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RESEARCH ARTICLE

Coordination of distinct but interacting rhythmic motor programs by a modulatory projection neuron using different co-transmitters in different ganglia

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SUMMARY

While many neurons are known to contain multiple neurotransmitters, the specific roles played by each co-transmitter within a neuron are often poorly understood. Here, we investigated the roles of the co-transmitters of the pyloric suppressor (PS) neurons, which are located in the stomatogastric nervous system (STNS) of the lobster *Homarus americanus*. The PS neurons are known to contain histamine; using RT-PCR, we identified a second co-transmitter as the FMRFamide-like peptide crustacean myosuppressin (Crust-MS). The modulatory effects of Crust-MS application on the gastric mill and pyloric patterns, generated in the stomatogastric ganglion (STG), closely resembled those recorded following extracellular PS neuron stimulation. To determine whether histamine plays a role in mediating the effects of the PS neurons in the STG, we bath-applied histamine receptor antagonists to the ganglion. In the presence of the antagonists, the histamine response was blocked, but Crust-MS application and PS stimulation continued to modulate the gastric and pyloric patterns, suggesting that PS effects in the STG are mediated largely by Crust-MS. PS neuron stimulation also excited the oesophageal rhythm, produced in the commissural ganglia (CoGs) of the STNS. Application of histamine, but not Crust-MS, to the CoGs mimicked this effect. Histamine receptor antagonists blocked the ability of both histamine and PS stimulation to excite the oesophageal rhythm, providing strong evidence that the PS neurons use histamine in the CoGs to exert their effects. Overall, our data suggest that the PS neurons differentially utilize their co-transmitters in spatially distinct locations to coordinate the activity of three independent networks.

Key words: FMRFamide, histamine, myosuppressin, neuromodulation, pyloric suppressor neuron, stomatogastric nervous system.

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INTRODUCTION

While neurons were originally thought to contain a single neurotransmitter, it is now well established that most, if not all, contain multiple signaling agents (Trudeau and Gutiérrez, 2007). However, how co-transmitters are distributed within neurons and used to affect different behavioral outputs is still under investigation. Dale's principle (Dale, 1935; Eccles et al., 1954; Eccles, 1976) proposed that all neuronal co-transmitters are uniformly distributed and co-released. While this may be true for some neurons, others are clearly capable of differentially packaging and trafficking neuroactive compounds to distinct compartments (Sossin et al., 1990; Hattori et al., 1991; Sámano et al., 2006; Kueh and Jellies, 2012) and/or differentially releasing co-transmitters in response to different activity patterns (Whim and Lloyd, 1989). Even when co-released, co-transmitter actions can be compartmentalized *via* the differential distribution of their receptors (Marder et al., 1995; Thurimulai and Marder, 2002).

Invertebrate nervous systems, including the crustacean stomatogastric nervous system (STNS), with its small number of large, uniquely identifiable neurons (Nusbaum et al., 2001; Skiebe, 2001; Nusbaum and Beenhakker, 2002; Fénelon et al., 2003; Fénelon et al., 2004; Hooper and DiCaprio, 2004; Marder and Bucher, 2007; Stein, 2009), have for many years provided important insights into our understanding of co-transmission (Kupfermann, 1991; Nusbaum et al., 2001; Nässel, 2009; Christie et al., 2010). In the present study, we identified the peptide co-transmitter in a pair of modulatory histaminergic projection neurons of the lobster STNS, then examined the roles played by this peptide and histamine in simultaneously modulating three different rhythmic motor patterns.

The pyloric suppressor (PS) neurons of homarid lobsters contain at least two co-transmitters: histamine (Mulloney and Hall, 1991; Le Feuvre et al., 2001) and an FMRFamide-like peptide (Fénelon et al., 1998). Their morphology is well characterized, with terminal endings in all four ganglia of the STNS [the oesophageal ganglion (OG), paired commissural ganglia (CoGs) and stomatogastric ganglion (STG)] (Meyrand et al., 1994). Stimulation of the PS neurons in the European lobster, *Homarus gammarus*, distinctly alters the gastric mill and pyloric patterns produced in the STG, as well as the oesophageal pattern, generated in the CoGs (Cazalets et al., 1987; Cazalets et al., 1990a; Cazalets et al., 1990b; Meyrand et al., 1991; Meyrand et al., 1994). Specifically, pyloric neurons start firing in longer bursts, more similar to gastric than pyloric timing (Cazalets et al., 1987). Furthermore, the cycle frequency of the oesophageal rhythm increases and becomes phase-locked with the combined gastric mill/pyloric pattern (Meyrand et al., 1994).

Overall, PS modulation in *H. gammarus* dismantles the three pattern generators, and re-assembles them into a single functional network (Meyrand et al., 1991).

Although the effects of the PS neurons on these motor patterns have been well documented, the roles of the individual co-transmitters utilized by the PS neurons to modulate these spatially segregated networks are unknown. To determine whether the co-transmitters exerted their effects equally in both the STG and the CoGs, we identified the FMRFamide-like peptide co-transmitter within the PS neurons, and then tested the hypothesis that the PS neurons differentially utilize their co-transmitters to coordinate three motor patterns produced by the spatially distinct ganglia into a single unified motor pattern.

MATERIALS AND METHODS

Animals

Adult American lobsters, *Homarus americanus* Milne-Edwards, were purchased from local seafood retailers (Brunswick and Bar Harbor, ME, USA). Males and females weighing 450–600 g were kept in tanks of re-circulating seawater at 10–12°C on a 12 h:12 h light:dark cycle. Lobsters were fed approximately weekly on a diet of chopped squid.

Dissection

Lobsters were cold-anesthetized by packing in ice for ~20–30 min prior to dissection. As the stomach was dissected out of the cephalothorax of the lobster, chilled (~4°C) physiological saline [composition in mmol l^{-1}: 479.12 NaCl, 12.74 KCl, 13.67 $CaCl_2$, 20.00 $MgSO_4$, 3.91 Na_2SO_4, 11.45 trizma base and 4.82 maleic acid; pH 7.45] was rinsed over it to keep it cold.

Once the stomach was removed, it was opened ventrally from the oesophagus to the pylorus and was pinned out, dorsal side up, in a Sylgard-coated dish filled with cold saline. The STNS (Fig. 1A), including all four ganglia and the oesophageal, gastric and pyloric motor nerves, was microdissected from the surrounding musculature and pinned out in a clear Sylgard-coated Petri dish. During the microdissection, the saline was changed periodically to maintain the temperature of the preparation at ~10°C. For reverse transcription-polymerase chain reaction (RT-PCR) profiling experiments (see below), the cardiac ganglion (CG) and samples of cardiac muscle were isolated from the heart by manual microdissection in chilled physiological saline.

RT-PCR tissue profiling

Total RNA was isolated from freshly dissected lobster CoGs, inferior ventricular nerves (*ivn*s), CGs or cardiac muscle using an SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA; catalog no. Z3100). For each experiment, two CoGs, four *ivn*s, four CGs or several small pieces of cardiac muscle were pooled to obtain sufficient starting material. Before RNA isolation, the pooled tissues were manually minced in the RNA lysis buffer with spring scissors and then further homogenized using a QIAshredder spin-column homogenizer (Qiagen, Valencia, CA, USA; catalog no. 79654). RNA concentration was determined using a Nanodrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). cDNA was synthesized from the isolated mRNA using a SuperScriptTMIII First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA; catalog no. 18080051). PCR was carried out on a DNA Engine thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) using gene-specific primers (forward: CGAACGTGAGTTAC-TGAGGCT; reverse: TCCTCCATGATCCCTGC) and GoTaq Master Mix (Promega; catalog no. M7138). To confirm the identity of the RT-PCR products (predicted to be a 459 bp amplicon), a sample of a given product was cloned into a pCR2.1 TOPO vector using a TOPO TA cloning kit (Invitrogen) and sequenced using vector-specific (M13 forward and reverse) sequencing primers.

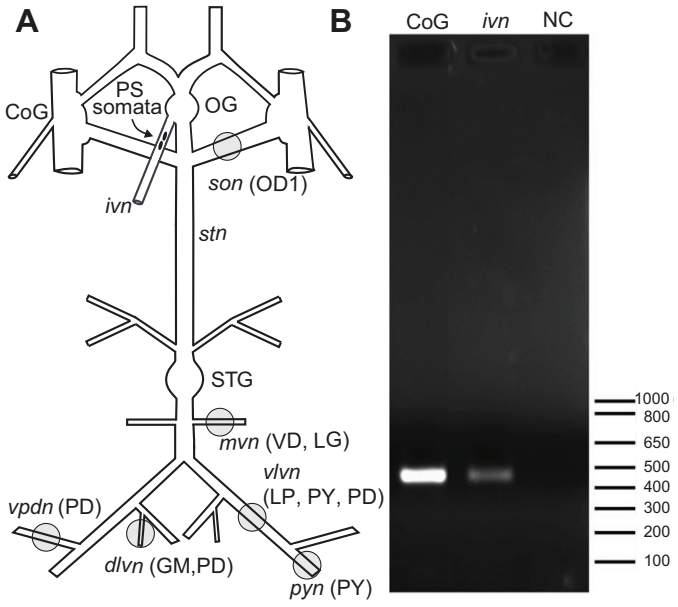

Fig. 1. Schematic representation of the *Homarus americanus* stomatogastric nervous system (STNS) and RT-PCR profiling of crustacean myosuppressin (Crust-MS) transcript in the pyloric suppressor (PS) neurons of the lobster. (A) Schematic representation of the lobster STNS showing the location of the PS neuron somata, as well as the locations of extracellular nerve recordings (grey circles) and the neurons recorded from each nerve. (B) RT-PCR profiling of Crust-MS transcript in the PS neurons indicates that the FLRFamide-like peptide in these neurons is Crust-MS. Using a gene-specific primer set, a robust band of the predicted Crust-MS PCR amplicon (459 bp in length) was consistently detected in the inferior ventricular nerve (*ivn*; Lane 2), the location of the PS neurons in *Homarus*, as well as in the commissural ganglion (CoG; Lane 1), a known source of Crust-MS peptide (Stemmler et al., 2007). In contrast, no product was detected in the negative (no cDNA) control (NC; Lane 3). Other abbreviations: *dlvn*, dorsal lateral ventricular nerve; GM, gastric mill neuron; LG, lateral gastric neuron; LP, lateral pyloric neuron; *mvn*, medial ventricular nerve; OD1, oesophageal dilator 1 neuron; OG, oesophageal ganglion; PD, pyloric dilator neuron; PY, pyloric neuron; *pyn*, pyloric nerve; *son*, superior oesophageal nerve; STG, stomatogastric ganglion; *stn*, stomatogastric nerve; VD, ventral dilator neuron; *vlvn*, ventral lateral ventricular nerve; *vpdn*, ventral pyloric dilator nerve.

Electrophysiological recordings

The STG and the CoGs were desheathed to allow Crust-MS and histamine to act directly on cells without interference from the sheath. The saline and peptide baths were superfused at ~5 ml min^{-1} and were kept cool (10–13°C) using a Peltier device (Warner Instruments, Hamden, CT, USA).

Small petroleum jelly wells were made surrounding sections of each motor nerve of interest, including: the dorsal lateral ventricular nerve (*dlvn*), the ventral lateral ventricular nerve (*vlvn*), the medial ventricular nerve (*mvn*), the pyloric nerve (*pyn*), the ventral pyloric dilator nerve (*vpdn*), the ventral posterior oesophageal nerve (*vpon*), the superior oesophageal nerve (*son*) and the *ivn*. Stainless steel pin electrodes were placed in the petroleum jelly wells to record or

stimulate extracellularly. Amplification of the signal was achieved using A-M Systems Model 1700 Differential AC Amplifiers (A-M Systems, Sequim, WA, USA), as well as Brownlee Precision Instrumentation Model 210A Amplifiers (Brownlee Precision Company, San Jose, CA, USA). Cambridge Electronic Design Power 1401 and Spike2 versions 6 and 7 (Cambridge Electronic Design, Cambridge, UK) were used for data recording.

Using a pin electrode and a petroleum jelly well on the *ivn*, the PS neurons, whose cell bodies are located within the *ivn*, and whose axons extend down the *ivn* and from there into the stomatogastric nerve (*stn*), as well as into the *son*s and the inferior oesophageal nerves (*ion*s), were stimulated extracellularly. Previous studies (e.g. Cazalets et al., 1990a) have shown that the only projection neurons activated by *ivn* stimulation in *Homarus* are the PS neurons. Stimuli were generated using a Grass S88 dual output square pulse stimulator (Astro-Med, West Warwick, RI, USA) and a Grass SIU 5 stimulus isolation unit. PS neuron stimulation consisted of 4.5 s trains of 0.5 ms pulses at ~30 Hz. Stimulations were performed while recording extracellularly from oesophageal, gastric and pyloric motor nerves. These stimulation parameters elicited a response that appeared to be not only qualitatively, but also quantitatively similar to that elicited by bath application of Crust-MS (10^{-6} mol l^{-1}).

Crust-MS (pQDLDHVFLRFamide) was custom synthesized by GenScript (Scotch Plains, NJ, USA) and stored as a 10^{-3} mol l^{-1} solution in deionized water at −20°C. It was diluted to 10^{-6} or 10^{-7} mol l^{-1} in cold saline just prior to use. In initial experiments, Crust-MS was superfused over the STG for ~10 min, which was sufficient to lead to a stable pattern. In subsequent experiments, Crust-MS was superfused over the STG for only 30–45 s, followed by a saline wash of approximately 20–30 min. This short bath application allowed us to focus on the immediate effects of the peptide application rather than its longer-term effects, a timeline consistent with the expected availability of the peptide following stimulation of the PS neurons.

Because histamine receptors desensitize rapidly with long-term or repeated exposure (Claiborne and Selverston, 1984), the release of histamine was mimicked using a Picospritzer (General Valve, Pine Brook, NJ, USA) to eject a short puff of histamine onto either the STG or the CoGs. Histamine dihydrochloride (Sigma-Aldrich, St Louis, MO, USA; catalog no. 53300) was dissolved directly into saline at a concentration of 10^{-4} mol l^{-1}, and was puffed at 100–140 kPa for 2–3 ms onto the STG and 6–7 ms onto the CoG in separate experiments to examine the effects of histamine on the neurons contained within each ganglion. The recorded response to histamine's effect also served as the indicator for when the histamine receptor antagonists had fully blocked histamine responses. Although most histamine responses that have been examined in crustaceans are blocked by the H_2 receptor antagonist cimetidine (Bayer et al., 1989; Callaway and Stuart, 1989; El Manira and Clarac, 1994; Christie et al., 2004; Cebada and García, 2007; McCoole et al., 2011), it failed to block the responses to puffed histamine in either the CoG or the STG of *H. americanus*. We therefore tested a series of other histamine receptor antagonists, and found that the responses to puffs of histamine were blocked most effectively by a cocktail of two antagonists, the H_1 receptor antagonist *trans*-triprolidine hydrochloride (Tocris Bioscience, Ellisville, MO, USA; catalog no. 0662) and the H_2 receptor antagonist ranitidine hydrochloride (Tocris Bioscience; catalog no. 1967), both of which have been shown to block some crustacean histamine receptors (Callaway and Stuart, 1989; Cebada and García, 2007); the cocktail consisted of 10^{-6} to 5×10^{-6} mol l^{-1} triprolidine and 10^{-5} mol l^{-1} ranitidine. Each solution was prepared fresh daily, and the mixture of the two antagonists was bath applied over either the STG or the CoGs until the response elicited by histamine puffs was blocked.

Data analysis

Neuronal responses to Crust-MS bath application, PS stimulation and histamine puff application were quantified by measuring the burst duration and cycle period for specific neurons of interest [pyloric (PY), pyloric dilator (PD) and oesophageal dilator 1 (OD1) neurons]. Burst parameters were analyzed using built-in functions of Spike2 v6 and 7 and custom-written scripts (available at http://www.whitney.ufl.edu/BucherLab/Spike2_Scripts2_box.htm).

The effects of Crust-MS in the STG were quantified from the time of the onset of modulator to the time at which neurons returned to a stable baseline pattern (i.e. when the neuron in question generated three bursts at control cycle frequency, with each burst consisting of no fewer than four spikes). Similarly, the modulatory effects resulting from stimulation of the PS axons in the *ivn* were measured from the end of the extracellular PS stimulation to the time at which neurons in the STG exhibited three bursts similar to the control pattern. Changes in neuronal activity produced by histamine application were measured from the end of the histamine puff to the time at which three bursts similar to the control pattern occurred, as with PS stimulation analysis.

To analyze the oesophageal pattern, the frequency of the five bursts immediately before each stimulation or application of neuromodulator was compared with the frequency of the five bursts directly after PS stimulation, histamine application or Crust-MS application. Pooled data were analyzed and graphed using Prism 5 (GraphPad Software, La Jolla, CA, USA).

For effects of all treatments on each of the three motor patterns, burst parameters under different conditions were compared using repeated-measures ANOVA, followed by *post hoc* Tukey's tests. Differences were considered significant if they reached an α-level of <0.05. Specific statistical values are presented in the associated figure legends. *N*-values refer to the number of individual preparations analyzed.

RESULTS

RT-PCR analysis suggests that Crust-MS is present in the lobster PS neurons

To date, over two dozen FMRFamide-like peptides have been identified in the nervous system of *H. americanus* (Trimmer et al., 1987; Stemmler et al., 2007; Dickinson et al., 2007; Ma et al., 2008); amongst these is pQDLDHVFLRFamide (Stemmler et al., 2007; Ma et al., 2008), a member of the Crust-MS subfamily. Immunohistochemistry using antibodies directed against the sequence FMRFamide has long suggested that at least a subset of these peptides is present within the lobster STNS (Fénelon et al., 1998), including within the cell bodies of the PS neurons (Fénelon et al., 1998). Given that at least some of the actions of the PS neurons within the STNS are inhibitory, specifically including a suppression of active pyloric cycling in *H. gammarus* (Cazalets et al., 1987; Cazalets et al., 1990a; Cazalets et al., 1990b; Meyrand et al., 1994), and that the insect peptide leucomyosuppressin also disrupts pyloric activity (Tierney et al., 1997), we hypothesized that the lobster myosuppressin pQDLDHVFLRFamide might be the FMRFamide-like peptide co-transmitter present in the lobster PS cells.

Although we were unable to obtain a myosuppressin-specific antibody to assess the presence or absence of pQDLDHVFLRFamide in the PS neurons of *H. americanus*, a previously identified lobster cDNA encoding Crust-MS (Stevens et al., 2009) allowed for molecular screening of these cells for the

peptide using RT-PCR. Specifically, mRNA isolated from the *ivn* (Fig. 1A), in which the PS somata are located, was used as a template to synthesize nerve-specific cDNA; transcript-specific primers were subsequently used to amplify a portion of the Crust-MS-encoding sequence from the cDNA. As can be seen in Fig. 1B, a band corresponding to the size of the expected Crust-MS fragment (459 bp) was consistently amplified (N=3 independent tissue extractions and PCR amplifications) from the *ivn*-specific cDNA, as well as from that of another portion of the nervous system known to contain Crust-MS (Stemmler et al., 2007), the CoG (Fig. 1A). No PCR product was detected in cDNA derived from tissues that do not stain for FMRFamide-like peptides using antibodies (i.e. the CG and cardiac muscle, data not shown) or in a negative (no cDNA) control (Fig. 1B). Sequence analysis of the *ivn* PCR product confirmed it to be identical to the targeted portion of the Crust-MS-encoding transcript (data not shown). As the PS neurons are the only somata that exhibit FMRFamide-like labeling in the *ivn*, and thus the only possible source of a FMRFamide-like peptide-encoding transcript, these results strongly support the hypothesis that pQDLDHVFLRFamide (Crust-MS) is the isoform of FMRFamide-like peptide produced by the PS neurons.

Prolonged bath application of Crust-MS to the STG uniquely modulates the gastric and pyloric rhythms

The central pattern generators (CPGs) contained within the STG are composed almost entirely of motor neurons; consequently, the activity of the CPGs can be readily monitored on motor nerves. These patterns are almost always constitutively active in the intact STNS preparation from *H. americanus*. The gastric mill motor pattern (Fig. 2A) includes the lateral gastric (LG) and gastric mill (GM) neurons, with a cycle period of approximately 8–20 s. The pyloric pattern, with a period of 1–2 s, includes the PD, lateral pyloric (LP), PY and ventricular dilator (VD) neurons. In the absence of modulation, these two motor patterns maintain independent rhythms (Fig. 2A).

Prolonged (10 min) bath application of Crust-MS to the STG distinctly modulated the gastric mill and pyloric patterns, such that the two independent networks merged to form a single functional network (N=11 of 11 preparations; Fig. 2B). Activity in some pyloric neurons, such as the PD and LP neurons, was completely suppressed, while activity in others, such as the PY and VD neurons, assumed a more gastric-like rhythmicity in all preparations. The gastric mill rhythm was enhanced overall, with the GM and LG neurons generally exhibiting more intense bursts than those recorded in control saline. However, the detailed effects on the gastric mill rhythm were more variable than those seen on pyloric neurons. In some preparations, burst duration of the GM neurons was relatively unchanged (N=2 of 7), while in others, GM burst duration increased (N=4 of 7) or decreased (N=1 of 7) during Crust-MS application. The effects of Crust-MS on cycle frequency were also somewhat variable, with virtually no change in some preparations (N=3 of 7), but clear cycle frequency increases in others (N=4 of 7).

PS stimulation, Crust-MS application and focal histamine application to the STG have overlapping effects on the pyloric and gastric mill patterns

The effects of PS neuron stimulation on the pyloric and gastric mill patterns recorded in *H. americanus* were essentially identical to those previously described in *H. gammarus* (Cazalets et al., 1987; Cazalets et al., 1990a; Meyrand et al., 1991; Meyrand et al., 1994). Specifically, some pyloric neurons, such as the PD and LP neurons (N=19 of 20; LP data not shown), were completely

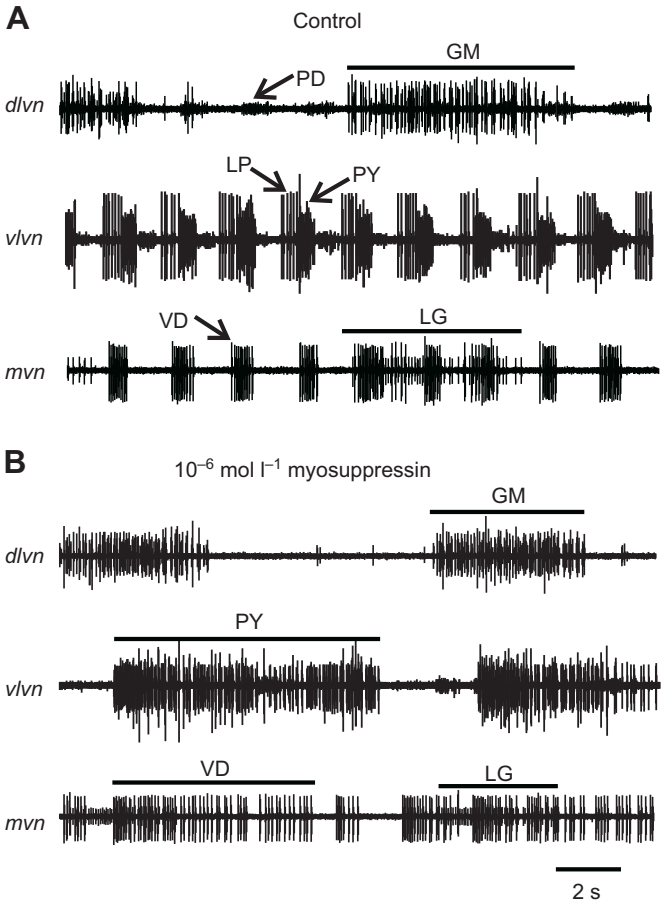

Fig. 2. Crust-MS modulates both the pyloric and gastric mill patterns, increasing the interactions between them, as seen in extracellular recordings from the dorsal lateral ventricular nerve (*dlvn*), ventral lateral ventricular nerve (*vlvn*) and medial ventricular nerve (*mvn*) in the stomatogastric nervous system (STNS) of *H. americanus*. (A) In control saline, lateral pyloric (LP), pyloric (PY), ventricular dilator (VD) and pyloric dilator (PD) neurons fired in pyloric time. Gastric mill (GM) and lateral gastric (LG) neurons fired in gastric time. (B) Bath application of 10^{-6} mol l^{-1} Crust-MS uniquely modulated the pyloric and gastric mill networks. PD and LP neurons were completely suppressed, whereas PY and VD neurons began firing in a more gastric timed pattern, coordinated with other gastric motor neurons (LG and GM shown). In this preparation, burst duration decreased in the GM and LG neurons; in some preparations, GM burst duration increased or remained the same.

inhibited immediately following extracellular stimulation of the PS neurons. Others, such as the PY (N=18 of 18) and VD (N=9 of 9) neurons, were excited and assumed a more gastric-like cycle period (Fig. 3A,B). In addition, the gastric mill pattern was enhanced, with the GM neurons (N=9 of 11) exhibiting a shorter cycle period and/or more intense bursts. Together, these effects created a unified rhythmic pattern with a cycle period that was usually intermediate to those of the two independent networks. Activity gradually returned to control patterns of firing after the stimulation.

The effects of PS stimulation and long-term Crust-MS bath application on the gastric and pyloric patterns were similar, but are difficult to compare because of the different time courses of activation. The PS neurons in *H. gammarus* have been shown to fire in repeated bursts (Meyrand et al., 1994); this would result in the release of Crust-MS for relatively short periods of time. We

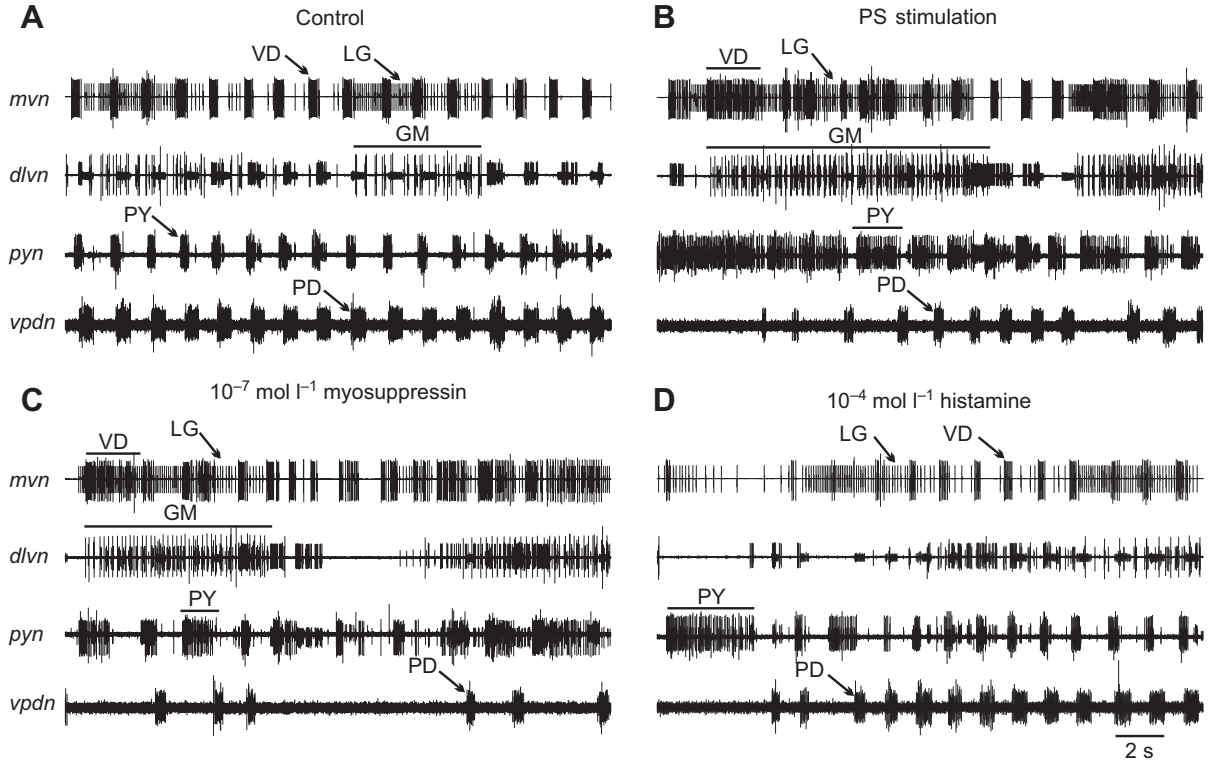

Fig. 3. The effects of extracellular stimulation of the pyloric suppressor (PS) neurons on the pyloric and gastric mill patterns overlap with effects of Crust-MS and histamine application, as seen in extracellular recordings from a single preparation. (A) In control saline, the pyloric (PY), pyloric dilator (PD) and lateral pyloric (LP) (not shown) neurons all fired with pyloric timing, while the gastric mill (GM) neuron fired tonically. (B) Immediately after extracellular stimulation of the PS neurons (4 s at 30 Hz), some pyloric neurons, such as PD and LP (not shown), were completely suppressed. Other neurons [PY and ventricular dilator (VD) shown] that fired in pyloric time prior to PS stimulation began to fire in longer bursts, similar to gastric activity. GM activity was enhanced following PS stimulation, with shorter, more defined bursts. (C) Similar to PS stimulation, transient application (30 s, 10^{-7} mol l^{-1}) of Crust-MS to the STG suppressed PD and LP (not shown) and excited PY and VD neurons. The GM neuron exhibited enhanced activity as well. (D) Focal histamine application (3 ms puff delivered just before the onset of this recording) to the stomatogastric ganglion (STG) produced both similar and distinct modulatory effects from those seen in B and C. Similar effects included the inhibition of the PD neuron and the excitation of the PY neurons, both of which returned to control levels within 10 s. The VD and GM neurons were inhibited following histamine application, which differs from PS stimulation and Crust-MS application.

therefore bath applied Crust-MS for 20–30 s to examine its transient effects. Many of the similarities observed between prolonged application of Crust-MS and PS stimulation were still present. The suppression of the PD neurons and the excitation of the PY and VD neurons persisted in all preparations (N=10), and GM neuron activity was likewise enhanced in most preparations (N=7 of 10; Fig. 3C). Thus, for the rest of this paper, Crust-MS effects refer to the effects of short-term bath application of Crust-MS.

The PS neurons contain Crust-MS, but histamine is also present, as shown previously (Mulloney and Hall, 1991; Le Feuvre et al., 2001). We therefore examined the effects of a 3 ms focal application of histamine to the STG and compared it with the modulation produced by Crust-MS application. The modulation of the pyloric and gastric mill motor patterns was both similar to and distinct from alterations produced by bath application of Crust-MS and by PS stimulation. Similarities included the inhibition of PD neuron firing and the excitation of PY neuron firing, observed in all preparations (N=16; Fig. 3D). In contrast to the effects of both Crust-MS application and PS stimulation, the GM (N=10 of 11) and VD (N=8 of 8) neurons were inhibited upon histamine application (Fig. 3D), whereas they were excited by PS neuron stimulation and Crust-MS bath application. Thus, while histamine application modulated the pyloric and gastric rhythms, Crust-MS application more closely reproduced the effects of PS stimulation.

Quantitative comparisons of the effects of Crust-MS application, PS stimulation and focal histamine application in the STG confirm similarities in effects on the PD and PY neurons

To further compare the modulatory effects of the three treatments (PS neuron stimulation, Crust-MS application and histamine focal application), we chose to quantify their effects on the PD and PY neurons, because the effects on these two neurons were the most consistent across preparations. In all three modulatory treatments, PD neuron bursting was initially suppressed; during recovery, the PD neurons began by producing weak bursts, then recovered to a normal oscillation pattern. We quantified the effects of the modulatory treatments by calculating the average PD cycle period during this period of recovery and compared these values with the control PD cycle period. The average cycle period increased significantly after PS stimulation, Crust-MS bath application and histamine application, but there were no significant differences between the effects of Crust-MS, PS stimulation and histamine (N=8; Fig. 4A, control saline bars), suggesting that all three modulatory treatments altered PD neuron firing similarly.

We quantified the excitation of the PY neurons after each of the three treatments by quantifying PY burst duration over the same period for which we analyzed PD cycle frequency. Like PD cycle frequency, PY burst duration increased significantly after all three treatments

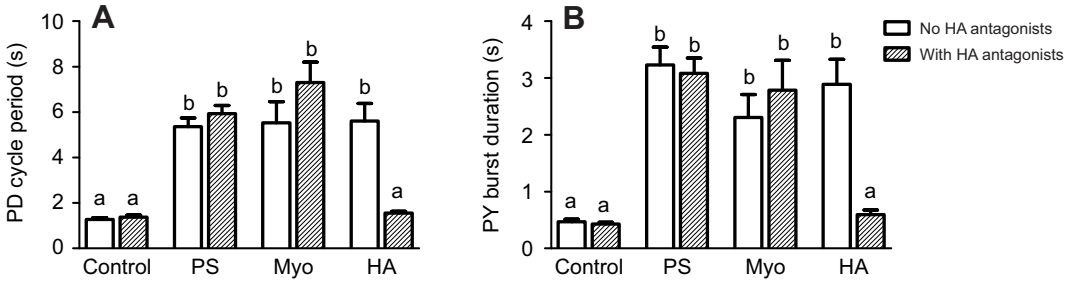

Fig. 4. Quantification of effects of pyloric suppressor (PS) stimulation, Crust-MS (Myo) application and histamine (HA) application in control *versus* histamine receptor antagonist conditions suggested that PS effects in the stomatogastric ganglion (STG) are mediated largely by Crust-MS. (A) In control saline (white bars), histamine, Crust-MS and PS stimulation significantly increased the cycle period of pyloric dilator (PD) bursting compared with the values measured before the treatments; there were no differences between the effects of the three treatments. However, in the presence of histamine receptor antagonists (10^{-6} mol l^{-1} triprolidine/10^{-5} mol l^{-1} ranitidine; striped bars), PD cycle period increased significantly after Crust-MS bath application and PS stimulation, but not after focal histamine application. Different letters indicate significant differences (repeated-measures ANOVA with Tukey's *post hoc* test, $P<0.0001$, $N=7$ preparations). (B) In control saline (white bars), histamine, Crust-MS and PS stimulation significantly increased burst duration when compared with values measured before treatment; effects of the three treatments did not differ significantly. In the presence of histamine receptor antagonists (striped bars), pyloric (PY) burst duration increased significantly immediately after both Crust-MS bath application and PS stimulation, but not after focal histamine application. Different letters indicate significant differences (repeated-measures ANOVA with Tukey's *post hoc* test, $P<0.0001$, $N=5$). Error bars represent ±s.e.m.

when compared with the control, with no significant differences among the three treatments ($N=7$; Fig. 4B, control saline bars), suggesting that the modulation of PY is similar in all three cases.

Thus, although the modulatory effects on the pyloric and gastric mill motor patterns observed after Crust-MS bath application to the STG more closely mimicked the changes seen after PS neuron stimulation, the response to histamine was not sufficiently different to rule it out as a putative transmitter released by the PS neurons in the STG. However, because Crust-MS bath application more closely mimicked all of the effects seen after stimulation of the PS neurons, we hypothesized that their modulatory effects on the pyloric and gastric mill patterns were mediated by their peptide co-transmitter, Crust-MS, in the STG.

Histamine antagonists do not block the ability of the PS neurons to modulate the pyloric and gastric mill patterns

To enable us to distinguish more clearly between the effects of Crust-MS and histamine in the STG, we bath applied H_1 and H_2 histamine receptor antagonists to the STG to block the modulation of the pyloric and gastric mill patterns by histamine. Not surprisingly, focal histamine application no longer affected the gastric mill or pyloric patterns (Fig. 5A,B), indicating that histamine receptors were effectively blocked. The effects of PS neuron stimulation were then re-examined in this blocked condition to determine whether modulation still occurred. PS stimulation produced modulatory effects similar to control levels, despite histamine receptors being blocked. Most notably, there was still a strong inhibition of PD and excitation of PY neuron firing (Fig. 5C). These results suggest that the primary neurotransmitter released in the STG by the PS neurons to modulate the pyloric and gastric mill patterns is Crust-MS rather than histamine.

Because the specificity of the histamine receptor antagonists is unknown in the lobster nervous system, it was important to ensure that the effects of Crust-MS were not altered in the presence of the antagonists. Qualitatively, we saw no difference in the responses of the gastric and pyloric patterns to Crust-MS in control saline *versus* in the presence of histamine antagonists (Fig. 3C and Fig. 5D, respectively).

To confirm our qualitative observations, we again measured the average cycle period of the PD neurons, as well as PY burst duration, during the recovery period following treatment to quantify the modulation produced by histamine application, PS neuron stimulation and Crust-MS application in the presence of histamine receptor antagonists. In the presence of antagonists, histamine did not increase either the PD neuron cycle period or the PY burst duration when compared with control cycle periods (Fig. 4, histamine antagonist bars), indicating that histamine was incapable of suppressing PD firing and activating PY bursting, unlike in control saline. These results indicated that the antagonists were functioning, and that the histamine response was blocked.

In contrast to the effects of histamine in the presence of histamine receptor antagonists, both PS stimulation and Crust-MS elicited modulatory effects that were consistent with the modulation produced in control saline. The increase in average PD neuron cycle period both after PS stimulation and in the presence of Crust-MS was conserved in the presence of antagonists; that is, the cycle period increased significantly in both cases (Fig. 4A, histamine antagonist bars). Moreover, the effects of both PS neuron stimulation and Crust-MS application on PD neuron cycle period did not differ significantly between control and antagonist saline (Fig. 4A, histamine antagonist bars). Similarly, PY neuron burst duration increased significantly in response to both PS stimulation and Crust-MS application in the antagonist saline (Fig. 4B, histamine antagonist bars); these effects were similar to those seen in control saline. Taken together, these results suggest that the PS effects on the PD and PY neurons were conserved when histamine was blocked in the STG. Although we did not quantify other effects of PS stimulation/Crust-MS application, qualitative observations suggested that other STG neurons also continued to respond similarly to PS stimulation and Crust-MS in the presence of histamine receptor antagonists.

These results indicate that the modulatory effects of histamine were eliminated in the STG in the presence of histamine receptor antagonists. In contrast, the modulatory effects of both PS stimulation and Crust-MS application to the STG were similar to those recorded in control saline, suggesting that the PS neurons' effects on the pyloric and gastric mill patterns are mediated primarily by Crust-MS.

Both PS stimulation and histamine application to the CoGs modulate the oesophageal rhythm, but Crust-MS does not

PS stimulation had pronounced effects on the pyloric and gastric mill patterns in the STG, but the PS neurons also modulate the

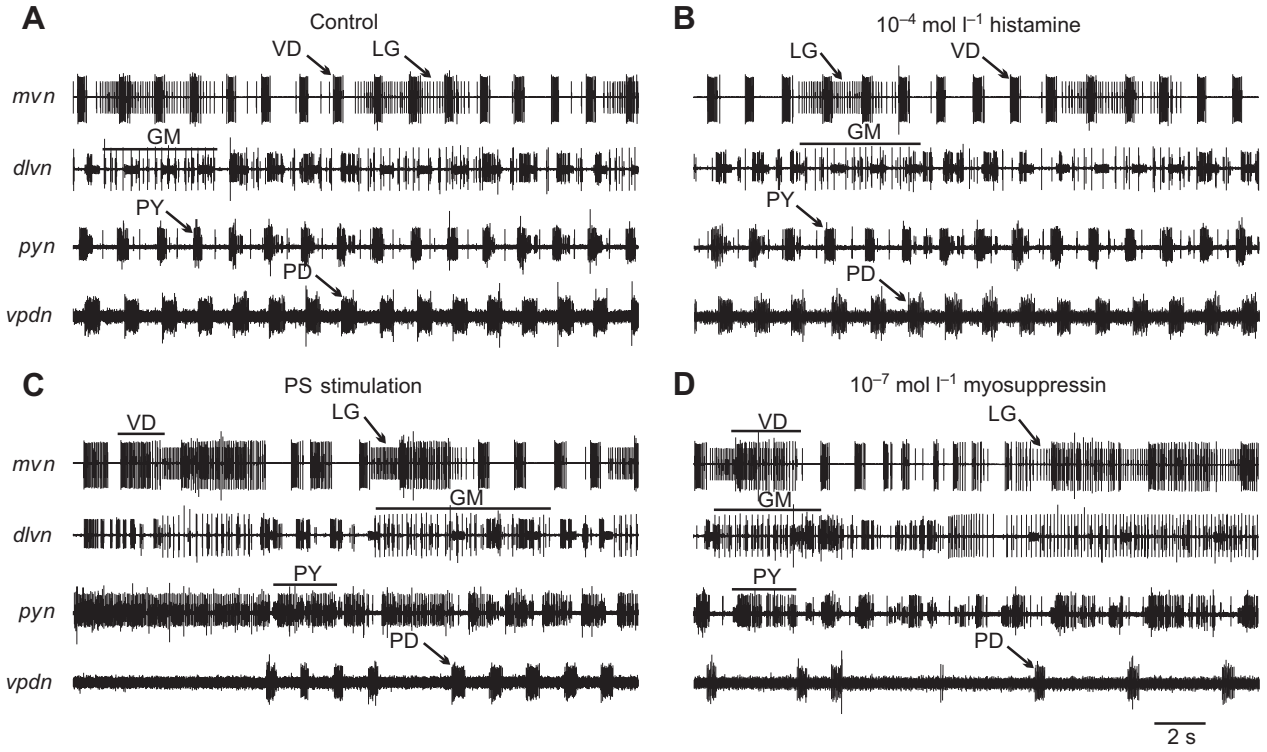

Fig. 5. Bath application of H_1 (10^{-6} mol l^{-1} triprolidine) and H_2 (10^{-5} mol l^{-1} ranitidine) histamine receptor antagonists blocked the effects of focal histamine application to the stomatogastric ganglion (STG), but did not block effects of either pyloric suppressor (PS) stimulation or Crust-MS. (A) In the presence of histamine receptor antagonists, pyloric and gastric mill activity resembled that recorded in control saline, i.e. independent pyloric and gastric mill patterns (cf. Fig. 3A). (B) Recordings of the pyloric and gastric mill patterns immediately after focal histamine application (3 ms puff, 10^{-4} mol l^{-1}) to the STG did not differ from control recordings, indicating that histamine had no effect in the presence of histamine receptor antagonists. (C) When histamine receptors were blocked, extracellular PS neuron stimulation (4 s at 30 Hz) exerted effects similar to those recorded in control saline on the pyloric and gastric mill patterns. Firing of the pyloric dilator (PD) neurons was suppressed, whereas the pyloric (PY) and ventricular dilator (VD) neurons were excited. Activity in the gastric mill (GM) neurons was also enhanced. (D) Crust-MS, like PS stimulation, continued to modulate the GM and PY neurons when histamine receptors were fully blocked.

oesophageal rhythm, which is generated in the CoGs. Specifically, following extracellular PS neuron stimulation, the burst frequency of the oesophageal rhythm increased (Fig. 6A,B). Again, we wanted to determine whether Crust-MS or histamine (or both) was responsible for this excitation of the oesophageal rhythm. In contrast to the effects of Crust-MS on the pattern generators in the STG, bath application of Crust-MS to the CoGs did not alter the oesophageal rhythm (Fig. 6C), suggesting that the PS neurons do not utilize Crust-MS in the CoGs. We then examined the effects of histamine puff application to the CoGs.

Unlike the results seen in the STG, the effects of focal histamine application in control saline varied considerably, both between preparations and between locations within the CoG of an individual preparation. Most preparations exhibited at least some excitatory (N=17 of 21) or inhibitory (N=10 of 21) responses to histamine that did not resemble the effects of PS stimulation. By trying multiple locations in each preparation, we were able to elicit excitatory responses that qualitatively resembled the effects of PS stimulation in approximately half of all preparations (N=11 of 21; Fig. 6D). The location within the CoG that evoked an excitatory response varied from preparation to preparation, but it was always in the medial half of the CoG.

In six of the preparations in which histamine puffs elicited excitatory effects that mimicked those of PS stimulation, we examined the effects of blocking histamine in the CoGs. H_1 and H_2 histamine receptor antagonists completely blocked the modulation of the oesophageal motor pattern by PS stimulation in those preparations in which histamine originally mimicked PS stimulation, suggesting that the effects of these neurons in the CoGs are mediated entirely by histamine rather than by Crust-MS (Fig. 7). These effects were confirmed by quantifying the cycle period of the OD1 neuron (Fig. 8). OD1 cycle period decreased significantly following both PS stimulation and histamine application in control saline (Fig. 8), but neither treatment had an effect on OD1 cycle period in the presence of histamine receptor antagonists.

Overall, our data suggest that the PS neurons modulate the oesophageal pattern in the CoGs using histamine, but that the primary neurotransmitter mediating the modulatory effects of the PS neurons on the gastric and pyloric patterns in the STG is the FMRFamide-like peptide Crust-MS.

DISCUSSION

While it is known that neurons can contain multiple co-transmitters, and that modulatory neuron activity can dramatically alter the interactions of functionally related motor patterns, relatively little is understood about how co-transmitters are specifically utilized to alter the interactions of different neuronal networks. Because both PS neurons in the lobster stomatogastric system contain two co-transmitters (LeFeuvre et al., 2001), the second of which is now identified, and are known to cause the functional re-configuration of three pattern generators into a single CPG, we were able to show that this pair of modulatory neurons uses its co-transmitters

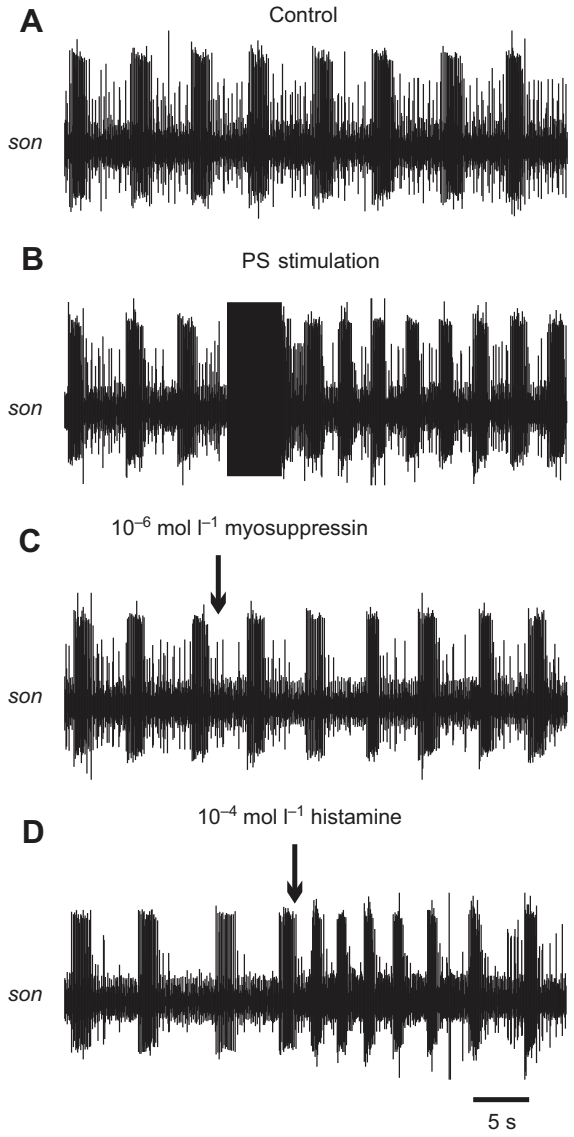

Fig. 6. The oesophageal pattern, characterized by regular bursting in the oesophageal motor neuron OD1 (oesophageal dilator 1), increased in frequency after both stimulation of the pyloric suppressor (PS) neurons and application of histamine, but not application of Crust-MS. (A) In control saline, OD1 exhibited characteristic oesophageal firing and frequency. (B) Following extracellular PS stimulation (4 s, 30 Hz), OD1 cycle frequency increased. (C) Bath application of 10^{-6} mol l^{-1} Crust-MS to the commissural ganglia (CoGs) did not affect the oesophageal rhythm. (D) After focal application of 10^{-4} mol l^{-1} histamine (6 ms) to one of the desheathed CoGs, oesophageal cycle frequency increased to levels similar to those recorded after PS stimulation. All recordings are from the superior oesophageal nerve (*son*) in a single preparation.

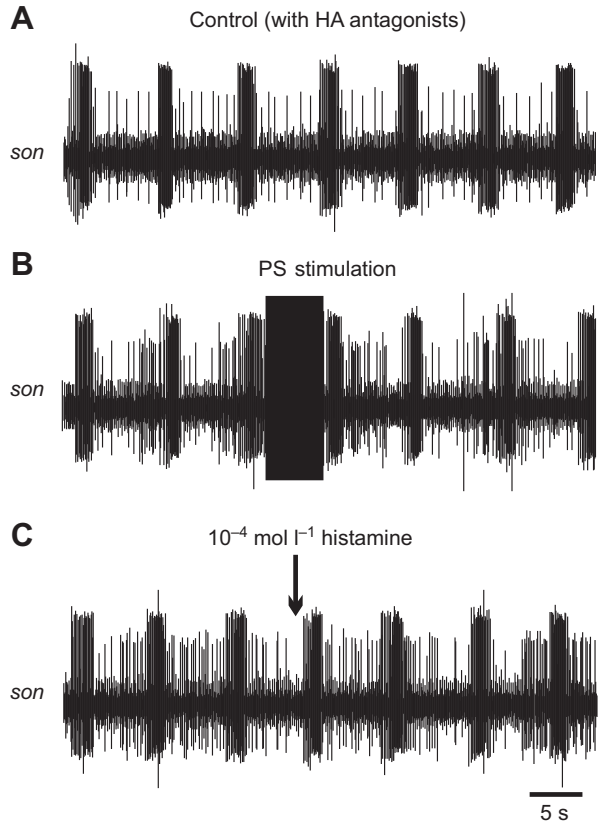

Fig. 7. Bath application of histamine receptor antagonists (10^{-6} mol l^{-1} triprolidine and 10^{-5} mol l^{-1} ranitidine) to the commissural ganglia (CoGs) blocked the excitation of the oesophageal pattern that was seen following pyloric suppressor (PS) stimulation and focal application of 10^{-4} mol l^{-1} histamine in control saline. (A) Oesophageal activity, seen as bursting of the oesophageal dilator 1 (OD1) neuron, recorded in saline containing histamine (HA) antagonists resembled activity recorded in control saline (cf. Fig. 6A). (B) Stimulation of the PS neurons (4 s, 30 Hz) did not alter the oesophageal pattern. (C) Focal application of histamine likewise had no effect on the oesophageal pattern in the presence of the histamine antagonists. All recordings are from the preparation shown in Fig. 6.

differentially. Specifically, these neurons alter the activity of two CPGs in the STG using largely a peptide co-transmitter, but alter the activity of the third CPG, in the CoGs, using a small molecule transmitter, histamine.

Prior to PS stimulation, the oesophageal, gastric mill and pyloric patterns were distinct, with different characteristic cycle periods and frequencies. After PS stimulation, and mediated by the peptide co-transmitter Crust-MS, gastric mill activity was enhanced, with cycle period generally decreasing. In addition, some pyloric neurons (PD and LP) were inhibited, whereas others (PY and VD) were excited and assumed a cycle period similar to that exhibited by the gastric mill neurons following PS neuron stimulation. In effect, this modulation created a unified rhythmic pattern with a cycle period intermediate to those of the two independent networks, similar to that seen following PS stimulation in *H. gammarus* (Meyrand et al., 1991; Meyrand et al., 1994).

Additionally, our data indicate that activity of the modulatory PS neurons in the American lobster, *H. americanus*, modulates the oesophageal rhythm in the CoGs by differentially utilizing histamine (Mulloney and Hall, 1991), but not Crust-MS, the PS neuron co-transmitter we identified in the present study through RT-PCR analysis. Our results, coupled with the documentation of PS neuron synaptic connectivity in the CoGs (Cazalets et al., 1990b; Meyrand et al., 1994), suggest that this modulation occurs directly within the CoGs. Thus, the combined activity of the co-transmitters in the PS neurons can produce the reorganization and coordination of spatially distinct motor patterns in the STNS. Although the coordination of the oesophageal, pyloric and gastric mill rhythms has been described previously, how the PS neurons utilize their co-transmitters to integrate networks in spatially distinct locations of a system was not previously known.

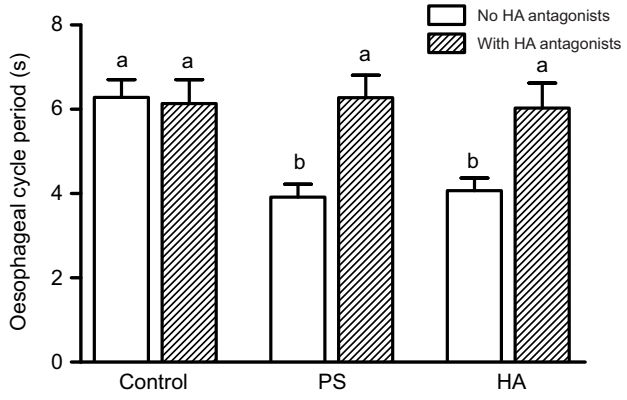

Fig. 8. Quantification of the effects of pyloric suppressor (PS) stimulation and histamine (HA) application suggest that the effects of the PS neurons on the oesophageal pattern are mediated primarily by histamine. Oesophageal cycle period was quantified by measuring the cycle period of bursts in the oesophageal dilator 1 (OD1) neuron. In control saline (white bars), OD1 cycle period decreased significantly, and to similar values after both extracellular PS stimulation (4 s, 30 Hz) and focal histamine application (6 ms, 10^{-4} mol l^{-1}). In the presence of histamine antagonists, both PS stimulation and focal histamine application failed to cause a decrease in the OD1 cycle period (striped bars). Different letters indicate significant differences (repeated-measures ANOVA with Tukey's *post hoc* test, $P<0.05$, $N=6$ preparations). Error bars represent ±s.e.m.

To date, we are aware of only one other published account of a modulatory neuron that differentially utilizes its co-transmitters on spatially distinct targets. In their earlier study, Blitz and Nusbaum (Blitz and Nusbaum, 1999) examined the simultaneous modulation of two of the three motor patterns examined in this study, the gastric mill and pyloric motor patterns, in the STNS of another crustacean, the crab *Cancer borealis*. They found that the motor patterns selected for by the modulatory proctolin neurons (MPNs) are a result of the direct modulation of the pyloric pattern by proctolin and indirect modulation of the gastric pattern by GABA. Specifically, within the STG, the modulation of the pyloric pattern by MPNs is mediated by proctolin; in the CoGs, MPNs utilize GABA to inhibit two modulatory projection neurons. Because these projection neurons normally activate the gastric mill rhythm, MPN stimulation results in the suppression of gastric activity.

The differential utilization of co-transmitters described in this paper is similar to the results of Blitz and Nusbaum (Blitz and Nusbaum, 1999) in several respects. First, each of the modulatory neurons contains and differentially utilizes one small molecule transmitter and one peptide co-transmitter. Second, the targets of the modulatory neurons are the same ganglia (i.e. the CoGs and the STG). However, our results differ in several important aspects. First, while MPNs target two CPG rhythms, the PS neurons target three. Second, the targets of MPN modulation are located in the same ganglion, but its co-transmitters target these patterns through a direct and indirect route. The PS neurons, in contrast, appear to directly modulate spatially segregated CPGs. Third, when modulated by the MPNs in *C. borealis*, the two motor patterns remain relatively independent and retain their distinct characteristics, whereas the PS neurons in *H. americanus* function to coordinate their three target patterns into a cohesive rhythm.

In addition to the two organizational patterns for differential use of co-transmitters described above [i.e. (1) spatially distributed modulation of several networks and (2) differential modulation of networks *via* direct and indirect pathways], a third pattern in the differential use of co-transmitters has also been reported in the crustacean stomatogastric system. In *H. americanus*, Thirumalai and Marder (Thirumalai and Marder, 2002) showed that a modulatory neuron contains two peptides, red pigment concentrating hormone and *C. borealis* tachykinin-related peptide. Each of these peptides modulates a specific subset of neurons within the pyloric pattern generator; co-application of the two peptides activates all of the neurons, resulting in a complete pyloric pattern. In this case, the two co-transmitters act on different elements within a single motor pattern to produce a global activation of that network.

While our results show that the main effects of PS stimulation stem from the release of Crust-MS in the STG and histamine in the CoG, more subtle effects of the co-transmitters may be present. Specifically, we noted a general trend toward an increase in the overall duration of the Crust-MS effect on pyloric neurons when histamine was blocked compared with the control effect (data not shown). This difference suggests the possibility that the histamine receptor antagonists may potentiate the response elicited by Crust-MS, or that histamine may have an endogenous role in the response of STG cells to Crust-MS, and therefore to PS activity. Consistent with this possibility, in *H. gammarus*, it has been shown that histamine mediates an immediate transient excitatory postsynaptic potential in several pyloric neurons, with a time course of a few milliseconds, prior to the longer-lasting hyperpolarization seen following PS stimulation (Cazalets et al., 1990a; Cazalets et al., 1990b). Because we analyzed the PS effect over several seconds, very rapid effects of histamine in the STG could have gone largely undetected in our study. Blocking the response to Crust-MS would have enabled us to determine whether there are any subtle alterations in the PS effect in the absence of Crust-MS; however, no antagonists to the Crust-MS receptor are currently available. Overall, our results suggest that Crust-MS is the main co-transmitter used by the PS neurons in the STG to alter the gastric and pyloric patterns, with known connections between the PS neurons and gastric/pyloric neurons suggesting that this modulation is direct (Cazalets et al., 1990b; Meyrand et al., 1994; Faumont et al., 2005).

What our data are currently unable to address are the mechanisms that underlie this differential use of transmitters. At least three possibilities exist. First, consistent with Dale's principle (Dale, 1935; Eccles et al., 1954; Eccles, 1976), the PS neurons might release histamine and Crust-MS equally at all terminals, but receptors could be differentially distributed (Marder et al., 1995). Second, the two co-transmitters might be present in the same terminals but released separately in response to different activity patterns (Whim and Lloyd, 1989). Lastly, the histamine and Crust-MS in the PS neurons might be differentially trafficked to the CoGs and the STG. It is clear in other systems that various neuronal components, including neurotransmitter-containing vesicles, can be differentially trafficked within neurons (Sossin et al., 1990; Hattori et al., 1991; Sámano et al., 2006; Kueh and Jellies, 2012). Regardless of the mechanism(s) involved, our data strongly support the hypothesis that a single modulatory neuron can use its co-transmitters differentially to modulate three spatially distinct target networks.

LIST OF ABBREVIATIONS

CG	cardiac ganglion
CoG	commissural ganglion
CPG	central pattern generator
dlvn	dorsal lateral ventricular nerve
GM	gastric mill neuron
HA	histamine
ion	inferior oesophageal nerve
ivn	inferior ventricular nerve

LG	lateral gastric neuron
LP	lateral pyloric neuron
MPN	modulatory proctolin neuron
mvn	medial ventricular nerve
OD1	oesophageal dilator 1 neuron
OG	oesophageal ganglion
PD	pyloric dilator neuron
PS	pyloric suppressor neuron
PY	pyloric neuron
pyn	pyloric nerve
RT-PCR	reverse transcriptase-polymerase chain reaction
son	superior oesophageal nerve
STG	stomatogastric ganglion
stn	stomatogastric nerve
STNS	stomatogastric nervous system
VD	ventral dilator neuron
vlvn	ventral lateral ventricular nerve
vpdn	ventral pyloric dilator nerve
vpon	ventral posterior oesophageal nerve

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AUTHOR CONTRIBUTIONS

M.A.K., E.R.G., K.E.H., M.C.C., A.E.C. and P.S.D. were involved in the conception, design and execution of the study and the interpretation of findings. M.A.K., A.E.C. and P.S.D. drafted and revised the article.

COMPETING INTERESTS

No competing interests declared.

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