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GABA AND RESPONSES TO GABA IN THE STOMATOGASTRIC GANGLION OF THE CRAB *CANCER BOREALIS*

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Summary

The multifunctional neural circuits in the crustacean stomatogastric ganglion (STG) are influenced by many small-molecule transmitters and neuropeptides that are co-localized in identified projection neurons to the STG. We describe the pattern of γ-aminobutyric acid (GABA) immunoreactivity in the stomatogastric nervous system of the crab *Cancer borealis* and demonstrate biochemically the presence of authentic GABA in *C. borealis*. No STG somata show GABA immunoreactivity but, within the stomatogastric nervous system, GABA immunoreactivity co-localizes with several neuropeptides in two identified projection neurons, the modulatory proctolin neuron (MPN) and modulatory commissural neuron 1 (MCN1). To determine which actions of these neurons are evoked by GABA, it is necessary to determine the physiological actions of GABA on STG neurons. We therefore characterized the response of each type of STG neuron to focally applied GABA. All STG neurons responded to GABA. In some neurons, GABA evoked a picrotoxin-sensitive depolarizing, excitatory response with a reversal potential of approximately −40 mV. This response was also activated by muscimol. In many STG neurons, GABA evoked inhibitory responses with both K^+- and Cl^--dependent components. Muscimol and β-guanidinopropionic acid weakly activated the inhibitory responses, but many other drugs, including bicuculline and phaclofen, that act on vertebrate GABA receptors were not effective. In summary, GABA is found in projection neurons to the crab STG and can evoke both excitatory and inhibitory actions on STG neurons.

Key words: crustacean, GABA receptor, projection neurone, crab, *Cancer borealis*, stomatogastric ganglion.

Introduction

Nervous systems employ many substances as neurotransmitters and neuromodulators. Frequently, several of these are co-localized (Brezina and Weiss, 1997; Kupfermann, 1991; Blitz et al., 1999). To understand the role of each co-localized substance released from an individual neuron, it is necessary to characterize the actions of these substances on their potential target neurons or muscles. The neuropil of the stomatogastric ganglion (STG) of the crab *Cancer borealis* contains more than 15 different neuromodulators (Marder et al., 1995, 1997; Marder and Weimann, 1992). These substances are found in approximately 20 pairs of modulatory neurons (Coleman et al., 1992) that project into the STG neuropil primarily from somata in the paired commissural ganglia (CoGs) and unpaired oesophageal ganglion (OG). This rich and extensive neuromodulatory control of the rhythmically active circuits in the STG raises the question of whether all the actions of a particular projection neuron on its target neurons in the STG result from the simple addition, or from a more complex interaction, of the individual actions of its cotransmitter complement.

The neurotransmitter γ-aminobutyric acid (GABA) is found in inputs to the STG in several decapod species (Blitz et al., 1999; Cazalets et al., 1987; Cournil et al., 1990a,b; Mulloney and Hall, 1990). It has been localized as a cotransmitter in two identified projection neurons, modulatory proctolin neuron (MPN) and modulatory commissural neuron 1 (MCN1), in the crab *C. borealis* (Blitz et al., 1999). In this paper, we document the presence of authentic GABA and its distribution in the stomatogastric nervous system of *C. borealis*. As the first step in understanding the role of GABA as a cotransmitter, it is necessary to characterize its individual actions on the STG neurons. For the first time, therefore, we have characterized the responses to GABA of each identified class of STG neuron.

Crustacean neurons exhibit several different classes of responses to GABA, some of which display pharmacological profiles that differ from those of the conventional vertebrate GABA receptors (Fuchs and Getting, 1980; Jackel et al., 1994;

Kerrison and Freschi, 1992; Marder and Paupardin-Tritsch, 1978; Miyata et al., 1997; Zhainazarov et al., 1997). We hope eventually to use pharmacological tools to dissect the role of GABA as a cotransmitter, so we have included a description of the pharmacological profile of the responses of the crab STG to GABA. Of particular interest is an excitatory response to GABA that does not correspond closely to any previously described GABA response. Some of this work has been published previously in abstract form (Golowasch et al., 1996).

Materials and methods

Experiments were conducted on the stomatogastric nervous system of the crab *Cancer borealis*. Animals were obtained from commercial suppliers (Boston, MA, USA) and from the Marine Biological Laboratory (Woods Hole, MA, USA) and were maintained in filtered, recirculating seawater tanks at 10–12 °C. Conventional stomatogastric nervous system dissections were performed (Harris-Warrick et al., 1992).

Immunocytochemistry

Wholemount immunocytochemistry was performed using techniques modified from those of Beltz and Kravitz (1983), as described by Christie et al. (1997). Specifically, the stomatogastric nervous system (Fig. 1) was dissected from the animal in chilled (approximately 4 °C) physiological saline (see below), fixed for 2–3 h in 4 % paraformaldehyde in 0.1 mol l^{-1} sodium phosphate buffer (pH 7.3–7.4), and rinsed five times over approximately 5 h in a solution of 0.1 mol l^{-1} sodium phosphate, pH 7.2 (*P*), containing 0.3 % Triton X-100 (*P*-Triton). Incubation with a polyclonal anti-GABA antibody (1:200 final dilution; Sigma, St Louis, MO, USA) was performed in *P*-Triton for 48–72 h (10 % goat normal serum was added to the reaction to reduce nonspecific binding). After incubation in anti-GABA, tissues were again rinsed five times over approximately 5 h in *P*-Triton and then incubated for 18–24 h in goat anti-rabbit IgG (1:300 final dilution in *P*-Triton) conjugated to either fluorescein (FITC) or rhodamine (Calbiochem, San Diego, CA, USA). After secondary antibody incubation, each preparation was rinsed five times over approximately 5 h in *P*. Preparations were then mounted between a glass microscope slide and coverslip in a solution of 80 % glycerine, 20 % 20 mmol l^{-1} sodium carbonate (pH≈9.0). Tissue was viewed using a Leica TCS NT laser scanning confocal microscope equipped with krypton and argon lasers and standard FITC and rhodamine filter sets (Christie et al., 1997). Preadsorption controls with the anti-GABA antibody in the stomatogastric system had been performed previously (Blitz et al., 1999).

To determine the axonal projection pattern of a pair of GABAergic neurons in the OG, Lucifer Yellow-CH (Sigma) backfills of the inferior oesophageal nerve were paired with GABA immunoprocessing (N=3 preparations). In these experiments, a Vaseline well was built around an inferior oesophageal nerve, and the saline within the well was replaced with distilled water. After several minutes, the distilled water was removed and replaced with a solution of 10 % Lucifer Yellow in distilled water, after which the nerve was transected within the well. The preparation was then incubated at 4 °C overnight (18–24 h), after which the Lucifer Yellow was removed from the well and the preparation subjected to GABA immunoprocessing as described above, with GABA-immunoreactivity visualized using a rhodamine-conjugated secondary antibody. Double-labeled preparations were imaged *via* laser scanning confocal microscopy (see above) using a standard FITC/rhodamine filter set as described by Blitz et al. (1999).

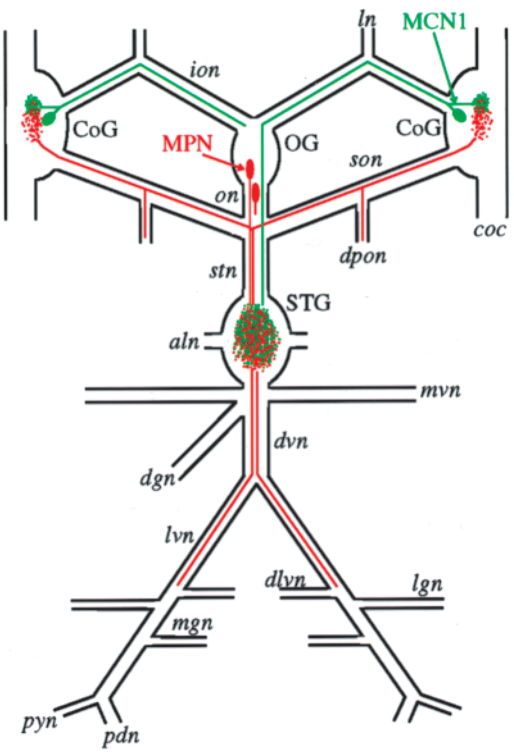

Fig. 1. A diagram of the stomatogastric nervous system, including its four ganglia with their connecting and peripheral nerves. Also shown are the two identified projection neurons, MCN1 and MPN, which both exhibit GABA immunoreactivity and innervate the crab stomatogastric ganglion. CoG, commissural ganglion; OG, oesphageal ganglion; STG, stomatogastric ganglion; *aln*, anterior lateral nerve; *coc*, circumoesophageal connective; *dlvn*, dorso-lateral ventricular nerve; *dpon*, dorsal posterior oesophageal nerve; *dvn*, dorsal ventricular nerve; *dgn*, dorsal gastric nerve; *ion*, inferior oesophageal nerve; *lgn*, lateral gastric nerve; *lvn*, lateral ventricular nerve; *mgn*, medial gastric nerve; *mvn*, medial ventricular nerve; *on*, oesophageal nerve; *pdn*, pyloric dilator nerve; *pyn*, pyloric nerve; *son*, superior oesophageal nerve; *stn*, stomatogastric nerve; MCN1, modulatory commissural neuron 1; MPN, modulatory proctolin neuron.

GABA purification and identification

Tissue extraction

The CoGs, OGs and STGs were collected separately for extraction and analysis. Extraction of GABA from these ganglia was performed by ganglionic homogenization under one of the following conditions: (i) acid extraction with either 0.1 mol l^{-1} HCl (pH<1.0) or 0.5 mol l^{-1} acetic acid (pH 1.0); (ii) methanol extraction (pH 4.5); (iii) frozen extractions with dichlorodifluoromethane; and (iv) microsonication with 0.5 mol l^{-1} acetic acid (pH 1.0). Several extractions were performed after heating the ganglia (80 °C for 5 min) to inactivate enzymes. All procedures gave comparable results.

Dansylation of GABA

To make GABA ultraviolet-detectable so that its presence could be monitored during purification and identification, it was dansylated (Ge et al., 1988). Extracted solutions were lyophilized and resuspended in $NaHCO_3$ (200 μl, 10^{-2} mol l^{-1}, pH 9.5) and 0.25 % dansyl chloride in acetone (300 μl) (Pierce, Rockford, IL, USA). Samples were incubated in a water bath (70 °C) for approximately 20 min and then passed through a SepPak C_{18} cartridge which had been conditioned by pre-injection of 5 ml of methanol (100 %). Water-soluble material was flushed from the cartridge by injection of 1 ml of water, after which dansylated GABA was eluted by injection of 1 ml of acetonitrile (CH_3CN; 70 %). The latter eluate was collected and lyophilized for subsequent purification and identification by reverse-phase high-performance liquid chromatography (HPLC).

HPLC

All HPLC analyses were performed using a computer-controlled Rabbit HPX dual-pump delivery system with a dynamic mixer (Rainin, Woburn, MA, USA), using Dynamax HPLC Method Manager (version 1.3, Rainin). All samples were analyzed with a Vydac C_{18} HPLC analytical column (0.46 cm×25 cm, 30 nm pore size) to which was fitted a Vydac C_{18} guard column and a precolumn filter. In the first purification step, the dansylated GABA samples were run on a linear gradient of CH_3CN with 0.1 % trifluoroacetic acid (TFA, pH 2; Aldrich, Milwaukee, WI, USA) of 20 % to 40 % for 10 min (2 % min^{-1}) at a flow rate of 1.0 ml min^{-1}. Collected samples were then further purified under isocratic conditions of 22 % CH_3CN, with 0.1 % TFA (pH 2.0). The final purification step was also under isocratic conditions of 40 % CH_3CN, 0.1 % tetraethylammonium (TEA^+), pH 5.0.

For identification of GABA during each experiment, under all conditions, injections were also made of both synthetic dansyl-GABA and synthetic GABA dansylated using the procedure described above. Control injections were also performed with dansylated water, saline, homocarnosine, taurine, glutamate, γ-amino-β-hydroxybutyric acid (GABOB) and GABAmide. Further control injections were made with synthetic dansyl-glutamate, dansyl-glycine and dansyl-aspartate. All these substances were obtained from Sigma (St Louis, MO, USA). Peaks were detected at 222 nm using a Knauer variable-wavelength ultraviolet detector controlled by Dynamax Method Manager software. All chromatograms were stored for analysis using the Method Manager software.

Electrophysiology

Stomatogastric nervous systems were pinned onto Sylgard-coated Petri dishes and superfused with *C. borealis* saline (below) at 10–13 °C. Intracellular recordings were performed using two-electrode current-clamp, two-electrode voltage-clamp or single-electrode discontinuous current-clamp (DCC). During current injections with DCC, sample rates were approximately 3 kHz (Axoclamp 2, Axon Instruments, Foster City, CA, USA). For intrasomatic recordings, glass microelectrodes were filled with either 0.6 mol l^{-1} K_2SO_4/20 mmol l^{-1} KCl or 4 mol l^{-1} potassium acetate/20 mmol l^{-1} KCl (15–30 MΩ). For chloride injections, electrodes were filled with 3 mol l^{-1} KCl (10–20 MΩ). Current–voltage (*I*/*V*) curves were generated in two ways: (i) cells were voltage-clamped, and the voltage was swept in a slow ramp from a hyperpolarized value (typically –90 mV) to a depolarized value (typically –20 mV), or (ii) cells were voltage-clamped, and the voltage was stepped from a holding potential to a series of test potentials.

Pharmacology

Application of GABA was by ionophoresis or pressure application. For ionophoresis, microelectrodes were filled with 1 mol l^{-1} GABA dissolved in water, pH 4.0. Pressure applications of GABA were performed using a Picospritzer (General Valve Corporation, Fairfield, NJ, USA). Pipettes were filled with 10^{-3} to 10^{-2} mol l^{-1} GABA in physiological saline (at physiological pH) and ejected 20–100 μm from the neuropil with pressures of 104–173 kPa. Baclofen, SKF97541, isoguvacine and 3-aminopropanesulphonic acid were all ionophoresed at 10^{-1} mol l^{-1} (pipette concentration), *trans*-4-aminocrotonic acid/(E)-4-amino-2-butenoic acid (TACA) and *cis*-4-aminocrotonic acid/(Z)-4-amino-2-butenoic acid (CACA) at 10^{-2} mol l^{-1} and (Z)-3-[(aminoiminomethyl)thio]prop-2-enoic acid (ZAPA) at 5×10^{-3} mol l^{-1}. All GABA antagonists were bath-applied at the concentrations indicated. All agonists and antagonists were obtained from Research Biochemicals International (Natick, MA, USA), Tocris Cookson Inc. (Ballwin, MO, USA) or Sigma (St Louis, MO, USA).

Saline composition

Normal saline consisted of (in mmol l^{-1}): 440 NaCl; 11 KCl; 13 $CaCl_2$; 26 $MgCl_2$; 12.4 Trizma base; 5.2 maleic acid. Mn^{2+}, low-Ca^{2+} saline consisted of (in mmol l^{-1}): 440 NaCl; 11 KCl; 1.3 $CaCl_2$; 11.7 $MnCl_2$; 26 $MgCl_2$; 12.4 Trizma base; 5.2 maleic acid. In 0.5[Na^+] saline, 220 mmol l^{-1} NaCl was replaced with equimolar *N*-methyl-D-glucamine and brought to pH using HCl. 2[K^+] saline and 0.5[K^+] saline contained 22 and 5.5 mmol l^{-1} KCl respectively. All solutions were adjusted to pH 7.4–7.6 at room temperature.

Data analysis

Data analysis was performed using the pCLAMP analysis package (Axon Instruments, Foster City, CA, USA) and statistical analyses were performed using the SigmaPlot and

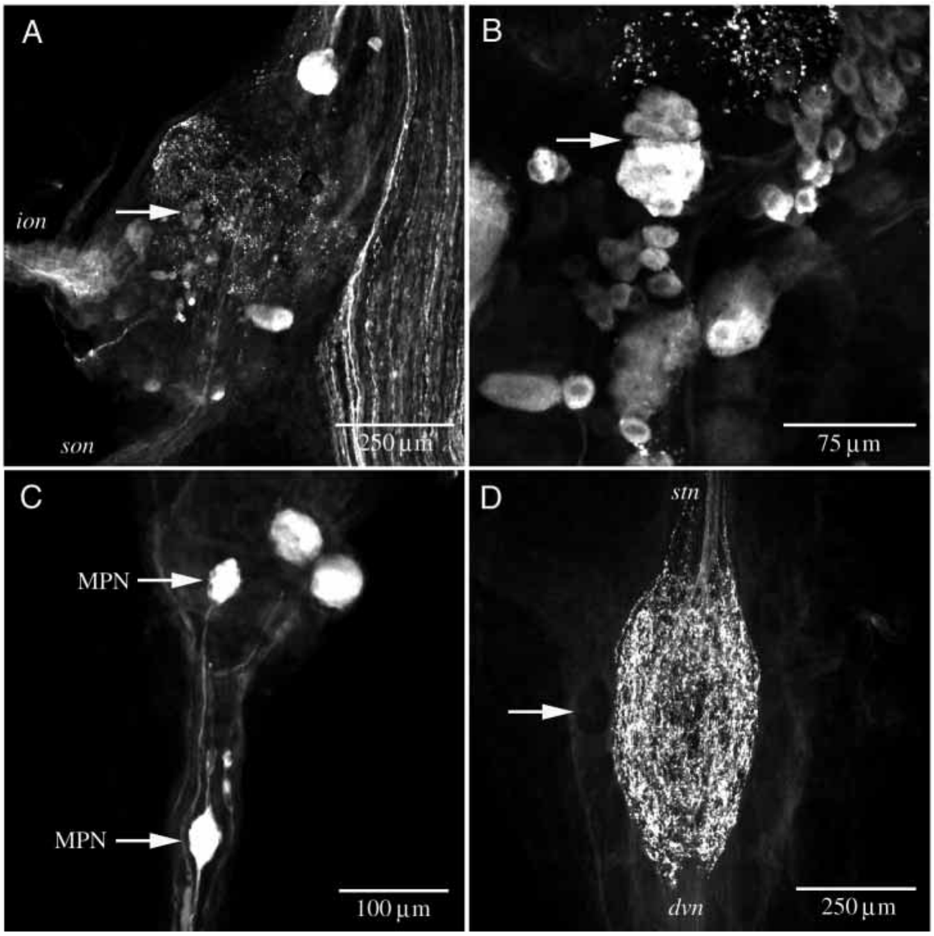

Fig. 2. GABA immunoreactivity (GABA-IR) in the ganglia of the crab stomatogastric nervous system. Each panel is a composite confocal micrograph obtained from ganglionic wholemounts. (A) Low-magnification image of GABA-IR in neuronal somata, neuropil and fibers in the commissural ganglion (CoG). Approximately 50 neuronal somata exhibited GABA-IR in this CoG. The extensive immunolabeled fiber tract at the lateral edge of the ganglion (right) is within the circumoesophageal commissure that connects the supraoesphageal and thoracic ganglia. The arrow points to a region of the CoG that is shown at higher magnification in B. This micrograph is a composite image of 42 optical sections taken at 2 µm intervals through the ganglion. (B) High-magnification image of the region in A illustrated with the white arrow. Composite image of 24 optical sections taken at approximately 1 µm intervals. (C) GABA-IR in the oesphageal ganglion (OG). Four neuronal somata exhibit GABA-IR in the OG, including a previously identified pair of projection neurons, the modulatory proctolin neurons (MPNs) (arrows). In this preparation, one MPN soma was located within the OG, as are the other two GABA-immunoreactive somata. The other MPN was in the oesophageal nerve, which connects the OG with the superior oesophageal (*son*) and stomatogastric (*stn*) nerves (see Fig. 1). This micrograph is a composite image of 16 optical sections taken at 1 µm intervals through the ganglion. (D) GABA-IR in the stomatogastric ganglion (STG). There is GABA immunolabeling of several axons entering the STG from the *stn* and extensive labeling of these axonal branches within the STG neuropil. Note that no STG somata exhibit GABA-IR. These somata form a single layer around the perimeter of the neuropil. One such soma is indicated by the arrow. This micrograph is a composite image of 32 optical sections taken at approximately 2 µm intervals through the ganglion. All micrographs shown in this figure were collected from the same preparation. *dvn*, dorsal ventricular nerve; *ion*, inferior oesophageal nerve.

SigmaStat software packages (Jandel Scientific, San Rafael, CA, USA).

Results are presented as means ± S.D.

Results

Distribution of GABA-immunoreactivity in the crab stomatogastric nervous system

The distribution of GABA immunoreactivity (GABA-IR) has been studied previously in several decapod species (Cournil et al., 1990a; Mulloney and Hall, 1990). Nonetheless, since many modulators show significantly different distributions in closely related crustacean species (Katz and Harris-Warrick, 1999; Katz and Tazaki, 1992; Mortin and Marder, 1991; Skiebe, 1999; Turrigiano and Selverston, 1991), we studied the distribution of GABA-IR throughout the stomatogastric nervous system in *C. borealis* in wholemount preparations imaged by laser scanning confocal microscopy. Confocal micrographs demonstrating GABA-IR in wholemounts of the CoGs, OG and STG are shown in Fig. 2.

As in other species, GABA-IR is present in neuronal somata in the CoGs and OG, but not in the STG somata.

Fig. 2A,B shows a wholemount view of a CoG, the location of the somata of many of the modulatory neurons that project into the STG (Coleman et al., 1992). There is extensive GABA-IR within the CoG neuropil, and approximately 50 of the CoG somata stain (54±17 somata, *N*=11 preparations, *N*=22 CoGs; range 21–86 somata). Fig. 2C shows the four GABA-immunoreactive neuronal somata (4±0, *N*=11) within the OG. Two of the four GABA-stained OG neurons have previously been identified as the modulatory proctolin neurons (MPNs) (Nusbaum and Marder, 1989a) (see below). Fig. 3 illustrates that the other two GABA-staining neurons in the OG project axons into the inferior oesophageal nerves.

The STG does not contain any GABA-immunoreactive somata, but the STG neuropil exhibits strong GABA-IR (Fig. 2D). The STG somata form a single layer around the perimeter of the neuropil. One such unstained soma is indicated by the arrow in Fig. 2D. The staining in the STG results from the neuropilar arborization of eight GABA-immunoreactive axons (7±1; range 6–8; *N*=11) that enter the STG from the stomatogastric nerve (Fig. 4A). These are a subset of the approximately 40 stomatogastric nerve axons that project to the STG (Coleman et al., 1992). Most of these axonal projections to the STG originate from bilaterally symmetrical neuronal somata in the CoGs, with one pair originating in the OG. As shown in Fig. 4B–D, the stained stomatogastric nerve axons are among the subset of GABA-immunoreactive fibers in the nerves that connect the four ganglia, including the oesophageal nerve and the superior and inferior oesophageal nerves (see Fig. 1).

The motor nerves that exit the STG also contain GABA-immunoreactive fibers (Fig. 4E). There are commonly four GABA-immunoreactive fibers in the dorsal ventricular nerve, which is the main motor nerve projecting from the posterior end of the STG. Two of these fibers project through each lateral ventricular nerve (Fig. 4E), at least as far as the dorsal branch of the lateral ventricular nerve. We were unable to determine the eventual destination of these fibers. A diagram summarizing GABA-IR in the crab stomatogastric nervous system is shown in Fig. 5.

GABA-containing projection neurons

Previous work (Blitz et al., 1999) has shown that two pairs of the GABA-immunoreactive neurons that innervate the STG are previously identified projection neurons. One pair of the GABA-immunoreactive neurons is the modulatory commissural neuron 1 (MCN1s). There is a single MCN1 in each CoG, and each one projects *via* the inferior oesophageal nerve and stomatogastric nerve to the STG (Coleman and Nusbaum, 1994). The other previously identified pair of GABA-immunoreactive projection neurons is the modulatory proctolin neurons (MPNs). The MPNs are found in the OG, from which they project *via* the oesophageal nerve and stomatogastric nerve into the STG (Nusbaum and Marder, 1989a).

Nusbaum and Marder (1989a) showed that each MPN projects two axons through the stomatogastric nerve. Each of these neurons projects a single axon from its soma to the junction of the oesophageal nerve, superior oesophageal nerves and stomatogastric nerve. At this juncture, the MPN axon divides so that a single axon projects through each superior oesophageal nerve and a pair of axons projects through the stomatogastric nerve. We investigated whether both stomatogastric nerve axons of each MPN exhibited GABA-IR. In four nervous systems stained for GABA-IR, we followed these axons from each MPN soma to their branch point at the oesophageal nerve/superior oesophageal nerve/stomatogastric nerve junction and then into the stomatogastric nerve for several additional millimeters. In all four preparations, both stomatogastric nerve axons of both MPNs showed GABA-IR (Fig. 6). Thus, the stomatogastric nerve axons of MCN1 and MPN account for six of the eight GABA-immunoreactive axons in the stomatogastric nerve and, therefore, appear to represent two of the three pairs of GABAergic neurons that innervate the *C. borealis* STG. The axonal projections of these neurons are shown schematically in Fig. 1.

The presence of GABA in the stomatogastric nervous system: HPLC analysis

Authentic GABA has been identified previously in the crustacean brain and abdominal nervous system (Dudel et al., 1963; Hall et al., 1970; Kravitz et al., 1963; Orona et al., 1990; Otsuka et al., 1966, 1967). However, it has not previously been identified in the stomatogastric nervous system of *C. borealis*. Therefore, to verify that the GABA-IR was likely to be labeling authentic GABA, we extracted material separately from CoGs, OGs and STGs and analyzed it for GABA content (see Materials and methods). We found a molecule that is chromatographically indistinguishable from native GABA in each ganglion of the crab stomatogastric system (Fig. 7; *N*=10 for the STG and OG; *N*=20 for the CoG). As controls, we determined the HPLC retention time of several small molecules that might be found in neural tissue and might produce false positives for GABA. The substances tested included glutamate, aspartate, glycine, taurine and several metabolic relatives of GABA (GABOB, GABAmide). None of these co-migrated with either the purified substance or synthetic GABA.

The effect of GABA on STG motor patterns

Fig. 8 illustrates the effect of GABA on the pyloric rhythm of *C. borealis*. This figure shows simultaneous intracellular recordings from the lateral pyloric (LP) and pyloric dilator (PD) neurons and extracellular recordings from two motor nerves. GABA was applied in puffs of varying duration from 40 to 100 ms. Note that a brief GABA puff caused an inhibition of the PD neuron and depolarization and tonic firing of the LP neuron. As will be shown below, the depolarization in the LP neuron was a direct consequence of the actions of GABA on this neuron and not simply a circuit consequence of shutting off the pyloric rhythm. As the duration of the puff was

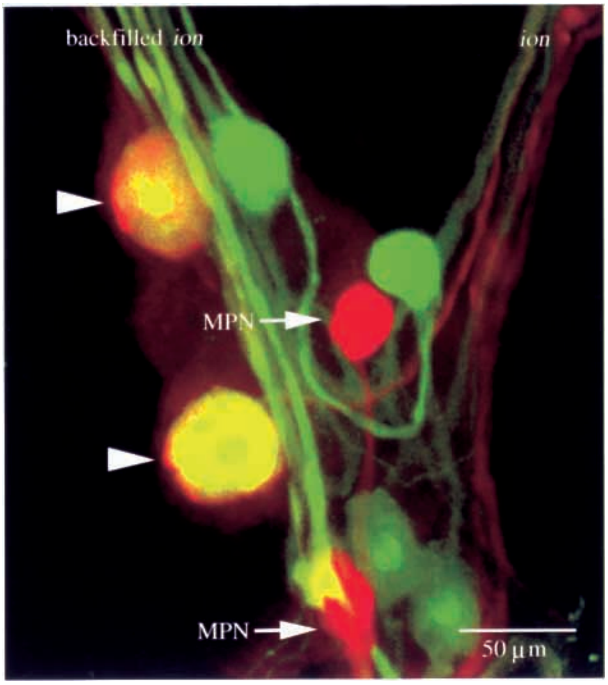

Fig. 3. Lucifer Yellow (LY) nerve backfill of the inferior oesophageal nerve (*ion*) paired with GABA immunoprocessing. LY backfills of this nerve routinely label six neuronal somata in the oesophageal ganglion (OG). Pairing these backfills with GABA immunoprocessing reveals that two of these OG neurons project axons into each *ion* and are GABA-immunoreactive (arrowheads). The modulatory proctolin neurons (MPNs, arrows), which are the only other GABAergic neurons in the OG, project through the *ions*. Labeling: green, LY dye-filling; red, GABA-immunoreactivity (GABA-IR); yellow (or shades of yellow to orange), co-localization of LY dye-filling and GABA-IR. Note that the yellow coloration seen overlying part of the modulatory proctolin neuron (MPN) at the bottom center of this image results not from co-localization of dye and immunoreactivity in a common optical section but from overlap of these moieties at distinct focal depths. This is a pseudocolored composite image of 42 optical sections collected at approximately 1 µm intervals.

increased (Fig. 8B,C), thus increasing the concentration of applied GABA, a more long-lasting inhibition of the pyloric rhythm was evoked. Under these conditions, the LP neuron still showed a depolarization, but did not fire. These effects were characteristic of those seen in 10 preparations.

Fig. 9 illustrates the effects of GABA on each identified cell type found in the STG. Each panel of Fig. 9 shows current-clamp recordings from the indicated cell type in response to GABA, applied either ionophoretically or by pressure. The LP neuron showed large depolarizations that often elicited firing. The ventricular dilator (VD) neuron showed modest depolarizations. The pyloric (PY) neurons showed a range of responses from excitatory (as shown) to inhibitory (not shown). All other pyloric cells, as well as all the gastric cells except interneuron 1 (INT1), showed strong inhibition. Often, neurons showing inhibition also exhibited a delayed excitation, as shown in Fig. 9 for the dorsal gastric (DG) and lateral gastric (LG) neurons. This is most evident in the cells that were weakly active prior to the application of GABA.

Table 1. *Reversal potentials of GABA responses in identified motor neurons*

Cell type	GABA reversal potential (mV)
Lateral pyloric (LP)	−41.3±6.4 (16)
Ventricular dilator (VD)	−41.7±7.7 (6)
Pyloric (PY)	−47.2±15.2 (9)
Median gastric (MG)	−51.8±6.5 (4)
Gastric mill (GM)	−61.9±12.1 (7)
Inferior cardiac (IC)	−65.1±8.9 (9)
Dorsal gastric (DG)	−70.3±2.5 (3)
Pyloric dilator (PD)	−70.7±8.8 (29)
Lateral gastric (LG)	−71.3±4.0 (3)
Lateral posterior gastric (LPG)	−74.5±4.0 (4)

Values are the means ± S.D. for each motor neuron type. The number of cells of each type in which values were measured is shown in parentheses.

All these data were collected in the presence of 10^{-7} mol l^{-1} tetrodotoxin.

Because there is extensive synaptic connectivity in the STG, it is impossible to determine from recordings such as those shown in Fig. 9 whether these responses are produced by direct actions of GABA or are a consequence of circuit interactions. In 12 experiments, therefore, we applied GABA to neurons in 10^{-7} mol l^{-1} tetrodotoxin (TTX) to block action potential generation and in a Mn^{2+}/low-Ca^{2+} solution to block graded synaptic release. An example of the responses of the LP and PD neurons under these conditions is shown in Fig. 10. In these solutions, the basic waveforms in response to the application of GABA stayed the same, suggesting that the GABA responses were not caused indirectly through chemical synapses. Nonetheless, some of these GABA responses could be a consequence of electrical synapses with other STG neurons or to terminals of the projection neurons. Given the distribution of response types and the pattern of electrical coupling within the STG, most of the responses are unlikely to result from electrical coupling. Notable exceptions are the PD neurons, the anterior burster (AB) cell and the lateral posterior gastric (LPG) neurons, because these neurons are strongly electrically coupled and all show hyperpolarizing responses.

Table 1 shows the reversal potentials for the GABA responses for most cell types in the STG measured in the presence of TTX. Note that the reversal potentials in the LP and VD neurons were the most depolarized, those of the PY and median gastric (MG) neurons were intermediate, and the remaining neurons had considerably more hyperpolarized reversal potentials.

Pharmacological dissection of GABA responses

Fig. 9 and Table 1 show clearly that different cell types respond differently to GABA. Therefore, we wished to

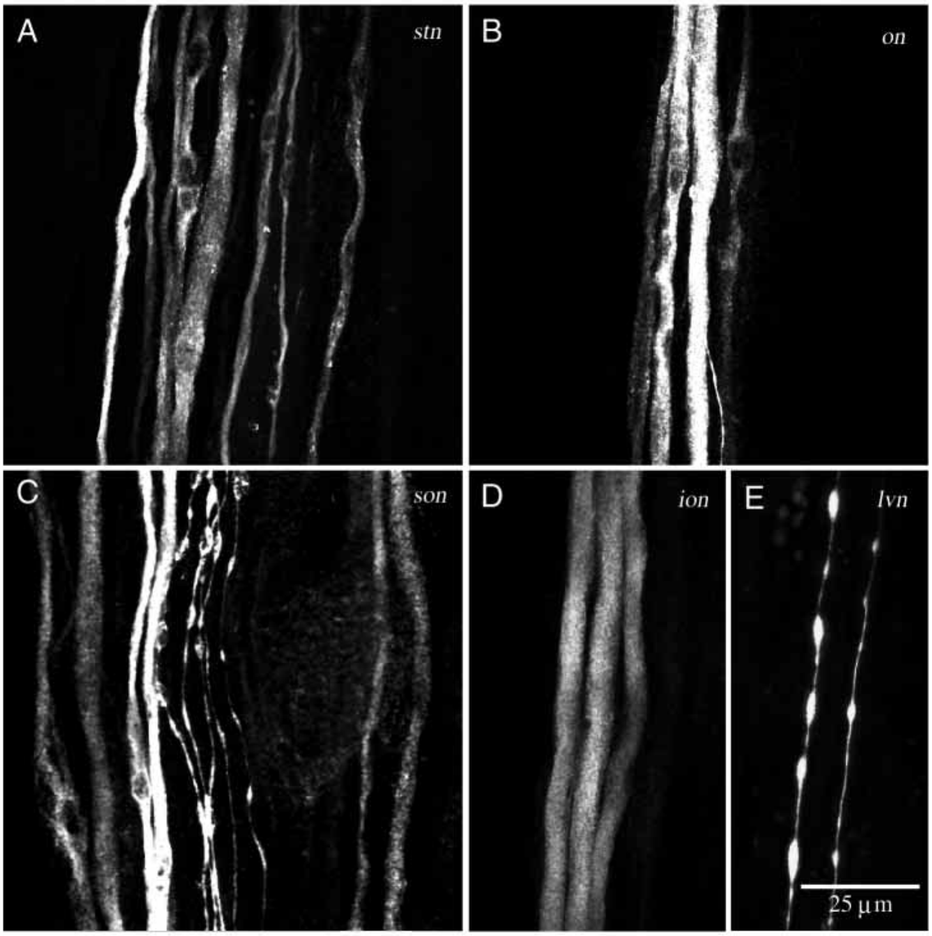

Fig. 4. GABA immunolabeling in neuronal fibers within the connecting nerves of the stomatogastric nervous system, including (A) the stomatogastric nerve (*stn*), (B) the oesophageal nerve (*on*), (C) the superior oesophageal nerve (*son*), (D) the inferior oesophageal nerve (*ion*) and (E) the lateral ventricular nerve (*lvn*). A–D are composite images of 36 optical sections taken at approximately 0.5 µm intervals and are from the same preparation. E is a composite image of 28 optical sections taken at 0.5 µm intervals. All images are shown at the same scale.

characterize these different responses in terms of their ionic dependence and pharmacological properties, with the hope of being able to use pharmacological tools to isolate different components of the synaptic potentials evoked by GABA-containing modulatory neurons.

Fig. 11 shows the GABA-evoked currents of an LP neuron and a PD neuron at different holding potentials, measured in two-electrode voltage-clamp mode. In this LP neuron, GABA evoked an outward current at holding potentials more depolarized than −50 mV and an inward current at more hyperpolarized holding potentials (Fig. 11A). When picrotoxin (10^{-5} mol l^{-1}) was applied to this preparation, the GABA-evoked outward currents at depolarizing holding potentials were enhanced, the reversal potential moved to approximately −75 mV and the amplitude of the GABA-evoked inward currents recorded at more hyperpolarized holding potentials

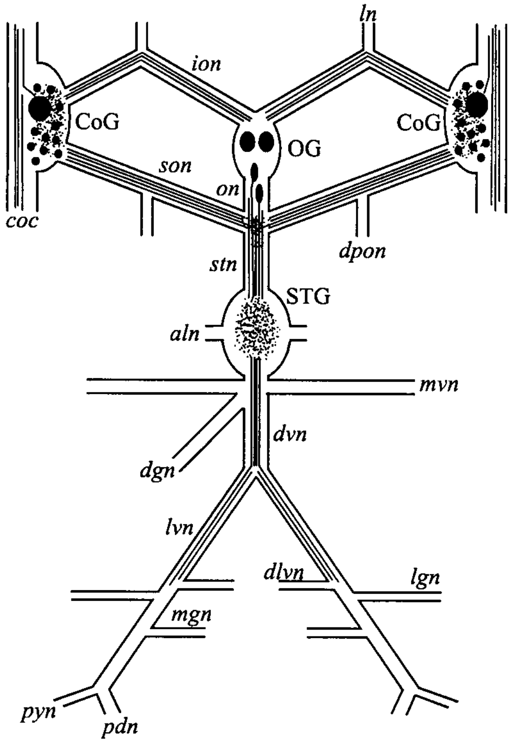

Fig. 5. Distribution of GABA immunoreactivity (GABA-IR) within the stomatogastric nervous system of *Cancer borealis.* Filled circles in the oesophageal ganglion (OG) and commissural ganglion (CoG) represent GABA-immunoreactive neuronal somata, lines within nerves represent GABA-immunoreactive axons, and speckled label in the stomatogastric ganglion (STG), CoGs and *stn*/*on* junction represents GABA-IR within neuropilar processes. Abbreviations as in Fig. 1.

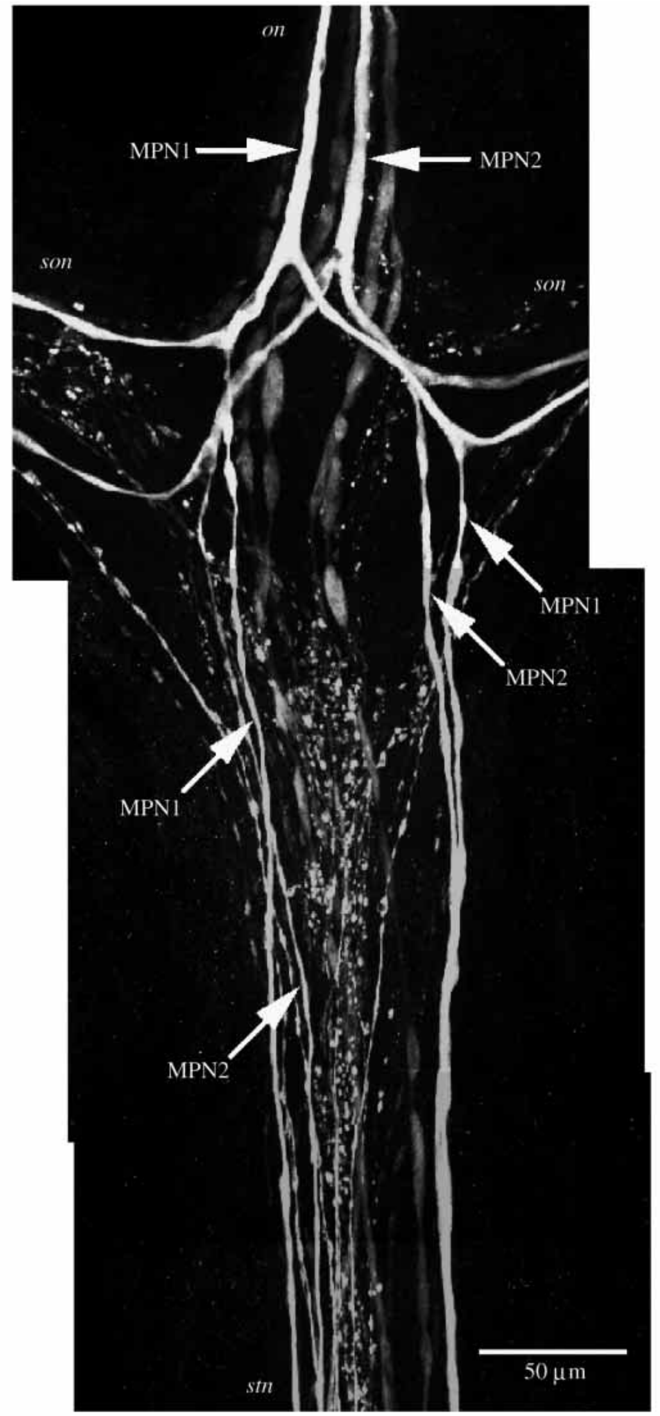

Fig. 6. Each of the two modulatory proctolin neurons (MPNs; described in Nusbaum and Marder, 1989a) projects two GABA-immunopositive axons through the stomatogastric nerve (*stn*) towards the stomatogastric ganglion. Each MPN projects a single axon through the oesophageal nerve (*on*) to where this nerve connects with the superior oesophageal nerve (*son*) and the *stn*. At this junction, the single axon divides into branches that project as a pair through the *stn* and singly through each *son*. All these axonal branches of both MPNs (labeled MPN1 and MPN2) exhibit GABA-immunoreactivity. This image is a montage of three sets of composite confocal images from the same preparation. Each composite consists of 41 optical sections collected at 1 µm intervals.

Table 2. *Response reversal potentials in the presence of various pharmacological agents*

Pharmacological substance(s)	Reversal potential (mV)	
	LP neuron	PD neuron
GABA	−41.3±6.4 (16)	−70.7±8.8 (29)
GABA/PTX	−71.1±4.0 (8)**	−68.8±8.1 (10)
Muscimol	−31.7±6.3 (9)**	−70.1±6.1 (10)
Muscimol/PTX	−72.8±15.5 (4)**	−72.0±10.6 (6)
β-GP	−73.8±5.7 (9)**	−72.6±4.7 (11)
β-GP/PTX	−77.4±11.2 (7)**	−70.3±4.8 (7)

Values are the means ± S.D.

The number of neurons studied *N* is given in parentheses for each set of measurements.

Reversal potentials of the responses are given for each of three agonists under control conditions and in the presence of $10\,\mu\mathrm{mol\,l^{-1}}$ picrotoxin (PTX).

For the LP neuron, there was a statistically significant difference for the reversal potentials between the responses under the various conditions tested and the GABA-evoked response (**$P<0.05$; one-way ANOVA followed by Dunnett's test). There was, however, no statistically significant difference between the shifts in reversal potential for β-GP, β-GP with PTX, GABA with PTX and muscimol with PTX ($P=0.581$; one-way ANOVA).

For the PD neuron, there was no statistically significant difference between any of the reversal potentials ($P=0.890$; one-way ANOVA).

LP, lateral pyloric neuron; PD, pyloric dilator neuron; β-GP, β-guanidinopropionic acid.

decreased (Fig. 11A). These data suggest that, in control saline, GABA evokes at least two different currents and that picrotoxin partially blocks a current with a more depolarized reversal potential. The effects of picrotoxin on the reversal potentials of GABA-evoked currents in the LP neuron are summarized in Table 2.

In the PD neuron shown in Fig. 11B, GABA evoked an outward current at holding potentials more depolarized than approximately −63 mV. At more negative holding potentials, GABA evoked an inward current. In this case, picrotoxin produced a relatively minor shift in the reversal potential and decreased the amplitude of the peak currents at both depolarized and hyperpolarized holding potentials. Note that some of these traces show a late inward current as well. The effects of picrotoxin on the reversal potentials of GABA-evoked currents in PD neurons are summarized in Table 2.

The data in Fig. 11 strongly suggest that GABA evokes two or more currents in STG neurons. Therefore, we hoped to find agonists that would preferentially activate these currents, thus allowing their ionic and pharmacological separation and characterization. To this end, we tested a number of substances that are known to be GABA agonists in other preparations. Fig. 12A shows the currents evoked by muscimol in a voltage-clamped LP neuron. In control saline, muscimol evoked a large inward current with a reversal potential more depolarized than −35 mV. In the presence of picrotoxin, the large inward current was blocked, revealing a small outward current that reversed

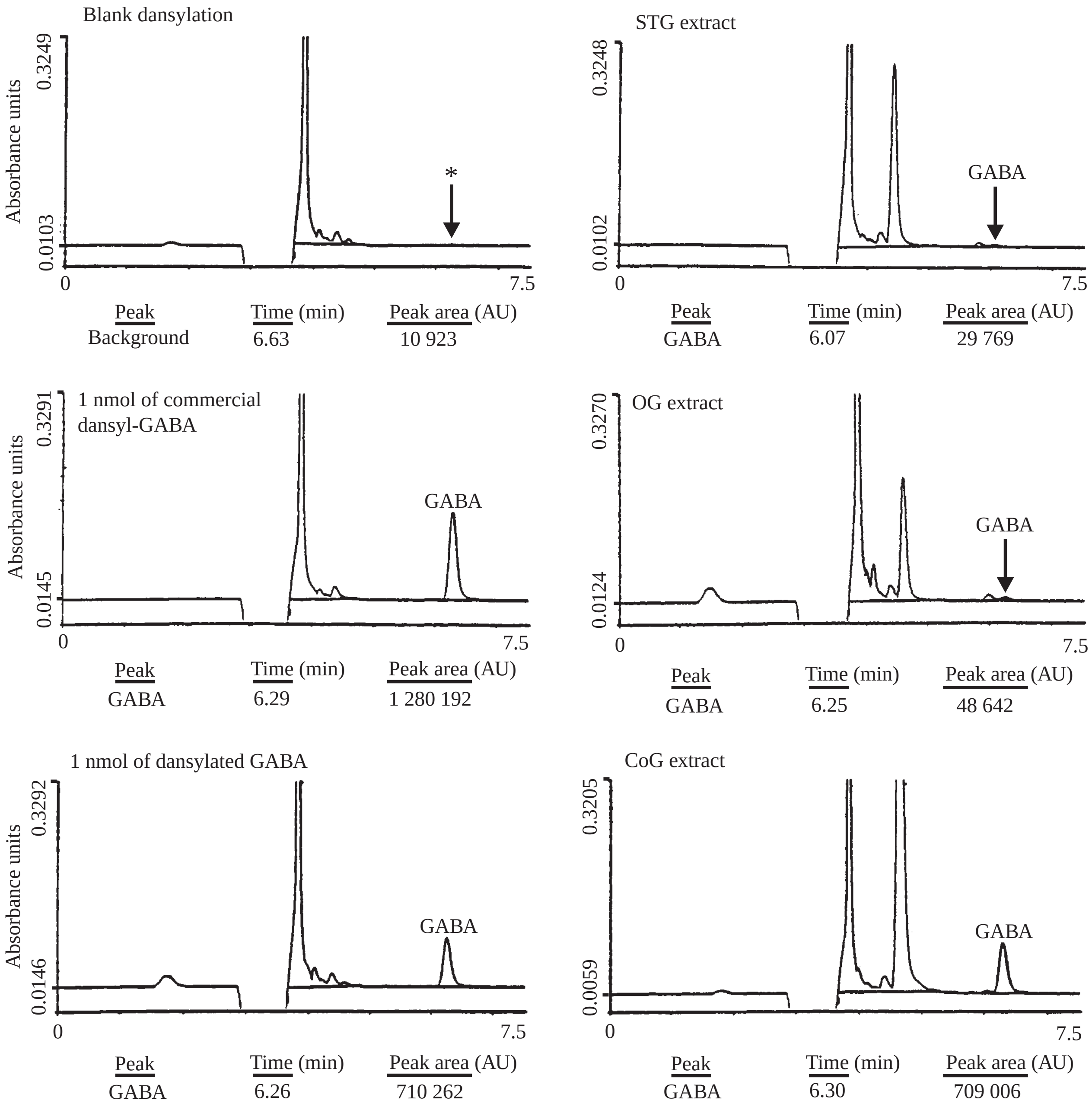

Fig. 7. The crab stomatogastric nervous system contains a molecule that is chromatographically indistinguishable from GABA. The solutions from separate extractions of a single stomatogastric ganglion (STG), oesophageal ganglion (OG) and commissural ganglion (CoG) were dansylated for processing and detection *via* HPLC to determine whether authentic GABA (detected as dansylated GABA) was present. After initial purifications *via* serial HPLC injections and collections using first a linear gradient and then isocratic conditions, both with trifluoroacetic acid as the counter-ion (not shown), the collected samples were run under isocratic conditions with TEA^+ as the counter-ion (see Materials and methods for details). The HPLC peaks from the latter isocratic conditions are shown. The GABA peak detected from samples of each ganglion showed the same retention time as both synthetic dansyl-GABA and dansylated synthetic GABA. An asterisk indicates the absence of a peak at the GABA position in the blank dansylated control. AU, absorbance units. *y*-axis values indicate baseline and peak of *y*-axis.

at approximately −85 mV. Our interpretation is that this small outward picrotoxin-resistant current was hidden by the much larger inward picrotoxin-sensitive current. The reversal potentials of the muscimol-evoked currents in the LP neuron in control saline and picrotoxin are shown in Table 2.

Fig. 12B shows the responses of a voltage-clamped PD neuron to muscimol in control saline and in the presence of picrotoxin. In control saline, muscimol evoked a current that reversed at approximately −70 mV. This current and its reversal potential were largely unaffected by picrotoxin (Table 2).

Previous work on the crab *Cancer pagurus* has shown that β-guanidinopropionic acid (β-GP) activated an increase in K^+ conductance (Marder and Paupardin-Tritsch, 1978). We

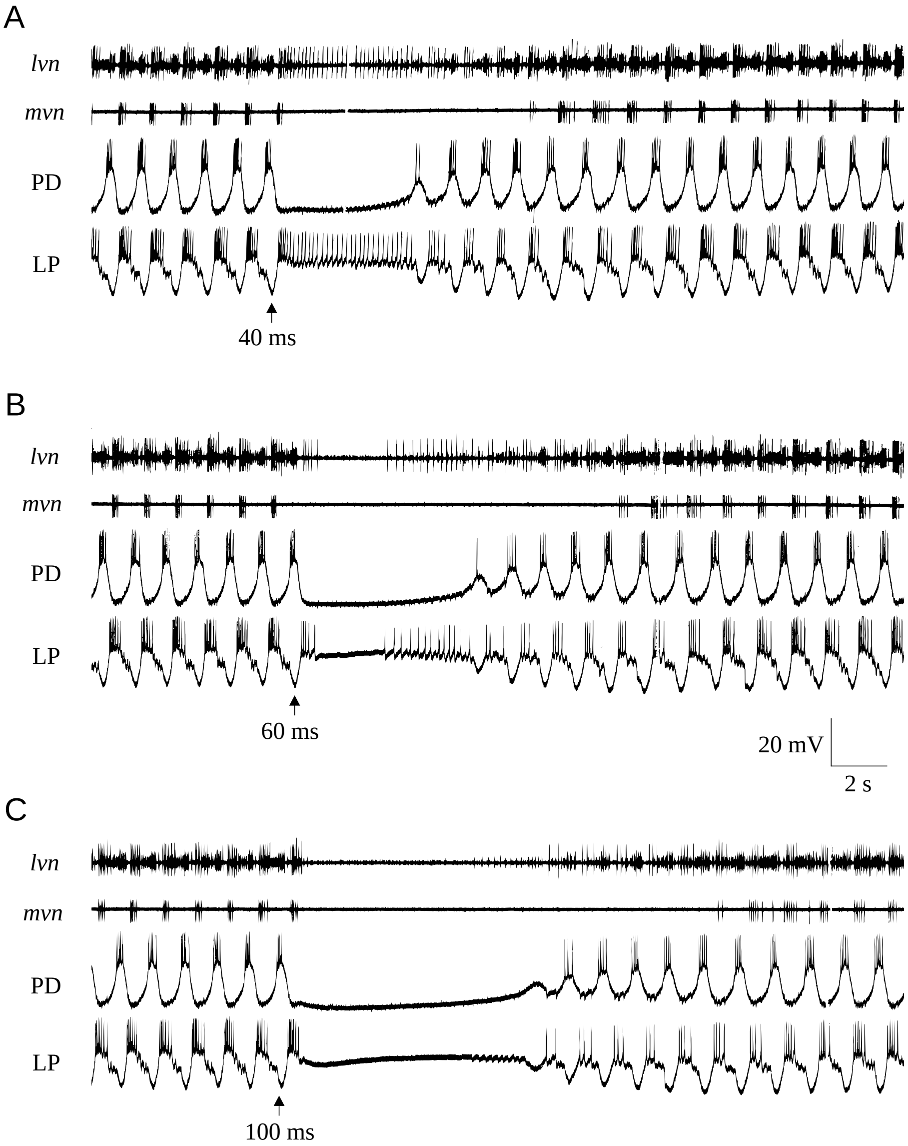

Fig. 8. The effects of GABA on the pyloric rhythm. In each panel, the top two traces are extracellular recordings from stomatogastric ganglion (STG) motor nerves, and the bottom two traces are simultaneous intracellular recordings from the pyloric dilator (PD) and lateral pyloric (LP) neurons. The three panels show the effects of increasing the dose of focally applied GABA by increasing the duration of GABA ($5\times10^{-3}\,\mathrm{mol\,l^{-1}}$ in the pipette) application (A for 40 ms; B for 60 ms; C for 100 ms). The onset of GABA application is indicated by the arrow under each set of recordings. Most hyperpolarized membrane potential: PD, −60 mV; LP, −48 mV. *lvn*, lateral ventricular nerve; *mvn*, medial ventricular nerve.

therefore applied β-GP to neurons in the STG. Fig. 13 shows that β-GP elicited a similar response in both the LP and PD neurons that reversed at relatively negative holding potentials. This response was also picrotoxin-insensitive in both cells (Table 2), suggesting that β-GP activates primarily the picrotoxin-insensitive outward component of the GABA response. We assume that this is the same component seen in the presence of picrotoxin when GABA or muscimol is used as an agonist.

In addition, we screened a large number of other substances that are known to be either effective agonists or antagonists of vertebrate GABA responses. Most antagonists were ineffective on the *C. borealis* GABA responses: bicuculline (up to $10^{-3}\,\mathrm{mol\,l^{-1}}$, N=5), phaclofen (up to $10^{-3}\,\mathrm{mol\,l^{-1}}$, N=5), 2-hydroxysaclofen ($10^{-4}\,\mathrm{mol\,l^{-1}}$, N=2) and SR-95531 ($10^{-4}\,\mathrm{mol\,l^{-1}}$, N=2). The effects of β-hydrastine ($10^{-5}\,\mathrm{mol\,l^{-1}}$, N=7) were similar effects to those of picrotoxin. The GABA agonists varied in their effectiveness. Some elicited GABA-like responses in LP and PD neurons [TACA (N=3) and isoguvacine (weak, N=6)], others elicited β-GP-like responses [ZAPA (N=7) and SKF97541 (N=6)] and still others elicited weak or variable responses that were difficult to classify [baclofen (N=6), 3-aminopropanesulphonic acid (N=3) and CACA (N=5)]. Our inability to isolate each GABA response completely complicated the following ionic characterization of these responses.

Reversal potentials and ionic selectivity of the GABA responses

To compare the reversal potentials of the responses evoked by GABA, muscimol and β-GP, we voltage-clamped neurons and applied each of these agonists in turn to the same neuron at a variety of membrane potentials. Fig. 14A shows a plot of the currents evoked in an LP neuron by all three agonists. Note that the reversal potentials of the currents evoked by these three agonists were distinct. The reversal potential of the β-GP

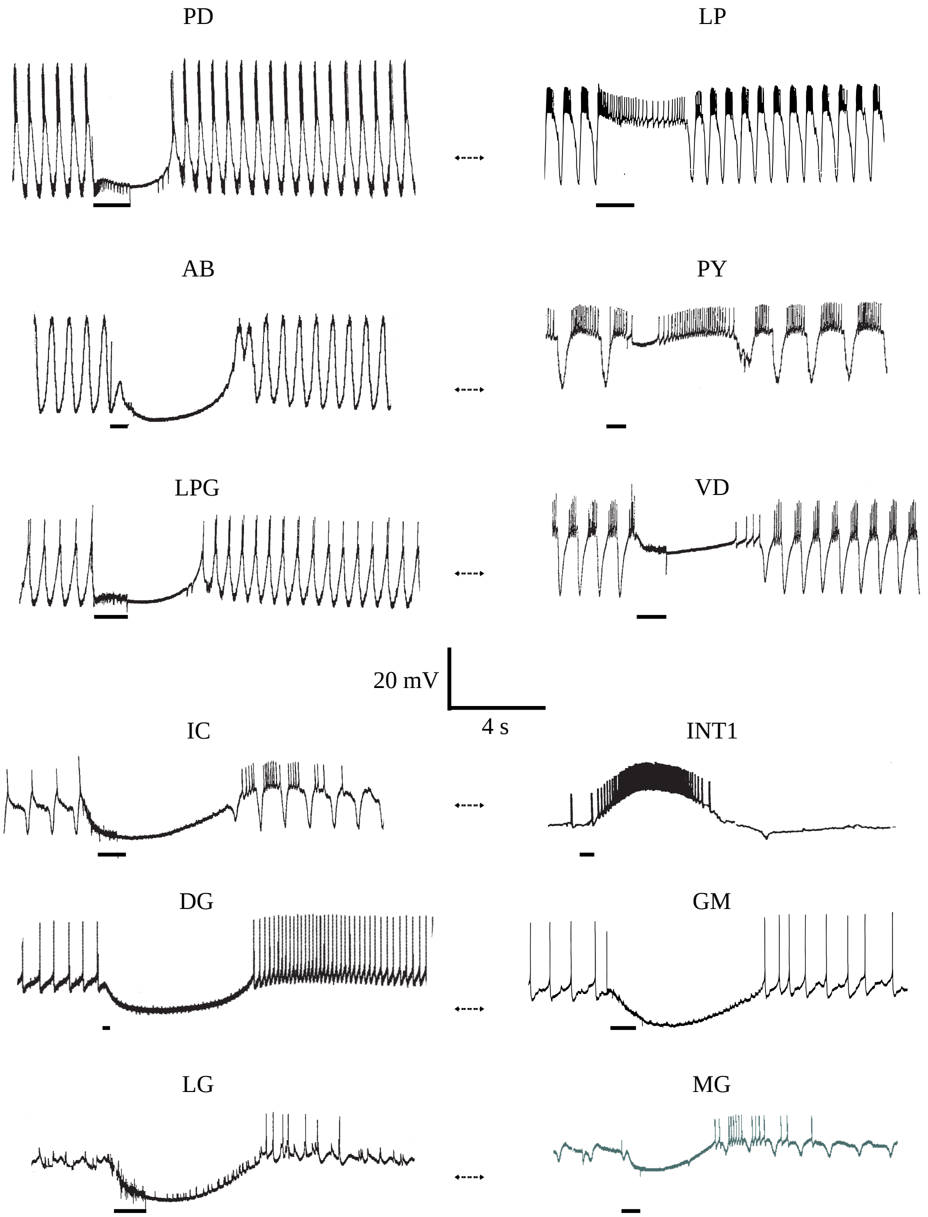

Fig. 9. Examples of the responses to GABA of each identified neuron in the stomatogastric ganglion (STG). Intracellular recordings of each neuron were made during saline superfusion with an ongoing pyloric rhythm but no gastric mill rhythm. GABA ($10^{-2}\,\mathrm{mol\,l^{-1}}$ in the pipette) was focally applied to the STG neuropil for the duration of the bar under each recording. Dashed lines with arrowheads indicate –50 mV. AB, anterior burster neuron; INT1, interneuron 1. All other neurons are defined in Table 1.

response in this neuron was –76 mV, while that evoked by GABA was –56 mV, and the reversal potential evoked by muscimol was –36 mV. This suggests that muscimol preferentially activates an inward current, that β-GP preferentially activates an outward current and that GABA activates both. The reversal potentials obtained with GABA will depend on the relative densities of these two components in a given neuron.

Fig. 14B shows that all three agonists evoke responses with similar reversal potentials in a PD neuron. To attempt to characterize the ionic conductances underlying these responses, we performed ion-substitution experiments on both LP and PD neurons with GABA, muscimol and β-GP as agonists, in the absence and presence of picrotoxin. The results of these experiments are summarized in Table 3. In the LP neuron, the reversal potentials of both the GABA and muscimol-evoked responses were shifted in low-Na^+ saline, while low-Na^+ saline had no effect on the reversal potentials of the β-GP response in the LP neuron or on the responses evoked by any of the agonists in the PD neuron. This suggests that the inward current evoked at depolarized holding potentials in the LP neuron is partially Na^+-dependent. The hyperpolarizing responses seen in current-clamp mode (or the outward currents seen at holding potentials close to the resting potentials of both neurons) seem to have both a K^+ and a Cl^- component because the shifts in reversal potential produced by changes in K^+ concentration are significantly smaller than those predicted by the Nernst equation for a pure K^+ conductance.

Discussion

Although the physiological actions of many neurotransmitters and modulators have been studied on the

Table 3. *Shifts in reversal potential produced by ionic substitutions and Cl$^-$ injection*

	Reversal potential shift (mV)		
	0.5[Na$^+$]	2[K$^+$] or 0.5[K$^+$]	Cl$^-$ injection
LP neuron			
GABA	−11.7±4.0 (3)	−8.0±1.0 (3)	5.3±3.5 (3)
GABA/PTX	1.3±1.5 (3)*	8.7±4.0 (3)	7.3±3.1 (4)
Muscimol	−11.2±3.3 (6)	−5.7±2.5 (3)	0.3±0.6 (3)
Muscimol/PTX	1.0±2.6 (3)*	9.0±2.6 (3)	6.0±2.0 (3)
β-GP	3.0±2.0 (3)*	−7.7±1.5 (3)	7.7±4.5 (3)
β-GP/PTX	2.3±3.5 (3)*	9.3±3.5 (3)	10.3±0.6 (3)
	0.5[Na$^+$]	2[K$^+$]	Cl$^-$ injection
PD neuron			
GABA	−0.2±1.5 (10)	11.2±5.8 (6)	10.8±6.4 (5)
GABA/PTX	2.3±0.5 (4)	8.8±2.6 (4)	11.3±7.1 (3)
Muscimol	1.3±1.2 (3)	8.8±1.5 (4)	11.0±7.0 (3)
Muscimol/PTX	1.5±1.7 (4)	7.8±1.7 (4)	14.7±3.8 (3)
β-GP	1.3±1.5 (4)	8.3±1.7 (4)	10.5±9.0 (4)
β-GP/PTX	0.8±1.7 (4)	7.8±2.4 (4)	9.3±2.5 (3)

Reversal potential shifts in response to substitutions of 0.5[Na$^+$], 0.5[K$^+$], 2[K$^+$] (see Materials and methods) or hyperpolarizing current injections with 3 mol l^{-1} KCl electrodes.

All values are means ± S.D. (*N*). For the lateral pyloric (LP) neuron, there was no statistically significant difference in reversal potential shifts in 0.5[Na$^+$] between GABA and muscimol (P=0.847; *t*-test). There was, however, a statistically significant difference in the reversal potential shifts in 0.5[Na$^+$] for β-guanidinopropionic acid (β-GP), β-GP with picrotoxin (PTX), GABA with PTX and muscimol with PTX compared with the shifts for GABA (P<0.05; one-way ANOVA followed by Dunnett's test). In addition, for the LP neuron, there was no significant difference between the reversal potential shifts in 0.5[Na$^+$] for β-GP, β-GP with PTX, GABA with PTX and muscimol with PTX (P=0.761; one-way ANOVA). There were no significant differences in the LP neuron for the shifts due to K$^+$ concentration substitutions (negative shifts were in 0.5[K$^+$], positive shifts were in 2[K$^+$]) (P=0.637; one-way ANOVA). All the responses in the LP neuron also showed dependencies on Cl$^-$ except for that to muscimol.

For the pyloric dilator (PD) neuron, there was no statistically significant difference for the shifts in 0.5[Na$^+$] (P=0.099; one-way ANOVA) or for the shifts in 2[K$^+$] (P=0.600; one-way ANOVA). All the responses in PD were Cl$^-$-dependent.

neurons of the stomatogastric ganglion of several decapod species, this is the first time that the effects of any neurotransmitter or neuromodulator have been studied on all the identified pattern-generating neurons in the stomatogastric ganglion of any species. Several interesting organizational principles emerge from our studies of the effects of GABA on the 12 different identified cell types in the STG (Fig. 12). All the STG neurons respond to GABA. This was not a foregone conclusion because other neurotransmitters and modulators are more selective in their actions on target neurons (Hooper and Marder, 1987; A. M. Swensen and E. Marder, in preparation). There are two identified pairs of modulatory projection neurons that contain GABA and innervate the STG in *C. borealis*. Both these neuron types also contain one or more neuropeptide cotransmitters (Blitz et al., 1999). Determining the actions of applied GABA in the STG will therefore facilitate future studies aimed at understanding the response of the neuronal network to co-released transmitters. Because GABA is released onto STG target neurons by descending modulatory projection neurons that act on multiple neural network targets in the STG, the actions of GABA at many sites within the pyloric and gastric mill networks are likely to play a role in determining how the modulatory neurons reshape the STG motor patterns.

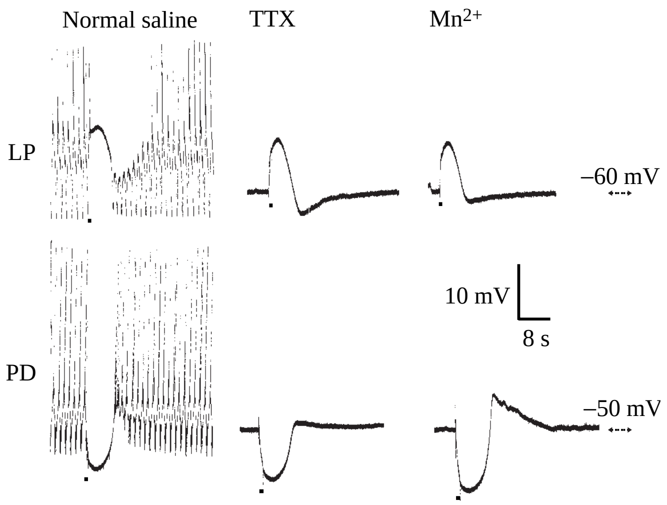

Fig. 10. GABA responses in the lateral pyloric (LP) and pyloric dilator (PD) neurons persist in the absence of synaptic transmission. Intracellular recordings from the LP and PD neurons in control saline, in the presence of tetrodotoxin (TTX; 10^{-7} mol l^{-1}) to eliminate action potentials, and in the presence of TTX in saline containing added Mn^{2+} (11.7 mmol l^{-1}) and reduced Ca^{2+} (1.3 mmol l^{-1}) to block transmitter release. GABA (10^{-2} mol l^{-1} in the pipette) was focally applied to the STG neuropil for the duration of the bar under each recording.

In the European lobster *Homarus gammarus*, Cazalets et al. (1987) found that bath application of GABA strongly inhibits the pyloric rhythm, and our data show that this is also the case in *C. borealis.* However, stimulation of both MCN1 and MPN, which contain GABA as one of their cotransmitters, results in strong excitation of both the pyloric and gastric mill rhythms. Thus far, neither of these projection neurons is known to elicit inhibitory responses from any STG neurons (Bartos and Nusbaum, 1997; Coleman and Nusbaum, 1994; Nusbaum and Marder, 1989a,b), although MPN does produce GABAergic inhibition in the CoGs (Blitz and Nusbaum, 1999). Some of the excitatory actions of these neurons could result from the depolarizing effects of GABA on some of the STG target neurons, such as on LP, PY and INT1. In fact, MCN1 and MPN both elicit transmitter-mediated, unitary excitatory postsynaptic potentials and slow modulatory responses in their STG target neurons (Coleman et al., 1995; Nusbaum and Marder, 1989a) that are mimicked by the peptide cotransmitters in these neurons (A. M. Swensen and

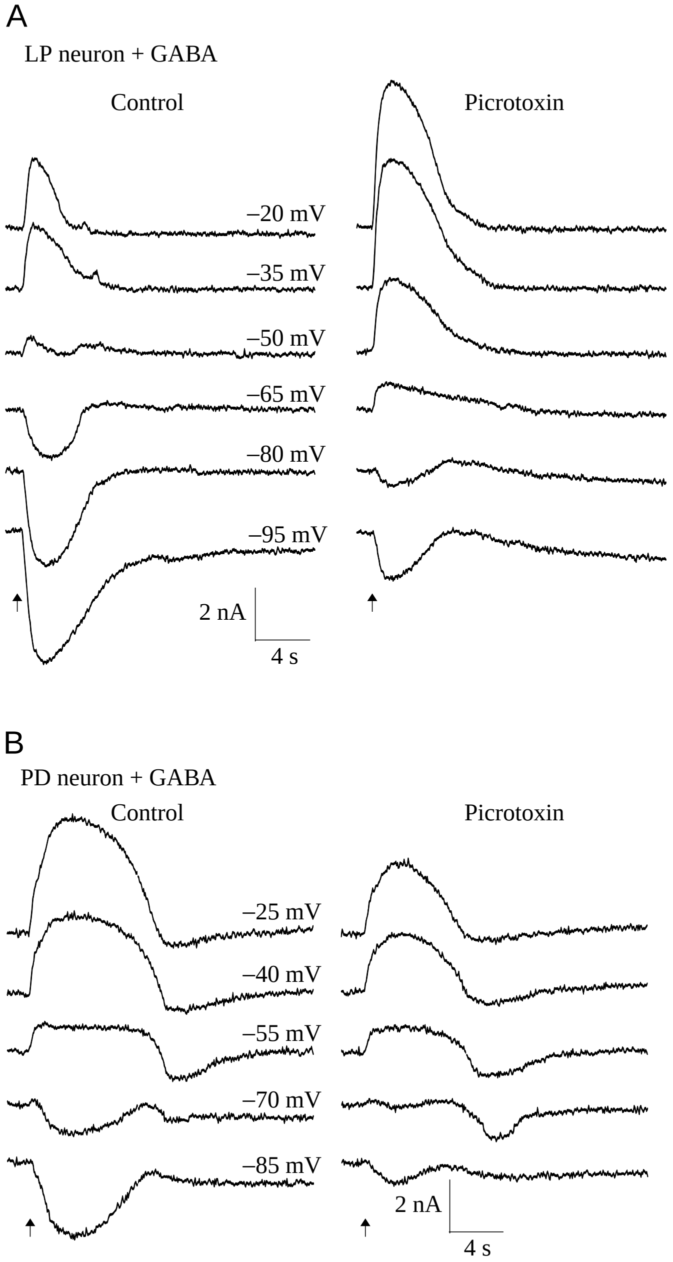

Fig. 11. The currents evoked by GABA in a lateral pyloric (LP) and a pyloric dilator (PD) neuron. (A) The GABA-evoked currents from an LP neuron in control saline and in saline containing picrotoxin (10^{-5} mol l^{-1}). In control saline, the LP response to GABA showed a reversal potential of approximately −50 mV. Picrotoxin altered the GABA-evoked current so that the net response now had a reversal potential of approximately −75 mV. (B) The GABA-evoked currents from a PD neuron in control saline and in saline containing picrotoxin (10^{-5} mol l^{-1}). In control saline, the PD response showed a reversal potential approximately −63 mV. In the presence of picrotoxin, the reversal potential of this response shifted to a slightly more hyperpolarized potential. Recordings were made in two-electrode voltage-clamp mode from various holding potentials. Focal GABA (5×10^{-3} mol l^{-1} in the pipette) applications to the stomatogastric ganglion neuropil are indicated by the arrows under the recordings. The saline contained tetrodotoxin (10^{-7} mol l^{-1}). Saline containing picrotoxin was superfused over the preparation.

E. Marder, in preparation). The role of the inhibitory responses to GABA displayed by many of the STG neurons (e.g. the GM, DG, AB and PD neurons) remains unexplained in the context of the actions of the GABA-containing modulatory neurons. These inhibitory responses may result from the actions of the third, as yet unidentified, GABA-containing projection neuron and/or from as yet unidentified inhibitory actions of MCN1 or MPN.

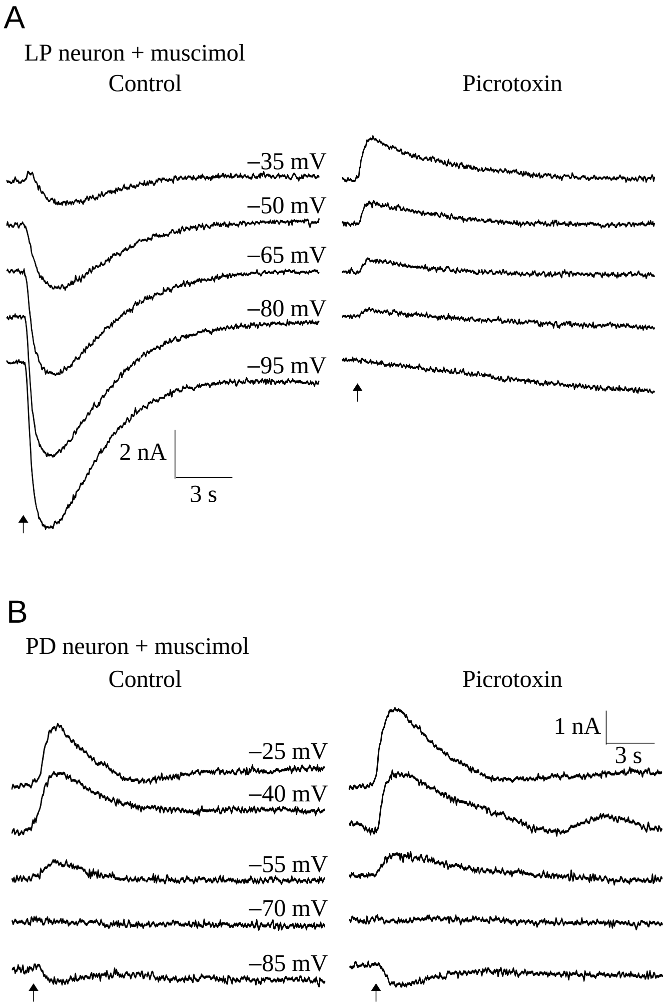

Fig. 12. The currents evoked by muscimol in a lateral pyloric (LP) and a pyloric dilator (PD) neuron. (A) The currents evoked in an LP neuron by a brief focal application of muscimol (5×10^{-3} mol l^{-1} in the pipette) to the stomatogastric ganglion neuropil in control saline and in saline containing picrotoxin (10^{-5} mol l^{-1}). In control saline, muscimol evoked a current showing a reversal potential more depolarized than −35 mV. With picrotoxin present in the superfused saline, the muscimol response reversed at a much more hyperpolarized membrane potential of approximately −85 mV. (B) The currents evoked in a PD neuron by a brief focal application of muscimol in control saline and in saline containing picrotoxin. In control saline, muscimol evoked a current with a reversal potential of approximately −70 mV. This response was unaffected by picrotoxin. Arrows under the recordings mark focal application of muscimol. Recordings were made in two-electrode voltage-clamp mode from various holding potentials. The saline contained tetrodotoxin (10^{-7} mol l^{-1}).

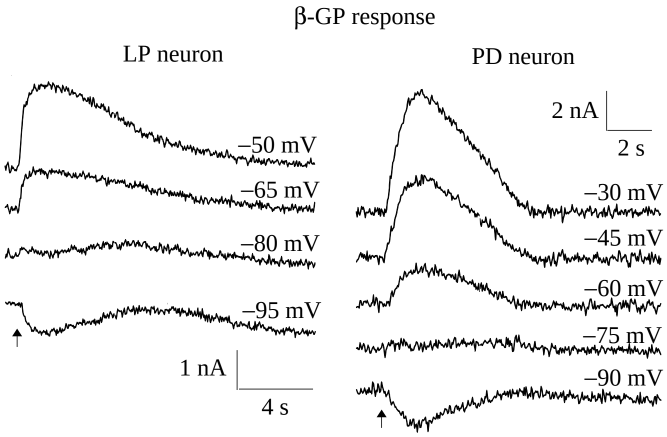

Fig. 13. The currents elicited by β-guanidinopropionic acid (β-GP) in a lateral pyloric (LP) and a pyloric dilator (PD) neuron. β-GP (2×10^{-2} mol l^{-1} in the pipette) was focally applied to the stomatogastric ganglion neuropil. Both cell types show responses with relatively hyperpolarized reversal potentials of approximately –75 mV. Arrows under the recordings mark focal applications of β-GP. Recordings were made in two-electrode voltage-clamp mode from various holding potentials. The saline contained tetrodotoxin (10^{-7} mol l^{-1}).

Whatever the case, the difference between the actions of bath-applied GABA and the actions of the modulatory neurons highlights the necessity of understanding in detail the responses elicited by all the cotransmitters released by a neuron on each of its targets.

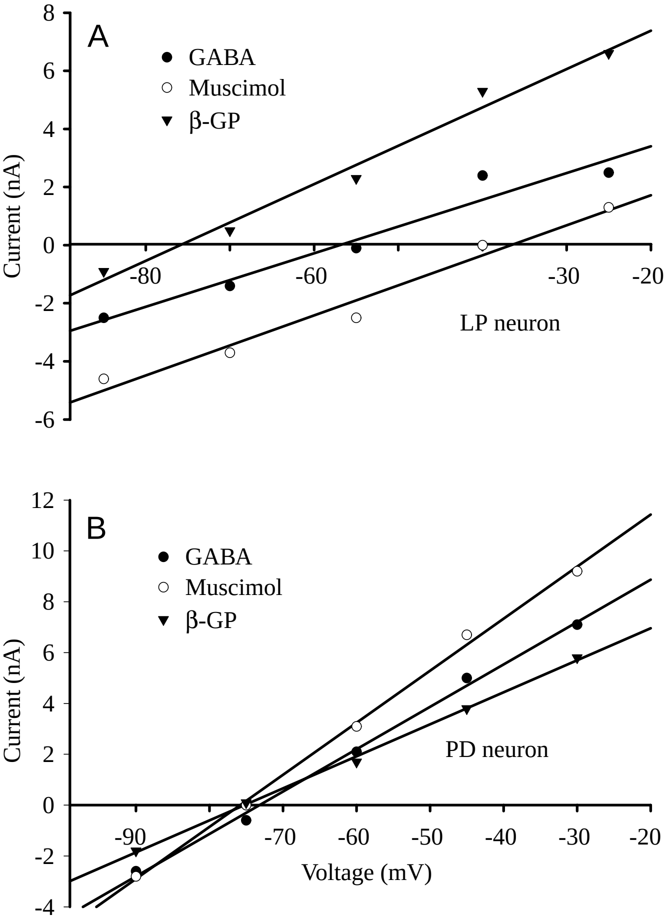

Fig. 14. The GABA-evoked, muscimol-evoked and β-guanidinopropionic acid (β-GP)-evoked currents with different reversal potentials in the lateral pyloric (LP) and pyloric dilator (PD) neurons. (A) Current *versus* voltage curves for the LP neuron response to GABA, muscimol and β-GP. Data points indicate that the LP neuron response to each of these substances involves a distinct mix of ionic conductances with different reversal potentials (V_{rev}). Muscimol elicited a response with a V_{rev} of –36 mV, GABA elicited a response with a V_{rev} of –56 mV and β-GP elicited a response with a V_{rev} of –76 mV. All data points are from the same experiment. (B) Current *versus* voltage curves for the PD neuron response to GABA, muscimol and β-GP. Application of all three of these substances elicited responses in the PD neuron that had essentially the same reversal potential (muscimol, V_{rev}=–76 mV; GABA, V_{rev}=–73 mV; β-GP, V_{rev}=–76 mV). All data points are from the same experiment. A and B are from different experiments. Recordings were made in two-electrode voltage-clamp mode from various holding potentials. The saline contained tetrodotoxin (10^{-7} mol l^{-1}). The points are peak measured currents. Lines were generated by linear regression using a least-squares fit (LP/GABA, r^2=0.949; LP/muscimol, r^2=0.971; LP/B-GP, r^2=0.982; PD/GABA, r^2=0.996; PD/muscimol, r^2=0.997; PD/B-GP, r^2=0.998).

There appear to be only three pairs of GABAergic projection neurons that innervate the crab STG. This statement is based on the presence of eight GABA-immunoreactive fibers in the stomatogastric nerve (this study), the fact that most or all projection neurons occur as bilaterally symmetrical pairs, including MCN1 and MPN (Coleman and Nusbaum, 1994; Nusbaum and Marder, 1989a), and that each MPN projects two axons through the stomatogastric nerve (Nusbaum and Marder, 1989a). Thus, there is likely to be one additional GABAergic projection neuron pair that innervates the STG. Cournil et al. (1990a) showed that the lobster stomatogastric system includes four GABAergic projection neuron pairs that innervate the STG. Their anatomical data show that both pairs of GABAergic neurons in the OG project to the STG. Thus, there is a difference between the two species: the MPN-equivalent neurons in the lobster, called GN1/2, only contribute one stomatogastric nerve axon each instead of two axons each as in the crab (Meyrand et al., 1994). However, each of the other pair of GABA-immunoreactive OG neurons in the lobster projects two axons through the stomatogastric nerve. In addition, the lobster system includes two paired CoG neurons that are GABA-immunoreactive and project to the STG. In the crab system, MCN1 is both GABA-immunoreactive and projects through the inferior oesophageal nerve and stomatogastric nerve to innervate the STG (Blitz et al., 1999). There are no physiological data available on the lobster neurons, however, so we cannot determine whether either of the lobster neurons is the species-equivalent version of MCN1. We have not yet determined whether the third and final GABA-immunoreactive projection neuron in the crab stomatogastric system is equivalent to the additional GABA-immunoreactive OG neuron or CoG neuron in the lobster.

A surprising and new feature of the immunocytochemical results reported here is the presence of GABA-immunoreactive fibers in the motor nerves exiting the STG. Unlike the case in many of the leg and abdominal muscles in crustaceans, there are no known inhibitory motor neurons that innervate the stomach muscles. Nonetheless, several of the stomach muscles show strong conductance changes in response to GABA (Albert et al., 1986; S. Gutovitz, J. Birmingham and E. Marder, unpublished observations), which have always been puzzling because, until now, there was no indication of a possible GABAergic innervation of these muscles. It is possible that at least some of these GABA-immunoreactive axons originate from MPN because MPN action potentials can be recorded in the nerves posterior to the STG (M. P. Nusbaum, unpublished observations). Thus, it is possible that either MPN or another neuron located in a ganglion outside the STG plays the role of a peripheral inhibitor. If MPN does project to some of the stomach muscles, this would give it two quite different functions, first as a modulator of the STG networks and second as an inhibitory motor neuron.

GABA is the most common inhibitory neurotransmitter found in invertebrate and vertebrate nervous systems, including those of crustaceans. Indeed, the first direct demonstrations of the role of GABA as a neurotransmitter were elegantly carried out using crustacean preparations (Dudel et al., 1963; Dudel and Kuffler, 1961; Hall et al., 1970; Kravitz et al., 1963; Otsuka et al., 1966, 1967). More recently, the genes encoding the receptors mediating GABA actions in a number of nervous systems have been cloned and sequenced (Bettler et al., 1998; Hosie et al., 1997). In vertebrates, GABA receptors are found in two major classes: the Cl^--permeable ionotropic $GABA_A$ receptors, and the metabotropic $GABA_B$ receptors that either decrease voltage-dependent Ca^{2+} currents or activate K^+ currents. In vertebrates, the pharmacology of these two major classes of GABA receptor has been extensively characterized, and numerous agonists and antagonists for these receptors are available.

In contrast, the pharmacology of invertebrate GABA receptors is less clear. For example, although in many arthropod species there is a receptor that resembles, both structurally and pharmacologically, the vertebrate $GABA_A$ receptor (Hosie et al., 1997), in some arthropod species there are GABA-mediated increases in Cl^- conductance that are not blocked by picrotoxin (Albert et al., 1986; Marder and Paupardin-Tritsch, 1978). It is possible that a single amino acid change in the structure of the receptor could be responsible for this loss of picrotoxin sensitivity, because insecticide- and picrotoxin-resistant strains of *Drosophila melanogaster* have a single amino acid difference from the picrotoxin-sensitive wild-type strains (Buckingham et al., 1996; ffrench- Constant, 1993; ffrench-Constant et al., 1993; Zhang et al., 1994, 1995). Bicuculline, a competitive $GABA_A$ antagonist in vertebrates, is often without effect in invertebrate preparations (Benson, 1988; Hosie et al., 1997; Zhainazarov et al., 1997), and some GABA receptors in invertebrates (Jackel et al., 1994) may more closely resemble the vertebrate bicuculline-insensitive $GABA_C$ receptors (Djamgoz, 1995; Lukasiewicz, 1996) than the $GABA_A$ receptor.

Given that single amino acid replacements can significantly alter the pharmacological profile of GABA receptors, it is perhaps not surprising that the responses to GABA reported in this paper were relatively refractory to simple pharmacological classification. That said, our inability to find agonists that would preferentially activate only a single class of receptor makes it difficult to determine unambiguously the ionic mechanisms underlying the effects of GABA on STG neurons in this species. The simplest interpretation of our data is that there are three classes of GABA responses displayed by STG neurons in *C. borealis*: a depolarizing response, and two separate hyperpolarizing responses, mediated by Cl^- and K^+ respectively. However, we were never able to isolate these three responses completely, one from another, and in all cell types GABA clearly activates more than one of these, and possibly all three, to a different degree.

If there are three different GABA responses, each with a different reversal potential, then the reversal potential elicited by GABA would be a weighted average of the reversal potentials of the responses. This would depend on the number and conductance of the channels gated by GABA in each cell type. For example, the GABA reversal potential in the LP neuron was quite depolarized (Table 1) in comparison with the GABA reversal potentials in the PD, LG and LPG neurons, suggesting that the depolarizing response was large in the LP neuron and relatively small in the PD, LG and LPG neurons. When the large inward current in the LP neuron was blocked by picrotoxin (e.g. Fig. 11A), there remained a large outward current previously hidden by the inward current. The shift of the GABA reversal potential from –41 mV to –71 mV produced by picrotoxin (Table 2) illustrates the extent to which the depolarization was contributing to the GABA reversal potential. In the LP neuron, muscimol appears to be slightly more selective than GABA in evoking the depolarizing response because the reversal potential seen in the presence of muscimol was more depolarized than that seen in the presence of GABA. Nonetheless, picrotoxin revealed a hyperpolarizing response also evoked by muscimol. Thus, muscimol, like GABA, was not entirely selective in its actions.

Previous work on the closely related crab *Cancer pagurus* has shown that GABA elicited two distinct inhibitory responses, one mediated by Cl^- and the other mediated by K^+ (Marder and Paupardin-Tritsch, 1978). However, in *C. borealis*, ionic substitution experiments failed to reveal any responses that appeared to be purely Cl^--dependent or purely K^+-dependent responses (Table 3). The earlier study used hand-made fine-tipped ionophoretic electrodes to deliver the GABA, and this study used more diffuse puffs of GABA, so it is possible that the previous study achieved better spatial restriction of the applied GABA, thus revealing different types of responses. Alternatively, it is possible that, in *C. borealis*, $GABA_A$-like Cl^--dependent and $GABA_B$-like K^+-dependent responses are routinely found in close proximity on all the neurons.

One of the most interesting unresolved issues in this study is the nature of the depolarizing response evoked by GABA and muscimol. GABA is known to elicit depolarizations by a number of mechanisms in other systems. (i) In many vertebrate neurons, GABA inhibitory responses are followed by a bicarbonate-mediated excitation (Perkins and Wong, 1996). Bicarbonate depolarizations have also been well-studied in crustacean preparations (Kaila, 1994; Kaila et al., 1989; Kaila and Voipio, 1987). Because our saline did not contain a bicarbonate buffer, there would be a large driving force for bicarbonate leaving the cell, making the bicarbonate responses depolarizing. We suggest that our early depolarizing GABA response is not completely attributable to bicarbonate because it is strongly Na^+-dependent. Nonetheless, the delayed inward current sometimes seen after the strong outward currents (Fig. 11B) is reminiscent of the bicarbonate responses that follow GABA responses in other preparations. (ii) In spinal motor neurons of *Xenopus laevis*, there is a Na^+-dependent Cl^- transporter that increases the intracellular Cl^- concentration, thus producing an inward Cl^- current that is sensitive to changes in Na^+ concentration (Rohrbough and Spitzer, 1996). This does not appear to be the case in our neurons because, after the depolarizing current had been blocked by picrotoxin (assuming that the transporter is picrotoxin-insensitive), the reversal potential of the remaining current was insensitive to changes in Na^+ concentration, but still retained sensitivity to both Cl^- and K^+ (Table 3). (iii) Na^+-dependent GABA uptake can produce a depolarization in crustacean neurons (Kaila et al., 1992). In these studies, muscimol was not taken up well by the transporter, and picrotoxin did not block the uptake system, arguing against this as an explanation for our data. (iv) There could be a GABA-activated mixed cation channel. This would be the most novel possibility. There are other reports of GABA-mediated depolarizations in invertebrates (Norekian, 1999; Zhang et al., 1997), including a depolarizing response in *Aplysia californica* attributed to an increase in cation conductance (Yarowsky and Carpenter, 1978) but, to the best of our knowledge, there are no unambiguously demonstrated GABA-activated mixed cation (Na^+/K^+) or Na^+ conductances.

On the basis of our present data, it is not possible to determine whether some of these or other mechanisms are giving rise to the depolarizing response. That said, the strong depolarization evoked by GABA in the LP and PY neurons of the pyloric circuit and in INT1 of the gastric mill circuit can play important roles in shaping the response of these circuits to GABA released as a cotransmitter from one of the modulatory projection fibers.

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