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Buccal neurons activate ciliary beating in the foregut of the pteropod mollusk *Clione limacina*

Aleksey Y. Malyshev* and Pavel M. Balaban
Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences, Moscow, Russia
*Author for correspondence (malyshev@ihna.ru)

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SUMMARY

Beating of cilia lining the foregut of gastropods facilitates the swallowing of food and, therefore, plays a role in feeding behavior. Despite the fact that neural control of feeding is well studied in mollusks, no neurons controlling ciliary beating in the foregut have been identified to date. Here we describe for the first time a pair of buccal neurons innervating the foregut of *Clione*. Intracellular stimulation of these neurons induced vigorous activation of cilia lining the foregut in a semi-intact preparation. Using immunochemistry labeling, buccal foregut cells were found to contain peptides similar to CNP neuropeptides of the terrestrial snail *Helix lucorum*. Application of DYPRL-amide, a member of the *Helix* CNP peptide family, mimicked the effect of buccal foregut cell stimulation on ciliary activity. Induction of fictive feeding in an isolated CNS preparation resulted in the activation of buccal foregut cells suggesting that these cells control ciliary beating in the foregut during feeding. Thus, cilia-activating buccal neurons may represent a new intrinsic element of the neural control of feeding in gastropods.

Key words: pteropod mollusk, feeding behavior, cilia, foregut, neuron.

INTRODUCTION

The neural control of feeding is probably one of the best studied areas in molluscan neuroethology and provides a remarkable example of how a relatively simple neural network can ensure complex and well coordinated movements of several anatomical structures recruited in feeding. Such coordination ensures the orderly production of complex behaviors and represents an important and universal principle of CNS functioning in both vertebrate and invertebrate species. Feeding in gastropods is a considerably more complex process than mere protraction and retraction of radula. For instance, in *Aplysia*, protraction and retraction of radula are synchronized with movements of the lips and jaws as well as with radula opening and closing (for a review, see Elliott and Susswein, 2002). It was also shown in *Aplysia* that cerebral motoneurons, innervating extrinsic buccal muscles, burst in phase with buccal radula motoneurons, thus providing coordinated movements of the whole buccal mass during feeding (Jahar-Parwar and Fredman, 1983). Buccal peptidergic neurons B1 and B2 in *Aplysia*, whose firing induced peristaltic contraction of the gut, were reported to fire during the swallowing cycle of each feeding movement (Lloyd et al., 1988). Homologous buccal neurons with similar functions were also described in *Lymnaea* and *Tritonia* (Lloyd and Willows, 1988; Perry et al., 1998). *Clione* is a highly specialized carnivore, which feeds only on two species of shelled pteropod mollusks of the genus *Limacina* (Lalli, 1970; Lalli and Gilmer, 1989; Wagner, 1885). As a consequence of their high feeding specialization, *Clione* and other mollusks from the order Gymnosomata have a unique feeding structure – chitinous hooks, whose functional role is to grab the soft body of *Limacina*, and pull it out of its shell and into their buccal cavity. We have shown in a previous study that the rhythmic movements of radula and hooks are highly coordinated in a phase-dependent manner (Malyshev and Norekian, 2002). This phase-dependent coordination was observed at a behavioral level and was always recorded at the motoneuronal level during spontaneous and induced rhythmic activity. Hook protractor neurons were active in the same phase as radula retractor neurons, whereas hook retractor neurons burst in phase with radula protractor neurons (Malyshev and Norekian, 2002).

The foregut of all gastropod mollusks is lined with a ciliated epithelium; beating of cilia drives the mucus with particles of food towards the stomach (Ruppert et al., 2004). Despite the fact that neural control of cilia has been described in gastropods (Audesirk, 1978), the precise neurons controlling cilia in the molluscan foregut and, therefore, involved in the neural control of feeding, have not been reported. Using electrophysiology and immunochemistry techniques we describe in the present study a pair of buccal neurons, named Bc-FG cells, controlling the activity of cilia lining the foregut of *Clione*. Bc-FG cells were tonically active during fictive feeding, suggesting that they can play a role in feeding behavior by facilitating swallowing *via* activation of ciliary beating in the foregut.

MATERIALS AND METHODS

Preparation

Adult specimens of *Clione limacina* Phipps 1774 were collected at the White Sea Marine Biological Station of the Zoological Institute (White Sea, Russia) and held in the laboratory individually in 1 l jars in a refrigerator at 10°C with daily water filtering. The animals were anesthetized in a 1:1 mixture of seawater and isotonic $MgCl_2$ and dissected in a Sylgard-coated Petri dish. The body wall in the neck region was carefully cut to expose the CNS. Semi-intact preparations consisted of wings, dissected head with the isolated buccal mass, and the attached CNS. All nerves running to the head, wings, buccal mass and body wall were left intact. In experiments with optical recording of ciliary beating a reduced semi-intact preparation consisting only of the CNS and the foregut was used. Prior to electrophysiological recording, the sheath of the central ganglia was softened by bathing the preparation in a 1 mg ml^{-1}

solution of protease (Sigma, type XIV; St Louis, MO, USA) for 5 min, followed by a 30 min wash.

Electrophysiological recordings

Intracellular recordings from individual neurons were made with glass microelectrodes (10–30 MΩ resistance, filled with 2 mol l^{-1} potassium acetate). Electrophysiological signals were amplified, displayed and recorded using conventional electrophysiological techniques (Bramp 01R amplifiers, NPI, Tamm, Germany; Digidata 1320 ADC board and Axoscope software, both from Axon Instruments, Union City, CA, USA). Intracellular stimulation was achieved *via* an amplifier bridge circuit. For morphological investigation of recorded neurons, a 10 mmol l^{-1} solution of AlexaFluor 594 (Invitrogen, Carlsbad, CA, USA) in 0.1 mol l^{-1} potassium chloride was iontophoresed *via* the recording electrodes (20–40 MΩ resistance) with 1–5 nA negative current pulses for 60 min. Preparations were then incubated for 48–72 h at +4°C. Processes of injected cells in the foregut tissue were observed live in the recording dish with an Olympus BX51W epifluorescence microscope (Olympus, Tokyo, Japan) or a Zeiss LSM 5 Live laser confocal microscope (Carl Zeiss, Jena, Germany) equipped with appropriate filters. DYPRL-amide solutions were prepared in filtered seawater immediately before use. Solutions were applied with an automatic pipette. The final concentration was estimated from the known volume of injected solution and the known volume of saline in the recording dish.

Optical recording of ciliary beating

Isolated patches of the foregut or whole foregut connected to the brain were pinned to Sylgard-coated Petri dishes with the ciliated surface uppermost and viewed with an Olympus BX51W microscope. An image of the beating cilia was projected through a ×40 Olympus long working-distance, fluid-immersion objective onto an 80×80 CCD camera (NeuroCCD, RedShirtImaging, Decatur, GA, USA). Ciliary beating was recorded optically with a frame rate of 125 Hz in parallel with intracellular recording using the NeuroCCD-SMQ imaging system and Neuroplex software (both from RedShirtImaging). Each photodetector of the CCD camera recorded changes in transmitted light corresponding to ciliary beating. In experiments with peptide applications fast Fourier transform (FFT) analysis was used to recover the dominant frequency of optically recorded oscillations. FFT spectra were then analyzed and only traces with a prominent peak, corresponding to the dominant frequency, were selected. Only traces with the FFT peak to the base of the peak ratio equal to or greater than the empirically chosen coefficient of 1.86 were selected for analysis. Ciliary beat frequencies were then averaged across selected traces.

Immunocytochemistry and double-labeling experiments

Because *Clione* is a relatively small mollusk, we used a whole-mount immunocytochemical procedure. Semi-intact preparations were fixed for 3±5 h in 4% paraformaldehyde solution saturated with picric acid at room temperature (20°C) and transferred to 3 ml vials, where they were washed for 12 h in phosphate-buffered saline (PBS) and preincubated in PBS containing 0.1% Triton X-100 to increase tissue permeability. The tissue was then exposed to 8% goat serum in PBS and 0.1% Triton X-100 for 6 h to reduce non-specific antibody binding. The primary antiserum was diluted in PBS–Triton–goat serum and was applied for 48 h at 5°C. Antiserum to the CNP4 neuropeptide from the mollusk *Helix lucorum* was produced in rabbits in our laboratory and used at a dilution of 1:200. Following incubation in the primary antibody, the tissue was washed in PBS for 12 h and then incubated for 24 h in a 1:40 dilution of secondary antibody in PBS (Alexa 488-conjugated goat anti-rabbit IgG; Invitrogen). The secondary antibody was removed with several PBS exchanges, and preparations were washed overnight. The tissues were then cleared in xylene, mounted in DPX, and examined whole mount with a Zeiss LSM-5 laser scanning fluorescence microscope. In preabsorption control experiments, the anti-CNP antibody was incubated for 4 h with 10^{-3} CNP4 peptide. After incubation, the mixture was applied to the tissue. No staining was observed in these control experiments. For double-labeling experiments, neurons were injected with neurobiotin (Vector Laboratories, Burlingame, CA, USA) *via* recording electrodes, which were filled with 5% neurobiotin solution in 0.2 mol l^{-1} potassium acetate. Neurobiotin was iontophoresed by applying positive current pulses of +2 nA amplitude for 20–30 min. The preparations were then fixed in 4% paraformaldehyde in PBS, and incubated for 12 h in Rhodamine-labeled streptavidin (Imtek, Moscow, Russia) to visualize neurobiotin-filled neurons. The preparations were then processed for the immunocytochemical experiments described above. By switching filters in the laser scanning confocal microscope for Rhodamine and FITC, neurons were identified as CNP immunoreactive.

RESULTS

CNP-like immunoreactivity in *Clione*

Staining the foregut tissue of *Clione* with antibodies raised against *Helix* CNP peptides revealed a very dense network of immunoreactive fibers (Fig. 1). As it was found in other gastropods that various neuropeptides play an important role in the neural control of the gut, we performed a search for CNP-like positive neurons in the CNS. These neurons could be the putative source of immunopositive fibers in the foregut tissue and thus involved in the neural control of the gut. Staining of whole-mount preparations of *Clione* brain with anti-CNP antibodies revealed very few immunoreactive cells (Fig. 2). Two pairs of relatively small CNP-like positive neurons (soma diameter ~30 μm) were found on the dorsal surface of the cerebral ganglia (Fig. 2A) and one unpaired cell was usually situated on the dorsal surface of the left pleural ganglion (Fig. 2B). The network of immunoreactive fibers was found in cerebral and intestinal ganglia. Only single immunoreactive fibers were usually stained in pedal and pleural ganglia. One pair of giant immunoreactive neurons was observed in the dorsal anterior region of buccal ganglia (Fig. 2C). These immunoreactive neurons had soma sizes around 75 μm and showed a bright fluorescence. Buccal immunoreactive neurons apparently gave rise to immunoreactive axons in the gastro-esophageal nerve that innervates the foregut (Fig. 2C). In addition, the neuropile of buccal ganglia contained a dense network of immunoreactive fibers (Fig. 2C).

Buccal foregut cells and their effect on the beating of cilia lining the gut

Buccal CNP-like immunoreactive cells, designated as buccal foregut cells (Bc-FG), were then studied intracellularly. Intracellular staining of Bc-FG cells with neurobiotin or AlexaFluor revealed that each cell sent one neurite to the contralateral ganglion, branching in both ipsilateral and contralateral ganglia, and another main neurite into the gastro-esophageal nerve which produce intense arborization in the ipsilateral half of the foregut (Fig. 3A,B; N=6). The Bc-FG cells usually showed spontaneous low frequency spiking activity (0.2–0.7 Hz) interrupted with high-amplitude IPSPs from unknown sources (Fig. 3C). The averaged membrane potential of Bc-FG neurons was –38.9±1.2 mV (N=16 cells). The identity of Bc-FG cells

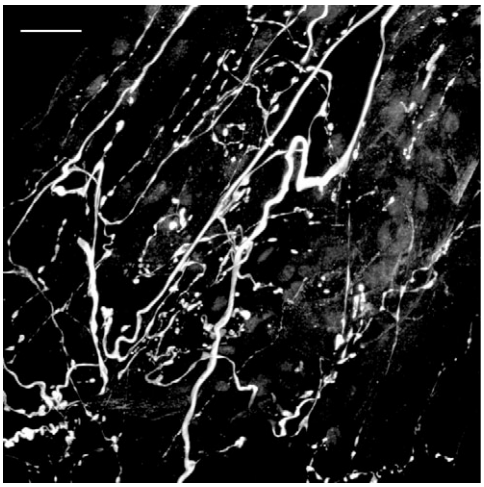

Fig. 1. CNP-like immunoreactivity in the foregut of *Clione*. Note the dense network of immunoreactive fibers. Scale bar, 20 μm.

was confirmed in a series of double-labeling experiments, in which the recorded cells were intracellularly stained with neurobiotin and the preparations were later stained with antibodies to the *Helix* CNP peptides (Fig. 4).

To determine the physiological role of the Bc-FG cells, we performed a series of experiments in semi-intact preparations consisting of buccal ganglia connected to the buccal mass and the foregut of the animal. The inner surface of the *Clione* gut is covered with a ciliated epithelium that always shows some background ciliary beating. We have found in pilot experiments that intracellular stimulation of the Bc-FG cells produced vigorous activation of cilia beating on the ipsilateral part of the foregut. Ciliary beating was visible through the semi-transparent wall of the foregut. To quantify the influence of Bc-FG cells on ciliary activity we developed a reduced semi-intact preparation consisting of the buccal ganglia connected *via* gastro-esophageal nerves to the foregut, which was cut longitudinally and tightly pinned to the Sylgard-coated dish with the ciliated epithelium facing upwards. Cilia beating was observed under the microscope with a ×40 Olympus long working-distance fluid-immersion objective. Beating cilia could be viewed through the oculars or projected to the CCD camera, mounted to the photo-output of the microscope. Cilia beatings were recorded optically at 125 frames s^{-1} in parallel with intracellular recording. It was found that intracellular stimulation of Bc-FG cells produced vigorous activation of quiescent cilia (Fig. 5A,B, traces 1–4) and increased the frequency of on-going cilia activity (Fig. 5A,B, traces 5–7; N=10). The effect of Bc-FG cell stimulation was extremely strong: even a few action potentials induced pronounced activation of cilia beatings lasting for several minutes. Brief intracellular stimulation of the Bc-FG cell in parallel induced a weak tonic contraction of the foregut, lasting for 1–2 min after stimulation (Fig. 5C). The foregut movements became apparent when the preparation was not pinned sufficiently to the bottom of the dish. Movements were recorded optically with the same CCD camera. Signals were low-pass filtered to remove oscillations corresponding to cilia beating.

To determine the pattern of activity of Bc-FG cells during feeding we performed another series of experiments in semi-intact preparations involving stimulation of the cerebro-buccal connective, which is known to induce fictive feeding. Fictive feeding was monitored by recording the intracellular activity of the buccal protractor interneuron (Bc-PIN) known to discharge rhythmically during the buccal rhythm (Arshavsky et al., 1993; Norekian and Malyshev, 2005). It was found that during fictive feeding induced by cerebro-buccal connective stimulation, the Bc-FG cells demonstrated prolonged depolarization with spike discharge (Fig. 6;

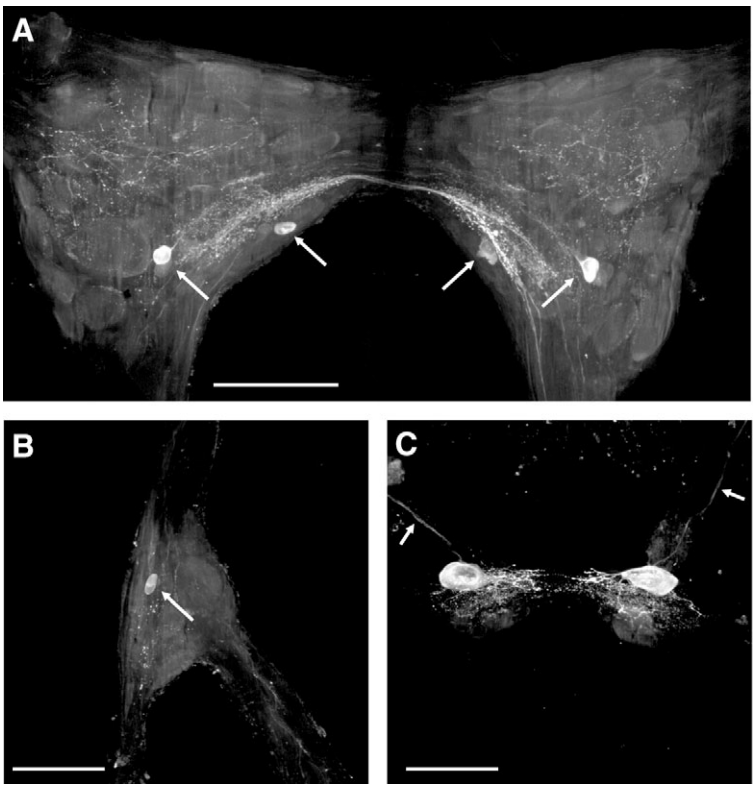

Fig. 2. CNP-like immunoreactivity in the CNS of *Clione*. (A) There are only two pairs of immunoreactive cells in the cerebral ganglia (marked by arrows). (B) A single unpaired cell was stained in the left pleural ganglion (arrow). (C) Two intensively stained neurons are localized on the dorsal surface of the buccal ganglia. Arrows indicate immunoreactive axons in gastro-esophageal nerves. Scale bars, 200 μm.

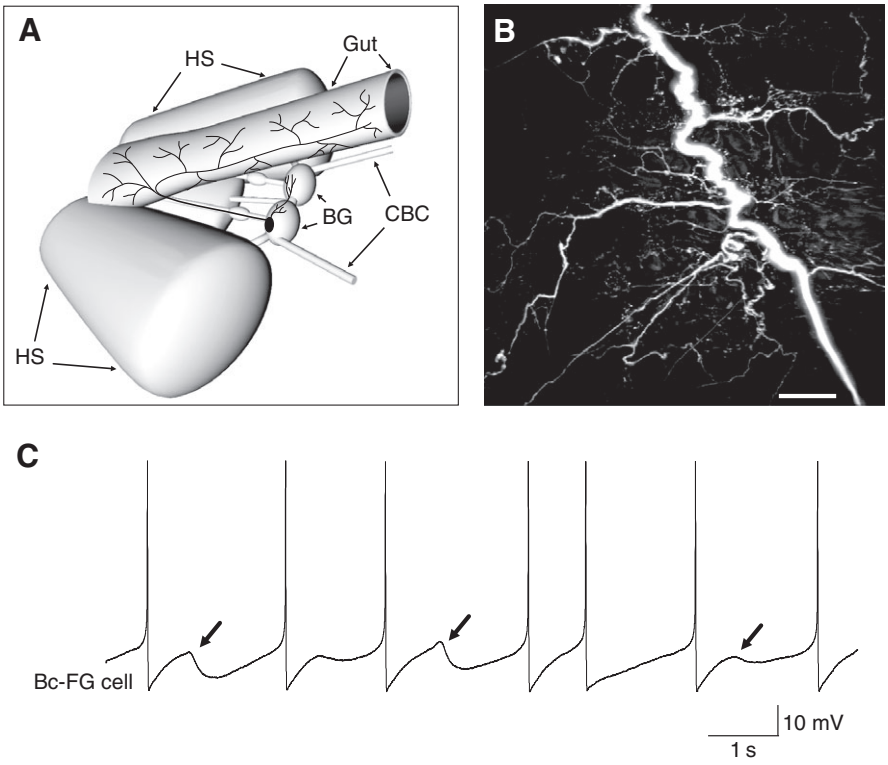

Fig. 3. Morphology and electrophysiology of buccal foregut (Bc-FG) cells. (A) Schematic drawing showing the innervation targets of the Bc-FG neurons. Bc-FG cells projected into the gastro-esophageal nerve and innervated the ipsilateral half of the pharynx and esophagus. HS, muscular hook sacs, containing retracted chitinious hooks; BG, buccal ganglia; CBC, cerebro-buccal connective. (B) Branching of a Bc-FG cell in the gut. The neuron was intracellularly stained with Alexa 488 and then the live preparation was subjected to confocal microscopy investigation. Scale bar, 100 μm. (C) Example of spontaneous activity of a Bc-FG cell in a semi-intact preparation. Large spontaneous IPSPs are seen (marked by arrows), which are typical for this cell.

N=5). The firing rate of the Bc-FG cells during fictive feeding was around 1–2 Hz. Interestingly, the Bc-FG cell did not demonstrate rhythmic activity during fictive feeding, unlike the majority of buccal neurons (Fig. 6, lower trace), but instead showed tonic regular spiking.

Role of the CNP peptide in mediating the effects of the Bc-FG cells

The Bc-FG cells demonstrated strong CNP-like immunoreactivity, which suggested that they contained a peptide similar to the *Helix* CNP peptides. This peptide could be used by the cell as a transmitter to influence cilia in the foregut. To test this possibility, we studied the effect of DYPRL-amide, one of the four CNP peptides described in *Helix*, on cilia activity in the isolated foregut preparation. To avoid arbitrariness during calculation of cilia beat frequency, we introduced an automated routine which calculated the Fourier spectrum on each of 6400 traces recorded by individual detectors of the CCD camera and, if there was a prominent peak on the spectrum corresponding to the dominant frequency, the routine procedure defined the frequency of the peak and then calculated an average frequency for a 96 s recording period (Fig. 7, right). We found that application of DYPRL-amide in the broad range of concentrations from 10^{-8} to 10^{-4} mol l^{-1} did not induce any significant changes in the activity of the cilia in the isolated patches of the foregut (Fig. 7A; *N*=10). However, we noted that cilia activity on the patches of the foregut was substantially different from that on the whole foregut connected to the brain. First, denervation of the foregut led to the disappearance of the area with quiescent cilia, which were usually present in the semi-intact preparation (Fig. 8); and second, the average frequency of cilia beating was significantly greater on the patches of the foregut compared with the foregut connected to the brain (10.09±0.75 Hz, *N*=9 *versus* 7.48±0.50 Hz, *N*=7; $P<0.05$, Mann–Whitney rank sum test). This suggests that the nervous system in general exerted inhibition of ciliary beating in the foregut of *Clione*. To eliminate the effect of denervation of the gut, we performed a series of experiments in semi-intact preparations

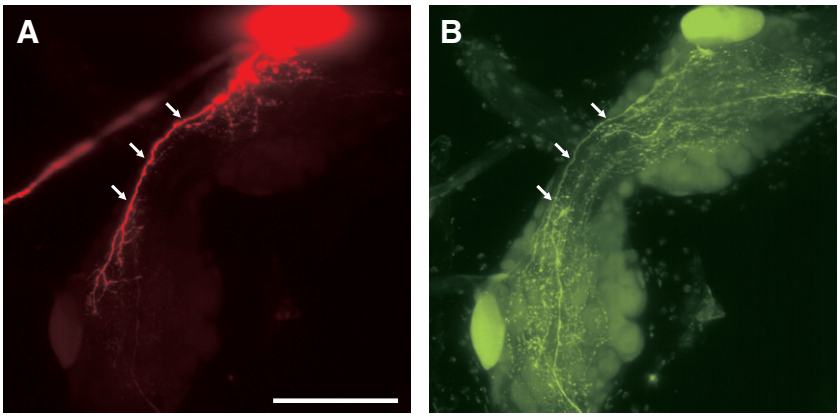

Fig. 4. Double-labeling experiments confirmed the CNP-like immunoreactivity of Bc-FG cells. (A) Confocal microscope image of buccal ganglia with the Bc-FG neuron filled with neurobiotin and visualized by Rhodamine. (B) Confocal image of the same buccal ganglia showing the CNP-like immunoreactivity (CNP antisera were labeled with AlexaFluor 488). Note the immunoreactive neurite of the Bc-FG cell projecting to the contralateral ganglion (marked by arrows). All images represent a composite confocal microscope reconstruction from several (not all) optical sections.

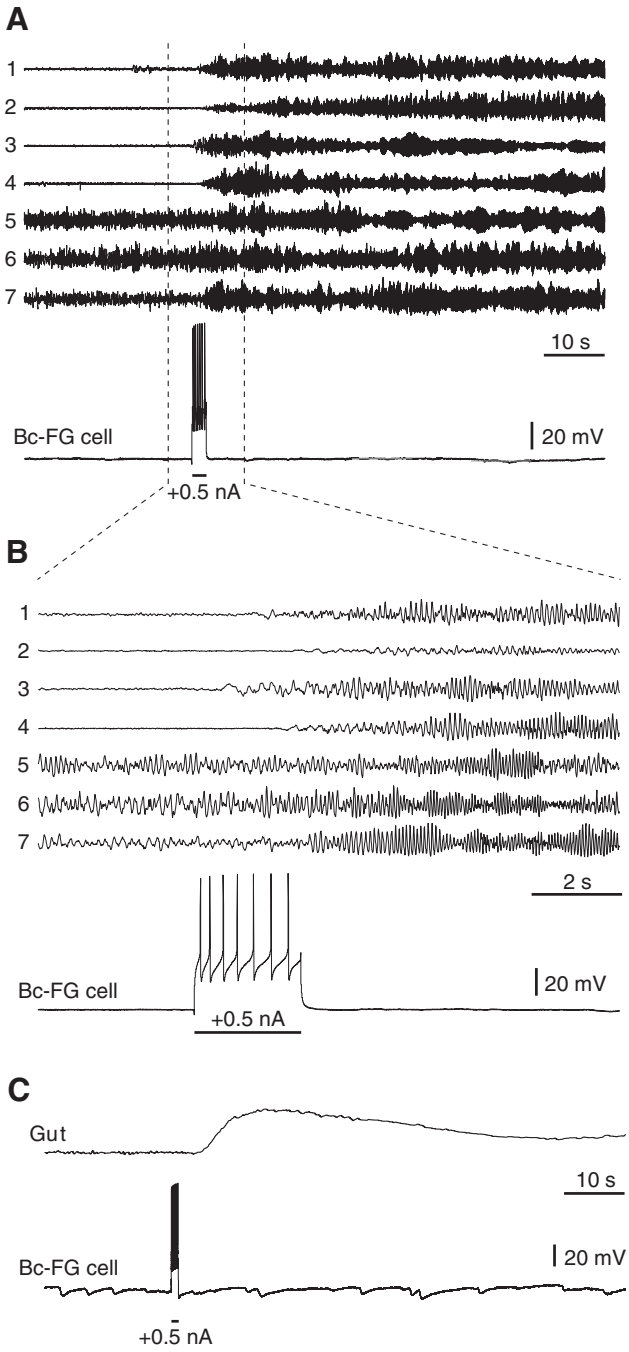

Fig. 5. Effect of intracellular stimulation of the Bc-FG cells. (A) Stimulation of Bc-FG cells produced vigorous activation of cilia lining the gut. Traces 1–7 represent ciliary beating recorded optically from different points in the foregut. In the points represented by traces 1–4 cilia were quiescent before cell stimulation, whereas those represented by traces 5–7 were chosen from the area with cilia activity existing before stimulation. (B) Section of the same recording as in A but shown with higher temporal resolution. (C) A short burst of spikes induced in a Bc-FG cell resulted in not only activation of ciliary beating but also a long-lasting tonic contraction of the foregut. Foregut movements were recorded optically; the signal was low-pass filtered to remove oscillations corresponding to ciliary beating.

where buccal ganglia and the foregut were placed in separate chambers but remained connected *via* the gastro-esophageal nerve passing through the Vaseline wall that divided the chambers. Such preparations, on the one hand, allowed easy application of the peptide to the foregut without affecting ganglia and, on the other hand, preserved a normal pattern of cilia activity, which disappeared in the isolated foregut preparation. It was found that bath application on the foregut of the DYPRL-amide at a final concentration of 10^{-5} mol l^{-1} induced a statistically significant increase in cilia beat frequency, on average from 7.48±0.50 Hz to 12.11±0.85 Hz (N=7; P<0.05, Wilcoxon signed rank test; Fig. 7B) in such preparations.

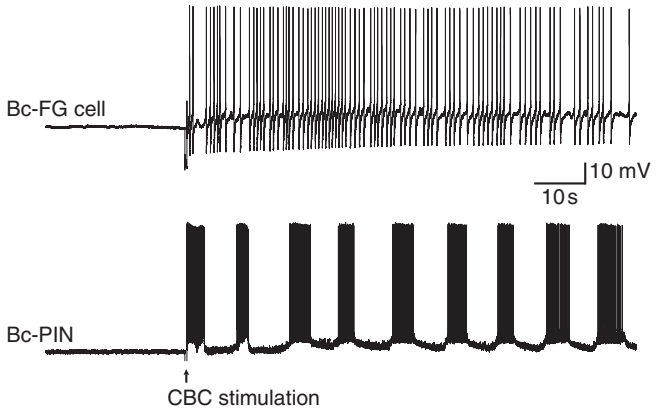

Fig. 6. Bc-FG cells were activated during fictive feeding. Fictive feeding was induced in the isolated CNS of *Clione* by brief electrical stimulation of the cerebro-buccal connective (CBC, arrow). Rhythmic activity of the identified buccal protractor interneuron (Bc-PIN) confirmed that fictive feeding was successfully initiated.

DISCUSSION

Neural control of ciliary beating in mollusks

Neural control of cilia beating was described in the nudibranch mollusk *Hermissenda* (Crow and Tian, 2003) and the freshwater snail *Planorbis corneus* (Deliagina and Orlovsky, 1990) as well as in gastropod veliger larvae (Mackie et al., 1976) and the embryo of *Helisoma trivolvis* (Goldberg et al., 2008). Neural control of cilia was intensively studied in the nudibranch mollusk *Tritonia diomedea*. Audesirk (Audesirk, 1978) was the first to report that intracellular stimulation of identifiable Pd12 pedal neurons activated cilia on the surface of the foot, where cilia are used for locomotion of the animal. It was found later that Pd12 most likely uses serotonin to increase cilia beating (Audesirk et al., 1979). Serotonin was also found to mediate neural control of ciliary beating in embryos of *Helisoma* (Diefenbach et al., 1991; Goldberg et al., 1994). The next important step in the story of neural control of cilia in *Tritonia* was purification of *Tritonia* pedal peptide (TPep). It was shown that application of TPep increased beating of the cilia covering the foot of *Tritonia* (Willows et al., 1997), while intracellular stimulation of the Tpep-containing neuron Pd5 induced an increase in locomotion speed (Popescu and Willows, 1999). Bath application of Tpep also increased the cilia beat rate of cells of the esophagus (Pavlova et al., 1999), while dense Tpep-like immunoreactivity was observed in the foregut of *Tritonia* (Gaston, 1998), suggesting that Tpep may play a role in the regulation of cilia lining the foregut. Therefore, buccal Tpep-immunopositive neurons in *Tritonia* may be homologous to Bc-FG of *Clione* described here. On the other hand, immunolabeling of *Tritonia* buccal ganglia with anti-TPep antibodies revealed a dozen relatively small cells (Gaston, 1998) in contrast to one pair of large Bc-FG neurons in the buccal ganglia of *Clione*. Unfortunately, no data on Tpep-like immunoreactivity in *Clione* buccal ganglia exist in the literature.

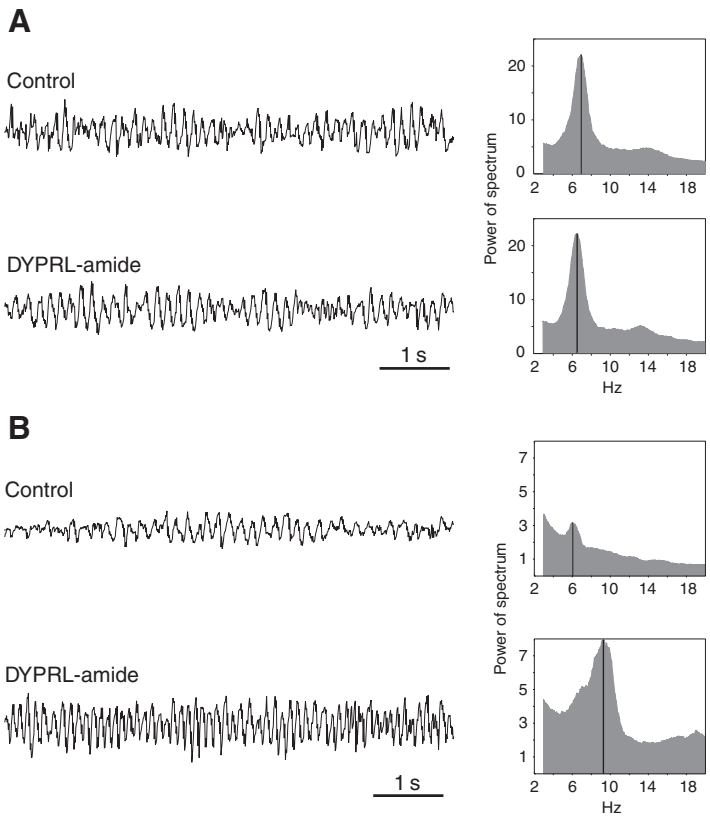

Fig. 7. Effect of bath application of DYPRL-amide on the activity of cilia in the innervated and denervated foregut. (A) DYPRL-amide did not induce any changes in cilia beating frequency on isolated patches of the gut (denervated preparation). Optical recording of cilia activity from one experiment before and after 10^{-5} mol l^{-1} DYPRL-amide application is shown. Right, Fourier spectra of corresponding optical traces showing the dominant frequency of cilia beating. (B) Application of DYPRL-amide at the final concentration of 10^{-5} mol l^{-1} on the gut connected to the buccal ganglia *via* the gastro-esophageal nerve (innervated preparation) induced an increase in cilia beat frequency.

Role of CNP-like peptides in the neural control of cilia in the gut

Bc-FG cells showed strong CNP-like immunoreactivity, as was confirmed by a series of double-labeling experiments. Furthermore, application of DYPRL-amide, one of the *Helix* CNP peptides, increased cilia beat frequency in the *Clione* foregut – an effect similar to that produced by Bc-FG cell stimulation. It is thus likely that some components of the Bc-FG cell-induced response could be mediated by the release of a peptide similar to *Helix* CNP. This effect was present only in preparations consisting of the foregut connected to the buccal ganglia and was absent in isolated patches of the foregut. We can see two possible explanations for this finding. The first explanation is that the *Clione* CNP-like peptide is probably not the only transmitter released by the Bc-FG cells. Thus, the CNP-like peptide-mediated effect on the cilia may require some co-transmitter which is released in semi-intact preparations due to spontaneous activity of the CNP-cells, and this contribution was revealed by the bath application of DYPRL-amide. A similar situation has been described for the buccal B2 neuron in *Lymnaea*. This buccal neuron

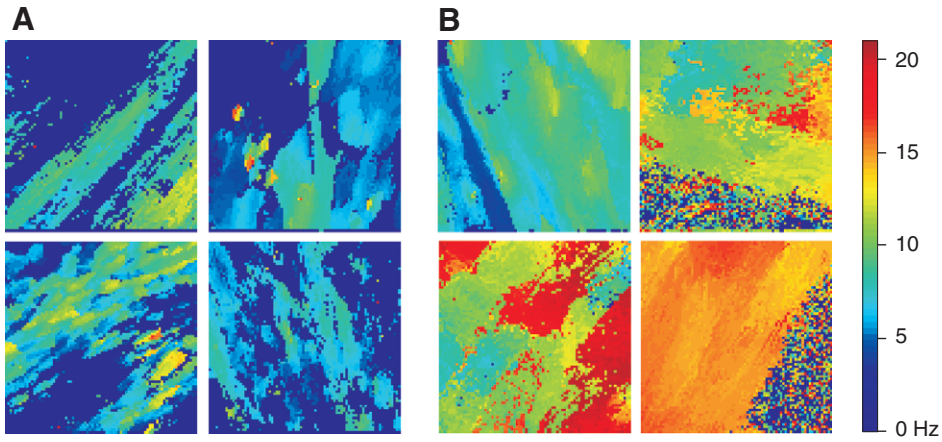

Fig. 8. Isolated patches of the foregut demonstrated a significantly higher level of cilia activity compared with that in the foregut connected to ganglia. Each square shown here represents the spatial distribution of cilia beating frequencies in the fragment of foregut (140 μm×140 μm) recorded optically during the experiment with the 64×64 CCD camera. Ciliary beat frequencies were calculated for each detector of the CCD camera using FFT analysis and encoded with pseudocolors: dark red corresponded to the maximal beating frequency whereas dark blue implied the absence of activity. (A) Four representative examples of cilia activity in the foregut connected to the ganglia in four different preparations. Note the large areas of quiescent cilia shown by the dark blue color. (B) Another four examples showing cilia activity in the isolated patches of the foregut from the four different animals. Ciliary beat frequency was in general greater on such preparations and they had virtually no zones with quiescent cilia.

contains both acetylcholine and myomodulin, and only their joint application can mimic the effect of B2 cell stimulation on foregut motility (Perry et al., 1998). The second explanation is based on the fact that the frequency of cilia beatings was significantly higher in isolated patches of the foregut compared with that in the foregut connected to buccal ganglia (10.1 and 7.5 Hz, respectively). It is quite possible that cilia are under constant inhibitory influence from the nervous system (from neurons other than Bc-FG cells). Denervation of the foregut thus removes this inhibitory effect and allows the cilia to beat at the maximum possible frequency, making a further increase in the frequency by CNP peptide impossible.

CNP peptides in invertebrates

A gene encoding the precursor of CNP peptides was originally found in the terrestrial snail *Helix lucorum* where it is predominantly expressed in parietal command neurons for withdrawal behavior (Bogdanov et al., 1998). This gene, named *HCS2*, encodes a propeptide containing a Ca^{2+}-binding protein of the EF-hand family and four putative neuropeptides (command neuron peptides) CNP1–CNP4 with a similar Tyr-Pro-Arg-X amino acid sequence at the C-terminus. The level of *HCS2* expression in the CNS is very flexible and could be up-regulated by noxious stimulation applied to the whole animal or by application of various neurotransmitters to isolated CNS preparations (Balaban et al., 2001). It was shown that application of CNP peptides on semi-intact preparations mimics the effect of command neuron activity on the neurons controlling the *Helix* respiratory system – one of the main targets of command neurons. Using anti-CNP antibodies, CNP-like immunoreactive neurons were found in other gastropods [*Aplysia* (Ierusalimsky et al., 2003) and *Lymnaea* (V. N. Ierusalimsky, unpublished observation)] as well as in *Drosophila* (Ierusalimsky and Balaban, 2007) and medical leech (Aseev et al., 2005). Moreover, a very close homolog of the HCS2 neuropeptide precursor containing three CNP-like neuropeptides and one EF-hand motif was described in *Aplysia* (Blast NCBI protein database, BLASTP 2.2.6, accession no. AAK56547). It is interesting that *Clione* is the only studied species which shows CNP-like immunoreactive neurons in the buccal ganglia. So, despite evidence for the presence of CNP-like peptides in a number of animals, the role of these peptides might be dissimilar in the different species.

Buccal foregut neurons in gastropods

Buccal peptidegic neurons innervating the foregut similar to that described here for Bc-FG cells were identified in several gastropod species: *Aplysia* (Lloyd et al., 1988), B2 in *Lymnaea* (Perry et al., 1998), B5 in *Helisoma* (Murphy and Kater, 1980; Murphy et al., 1985) and B11/B12 in *Tritonia* (Lloyd and Willows, 1988). All these cells have a similar morphology: a large cell body and main neurite leaving the CNS through the gastro-esophageal nerve and innervating the foregut. These neurons were shown to contain SCPb (small cardioactive peptide b), myomodulin and some other peptides, and regulated motility of the gut (Lloyd et al., 1988; Lloyd and Willows, 1988; Perry et al., 1998). Unfortunately, no information about their effect on cilia activity was reported. It is interesting that *Clione* also has two large neurons in buccal ganglia immunoreactive to myomodulin (Norekian and Satterlie, 1997). Although it requires further study, it is quite possible that the identified Bc-FG cells also contain myomodulin and, therefore, could be homologs of the peptidergic neurons regulating gut motility described in other species. An interesting prediction could be made based on the assumption of this homology: it is possible that, as described in *Aplysia*, *Lymnaea* and *Tritonia*, foregut cells could be also involved in the control of cilia lining the gut. However, further studies are required to confirm or reject this hypothesis.

Role of Bc-FG cells in the feeding behavior of *Clione*

The beating of cilia lining the foregut of gastropods drives mucus with particles of food towards the stomach and, therefore, facilitates swallowing of the food (Ruppert et al., 2004). We have shown here that the Bc-FG cells, stimulation of which produced powerful activation of ciliary beating in the foregut, were active during fictive feeding in semi-intact preparations. This finding suggests that activation of ciliary beating in the foregut by the Bc-FG cells is likely to occur during feeding behavior of *Clione*. *Clione* does not bite small pieces from the prey during feeding as many other animals do, but pulls the entire prey from its shell using specialized feeding structures, chitinous hooks and toothed radula (Lalli, 1970; Lalli and Gilmer, 1989; Wagner, 1885). Therefore, activation of cilia in the foregut of *Clione* during feeding may help with swallowing the prey and facilitate its extraction from the shell. Thus, cilia-activating buccal neurons may represent a new element of neural control of feeding in gastropods.

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