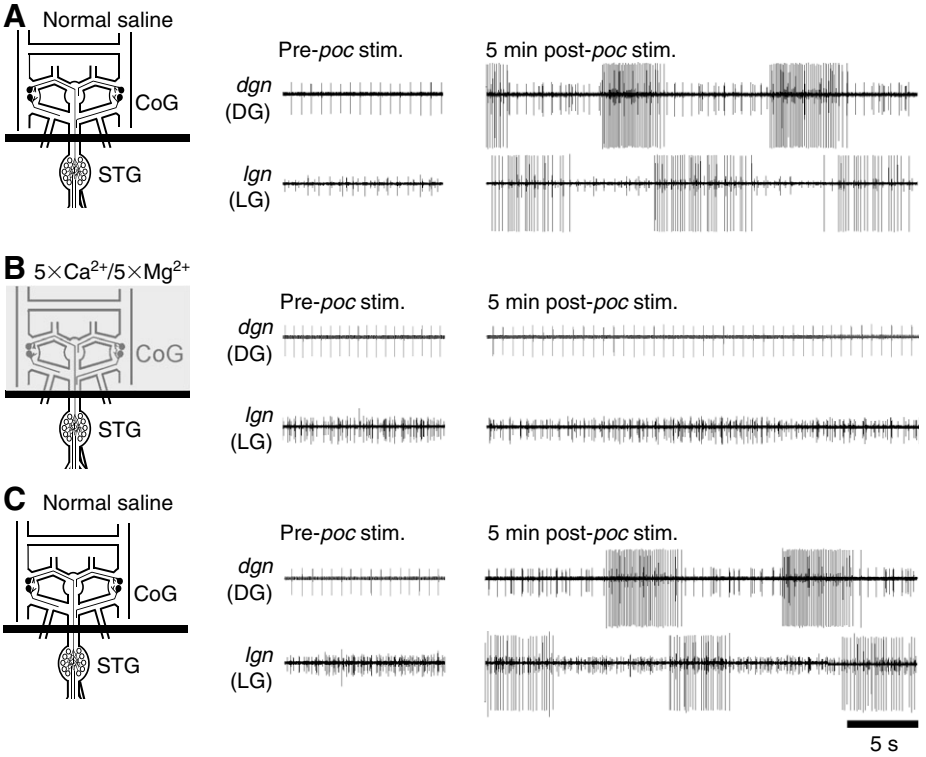

Fig. 4. The *poc*-triggered gastric mill rhythm requires the activation of CoG projection neurons. (A) During normal saline superfusion of the CoGs, tonic *poc* stimulation (15 Hz, 30 s) triggered the gastric mill rhythm. (B) During superfusion of 5× Mg^{2+}/5× Ca^{2+} saline selectively to the CoGs and OG (grey shading in STNS schematic), the same *poc* stimulation did not trigger the gastric mill rhythm. (C) After washout of the 5× Mg^{2+}/5× Ca^{2+} saline, *poc* stimulation again triggered the gastric mill rhythm. Note that the black bar in each STNS schematic represents a Vaseline wall that enabled separate saline superfusion of the anterior (CoGs, OG) and posterior (STG) aspects of the STNS. All panels are from the same preparation.

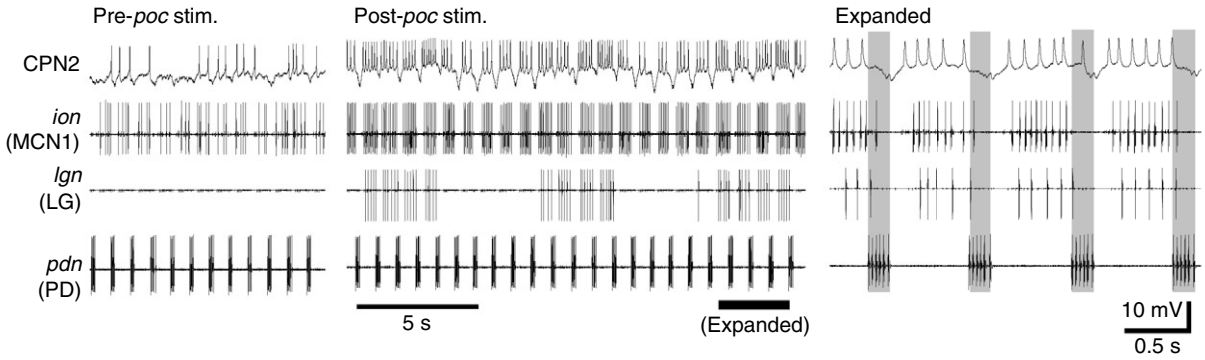

Fig. 5. Activation of the CoG projection neurons CPN2 and MCN1, as well as the gastric mill rhythm, is triggered by *poc* stimulation. (Left) Before stimulation, CPN2 and MCN1 were weakly active and there was an ongoing pyloric rhythm (*pdn*) but no gastric mill rhythm (*lgn, dgn*). (Middle) After *poc* stimulation (15 Hz, 30 s), CPN2 and MCN1 were excited and the gastric mill rhythm was triggered. (Right) Expanded section from the middle panel showing that the activity of LG, MCN1 and CPN2 is interrupted in pyloric-time. Note that each such interruption occurs during activity of the pyloric pacemaker PD neuron (grey shading). Most hyperpolarized membrane potential: CPN2, –45 mV.

protraction not significantly different from retraction, $P>0.05$, RM one-way ANOVA and Student–Newman–Keuls test of multiple comparisons).

A key feature of the MCN1 and CPN2 activity pattern is that their activity was terminated for a portion of each pyloric cycle during both protraction and retraction (Fig. 5). We therefore determined the percentage of the pyloric cycle period during which the projection neurons were active (see Materials and methods). MCN1 and CPN2 were always silent during the pyloric pacemaker neuron burst, which extended from the onset of each pyloric cycle (0%) until approximately the 20% point of each cycle (protraction: 0–20.1±0.4%; retraction: 0–20.2±0.6%; N=6). During the POC-triggered gastric mill rhythms, activity in these two projection neurons generally commenced with a delay after each pyloric pacemaker neuron burst. For example, MCN1 was active for ~65% of each pyloric cycle during protraction (onset: 34.9±2.9%; offset: 100.0±2.5%) and for ~58% of each pyloric cycle during retraction (onset: 39.4±3.1%; offset: 98.3±0.6%; N=6). Comparably, CPN2 was active for ~72% of each pyloric cycle during protraction (onset: 30.3±0.9%; offset: 102.4±0.8%) and was active for ~47% of each pyloric cycle during retraction (onset: 42.3±1.3%; offset: 88.4±7.2%; N=3). MCN1 and CPN2 were presumably silent during the pacemaker burst due to feedback inhibition in the CoGs from the anterior burster, the pyloric pacemaker interneuron (Coleman and Nusbaum, 1994; Norris et al., 1994; Wood et al., 2004).

We tested the hypothesis that the anterior burster neuron feedback to MCN1 and CPN2 in the CoGs was responsible for the pyloric-timed activity pattern of these projection neurons during the POC-triggered gastric mill rhythm. Specifically, we used hyperpolarizing current injection into the pyloric pacemaker neurons to suppress their activity and, consequently, that of the pyloric rhythm. The pyloric pacemaker neurons are a group of electrically coupled neurons that include the single anterior burster neuron plus the paired PD and lateral posterior gastric (LPG) neurons (Weimann et al., 1991; Weimann and Marder, 1994). When the pyloric rhythm was suppressed during the POC-triggered gastric mill rhythm, MCN1 and CPN2 activity switched from pyloric-timed to tonic (N=4; Fig. 6). At these times, the LG neuron activity pattern also switched from pyloric-timed to tonic, presumably because its activity was driven by these projection neurons (Beenhakker and Nusbaum, 2004). There is no direct synapse from the pyloric pacemaker neurons to LG (Bartos et al., 1999). In contrast to our findings, in the European lobster *Homarus gammarus* the pyloric-like activity of some CoG projection neurons can persist when the pyloric feedback is eliminated (Cardi and Nagy, 1994).

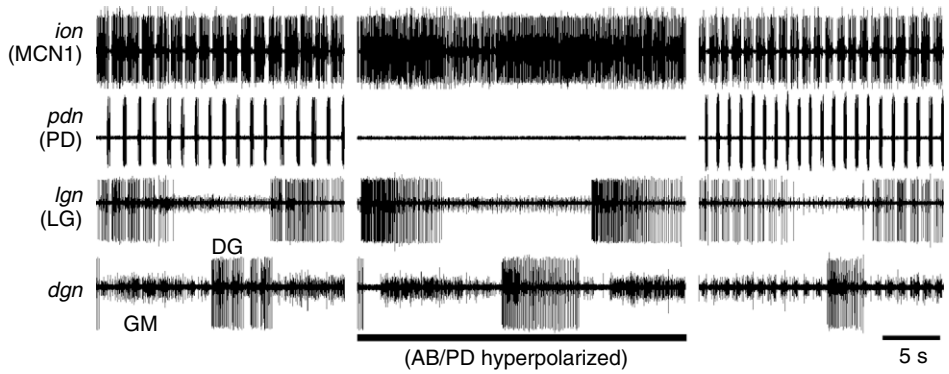

Fig. 6. The pyloric rhythm in the STG is responsible for the pyloric-timed activity of the CoG projection neuron MCN1 and the gastric mill protractor neuron LG during the POC-triggered gastric mill rhythm. (Left) During the POC-triggered gastric mill rhythm, MCN1 and LG exhibited pyloric-timed activity. (Middle) When the pyloric rhythm was suppressed, by hyperpolarization of the pyloric pacemaker neurons, the POC-triggered gastric mill rhythm persisted but the activity of MCN1 and LG changed from pyloric-timed to tonic. (Right) After releasing the pyloric pacemaker neurons from hyperpolarization, the pyloric rhythm resumed and MCN1 and LG returned to exhibiting pyloric-timed activity.

In previously studied gastric mill rhythms (Bartos et al., 1999; Wood et al., 2004), the gastric mill cycle period was regulated by the pyloric rhythm. Specifically, suppressing the pyloric rhythm increased the gastric mill cycle period. This was due to both inter-circuit interactions within the STG and to the pyloric-timing of MCN1 activity. Thus, we tested whether the cycle period of the POC-triggered gastric mill rhythm was also regulated by the pyloric rhythm. We found that the POC-triggered gastric mill cycle period was indeed increased when the pyloric rhythm was suppressed, from 12.3±1.8 s to 19.4±2.7 s (N=4; P<0.05, paired t-test).

The POC neurons project through the medial aspect of the coc_{TG} to innervate the CoGs

As a step towards localizing the POC neurons, we determined whether their axons preferentially projected through the lateral or medial aspect of the coc_{TG}. We anticipated that the POC neurons projected through the medial coc_{TG}, by analogy with the fact that most projections through the coc_{B} that innervate the CoG do so *via* the medial coc_{B} (Kirby and Nusbaum, 2007). To determine if this was indeed the case for the POC neurons, we first stimulated the *poc* with the entire coc_{TG} intact, to ensure the ability of this input to trigger the gastric mill rhythm in these preparations (Fig. 7). We then selectively transected either the lateral (N=3) or medial (N=3) aspect of the coc_{TG}, after which we again assessed the ability of *poc* stimulation to trigger the gastric mill rhythm (Fig. 7). There were no landmarks to enable precise transection of exactly one half of each coc_{TG}. Therefore, these transections were done in a fashion to ensure the retention of the lateral-most or medial-most coc_{TG}, with a variable degree of transection of the central aspect of this nerve from preparation to preparation.

The gastric mill rhythm was never triggered by *poc* stimulation after medial coc_{TG} transection (N=3) (Fig. 7B). By contrast, *poc* stimulation consistently triggered the gastric mill rhythm in every preparation after the lateral coc_{TG} was transected (N=3). The resulting motor pattern retained its characteristic pyloric-timed activity pattern during the protractor phase (Fig. 7C). In these latter experiments, the resulting gastric mill rhythm continued to persist for a long duration, ranging from 8 to 24 min (N=3).

To ensure that the CoG projection neurons and STG circuit neurons were still capable of generating the gastric mill rhythm after medial coc_{TG} transection, we stimulated the VCN neurons (Beenhakker et al., 2004; Beenhakker and Nusbaum, 2004). The VCN-triggered gastric mill rhythm was readily elicited in each of the three preparations after the medial coc_{TG} was transected (not shown).

The POC neurons appear to contain the peptide transmitter CabTRP Ia

There is a dense CabTRP Ia-immunoreactive (CabTRP Ia-IR) arborization within the anterior CoG neuropil, called the anterior commissural organ (ACO; Fig. 8A) (Messinger et al., 2005). The ACO innervates each CoG *via* a population of small diameter axons that project as a bundle through the medial aspect of the coc_{TG} (Goldberg et al., 1988; Messinger et al., 2005). This CabTRP Ia-IR bundle does not project through the coc_{B} (Fig. 8A) (Goldberg et al., 1988; Messinger et al., 2005). Based on the results of the coc_{TG} transection experiments reported above, and the fact that MCN1 and CPN2 arborize in the anterior CoG neuropil (Coleman and Nusbaum, 1994; Norris et al., 1994), we examined whether the ACO axons projected through the *poc* and therefore might be the axons of the POC neurons.

Whole-mount immunocytochemistry revealed that the ACO axon population did indeed project through the *poc* (Fig. 8B). Specifically, at the junction between the coc_{TG} and *poc*, a fraction of the CabTRP Ia-IR axon bundle in the medial coc_{TG} separated and projected through the *poc*, whereas the remainder projected posteriorly past the *poc* as a tight bundle along the medial coc_{TG} and terminated as the ACO in the ipsilateral CoG (N=16; Fig. 8B). This CabTRP Ia-IR fiber bundle projection continued in the medial coc_{TG}, past the *poc*, towards the TG (Fig. 8B; N=16).

As further support that the POC neurons were likely to be the source of the ACO, we determined whether the CabTRP-IR bundle in the medial coc_{TG} was transected or retained in each of the above

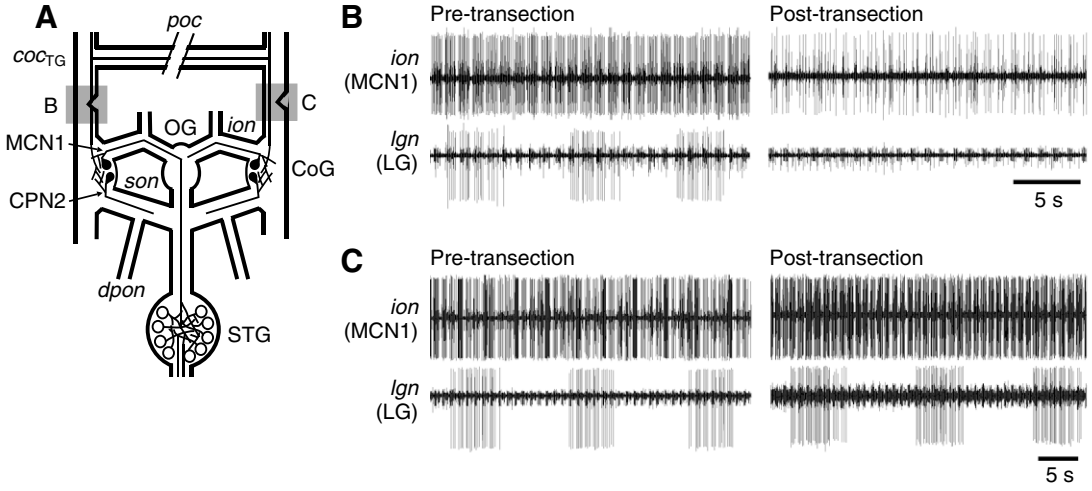

Fig. 7. The POC neurons project through the medial aspect of the coc_{TG} to influence MCN1 and CPN2 in the CoG. (A) STNS schematic indicating the location and extent of the coc_{TG} transections (grey boxes), the results of which are shown in B and C. (B) Transecting the medial aspect of the coc_{TG} eliminated the ability of *poc* stimulation to trigger the gastric mill rhythm. (Left) Before medial coc_{TG} transection, *poc* stimulation triggered the gastric mill rhythm. (Right) After medial coc_{TG} transection, *poc* stimulation did not trigger the gastric mill rhythm. (C) Transecting the lateral aspect of the coc_{TG} did not alter the ability of *poc* stimulation to trigger the gastric mill rhythm. The gastric mill rhythm was triggered both (left) before, and (right) after lateral coc_{TG} transection by *poc* stimulation. B and C are from different preparations.

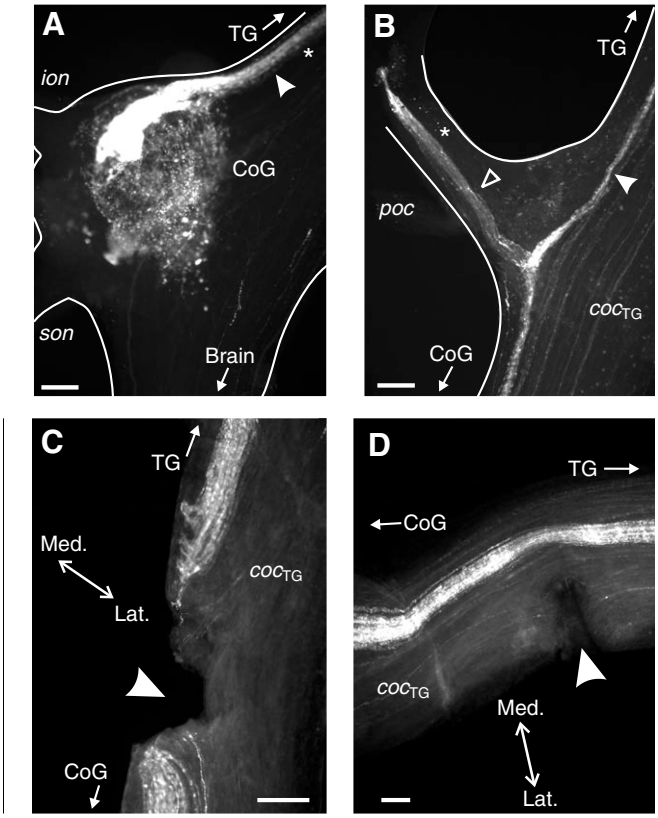

Fig. 8. A CabTRP Ia-immunoreactive axon bundle projects through the *poc* and medial aspect of the anterior coc_{TG} to form terminal arborizations in the CoG. (A) CabTRP Ia immunoreactivity (CabTRP Ia-IR) occurred in a tightly associated axon bundle in the medial aspect of the coc_{TG} (arrowhead) that terminated as a dense arborization in the antero-medial CoG. There was also more diffuse CabTRP Ia-IR throughout the CoG neuropil and in a subset of CoG neuronal somata. Asterisk indicates area examined to determine the number of CabTRP Ia-IR fibers present in the coc_{TG} (see text). (B) The CabTRP Ia-IR axon bundle in the medial aspect of the coc_{TG} (filled arrowhead) projected past the *poc* towards the TG, and also projected through the *poc* (open arrowhead). Asterisk indicates area examined to determine the number of CabTRP Ia-IR fibers present in the *poc* (see text). (C) CabTRP Ia-IR bundle was transected in a preparation in which the medial coc_{TG} was transected (arrowhead). (D) CabTRP Ia-IR bundle was not transected in a preparation in which the lateral coc_{TG} was transected (arrowhead). Spatial axes in C are for A–C. All scale bars, 150 μm.

coc_{TG} transection experiments. We found that, in each experiment in which the medial coc_{TG} was transected and *poc* stimulation no longer triggered the gastric mill rhythm, the CabTRP Ia-IR bundle had been transected (Fig. 8C; *N*=3). Conversely, the CabTRP Ia-IR bundle remained intact in preparations in which the lateral coc_{TG} was transected and *poc* stimulation still triggered the gastric mill rhythm (Fig. 8D; *N*=3).

We also combined CabTRP Ia immunocytochemistry and confocal microscopy to determine the number and distribution of axon diameters for the CabTRP Ia-IR axons in the *poc* and medial coc_{TG} bundle. In the *poc*, as well as in the coc_{TG} adjacent to the CoG, the CabTRP Ia-IR axons were of small diameter (<1 μm) and often tightly fasciculated. Their relatively small diameter and tight fasciculation made it difficult to unambiguously determine the number of individual axons present. However, we counted the fibers to the best of our ability in order to obtain an estimate of the population size. We obtained a distribution of CabTRP Ia-IR axon counts from the left coc_{TG} (88±5, *N*=5) and right coc_{TG} (83±6, *N*=5). In the same five preparations, the distribution of axon counts in the *poc* suggested a smaller number of CabTRP Ia-IR axons (66±4), supporting our observation that only a subset of the CabTRP Ia-IR bundle in each coc_{TG} projected through the *poc*. In no preparation was branching from the axon bundles seen within the coc_{TG} or *poc*.

In all preparations examined, the CabTRP Ia-IR bundle in the medial coc_{TG} was traced to the junction of the coc_{TG} with the TG (*N*=5). At this location, the POC axon bundle was less tightly fasciculated, often fanning out and covering a large portion of the nerve (not shown). In five separate preparations, we obtained similar axon counts to those from the coc_{TG} near the CoG (left coc_{TG}: 78±4; right coc_{TG}: 73±9). Owing to the density and intensity of CabTRP Ia-IR within the TG, it was not possible to identify the destination of the POC axons within this ganglion. Although CabTRP Ia-IR

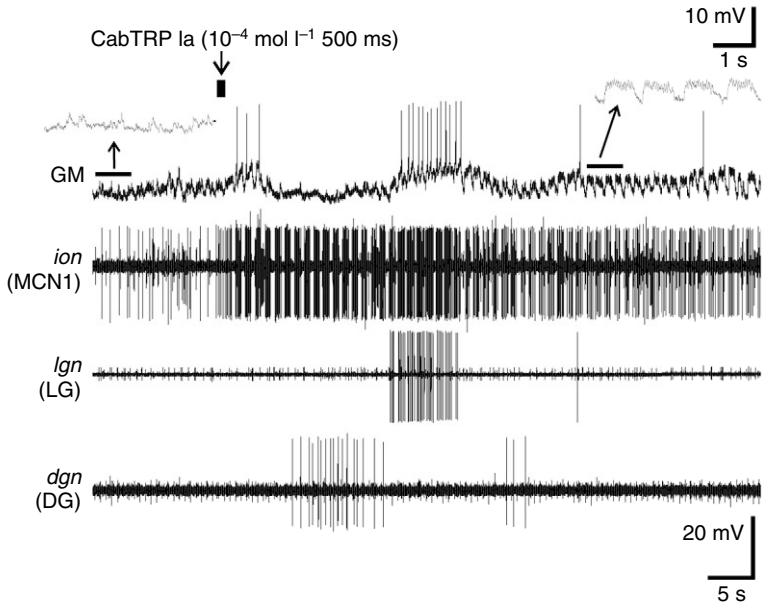

Fig. 9. Exogenous CabTRP Ia mimics the POC activation of MCN1 and CPN2. A brief (500 ms) puff of CabTRP Ia (10^{-4} mol l^{-1}) into the antero-medial aspect of the CoG neuropil excited MCN1 and CPN2 (monitored as excitatory postsynaptic potentials in GM; see text), and subsequently activated LG, GM and DG. Note that CabTRP Ia triggered pyloric-timed activity in MCN1, CPN2 and LG. Insets, showing an expanded time scale, indicate that the GM membrane potential was not pyloric-timed before CabTRP Ia application but exhibited barrages of excitatory postsynaptic potentials that were interrupted in pyloric-time after this application. Most hyperpolarized membrane potential: GM, –67 mV.

somata within the TG may well be the origin of the POC axons, no discrete clusters of 50-100 CabTRP Ia-IR somata were identified within this ganglion to support that possibility (data not shown).

The POC neurons appear to use the peptide transmitter CabTRP Ia

To determine whether ACO-released CabTRP Ia mediated the long-term actions of the POC neurons on MCN1 and/or CPN2, we examined whether focal application of CabTRP Ia mimicked the POC excitation of these projection neurons. In some of these experiments (e.g. Fig. 9), CPN2 activity was monitored *via* intracellular GM neuron recordings. CPN2 is the sole source of discrete excitatory postsynaptic potentials in the GM neuron (Norris et al., 1994).

Brief, focal application of CabTRP Ia (10^{-4} mol l^{-1}: 500 msec) into the anterior CoG neuropil triggered increased activity in MCN1 and CPN2 (*N*=4; Fig. 9). This increased activity was consistently pyloric-timed. In some preparations, the CabTRP Ia-triggered excitation of MCN1 and CPN2 led to the equivalent of a single gastric mill cycle, including an action potential burst in the retractor DG neuron preceding a burst in the protractor LG and GM neurons (Fig. 9).

To further assay whether CabTRP Ia mediated the actions of the POC neurons on MCN1 and/or CPN2, we determined whether suppressing the extracellular peptidase-mediated degradation of this peptide would prolong the POC influence on these projection neurons. To this end, we applied the endopeptidase inhibitor phosphoramidon (10^{-5} mol l^{-1}), which effectively prolongs the actions of both focally applied and neuronally released CabTRP Ia (Wood et al., 2000; Stein et al., 2007). Because the *poc* stimulus protocol used to trigger the gastric mill rhythm had such a long-lasting effect, we used briefer *poc* stimulations (15 Hz, 15 s) to achieve relatively brief control responses. These control stimulations triggered increased activity in MCN1 and CPN2 as well as a relatively short-lasting gastric mill rhythm (duration: 0.5–13 min, *N*=5; Fig. 10A). For example, in Fig. 10A the projection neuron activity was subsiding and the gastric mill rhythm had terminated by 90 s post-POC stimulation. Although phosphoramidon alone did not alter CPN2 or LG activity prior to *poc* stimulation (e.g. Fig. 10A, middle left panel), the POC-triggered rhythm during

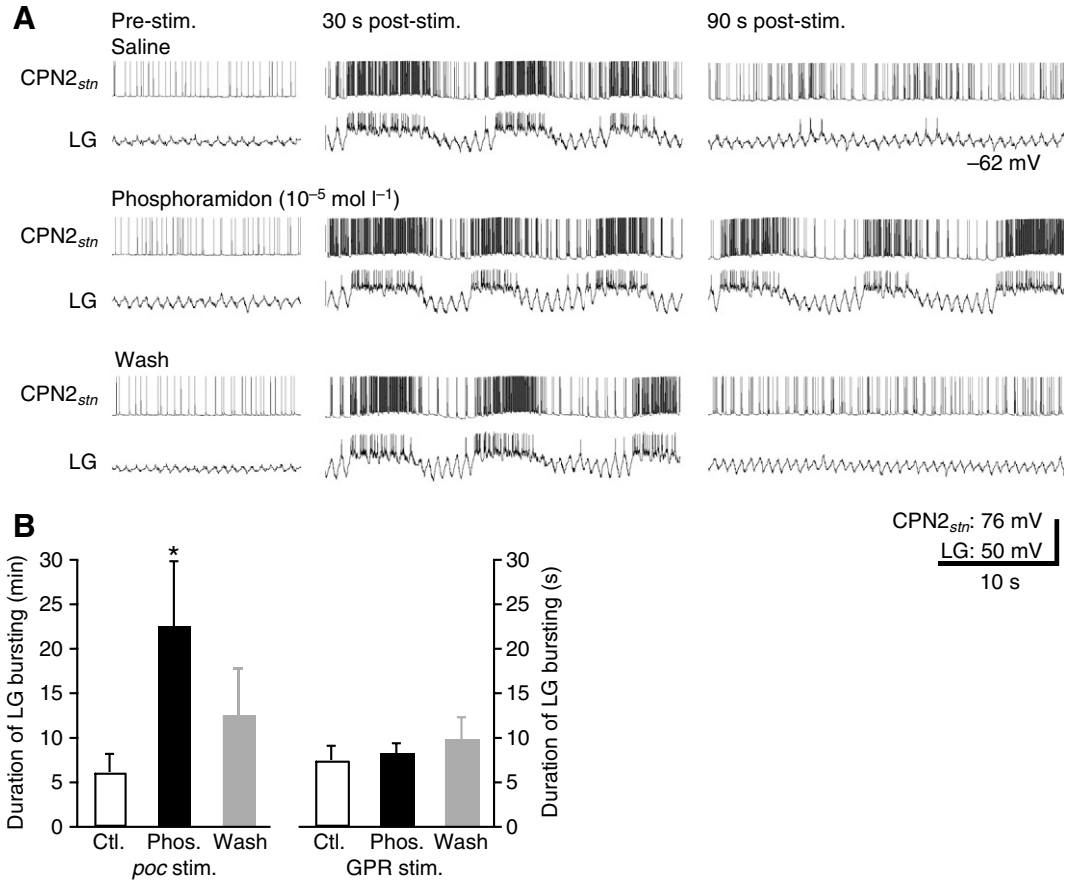

Fig. 10. Blocking extracellular peptidase-mediated degradation of CabTRP Ia prolongs the actions of the POC neurons. (A) Before, during and after superfusion of the endopeptidase inhibitor phosphoramidon (10^{-5} mol l^{-1}) to the CoGs, CPN2 was weakly active before *poc* stimulation and LG was silent (left panel: top, middle, bottom). CPN2 activity was monitored with an intra-axonal recording near the entrance to the STG (Beenhakker and Nusbaum, 2004). 30 s after *poc* stimulation (15 Hz, 15 s), the gastric mill rhythm was triggered (as indicated by the rhythmic LG bursting) and CPN2 activity was strengthened (middle panel: top, middle, bottom). 90 s after *poc* stimulation, the gastric mill rhythm had terminated and CPN2 activity had subsided during saline superfusion, both before and after phosphoramidon application (right panel: top, bottom). By contrast, 90 s after *poc* stimulation during phosphoramidon superfusion, CPN2 activity remained strong and the gastric mill rhythm persisted. (B) (Left) There was a significant increase in the duration of LG bursting after *poc* stimulation in the presence of phoshoramidon (Phos.,10^{-5} mol l^{-1}; $P<0.05$, *N*=5), compared with its bursting duration in saline before phosphoramidon application (Ctl.). (Right) By contrast, phosphoramidon (10^{-5} mol l^{-1}) did not alter the duration of LG bursting after stimulation of the proprioceptor sensory GPR neuron. Most hyperpolarized membrane potentials: $CPN2_{stn}$, –73 mV; LG, –63 mV.

phosphoramidon superfusion persisted for more than 90 s post-stimulation. After washout of the phosphoramidon, the POC action on CPN2 and the gastric mill rhythm returned to pre-application levels (Fig. 10A). In all cases, when phosphoramidon (10^{-5} mol l^{-1}) was superfused selectively to the CoGs, *poc* stimulation triggered a more prolonged excitation of MCN1 (not shown) and CPN2 and triggered a longer-lasting gastric mill rhythm (N=5).

We quantified the influence of phosphoramidon on the duration of POC actions by measuring the time during which the LG neuron generated rhythmic bursts after *poc* stimulation. Specifically, phosphoramidon application reversibly increased the duration of LG bursting by approximately fourfold (Fig. 10B; saline: 6.1±1.9 min, phosphoramidon: 22.5±6.7 min, wash: 12.5±4.8 min; N=5; P<0.05, RM one-way ANOVA and Student–Newman–Keuls test of multiple comparisons).

To control for the specificity of phosphoramidon action, we examined the influence of phosphoramidon on the duration of LG bursting after stimulating the gastro-pyloric receptor neuron (GPR) (Katz et al., 1989; Katz and Harris-Warrick, 1990). GPR stimulation excites MCN1 and CPN2 and thereby elicits the gastric mill rhythm (Blitz et al., 2004). GPR does not, however, contain CabTRP Ia but instead contains the co-transmitters acetylcholine, serotonin and allatostatin (Katz and Harris-Warrick, 1990; Skiebe and Schneider, 1994). Phosphoramidon (10^{-5} mol l^{-1}) superfusion did not change the duration of LG bursting after GPR stimulation (Fig. 10B; N=4, P>0.5 RM one-way ANOVA).

DISCUSSION

We have identified an extrinsic input, the POC neurons, that triggers a long-lasting activation of identified CoG projection neurons and thereby initiates a distinct version of the gastric mill rhythm in the *C. borealis* STG. The POC axons project as a tightly associated bundle through the medial aspect of each coc_{TG}, from the direction of the TG, to innervate the ipsilateral CoG. A subset of these axons also project through the *poc*, enabling them to innervate the contralateral CoG. The long-term activation of the CoG projection neurons MCN1 and CPN2 by POC stimulation is probably mediated by the peptide transmitter CabTRP Ia.

The POC neurons appear to be the source of the extensive CabTRP Ia-IR arborization in the anterior CoG neuropil (Goldberg et al., 1988). This arborization was recently characterized as a neurohemal organ, the ACO, which is well-situated to release CabTRP Ia into the hemolymph as a circulating hormone in the related species *Cancer productus* (Messinger et al., 2005). In that study, the ACO was also studied extensively for the presence of co-transmitters but none were identified. One function of circulating hormones, including CabTRP Ia, is to modulate the properties of muscles that mediate movements of the foregut (Jorge-Rivera and Marder, 1996; Messinger et al., 2005). Therefore, POC-mediated release of CabTRP Ia may well coordinately trigger the gastric mill rhythm and modulate the response of gastric mill muscles to the incoming motor pattern. Recently, a second isoform of CabTRP (CabTRP II) was isolated from the STNS, including the CoGs (Stemmler et al., 2007). Both CabTRP isoforms are recognized by the same antibody and have similar actions on the pyloric rhythm (Stemmler et al., 2007). Thus, either or both CabTRP peptides may mediate the POC actions in this system.

The likelihood that the CabTRP Ia released from the ACO terminals locally excites MCN1 and CPN2 supports the hypothesis that this neuronal population has both paracrine and endocrine functions. Given the sensitivity of MCN1 and CPN2 to relatively brief POC stimulation, there may well be times when this input acts largely or exclusively as a local modulator of neuronal activity, whereas at other times its activation results in both paracrine and endocrine actions. Previous studies in other systems have established the ability of the same neurons to release signaling molecules that act both locally, in a paracrine fashion, and as circulating hormones (Mayeri, 1979; Sigvardt et al., 1986; Jung and Scheller, 1991; Loechner and Kaczmarek, 1994; Ludwig and Pittman, 2003; Fort et al., 2004; Oliet et al., 2007).

We have not yet identified the location of the POC neuronal somata. These somata may be located within the TG, in which the coc_{TG} terminates. In *C. borealis*, the entire ventral nerve cord is compressed into the single TG (Horridge, 1965). However, the POC somata may instead be located within one or more peripheral nerves or related structures, as is common for muscle- and abdominal-stretch sensitive sensory neuron populations in decapod crustaceans (Alexandrowicz, 1951; Cattaert et al., 2002; Katz et al., 1989; Beenhakker et al., 2004). Whether these neurons originate in the TG or a peripheral structure, their point of origin appears likely to be outside the STNS. Thus, the POC neurons may help to coordinate the chewing of food with other behaviors, perhaps acting as a trigger for chewing in response to cues from other regions of the animal. In addition, these neurons may well contribute to the long-term maintenance of chewing in the intact crab and lobster insofar as the gastric mill rhythm can persist for hours after food is ingested (Fleischer, 1981; Turrigiano and Selverston, 1990). Similarly, there are long-lasting actions of the vertebrate tachykinin peptide, substance P, on rhythmic locomotor activity in the vertebrate CNS (Treptow et al., 1983; Parker and Grillner, 1999). Further, short-duration sensory stimuli can trigger long-term activation of descending reticulospinal neurons that drive locomotion in lamprey (Di Prisco et al., 1997).

The POC-elicited gastric mill rhythm is qualitatively different from gastric mill rhythms elicited by other extrinsic inputs in *C. borealis*. Specifically, the protraction phase activity pattern of MCN1, CPN2 and LG is pyloric-timed during the POC-triggered rhythm whereas these neurons exhibit tonic protraction phase activity during other gastric mill rhythms (Beenhakker and Nusbaum, 2004; Blitz et al., 2004; Christie et al., 2004; Saideman et al., 2007b). The LG-innervated muscles mediate protraction of the lateral teeth within the gastric mill. Thus, the distinct LG neuron activity pattern during the POC-triggered gastric mill rhythm could result in a different mode of chewing relative to the previously characterized gastric mill rhythms. In fact, both smooth protraction and pyloric-timed movements of the lateral teeth occur during *in vivo* endoscopic recordings of these teeth movements in *Cancer* crabs (Heinzel et al., 1993). Future work will be needed to establish whether the pyloric-timed LG neuron pattern is retained at the level of the LG-innervated muscles during the POC-triggered rhythm.

The distinct activity pattern of MCN1 during the POC rhythm also has consequences for motor pattern generation and inter-circuit coordination. For example, the pyloric circuit feedback to MCN1 during the protractor phase of the spontaneous POC-like gastric mill rhythm enables the pyloric rhythm to regulate the speed and pattern of the gastric mill rhythm, as well as its coordination with the pyloric rhythm (Wood et al., 2004). This is also evident in the present study from the change in gastric mill cycle period that occurred when the pyloric rhythm was suppressed. This pyloric regulation of the gastric mill rhythm during the protractor phase, *via* feedback inhibition of MCN1 and CPN2, occurs only during the POC-type of gastric mill rhythm (Beenhakker and Nusbaum, 2004; Blitz et al., 2004; Christie et al., 2004). Previous work documented additional cellular and synaptic mechanisms

underlying inter-circuit regulation during other versions of the gastric mill rhythm (Bartos and Nusbaum, 1997; Clemens et al., 1998; Bartos et al., 1999; Wood et al., 2004). Although coordination between different behaviors, such as locomotion and respiration, occurs in many animals (Bramble and Carrier, 1983; Syed and Winlow, 1991; Kawahara et al., 1989; Morin and Viala, 2002; Saunders et al., 2004), the underlying cellular mechanisms remain to be determined in these other systems.

It appears likely that the POC-like gastric mill rhythm previously studied by Wood et al. (Wood et al., 2004) does represent POC-triggered rhythms, presumably resulting from POC activation that occurred during the dissection. In both cases there was a prominent activation of MCN1, and they further share the distinct pyloric-timed activity pattern during the protractor phase. CPN2 activity, however, was not studied in the earlier work (Wood et al., 2004). Wood et al. (Wood et al., 2004) did establish that pyloric-timed MCN1 stimulation elicited a gastric mill rhythm that was comparable to the spontaneous POC-like rhythm.

Given that MCN1 and CPN2 are necessary and sufficient to elicit the VCN- and GPR-elicited gastric mill rhythms (Blitz et al., 2004; Beenhakker and Nusbaum, 2004), it is likely that they play pivotal roles during the POC-triggered rhythm as well. Addressing this issue will provide insight into the extent to which this system uses convergent activation of the same projection neurons to elicit distinct activity patterns. This would be in contrast to the prevalent hypothesis in other model systems that the generation of distinct but related movements results from the activation of distinct but overlapping sets of projection neurons (Georgopoulos, 1995; Kristan and Shaw, 1997; Lewis and Kristan, 1998; Liu and Fetcho, 1999).

LIST OF ABBREVIATIONS

ACO	anterior commissural organ
AGR	anterior gastric receptor
AM	anterior median
CabTRP Ia	*Cancer borealis* tachykinin-related peptide Ia
CabTRP Ia-IR	*Cancer borealis* tachykinin-related peptide Ia immunoreactivity/immunoreactive
coc	circumoesophageal connective
coc_B	circumoesophageal connective projecting from the CoG to the brain
coc_{TG}	circumoesophageal connective projecting from the CoG to the TG
CoG	commissural ganglion
CPN2	commissural projection neuron 2
DG	dorsal gastric
dgn	dorsal gastric nerve
dpon	dorsal posterior oesophageal nerve
EPSPs	excitatory postsynaptic potentials
GM	gastric mill
GPR	gastro-pyloric receptor
IC	inferior cardiac
Int1	interneuron 1
LG	lateral gastric
lgn	lateral gastric nerve
LPG	lateral posterior gastric
MCN1	modulatory commissural neuron 1
MG	medial gastric
OG	oesophageal ganglion
PD	pyloric dilator
pdn	pyloric dilator nerve
poc/POC	post-oesophageal commissure
STG	stomatogastric
STNS	stomatogastric nervous system
TG	thoracic ganglion
VCNs	ventral cardiac neurons
VD	ventricular dilator

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