RESEARCH ARTICLE

Contextual modulation of a multifunctional central pattern generator

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ABSTRACT

The multifunctional buccal central pattern generator in snails, which controls different oral behaviors, has been well characterized. In this work we propose a role for the group of about 40 electrotonically coupled buccal A cluster cells as a context-dependant switch for the buccal central pattern generator, modulating motor patterns that elicit different oral behaviors. We characterize these cells based on location and morphology, and provide evidence for their selective activation under two different stimuli – Listerine perfusion and intestinal nerve stimulation – triggering buccal motor patterns putatively underlying egestion and substrate cleaning. A new role for these electrotonically coupled buccal A cluster neurons is shown. They serve as a context-dependant switch that alters buccal motor patterns depending on input stimuli, thereby eliciting the appropriate behavioral response.

KEY WORDS: CPG, Buccal ganglia, Snail, Sensory motor, *Helisoma*

INTRODUCTION

Rhythmic movements in animals underlie a vast array of behaviors, such as locomotion, respiration, mastication, etc. The neural networks controlling such programmed behaviors are termed central pattern generators (CPGs) and they have the potential to produce different motor outputs depending on the current external and internal environment of the organism (McCrea and Rybak, 2008; Rossignol et al., 2006; Selverston, 2010; Serrano et al., 2007). In some cases the functional reorganization of a multi-functional CPG is under the influence of projection neurons (Benjamin and Elliott, 1989; Combes et al., 1999; Croll et al., 1985; Jing and Weiss, 2001; Meyrand et al., 1994; Rosen et al., 1991) or sensory neurons (Bässler and Büschges, 1998; Blitz and Nusbaum, 2011; Smotherman, 2007). Neuromodulator action has also been shown to alter CPG outputs of both invertebrates and vertebrates (Cazalets et al., 1992; Chambers et al., 2011; Gray et al., 1999; Harris-Warrick, 2011; Katz, 1998; Selverston, 2010).

In the pulmonate snail *Helisoma*, a group of about 40 electrotonically coupled cells, termed 'cyberchron' neurons, were identified in the buccal ganglia (Kater, 1974; Kaneko et al., 1978; Merickel et al., 1978; Merickel and Gray, 1980). This cyberchron network [since renamed buccal A cluster cells (BACs) (Murphy, 2001; Murphy, 1991)] was initially thought to be the pattern generator but its function remains unclear. While a modulatory role has been suggested for these BAC cells in altering the

multifunctional buccal CPG, there has not been a comprehensive study addressing their neuromodulatory role (Murphy, 1991). Here, we morphologically characterize these BAC neurons and define a role for them as a neuromodulatory switch for the buccal motor pattern.

The electrotonic nature of the BAC cells, the electrical coupling between the BAC neurons and the changes in coupling during the bursting of these cells have been extensively characterized by Kaneko and others (Kater, 1974; Kaneko et al., 1978). The burst generation properties of this neural network have been studied using single-electrode voltage clamp techniques (Merickel and Gray, 1980) and computer simulation models (Merickel et al., 1978). Although these experiments elegantly described the electrical properties of the cells, morphological characterizations of the BAC cells were not very clear because of a lack of good intracellular dyes at that time. In this study, we describe the morphology of BAC cells and classify this diverse population of neurons.

While a clear role for the BAC cells in the buccal system has yet to emerge, BAC-cell-like inputs have been routinely observed in experiments that alter the regular buccal CPG patterns using mechanical, emetic and nerve-stimulation protocols (S.R., B.A. and A.D.M., unpublished observations). In this study, we show the effects of BAC cells on buccal motor patterns in *Helisoma trivolvis* Say 1817 pond snails. We address two different sensory triggers, emetic injection and intestinal nerve stimulation, that activate the BAC network and possibly switch the buccal motor pattern towards two distinct behaviors: regurgitation and substrate cleaning, respectively. We show that the BAC cell cluster plays an important role as a sensory–motor interface, serving as a context-dependent switch to alter buccal motor patterns.

RESULTS
Revisiting the buccal CPG

Initial work on the BAC neurons (then termed 'cyberchrons') proposed that they both timed and drove a bi-phasic feeding pattern generator (Fig. 1) (Kater, 1974; Kaneko et al., 1978). Subsequent studies in *Helisoma*, as well as the closely related pulmonate *Lymnaea*, have revealed that the feeding CPG in these snails is triphasic and comprises three semi-independent neuronal oscillators (S1, S2 and S3) controlling protraction, rasp and swallow (Fig. 1B) (Benjamin and Rose, 1979; Murphy, 2001; Quinlan and Murphy, 1991; Quinlan and Murphy, 1996; Rose and Benjamin, 1981; Rose and Benjamin, 1979). The 'cyberchron' network was initially thought to be the pattern generator but appears to be neither necessary nor sufficient for the generation of the standard feeding pattern (Murphy, 2001; Murphy, 1991).

Basic identification and location of BAC cells

BAC neurons are defined based on one or more of the following criteria (Kaneko et al., 1978): (1) bursts of action potentials correlate with large hyperpolarizations in 'protractor motor neurons' (now

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List of symbols and abbreviations

5-HT	5-hydroxytryptamine (serotonin)
AVT	arginine vasotocin
BAC	buccal A cluster
CBC	cerebro-buccal connective
CDC	caudodorsal cell
CPG	central pattern generator
EPSP	excitatory postsynaptic potential
ET	esophageal trunk
GnRH	gonadotropin-releasing hormone
IN	intestinal nerve
IPSP	inhibitory postsynaptic potential
LBN	lateral buccal nerve
PBN	posterior buccal nerve
PSP	postsynaptic potential
VBN	ventral buccal nerve

known to be hyper-retractor motor neurons); (2) brief intracellular depolarizations result in characteristic activity that outlasts the depolarization; (3) stimulation of the esophageal nerve trunk results in a short latency 'anti-dromic' response (normal direction of action potential propagation is yet undemonstrated); and (4) electrical coupling with other BAC neurons. We located and recorded from snail BAC cells physiologically identified based on the criteria stated above. There are about 20 BAC cells, with soma sizes ranging from 20 to 40 μm in diameter, located in each of the paired buccal ganglia.

The location of BAC cells relative to some uniquely identifiable buccal neurons is indicated in Fig. 2. Neuron B5, one of the largest cells in the buccal ganglia (diameter 100–120 μm), is an esophageal motor neuron and is found usually towards the center of the ganglion. B19, a phase 3 motor neuron situated slightly ventral and medial to cell B5, is frequently used as a CPG monitor. The BAC cells were primarily located medial to cell B5 with a few near the buccal commissure and towards the dorsal edge of the ganglion (Fig. 2). Some BAC neurons were found along the rim of the cell B5, mostly along its ventral edge towards B19.

Morphology
Intracellular injections of Lucifer Yellow dye were used to characterize the morphology of BAC neurons (N=80). BAC cells

usually had axonal projections into three or more of the following buccal nerves (Fig. 3): the ipsilateral and contralateral esophageal trunks (ETs); ipsilateral and contralateral posterior buccal nerves (PBNs); ipsilateral and contralateral latero-buccal nerves (LBNs); and sometimes into the ipsilateral or contralateral cerebro-buccal connectives (CBCs). None of the BACs had axons in the ventro-buccal nerves (VBNs) (N=80) (Fig. 3A–C). All BAC neurons had an axon in the ipsilateral ET, and in at least two other buccal nerves mentioned above. Branches in the CBCs occurred less frequently than those in the other buccal nerves. Furthermore, BAC cells seemed to have axons with smaller diameters relative both to identified motorneurons and to interneurons of similar soma size (Fig. 3D). The dendritic arbor of BAC cells was minimal and sparse and usually restricted to the ipsilateral ganglion (N=80) (Fig. 3). Note that the VBNs, the only buccal nerves that never contain BAC neuron axons, innervate muscles (e.g. supralateral radular tensor) involved in hyper-retraction of the radula/odontophore (i.e. swallowing). The other buccal nerves that do contain BAC neuron axons all innervate muscles that envelop some portion of the oral cavity. Hence they have access to chemical and mechanical stimuli, which is consistent with our hypothesis that BAC neurons can be primary or secondary sensory neurons.

Classification of BAC cells based on morphological evidence
Based on their axonal projections into different buccal nerves, BAC cells were organized into 17 different morphological types (Fig. 4). The cells ascribed to a certain morphological type were not restricted to one position within the ganglion. The distribution of different BAC cell types in the buccal ganglion relative to uniquely identifiable cells B5, B4 and B19 is shown in Fig. 5. At present, it is difficult to predict with certainty the morphological type of the BAC cell based on its location and physiology alone. There is great variability in the distribution of these morphological types across snails (S.R., unpublished observations). As yet, BAC cell type 1 is the only cell that was found repeatedly (at least 10 times) in the specific location between cells B5 and B19. But this could be attributed to the fact that cells were chosen for physiological experiments with greater frequency in that area.
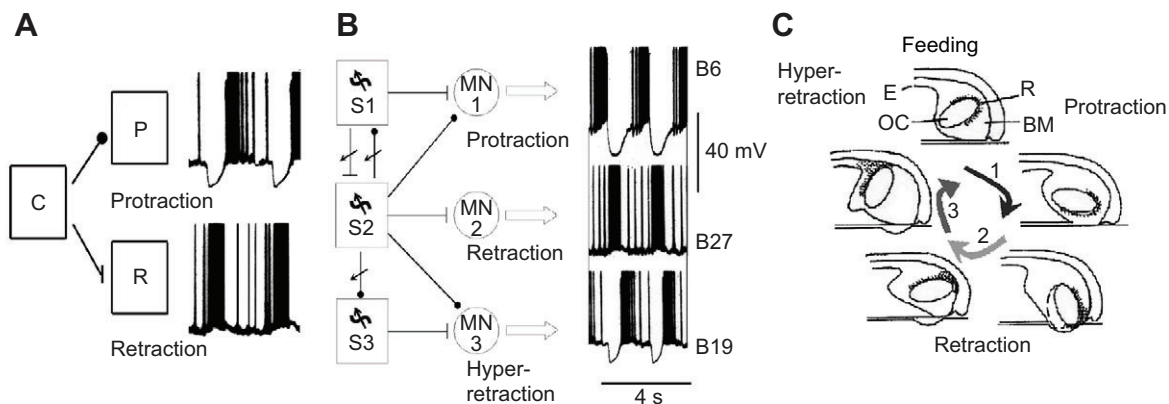

Fig. 1. Comparison of the current conditional oscillator model for the multifunctional buccal central pattern generator (CPG) in snails with the earlier cyberchron model. (A) In the earlier cyberchron model, the network of electrotonic cells called 'cyberchrons' was the CPG that timed and drove the activity of feeding motor neurons (after Kater, 1974). (B) The current model has three conditional oscillators (S1, S2 and S3) composed of interneurons that activate the motor neurons of phases 1, 2 and 3 (MN1–MN3) sequentially. This results in the motions of protraction, retraction/rasp or hyper-retraction/swallow, culminating in feeding. The 'protraction' neurons under the cyberchron model are actually the hyper-retraction phase 3 neurons under the current model (Murphy, 2001). A schematic of the firing patterns of protractor, retractor and hyper-retractor during feeding is shown on the right measured in B6, B27 and B19 cells, respectively. (C) Schematic of the movements of the radula/odontophore during the triphasic feeding motor pattern (1–3). E, esophagus; BM, buccal mass; OC, odontophore cartilage; R, radula.
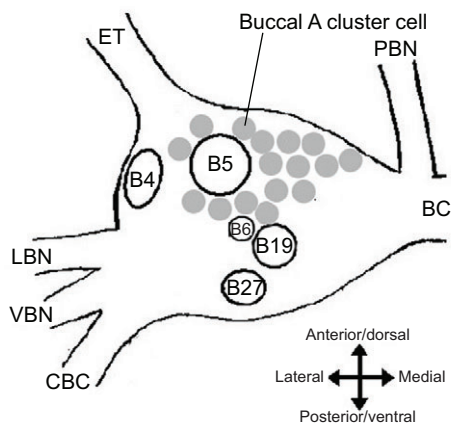
Fig. 2. Location of buccal A cluster cells with respect to principal neurons of the snail buccal ganglia. B5, the largest cell in the ganglion, is found near the center of the ganglion. B19 is situated ventral and medial to B5. The BAC cells can usually be found medial to cell B5 and towards the dorsal edge of the buccal ganglion. Other BAC neurons can be located all around the B5 cell, especially around its ventral edge towards cell B19. BC, buccal commissure; CBC, cerebrobuccal connectives; ET, esophageal trunk; LBN, latero-buccal nerve; PBN, posterior buccal nerve; VBN, ventral buccal nerve.

Effect of BAC cells on the buccal motor pattern

Intracellular recordings were made from BAC neurons in conjunction with uniquely identifiable buccal motor neurons. Typically, BAC neurons had a resting potential of about −50 mV, ranging from −35 to −70 mV (N=22). In quiescent preparations, BAC cells were usually silent, showing very little, if any, spike activity (e.g. Fig. 6). Fig. 6 shows a simultaneous intracellular recording of a BAC neuron with the phase 3 motor neuron B19. Initially, both neurons were quiescent. Upon stimulation with watermelon juice, the buccal feeding pattern is activated and neuron B19 shows a characteristic inhibitory postsynaptic potential (IPSP) in phase 2 of the feeding motor program (rasp) and bursts during phase 3 (swallow). The BAC neuron is silent, except for small EPSPs (~2 mV in amplitude) that correspond to the phase 2 IPSPs in B19 (Fig. 6). These EPSPs are electrotonic and cannot be enhanced by hyperpolarization of the BAC neuron.

Repetitive stimulation of BAC cells by applying depolarizing pulses elicited a barrage of action potentials (Fig. 7). In Fig. 7, constant depolarizing pulses were applied to a BAC cell, causing a steady rise in baseline membrane potential. After the fifth stimulation, spontaneous firing of BAC cells can be seen. This was followed by a BAC discharge indicated by the barrage of action potentials. Activity in other cells of the electrotonic network can be seen as small amplitude spikes in the BAC cell (Fig. 7). BAC discharges lasted for about 30 s and were accompanied by a shut down of bursting in cell B19. Compound IPSPs distinct from regular phase 2 IPSPs can also be seen in B19.

The effects of BAC cell action potentials on the buccal motor pattern are shown in Fig. 8. The preparation, on bath application of 10 μmol l^{-1} serotonin, exhibited a phase 2–3 pattern of activity, characterized by phase 2 IPSPs followed by phase 3 bursting in B19. During the initial phase of the recording, the BAC cell was held hyperpolarized. Releasing the BAC cell back to resting potential (Fig. 8, RP) elicited a barrage of action potentials. Initially, these were off-screen because of a change in bridge balance and were shifted back on screen where indicated (Fig. 8, *). The BAC discharge immediately shut down phase 3 bursting in B19 (Fig. 8). Closer examination of synaptic inputs on the motor neuron revealed
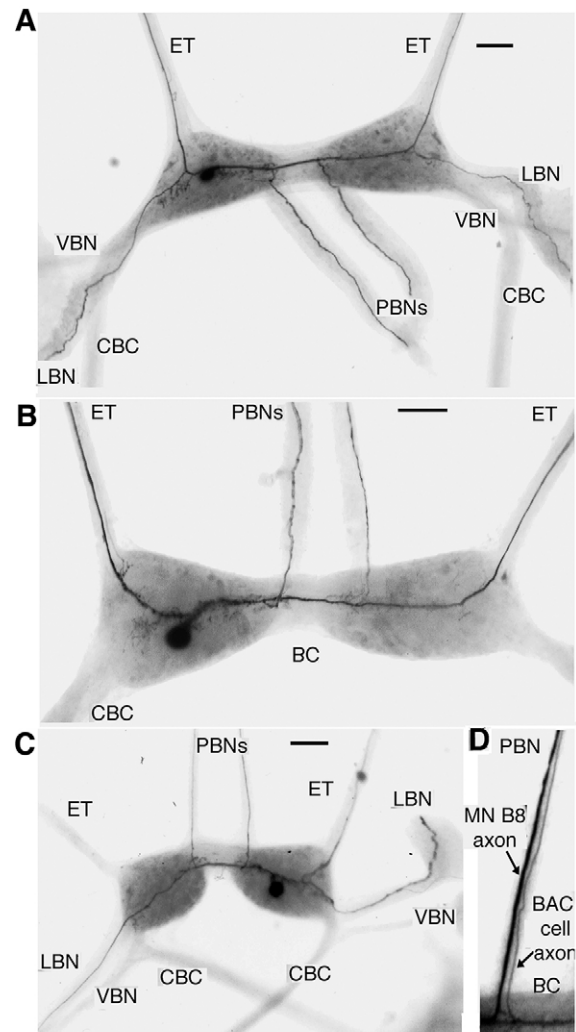

Fig. 3. Morphology of three BAC cells from the snail. (A) Axonal projections through the ipsi and contralateral ETs, PBNs and LBNs (type 1). (B) Axons in ipsilateral and contralateral ETs, and ipsilateral and contralateral PBNs. Note the sparse dendritic arbor near the soma (type 14). (C) BAC with axons in ipsilateral ET, ipsilateral and contralateral LBNs and PBNs (type 16). (D) Comparison of BAC axon with that of motor neuron B8. BAC cells have soma sizes comparable to that of motor neuron (MN) B8, but have much finer axons. Scale bars: 100 μm.

the presence of non-phase-2 inhibition that was distinct from regular phase 2 IPSPs (Fig. 8, dotted lines versus 2). The non-phase-2 PSPs had one-for-one correlation with the BAC spikes (Fig. 8, dotted lines).

BAC-like effects observed in buccal motor pattern are induced by Listerine perfusion and intestinal nerve stimulation

BAC cell barrages result in two main effects on the buccal motor pattern: a suppression of phase 3 firing and the induction of non-phase-2 synaptic inputs onto buccal motor neurons. These two phenomena are observed under two different experimental paradigms that stimulate distinct oral behaviors: regurgitation and substrate cleaning.

Listerine has been shown to be an aversive stimulus that elicits an emetic response in snails (Arnett, 1996). Perfusion of the esophagus with Listerine was performed while recording from the buccal motor
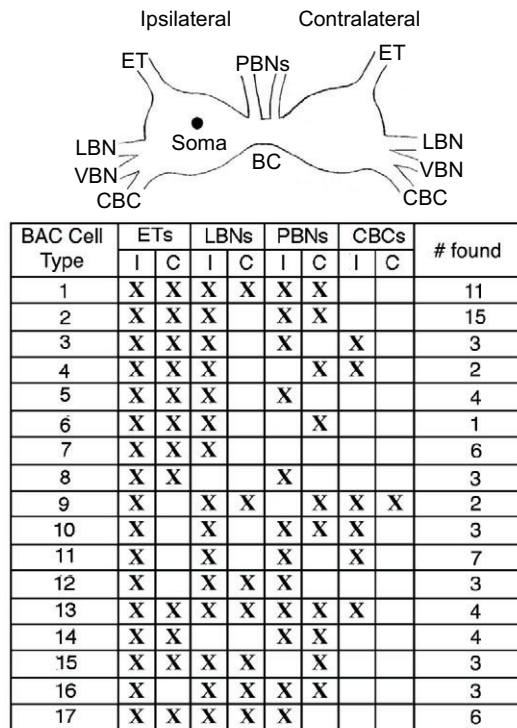
BAC Cell Type	ETs I	ETs C	LBNs I	LBNs C	PBNs I	PBNs C	CBCs I	CBCs C	# found
1	X	X	X	X	X	X			11
2	X	X	X		X	X			15
3	X	X	X		X		X		3
4	X	X	X			X	X		2
5	X	X	X		X				4
6	X	X	X			X			1
7	X	X	X						6
8	X	X			X				3
9	X		X	X		X	X	X	2
10	X		X		X	X	X		3
11	X		X		X		X		7
12	X		X	X	X				3
13	X	X	X	X	X	X	X		4
14	X	X			X	X			4
15	X	X	X	X		X			3
16	X		X	X	X	X			3
17	X	X	X	X	X				6

Fig. 4. Morphological analysis of BAC cells from the snail. Numbers on left indicate the BAC cell type. 'X' indicates an axonal projection in that buccal nerve. I, ipsilateral; C, contralateral. Numbers of different BAC neural types located and filled are indicated.

neurons in two kinds of semi-intact preparations: one with the buccal mass, esophagus and associated buccal nerves intact and the other with just the esophagus attached (see Materials and methods). Recordings from buccal motor neuron B19 during Listerine perfusion is shown in Fig. 9. Effects of Listerine were observed both following induced fictive feeding [by applying 1 μmol l^{-1} serotonin
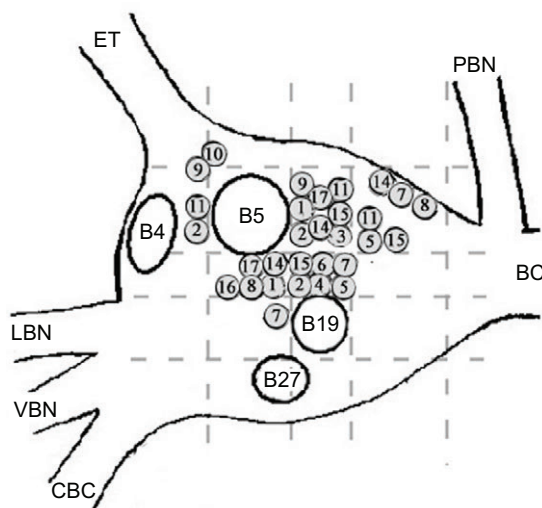

Fig. 5. Distribution of buccal A cluster cell morphological types in the buccal ganglia relative to uniquely identifiable cells B5, B19 and B4. Data from 80 different preparations was used. Grid lines were drawn using the vertical and horizontal spread of B19 and B5 as markers. Gray circles indicate BAC cells. Numbers within gray circles identify the specific morphological type (see Fig. 4).
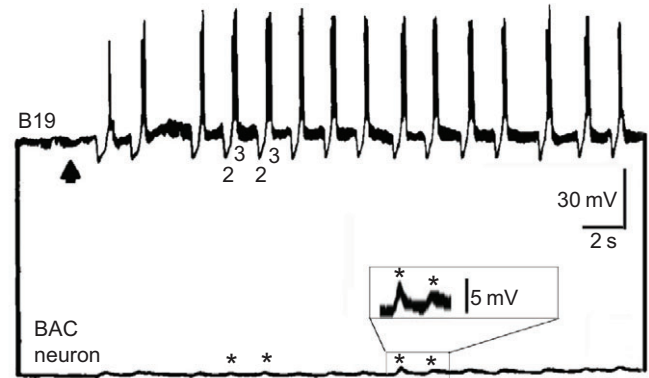

Fig. 6. Simultaneous recordings from a BAC cell neuron (morphological type 4) and B19. On stimulation with watermelon juice, B19 responds with a feeding rhythm characterized by phase 2 IPSPs (2) and phase 3 excitation (3). Most BAC neurons are usually quiet, exhibiting small electrotonic EPSPs (*) in conjunction with phase 2 IPSPs of B19 (inset).

(5-HT)] and under quiescent conditions. Listerine perfusion suppressed bursting in phase 3 motor neuron B19 (N=5) (Fig. 9A). Furthermore, Listerine also elicited a succession of IPSPs in B19 consisting of both phase 2 and non-phase-2 components (N=5) (Fig. 9B, arrowheads). These responses to Listerine were almost identical to those observed on stimulating BAC cell discharges (compare Fig. 9 to Figs 7, 8).

The intestinal nerve has been shown to be both necessary and sufficient for substrate cleaning behavior prior to egg-laying in the pulmonate snail (Ferguson et al., 1993; Hermann et al., 1994). Lesions to this nerve, with all other peripheral nerves intact, effectively abolish substrate cleaning behavior without affecting other oral behaviors (Ferguson et al., 1993). Fig. 10 shows the effect of intestinal nerve stimulation on the buccal motor pattern both following induced fictive feeding (by applying 1 μmol l^{-1} 5-HT) and under quiescent conditions. A phase 2–3 buccal motor pattern was induced by bath application of 1 μmol l^{-1} 5-HT, monitored by the phase 2 IPSPs followed by phase 3 bursts in B19 (Fig. 10A). Intestinal nerve stimulation shut down phase 3 bursting in B19 (Fig. 10A). Furthermore, stimulation of the intestinal nerve also triggered PSP barrages with non-phase-2 synaptic inputs apart from regular phase 2 inhibition (N=20) (Fig. 10A,B, arrowheads). Analysis of PSP barrages triggered after intestinal nerve stimulation (3–4 cycles each from five snails) revealed that 53.6% of these PSP barrages contain non-phase-2 components along with regular phase 2 activity.

Effect of Listerine perfusion and intestinal nerve stimulation on BAC cells

Both Listerine perfusion in the esophagus and intestinal nerve stimulation caused changes in the buccal motor pattern that were suggestive of BAC-cell-like effects. Intracellular recordings of BAC neurons were used to determine whether they were activated during these two stimulation paradigms. The morphological type of the BAC cells examined was identified using intracellular Lucifer Yellow.

BAC cells were activated and responded with a barrage of action potentials upon Listerine perfusion in the esophagus (N=11) (Fig. 11). In Fig. 11, the BAC neuron was initially silent but showed a burst of action potentials in response to Listerine application. These corresponded with the lengthened compound IPSP in cell B19 (Fig. 11A). While all the BAC cells examined responded to Listerine
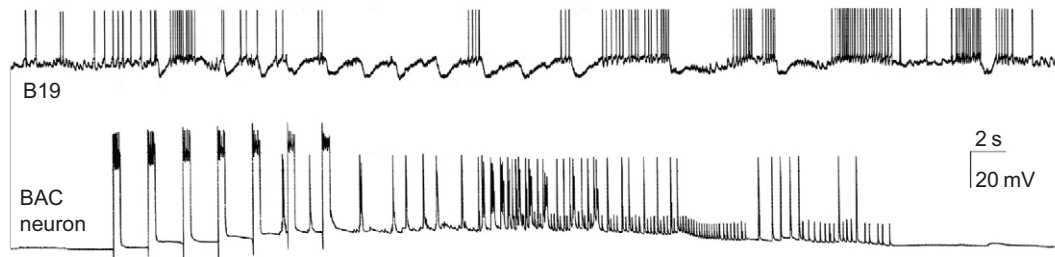
Fig. 7. A BAC cell discharge (morphological type 2). Seven constant depolarizing pulses were injected into the BAC cell. This caused a steady rise in resting potential and elicted a barrage of action potentials in the BAC cell. Small amplitude spikes reflect activity in other members of the BAC network. The BAC barrage shuts down B19 and elicits long compound IPSPs distinct from the regular phase 2 IPSP in B19.

application, there was variability in individual responses. Some BAC neurons discharged with short latency on Listerine application, coinciding with the compound IPSP in cell B19 (Fig. 11A). Other BAC neurons, had a longer latency for discharge, but their spikes evoked one-for-one PSPs that followed after the initial compound IPSP (Fig. 11B). The burst of a BAC cell such as that seen in Fig. 11A, is accompanied by simultaneous electrotonic spiking in other BAC cells in the network similar to that in Fig. 11B. Of the BAC cells that responded to Listerine perfusion, five were of morphological type 1, three of type 2 and one each of types 12, 13 and 14.

Intestinal nerve stimulation also elicited responses from the BAC cells (N=7). Fig. 12 shows a representative recording of a BAC cell in conjunction with neuron B19. Upon intestinal nerve stimulation, the BAC cell responded by robust bursting, which was correlated with one-for-one PSPs on B19 (Fig. 12A,B, dotted lines). Other BACs responded with pronounced bursting upon nerve stimulation in conjunction with long compound IPSPs in motor neuron B19 (Fig. 12C). This reiterates the fact that while the BAC network is activated by intestinal nerve stimulation, different BACs show different responses. The BAC cells from which recordings were made during the intestinal nerve stimulation experiments were of the following morphological types: 2, 4, 5, 8, 15, 16 and 17.

Long-term recordings from BAC cells indicate that after intestinal nerve stimulation the BAC cells periodically produce an action potential discharge. This was always associated with a barrage of

PSPs in the buccal motor neurons (N=3). An example of a long-term BAC barrage induced after intestinal nerve stimulation is shown in Fig. 13. Here B5, an esophageal motor neuron, shows compound IPSPs during the bursts in the BAC cell.

DISCUSSION

The multi-functional pattern generator underlying oral behaviors of pulmonate snails has been well studied and the component muscles, motor neurons and interneurons have been characterized (Murphy, 2001). Apart from regular feeding, the snail uses its oral apparatus for two other distinct behaviors – regurgitation and substrate cleaning prior to egg-laying. When a snail ingests an aversive stimulus, it rejects it immediately. This rapid change in behavioral output to regurgitation would be a reflection of a fast alteration in the buccal CPG. Egg-laying in pulmonate snails is an all-or-nothing event that is triggered by the discharge from the caudo-dorsal cells in response to appropriate environmental cues. Prior to oviposition, the snail performs substrate cleaning, where it makes tiny grooves on which to lay eggs, by rasping a small area of the substrate. This behavior lasts for almost an hour. Thus, the buccal system of snails must accommodate these different behavioral outputs and have the capacity to switch in an immediate or time-dependent sustainable manner to produce them reliably. The BAC system, a group of electrotonically coupled cells located in the buccal ganglia, is a prime candidate to function as this oral behavioral switch. While the ability of the BAC cell network to alter the buccal CPG has been well recorded, its purported role (Kater, 1974) as the CPG has been refuted (Murphy, 2001). Here, we have shown that the BAC cell network has the ability to act as a behavioral switch modifying buccal motor output.

BAC cells, showing little or no activity in regular preparations, can be excited into a discharge of action potentials (Kaneko et al., 1978) (also see Results). This exerts a pronounced change on the buccal motor neurons, causing a shut down of phase 3 excitation and eliciting PSPs that are not part of the regular CPG activity. Both Listerine perfusion and intestinal nerve stimulation trigger similar responses in the buccal motor pattern. We also show that BAC cells are activated under both Listerine perfusion and intestinal nerve stimulation. In both these cases, BAC cells switched the buccal motor pattern by suppressing phase 3 excitation. Furthermore, under both paradigms, BAC cell action potentials had one-for-one correlation with the non-phase-2 IPSPs observed in neuron B19.

Previous work had indicated that different cells within the BAC network show differential coupling and variability in bursting patterns (Merickel and Gray, 1980). Some of them were classified as primary burst generators and others as secondary followers. Thus, the network of BAC cells is not a homogeneous population. This property of BAC cells was reiterated during our experiments. Some BAC cells responded with lengthy bursts to the application of
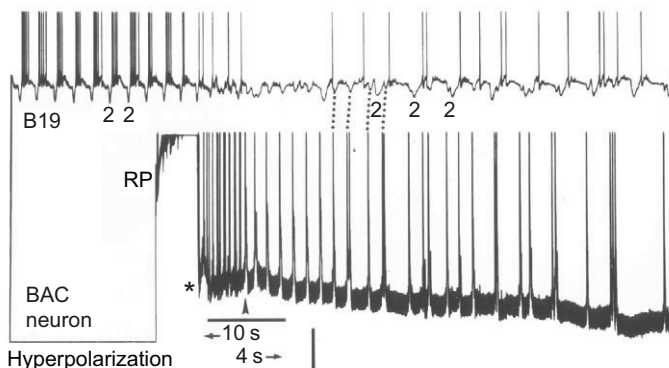

Fig. 8. Effect of BAC cell action potentials on buccal motor neuron B19. Bath application of 1 µmol l^{-1} 5-HT elicits a phase 2–3 buccal motor pattern. B19 shows phase 2 IPSPs (2) followed by phase 3 bursting. The BAC cell was initially held hyperpolarized (H). Anode break back to resting potential triggered a barrage of action potentials (RP) (off screen and shifted back on screen at *). B19 stops phase 3 bursting on BAC cell discharge. Arrowhead indicates a shift in recording speed from 10 s to 4 s. Non-phase-2 synaptic inputs observed in B19 show one-for-one correspondence to BAC cell spikes (dotted lines). These are distinct from regular phase 2 IPSPs (2). Morphological analysis indicates BAC cell type 10.
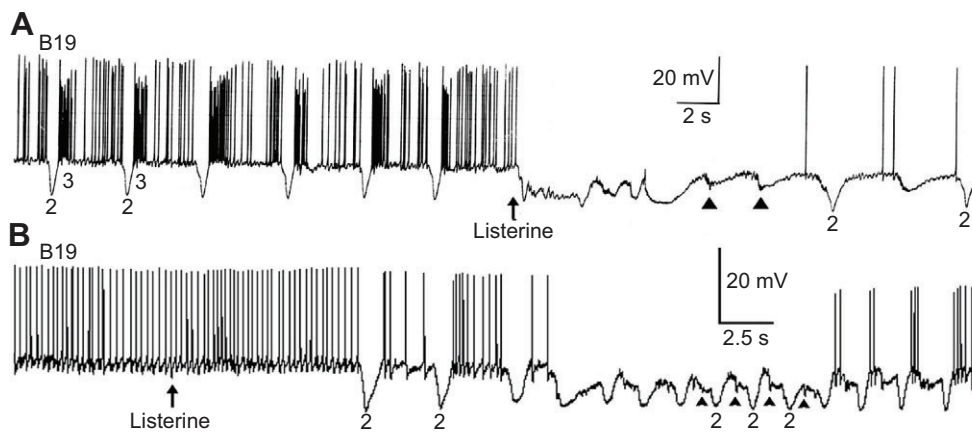
Fig. 9. Listerine perfusion shuts down phase 3 and elicits non-phase-2 synaptic potentials in buccal motor pattern. Recordings were made during fictive feeding (A) and in quiescent preparations (B). A regurgitation pattern was triggered in both preparations. (A) B19 shows a regular feeding pattern with phase 2 IPSPs (2) followed by phase 3 bursting induced by application of 1 µmol l⁻¹ 5-HT. On Listerine application, the phase 3 bursting is suppressed and non-phase-2 PSPs are triggered (arrowheads). (B) Listerine perfusion in the esophagus triggers non-phase-2 IPSPs in cell B19 (arrowheads) that are distinct from regular phase 2 inhibition. Arrows indicate point of Listerine perfusion.

Listerine or intestinal nerve stimulation, coinciding with the pronounced compound IPSPs observed in the phase 3 motor neurons. In other BAC cells, action potentials that evoked one-for-one PSPs in motor neurons were observed upon stimulation.

Earlier work on these cells did not give much morphological evidence because of a lack of good intracellular dyes. Intracellular fills of over 80 BAC cells with Lucifer Yellow dye allowed us to identify 17 different morphological types with variable axonal projections into multiple buccal nerves. Despite this, with the exception of the BAC type 1 neuron with soma between those of neurons B5 and B19, it is as yet unclear whether a certain morphological type can be reliably located across different snails in the same spot. This makes it difficult to uniquely identify a certain BAC cell of a particular morphological type based on location alone.

Regurgitation and substrate cleaning behaviors have two main differences: the latency of onset and the sustainability of the pattern. While regurgitation needs to be triggered immediately on encountering the aversive stimulus, substrate cleaning has a greater leniency for onset. Prior to substrate cleaning during egg-laying, the snail shows almost an hour long period of low oral and locomotor activity (ter Maat et al., 1989). Once the snail begins substrate cleaning however, it will continue to clean a small area for about 40–60 min (ter Maat et al., 1989). Both behaviors activate a phase 1–2 pattern in the buccal CPG. The BAC cell network is activated in both cases. How does the BAC network activation alter the CPG towards regurgitation in one case and substrate cleaning in the other?

During both Listerine perfusion and intestinal nerve stimulation, we examined the morphological types of the BAC neurons that responded to the treatments. So far, only BAC cells of morphological type 2 show overlapping physiology by responding to both intestinal nerve stimulation and Listerine perfusion. But we cannot assert that different BAC cell types respond to one or the other kind of stimulation. It is possible that there are distinct subpopulations of BAC cells that are activated in one behavior versus the other. It is equally likely that the entire BAC network is activated in both regurgitation and substrate cleaning.

Studies in other systems have revealed that neural circuits trigger different behaviors based on the neuromodulators that activate them (Chambers et al., 2011; Harris-Warrick, 2011; Katz, 1998; Selverston, 2010). It is possible that the BAC neurons could be activated by different sensory inputs to effect the distinct motor outputs – signals from the esophagus evoking regurgitation while internal signals from the reproductive system through the intestinal nerve prompt substrate cleaning. Indeed, immunohistochemical evidence points to two peptides – arginine vasotocin (AVT) along the esophagus and gonadotropin-releasing hormone (GnRH) along the intestinal nerve – that may be involved in these signaling pathways (data not shown) (Young et al., 1999; Richmond et al., 1985).

Thus, it might be that the BAC cells are activated by two different sensory modalities using different peptidergic cues to switch the behavior towards either regurgitation or substrate cleaning (Fig. 14).
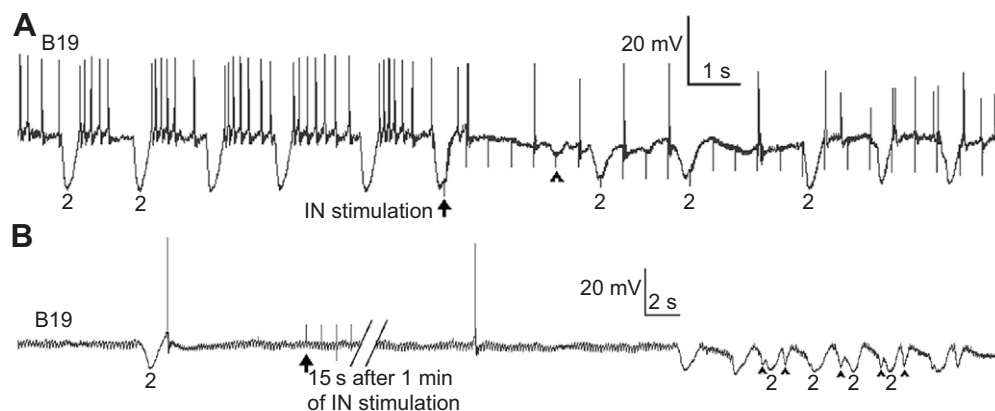

Fig. 10. Intestinal nerve stimulation elicits a response similar to that induced by BAC cell discharge. Recordings were made during fictive feeding (A) and in a quiescent preparation (B). (A) Application of 1 µmol l⁻¹ 5-HT triggers a phase 2–3 motor pattern in B19, characteristic of feeding. On intestinal nerve (IN) stimulation, phase 3 is virtually abolished, characteristic of substrate cleaning. (B) Initially B19 is quiet. On nerve stimulation, a barrage of IPSPs with distinct phase 2 (2) and non phase 2 components (arrowheads) can be observed, characteristic of a substrate cleaning motor pattern. Artifacts of extracellular nerve stimulation are seen as small downward vertical bars along the trace. Arrows indicate first instance of stimulation.
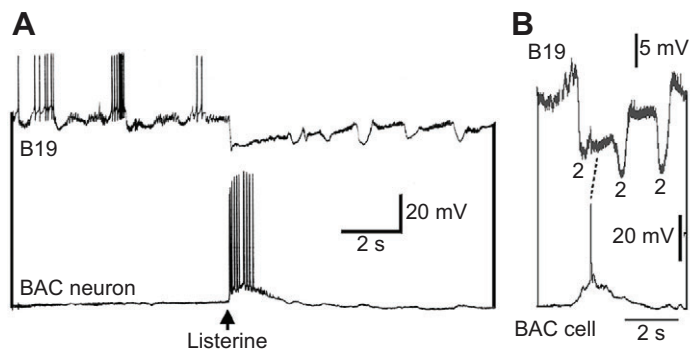
Fig. 11. The effect of Listerine on BAC cells. (A) Listerine triggers a BAC cell barrage which coincides with the large compound IPSP in B19. (B) The BAC cell response to Listerine elicits 1:1 IPSP in cell B19 (dotted line), which is different from regular phase 2 inhibition (2). Cell in A is of morphological type 1 and that in B is type 13.

Emetic stimuli to the esophagus may trigger the cells along the gut to send AVT signals up the esophageal trunk to the BAC neurons altering the motor pattern towards regurgitation. Movement of eggs through the reproductive tract causes stimulation of the intestinal nerve. These signals may be passed on to the central nerve ring through GnRH and activate BAC cells, resulting in substrate cleaning behavior. At this juncture, it is difficult to postulate whether both pathways target the entire group of BAC neurons, activating them as a whole, or whether there is a dedicated subpopulation of BAC cells for each behavior.

Morphological characterization of the BAC neurons shows that they innervate areas of the buccal mass near the oral cavity where they could have access to both chemo- and mechanostimuli. However, BAC neurons never have axons in the VBNs that innervate muscles involved in hyper-retraction of the radula, which are activated during swallowing. Furthermore, many BAC neurons have axonal projections in the ETs and the CBCs, which may serve as pathways to relay sensory information from the esophagus and the cerebral ganglia (Fig. 14). How such sensory information is relayed to the BAC neurons is as yet unknown and is an interesting area for future studies.

Many pattern generators associated with rhythmic outputs are capable of producing more than one behavior under different circumstances (Berkowitz et al., 2010; Latorre et al., 2013). The 'choice' involved in this switch from the pattern underlying one behavior to the other has been under a lot of scrutiny. Some claim that these behavioral choices are hierarchical, where neurons activating one behavior are inhibited by those activating the other (Kovac and Davis, 1977; Kovac and Davis, 1980). Such behavioral choices have been reported in many systems: struggling versus swimming in frog tadpoles (Green and Soffe, 1998; Soffe, 1993); withdrawal versus hunting in *Clione* sea slugs (Norekian and Satterlie, 1996); feeding versus tactile responses in leeches (Misell et al., 1998); and regurgitation versus feeding in *Aplysia* sea slugs (Jing and Weiss, 2001). Some decision neurons and circuits have been found to be active in both behavioral paradigms as seen in the swim/crawl system of the leech (Briggman et. al, 2005; Esch et al., 2002) and *Tritonia* sea slugs (Popescu and Frost, 2002), limb control in turtles (Stein, 2005) and the premotor neurons of the swallowing/air-way protection CPG in vertebrates (Broussard and Altschuler, 2000). Neurons with switching properties have also been identified in the medullary raphae magnus and nucleus reticularis magnocellularis, which respond to noxious colorectal distension, evoking cardiovascular and somatomotor responses in rats (Brink and Mason, 2004). Electrotonic coupling has been used in neural circuits for a variety of reasons: (1) to maintain synchronicity among motor neurons as occurs in swimming or respiration (Anderson and Mackie, 1977; Arshavsky et al., 1985; Li et al., 2009; Nusbaum et al., 1987); (2) to produce an immediate and effective behavioral change, as used in whole body withdrawal in *Lymnaea,* or prey capture in *Clione* and *Navanax* (Ferguson and Benjamin, 1991; Syed and Winlow, 1991; Norekian and Satterlie, 1993; Levitan et al., 1970; Spira et al., 1980); or (3) to create long-lasting changes triggered in an all-or-nothing event such as egg-laying in *Aplysia* or *Lymnaea* (Bodmer et al., 1988; ter Maat et al., 1989).

Indeed there are distinct similarities between the BAC neuron responses and that seen during the bag cell discharge in *Aplysia* (Kupfermann, 1970) and the caudo-dorsal cell discharge in *Lymnaea* (ter Maat et al., 1989). Both the bag cells and CDC neurons are electrotonically coupled and normally quiescent, and can be stimulated to evoke a barrage of action potentials during a discharge.
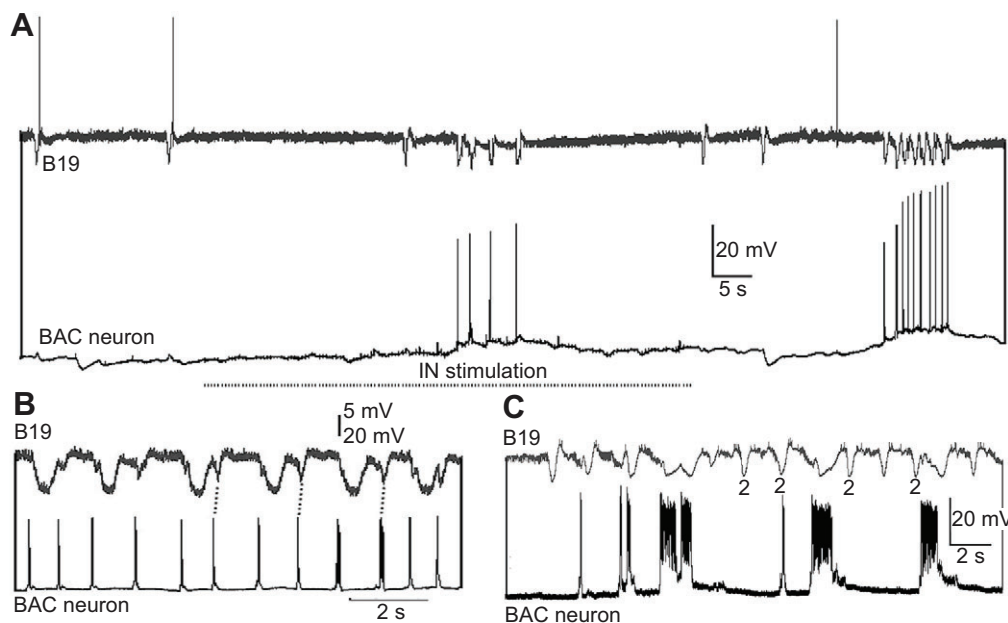

Fig. 12. Effect of intestinal nerve stimulation on BAC cells. (A) Both B19 and the BAC cell are initially quiet. Stimulation of intestinal nerve (indicated by small vertical lines below traces) raised the BAC cell resting potential and triggered a burst of action potentials. These corresponded with the barrage of IPSPs seen in B19. (B) The IPSP barrage has both phase 2 and non-phase-2 components. The latter show 1:1 correspondence with BAC cell spikes (dotted lines). (C) Recording from a different BAC cell during intestinal nerve (IN) stimulation elicits robust bursting in correlation with long compound IPSPs in B19. Cells in A and B are morphological type 4 and the BAC cell in C is type 17.
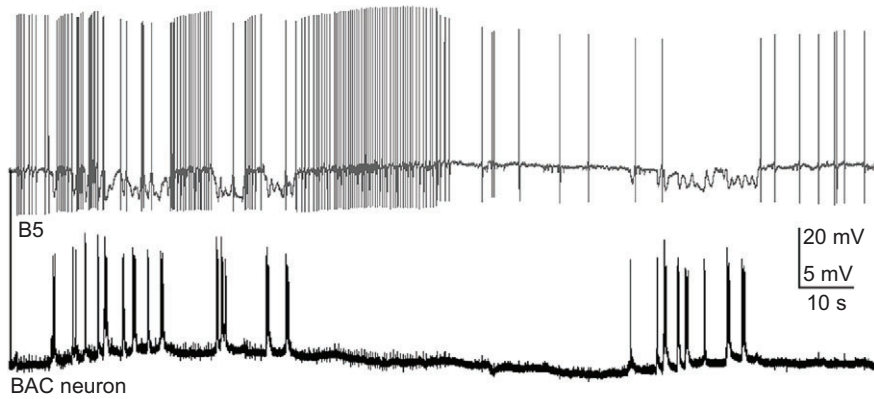
Fig. 13. Intestinal nerve triggers a long-term BAC response. Trace ~15 min after first stimulation. The BAC cell bursts repeat themselves periodically. These coincide with the PSP barrages seen in cell B5.

This is accompanied by a release of a cocktail of peptides that then modulate a cascade of events that lead to egg-laying. It is possible that the BAC cell network is involved in a similar all-or-nothing discharge event, albeit on a much smaller time scale, leading to local dynamic alterations of the buccal motor pattern. Recent findings also point towards a neuropeptide phenylalanine (NPF) as the peptidergic signal from the BAC neuronal system, with effects on the buccal motor pattern (Sato et al., 2010).

Here, we provide evidence to assign a similar function of behavioral switching to the network of BAC neurons. These cells are activated to discharge by two different experimental paradigms underlying two distinct behaviors. We have also shown that such BAC discharges evoke significant changes in the buccal motor pattern, ultimately reflecting a changed motor output. Furthermore, how does activation of the BAC system result in regurgitation on one hand and substrate cleaning on the other? The answer could lie in the motivational state of the animal and the state of the nervous system. Command systems are frequently modulated to target specific motor functions by using specific neurotransmitters and peptides. The ultimate effect of the BAC network in altering the buccal system to one behavior or the other may arise from both where the activating signal is coming from and what these signal peptides are.

MATERIALS AND METHODS
Snails
A laboratory-reared albino variety of *Helisoma trivolvis* Say 1817 pond snails were used in the experiments. On average, adult snails of ~10–12 mm shell diameter were used. All snails were anesthetized using cold saline and de-shelled prior to dissection. The brain proper and the buccal mass were exposed using a midline dorsal incision. The buccal ganglia were exposed

by cutting the esophagus and pulling it forward. All nerves except the esophageal trunks, the cerebro-buccal connectives and the intestinal nerve were cut to isolate the nervous system. The intestinal nerve, which innervates the reproductive tract, was gently removed of connective tissue and pinned down using a small piece of the reproductive tract.

Solutions
Standard physiological *Helisoma* saline was used. Specifically, *Helisoma* saline (pH 7.3) was composed of (in mmol l^{-1}) 51.3 NaCl, 1.7 KCl, 1.5 MgCl$_2$, 4.1 CaCl$_2$ and 5.0 Hepes buffer.
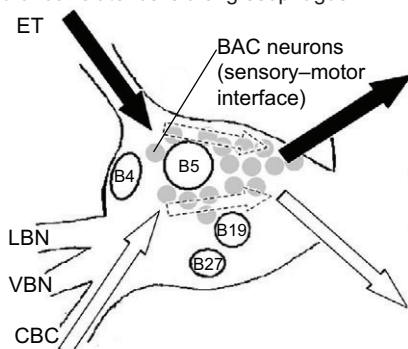
Perfusion of the esophagus
A semi-intact preparation of just the nervous system with the esophagus attached was used for perfusion experiments. Polyethylene plastic tubing (Intramedic PE-160, Gentofte, Denmark) was pulled to a fine diameter over an open flame to form a cannula. This was then inserted into the cut end of the esophagus and secured in place using silk thread. The cannula was attached to a t-valve whose inlets were connected to tuberculin syringes (Fisher Scientific, Pittsburgh, PA, USA) – one containing regular *Helisoma* saline and the other 20% Listerine. Injections were monitored under a Wild dissection microscope (Wild Heerbrug AG, Germany) and care was taken to not distend the esophagus.

Intracellular recordings and staining
Isolated brain preparations were pinned down in a Sylgard dish recording chamber (Fisher Scientific). Standard electrophysiological techniques were used. Glass microelectrodes made using the KOPF vertical microelectrode puller (KOPF Instruments, Tujunga, CA, USA) were used for intracellular recordings. Electrodes were filled with either potassium acetate (20–40 MΩ) or 3% Lucifer Yellow CH (Sigma, St Louis, MO, USA) (120–200 MΩ). Signals were amplified using AM systems Neuroprobe Amplifier (Model 1600; AMSystems, Sequim, WA, USA) and recorded using PowerLab/8SP (AD Instruments, Colorado Springs, CO, USA) or the Gould 2-channel chart
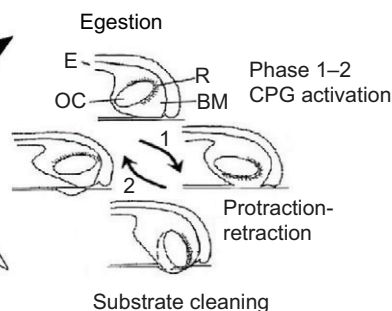

Fig. 14. Model for BAC cells as CPG switches. Inputs from the reproductive tract through the intestinal nerve and those from the gut through the esophageal nerve both activate the BAC cells. The former triggers substrate cleaning, while the latter triggers regurgitation. Both of these protraction/retraction rhythms activate a phase 1–2 buccal motor pattern.

recorder (US Instruments Services, Southlake, TX, USA). Where morphology of the cell was to be determined, Lucifer Yellow dye was injected into the cell using hyperpolarizing pulses (Murphy et al., 1983). These preparations were then fixed overnight in Zamboni's fixative (7.5% picric acid, 4% paraformaldehyde in PBS), followed by alcohol dehydration and cleared on a slide using methyl salicylate. Mounted preparations were viewed under a Zeiss microscope (Jena, Germany) under a filter set designed for Lucifer yellow dye observation.

Extracellular nerve stimulation

Extracellular thin wire electrodes made out of 0.0008 in Teflon-coated copper wires or 0.0007 in Teflon-coated silver wires (Fisher Scientific) were used for stimulation. These were connected to a Grass S88 stimulator through a Grass SIU5 stimulus isolation unit (Grass Technologies, Warwick , RI, USA). Stimulation paradigm was adapted from that of Hermann et al. (Hermann et al., 1997). The intestinal nerve was placed between the ends of the electrode within the recording dish, and current pulses of 0.2 ms period, 0.6 ms duration and amplitude of 5 µA were delivered for stimulation.

Figures

All figures were made using Adobe Photoshop (versions 5.5 and 7). Photographs taken of stained cells were scanned as film positives using a Canon scanner (Tokyo, Japan). The color contrast of these pictures was then adjusted using Adobe Photoshop.

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