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RESEARCH ARTICLE

Peptidergic modulation of a multi-functional central pattern generator in the pulmonate snail

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ABSTRACT

Egg laying in pulmonate snails is a well-orchestrated process that involves a period of reduced locomotion, followed by substrate cleaning with rhythmic rasping of the surface to make tiny grooves, into which eggs are deposited. Although the neurohormonal control of initiating egg laying has been well established, the signals that modulate the buccal central pattern generator to substrate cleaning during egg laying are not known. Neuropeptides of the invertebrate gonadotropin-releasing hormone/corazonin family (invGnRH/CRZ) have been shown to be involved in reproduction and allied behaviors in many vertebrates and invertebrates. Here, we show that the buccal motor pattern underlying substrate cleaning during egg laying is altered by a vertebrate GnRH agonist. Signals from the intestinal nerve innervating reproductive structures, previously shown to be both necessary and sufficient for egg-laying behaviors, are blocked by a vertebrate GnRH antagonist. Further, the vertebrate GnRH-triggered response elicits rhythmic, phase 2 and non-phase 2 activity in the buccal motor pattern, with a shutdown of phase 3, indicative of repetitive rasping without accompanied swallowing behavior. Using immunohistochemistry, intracellular electrophysiology and extracellular nerve stimulation, we show that a member of the invGnRH/CRZ family of neuropeptides could be the signal that contextually switches the multifunctional buccal CPG to a biphasic rasping rhythm that underlies substrate cleaning behavior during egg laying in the pulmonate snail *Planorbella* (*Helisoma*) *trivolvis*.

KEY WORDS: InvGnRH/CRZ, CPG, Egg laying, Electrophysiology, *Planorbella*

INTRODUCTION

Snails display a variety of oral behaviors such as feeding, swallowing, egestion and substrate cleaning during egg laying that are controlled by the central pattern generator (CPG) in their buccal ganglia (Murphy, 2001; Benjamin, 2012; Benjamin and Crossley, 2020). The buccal CPG, composed of oscillators, interneurons and motor neurons, controls the muscles of the buccal mass and the radular odontophore and has three phases: (1) protraction, (2) retraction (rasp) and (3) hyper-retraction (swallow). The triphasic pattern results in feeding behavior, and can be modulated depending on the nature of the food (Murphy, 2001; Benjamin, 2012; Benjamin and Crossley, 2020). The buccal CPG is multifunctional and can be modulated to trigger behaviors other than feeding, such as swallowing or regurgitation, depending on varying sensory inputs (Murphy, 2001; Kemenes and Benjamin, 2009; Benjamin and Crossley, 2020). The electrotonically coupled buccal A cluster (BAC) cells have been shown to use sensory information to switch from one kind of motor pattern to another depending on context; for example, when presented with watermelon juice, the snail responds by eating with a 1–2–3 triphasic buccal motor pattern, but when presented with Listerine®, it regurgitates using a 1–2 biphasic pattern (Murphy, 2001; Ramakrishnan et al., 2014).

Egg laying in pulmonate snails is a programmed behavior that is all-or-nothing triggered by a neurohormonal cascade from the caudodorsal cells in response to appropriate environmental stimuli (de Vlieger et al., 1980; Ter Maat, 1992; Hermann et al., 1994; Jansen et al., 1997). Egg-laying behavior consists of four phases, three of which – resting, turning and oviposition – each last over an hour (Koene, 2010). The turning phase is also accompanied by repetitive rasping of the surface, termed as substrate cleaning, which lasts approximately 40–60 min (Ter Maat et al., 1989). This is essential, as without this behavior, the eggs are improperly attached to the surface and have a greater probability of predation and mortality (Ter Maat, 1992). It has been shown that the primary function of the rasping behavior accompanying egg laying is to clean the substrate and not to feed, and posited that it only involves a biphasic 1–2 buccal motor pattern, with the absence of the hyper-retraction or swallow (Ter Maat et al., 1989; Ter Maat, 1992; Hermann et al., 1994). Thus, phase 3 of the buccal CPG is inactivated, and phase 2 is activated. The signal molecules involved in the modification of the buccal CPG underlying this behavior are as yet unknown.

The neuroendocrine control of egg laying in pulmonate snails has been extensively studied over the years (de Vlieger et al., 1980; Ter Maat, 1992; Jiménez et al., 2004). It is well established that a discharge from the caudodorsal cells triggers an all-or-nothing response that leads snails into a series of rhythmic behaviors including substrate cleaning behavior in preparation for egg laying, culminating in oviposition and egg deposition (Ter Maat, 1992; Ferguson et al., 1993). The intestinal nerve (IN) that extends from the central visceral ganglion of the snail along the vagina and the egg tract has been shown to be both necessary and sufficient for triggering substrate cleaning during egg laying (Ferguson et al., 1993; Hermann et al., 1994). Further, stimulation of the IN has been shown to trigger a biphasic 1–2 buccal CPG activation, suggesting that this underlies the repetitive rasping accompanying egg laying (Ramakrishnan et al., 2014). IN stimulation has also been shown to alter the BAC neurons (Ramakrishnan et al., 2014). However, the signal molecules that mediate the IN-triggered buccal CPG modulation towards substrate cleaning are not yet known.

Members of the invertebrate gonadotropin-releasing hormone/corazonin family of neuropeptides (invGnRH/CRZ) have been implicated in reproduction and related behaviors in vertebrates and

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many invertebrates (Sakai et al., 2020). InvGnRH/CRZ-like peptides have been identified in molluscs and have been shown to have both overlapping and distinct functions (Hauser and Grimmelikhuijzen, 2014; Tsai, 2018; Zandawala et al., 2018; Sakai et al., 2020). GnRH-like peptides have been located in molluscs, including pulmonate snails (Tsai, 2006), and have been shown to suppress locomotion and trigger egg-laying behavior upon injection into the great pond snail (*Lymnaea stagnalis*) (Fodor et al., 2021). As the nomenclature and function of invGnRH/CRZ in molluscan systems is still under flux and there are as yet no sequence data from *Planorbella trivolvis*, we will refer to the peptide to be investigated as part of the invGnRH/CRZ family. To date there is no information on the effects of GnRH/CRZ-related peptides on the buccal motor pattern, and its significance in the neural control of egg-laying.

In this study using the pulmonate snail *Planorbella* (*Helisoma*) *trivolvis*, we performed immunohistochemistry (IHC) with an antiserum raised against the invGnRH/CRZ peptide of the common octopus, *Octopus vulgaris* (oct-GnRH/CRZ). Moreover, we examined the buccal motor pattern using electrophysiological recordings of monitor motor neurons in the buccal ganglia in the presence of vertebrate GnRH agonists and antagonists to determine effects on neural activity. We show the presence of invGnRH/CRZ-like immunoreactivity (invGnRH/CRZ-li) in the pulmonate system, suggesting that *Planorbella* (*Helisoma*) invGnRH/CRZ (pla-GnRH/CRZ) peptide is present in *P. trivolvis*. Moreover, based on our preliminary electrophysiological experiments, we suggest that pla-GnRH/CRZ acts as a neuromodulatory regulator from the intestinal nerve altering the buccal CPG during egg-laying behavior.

MATERIALS AND METHODS

Snails

Laboratory-reared *Planorbella* (*Helisoma*) *trivolvis* (Say 1817) (albino variety) were used in the experiments. On average, adult snails of ∼10–12 mm shell diameter were used for electrophysiology experiments and 4–6 mm diameter for IHC. 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 IN, 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 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer.

Intracellular recordings and staining

Isolated brain preparations were pinned down in a Sylgard dish recording chamber. Standard electrophysiological techniques were used. Glass microelectrodes made using the KOPF vertical microelectrode puller were used for intracellular recordings. Electrodes were filled with either potassium acetate (20–40 MΩ) or 3% Lucifer Yellow CH (Sigma-Aldrich) (120–200 MΩ). Signals were amplified using an AM Systems Neuroprobe Amplifier (model 1600) and recorded using PowerLab/8SP (AD Instruments) or the Gould 2-channel chart recorder. Where morphology of the cell was to be determined, Lucifer Yellow dye was injected into the cell using hyperpolarizing pulses. 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 under a filter set designed for Lucifer Yellow dye observation.

For electrophysiological recordings in the presence of the agonist or antagonist, there was an initial 10 min pre-application recording followed by 15–20 min of recording in the presence of the agonist or antagonist. In the case of IN stimulation (as described in Ramakrishnan et al., 2014), a 10 min pre-stimulation recording was followed by a 20 min post-stimulation recording. Applied chemicals were washed off with regular saline 3–4 times, and post-wash effects were recorded for 10 min. Pre- and post-stimulation recordings were compared in the same preparation.

Agonists and antagonists

Given that the coding region of the Pla-GnRH/CRZ prepropeptide has not yet been sequenced, we used a commercially available mammalian GnRH peptide and its antagonist in our experiments. The mammalian GnRH peptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2), heretofore referred as vertebrate GnRH agonist, was obtained from Bachem (prev. Peninsula Laboratories, Inc.). It was reconstituted in water and stored at −80°C. Final dilutions to 50 µmol l^{-1} were made in regular saline. The GnRH antagonist, heretofore referred as vertebrate GnRH antagonist [sequence: Ac-D-2-Nal-p-chloro-D-Phe-β-(3-pyridyl)-D-Ala-Gly-Arg-Pro-D-Ala-NH_2 trifluoroacetate salt], was procured from Bachem. It was reconstituted in water and stored at −80°C. Final dilutions to 100 µmol l^{-1} were made in regular saline for bath application.

Immunohistochemistry

Solutions

Zamboni's fixative was prepared by combining 4 g paraformaldehyde in 70 ml H_2O, 0.4 g $Na_2HPO_4{\cdot}H_2O$ and 0.65 g Na_2HPO_4 in 20 ml H_2O with 7.5 ml super saturated picric acid and made up to final volume of 100 ml with H_2O after filtration. Stock antibody solutions were diluted to a maximum of 1:10 and stored frozen at −80°C. The diluting fluid consisted of 0.1 g bovine serum albumin and 0.01 g sodium azide (an anti-bacterial agent), dissolved in 10 ml of 0.187 mol l^{-1} phosphate buffered saline (PBS, pH 7.2). One liter of 0.187 mol l^{-1} PBS contained 8 g of NaCl and 7.1 g of Na_2HPO_4. Primary antibody was diluted in a Triton/azide/PBS solution (TAPS) that contained 0.3% Triton X-100 and 0.02% sodium azide in PBS. Goat serum was added to the initial dilution to prevent non-specific binding of the secondary antibody. All primary antibodies were used at a 1:200 final dilution in TAPS. Secondary antibodies were diluted in PBS to a final concentration of 1:200. Glycerine mounting fluid was prepared by adding 0.2 g N-propyl gallate to 5 ml of distilled water and 15 ml of glycerol.

The rabbit anti-octopus GnRH/CRZ antisera (anti-oct-GnRH/CRZ) was kindly given to us by Dr Hiroyuki Minakata (Suntory Institute of Bioorganic Research, Osaka, Japan) (Iwakoshi-Ukena et al., 2004). All other chemicals and secondary antibodies were obtained from Sigma-Aldrich.

Procedure

Adult snails with maximum shell diameter between 6 and 10 mm were used for all immunostaining experiments. Isolated brain preparations of the buccal ganglia, central nerve ring, along with a piece of the intestinal nerve were pinned in a Sylgard dish. The

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cerebral–pedal and cerebral–pleural commissures were cut on one side of the central nerve ring. The pedal, pleural, parietal and visceral ganglia were then pulled from under the cerebrals and laid out in an adjacent position. This placed the cerebrals dorsal side up and the pedals ventral side up or vice versa. In some cases just the pedal commissure was cut and the pedals were laid out ventral side up on either side of the cerebrals.

The brains were washed in PBS, before applying 0.1% protease on the preparations. Depending on the duration of protease treatment (between 8 and 12 min), differential staining was obtained in either the axons or the cell bodies. After rinsing the protease with PBS, the preparations were fixed overnight in Zamboni's fixative and stored at 4°C. The following day, preparations were rinsed with PBS at 1 h intervals over an 8 to 10 h period. They were then carefully dried and incubated with the primary antibody for 48 h at room temperature. After repeated rinses with PBS, the brains were immersed in the secondary antibody conjugated with rhodamine (at 1:200 dilution). After an overnight incubation with the secondary antibody at room temperature, the preparations were once again rinsed with PBS. Finally, the brains were mounted on a microscope slide with glycerin and viewed under a Zeiss scope with the Rhodamine filter.

Two kinds of control staining procedures were used: (i) eliminating the primary antibody and (ii) pre-absorption of the primary antibody (anti-oct-GnRH/CRZ) with 0.2 mmol l^{-1} of the vertebrate GnRH agonist that was used in the electrophysiological experiments (n=5 each). All other steps remained identical to those of regular staining.

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. The color-contrast of these pictures was then adjusted on Adobe Photoshop.

RESULTS

InvGnRH/CRZ-like immunoreactivity is present in the nervous system of *P. trivolvis*

To identify whether pla-GnRH/CRZ-like peptide is present along the reproductive–buccal axis in *P. trivolis*, we performed IHC using anti-oct-GnRH/CRZ antisera. InvGnRH/CRZ-like immunoreactivity (invGnRH/CRZ-li) was found in the main regions of the nervous system (n=9) associated with the reproductive–cerebral–buccal axis (Fig. 1) including the intestinal nerve (Fig. 1A), the cerebro-buccal connectives (Fig. 11D), neurons in the cerebral and pedal ganglia (Fig. 1B,C), as well as the buccal ganglion (Fig. 1E). The locations of invGnRH/CRZ-li cell bodies and fibers are indicated in the schematic in Fig. 1H. Staining of neurons in the paired ganglia were usually bilaterally symmetrical in any given preparation. InvGnRH/CRZ-li fibers were found using both antisera in the esophageal trunks (ET), cerebro-buccal connectives, the buccal and cerebral commissures and the intestinal nerve (Fig. 1H). InvGnRH/CRZ-li cells in the buccal ganglia were consistently observed: (i) on the anterior edge of the ganglion near the ET; (ii) slightly lateral to and underneath (rostral) the posterior shoulder of cell B5; and (iii) in clusters medial to cell B5 and towards the posterior edge of the ganglion. A fairly large cell (~40–60 µm) was observed in the cerebral ganglia near the cerebral–pedal commissure (Fig. 1C). Staining of cells or fibers of the nervous system was absent in the control experiments (n=5; Fig. 1F,G). Thus, a pla-GnRH/CRZ-like peptide is located in the snail central nervous system and is associated with the structures connecting the reproductive structures with the central nerve ring and the buccal ganglia.

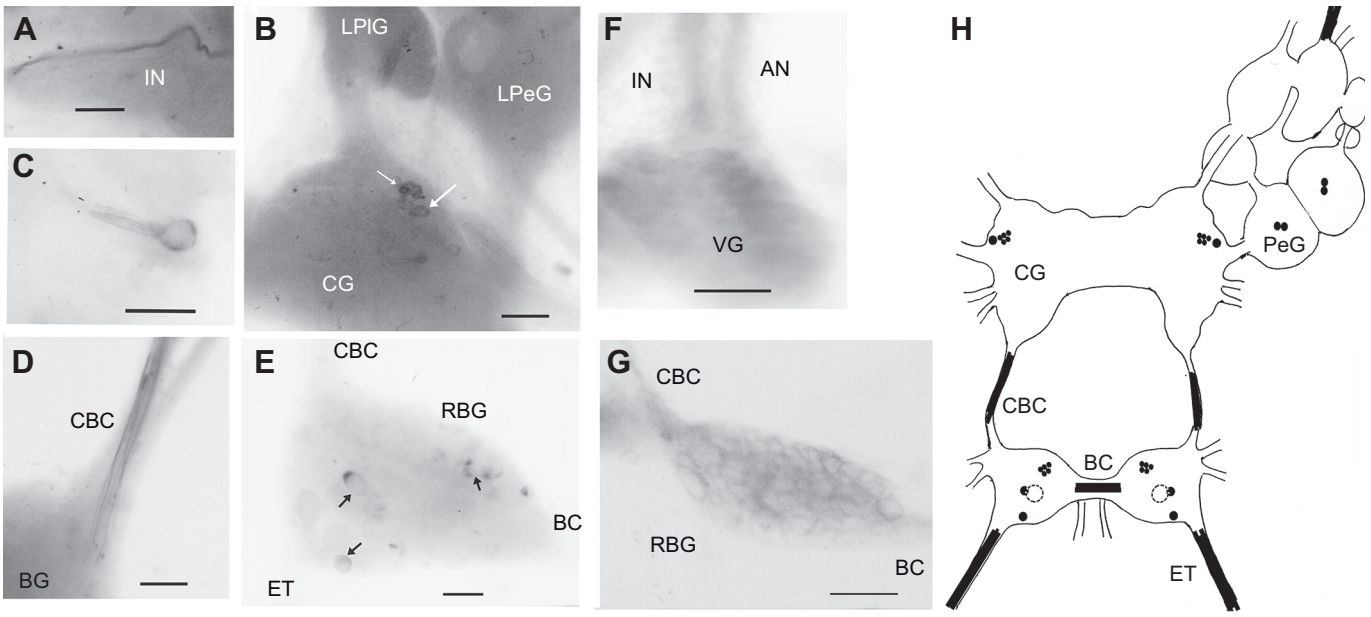

Fig. 1. InvGnRH/CRZ-like immunostaining in *Planorbella trivolvis* with schematic indicating presence of cell bodies and fibers. (A) Intestinal nerve of *P. trivolvis* with invGnRH/CRZ-like immunopositive fibers. (B) Cerebral ganglia with cell bodies showing invGnRH/CRZ-li staining (arrows). (C) Magnified version (25×) of invGnRH/CRZ-positive cerebral cell showing soma and proximal axon. (D) InvGnRH/CRZ-li fibers in the cerebro-buccal connective (CBC). (E) Buccal cells showing invGnRH/CRZ-li immunoreactivity (arrows). (F,G) Control staining after eliminating the primary antibody. (H) Schematic of snail brain with invGnRH/CRZ-li neurons (circles) and processes (lines), indicating immunoreactivity for invGnRH/CRZ using anti-oct-GnRH/CRZ antibodies (n=9). L, left; R, right; AN, anal nerve; IN, intestinal nerve; BG, buccal ganglion; BC, buccal commissure; CG, cerebral ganglion; CC, cerebral commissure; ET, esophageal trunk; PBN, posterior buccal nerve; PeG, pedal ganglion; PIG, pleural ganglion; VG, visceral ganglion. Scale bars: 100 µm.

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Vertebrate GnRH agonist alters the buccal motor pattern, and effects a BAC response

To determine whether vertebrate GnRH-agonist application has effects on the buccal motor pattern, 50 µmol l^{-1} vertebrate GnRH agonist was bath applied on isolated brains, where motor neurons such as B19 were used to monitor the phase of the buccal CPG. Fig. 2 shows a recording from cells B5 and B19, approximately 10 min before (Fig. 2A) and 10 min after (Fig. 2B) the bath application of vertebrate GnRH agonist on the isolated brain. The vertebrate GnRH agonist triggered a long-term rhythmic pattern in the buccal neurons (86%, *n*=22). Prior to agonist application, phase 3 bursts followed by phase 2 inhibitory postsynaptic potentials (IPSPs) can be routinely seen in cell B19 (Fig. 2Ai,ii). After vertebrate GnRH-agonist application, the bursting in B19 is shut down. Barrages of IPSPs can be seen in both cells B5 and B19 (Fig. 2B). On average, barrages were triggered within 5–6 min after the application of vertebrate GnRH agonist. Further, non-phasic synaptic inputs, not seen during feeding buccal rhythms, appear within a minute of vertebrate GnRH-agonist application.

Previously, the non-phasic inputs seen in the buccal CPG were shown to be activated by BAC neurons in the buccal ganglia (Ramakrishnan et al., 2014). To determine whether the non-phase 2 IPSPs seen in the buccal motor pattern were BAC-triggered, we recorded simultaneously BAC neurons in conjunction with buccal CPG monitors. Bath application of vertebrate GnRH agonist triggered a BAC cell discharge, which induced a change in the buccal pattern (*n*=5). Fig. 3 shows a 1–1 correspondence in non-phase 2 IPSPs seen in B19 with BAC action potentials. Bursts of action potentials in the BAC cell correspond with the lengthy non-phase 2 IPSPs in cell B19. Apart from these, smaller non-phase 2 IPSPs in the B19 can be correlated one-for-one with BAC cell action potentials.

Vertebrate GnRH-agonist-triggered buccal response resembles that of IN stimulation

To determine whether vertebrate GnRH-agonist-triggered buccal activation resembled that induced by IN stimulation (Ramakrishnan et al., 2014), we compared long-term buccal activation upon both agonist application and IN stimulation (Fig. 4). Vertebrate GnRH-agonist application elicited similar long-term cyclical activity in B5 and B19 (Fig. 4A), including suppression of phase 3 activity, and induction of repetitive IPSP barrages that involved non-phase2 BAC responses (Fig. 4B). The time course of these repetitive activity was also similar, with barrages repeating on average every 70 s.

Vertebrate GnRH antagonist blocks the IN response

To determine whether the buccal CPG activation by stimulation of the IN was indeed mediated by GnRH/CRZ, buccal neurons (B27, B91, B5) were recorded during IN stimulation in the presence of a GnRH antagonist. Bath application of vertebrate GnRH antagonist blocked the alteration of the buccal CPG that is usually triggered by IN stimulation (*n*=4; Fig. 5B). There was no inducement of IPSP barrages or non-phase 2 IPSPs in the buccal motor pattern.

This activation by the IN was restored upon washing off the antagonist and replacing with regular saline (Fig. 5C). Further, concomitant application of the antagonist with the vertebrate GnRH agonist blocked the agonistic effect on the buccal motor pattern (*n*=6). Barrages of PSPs consistently seen upon agonist application were not observed in the presence of the antagonist. Further, agonist application in the presence of the vertebrate GnRH antagonist also did not induce any BAC cell discharges or non-phase 2 IPSPs in the buccal pattern (Fig. 6).

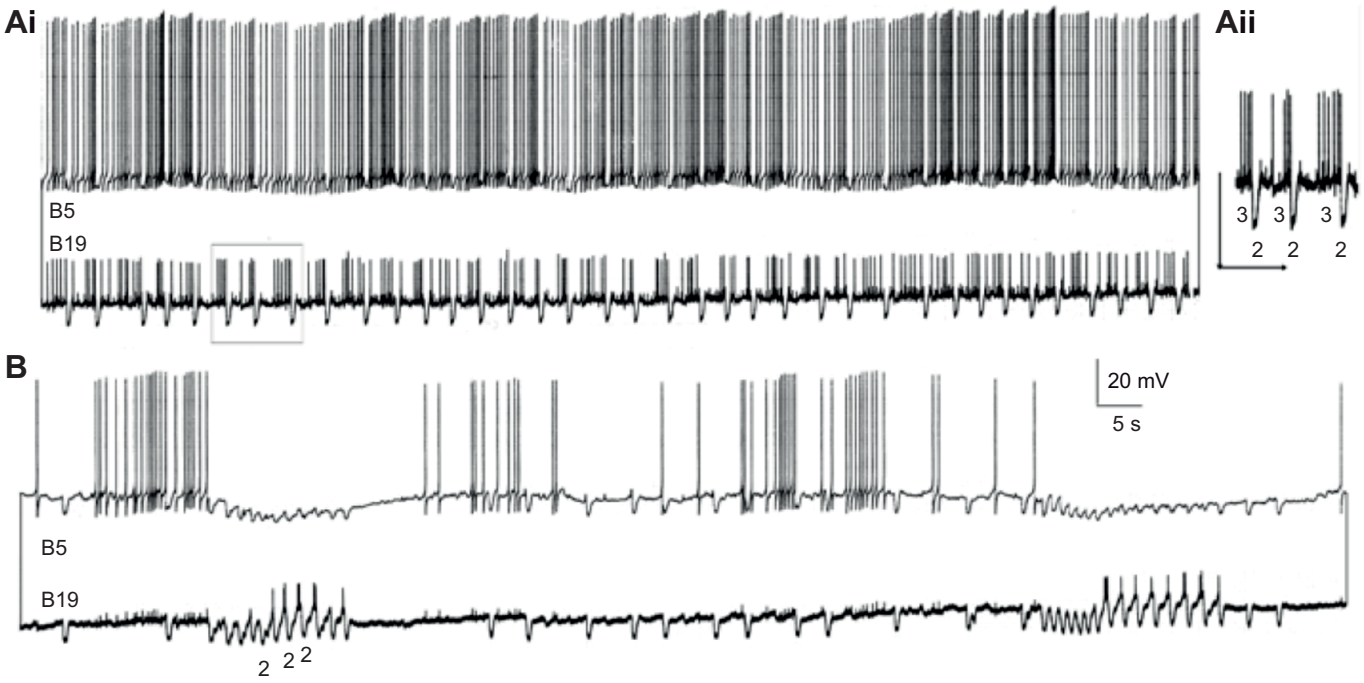

Fig. 2. Effect of bath application of 50 µmol l^{-1} vertebrate GnRH agonist on the buccal motor pattern of *P. trivolvis*. (Ai) Recording from cells B19 and B5, ∼10 min before the application of vertebrate GnRH. B5 shows tonic spiking, whereas B19 exhibits a rhythmic 3–2 activity, indicated by the phase 3 bursts, followed by the phase 2 inhibitory postsynaptic potentials (IPSPs) (*n*=22). (Aii) Enlarged version of boxed area in Ai showing phases 2 and 3. (B) Traces 10 min after application of vertebrate GnRH agonist in the same preparation. B19 bursting is abolished. Rhythmic barrages of IPSPs can be seen in both B19 and B5. Tonic firing in B5 has been reduced.

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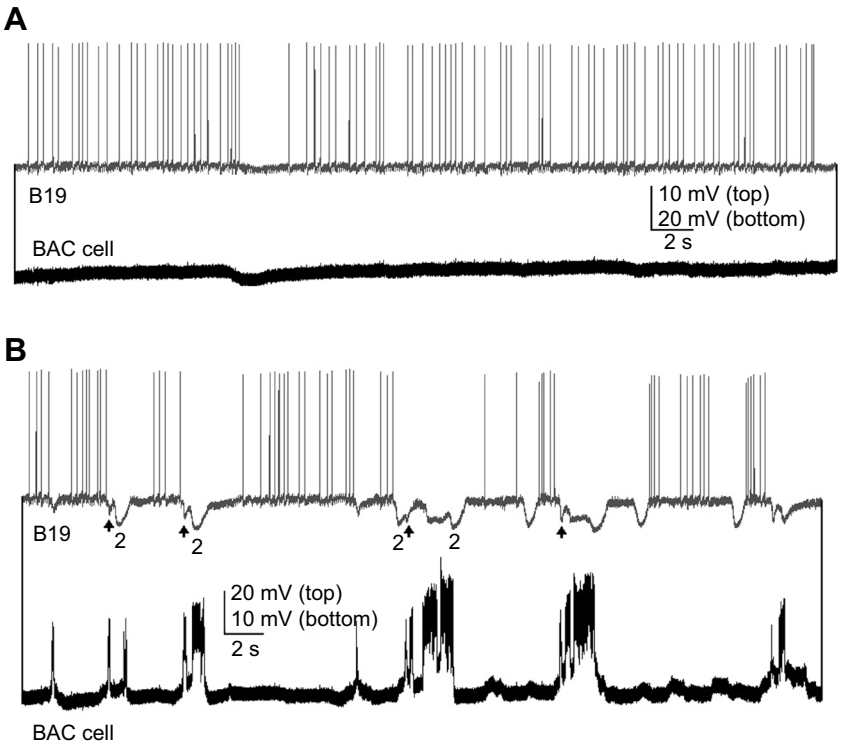

Fig. 3. Vertebrate GnRH application activates buccal A cluster (BAC) cells. (A) The BAC cell is typically quiet and B19 shows some spike activity. (B) On vertebrate GnRH application, a BAC discharge is triggered (~8 min after application). Bursts in the BAC cell correspond with the lengthy IPSP in B19. Other PSPs show one-for-one correlation with BAC cell action potentials (arrows). These are distinct from regular phase 2 IPSPs (labelled '2') in B19 (n=5).

Contextual switch: vertebrate GnRH antagonist does not block AVT-triggered buccal CPG modulation

Listerine® perfusion in the esophagus elicits regurgitation behavior in snails and modulates the buccal motor pattern to a biphasic 1–2 rhythm (Ramakrishnan et al., 2014). Arginine vasotocin (AVT), identified along the esophagus, could potentially be the peptide that modulates emetic responses in snails (Richmond et al., 1987). To determine whether the vertebrate GnRH is acting on BAC cells via an AVT pathway, we recorded from buccal neurons and applied 20 µmol l^{-1} AVT in conjunction with the vertebrate GnRH antagonist. The vertebrate GnRH antagonist was not able to block the AVT-triggered BAC discharge and alterations in the buccal

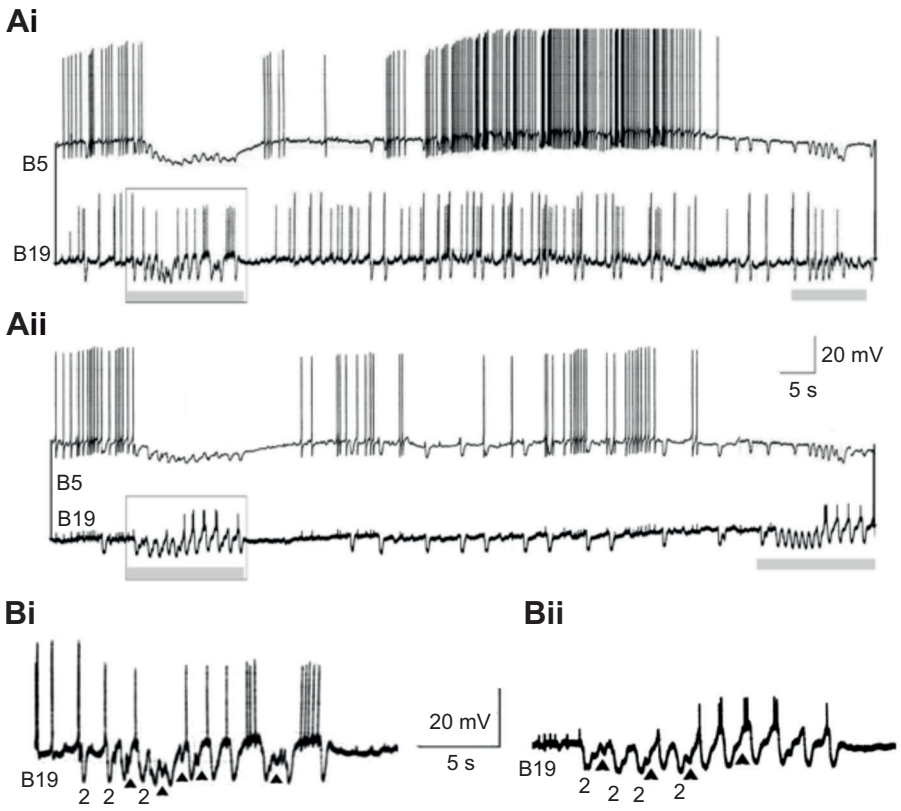

Fig. 4. Comparison of buccal motor neuron physiology between intestinal nerve (IN) stimulation and vertebrate GnRH agonist application. (A) Physiology from B5 and B19 in *P. trivolvis* on (i) IN stimulation and (ii) vertebrate GnRH application. Repetitive barrages of IPSPs can be seen in both B19 and B5 under both stimulation paradigms (gray bars). In both cases bursting in B19 is absent after the experimental paradigm. (B) Expanded view of respective boxed areas in Ai and Aii. Under IN stimulation (i) and agonist application (ii) the PSP barrages contain phase 2 components along with non-phase 2 inputs (arrowheads).

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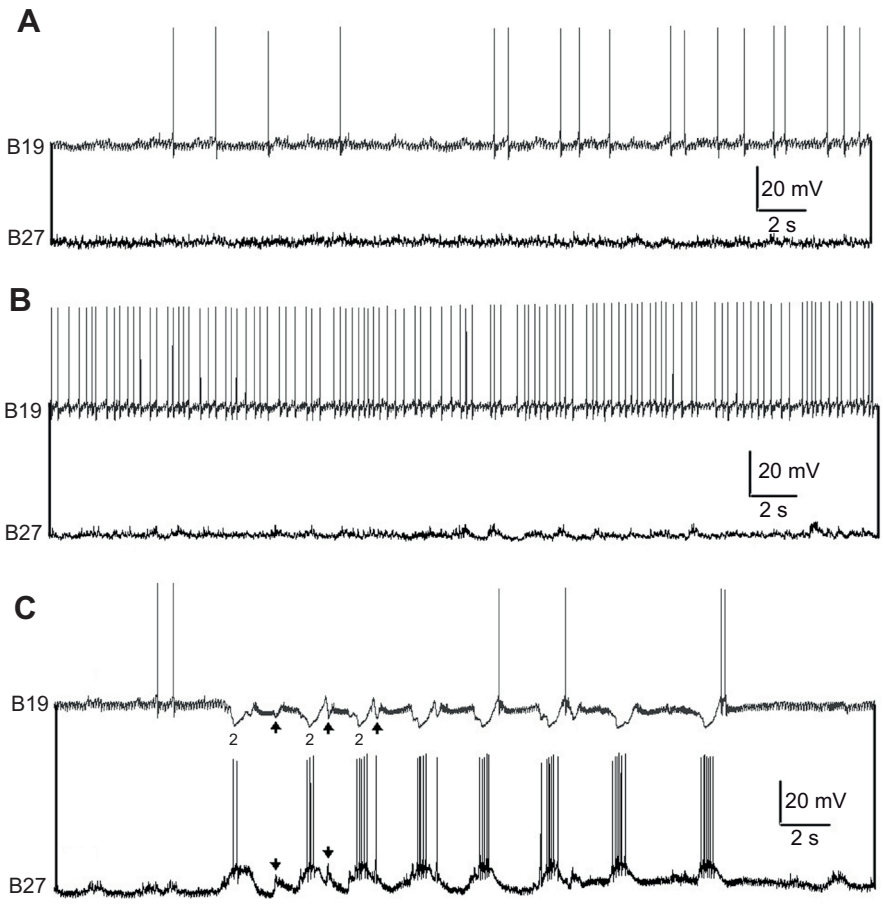

Fig. 5. Vertebrate GnRH antagonist blocks IN stimulation effects on buccal motor pattern. (A) Physiology in cells B19 and B27, 8 min after antagonist application. B27 is quiet, showing lack of phase 2 excitation. B19 shows little activity too. (B) Trace ∼15 min after IN stimulation in the presence of the antagonist. B19 shows increased firing but no IPSPs, but B27 is still silent. (C) Recording ∼10 min after first wash of antagonist with normal saline and 5 min following nerve stimulation after wash. B19 shows phase 2 IPSPs that correspond with bursts in B27. Non-phase 2 synaptic inputs can also be seen in both cells (a few labeled by arrows). *N*=4.

motor pattern (*n*=5; Fig. 7). Thus, although AVT activates a BAC response and switches the buccal motor pattern, it is not blocked by the vertebrate GnRH antagonist.

DISCUSSION

Presence of invGnRH/CRZ in the pulmonate nervous system

InvGnRH/CRZ-immunopositive neurons and fibers have been located in molluscs (*Aplysia*, *Lymnaea*, octopus, scallops, shrimp, abalone) including pulmonates (Iwakoshi et al., 2002; Iwakoshi-Ukena et al., 2004; Tsai, 2006; Ngernsoungnern et al., 2008; Johnson et al., 2014; Jung et al., 2014; Nuurai et al., 2014; Nagasawa et al., 2015; Fodor et al., 2020b). Using two different kinds of mammalian GnRH antibodies, GnRH-ir neurons and processes were previously identified in *Helisoma trivolvis* in the circumesophageal ganglia, in the buccal ganglia, cerebro-buccal connectives, the intestinal nerve and various reproductive structures (Young et al., 1999). Recently, a GnRH/CRZ peptide was identified in the closely related *Biomphalaria glabrata* (Rosa-Casillas et al., 2021), and IHC using antibodies against this peptide identified both cell bodies and processes in the circumesophageal ganglia, and just processes but no cell bodies in the buccal ganglia. Although the authors mention the location of cell bodies and processes in the ovotestis, oviduct and albumen gland, no specific mention is made of the intestinal nerve, though figures do indicate immunoreactivity in the nerve from the

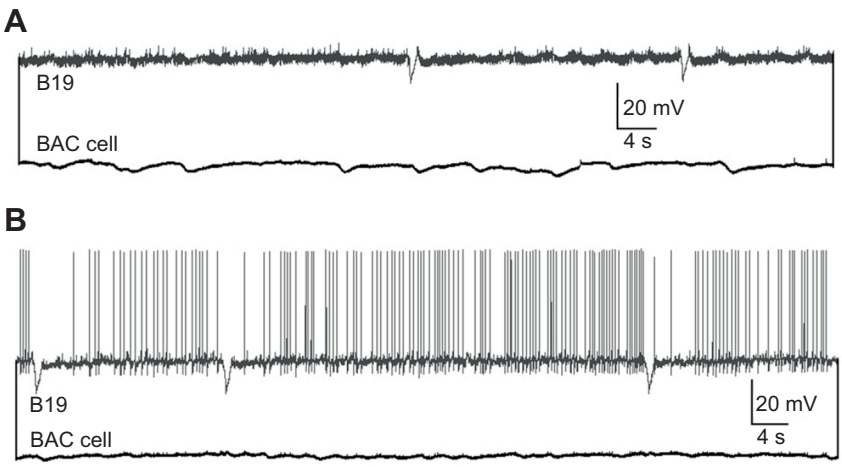

Fig. 6. Effects of vertebrate GnRH application were blocked by the vertebrate antagonist, including the induction of BAC-cell-induced IPSP barrages. (A) Recording in just the presence of the vertebrate GnRH-antagonist. (B) 5 min after concomitant application of agonist with the vertebrate antagonist.

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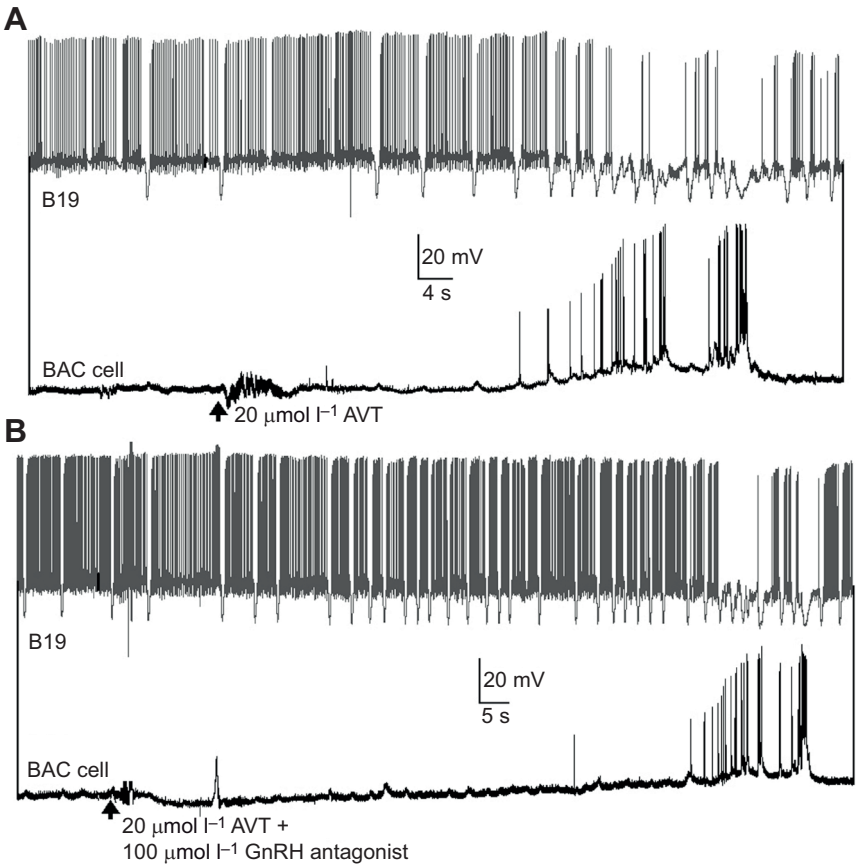

Fig. 7. Vertebrate GnRH antagonist does not block arginine vasotocin (AVT) activation of BAC cells. (A) A BAC cell discharge is triggered on application of 20 μmol l^{-1} AVT. This corresponds with the shutdown of phase 3 bursts in B19 along with incidence of phase 2 IPSPs and non-phase 2 synaptic inputs. (B) The vertebrate GnRH antagonist does not block the AVT-triggered effect (n=5). An application of 20 μmol l^{-1} AVT in conjunction with 100 μmol l^{-1} vertebrate GnRH antagonist was still able to elicit the same response seen in A. Arrows indicate beginning of solution change.

visceral ganglion, which could be the IN. However, ir-staining of buccal neurons B3/B4 in *Lymnaea*, another closely related pulmonate, was seen in a different study using a specific invGnRH/CRZ antiserum (Fodor et al., 2020b). We also observed GnRH/CRZ-li staining in *Lymnaea*, in the buccals, cerebrals and the cerebro-buccal connectives (data not shown). Here, we used an oct-GnRH/CRZ antibody to show the presence of invGnRH/CRZ-like immunoreactive cell bodies in the buccal ganglia of *P. trivolvis*, fibers in the cerebro-buccal connectives and the IN (which innervates reproductive structures) and cell bodies in the circumesophageal ganglia, including the cerebral and pedal ganglia. Differential staining of GnRH/CRZ neurons and processes using different antibodies is not uncommon, as multiple forms of GnRH and CRZ are expressed in many species, and these are located in distinct locations in the nervous system (Tsai et al., 2003; Pandolfi et al., 2005; Soga et al., 2005; Whitlock, 2005; Ogawa and Parhar, 2020; Ogawa et al., 2022). Aligning the available relevant sequences (e.g. *Aplysia*, *Lymnaea*, *Haliotis*, *Biomphalaria*, *Deroceras*), the general active invGnRH/CRZ peptide sequence is Q-N-Y-H-F-S-N-G-W-variable (usually Y)-variable (usually A) in gastropods. Hence, we suppose that the sequence of the active pla-invGnRH/CRZ peptide is QNYHFSNGWxx, which differs from the active oct-GnRH/CRZ peptide (QNYHFSNGWHPG). A previous study demonstrated that even such a small sequence difference can cause non-specific staining during IHC (Tsai et al., 2010). Hence, some immunopositive signals seen here might have been non-specific. Keeping this in mind, identification of the coding region of pla-GnRH/CRZ prepropeptide is highly warranted in the future to develop specific antibody for the active peptide. Nevertheless, our IHC results suggest that an invGnRH/CRZ peptide is present in *P. trivolvis*.

Role of invGnRH/CRZ in the molluscan system

As evidence has accumulated through sequencing and functional characterization of various invGnRH/CRZ peptides (originally termed invGnRHs) from several molluscs (reviewed by Sakai et al., 2020), the current consensus suggestion is that these peptides should be classified as CRZs (reviewed in Tsai, 2018). In molluscs, multiple roles in reproduction have been suggested for the invGnRH/CRZ peptides, such as oviduct contraction in the octopus (Iwakoshi-Ukena et al., 2004), sperm cell proliferation in scallops (Nagasawa et al., 2015), oocyte proliferation in abalone (Nuurai et al., 2014) and sometimes both sperm and oocyte proliferation (Sharker et al., 2021). A putative GnRH-like peptide stimulated spermatogonial cell division in cultured scallop testis (Treen et al., 2012).

In gastropods, there have been different results. Although injection of ap-GnRH/CRZ did not stimulate egg laying or any acute reproductive activity in *Aplysia californica*, it inhibited feeding (Tsai et al., 2010; Johnson et al., 2014). Recent studies in the pulmonate *L. stagnalis* showed that injection of GnRH into adult, sexually mature snails inhibited locomotion within 15 min of injection and accelerated egg laying, with no effect on feeding (Fodor et al., 2021). In this study, we showed immediate and long-term rhythmic effects of vertebrate GnRH-agonist application on the buccal motor pattern, putatively triggering the rasping behavior accompanying egg laying. It reduces the activity in phase 3 of the pattern generator, shown by the reduction in activity in phase 3 motor neuron B19, potentially dampening the ‘swallow’ phase of the oral rhythm, thereby inhibiting feeding. Indeed, if this was the signal underlying substrate cleaning, where the goal of rasping is not to eat, but to make tiny grooves along the surfaces to stick eggs (Ter Maat et al., 1989; Ter Maat, 1992), then we should see reduced

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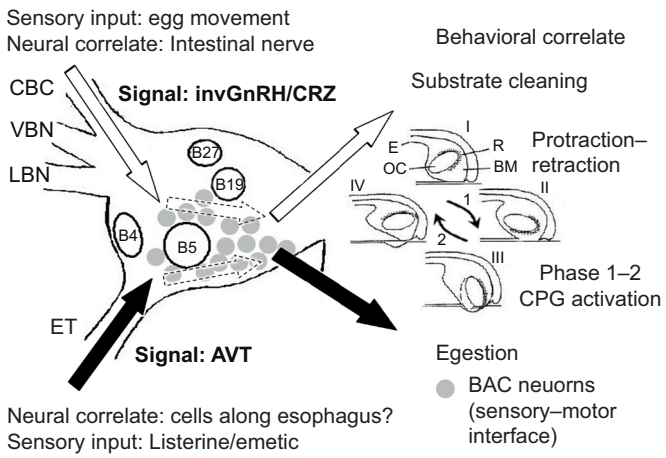

Fig. 8. Schematic of the peptidergic modulation of the multifunctional oral central pattern generator (CPG) in snails. InvGnRH/CRZ signals from the intestinal nerve target BAC cells in the buccal ganglia to modulate the CPG towards a 1–2 biphasic substrate cleaning pattern. AVT signals from the esophagus alter the buccal pattern towards a similar 1–2 pattern underlying egestion.

phase 3 buccal activation. In addition to the reduced phase 3 activation, we also observed repetitive phase 2 and non-phase 2 IPSP barrages in B19 as well as activation of B27, a phase 2 motor neuron (Fig. 5), indicative of repetitive rasping. This indicates that vertebrate GnRH application is not just inhibiting feeding, but also triggers rasping behavior underlying substrate cleaning during egg laying. The use of a mammalian GnRH peptide to trigger electrophysiological responses in molluscs raises some concerns, as the sequence of the neuropeptides are different [QNYHFSNGWxx (usually QNYHFSNGWYA) in molluscs versus QHWSYGLRPG in humans]. This is potentially why a concentration of 50 µmol l^{-1} mammalian GnRH is required to elicit the molluscan response. We suggest that there is enough similarity between the peptides that there is indeed an effect of mammalian GnRH agonists and antagonists on the molluscan receptors. This is, in effect, yet another example of promiscuity of another typical ‘lock and key’ mechanism, i.e. vertebrate GnRH peptides are similar enough in structure to the natural molluscan invGnRH/CRZ peptide ligands to be able to bind to their receptors (Fodor et al., 2020a). The consistent response through all preparations, the similarity in response to that physiologically triggered by the intestinal nerve and the nature of alteration of the buccal motor pattern all allude to a veritable, elicited activation by the vertebrate GnRH agonist. It would be ideal to identify the coding sequence of the pla-GnRH/CRZ prepropeptide to make a specific synthetic active peptide for further *in vitro* and *in vivo* experiments.

InvGnRH/CRZ as the signal molecule from pulmonate reproductive organs

Previous work has shown that the IN from the visceral ganglion that innervates different parts of the reproductive tract is both necessary and sufficient for substrate cleaning behavior during egg laying (Ter Maat et al., 1989; Ferguson et al., 1993; Hermann et al., 1994). Lesions to this nerve with other peripheral nerves intact eliminates substrate cleaning behavior (Ferguson et al., 1993; Hermann et al., 1994). Our previous findings (Ramakrishnan et al., 2014) reported that the stimulation of the IN induced a long-term rhythm in the buccal ganglia, with reduced phase 3 activation and induction of both phase 2 and BAC-triggered non-phase 2 activity. Here, we show that this IN-induced rhythm induction is blocked in the presence of the vertebrate GnRH antagonist and is regained after it is washed off. Vertebrate GnRH-agonist application and IN stimulation both trigger very similar buccal motor activation, with similar long-term rhythms. Thus, this study indicates that an invGnRH/CRZ-like peptide is the signal molecule from the IN that alters the buccal motor pattern towards substrate cleaning behavior during egg laying in *P. trivolvis*. Beyond priming animals for reproduction, we suggest a role for an invGnRH/CRZ peptide in the active, immediate neural modulation that directly affects an important aspect of egg-laying behavior in the snail.

InvGnRH/CRZ as the contextual switch

Hormonal regulation of CPGs has been found in other animals regulating a variety of behaviors, including sound vocalization circuits in teleosts and amphibians (Bass and Remage-Healey, 2008; Barkan et al., 2021), feeding circuits of stomatogastric ganglia in crabs (Cook and Nusbaum, 2021), cardiac CPGs in lobsters (Dickinson et al., 2016), spinal locomotion circuits in fish (Berg et al., 2018), electric organ discharge in weakly electric fish (Borde et al., 2020) and the abdominal ganglia during ecdysis in *Manduca* (Wells et al., 2006). This is one of the first studies to show the direct effect of the neuropeptide GnRH on altering a CPG.

Multifunctional CPGs with contextual modulators have been found in different animal species (Briggman and Kristan, 2008), many of which involve the concerted activity of groups of neurons (Staras et al., 1998; Hooper and DiCaprio, 2004; Grillner, 2021). Projection systems bringing in sensory stimuli offer context to these multifunctional circuits, triggering appropriate behavioral output (Briggman and Kristan, 2008). Neuromodulators released locally or diffused systemically have been shown to have both short- and long-term effects on these pattern-generating circuits (Dickinson, 2006; Briggman and Kristan, 2008; Sakurai and Katz, 2015). Although a role for the electrotonically coupled BAC cells as the contextual modulators that altered buccal CPG rhythms has been suggested (Ramakrishnan et al., 2014), not much is known about the signals that activate these network switches. Neuropeptides such as phenylalanine (NPF) have been implicated as a potential signal from the BAC neuronal system with effects on the buccal motor pattern (Sato et al., 2010). Here, we show that the IN-triggered BAC response is similar to the vertebrate GnRH-triggered response, and is effectively blocked by the vertebrate GnRH antagonist. In a different context, esophageal signals sensing emetics such as Listerine would trigger a regurgitation response using the BAC neurons (Ramakrishnan et al., 2014), potentially using AVT as the signal molecule from the esophageal lining (Richmond et al., 1987). If signal molecules such as invGnRH/CRZ and AVT are indeed context mediators of the BAC response, activation by one signal molecule should not be affected by blocking the other. Indeed, we show that the AVT-triggered BAC response was not affected by the vertebrate GnRH antagonist. At this moment we are unable to say whether specific types of BAC neurons have specific roles in mediating one or the other signal response. Thus, the modulation of the buccal CPG mediated by the BAC neurons may respond to emetic signals from the esophagus that trigger AVT release sent via the esophageal nerve trunks by triggering regurgitation; either the same BAC cluster or a different one would respond to the movement of eggs in the reproductive tract that send invGnRH/CRZ signals via the IN and cerebro-buccal connectives to trigger substrate cleaning behavior. While both of these trigger 1–2 biphasic buccal motor

rhythms, they are very specific behavioral responses to the context (Fig. 8).

Conclusions

The multifunctional buccal CPG in snails controls oral behaviors such as feeding, swallowing, egestion and substrate cleaning during egg laying. With this study, we show that an invGnRH/CRZ-related peptide is the signal molecule via the IN from the reproductive tract that modulates the buccal motor rhythm towards a biphasic 1–2 pattern underlying substrate cleaning during egg-laying behavior. Although the role of the invGnRH/CRZ family of peptides in directly affecting reproduction is unclear in other molluscan species, in the pulmonate snail *P. trivolvis*, it serves as the contextual switch in modulating behavior towards substrate cleaning underlying egg-laying behavior.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.R., A.D.M.; Methodology: S.R.; Validation: S.R.; Investigation: S.R.; Resources: A.D.M.; Writing - original draft: S.R.; Writing - review & editing: S.R.; Visualization: S.R.

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