A Composite Modulatory Feedforward Loop Contributes to the Establishment of a Network State

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Key Words: Aplysia, feedforward loops, feeding, neuromodulation, network states.
Feedforward loops (FFLs) are one of many network motifs identified in a variety of complex networks, but their functional role in neural networks is not well understood. We provide evidence that combinatorial actions of multiple modulators may be organized as FFLs to promote a specific network state in the *Aplysia* feeding motor network. The *Aplysia* feeding central pattern generator (CPG) receives two distinct inputs — a higher-order interneuron CBI-2 and the esophageal nerve (EN), that promote ingestive and egestive motor programs, respectively.

EN stimulation elicits a persistent egestive network state, which enables the network to temporarily express egestive programs following a switch of input from the EN to CBI-2. Previous work showed that a modulatory CPG element, B65, is specifically activated by the EN and participates in establishing the egestive state by enhancing activity of egestion-promoting B20 interneurons while suppressing activity and synaptic outputs of ingestion-promoting B40 interneurons. Here, a peptidergic contribution is mediated by small cardioactive peptide (SCP).

Immunostaining and mass spectrometry show that SCP is present in the EN, and is released upon EN stimulation. Importantly, SCP directly enhances activity and synaptic outputs of B20, and suppresses activity and synaptic outputs of B40. Moreover, SCP promotes B65 activity. Thus, the direct and indirect (through B65) pathways to B20 and B40 from SCPergic neurons constitute two FFLs with one functioning to promote egestive output, and the other to suppress ingestive output. This composite FFL consisting of the two combined FFLs appears to be an effective means to co-regulate activity of two competing elements which do not inhibit each other, thereby contributing to establish specific network states.
A common theme emerging from system-level studies of network functions is that most neural networks are multifunctional. This is true both for simple invertebrate motor networks (Briggman and Kristan 2008; Getting 1989; Jing 2009; Jing and Weiss 2005; Jing et al. 2009; Marder et al. 2005; Nusbaum and Beenhakker 2002) and for vertebrate neural networks (Grillner 2006; Lieske et al. 2000) including cortical networks (Haider and McCormick 2009; Yuste et al. 2005). In part, distinct network outputs stem from combinatorial actions of a variety of neuromodulators that sculpt a specific functional pattern from the anatomical connectivity of the network. Currently, well understood mechanism for diverse network actions is the convergence and divergence of modulation (Brezina and Weiss 1997; Nusbaum et al. 2001). However, identification of additional organizational principles, e.g., network motifs in other networks (Alon 2007; Bullmore and Sporns 2009), raises the possibility that these principles may be applicable to combinatorial actions of modulators.

Specifically, recent work suggests that different complex networks may utilize, as building blocks, similar network motifs (Alon 2007; Milo et al. 2002). Network motifs are interacting pathways formed by a small number of network elements/nodes. Initially, these network motifs are identified using a combination of graph theory and statistic method, and their functions are best understood in transcription regulation networks (Alon 2007; Bullmore and Sporns 2009). One common motif that has been identified in several types of complex networks, particularly information processing networks, is the feedforward loop (FFLs) that is formed by three network nodes, $X$, $Y$, $Z$, in which there is a direct interaction from $X$ to $Z$ ($X \rightarrow Z$) and an indirect
feedforward interaction from X to Z through Y (X → Y → Z) (Fig. 1). The FFL is defined as coherent if the signs of the direct and indirect pathways onto Z are the same, or incoherent if they are the opposite (Alon 2007). Although evidence suggests that network motifs including the FFLs are present in the neural networks (Milo et al. 2002; Song et al. 2005; Sporns and Kotter 2004), specific function of this network motif is not well understood. Here we provide evidence suggesting that, in *Aplysia* feeding motor network, actions of multiple neuromodulators may be organized to create FFLs. Moreover, this *Aplysia* network appears to use a combination of two coherent FFLs, with one FFL enhancing the synergist output and the other FFL suppressing the competing output with their combination promoting the required network state.

The *Aplysia* feeding central pattern generator (CPG) can be activated by two distinct inputs, cerebral-buccal interneuron-2 (CBI-2) and the esophageal nerve (EN), that, in steady state, can evoke two competing responses, ingestive vs. egestive motor programs, respectively (Chiel et al. 1986; Jing and Weiss 2005; Morgan et al. 2002; Proekt et al. 2004; Zhurov et al. 2005). However, recent studies showed that for several minutes following EN stimulation, CBI-2 stimulation elicits egestive programs rather than ingestive programs (Friedman et al. 2009; Proekt et al. 2004, 2007, 2008). Thus, EN stimulation induces a persistent egestive state that endows the network with slow dynamics to temporarily override the switch in the input.

Proekt et al. (2007) showed that a modulatory CPG interneuron B65 plays a role in the network dynamics. Specifically, B65 is activated by EN stimulation, and B65 activity modulates the synaptic output and activity of critical CPG elements thus contributing in the development of the egestive network state. However, other work (Due et al. 2004) indicated that, even in the
absence of B65 activity, the feeding CPG may express egestive-like programs in response to EN stimulation. This suggested that additional modulators may also contribute to the establishment of the egestive network state. Previous work indicated that small cardioactive peptides (SCPs) (Lloyd et al. 1985, 1987b, 1988; Mahon et al. 1985; Sossin et al. 1987) are present in the EN (Jing et al. 2007). Here, we present evidence that SCP is released upon EN stimulation and it can convert CBI-2-elicited ingestive motor programs to egestive ones. More importantly, SCP directly increases the activity and synaptic output of the egestion-promoting CPG interneuron (B20), while at the same time decreasing the activity and synaptic output of the ingestion-promoting CPG interneuron (B40). These actions are largely similar to actions of B65. In addition, SCP directly promotes B65 activity. Thus, the SCP direct pathway and the indirect pathway through B65 to B20 and B40 form two coherent FFLs that act with opposite signs to promote the egestive output of B20 and to suppress the ingestive output of B40. Because the modulators (SCP and B65) are shared in the two FFLs, the combined FFL may be considered as a composite FFL that functions to control the balance of two competing network outputs towards the purpose of promoting the egestive network state.
Aplysia californica were obtained from Marinus Scientific (Garden Grove, CA) and maintained in circulating artificial seawater (ASW) at 14–15°C.

Immunocytochemistry

Rat antibody to SCPβ was made as described previously (Vilim et al. 1996).

Immunocytochemistry was performed as described previously (Furukawa et al. 2001). Tissues were fixed in 4% paraformaldehyde, 0.2% picric acid, 25% sucrose, and 0.1 M NaH₂PO₄ (pH 7.6) for either 3 h at room temperature or overnight at 4°C. After washes with PBS, ganglia were desheathed to expose the neurons except in ganglia from small animals (10 - 15 g). All subsequent incubations were done at room temperature. Tissue was permeabilized and blocked by overnight incubation in blocking buffer (BB; 10% normal donkey serum, 2% Triton X-100, 1% BSA, 154 mM NaCl, 50 mM EDTA, 0.01% thimerosal, and 10 mM Na₂HPO₄, pH 7.4). The primary antibody was diluted 1:250 in BB and incubated with the tissue for 4–7 d. The tissue was then washed twice per day for 2–3 d with washing buffer (WB; 2% Triton X-100, 1% BSA, 154 mM NaCl, 50 mM EDTA, 0.01% thimerosal, and 10 mM Na₂HPO₄, pH 7.4). The tissue was then incubated with a 1:500 dilution of secondary antibody (lissamine rhodamine donkey anti-rat; Jackson ImmunoResearch, West Grove, PA) for 2–3 d. The tissue was then washed twice with WB for 1 d and four times with storage buffer (1% BSA, 154 mM NaCl, 50 mM EDTA, 0.01% thimerosal, and 10 mM Na₂HPO₄, pH 7.4) for 1 d.

In nerve crush experiments, the buccal ganglion and part of the gut, particularly the esophagus, that remained innervated by the buccal ganglion through the bilateral EN, were
dissected and pinned out in Petri dish filled with sylgard. The anterior and posterior branches of the bilateral EN were crushed with forceps, but the two sides of the crushed nerve remained attached due to the sheath remaining intact. The preparations were incubated in the ASW at 15°C overnight, and fixed and processed for immunocytochemistry the next day.

Electrophysiology

Conventional electrophysiological recordings were performed as described previously (Jing and Weiss 2005; Wu et al. 2007). The cerebral and buccal ganglia were desheathed. The preparation was then transferred to a recording chamber, lined with sylgard, containing ~ 1.5 ml ASW (in mM: 460 NaCl, 10 KCl, 55 MgCl₂, 11 CaCl₂, and 10 HEPES buffer, pH 7.6), continuously perfused with ASW at 0.3 ml/min and maintained at 14–17°C. In order to suppress polysynaptic activation, a high-divalent cation saline (Hi-Di) (in mM: 368 NaCl, 10 KCl, 13.8 CaCl₂, 101 MgCl₂, and 10 HEPES at pH 7.6) was used. The peptide, SCP_B (Anaspec, Fremont, CA), was dissolved in either ASW or Hi-Di (as indicated in Results) immediately before each application. Nerve stimulation and recording were done with polyethylene suction electrodes.

Identification of motor programs

The *Aplysia* feeding CPG is localized in the buccal ganglion and can generate two major types of motor programs, ingestive and egestive. Each cycle of the motor program, irrespective of program type, consists of a radula protraction-retraction sequence that is coupled to the opening-closing movements (Brembs et al. 2002; Hurwitz et al. 1997, 2005; Jing et al. 2004; Jing and Weiss 2001; Morgan et al. 2002; Morton and Chiel 1993a, b; Nargeot et al. 1997;
Sasaki et al. 2007, 2008; 2009; Wu et al. 2007). In ingestion, closing movement occurs during retraction in order to pull food objects into the buccal cavity. In egestion, closing movement occurs during protraction to push inedible objects out.

Two external inputs can activate the buccal CPG. One input originates in a higher-order interneuron, e.g., cerebral-buccal interneuron-2 (CBI-2), located in the cerebral ganglion. CBI-2 receives sensory inputs, e.g., food, from the head sensory organs (Jing and Weiss 2005; Rosen et al. 1991). Stimulation of CBI-2 evokes ingestive-like behaviors (Jing and Weiss 2005). In isolated CNS, repetitive activation of CBI-2 triggers ingestive motor programs. Specifically, the ingestive programs were elicited by stimulation of CBI-2 with short current pulses, each of which elicits a single action potential. The other input is from a buccal nerve, the EN, that innervates the gut, and may transmit information about the stretch of the esophagus (Chiel et al. 1986; Church and Lloyd 1994; Schwarz and Susswein 1986). Stimulation of the EN evokes egestive-like behaviors (Chiel et al. 1986; Church and Lloyd 1994). In isolated CNS, repetitive activation of the EN elicits egestive programs (Due et al. 2004; Morgan et al. 2002; Proekt et al. 2004). Specifically, the egestive motor programs were evoked by stimulation of the posterior branch of the EN (see Supplemental Fig. 1 of Jing et al. 2007) with 8–15 V and 3 ms pulses at 2 Hz.

Solid-phase extraction (SPE) bead sampling of peptidergic releasates for Matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF/TOF) mass spectrometry

Protocols used for neuropeptide sampling with SPE beads have been described previously (Hatcher et al. 2005, 2008; Jing et al. 2007). Briefly, solid-phase extraction beads
(30-50 μm diameter, Resin “D” type, International Sorbent Technology, Ltd., Hengoed, UK) were wetted in 50% acetonitrile (ACN) and equilibrated in ASW. Individual beads were transferred between solutions or *Aplysia* ganglions by hand-held borosilicate capillary pipettes (World Precision Instruments, Sarasota, FL) with tips pulled to ~ 200 μm in diameter. Beads were positioned on the rostral surface of isolated buccal ganglia (see Supplemental Fig. 1 of Jing et al. 2007). To limit loss of releasates in the bath, superfusion was briefly suspended during bead sampling intervals. Bead samples were collected for 30 min intervals before and during EN-elicited egestive motor programs; new beads were repositioned in the same location for each prestimulation/stimulation sampling period. Following collection, beads were removed, washed of salts in Millipore H₂O, and transferred to MALDI targets. Bead-bound releasates were eluted onto the MALDI target surface by rinsing first with 70% ACN/H₂O (~ 0.2 μl) followed by addition of CHCA MALDI matrix dissolved in 70% ACN (~ 0.2 μl). Reagents were obtained from Sigma-Aldrich.

**MALDI TOF/TOF mass spectrometry**

MALDI TOF/TOF mass spectrometry was performed with either a Voyager-DE-STR mass spectrometer (Applied Biosystems Inc., ABI, Foster City, CA) or an Ultraflex II MALDI TOF mass spectrometer (Bruker Daltonics, Billerica, MA). Releasate mass spectra were obtained in either linear or reflectron modes in order to optimize either limit of detection or mass resolution respectively. Mass spectra were accumulated and averaged from 300 to 400 laser shots and externally calibrated. SCP A and B were identified based on mass-to-charge ratio (m/z) values; mass peaks were all within 100 ppm accuracies. All paired prestimulation and post-stimulation samples were acquired with the same instrument settings and in the same acquisition.
day. Data were analyzed using either Data Explorer (ABI) or Biotools (Bruker Daltonics) software packages.

Data analysis

Electrophysiological recordings were digitized on-line with Axoscope and plotted with CorelDraw (Ottawa, Ontario, Canada). Data graphs were plotted using SigmaPlot 8 (SPSS, Chicago, IL) or Axum (Mathsoft, Cambridge, MA). Data are expressed as mean ± SEM. Differences between two data groups were tested with two-tailed paired or unpaired t-test. Data with more than two groups were first analyzed with one way repeated-measures ANOVA. If the data show overall significant difference among these groups, individual comparisons between groups were subsequently made with Bonferroni corrections. All statistical tests were performed using GraphPad Prism 4.0 (San Diego, CA).
Recent work suggests that EN stimulation can establish a persistent egestive network state that allows the CPG network to temporarily disregard the switch in the input (from the EN to CBI-2). Moreover, B65, an input-representing neuron (activated specifically by the EN, but not by CBI-2) contributes to the establishment of the network state by modulating the critical elements of the feeding CPG (Proekt et al. 2007) (The schematic diagram of the network (see Fig. 8) may be consulted for the understanding of network actions of specific neurons). In this paper, we examine peptidergic mechanisms that may contribute to the establishment of the egestive network state.

**Localization and release of SCP**

We focus on the SCP peptides. Previous work (Plummer and Kirk 1990; Sossin et al. 1987) suggests that bath application of SCP can activate the feeding CPG in *Aplysia*. More recently, we have shown that both forms of SCP, i.e., SCP\(_A\) and SCP\(_B\), are present in the EN based on peptide detection of the tissue extract of the EN using MALDI mass spectrometry (Jing et al. 2007). Here, we performed additional experiments to determine the localization and release of SCP. First, we used immunocytochemical methods as an independent means to verify the presence of SCP in the EN. Figure 2A shows the distribution of SCP in the buccal ganglion (the rostral view (*A1*) and caudal view (*A2*)). Rostral view shows a cluster of small neurons, that have been identified as radula mechanooafferent neurons (Miller et al. 1994; Rosen et al. 2000). Caudal view shows staining of large motor neurons including the two largest cells in the buccal
ganglion, motoneurons B1 and B2, consistent with earlier findings (Lloyd et al. 1987a, 1988). In addition, these immunohistochemical images indicate that the EN was strongly stained with SCP (see also Fig. 2B), consistent with the observation in MALDI mass spectrometry experiments (Jing et al. 2007).

SCP in the EN detected by MALDI mass spectrometry and immunostaining may have two sources of origin. One source may be from the CNS because the SCP containing esophageal-cluster neurons on the caudal surface (Fig. 2A2), which include neurons B1 and B2, send their axons to the periphery through the EN (Lloyd et al. 1987a, 1988). Note that the radula mechanoafferent neurons in the SCP-containing sensory neuron cluster on the rostral surface of buccal ganglion do not project to the EN. Instead, they project to the radula nerve (RN) (Evans et al. 2003; Miller et al. 1994; Rosen et al. 2000). Consequently, EN stimulation does not directly trigger activity in these sensory neurons (Ludwar et al. 2009).

Another source of SCP in the EN could be the neurons located in the periphery, specifically in the gastrointestinal (GI) system that the EN innervates. The major components of the GI system of Aplysia include: esophagus, crop, anterior gizzard (or triturating stomach), posterior gizzard (or filter chamber) and intestine. Previous work has reported isolated cells or small groups of cells in the gizzard (stomach) (Lloyd et al. 1988). We confirmed this observation, and in addition, we observed SCP-immunoreactive neurons and fibers in the esophagus (Fig. 2C), crop (Fig. 2D), and filter chamber (Fig. 2F). Furthermore, we observed a number of clustered SCP-positive neurons in the stomatogastric ring (Fujisawa et al. 1999; Furukawa et al. 2001; Sweedler et al.
that is located between the crop and anterior gizzard (Fig. 2E). Thus, it appears that SCPergic fibers in the EN may originate both centrally and peripherally.

To determine whether at least some of the peripheral SCPergic neurons project centrally, we determined the directionality of SCP transport in the EN using a nerve ligation approach (Jing et al. 2007; Li et al. 1998). Specifically, we investigated whether SCP is transported from the periphery to the buccal ganglion using immunocytochemistry and nerve crush on the EN in a reduced preparation with part of the GI tract (the esophagus) attached with the buccal ganglion through the EN (bilateral nerve crushes in 4 animals). We incubated the preparations in the ASW overnight to allow the transport of peptides to be determined, and then fixed the preparations for SCP immunohistochemistry. Fig. 2B illustrates one typical experiment, which shows that SCP is accumulated at the distal ends of the EN following nerve crush (the bottom part of the nerves in Fig. 2B1, B2. See also panel B4.). Fig. 2B3, B4 show the proximal (B3) and distal (B4) ends of a posterior branch in higher magnification. The data support the notion that peripheral SCPergic neurons may transport SCP centrally to the buccal ganglion and potentially act on the feeding CPG. Not surprisingly, we also observed SCP accumulation at the proximal ends of the EN following nerve crush (top part of the nerves in Fig. 2B1, B2. See also Fig. 2B3.) because motoneurons located in the buccal ganglion are known to transport SCP toward the periphery through the EN (Lloyd et al. 1984, 1988).

In addition, we used MALDI mass spectrometry to examine whether stimulation of the EN results in SCP release. SPE beads have been used previously as extracellular neuropeptide sampling probes that can be combined with MALDI MS to characterize neuronal releasates from
discrete regions in the isolated *Aplysia* ganglia preparation (Hatcher et al. 2005; Jing et al. 2007).

Beads were placed in neuropilar regions of the rostral surface of the buccal ganglion as previously described (Jing et al. 2007). We compared analytes collected with SPE beads before and during stimulation of proximal ends of the EN to differentiate stimulation-dependent releasates from endogenous background compounds. Prior to EN stimulations, low levels of SCP\textsubscript{A} were observed in 2 of 4 bead collections, while SCP\textsubscript{B} was not detected in any of the samples (Fig. 3, top). After EN stimulation, we found that peaks of SCP\textsubscript{A} and SCP\textsubscript{B} were markedly elevated in all bead collections ($n = 4$) (Fig. 3, bottom). In the 2 of 4 preparations where SCP\textsubscript{A} was observed in prestimulation bead collections, SCP\textsubscript{A} peaks observed in stimulation conditions were larger. Thus, the data suggest that although there may be a low level of SCP detected in control conditions, both SCP\textsubscript{A} and SCP\textsubscript{B} are released as a result of EN stimulation.

SCP release during EN stimulation may have either a central or peripheral origin. Centrally located SCPergic neurons could include the motoneurons that project to the EN as mentioned above. However, these neurons are located on the caudal surface, while our SPE beads were placed on the rostral surface. In principle, the mechanosensory neurons on the rostral surface may also contribute to SCP release, but they do not project to the EN and are not activated during EN stimulation. Thus, contributions from these two groups of neurons to the SCP release are expected to be small. In contrast, given that SCP accumulated in the distal end of the EN after a nerve crush (Fig. 2B1, B2 and B4), it is likely that at least some of the released SCP has a peripheral origin. Regardless of the exact source of the released SCP, the fact that EN
stimulation results in SCP release provides a basis for studying the central actions of SCP (i.e., in the buccal ganglion) in the context of EN stimulation.

Modulatory actions of SCP on the feeding motor programs

To examine potential actions of SCP on the generation of feeding motor programs, we took advantage of the isolated CNS preparations in which SCP can be superfused, while many essential features of the feeding motor programs can be simultaneously monitored. Specifically, in the isolated CNS, protraction is monitored by activity in the I2 nerve which contains the axons of protraction motoneurons (Hurwitz et al. 1996). Retraction can be monitored by activity in the retraction interneuron B64 (Hurwitz and Susswein 1996; Wu et al. 2007). Radula closing can be monitored by activity in closing motoneuron B8. Thus, we can determine which type of motor programs is expressed based on the phasing of activity of B8 relative to protraction-retraction.

When B8 is predominantly active during retraction, the programs are classified as ingestive. When B8 is active predominantly during protraction, the programs are classified as egestive (Morgan et al. 2002; Morton and Chiel 1993a, b).

We examined the effects of SCP on the feeding motor programs elicited by CBI-2. We focused on the short form of the SCP, i.e., SCPB, in part because previous work has indicated that the bioactivity of SCP primarily originates from the c-terminal sequence of SCP, which SCPA and SCPB share (Lloyd 1986). We stimulated CBI-2 with short current pulses at 10 Hz to elicit single cycle programs at regular intervals (1 min). CBI-2 was stimulated for the duration of the protraction phase. After 4-8 single cycle motor programs were elicited, parametric features
(particularly B8 firing frequency during protraction and retraction) were stable and the motor
programs were ingestive because B8 was firing strongly during retraction and only weakly
during protraction. Fig. 4A1 illustrates a control motor program elicited in this fashion. While
continuing to stimulate CBI-2, we initiated superfusion of $10^{-6}$ M SCP$_B$ for 6-10 min.
Subsequently, we applied $10^{-5}$ M SCP$_B$ for 6-10 min. One typical motor program elicited by
CBI-2 in the presence of $10^{-5}$ M SCP$_B$ is shown Fig. 4A2. Longer superfusion of $10^{-5}$ M SCP$_B$
made the programs less robust that are represented in weaker firing frequency of motoneurons
including B8 (not shown). Finally, SCP$_B$ was washed-out until parametric features of the motor
program returned to the control levels as shown in Fig. 4A3.

The example shown in Fig. 4A2 suggests that the major effects of SCP$_B$ on motor programs is to
increase B8 firing frequency during protraction and to decrease B8 firing frequency during
retraction, i.e., to shift the nature of motor programs from ingestive towards more egestive. We
illustrate the individual experiments (Fig. 4B) and the mean data (Fig. 4C) ($n = 14$) of this effect
in 2-D graphs plotting B8 firing during protraction as x-axis, and B8 firing during retraction as y-
axis. In the graphs, ingestive programs are clustered in the upper left corner because B8 is mostly
active during retraction; and egestive programs are clustered in the lower right corner because B8
is mostly active during protraction (Jing et al. 2007; Jing and Weiss 2002; Morgan et al. 2002;
Proekt et al. 2004, 2007; Sasaki et al. 2008, 2009; Wu et al. 2007). In our experiments, the
individual and the mean data points for the control motor programs were mostly ingestive
because they were located in the upper left corner. In contrast, with $10^{-6}$ M SCP$_B$ superfusion,
motor programs shifted towards the lower right corner, as B8 became more active during
protraction and less active during retraction. Finally, $10^{-5}$ M SCP$_B$ superfusion shifted the
average data point to the egestive cluster, suggesting that the programs were converted into
egestive ones. Group data showed that SCPB had a significant effect on B8 activity during
protraction \((F_{(3,39)} = 16.52, P < 0.0001, n = 14, \text{Fig. 4D1})\) as well as B8 activity during retraction
\((F_{(3,39)} = 47.52, P < 0.0001, n = 14, \text{Fig. 4D2})\).

SCP targets in the feeding CPG and its modulatory pathways

The data presented above suggest that SCP plays a major role in promoting an egestive network
state as SCP is released upon EN stimulation, and SCP superfusion converts CBI-2-elicited
ingestive programs to egestive ones. To gain insights into the neural mechanisms of SCP actions,
we sought to identify the neural targets of SCP within the feeding CPG.

Earlier work (Jing et al. 2003; Jing and Weiss 2001, 2002) showed that B8 activity is controlled
by the feeding CPG interneurons. In particular, two CPG elements B20 and B40 are critical.
Both B20 and B40 are active during protraction, but B20 is preferentially active in egestive
programs, whereas B40 is preferentially active during ingestive programs. Furthermore, B20 and
B40 exert different actions on B8. B20 monosynaptically excites B8, therefore promoting B8
activity during protraction, the parametric feature that is prominent in egestive programs. In
contrast, B40 exerts fast inhibitory and slow excitatory effects on B8, therefore promoting B8
activity during retraction, the parametric feature that is prominent in ingestive programs. Because
SCPB appears to promote an egestive state, we sought to determine if SCPB does so by acting
directly on these CPG elements.
First, we sought to determine whether SCPB has a direct effect on the activity of B20 and B40. To reduce polysynaptic actions, these experiments were performed in high-divalent saline. We applied 2 s current pulses in B20 every 30 s. As illustrated for a single preparation in Fig. 5A, B20 fired 13 spikes in control, but fired 18 spikes during superfusion of 10^{-6} M SCPB, and 34 spikes during superfusion of 10^{-5} M SCPB. After washout of SCPB, B20 fired 10 spikes, similar to the control. Group data showed that B20 firing frequency was increased by SCPB in a concentration-dependent manner (F_{(3,12)} = 18.86, P < 0.0001, n = 5). In contrast, we found that 10^{-6} M SCPB reduced B40 firing frequency (F_{(2,10)} = 66.44, P < 0.0001, n = 6, Fig. 5B). Thus, consistent with its role in promoting egestive programs, SCP had opposite actions on the B20 and B40 firing frequency. The increase in B20 activity was associated with a slight depolarization of this neuron (Control: -59.64 ± 1.89 mV; 10^{-6} M SCPB: -58.39 ± 1.60 mV; 10^{-5} M SCPB: -57.29 ± 1.74 mV; Wash: -59.49 ± 1.83 mV; ANOVA: F_{(3,12)} = 9.78, P < 0.01; Bonferroni post-tests: 10^{-5} M SCPB group is different from the Control and Wash groups: P < 0.05). In contrast, the decrease in B40 activity, was associated with a slight hyperpolarization of this neuron (Control: -48.47 ± 1.89 mV; 10^{-6} M SCPB: -49.75 ± 0.74 mV; Wash: -48.7 ± 0.65 mV; ANOVA: F_{(2,10)} = 11.5, P < 0.01; Bonferroni post-tests: 10^{-6} M SCPB group is different from the Control and Wash groups: P < 0.05). These changes in the membrane potential may contribute to the changes in firing frequency.

We then examined the effects of SCPB on the synaptic connections from B20 and B40 to B8. In order to prevent influences of voltage dependence on the amplitude of PSPs, we applied DC injections in B8 during SCP superfusion so that the membrane potential of B8 remained the same as the control period. Under this condition, SCPB increased the amplitude of fast EPSPs from
B20 to B8 in a concentration-dependent manner \((F_{(3,9)} = 14.29, P < 0.001, n = 4, \text{Fig. 6A})\). In contrast, we found that SCPB reduced the amplitude of the slow EPSPs from B40 to B8. Group data show that this effect was significant \((F_{(2,6)} = 34.68, P < 0.001, n = 4, \text{Fig. 6B})\). Thus, SCP also had opposite effects on the synaptic outputs of B20 and B40 to B8.

Finally, previous work (Due et al. 2004; Proekt et al. 2007) has shown that the CPG interneuron B65 is strongly active during the egestive motor programs elicited by EN stimulation. B65 activity promotes the development of the egestive network state by enhancing activity of B20 and suppressing activity B40. As we showed in the above experiments that SCP also acts on B20 and B40, it is important to determine if SCP also acts on B65 to form a FFL. Indeed, SCPB increased B65 firing frequency in a concentration-dependent manner \((F_{(3,12)} = 36.28, P < 0.0001, n = 5, \text{Fig. 7A})\). The increase in B65 firing was associated with a slight depolarization of this neuron (Control: -56.3 ± 1.07 mV; 10^{-6} M SCPB: -55.75 ± 0.99 mV; 10^{-5} M SCPB: -54.17 ± 1.07 mV; Wash: -56.36 ± 1.03 mV; ANOVA: \(F_{(3,12)} = 4.73, P < 0.05\); Bonferroni post-tests: 10^{-5} M SCPB group is different from the Control and Wash groups: \(P < 0.05\)). The data support the notion that direct and indirect (through B65) actions of SCP on B20 and B40 form two FFLs.

Since SCP was released upon EN stimulation, we sought to determine if EN stimulation may also increase firing frequency of B65. We applied 3 s current pulses every 30 s to obtain relatively constant firing rate of B65. Between some tests in B65, we stimulated the EN for 10 s, so that the next test current pulse in B65 initiated 10 s later. Fig. 7B (top) shows an example of one such experiment, which suggests that B65 firing frequency increased 10 s after EN stimulation (Post EN), which recovered later. Group data indicate that increase in B65 activity
is statistically significant ($F_{(2,6)} = 22.2, P < 0.01, n = 4$, Fig. 7B). The increase in B65 activity was associated with a slight depolarization of this neuron (Control: $-54.48 \pm 3.27$ mV; Post EN: $-52.92 \pm 3.36$ mV; Recovery: $-54.36 \pm 3.34$ mV; ANOVA: $F_{(2,6)} = 12.6, P < 0.01$; Bonferroni post-tests: Post EN group is different from the Control and Recovery groups: $P < 0.05$). Taken together, SCP mimics actions of EN stimulation and it appears that SCP contributes, at least partially, to the increase in B65 activity in response to EN stimulation.
A composite modulatory feedforward loop mediated by SCP

We sought to identify multiple modulatory pathways that contribute to the establishment of an egestive network state resulting from EN stimulation. Previous work identified a modulatory CPG element B65 that is specifically activated by EN stimulation, and is important in this process (Proekt et al. 2007). Here we showed that a neuropeptide, SCP, is released during EN stimulation, and is capable of converting CBI-2-elicited ingestive programs to egestive ones. The data support the idea that multiple neuromodulators, the neuropeptide SCP and interneuron B65, may contribute to establishment of a network state.

Interestingly, the network organization of the combinatorial actions of these two types of modulators, SCP and B65, that affect the feeding CPG correspond to feedforward loops, similar to other FFLs identified in complex networks (Alon 2007). The CPG interneurons that are targeted by both SCP and B65 are the interneurons B20 and B40. Previous work (Jing et al. 2003; Jing and Weiss 2001, 2002) has shown that B20 and B40 functionally oppose each other because B20 promotes egestion while B40 promotes ingestion (Fig. 8A,B). Moreover, Proekt et al. (2007) have shown that, in order to promote egestive responses, B65 promotes activity of B20, while at the same time it suppresses activity and synaptic outputs of B40 (Fig. 8A). Here we show that SCP also promotes B65 activity. Thus, the modulatory pathways from SCPergic neurons acting through B65 on both B20 and B40 constitute two feedforward pathways. More importantly, we show that SCP also directly promotes activity and synaptic outputs of B20 and suppresses
activity and synaptic outputs of B40 (Fig. 8A). The direct effects of SCP on B20 and B40 provide an explanation for the finding that when bilateral B65 are hyperpolarized to prevent them from firing, EN stimulation still elicits egestive programs (Due et al. 2004). More generally, the two pathways to B20 (Fig. 8C1) and B40 (Fig. 8C2), originating from a direct effect of SCP and an indirect effect through B65, constitute two FFLs.

The existence of these two FFLs may be better understood in the context of a notable feature of the feeding CPG. That is, although the ingestive vs. egestive nature of the motor program is determined by the balance of activity of interneurons B20 and B40, there is no direct inhibitory connection between B20 and B40 (Jing and Weiss 2002). Consequently, if a given modulator were to promote one specific type of program and its actions were limited to an enhancement of activity and outputs of only one of the two interneurons, this might not be sufficient to achieve the required activity. Rather, it would be more effective if the modulator targeted both neurons to change the balance of outputs of B20 and B40. Indeed, the two FFLs, from SCPergic neurons to B20 and from SCPergic neurons to B40, functionally oppose each other. The loop to B20 is effectively excitatory, and has been defined as a coherent FFL type 1 (Fig. 8C1); while the loop to B40 is effectively inhibitory, and has been defined as a coherent FFL type 3 (Fig. 8C2) (Alon 2007). Moreover, because SCP and B65 are shared, the two FFLs are not independent, but perhaps can be considered as a single composite FFL (Fig. 8C3) that acts in concert to promote the desired network state.

Previous work suggests that B65 may contribute to the establishment of the egestive state because its effects on B20 and B40 persist even after B65 stops firing (Proekt et al. 2007). This
provides a partial explanation of how CBI-2 elicits egestive, rather than ingestive, programs following EN stimulation. Here, our finding that SCP modulates B20 and B40, through the direct arm of the composite FFL, provides an additional mechanism by which the feeding CPG is maintained in an egestive network state. Moreover, considering the fact that CBI-2 directly inhibits B65 (Jing and Weiss 2005), SCP actions may counteract this inhibition and boost the persistence of the egestive state in the absence of B65 firing (Fig. 8B2). One interesting question that remains to be answered is whether the modulatory actions of B65 and SCP are additive or supralinear.

The effects of SCP on the firing frequency of its target neurons might be attributable to a change in the excitability of these neurons. Regarding the underlying cellular mechanisms, there is a multitude of possibilities. For instance, to increase the excitability of its followers: (1) SCP could elicit an inward current that depolarizes the target neurons, similar to that demonstrated for proctolin in stomatogastric nervous systems (Golowasch and Marder 1992). (2) SCP could also reduce a potassium current in the target neurons, similar to that demonstrated for 5-HT in Aplysia (see review by Byrne and Kandel 1996). Thus, it is clear that peptidergic modulation of excitability can be associated with a membrane depolarization. In addition, it is likely that modulators can act through multiple, rather than a single, mechanism to exert their effects (cf. Byrne and Kandel 1996). It will be an interesting subject for future research to determine specific mechanisms of SCP actions.

Earlier work (Abrams et al. 1984) has shown that, in the CNS, SCP facilitates monosynaptic EPSPs from sensory neurons to motor neurons, our finding extends the action of SCP to the
modulation of synapses made by interneurons (i.e., B20 and B40). Interestingly, the sensory to motor neuron synapse in the earlier work is a well-known site of plasticity that may underlie different forms of learning and memory (Byrne and Kandel 1996; Kandel 2001). Thus, these studies suggest that a single modulator (SCP) may act broadly in multiple sites of plasticity in the central nervous systems, so as to promote network states and various forms of learning in Aplysia. Multiple neuropeptides promoting a single network state

It is intriguing to note that SCP is functionally similar to other peptides: e.g., apNPY (Jing et al. 2007; Rajpara et al. 1992), FRFamide and FMRFamide that are derived from different precursors (Vilim et al. 2010). Specifically, these three classes of peptides appear to promote egestive motor programs by converting CBI-2-elicited ingestive programs into egestive ones, i.e., to increase B8 activity during protraction and to decrease B8 activity during retraction. However, they show some important differences. First, program-converting actions of apNPY (Jing et al. 2007) and SCP are sufficient with application of either peptide alone. In contrast, a combination of FRFamide and FMRFamide is required to achieve a conversion. This is because each peptide by itself acts on B8 activity during protraction vs. retraction separately (Vilim et al. 2010). In addition, as described in the above section, mechanistically, SCP mimics the actions of EN stimulation on specific elements of the CPG network (Proekt et al. 2004, 2007), thus constituting a critical mechanism for implementing the actions of EN stimulation. In contrast, the mechanisms of apNPY and RFamide actions (Jing et al. 2007; Vilim et al. 2010) are presently unclear, but our preliminary work suggests that apNPY and RFamide may act through different means. A recent study (Martínez-Rubio et al. 2009) showed that dopamine is also present in the
EN, which could potentially contribute to some effects of EN stimulation. However, it is not yet
known whether dopamine is in fact released upon EN stimulation. Moreover, dopamine is
apparently present predominantly in the anterior branch of the EN, while our studies showed that
SCP and ANPY are localized predominantly to the posterior branch – the branch that we
stimulated in our study.

Second, at least in some neurons, the distributions of apNPY, RFamides, and SCP appear to be
distinct. This can be clearly seen in the CNS. For example, FMRFamide and FRFamide are
preferentially localized in the sensory neurons with centrally localized somata, i.e., S1 and S2
sensory clusters in the buccal ganglion (Vilim et al. 2010), while apNPY and SCP are not
localized in these cells. SCP, but not apNPY or RFamide, is localized in a separate sensory
neuron cluster on the rostral surface of the buccal ganglion (Fig. 2) (Miller et al. 1994). Similarly,
apNPY, but not SCP or RFamide, is located in B18 interneuron (Jing et al. 2007). Furthermore,
immunostaining suggests that many additional apNPY and SCP positive neurons originate from
the periphery, e.g., the gut. We found that SCP is located in more anterior parts of the gut (i.e.,
esophagus, crop and gizzard) (Fig. 2), whereas apNPY originates from more posterior parts of
the gut (i.e., in posterior gizzard, intestines and rectum) (Kupfermann et al. 2001). Thus it may
be interesting for future research to investigate more fully whether peripheral sources of apNPY,
SCP or even RFamide are distributed in distinct areas of the gut, and whether they may convey
different information from the periphery to the CNS.

Roles of modulatory peptides in input neurons: comparisons with other peptides and systems
Recent work indicates that the *Aplysia* feeding network is advantageous for studying central peptidergic modulation in the context of network multifunctionality (Jing et al. 2007; Jing and Weiss 2001; Koh et al. 2003; Koh and Weiss 2007; Morgan et al. 2002; Proekt et al. 2005; Sasaki et al. 2009; Sweedler et al. 2002; Vilim et al. 2010). In particular, these studies highlighted the essential roles of peptides in input neurons that are extrinsic to the CPG network. Specifically, other than the earlier peptide localization to motoneurons (Weiss et al. 1992), neuropeptides appear to be most prominently localized in input neurons that act on the CPG, i.e., sensory neurons (Furukawa et al. 2001; Miller et al. 1994; Vilim et al. 2010), higher-order interneurons (Koh et al. 2003; Morgan et al. 2002; Sweedler et al. 2002), as well as nerve fibers, such as apNPY, SCP and RFamide in the EN; but are largely absent in CPG neurons. A similar situation may be present in the stomatogastric nervous system (Marder and Bucher 2007; Nusbaum et al. 2001). Possibly in these CPGs, the fast and slow actions of the classical neurotransmitters implement pattern-generation on a cycle-cycle basis, while the slower acting peptides are primarily used to exert a more global modulatory control.

Localization of neuromodulators to specific neurons made it possible to change the focus of research from studying overall actions of modulators on CPG networks to more specific studies of how neuromodulators may account for the actions of individual neurons that contain them (e.g., Harris-Warrick and Marder 1991; Jing et al. 2007, 2008, 2009; Jing and Weiss 2001; Koh et al. 2003; Nusbaum and Beenakker 2002; Sakurai and Katz 2009). Such studies are well illustrated by the actions of two modulatory pathways in *Aplysia*: one of which originates in the higher-order interneuron, CBI-3 (Jing and Weiss 2001; Morgan et al. 2002) and the other in the EN. Modulatory actions of CBI-3 and the EN oppose each other as the former promotes
ingestive programs, while the latter promotes egestive programs. Mechanistically, the network actions of the EN and CBI-3 also oppose each other. The EN uses SCP to promote activity of B40 and suppress activity of B20. In contrast, CBI-3, active during protraction, uses fast excitation to promote activity of B40 and fast GABAergic inhibition to suppress activity of B20 (Jing et al. 2003; Jing and Weiss 2001). Moreover, CBI-3 uses neuropeptide APGWamide to suppress activity of egestion-promoting CPG neurons B4/5 and B70 that are active during retraction (Jing and Weiss 2001; Sasaki et al. 2009). Thus, neuromodulators, in conjunction with classical transmitters, act on specific CPG elements to configure the network for the expression of motor programs that are appropriate for specific situational demands.

Summary

We have identified peptidergic modulatory mechanisms that may contribute to the establishment of the egestive network state. More generally, we showed that the peptide modulator (SCP) and a modulatory CPG interneuron form a composite FFL that is a combination of two coherent FFLs, with one FFL functioning to enhance the synergist output while the other FFL to suppress the competing output. Because network motifs such as FFLs have been shown to have important functions in other complex networks such as transcription regulation networks (Alon 2007), it is possible that the composite FFL identified in this paper may operate and fulfill similar functions in other networks.
ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health grant NS070583 (KRW), DA 13330 (FSV) and NS 031609 (JVS), and by National Institute on Drug Abuse Award P30 DA 018310 to the University of Illinois at Urbana–Champaign Neuroproteomics Center.


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FIGURES LEGENDS

FIG. 1. Schematic drawings of coherent and incoherent feedforward loops (FFLs). Feedforward loops involve two interacting pathways from X to Z: one is direct, one is indirect through Y. In a coherent FFL, indirect and direct pathways have the same signs on Z, while in an incoherent FFL, the two pathways have different signs on Z. Illustrated are two examples, several other possible schemes are possible (see Alon 2007). Arrows, excitatory; Bars, inhibitory.

FIG. 2. SCP immunostaining in the buccal ganglion and gut. A: Distribution of SCP immunoreactive neurons and fibers in the buccal ganglion. A1: rostral surface, showing a cluster of small immunoreactive mechanoafferent neurons as identified before. A2: caudal surface, showing immunoreactive esophageal cluster including B1 and B2 (arrow) as described before. Note that immunoreactive fibers are also present in the two peripheral nerves, i.e., the radula nerve (RN), and most prominently in the esophageal nerve (EN). B: Immunostaining of the anterior branch (AB) and the posterior branch (PB) of the EN following nerve crush of both branches. Top part of B1 and B2 is the proximal ends of the nerves that are connected with the buccal ganglion, while the bottom part of B1 and B2 is the distal ends of the nerves that are connected with the sections of the gut. There is accumulation of SCP (arrows) in proximate ends and distal ends of both branches of the EN, but most obvious in the PB. B1: Left esophageal nerve crush, B2: Right esophageal nerve crush. B3: The proximal end of a posterior branch in higher magnification. Crush end at right. B4: The distal end of a posterior branch in higher magnification. Crush end at left. C-F: SCP immunoreactive fibers and somata are present in the gut, including the esophagus (C), crop (D), stomatogastric ring (E), and filter chamber (F).
Arrowheads point to the isolated SCP containing somata in C and F. In the filter chamber, there are a number of SCP containing somata. SCP immunofluorescence is shown as negative image to enhance visibility. Scale Bar is 350 µm for Part A, 250 µm for Part B1,B2, 40 µm for Part B3,B4, and 100 µm for Part C-F.

FIG. 3. SCP release in the buccal ganglion increases following EN stimulation. (Top) MALDI TOF mass spectra obtained from buccal SPE bead samples over the course of 30 minutes prior to stimulation of the EN show marginal release of SCPs. (Bottom) Bead samples of releasates collected over the course of 30 minutes following stimulation of the ipsilateral EN demonstrate marked release of SCP A and B as well as other unidentified compounds observed at 1175.5 and 1293.5 m/z.

FIG. 4. SCP converts ingestive programs elicited by CBI-2 into egestive ones. A: Representative examples. Every 1 minute, CBI-2 was stimulated with short current pulses at 10Hz for the duration of protraction to elicit single cycle of program regularly. A1: A cycle of an ingestive motor program elicited by CBI-2 in which B8 fired more strongly during retraction (filled bar) and weakly during protraction (open bar). A2: A cycle of a CBI-2-elicited motor program in the presence of 10⁻⁵ M SCPB. The program became egestive because B8 fired only during protraction. A3: A cycle of a CBI-2-elicited program after washout of SCP in which B8 activity returned to the control level as in A1. B: 2-D graph showing B8 activity during protraction vs. retraction in 14 individual experiments under “control” (large circles), “10⁻⁶ M SCPB” (medium circles), “10⁻⁵ M SCPB” (small circles) conditions. C: 2-D graph showing mean activity of B8 during protraction vs. retraction in motor programs elicited under “control”, “10⁻⁶ M SCPB” and
“10^{-5} \text{ M SCP}_{\text{B}}” \text{ conditions, illustrating that B8 activity during protraction increased, while B8 activity during retraction decreased.}  D: \text{ Group data showing separately B8 activity during protraction (D1) or retraction (D2), illustrating that SCP}_{\text{B}} had a significant effect on B8 activity during the two phases. Bonferroni post-hoc tests: *, \text{ } P < 0.05; **, \text{ } P < 0.01; ***, \text{ } P < 0.001. \text{ Error bars indicate SEM.}

FIG. 5. SCP has opposite effects on firing frequency of B20 and B40.  A: SCP_{\text{B}} increased B20 firing frequency in a concentration dependent manner. Bottom: group data.  B: SCP_{\text{B}} at 10^{-6} \text{ M suppressed B40 firing frequency. Bottom: group data. Bonferroni post-hoc tests: *, } P < 0.05; **, \text{ } P < 0.01; ***, \text{ } P < 0.001. \text{ Error bars indicate SEM.}

FIG. 6. Effects of SCP on synaptic connections from B20 to B8 and from B40 to B8.  A: SCP_{\text{B}} increased the amplitude of fast EPSPs from B20 to B8. Bottom: group data. For the group data, amplitude of the six EPSPs in a single trial was averaged.  B: SCP_{\text{B}} at 10^{-6} \text{ M decreased the amplitude of the slow EPSPs from B40 to B8. Bottom: group data. Bonferroni post-hoc tests: **, } P < 0.01; ***, \text{ } P < 0.001. \text{ Error bars indicate SEM.}

FIG. 7. Effects of SCP and EN stimulation on B65 firing frequency.  A: SCP_{\text{B}} increased B65 firing frequency in a concentration dependent manner. Bottom: group data.  B: B65 firing frequency was increased 10 s following EN stimulation (Post EN). Bottom: group data. Bonferroni post-hoc tests: *, \text{ } P < 0.05; **, \text{ } P < 0.01; ***, \text{ } P < 0.001. \text{ Error bars indicate SEM.}
FIG. 8. Schematic diagrams of modulatory actions of neuropeptide SCP and the modulatory interneuron B65, and the feedforward loops (FFLs) formed by these two modulators. A,B: The Aplysia feeding CPG consists of protraction interneurons (P, e.g., B63) and retraction interneurons (R, e.g., B64) that inhibit each other, and mediate alternating protraction-retraction. The switch from protraction to retraction is mediated in part by the slow excitation of R neurons by P neurons. Three CPG interneurons, B65, B40 and B20 receive both chemical and electrical excitation from P neurons, and are also active during protraction. One major function of these neurons is to control activity of B8. B40 exerts fast inhibition and slow excitation on B8 to promote B8 activity during retraction in ingestive programs (see B1). B20 exerts fast excitation on B8 to promote B8 during protraction in egestive programs (see A, B2). A: During EN stimulation, B65 promotes egestive programs by enhancing B20 activity and suppressing B40 activity and synaptic output. As shown in the paper, SCP also promotes egestive programs by enhancing B20 activity and synaptic output and suppressing B40 activity and synaptic output (red). B1: During CBI-2 stimulation, CBI-2 promotes ingestive programs by providing larger excitation to B40 than to B20. In addition, CBI-2 inhibits B65 activity. B2: Following EN stimulation (Post EN), CBI-2 stimulation elicits egestive programs, in part because modulatory effects of B65 persist, even though B65 stops firing due to inhibition from CBI-2. Furthermore, modulatory actions of SCP also contribute to establishment of the egestive network state. Some connections (e.g., fast excitation from EN stimulation to B20) are omitted for clarity. C: Summary diagrams of the FFLs from SCPergic neurons to B20 and B40. C1: The FFL to B20 is excitatory. C2: The FFL to B40 is inhibitory. C3: The composite FFL from a combination of two loops shown in C1 and C2 acts in concert to promote egestive output over ingestive output by changing the balance of activity of B20 over B40. Open triangle: excitation; filled
circle: inhibition; resistor symbol, electrical connection. S: slow connections, M: modulation.

All symbols in C represent modulation.
Coherent FFL

\[ X \rightarrow Y \rightarrow Z \rightarrow X \]

Indirect

Direct

Incoherent FFL

\[ X \rightarrow Y \rightarrow Z \rightarrow X \]

Indirect

Direct
A  Egestive

B1  Ingestive

B2  Egestive (Post EN)

C1  Coherent FFL type 1

C2  Coherent FFL type 3

C3  A composite FFL