The Journal of Experimental Biology 211, 2624-2637
Published by The Company of Biologists 2008
doi:10.1242/jeb.019711

Characterization of a descending pathway: activation and effects on motor patterns in the brachyuran crustacean stomatogastric nervous system

Ulrike B. S. Hedrich and Wolfgang Stein*
Institute of Neurobiology, Ulm University, D-89069 Ulm, Germany
*Author for correspondence (e-mail: wstein@neurobiologie.de)

Accepted 2 June 2008

SUMMARY

The regulation of motor patterns by higher-order neuronal centers ensures appropriate motor function and behavior, but only a few studies have characterized this regulation at the cellular level. Here, we address motor pattern regulation in the stomatogastric nervous system (STNS) of the crab *Cancer pagurus*. This easily accessible model system is an extension of the central nervous system and contains the motor circuits that generate the rhythmic motor patterns for ingestion (esophageal rhythm) and processing of food (gastric mill and pyloric rhythms).

We have documented the actions of two identified neurons located in the brain on the STNS motor circuits. We show that these neurons provide exteroceptive chemosensory information to the motor circuits and we outline their axonal projection patterns, their firing activity and their effects on three motor patterns. Backfill stainings and activity measurements *in vivo* and *in vitro* show that two neurons located in cluster 17 of the brain project *via* the inferior ventricular (IV) nerve to the STNS. These IV neurons started to burst rhythmically when chemosensory stimuli were applied to the first antennae. When rhythmically activated *in vitro*, gastric mill rhythms were elicited or, if already active, entrained by the IV neuron activity. In addition, IV neuron stimulation excited the esophageal motor neuron and inhibited several pyloric neurons such that the timing of the IV neuron activity was imposed on all motor rhythms. The IV neurons were thus capable of synchronizing the activities of different motor circuits, which demonstrates the regulation of motor patterns by higher-order neuronal centers.

Key words: central pattern generation, *Cancer* crabs, stomatogastric ganglion, descending control.

INTRODUCTION

Rhythmic motor patterns such as walking (Bässler, 1993; Pearson, 2004), flying (Wilson, 1961), breathing (Lieske et al., 2000) and the grinding of food (Marder, 2000; Marder and Bucher, 2001; Marder and Calabrese, 1996) are usually produced by central pattern generators (CPGs). These CPGs are often multifunctional and comprise a large number of different motor outputs. They are controlled by sensory feedback and upstream neural structures (Brodfuehrer and Thorogood, 2001; Fleischer, 1981; Perrins et al., 2002; Rossignol et al., 2006; Spirito, 1975) in order to generate the appropriate behavior for the situation. The regulation of motor activity by higher neuronal centers, particularly those in the brain, is as yet poorly understood.

There are only a few systems in which CPG circuit neurons, their connectivity and their input pathways have been studied in sufficient detail to allow an investigation of motor pattern regulation at the cellular level. One such system is the stomatogastric nervous system (STNS) of decapod crustaceans (Marder and Bucher, 2001; Marder and Bucher, 2007; Nusbaum, 2002; Selverston and Moulins, 1987). The STNS is an extension of the central nervous system and contains several CPGs that control rhythmic movements of the crustacean foregut. Two of these CPGs are located in the stomatogastric ganglion (STG): the gastric mill circuit controls the chewing movements of three teeth in the gastric mill, and the pyloric CPG generates the rhythmic movement of the pyloric filter apparatus (Hartline and Maynard, 1975; Maynard and Dando, 1974).

The motor circuits are heavily modulated by projection neurons that descend from the paired commissural ganglia (CoG; see Fig. 1B) (Nusbaum and Beenhakker, 2002), which in turn receive input from the brain (Fleischer, 1981; Kirby and Nusbaum, 2007). The inferior ventricular nerve (*ivn*) is an unpaired direct connection between the brain and STNS (Fig. 1A,B) and it contains only a few axons (Böhm et al., 2001; Dando and Selverston, 1972; Orlov, 1929). In crayfish, the activity on the *ivn* correlates with the amount of ingested food (Böhm et al., 2001), inspiring the hypothesis that some of the neurons on the *ivn* transmit motor pattern-associated information either from the STNS to the brain or *vice versa*. The somata of two *ivn* axons, called pyloric suppressor (PS) neurons or inferior ventricular neurons (IV neurons), are located either in the *ivn* or in the brain, close to the insertion point of the *ivn*. While it has been shown that these neurons can affect STNS motor patterns (Christie et al., 2004; Claiborne and Selverston, 1984; Marder and Eisen, 1984a), it is unclear whether they receive information from the STNS or relay information to it, and what kind of information they process.

Here, we studied the activation of the IV neurons by chemosensory stimuli in the crab *Cancer pagurus*. We outline their axonal projection patterns and we demonstrate that by relaying sensory information from the brain they regulate the activities of three motor circuits in the STNS.

MATERIALS AND METHODS

Animals and preparation

Cancer pagurus L. were obtained from commercial sources (Feinfisch GmbH, Neu-Ulm, Germany). Crabs were kept in filtered, aerated artificial seawater (10–12°C). Before dissection, animals

were anesthetized by packing them in ice for 20–40 min. Experiments were performed on the intact animal and on the isolated nervous system (see below). Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and with the guidelines laid down by the NIH in the USA regarding the care and use of animals for experimental procedures.

Solutions

C. pagurus physiological saline had the following composition (mmol l^{-1}): NaCl, 440; $MgCl_2$, 26; $CaCl_2$, 13; KCl, 11; Trizma base, 10; maleic acid, 5, pH 7.4–7.6. In some experiments, high divalent saline (5× Ca^{2+}/5× Mg^{2+}) was applied exclusively to the CoG to block polysynaptic connections (Blitz and Nusbaum, 1999; Smarandache and Stein, 2007).

Electrophysiology and preparations

Electrophysiological experiments were performed using standard methods as described previously (Bartos and Nusbaum, 1997; Blitz and Nusbaum, 1997; Stein et al., 2006).

Intact animal preparation (Fig. 1A)

In *in vivo* experiments the activities of the dorsal ventral nerve (*dvn*) and the *ivn* were recorded extracellularly. For identification of the IV neuron spikes, we determined the conduction direction of spikes on the *ivn* with two hook electrodes attached to the *ivn* (see Fig. 1C,D). Silver wire (diameter 75 μm) with PTFE isolation (Goodfellow, Cambridge, UK) was used for the hook electrodes. The activities of the motor neurons of the pyloric and gastric CPGs were monitored *via* a single extracellular hook electrode at the *dvn*. Differential signals were recorded, filtered and amplified with an AM Systems amplifier (model 1700; Carlsborg, WA, USA). In these experiments, crabs were mounted in a turnable holder (courtesy of H. G. Heinzel, University of Bonn, Germany) to access *dvn* and *ivn* on the dorsal and ventral side of the crab, respectively. During the experiments, crabs were chilled to 12–14°C by surrounding the carapace with ice. Crabs were not in contact with seawater. Previous studies (Heinzel et al., 1993) have demonstrated that in these experimental conditions gastric mill and pyloric activity are generated consistently and neuromodulators keep their effects on the network. In general, activity generated under these conditions is similar to that in the intact animal and stress levels are usually sufficiently low to permit data collection. After dissection, animals remained undisturbed in the holder for at least 20 min to allow acclimatization. Only animals that showed normal eye and antennal movements as well as a typical motor activity (as estimated by the pyloric rhythm) were used in these experiments. For recording from the *dvn*, the dorsal carapace and the hypodermis above the STNS were opened. To test what kind of information the IV neurons relay from the brain to the motor circuits in the STG, we applied visual, tactile and chemosensory stimuli. Fleischer showed that the gastric mill rhythm is enhanced in the dark and suppressed when the crabs are illuminated (Fleischer, 1981). Therefore, we used a very simple stimulation protocol, namely turning the illumination on and off to estimate the impact of visual stimuli on *ivn* activity. Tactile stimuli were presented by touching the first antenna, the second antenna or the mouth area, in particular the maxillae and maxillipeds. Chemosensory stimuli were given by applying a mixture of seawater and crab food to the first antennae [which contain olfactory as well as chemosensory receptors (Brock, 1930)] and to the chemoreceptors around the mouth (Garm et al., 2005). Here, we did not distinguish between olfactory and chemoreceptive function. Chopped crab meat was used to stimulate the chemoreceptors and to feed the crabs, while seawater served as a control in these experiments. We tested the impact of food intake on *ivn* activity in feeding experiments. For this, a tube was inserted through the esophagus into the foregut and food was directly pressure injected into the stomach. Pressure injection rather than voluntary food intake was used to avoid olfactory and mechanical stimuli to antennae and claws and to avoid visual cues that could interfere with the measurements.

Isolated nervous system preparation (Fig. 1B)

The dissection of the STNS was performed in physiological saline at ~4°C as described previously (Stein et al., 2005). Briefly, the isolated STNS was pinned down in a silicon elastomer-lined (Elastosil RT-601, Wacker, Munich, Germany) Petri dish and continuously superfused (7–12 ml min^{-1}) with chilled physiological saline (10–13°C). In preparations with intact brains, the ophthalmic artery that contains the STG and carries hemolymph to the brain was superfused with chilled saline for 5–10 min prior to the dissection of the STNS to keep the brain alive (M. P. Nusbaum, personal communication).

Extracellular recordings *in vitro* were obtained as described previously (Smarandache and Stein, 2007); that is, by electrically isolating individual sections of STNS nerves from the bath by building a petroleum jelly-based cylindrical compartment around a section of the nerve. The action potentials propagating through the nerve were recorded by placing one of two stainless steel electrode wires within this compartment. The second wire was placed in the bath as a reference electrode. The differential signal was recorded, filtered and amplified with an AM Systems amplifier (model 1700).

To facilitate intracellular recordings the STG was desheathed and visualized with white light transmitted through a darkfield condenser (Nikon, Tokyo, Japan). Microelectrodes (15–25 MΩ) were filled with a solution containing 0.6 mol l^{-1} K_2SO_4 and 0.02 mol l^{-1} KCl. Intracellular current injections were accomplished using an NPI NEC 10L amplifier (NPI, Tamm, Germany) in bridge or single-electrode discontinuous current-clamp mode. Sample rates in discontinuous current-clamp mode ranged from 2 to 4 kHz. STG neurons were identified by estimating their activity patterns, synaptic interactions and axonal projection pathways in combination with current injections, as described previously (Bartos and Nusbaum, 1997; Blitz and Nusbaum, 1997; Weimann et al., 1991).

The pyloric cycle period was defined as the duration between the onset of impulse bursts of the two pyloric dilator (PD) neurons and the onset of the subsequent PD neuron bursts. Intracellular recordings were exclusively used to determine the mean number of action potentials per burst for several pyloric neurons, including the PD neurons and the lateral pyloric (LP, one cell) and pyloric constrictor (PY, 3–5 cells) neurons, and inferior cardiac (IC, one cell) and ventricular dilator (VD, one cell) neurons. The activities of the LP and PY neurons could also be monitored on the lateral ventricular nerve (*lvn*), while IC and VD neuron activities were recorded from the medial ventricular nerve (*mvn*; Fig. 1B). The activities of the PD neurons were monitored on the pyloric dilator nerve (*pdn*).

The gastric mill rhythm was monitored by the activity of the lateral gastric (LG, one cell) neuron, the dorsal gastric (DG, one cell) neuron and the gastric mill motor neurons (GM, four cells). The latter were exclusively measured intracellularly. The gastric mill rhythm was considered to be spontaneously active when the LG neuron (a member of the CPG) produced bursts of action potentials. The gastric mill cycle period was defined as the duration between the onset of a LG neuron burst and the onset of the

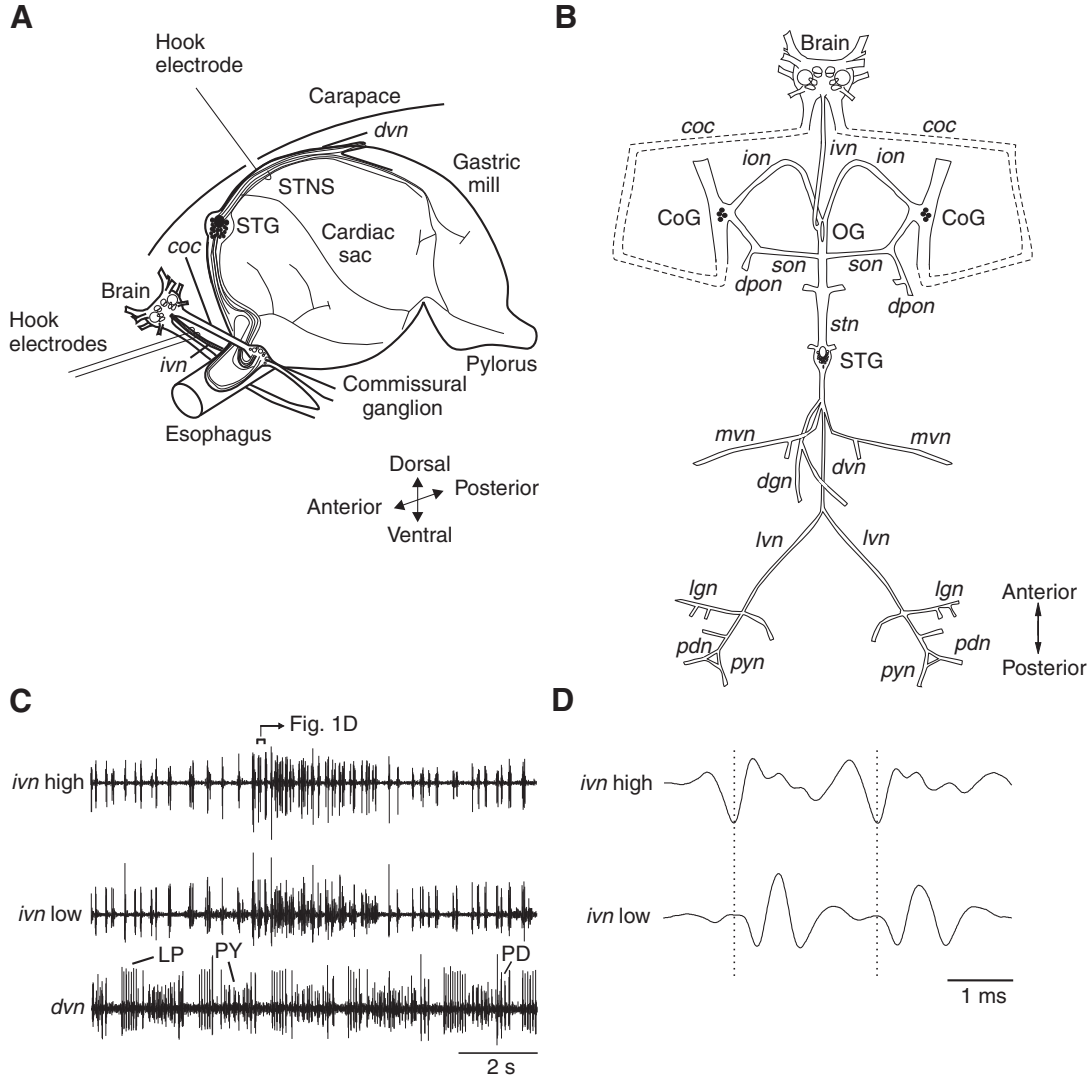

Fig. 1. Projections and activity patterns of the inferior ventricular (IV) neurons. (A) Schematic drawing of the foregut of the crab *C. pagurus* with the brain and the stomatogastric nervous system (STNS) attached. The foregut consists of four chambers with different functions: the esophagus (swallowing), the cardiac sac (storage of food), the gastric mill (chewing of food) and the pylorus (filtering of food). The STNS is located on the dorsal side of the foregut, and the brain on the dorsal side of the esophagus. The dorsal ventricular nerve (*dvn*) and the inferior ventricular nerve (*ivn*) were recorded extracellularly. For the *ivn*, we used a pair of hook electrodes to determine the direction of spike propagation. Adapted from Katz et al. (Katz et al., 1989). (B) Representation of the isolated STNS and the brain in *C. pagurus*. The STNS consists of four ganglia, including the unpaired stomatogastric ganglion (STG, 26 cells) and esophageal ganglion (OG, 15–16 cells) and the paired commissural ganglia (CoG, each about 500 cells). The brain and the STNS are connected *via* the unpaired *ivn* and the paired circumesophageal commissures (*coc*, dashed lines). In preparations using the isolated STNS, the *coc* were transected. *dgn*, dorsal gastric nerve; *dpon*, dorsal posterior esophageal nerve; *ion*, inferior esophageal nerve; *lgn*, lateral gastric nerve; *lvn*, lateral ventricular nerve; *mvn*, medial ventricular nerve; *son*, superior esophageal nerve; *stn*, stomatogastric nerve. (C) Extracellular recordings of the *ivn* (top and middle traces) and the *dvn* (bottom trace) in the intact animal. Both the *ivn* recording closer to the brain (*ivn* high; top trace) and the *ivn* recording closer to the STNS (*ivn* low; middle trace) show the activities of neurons that descend from and ascend to the brain. LP, lateral pyloric neuron; PD, pyloric dilator neurons; PY, pyloric constrictor neurons. (D) Magnification of Fig. 1C, showing the spikes of two neurons that descend from the brain to the STNS. The descending spikes first occurred on the *ivn* high recording (top trace) and then on the *ivn* low recording (lower trace).

subsequent LG neuron burst. The LG neuron was recorded either intracellularly from its soma or extracellularly from the lateral gastric nerve (*lgn*). The DG neuron was recorded either intracellularly or extracellularly from the dorsal gastric nerve (*dgn*; Fig. 1B).

The esophageal motor output was monitored by intra- and extracellular recordings from the esophageal motor neurons (OMNs). OMNs are a pair of bilaterally symmetrical neurons with one soma located in each CoG (Fig. 1B). Their axons project through the inferior esophageal nerve (*ion*) and can usually be identified by their large spike amplitudes.

Activities were measured as the number of action potentials (spikes) per burst, instantaneous spike frequency or as a sliding average with a bin width of 1 s. Mean values were determined from 10 consecutive cycles of gastric mill or pyloric activity. To activate the IV neurons, we stimulated the *ivn* extracellularly with 10 consecutive stimulus trains of 40 or 20 Hz stimulation frequency and different train durations (2–6 s) and intertrain intervals (1–20 s). For these experiments we used both preparations without brains and preparations with brains attached. In the latter preparations the STNS and the brain were exclusively

connected *via* the *ivn*. The paired circumesophageal commissures (*coc*) had been transected.

Data analysis

Data were recorded onto computer hard disk using Spike2 (version 5.03–6.04; CED, Cambridge, UK) and a micro 1401AD board (CED). Data were analyzed using Spike2 script language. Individual scripts are available at http://www.neurobiologie.de/spike2. Final figures were prepared with CorelDraw (version 12 for Windows). Graphics and statistics were generated using Excel (Microsoft) or Plotit (version 3.2, Scientific Programming Enterprises, Haslett, MI, USA). All data were tested for normal distribution. Statistical tests for data were one-way ANOVA for repeated measures (in the case of normal distribution) and Wilcoxon signed-rank test for non-parametric data. For the former, data are presented as means ± s.d. and for the latter as box-and-whisker plots showing medians, lower and upper quartiles, and minimums and maximums. *N* refers to the number of animals, while *n* gives the number of trials. For all statistical tests, significance with respect to control is indicated on the figures using the following symbols: $*P<0.05$, $**P<0.01$, $***P<0.001$.

Nerve fills

We used Lucifer Yellow-CH (Sigma-Aldrich, Munich, Germany), $CoCl_2$ and $NiCl_2$ fills of the *ivn* and other nerves within the STNS to determine the projections of the IV neurons (Kirby and Nusbaum, 2007). Both anterograde and retrograde axon fillings were observed. In these experiments, a Vaseline™ well was built around the *ivn*, *ion*, superior esophageal nerve (*son*) or stomatogastric nerve (*stn*), and the saline within the well was replaced with distilled water. Then, we transected the nerve within the well and, after several minutes, removed the distilled water and replaced it with a solution of 10% Lucifer Yellow, 10% $CoCl_2$ or 10% $NiCl_2$ in distilled water. The preparation was then incubated at 4°C for 12–72 h. Finally, the dye was removed from the well and the preparation was fixed in 4% paraformaldehyde (Merck, Hohenbrunn, Germany) in phosphate buffer, dehydrated in an ascending ethanol series and mounted in methyl salicylate (Fluka Chemie GmbH, Buchs, Switzerland) for viewing.

The exact location of the IV neurons in the brain was determined from frontal sections of the brain after Lucifer Yellow backfills of the *ivn*. For this, the brain was embedded in paraffin and sectioned on a microtome. The thickness of the brain slices was 10 µm.

RESULTS

The *ivn* connects the brain to the STNS. The localization of neuron somata in the brain and the arborization of the *ivn* fibers have not been determined in *C. pagurus*. We thus used a total of 57 Lucifer Yellow, $CoCl_2$ and $NiCl_2$ backfills to determine the number of axons contained within the *ivn* and their axonal projections.

The locations of the somata that project towards the brain (ascending fibers) within the *ivn* were determined with $NiCl_2$ backfills. Somata were located in the esophageal ganglion (OG) and in the CoG. In seven animals, we transected the CoG and filled the *ivn* towards the STNS. In five of the seven animals we found three stained somata in the OG. In two animals only two somata in the OG were stained. In five animals, the *ivn* was filled towards the STNS with intact CoG. In two preparations, the dye did not reach the CoG, but in three preparations one soma in each CoG was clearly visible.

We then filled the *ivn* towards the brain and determined the number of axons within the *ivn*. In *N*=9 of 11 frontal sections of *ivn* backfills, we found eight axons (Fig. 2C). In two animals the *ivn* contained seven axons. In each of the 11 backfills, we observed two large stained somata in the brain. Their axons could be traced to the *ivn*. No other somata were stained, which indicates that only two neurons descended from the brain to the STNS.

These neurons, called the IV neurons or PS neurons, had previously been identified in different crustacean species (Böhm et al., 2001; Cazalets et al., 1987; Christie et al., 2004; Claiborne and Selverston, 1984; Dando and Selverston, 1972; Sigvardt and Mulloney, 1982a; Sigvardt and Mulloney, 1982b). The IV neuron somata of *C. pagurus* were located in the brain, similar to the situation in *C. borealis* (Christie et al., 2004). They were located in the dorso-medial area of the brain (cell 1: 178.57±47.1 µm; cell 2: 221.43±48.5 µm; total thickness of brain: 575±67.7 µm; *N*=9; Fig. 2B) in cell cluster 17 [nomenclature after Sandeman et al. (Sandeman et al., 1992)]. There were no obvious dendritic ramifications of the IV neurons within the brain. We used $NiCl_2$ backfills of the *ivn* to quantify the size of IV somata. Cell bodies had an average length of 119.48±28.8 µm and a width of 90.89±19.3 µm (*N*=21). Fig. 2D shows a schematic summary drawing of the axonal projections of all *ivn* units in the brain and the location of the IV neuron somata in cluster 17.

Our *ivn* backfills (*N*=8) towards the STNS indicated that axons contained within the *ivn* projected to the *ion*, *son* and *stn*. To determine the axonal projections of the IV neurons we thus backfilled the *ion*, *son* or *stn* with $CoCl_2$ or $NiCl_2$ and followed the stained axons to the brain. In each backfill of the *son* (*N*=3), *ion* (*N*=9) and *stn* (*N*=5), the somata of the IV neurons were stained. In addition, when we traced the stained axons, we found that axons that connected to the IV neuron somata also projected to the *son*, *ion* and *stn* in each preparation (Fig. 2E). Together, these results show that the IV neurons projected *via* the *son* and *ion* to the CoG and *via* the *stn* to the STG.

Activation of the descending IV neurons in the intact animal

As yet, it is unclear whether and if so what kind of information the IV neurons relay from the brain to the STNS. Data from the crayfish *Orconectes limosus* indicate that the activity on the *ivn* increases after food intake and that this increase correlates with the amount of food intake (Böhm et al., 2001). It is, however, unclear whether this increase resulted from the activity of the descending IV neurons (and thus originated in the brain) or whether ascending *ivn* units started firing to a greater extent. Furthermore, since voluntary food intake activates a variety of sense organs, such as tactile, visual and olfactory organs, the sensory modalities stimulated during food intake are unknown. We thus recorded the *ivn* extracellularly in intact animals with two hook electrodes and used the delay between the spikes on both electrodes to determine their conduction direction and to differentiate between ascending and descending units. Corresponding to the results of our backfill stainings, all of our recordings (*N*=15) showed that only two neurons descended from the brain to the STNS. All other spikes ascended from the STNS to the brain (Fig. 1C).

We then tested the impact of mechanical, visual and chemosensory stimuli and that of food intake on the activity of the *ivn* units. First, we repeated the experiments previously reported on *O. limosus*, and fed the animal. Instead of relying on voluntary food intake we injected food directly into the stomach *via* a tube inserted into the esophagus, thus circumventing the activation of sense organs outside of the stomach. When we injected food, the activity of the neurons on the *ivn* recording remained unchanged (*N*=12; Fig. 3A and B right). By contrast, the pyloric rhythm sped up (Fig. 3A). In

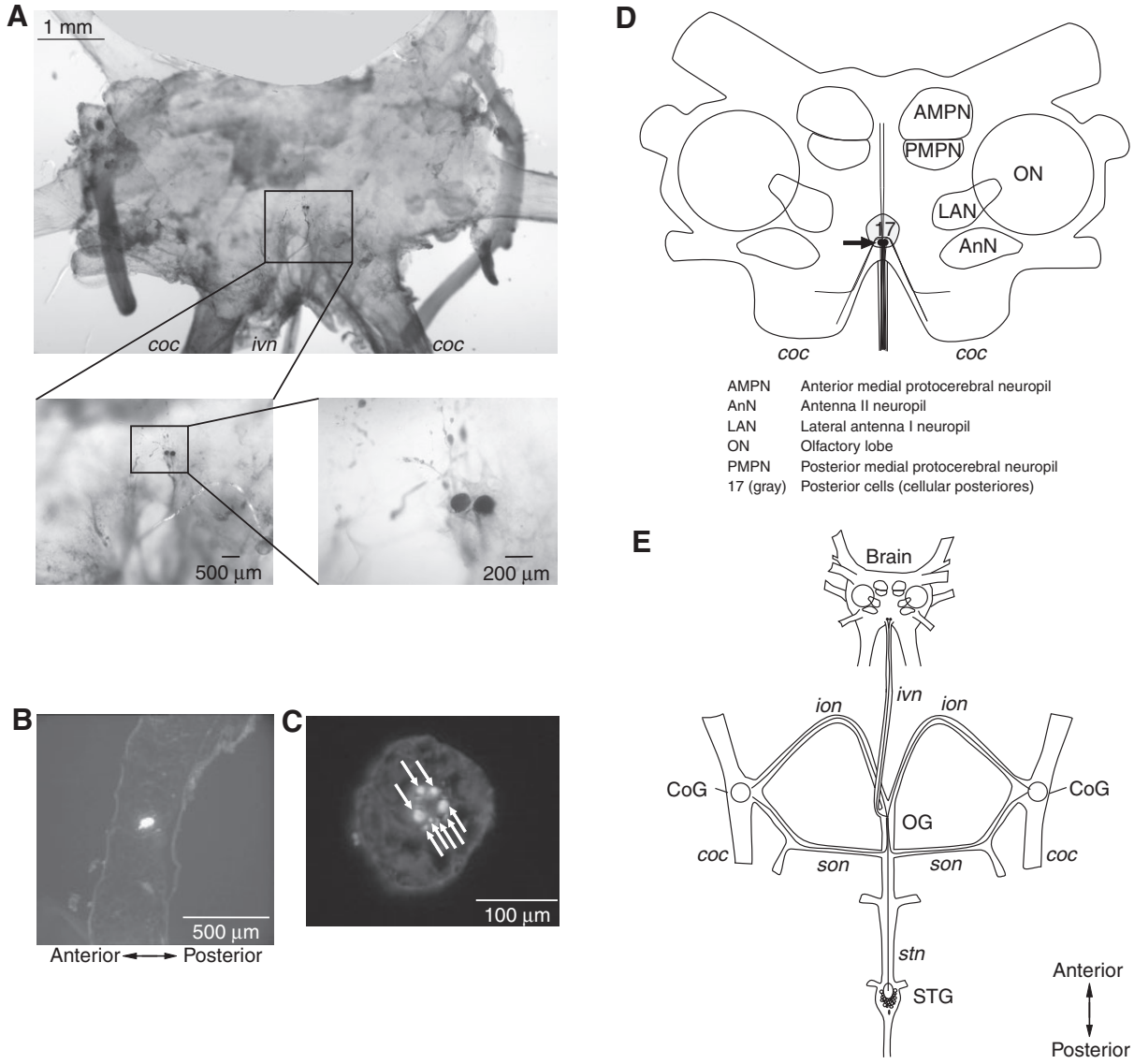

Fig. 2. Anatomical features of IV neurons. (A) *C. pagurus* brain with stained IV neurons after backfilling the *ivn* with $NiCl_2$. The areas indicated by the black boxes are magnified below the main image, and bottom right shows the somata of the IV neurons in area 17 [nomenclature after Sandeman et al. (Sandeman et al., 1992)]. No arborizations within the brain were observed. (B) Frontal section of a brain showing the dorso-medial location of the IV neuron somata. Somata were stained with a Lucifer Yellow backfill of the *ivn*. (C) Cross-section of the *ivn*. Eight axons were visible (arrows), two of which belonged to the IV neurons. (D) Schematic drawing of *ivn* projections in the brain, as revealed by *ivn* backfills. The somata of the IV neurons marked by the arrow were located in area 17 of the brain. Two (ascending) axons projected along the midline towards the anterior part of the brain, but could not be followed further than the AMPN (anterior medial protocerebral neuropil). The four other axons projected towards the *coc*. (E) Schematic drawing of IV neuron projections (summary). The IV neurons project *via* the *son* and the *ion* to the CoG and *via* the *stn* to the STG.

the example shown in Fig. 3A, the pyloric cycle period decreased from 1.06 s before feeding to 0.77 s after feeding. The median pyloric cycle period decreased significantly from 1.62 s (lower quartile 1.25 s; upper quartile 1.91 s) to 1.28 s (lower quartile 1.02 s; upper quartile 1.56 s; $P<0.01$, $N=12$, Wilcoxon signed-rank test). The phasing of the LP neuron also changed. Both the onset and the end of its activity were significantly delayed. The median phase onset changed from 0.36 before feeding to 0.38 after feeding ($N=12$, $P<0.04$; Fig. 3B left); the end of its activity phase changed from 0.66 before feeding to 0.70 after feeding ($N=12$, $P<0.01$, Wilcoxon signed-rank test; Fig. 3B left). The phasing of the PD neurons did not change. Together, these results indicate that filling the stomach with food activated sense organs within the stomach that in turn affected the pyloric circuit in the STG. This effect, however, was not mediated *via* the *ivn*.

To examine whether the IV neurons relay sensory information from the brain to the STNS we tested visual (1), tactile (2) and chemosensory stimuli (3). For all sensory modalities, we used very simple stimulation procedures. (1) Fleischer has shown that the gastric mill rhythm is very sensitive to changes in illumination; that is, it is suppressed in bright light and enhanced in darkness (Fleischer, 1981). Therefore, we used a very simple stimulation protocol, namely turning the illumination on and off, to test whether changes in illumination affected the activity of the IV neurons. However, in all 13 tested animals, the IV neurons did not respond to these illumination stimuli. (2) Similarly, tactile

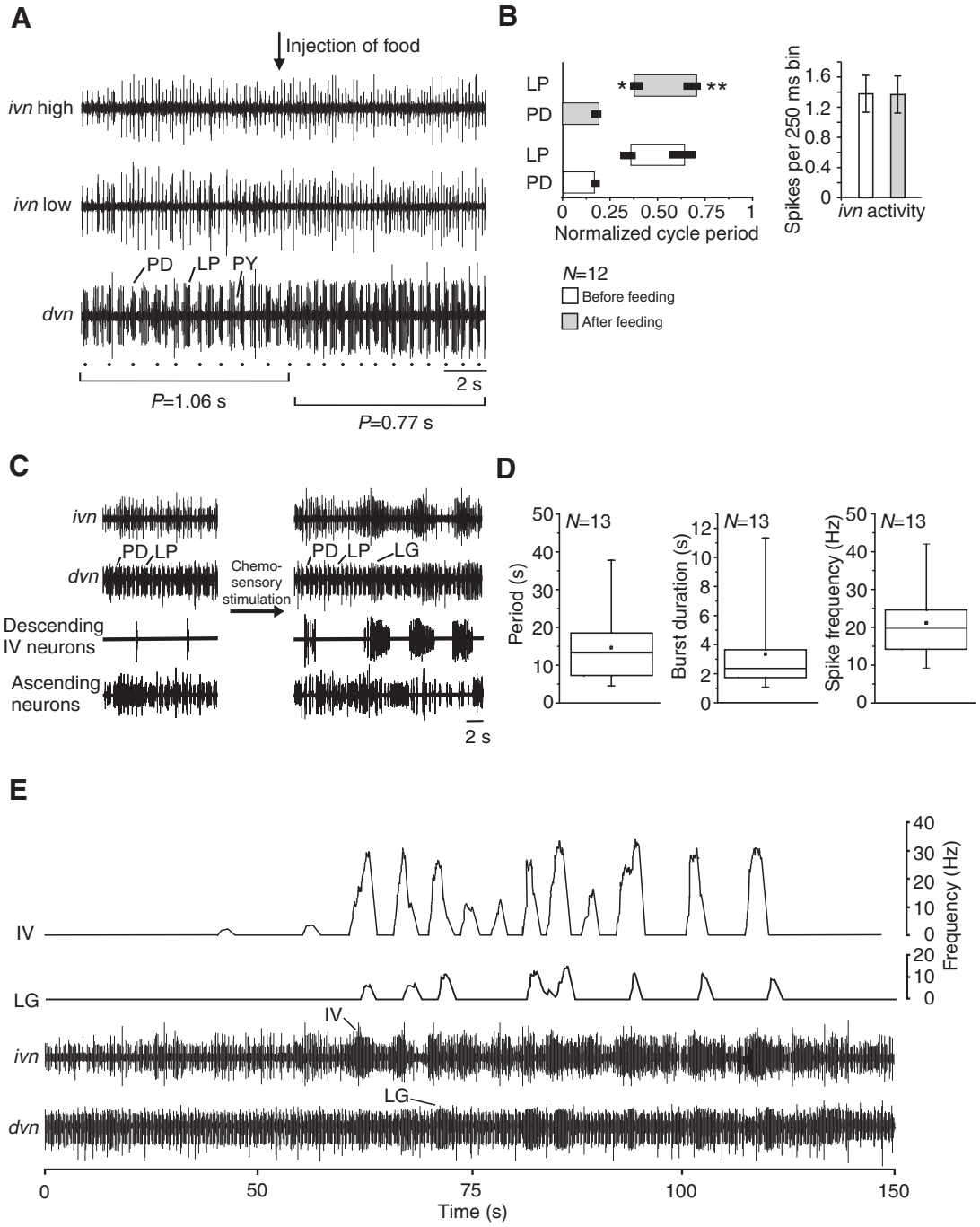

Fig. 3. Sensory-induced activity in the intact animal. (A) Extracellular recordings of the *ivn* and the *dvn* before and after food injection. Top: *ivn* recording close to the brain. Middle: recording of the *ivn* close to the STNS. Bottom: pyloric rhythm on the *dvn*. Each dot labels the beginning of a new pyloric cycle. The pyloric period increased from 1.06 s before feeding to 0.77 s after feeding. (B) Left: phase plot of the pyloric neurons PD and LP before (open boxes) and after feeding (filled boxes). Medians of the beginning and end of the activity phase are given for each neuron. Black boxes show upper and lower quartiles. **P*<0.05; ***P*<0.01 (Wilcoxon signed-rank test). Right: number of spikes per bin (250 ms) of the IV neurons before (open boxes) and after (filled boxes) feeding. (C) Extracellular recordings of *ivn* and *dvn* before (left) and after (right) chemosensory stimulation (upper two traces). In the two lower traces *ivn* activity was separated into the descending and ascending neurons by computer analysis. After chemosensory stimulation the descending (IV) neurons started to burst, whereas the activity of the ascending neurons appeared to remain unchanged. The bursts of the descending neurons were time locked with LG neuron bursts on the *dvn*. (D) Box plots (minimum, lower quartile, median, upper quartile, maximum) of period, burst duration and mean intraburst spike frequency of the rhythmic activity of the descending neurons. The square represents the mean. (E) Extracellular recordings of *dvn* and *ivn in vivo* (two bottom traces) during rhythmic IV neuron activity (top trace). LG neuron bursts coincided with each burst of the IV neurons that possessed a spike frequency of 30 Hz or more. The firing frequencies of LG and the IV neurons were measured as a sliding average with a bin width of 1 s (top two traces).

stimuli applied with a paintbrush or a pair of forceps to the antennae or the mouth area did not have any effect (*N*=13). (3) By contrast, we found that during chemosensory stimulation the IV neurons started to burst in *N*=13 of 15 animals immediately after the onset of stimulation (Fig. 3C). Action potentials in *ivn* originating in the STNS did not occur rhythmically and their

activity remained unchanged (Fig. 3C). In these experiments, we applied a mixture of seawater and crab food to the first antennae. The maximum period of the rhythmic IV bursts was 37.66 s and the minimum 4.43 s, the maximum burst duration of the IV neurons was 11.39 s and it was 1.13 s at minimum. The maximum spike frequency of both IV neurons was 42.03 Hz and the minimum was 9.23 Hz (N=13; Fig. 3D). By contrast, when we applied seawater only in control, the activity of the IV neurons remained unchanged (N=13). In three of the 13 animals that responded to chemosensory stimulation, a gastric mill rhythm occurred at the same time (Fig. 3C,E). In these rhythms a LG neuron burst occurred with each IV burst, in cases where IV neuron spike frequency exceeded 30 Hz (Fig. 3E). The delay between the first IV spike within a burst and the first action potential of the LG neuron within its burst averaged 1.91±0.7 s (N=3).

Together, these results indicate that the IV neurons started to burst rhythmically when chemosensory stimuli were applied to the first antennae. In a subset of experiments, rhythmic IV burst coincided with rhythmic activity of the gastric mill neuron LG. Changes in illumination and tactile stimuli applied to the antennae and mouth did not affect the IV neurons.

In vitro oscillations of the IV neurons

To investigate the actions of an enhanced IV neuron activity on the motor circuits in the STNS, we used the isolated nervous system preparation (Christie et al., 2004). In 34 preparations, the brain was left attached to the STNS, connected solely *via* the *ivn*. The *coc* were transected. In 64 additional preparations, the brain was removed. We recorded from the *ivn*, the *son*, the *ion*, the *stn* and the different motor nerves of the STG. To identify IV neuron spikes, we measured the direction of spike propagation on the *ivn* with two extracellular electrodes. The spikes of the IV neurons could also be monitored on the *son* and the *stn* recordings (Fig. 4A). Intriguingly, we were unable to monitor IV activity on the *ion* (Fig. 4A; but see Discussion), although the backfills showed that the IV axon projects through that nerve. Nevertheless, the spikes observed on the *ivn*, *son* and *stn* could be attributed to the IV neurons, because our backfills of the *son* and *stn* demonstrated that only the IV neurons project axons through the *ivn*, *son* and *stn*.

In 19 of 34 preparations with brains attached we observed rhythmic bursts of the IV neurons (example shown in Fig. 4A). This was never found in preparations where the brain had been removed (N=64). The IV neuron rhythms were not consistently active during the experiments. Rather, they appeared spontaneously in the course of the experiments. The cycle period of these rhythms was between 47.85 s, at the maximum, and 2.99 s, at the minimum (Fig. 4B; N=13). Burst durations were 4.75 s at the maximum and 0.43 s at the minimum. The firing frequency ranged from 42.19 to 10.59 Hz, which corresponded well to the frequencies measured in intact animals. In fact, cycle period, burst duration and spike frequency were not significantly different from those obtained *in vivo*. In three animals a gastric mill rhythm coincided with the IV neuron bursts (Fig. 4C). Here, the IV neuron bursts and the LG neuron bursts were time locked, such that the IV neuron bursts preceded those of the LG neuron. The latency between IV and LG neuron bursts in the example shown was 1.42±0.5 s. The mean latency of all animals was 1.55±0.6 s. Similar to the intact animal (Fig. 3E), the LG neuron was activated only when the IV neuron firing frequency exceeded 30 Hz. In preparations where the IV neuron firing frequencies stayed below 30 Hz, no gastric mill rhythms occurred.

ivn stimulation inhibits the pyloric rhythm

In other crustacean species, IV neuron activity is known to weaken or terminate the pyloric rhythm (Christie et al., 2004; Dando and Selverston, 1972; Marder and Eisen, 1984a; Sigvardt and Mulloney, 1982a; Sigvardt and Mulloney, 1982b). To examine the effects of the IV neurons on the motor circuits in *Cancer pagurus*, we stimulated the *ivn* extracellularly (Christie et al., 2004). These experiments were performed in the isolated nervous system either with the brain attached or without the brain. According to the IV neuron spike frequencies observed in intact animals and in isolated preparations, we used stimulation frequencies between 10 and 40 Hz. We then characterized the effects on the pyloric rhythm by measuring pyloric cycle period and the activities of the different pyloric neurons. Since the action potentials of some pyloric neurons are difficult to separate in extracellular recordings, we used intracellular recordings of single pyloric neurons to determine their response to *ivn* stimulation.

Stimulation frequencies of 10 and 15 Hz (10 Hz: N=2; 15 Hz: N=5) did not yield significant changes in the pyloric motor pattern (data not shown). Stimulation frequencies of 20 Hz and above, by contrast, clearly affected the pyloric rhythm. We found consistent results for both 20 and 40 Hz stimulation. *ivn* stimulation caused a small but significant decrease in pyloric cycle period from 1.08±0.4 to 1.04±0.3 s (N=11, P<0.005, ANOVA) during 20 Hz stimulation and from 1.05±0.3 to 0.95±0.2 s (N=14, P<0.05, ANOVA; Fig. 5B) during 40 Hz stimulation. Conversely, the activity of the PD motor neuron, which is electrically coupled to the pacemaker neuron anterior burster (Eisen and Marder, 1982; Maynard and Selverston, 1975; Miller and Selverston, 1982) decreased from 4.3±2.6 spikes burst^{-1} to 3.96±2.4 spikes burst^{-1} (N=11, P<0.002, ANOVA) during 20 Hz stimulation and from 4.12±1.9 to 3.47±1.6 spikes burst^{-1} (N=14, P<0.001, ANOVA; Fig. 5A,B) during 40 Hz stimulation. The activity of the LP motor neuron also decreased from 5.04±2.1 to 4.04±2.0 spikes burst^{-1} (N=15, P<0.001, ANOVA) during 20 Hz *ivn* stimulation and from 4.82±2.2 to 2.88±2.1 spikes burst^{-1} (N=17, P<0.001, ANOVA; Fig. 5A,B) during 40 Hz stimulation.

While we found consistent results for PD and LP neurons, we observed varying effects on the PY motor neurons. In 14 animals, PY neuron activity decreased from 2.25±1.1 to 1.84±1.2 spikes burst^{-1} (P<0.005, ANOVA) during 20 Hz stimulation and from 2.60±1.6 to 1.76±1.6 spikes burst^{-1} (P<0.001, ANOVA; Fig. 5B) during 40 Hz stimulation. By contrast, in three of 17 animals, PY neuron activity increased during 40 Hz *ivn* stimulation from 1.64±0.4 to 2.17±0.3 spikes burst^{-1}. In four animals, PY neuron activity remained unchanged during both 20 and 40 Hz stimulation (data not shown).

The activity of the pyloric neurons VD and IC decreased during *ivn* stimulation. In the VD neuron, the number of spikes per burst decreased from 2.48±1.6 to 1.28±0.7 spikes burst^{-1} (N=11, P<0.05, ANOVA) during 20 Hz stimulation and from 3.03±1.8 to 0.82±0.6 spikes burst^{-1} (N=13, P<0.05, ANOVA; Fig. 5B) during 40 Hz stimulation. The activity of the IC neuron changed from 4.80±1.1 to 4.23±1.2 spikes burst^{-1} (N=7, P<0.05, ANOVA) during 20 Hz stimulation and from 5.48±1.3 to 3.16±1.0 spikes burst^{-1} (N=10, P<0.001, ANOVA; Fig. 5B) during 40 Hz stimulation.

In contrast to findings in other crustacean species, we found no postsynaptic potentials in pyloric neurons that were elicited by the *ivn* stimulation (see Discussion).

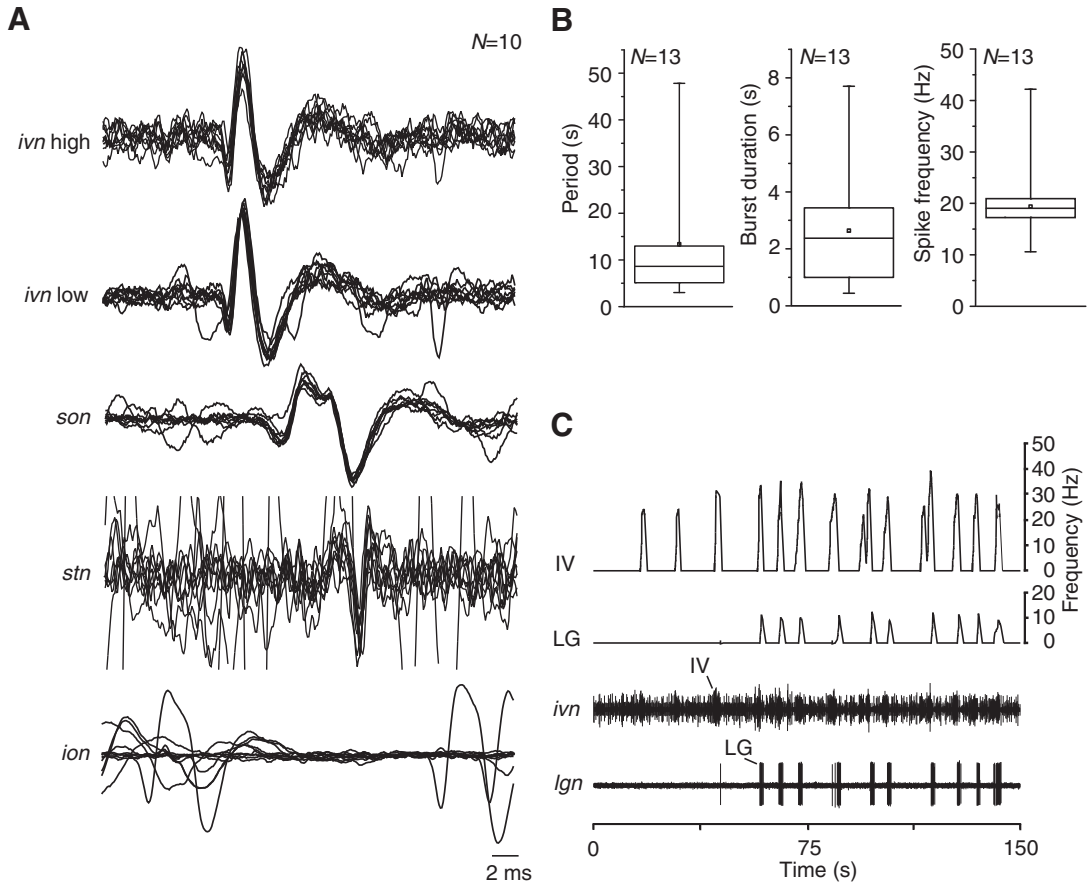

Fig. 4. IV neuron activity in the isolated STNS. (A) Multisweep recordings (n=39) of *ivn* high and low, *son*, *stn* and *ion,* triggered on the IV action potential on *ivn* high during rhythmic IV activity. The IV neuron action potential was observed in all nerves, except the *ion*. Action potentials on the *stn* are truncated. (B) Box plots (minimum, lower quartile, median, upper quartile, maximum) of period, burst duration and mean intraburst spike frequency of rhythmic IV activity in the isolated STNS. The square gives the mean. (C) Extracellular recordings of *lgn* and *ivn in vitro* during rhythmic IV activity (bottom two traces). With every IV burst, its spike frequency exceeding more than 30 Hz, a LG neuron burst occurred (sliding average with a bin width of 1 s). IV neuron bursts and LG neuron bursts were time locked.

ivn stimulation elicits gastric mill rhythms

Gastric mill rhythms typically have a 10-fold longer cycle period than pyloric rhythms (Bartos et al., 1999; Weimann and Marder, 1994; Weimann et al., 1991). Our results demonstrate that the cycle period of the rhythmic IV neuron activity in intact animals and in isolated ganglion preparations ranged within the same order of magnitude. For investigating the effects of such rhythmic IV neuron activity on the gastric mill rhythm, we thus applied 10 trains of *ivn* stimuli with 20 or 40 Hz intratrain frequency and durations between 2 and 6 s, respectively. Intertrain intervals ranged from 1 to 20 s, resulting in stimulation periods of 3 to 26 s. The gastric mill rhythm was monitored with either extracellular or intracellular recordings.

In all preparations without spontaneously active gastric mill rhythms (N=31), 40 Hz *ivn* stimulations elicited gastric mill rhythms, when the stimulation period was longer than 4 s (example shown in Fig. 6A). Stimulation periods shorter than 4 s or train durations of 3 s or less did not elicit gastric mill rhythms (Fig. 6Bi). If present, gastric mill rhythms persisted for the duration of the stimulation. Every stimulus train elicited a LG neuron burst, such that LG neuron was time locked to the stimulus, with the stimulus preceding the LG neuron bursts. In the example shown in Fig. 6A, the delay between the start of the *ivn* stimulus and the LG neuron burst was 2.68±0.29 s (n=10). For all animals, the delay averaged 3.65±1.1 s (N=31). As a consequence, the gastric mill period corresponded to the stimulus period. This was true over a broad range of stimulus periods, as shown in Fig. 6Bi ($6 \leq N \leq 32$; regression line slope 1.137, R^2=0.978).

In the lobsters *P. argus* and *P. interruptus*, *ivn* stimulation inhibits the GM neurons (Dando and Selverston, 1972; Sigvardt and Mulloney, 1982a; Sigvardt and Mulloney, 1982b). By contrast, we observed that in *C. pagurus*, *ivn* stimulation excited the GM neurons (Fig. 6A,B; N=13). The GM neurons started to burst during *ivn* stimulation, in time with the LG neuron (Fig. 6Bii). The phase plot for the gastric mill neurons LG, DG and GM, during *ivn* stimuli (stimulus duration 6 s, intertrain interval 6 s) is shown in Fig. 6Bii (N=13). Here, the beginning of the LG neuron burst was defined as the start of a gastric mill cycle. On average, the LG neuron burst ended at phase 0.22±0.09 (N=31). The onset of GM neuron activity averaged at 0.04±0.02 and its end at 0.20±0.03 (N=13), revealing that the gastric mill neurons LG and GM were active simultaneously. The burst of the antagonistic DG neuron started at phase 0.34±0.09 and ended at 0.51±0.13 (N=29). The small standard deviations show that the gastric mill rhythms of different animals were quite similar when identical stimulus protocols were used.

We also measured burst durations and spike frequencies of LG, DG and GM neurons during stimulus trains (Fig. 6Biii; stimulus regime as in Fig. 6Bii). The burst duration during *ivn* stimulation was 2.55±1.0 s for the LG neuron (N=31), 1.77±0.6 s (N=29) for the

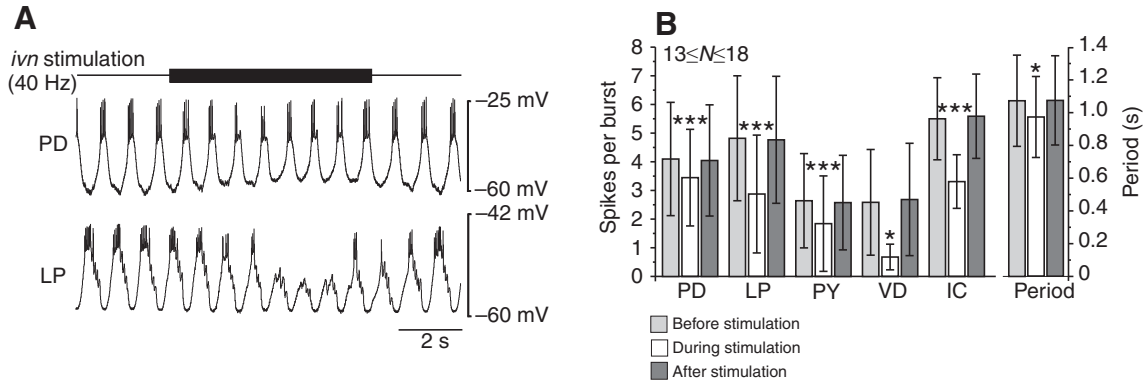

Fig. 5. IV neuron effects on the pyloric rhythm. (A) Intracellular recordings of PD and LP neurons. The *ivn* was extracellularly stimulated at 40 Hz. The spike activities of both PD and LP neurons diminished during the stimulus. (B) Mean number of spikes per burst of the pyloric neurons PD, LP, PY, VD (ventricular dilator) and IC (inferior cardiac), and pyloric cycle period before (light gray boxes), during (open boxes) and after stimulation (dark gray boxes). **P*<0.05, ****P*<0.001, significantly different from pre- and post-control (ANOVA).

DG neuron and 2.63±0.9 s for the GM neuron (N=13); the spike frequency was 7.83±3.8 Hz for the LG neuron (N=21), 11.39±4.4 Hz (N=21) for the DG neuron and 3.55±2.2 Hz for the GM neuron (N=13).

While we observed many postsynaptic potentials (PSPs) in GM neurons during *ivn* stimulation, none were time locked to single *ivn* stimuli, indicating that the GM neurons were activated indirectly *via* interneurons. Similarly, the gastric mill neurons LG and DG showed no PSPs that were time locked to the stimulus, although both neurons exhibited marked depolarizations caused by the *ivn* stimulus trains.

In contrast to the 40 Hz stimulations, 20 Hz stimuli did not elicit gastric mill rhythms. LG and the GM neurons, however, received

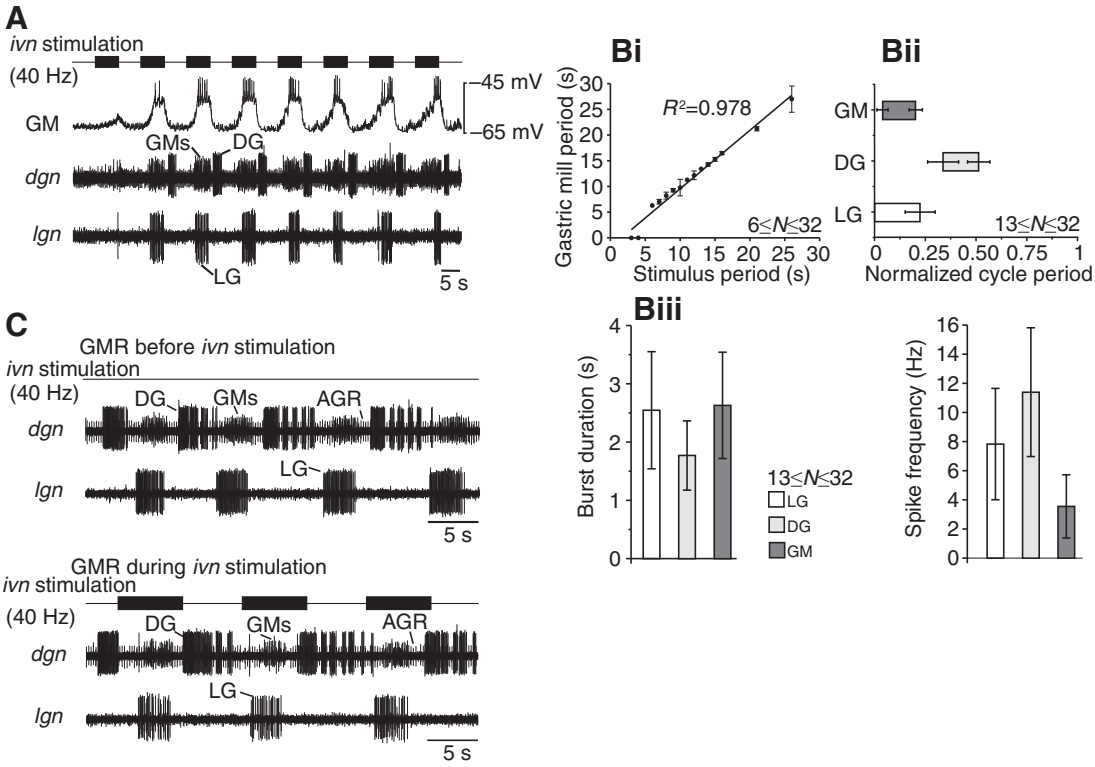

Fig. 6. *ivn* stimulation elicits gastric mill rhythms. (A) In preparations without spontaneous gastric mill rhythm, rhythmic *ivn* stimulation (40 Hz intratrain frequency) elicited a gastric mill rhythm which included activities of LG (lower trace, extracellular recording of *lgn*), DG (middle trace, extracellular recording of *dgn*) and gastric motor (GM) neurons (*dgn* recording and intracellular recording of GM neuron). The gastric mill rhythm lasted for the duration of the stimulation. (Bi) Stimulation period determined the period of the rhythm: plot of gastric mill period over stimulus period. Means of 6≤N≤32 animals. Regression line: slope 1.37, R^2=0.98. (Bii) Phase plot of the gastric mill motor neurons LG, DG and GM during 40 Hz *ivn* stimulation with train durations and intertrain intervals of 6 s, respectively. (Biii) Burst durations (left) and mean intraburst spike frequencies (right) of LG, DG and GM neurons (stimulus parameters as in Bii). (C) Entrainment of the gastric mill rhythm. Extracellular recordings of *lgn* and *dgn* before (top) and during *ivn* stimulation. The period of the gastric mill rhythm fell into synchrony with the *ivn* stimulation; it was entrained. AGR, anterior gastric receptor.

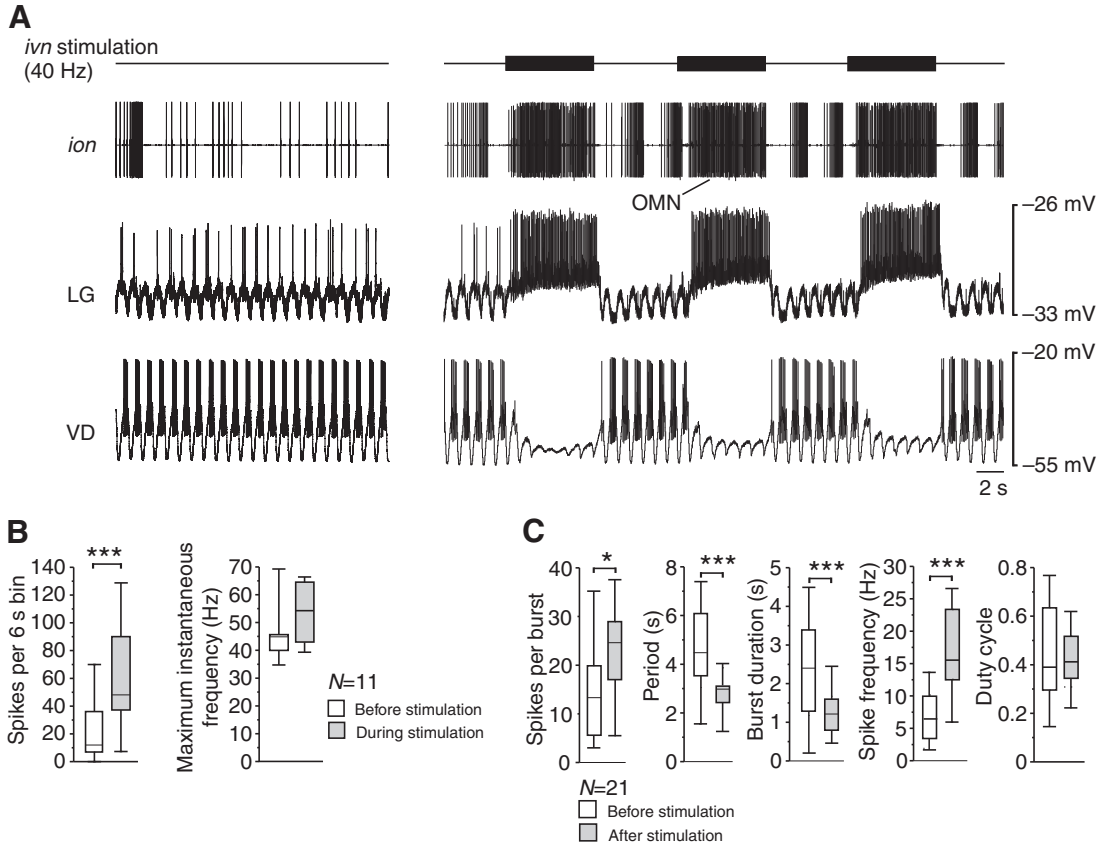

Fig. 7. Effects of *ivn* stimulation on the esophageal rhythm. (A) Intracellular recordings of the VD motor neuron (lower trace) and the LG neuron (middle trace), and an extracellular recording of the *ion,* illustrating the activity of the esophageal motor neuron (OMN; upper trace). *ivn* stimulation enhanced OMN activity. (B) Box plots (minimum, lower quartile, median, upper quartile, maximum) of OMN activity (left, bin width 6 s) and maximum instantaneous firing frequency (right) before (open boxes) and during (filled boxes) *ivn* stimulation. (C) Box plots showing spikes per burst, period, burst duration, mean intraburst spike frequency and duty cycle of OMN before (open boxes) and after *ivn* stimulation (filled boxes). **P*<0.05, ***P*<0.01, ****P*<0.001 (Wilcoxon signed-rank test).

excitation, but were not depolarized above spike threshold (LG: *N*=31, GM: *N*=13; data not shown).

In experiments with spontaneously active gastric mill rhythms, *ivn* stimulation with 40 Hz entrained the gastric mill rhythm (*N*=19; Fig. 6C). In this figure, the gastric mill period was 8.43±0.3 s before stimulation (*n*=10). During *ivn* stimulation with a period of 12 s, the stimulus period and the period of the gastric mill rhythm (12.2±0.6 s; *n*=10) fell into synchrony. After the end of the stimulation the gastric mill rhythm sped up again and its period corresponded to the gastric mill period measured prior to stimulation.

When we transected the CoG, *ivn* stimulation no longer elicited gastric mill rhythms (*N*=10, data not shown). It thus appears that the actions of the IV neurons on the gastric mill rhythm were mainly mediated *via* their projections to the CoG (see Fig. 2E), indicating an involvement of descending projection neurons located in the CoG.

ivn stimulation affects the esophageal rhythm

Our backfills revealed that the IV neurons project axons not only to the STG but also to the CoG (*via* the *son* and *ion*; Fig. 2E). In the CoG, information from the brain, the thoracic ganglion and several sense organs converges (Beenhakker and Nusbaum, 2004; Kirby and Nusbaum, 2007). In addition, the motor control circuit for the esophageal rhythm is located in the CoG (Spirito, 1975). This rhythm can be monitored by the activity of the OMNs. Like the pyloric rhythm, the esophageal rhythm is usually spontaneously active in the isolated STNS (Stein et al., 2005). The OMNs are a bilaterally symmetrical pair of neurons, with one soma residing within each CoG. Each OMN projects *via* the *ion* to the OG (Fig. 1B) to innervate esophageal muscles in the region of the OG (D. M. Blitz, M. P. Nusbaum and W.S., unpublished observation). Its activity can be recorded either with extracellular recordings of the *ion* or with intracellular recordings from the OMN somata.

When we stimulated the *ivn* with 10 stimulus trains (40 Hz intratrain stimulation frequency; duration 6 s, intertrain interval 6 s), we observed that IV neuron activity affected the esophageal rhythm. The original recording of OMN on the *ion* in Fig. 7A shows that each *ivn* stimulus train tonically activated OMN. OMN activity started on average 0.7±0.4 s (*N*=22) after the beginning of IV neuron stimulation. As a consequence of its mostly tonic activity, OMN burst duration corresponded to the duration of the stimulus train. The number of spikes per time bin (6 s) increased significantly from 12.0 (median) during rhythmic OMN activity before stimulation to 51.4 (median) during stimulation (Fig. 7B, left; *N*=11, *P*<0.001, Wilcoxon signed-rank test).

In between stimulus trains and after the end of the stimulation, OMN resumed its regular bursting activity. The maximum instantaneous firing frequency of OMN, as measured during the last burst before stimulation and during stimulation, did not change significantly (*N*=11; Fig. 7B, right). The excitation of OMN appeared

to be mediated monosynaptically, since it persisted when the CoG were bathed in high divalent saline (N=3, data not shown).

It is currently unknown whether or not OMN is a member of the esophageal CPG. Hence, the excitation of OMN may not reflect an influence of the IV neurons on the esophageal CPG. Indeed, we did not find a consistent effect of *ivn* stimulation on the immediate phasing of the esophageal rhythm (N=11). By contrast, when we compared OMN activity before and after the end of the stimulus trains, we found that the number of OMN spikes per burst was significantly increased after stimulation (Fig. 7C; median OMN spikes per burst pre-stimulation, 14.41; median post-stimulation, 24.88; N=21, P<0.05, Wilcoxon signed-rank test). The esophageal rhythm also sped up after *ivn* stimulation; its period decreased significantly from 4.82 s (median) before to 2.96 s (median) after stimulation (Fig. 7C; N=21, P<0.001, Wilcoxon signed-rank test). Additionally, OMN burst duration was reduced (Fig. 7C; median pre-stimulation, 2.44 s; median post-stimulation, 1.26 s; N=21, P<0.001, Wilcoxon signed-rank test) and intraburst spike frequency was enhanced (Fig. 7C; from 6.72 Hz (median) to 16.34 Hz (median), N=21, P<0.001, Wilcoxon signed-rank test). By contrast, OMN duty cycle did not change (Fig. 7C; P>0.2, N=21, Wilcoxon signed-rank test).

As a consequence of the excitatory influences of the IV neurons on OMN and the gastric mill neuron LG, and their inhibitory actions on pyloric rhythm, the timing of the IV neurons was imposed on all investigated motor patterns. This is shown in Fig. 7A by the simultaneous activation of the OMN and LG neurons and the concurrent inhibition of the pyloric neuron VD. Thus, IV neuron activity synchronized the motor activities of the pyloric, gastric mill and esophageal rhythms.

DISCUSSION

In this investigation, we characterized a neural pathway that descends from the brain of the crab *Cancer pagurus* to the motor circuits in the STNS. We showed that two identified neurons (the bilateral pair of IV neurons) respond to chemosensory stimulation and that they are involved in the regulation of the motor patterns generated in the STNS.

The activity of the motor circuits in both vertebrates and invertebrates is regulated by sensory feedback and upstream neural structures (Brodfuehrer and Thorogood, 2001; Fleischer, 1981; Perrins et al., 2002; Spirito, 1975), ensuring that the resulting behavior is appropriate for the situation at hand (for reviews, see Grillner, 2003; Rossignol et al., 2006). In the STNS, most of the regulatory neurons are located in the CoG, which are connected to the brain and to the thoracic ganglion *via* the *coc*. Backfills of the *coc* have localized the brain neurons that are likely candidates for influencing the STNS motor patterns (Kirby and Nusbaum, 2007). Indeed, stimulating axons within the *coc* affects motor activity, for example, during locomotion (Atwood and Wiersma, 1967; Bowerman and Larimer, 1974a; Bowerman and Larimer, 1974b). The individual neurons responsible for these effects, as well as their actions on the motor circuits and their input from the brain, remain to be identified in most cases.

By contrast, previous work has demonstrated the influence of two identified neurons in the brain, the IV (or PS) neurons, on the STNS motor patterns in various lobster and crab species (Christie et al., 2004; Dando and Selverston, 1972; Hooper et al., 1990; Meyrand et al., 1994; Sigvardt and Mulloney, 1982a; Sigvardt and Mulloney, 1982b). The IV neurons project to the STNS *via* a unilateral nerve, the *ivn*. Here, we show for the first time that their somata are located in cell cluster 17 of the brain and that these neurons provide exteroceptive sensory information to the motor circuits in the STNS. We also outline their axonal projection pattern in the crab, *C. pagurus*. The IV neurons respond with rhythmic bursts to chemosensory stimulation of the first antennae. They project to the CoG and the STG, and they have a pronounced impact on the esophageal, gastric mill and pyloric motor patterns. Their effects include a synchronization of these rhythms by providing excitation to the esophageal and gastric mill rhythms and inhibition to pyloric neurons.

IV neurons relay exteroceptive information from the brain to the motor circuits in the STNS

So far, it has not been clear whether the IV neurons transfer information from the brain to the STNS and, if so, what kind of information they relay. In only one study was the activity of neurons projecting through the *ivn* assessed in a behavioral approach: in *O. limosus*, the activities of all neurons on the *ivn* were measured during feeding. *ivn* activity increased during food intake (Böhm et al., 2001). Yet the different spikes were not classified into descending and ascending spikes, leaving the question of whether the IV neurons or neurons that ascend from the STNS to the brain responded to the food intake. In addition, animals may have been exposed to several different sensory stimuli during feeding, such as mechanical, olfactory and visual stimuli. We thus used the conduction delay between two hook electrodes attached to the *ivn* in intact animals to identify the IV neuron action potentials while we applied different sensory stimuli in order to determine whether the IV neurons indeed relay information from the brain to the STNS.

First, we tested the impact of food intake on the *ivn* units. In contrast to the experiments on *O. limosus* where food intake was voluntary, we injected food directly into the stomach. This circumvented the activation of sense organs outside of the stomach. In contrast to *O. limosus*, neither the descending IV neurons nor the ascending neurons were activated (Fig. 3A,B). However, the pyloric rhythm sped up and the phasing of the pyloric neurons was altered. Clearly, since no neurons on the *ivn* were affected in *C. pagurus*, the effect on the pyloric rhythm must have been mediated *via* different pathways. The most likely scenario is that local sensory feedback from within the stomach, such as that *via* the mechanosensitive ventral cardiac neurons (Beenhakker et al., 2004), was activated and thus affected the pyloric rhythm.

While neither mechanosensory stimuli applied to the mouth and antennae nor changes in illumination altered the activity of the *ivn* units, chemosensory stimulation of the first antennae activated the IV neurons. The first antennae are responsible for olfactory and chemosensory perception (Brock, 1930). In these experiments, we applied a mixture of seawater and crab food to the antennae. In a subset of these applications, a gastric mill rhythm started shortly after the beginning of the application. The finding that the bursts of the gastric mill protractor motor neuron LG were time locked to the IV neuron bursts (Fig. 3E), and that IV neuron bursts preceded those of the LG neuron argues for the hypothesis that the IV neurons contributed to the activation of the LG neuron. An indirect activation of the gastric mill rhythm, however, cannot be excluded, because in intact animals the brain and the STNS are connected not only *via* the *ivn* but also *via* the *coc*. In fact, it is also possible that the IV neurons were entrained into the gastric mill *via* ascending pathways from the STNS. This scenario appears unlikely, though, since in most experiments the IV neurons showed rhythmic activity even in the absence of a gastric mill rhythm.

Further support for the hypothesis that the IV neurons elicited the gastric mill rhythm comes from our experiments with isolated

nervous systems. In these experiments the *coc* had been transected, leaving the *ivn* as the only connection between the brain and STNS. Although we never found rhythmic activity of ascending units on the *ivn* and, thus, information related to the gastric mill rhythm could not have been transferred from the STNS to the brain, the IV neurons were rhythmically active in more than half of the preparations. In three preparations, the gastric mill motor neuron LG was activated and a gastric mill rhythm started whenever IV spike frequency exceeded 30 Hz. The isolated preparation thus showed the same frequency dependence as the intact animal. A possible reason for this dependence could be the neurotransmitter complement of the IV neurons. In the close relative *C. borealis* these neurons contain the co-transmitters histamine and the peptide FLRF-amide (Christie et al., 2004). Peptide release often requires high-frequency trains of presynaptic action potentials (Marder, 1998).

Our *in vivo* experiments yielded a low occurrence of gastric mill rhythms during chemosensory stimulation. Fleischer showed that optical information can suppress the gastric mill rhythm for periods of up to 40 h (Fleischer, 1981). It is thus conceivable that even the dissection of the animal or the illumination applied when implanting the electrodes reduced the occurrence of gastric mill rhythms and that this effect lasted throughout the duration of the experiment, possibly *via* a reduction of IV neuron firing rate.

Identification of IV neurons

Our extracellular recordings in the isolated STNS show that action potentials originating in the brain descend *via* the *ivn* to the STNS and that they occur either tonically or rhythmically. These action potentials could be generated by the IV neurons or by one of the other six neurons projecting axons through the *ivn.* In the latter case action potentials would travel towards the soma (antidromically), a situation known to exist in the STNS (Bucher et al., 2003). Several facts argue against this hypothesis, though. For example, in lobsters, rhythmic activity on the *ivn* was identified to be generated by the IV neurons with intracellular recordings from the IV neuron somata. Furthermore, in those species where the IV neuron somata are located in the *ivn* root rather than in the brain, rhythmic activity was present even in the absence of the brain. By contrast, in *C. borealis* and *C. pagurus*, where the IV neuron somata are located in the brain, rhythmic activity was never present if the brain was removed (*C. borealis*: W.S., unpublished observations). In addition, our extracellular recordings reveal that spikes that descended from the brain *via* the *ivn* were present on both *son* and the *stn*. At the same time, backfills from the *son* to the brain prove that only two axons project from the *son* to the brain, namely those of the IV neurons. Thus, the descending action potentials on the *ivn* must indeed be generated by the IV neurons.

The backfill stainings demonstrate that the IV neurons project one axon each *via* the *son* and *ion* to the CoG. Yet we were unable to detect the IV neuron spike on the *ion*. This corresponds to findings in *C. borealis* (Christie et al., 2004) where stimulation of the IV neurons activates the modulatory projection neuron 1 (MCN1) in the CoG solely *via* its axon in the *son* but not the *ion* (Christie et al., 2004). In *H. americanus* Lucifer Yellow staining of the PS neurons show that these neurons project an axon through the *ion*, but *ion* spikes that correlate with the PS soma potentials have never been observed (Cazalets et al., 1990). Furthermore, action potentials elicited by antidromic stimulation of the *ion* do not invade the soma. The function of the axon that projects through the *ion* is thus unclear. One possibility could be that this axon is functionally inactivated unless the adequate neuromodulatory environment is present. The region of the STNS where the IV neuron axon divides into *stn* and *ion* branches is under modulatory control (Goaillard et al., 2004), which may lead to a functional compartmentalization of the IV neurons. An example of such a compartmentalization is found in *Aplysia californica.* In the mechanosensory neuron B21 of this mollusk, spike initiation and propagation is regulated *via* synaptic control (Cropper et al., 2004; Evans et al., 2003). Depending on synaptic activity and the local membrane potential, action potentials are either actively propagated to a lateral process of the cell or do not enter this part of the neuron. A similar mechanism in the IV neurons could gate spike propagation to the *ion*, depending on the neuromodulatory conditions. Neuromodulators like octopamine, for example, act on active properties of axons in this region of the STNS and they affect spike propagation (Goaillard et al., 2004).

Effect on the motor patterns

The effects of the IV (or PS) neurons on the different motor patterns (pyloric, gastric mill and esophageal rhythms) were described previously in different crustacean species (Claiborne and Selverston, 1984; Dando and Selverston, 1972; Faumont et al., 2005; Hooper et al., 1990; Marder and Eisen, 1984a; Marder and Eisen, 1984b; Russell and Hartline, 1981; Sigvardt and Mulloney, 1982a; Sigvardt and Mulloney, 1982b). It appears that the effects of the IV neurons on the pyloric rhythm are more similar among brachyuran species than between Brachyura, Astacidea and Palinura. For example, we did not observe any PSPs in pyloric neurons, which is similar to *C. borealis*, but is in contrast to the findings for the spiny lobster *P. interruptus* (Hooper et al., 1990; Marder, 1984; Russell and Hartline, 1981; Sigvardt and Mulloney, 1982a) and *H. gammarus* (Cazalets et al., 1987; Faumont et al., 2005; Meyrand et al., 1991; Meyrand et al., 1994). Also, in *C. pagurus* and *C. borealis*, *ivn* stimulation inhibited VD and IC neurons, which contrasts with the findings for *P. interruptus* (Hooper et al., 1990; Russell and Hartline, 1981; Sigvardt and Mulloney, 1982b). In all species (Christie et al., 2004; Dando and Selverston, 1972; Sigvardt and Mulloney, 1982b), however, the activity of the PD motor neuron decreased in response to IV activation (Fig. 5A,B).

While the effects of the IV neurons on most pyloric neurons were consistent in all experiments, the PY neurons showed three different responses to *ivn* stimulation: PY activity increased during *ivn* stimulation or it decreased or it did not change. Different responses of the PY neurons to *ivn* stimulation have also been reported in the lobster *P. vulgaris* (Hooper et al., 1990): here, *ivn* stimulation caused long-term inactivation, long-term depolarization or no long-lasting response in different PY neurons. The findings in *C. borealis* were different, though. Here, the PY neurons started to fire tonically during *ivn* stimulation (Christie et al., 2004). In this study, however, PY activity was analyzed extracellularly, so that possible differences in PY responses may not have been detected. Until now, little was known about the interactions between the PY neurons and about their different actions, but they have been divided into subsets in *Panulirus* (Hartline et al., 1987) on the basis of their responses to stimulation of an afferent pathway within the STNS. Our results further support this subdivision.

One of the particularly noticeable effects of the PS neuron in the lobster *H. gammarus* is its action of combining the pyloric, gastric mill and esophageal rhythms into a single, conjoint motor pattern (Meyrand et al., 1991), which is entrained by PS activity. In *C. pagurus*, IV neuron stimulation either started or entrained the gastric mill rhythm (Fig. 6A,B) such that each LG neuron burst was time locked to the *ivn* stimulus trains. Thus, the gastric mill period corresponded to the stimulus period. The pyloric rhythm, by contrast, continued in between *ivn* stimulus trains, but received

phasic inhibitory input during the trains. While the pyloric and gastric mill circuits are located in the STG, the third spontaneously active motor rhythm in the isolated STNS of the crab – the circuit generating the esophageal rhythm – resides within the CoG (Spirito, 1975). We found that the IV neurons projected *via* the *son* and *ion* to the CoG and that they monosynaptically excited the OMN such that OMN activity increased during *ivn* stimulation (Fig. 7B). These results correspond to the findings in *C. borealis* (Christie et al., 2004). The excitation of OMN did not change the phasing of the esophageal rhythm, however. Similar to the pyloric rhythm, the esophageal rhythm continued to oscillate in between *ivn* stimulus trains. It thus appears that in *C. pagurus*, the IV neurons cannot conjoin the pyloric, gastric mill and esophageal rhythms. Rather, they impose their timing on the pyloric and esophageal rhythms by providing inhibitory and excitatory drive, respectively, to the motor neurons. Nevertheless, we found that the properties of the esophageal rhythm were different after the end of stimulation from what they had been before stimulation (Fig. 7B). This indicates that the IV neurons possessed (limited) access to the esophageal CPG, the effects of which outlasted the stimulation.

In preparations where the CoG, and thus also the IV neuron projections to these ganglia had been removed, *ivn* stimulation did not elicit a gastric mill rhythm. One possible explanation for this absence of the gastric mill rhythm is that the rhythm depended on excitation provided by one of the CoG projection neurons (Nusbaum and Beenhakker, 2002) that were activated during *ivn* stimulation. In *C. borealis*, two identified projection neurons, the modulatory commissural neuron 1 (Coleman and Nusbaum, 1994; Coleman et al., 1992) and the commissural projection neuron 2 (Blitz and Nusbaum, 1999), appear to contribute to the response of the gastric mill neurons (Christie et al., 2004). The fact that we did not observe PSPs in gastric mill neurons that were time locked to the *ivn* stimulus supports the hypothesis of an indirect excitation *via* projection neurons, especially since the GM neurons received many excitatory PSPs during the *ivn* stimulus train that were not time locked to the stimulus. These PSPs most probably originated from commissural projection neuron 2 (Blitz and Nusbaum, 1999), the only known source of excitatory input to the GM neurons (Stein et al., 2005). Our results thus indicate that projection neurons located in the CoG are involved in the processing of exteroceptive sensory information. These neurons are well known to process proprioceptive and mechanosensory information (Beenhakker and Nusbaum, 2004; Simmers and Moulins, 1988a; Simmers and Moulins, 1988b; Smarandache and Stein, 2007) from sense organs within the stomatogastric system. It is thus conceivable that they represent a point of multisensory convergence, processing information from proprioceptors and exteroceptors. Our study provides an initial characterization of identified neurons located within the *C. pagurus* brain that relay exteroceptive chemosensory information to the STNS *via* CoG neurons known to regulate STNS motor patterns. More generally, we provide a framework for future electrophysiological experiments aimed at characterizing the processing of descending information from the brain, which should, in turn, benefit our understanding of the principles that underlie the regulatory control of motor pattern generation at the cellular level.

LIST OF ABBREVIATIONS

coc	circumesophageal commissures
CoG	commissural ganglion
CPG	central pattern generator
DG neuron	dorsal gastric neuron
dgn	dorsal gastric nerve
dvn	dorsal ventral nerve
GM neuron	gastric motor neuron
IC neuron	inferior cardiac neuron
ion	inferior esophageal nerve
IV neuron	inferior ventricular neuron
ivn	inferior ventricular nerve
LG neuron	lateral gastric neuron
lgn	lateral gastric nerve
LP neuron	lateral pyloric neuron
lvn	lateral ventricular nerve
mvn	medial ventricular nerve
OG	esophageal ganglion
OMN	esophageal motor neuron
PD neuron	pyloric dilator neuron
pdn	pyloric dilator nerve
PS neuron	pyloric suppressor neuron
PSP	postsynaptic potential
PY neuron	pyloric constrictor neuron
son	superior esophageal nerve
STG	stomatogastric ganglion
stn	stomatogastric nerve
STNS	stomatogastric nervous system
VD neuron	ventricular dilator neuron

This work was supported by DFG STE 937/2-1 and STE 937/2-2. We would like to thank Professor Hans-Georg Heinzel for providing us with a turnable holder for the animal as well as Professor Dr Harald Wolf and Ursula Seifert for their support and helpful comments on the paper. We would also like to thank Christina Eberle for providing preliminary data for this study.

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