Interneuronal and Peptidergic Control of Motor Pattern Switching in *Aplysia*

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Morgan, Peter T., Jian Jing, Ferdinand S. Vilim, and Klaudiusz R. Weiss. Interneuronal and peptidergic control of motor pattern switching in *Aplysia. J Neurophysiol* 87: 49–61, 2002; 10.1152/jn. 00438.2001. It has been proposed that a choice of specific behaviors can be mediated either by activation of behavior-specific higher order neurons or by distinct combinations of such neurons in different behaviors. We examined the role that two higher order neurons, CBI-2 and CBI-3, play in the selection of motor programs that correspond to ingestion and egestion, two stimulus-dependent behaviors that are generated by a single central pattern generator (CPG) of *Aplysia*. We found that CBI-2 could evoke either ingestive, egestive, or ambiguous motor programs depending on the regime of stimulation. When CBI-2 recruited CBI-3 firing via electrical coupling, the motor program tended to be ingestive. In the absence of CBI-3 activation, the program was usually egestive. When CBI-2 was stimulated to produce ingestive programs, hyperpolarization of CBI-3 converted the programs to egestive or ambiguous. When CBI-2 was stimulated to produce egestive or ambiguous programs, co-stimulation of CBI-3 converted them into ingestive. These findings are consistent with the idea that combinatorial commands are responsible for the choice of specific behaviors. Additional support for this view comes from the observations that appropriate stimulus conditions exist both for activation of CBI-2 together with CBI-3, and for activation of CBI-2 without a concomitant activation of CBI-3. The ability of CBI-3 to convert egestive and ambiguous programs into ingestive ones was mimicked by application of APGWamide, a neuropeptide that we have detected in CBI-3 by immunostaining. Thus combinatorial actions of higher order neurons that underlie pattern selection may involve the use of modulators released by specific higher order neurons.

INTRODUCTION

Many behaviors consist of a stereotyped pattern of muscle activation (Delcomyn 1980). These patterned behaviors (e.g., respiration, mastication, locomotion, and scratching) are generated by ensembles of interconnected neurons named central pattern generators (CPGs) (Delcomyn 1980; Marder and Calabrese 1996). Often, individual CPGs can generate multiple patterned motor outputs. Neuronal mechanisms that are responsible for the ability of CPGs to generate multiple motor outputs have been of significant theoretical and experimental interest (e.g., Croll and Davis 1982; Dickinson et al. 1990; Flamm and Harris-Warrick 1986a,b; Hooper and Marder 1987; Katz and Frost 1995, 1997; Wood et al. 2000).

Most of the experimental work on the ability of CPGs to generate multiple motor patterns has been performed in preparations that are either autoactive or are stimulus-autonomous, i.e., they produce a motor rhythm even when the triggering stimulus is no longer present. This work has suggested that activity of partially overlapping sets of higher order neurons (such as command neurons, trigger neurons, or gating neurons) may be responsible for initiation of distinct autoactive or stimulus-autonomous motor programs (e.g., Combes et al. 1999a,b). It is still not clear, however, whether overlapping or nonoverlapping sets of higher order neurons integrate sensory inputs and generate the distinct motor patterns that mediate stimulus-dependent behaviors. Indeed, in one case where this question was studied, it has been hypothesized that distinct sets of neurons activate the CPG for each behavior (Croll et al. 1985). However, this hypothesis is tentative as some of the critical neurons have not been identified in this study. Thus the alternative possibility that intersecting sets of higher order neurons activate the CPG for each stimulus-dependent behavior remains.

A model CPG that generates multiple stimulus-dependent behaviors and permits the study of these questions is the *Aplysia* feeding CPG (Hurwitz and Susswein 1996; Kupfermann 1974; Perrins and Weiss 1998; Susswein and Byrne 1988). Higher order neurons, the cerebral-to-buccal interneurons (CBIs), that may be involved in CPG pattern selection have been identified. The CBIs are not spontaneously active but respond to feeding-related sensory inputs (Rosen et al. 1991), and they generate feeding-like motor patterns when stimulated (e.g., Church and Lloyd 1994; Morgan et al. 2000; Rosen et al. 1991; Sanchez and Kirk 2000). We sought to characterize the role that individual CBIs and combinations of CBIs play in the generation of ingestive and egestive motor patterns. In this paper, we present evidence that activation of different combinations of higher order neurons rather than activation of behavior-dedicated higher order neurons may be responsible for selection of different motor patterns in the feeding CPG of *Aplysia*.

A fundamental question that remains, independent of the specifics of circuitry that may allow higher order neurons to determine which motor pattern is generated, is how the complex reorganization of motor outputs of CPGs is achieved. A major hypothesis that has emerged from the work on autoactive

or stimulus-autonomous behaviors is that the ability of CPGs to produce multiple motor outputs may be due to actions of modulators that are released from higher order neurons (e.g., Wood et al. 2000). These modulators may determine pattern selection by modifying the biophysical characteristics and synaptic connections of CPG elements (Harris-Warrick and Marder 1991). In contrast to the extensive research that has been done on autoactive or stimulus autonomous circuits, little information is available about the role of modulation in motor pattern selection for stimulus-dependent behaviors. Therefore we sought to determine whether modulators contained in the CBIs could participate in the process of motor program selection. We find that neuropeptide APGWamide, which we have localized to neuron CBI-3, mimics the program-switching actions of this neuron. Thus peptidergic transmission may contribute to selection of specific motor programs in the feeding CPG of *Aplysia*.

METHODS

Animals

Experiments were performed on *Aplysia californica* weighing 100–250 g. The animals were obtained from Marinus (Long Beach, CA) and from the National Resource for *Aplysia* at the University of Miami. They were maintained at 14–15°C in holding tanks for 3–7 days then transferred to room temperature tanks (22–24°C) and kept there for 2 days prior to use. This treatment was shown to increase the probability of feeding behavior being elicited in semi-intact preparations (Weiss et al. 1986). The animals were anesthetized by the injection of isotonic $MgCl_2$ solution (50% of body wt) into the body cavity. In the isolated ganglia preparation, the cerebral and buccal ganglia were removed with the cerebral-to-buccal connectives (CBCs) intact. The ganglia were pinned to a silicone elastomer (Sylgard; Dow Corning, Midland, MI)–bottomed dish filled with a dissection solution consisting of 50% artificial sea water (ASW) and 50% isotonic $MgCl_2$ solution at room temperature. Cerebral ganglia were pinned ventral surface up, and buccal ganglia were pinned with either the rostral or the caudal surface up. The connective tissue sheath that covers the neurons was surgically removed from all ganglia from which intracellular recordings were to be made, and the dissection solution was replaced with 100% ASW (in mM: 460 NaCl, 10 KCl, 11 $CaCl_2$, 55 $MgCl_2$, and 10 HEPES, at pH 7.6). All salts were obtained from Sigma, St. Louis, MO. The cerebral and buccal ganglia were separated into two compartments by the placement of a polyethylene ring around the buccal ganglion. Silicone vacuum grease (Dow Corning, Midland, MI) was used to create a water-tight seal between the separator ring and the Sylgard without damaging the CBCs.

In the periphery-attached preparation, the structures of the head innervated by the upper labial, lower labial, and anterior tentacular nerves of the cerebral ganglion (including the lips, jaws, peri-oral area, and tentacles) as well as the structures of the head innervated by all the nerves of the buccal ganglion (the buccal mass and the proximal esophagus) were removed along with the cerebral and buccal ganglia. The pleural and pedal ganglia were also dissected with the cerebropleural and cerebropedal connectives intact.

Electrophysiology

Intracellular recordings were made using single-barreled microelectrodes filled with 2 M potassium acetate and beveled to a resistance of 6–8 MΩ. An Axoclamp 2A (Axon Instruments, Burlingame, CA) and two homemade amplifiers were used for the recordings. Neurons were identified based on size, morphology, membrane properties, and synaptic connections. Extracellular recordings were made using polyeth-ylene suction electrodes placed on the nerve of interest (Morton and Chiel 1993a,b) and connected to a model P15 AC amplifier (Grass Medical Instruments, Quincy, MA). Neurons were stimulated either by the injection of a constant, DC current, or by the injection of short DC pulses (each of which elicited a single spike) with frequencies ranging from 5 to 15 Hz produced by a model S88 stimulator (Grass Medical Instruments). The esophageal nerve was stimulated by injecting 3-ms, 1-V pulses at 1 Hz generated by the S88 stimulator through the differential AC amplifier model 1700 (A-M Systems, Carlsborg, WA) into a polyethylene suction electrode placed on the nerve. The bathing solution was kept at 16–18°C during the electrophysiological experiments.

Immunocytochemistry

The rat antibodies to APGWamide were obtained using previously described methods (Vilim et al. 1996). Briefly, the antigen was prepared by coupling 2 mg of APGWamide (AnaSpec, San Jose, CA) to 10 mg of BSA (SIGMA A0281) using 20 mg of 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC; SIGMA E7750) in a 0.5-ml volume of 50 mM NaH_2PO_4 (pH 7.2). The mixture was incubated overnight at 4°C, then purified and concentrated using a Centricon-10. The retentate was washed four times with 2 ml of 50 mM NaH_2PO_4 (pH 7.2), then resuspended in 0.5 ml of the same buffer and transferred to a new tube. This antigen was used to immunize two male Sprague Dawley rats (Teconic, 250–300 g) by intraperitoneal injection in an emulsion of 0.5 ml phosphate-buffered saline (PBS) and 0.5 ml of Freund's complete adjuvant. At 21 days and 42 days post initial injection, the rats were boosted by intraperitoneal injection with antigen in an emulsion of 0.5 ml PBS and 0.5 ml of Freund's incomplete adjuvant. One rat received 250 μg of antigen initially followed by 125 μg antigen for each boost; the other rat received 100 μg antigen initially and 50 μg antigen for each boost. The animals were killed by decapitation at 49 days post initial injection, and the blood was collected and processed for serum. Sera were aliquoted, frozen, and lyophilized, or stored at 4°C with EDTA (25 mM final) and thimerosal (0.1% final) added as stabilizers. Of the two antibodies, the higher dosage gave better immunostaining and was used for all the experiments.

Backfills of the CBC were performed using biocytin (Sigma). The cut end of the CBC was pinned inside a subchamber that was isolated from the fluid bathing the ganglion (sterile filtered 50% hemolymph/ 50% ASW) using a silicone grease seal. The cut end of the CBC was then osmotically shocked using a dH_2O wash followed by application of 10 μl biocytin saturated dH_2O. The preparation was incubated in a humidified chamber overnight at 15°C to allow for transport of the biocytin. The ganglia were then washed with several changes of 50% ASW/50% isotonic $MgCl_2$ and fixed with freshly prepared fixative (4% paraformaldehyde, 0.2% picric acid, 25% sucrose, 0.1 M NaH_2PO_4, pH 7.6) for either 3 h at room temperature or overnight at 4°C. The ganglia were then processed as described below for CP-1 and APGWamide immunocytochemistry except that biocytin was visualized using fluorescein coupled streptavidin (Jackson Immuno Research, West Grove, PA) and the ganglia were cleared with 50% glycerol, 50% PBS prior to photodocumentation.

After electrophysiological identification, neurons were filled with 3% 5(6)-carboxyfluorescein in 0.1 M potassium citrate, titrated to pH 8.0 with KOH (Rao et al. 1986), by iontophoresis (10–15 min of 500 ms, 2-nA pulses at 1 Hz). The immunohistochemical methods used were previously described (Morgan et al. 2000). Ganglia were fixed with 4% paraformaldehyde in phosphate-sucrose buffer for 2–4 h at room temperature. The ganglia were washed repeatedly in phosphate buffer and were placed in triton-azide-phosphate buffer and kept at 4°C. The ganglia were exposed to 0.5% normal goat serum (Jackson Immuno Research) for 2 h and subsequently to a 1:200 dilution of primary antibody either APGWamide or CP-1 (rabbit anti-CP-1 was a kind gift of Dr. Philip E. Lloyd, University of Chicago) was added. Following a 24-h incubation, the ganglia were washed repeatedly in phosphate buffer and left in

phosphate buffer for 24 h. The ganglia were then placed in triton-azide-phosphate buffer with a 1:100 dilution of rhodamine-conjugated secondary antibody (Jackson Immuno Research) for 24 h. Following this incubation the ganglia were again washed repeatedly in phosphate buffer and left for 48 h in phosphate buffer. During this 48-h period, the phosphate buffer solution was exchanged every 12 h to facilitate removal of the secondary antibody. Ganglia so prepared were mounted on depression slides in the phosphate buffer (as neurons of interest are superficial, no clearing agents were needed) and examined on a microscope equipped with filter packs for viewing rhodamine and carboxyfluorescein epifluorescence [buffers: phosphate buffer (in mM): 20 K_2HPO_4, 40 KH_2PO_4, and 140 Na_2HPO_4; phosphate-sucrose buffer: phosphate buffer diluted 1:2 in H_2O with 30% sucrose final concentration; phosphate-triton-azide buffer: phosphate buffer diluted 1:2 in H_2O with 2% Triton X-100 and 0.1% NaN_3, both final concentrations; all salts and sucrose from Sigma].

Identification of motor patterns

Both ingestive and egestive behaviors in *Aplysia* are implemented by a common set of muscles, but the sequence of muscle contractions and of activity of motor neurons that control these muscles in the two behaviors is different. The most salient difference between the two classes of behavior is manifested in the relative timing of the radula opening/closing versus radula protraction/retraction (Church and Lloyd 1994; Cropper et al. 1990; Morton and Chiel 1993a,b; Rosen et al. 1998). In ingestive behaviors, the radula closes during retraction and opens during protraction, while in egestive behaviors, the radula closes during protraction and opens during retraction.

Several motor neurons that control radula opening/closing and radula protraction/retraction have been identified (e.g., Church and Lloyd 1994; Cohen et al. 1978; Evans et al. 1996; Hurwitz et al. 1996; Morton and Chiel 1993a,b). Their firing patterns, as well as the firing patterns of several interneurons, have been characterized during ingestive and egestive behaviors elicited in intact and/or semi-intact preparations (e.g., Church and Lloyd 1994; Cropper et al. 1990; Hurwitz and Susswein 1996; Hurwitz et al. 1996; Morton and Chiel 1993a,b; Rosen et al. 1991). We used this information to distinguish between ingestive and egestive motor patterns that were elicited in the isolated nervous system.

In ingestive behaviors, radula closers B8 and B16 fire strongly during the retraction phase; in egestive behaviors, they fire strongly during protraction. In addition, in ingestive behaviors, a smooth depolarization leading to different amounts of firing is recorded in these two motor neurons during protraction (Church and Lloyd 1994). Protraction and retraction can be identified by the activity of two nerves, the interneuron B4, as well as the characteristic behavior of motor neuron B8, and the command like-neuron CBI-2. In buccal nerve 2 (BN2), the beginning of protraction is accompanied by the increase in frequency over baseline of a single, characteristic unit, and retraction is indicated by the presence of multiple units including a large unit corresponding to retraction motor neuron B10 (Morton and Chiel 1993a,b). Also, motor neuron B8 displays a slow depolarization during protraction phase (Church and Lloyd 1994), and this depolarization can be used to monitor the protraction phase. In nerve I2, the only motor neurons that send their axons via this nerve are protraction motor neurons (B31/32 and B61/62), and thus the protraction phase is demarcated by the presence of activity in this nerve (Hurwitz et al. 1996). In B4, the retraction phase is clearly demarcated by the presence of a large depolarization from baseline and the presence of action potentials [the presence and frequency of which depends on the nature of the motor pattern (Church and Lloyd 1994; Warman and Chiel 1995)]. We also used the inhibition of CBI-2 as an indicator of the retraction phase (Church and Lloyd 1994; Rosen et al. 1991).

Measurements of protraction and retraction phase durations were made using the characteristics of the phases described above. In experiments in which we were able to use more than one criterion to define specific phases of the program, we found an excellent correspondence between the criteria we used. In the figures, the protraction phase is indicated by the open bar, and the retraction phase is indicated by the closed bar.

To classify motor programs as ingestive, egestive, or ambiguous, we performed a cluster analysis on the firing of radula closer motor neurons B8 and B16 during the protraction and retraction phases of the motor programs (Fig. 1). In the figures, ingestive programs are indicated by an open circle within the protraction bar, while egestive programs are indicated by a closed circle within the protraction bar.

RESULTS

Classification of motor patterns

Ingestive and egestive motor programs differ from each other in that during ingestive programs, the radula closes during retraction phase, while during egestive programs, the radula closes during protraction phase. Therefore, to distinguish between motor patterns, we analyzed the firing of radula closing motor neurons B8 and B16 during protraction and retraction phases. Specifically, cycles were assessed to be ingestive-like or egestive-like based on the firing frequency of B8 or B16 during the protraction and retraction phases. An analysis of 243 cycles from CBI-2–induced motor patterns revealed three distinct groups of B8 or B16 activity (Fig. 1).

To perform cluster analysis, we first plotted the data separately for firing of the two radula closure motor neurons, B8 and B16, as shown Fig. 1A. For each cycle, we plotted the frequency of motor neuron firing during protraction (*x*-axis) versus retraction (*y*-axis). Visual inspection of these plots suggested the presence of three distinct clusters of firing of B8 and B16. Two of the clusters were large, and one was small. For the purpose of cluster analysis, these data can be grouped in four ways. First, the data can be treated as one group. Second, the data can be treated as three groups as suggested by visual inspection (see Fig. 1*A1* for B8 and Fig. 1*A2* for B16, in which separate clusters are encircled). Third, the data can be treated as two groups in which case the smallest visually apparent group is added to either of the larger groups. Fourth, three groups can be formed by random assignment of the data points, and we performed two such random assignments.

In our cluster analyses, we calculated the sums of squares and products matrices for each grouping (separately for B8 and for B16 data). The determinants of the matrices were taken and divided by the total number of data points, and the values (W) were compared.

The data taken as the three groups, as suggested by visual inspection, resulted in the smallest of all the Ws calculated (0.95 for B8 and 0.74 for B16). In comparison, the calculated Ws, when the data were treated as one group, were 33.5 for B8 and 29.6 for B16. For the two group analyses the Ws were 3.7 and 6.5 for B8 and 2.1 and 4.3 for B16. For the random three group analyses, the Ws were 9.7 and 15.3 for B8, and 4.2 and 6.6 for B16. Hence, the visual indication that three groups are present is supported by cluster analysis. Thus ingestive-like, egestive-like, and ambiguous cycles can be defined by the relative firing frequencies of radula closure motor neurons during protraction and retraction phases of the motor program.

Specifically, ingestive-like cycles were defined by an average firing frequency of B8 during protraction of less than 3.5 Hz (B16 less than 4.5 Hz) and during retraction of over 4.5 Hz (B16 over 5.5 Hz); the ratio of protraction to retraction phase
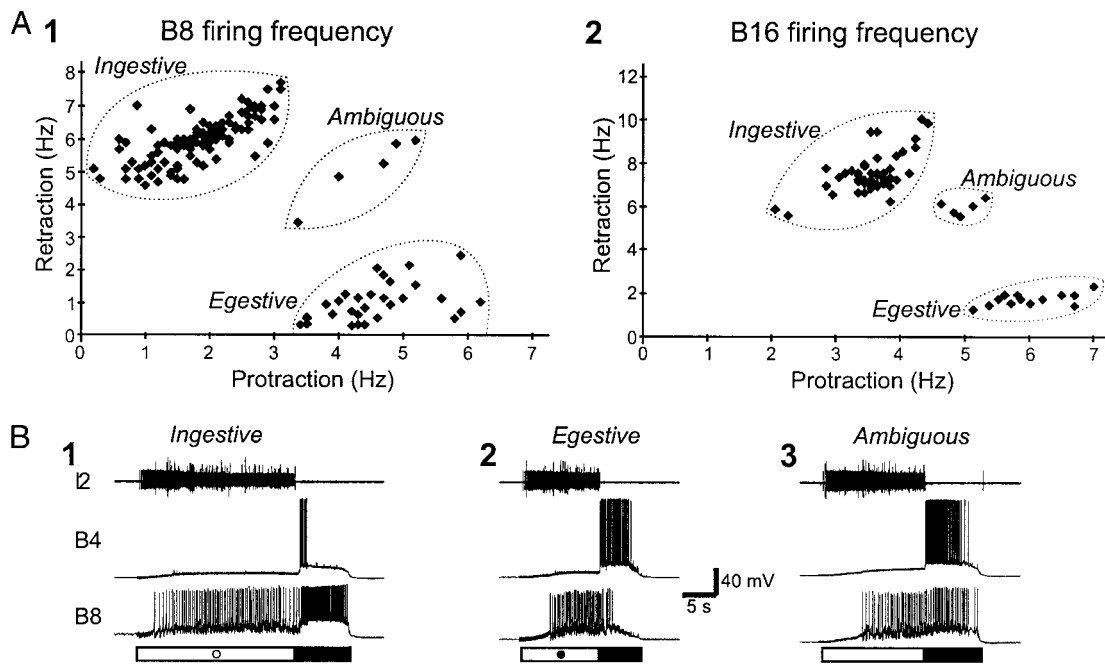
FIG. 1. Classification of motor programs. *A*: graphical representation of distribution of B8 (*A1*) and B16 (*A2*) firing frequency during protraction and retraction phases of the CBI-2–induced motor program. The average firing frequency was calculated during the burst of B8 and B16 firing, rather than the entire duration of each phase. In both panels, 3 groups of firing frequencies are apparent: the largest group with low protraction-phase firing frequency and high retraction-phase firing frequency is consistent with the characteristics of ingestive motor programs; the next largest group with high protraction phase firing frequency and low retraction phase firing frequency is consistent with egestive motor programs; the smallest group with high firing frequency in both protraction and retraction cannot be clearly assigned to either ingestive or egestive motor programs, and these programs are defined as ambiguous. *B*: examples of different motor programs (*B1*, ingestive program; *B2*, egestive program; *B3*, ambiguous program). In ingestive programs, B8 firing frequency is higher in the protraction phase than in the retraction phase. In egestive programs, B8 is firing at higher frequencies during protraction than during retraction. In ambiguous programs, B8 is firing at similar rates during protraction and retraction. Protraction was monitored by neural activity in the I2 nerve. Retraction was monitored by the sustained depolarization of neuron B4 that occurs after the termination of protraction phase.

average firing frequency is less than 0.65 (less than 0.75 for B16). Egestive-like cycles were defined by an average firing frequency of B8 during protraction of greater than 3.5 Hz (B16 > 5 Hz) and during retraction of less than 2.5 Hz (B16 less than 2.5 Hz); the ratio of protraction to retraction phase average firing frequency is greater than 2.0 (greater than 2.5 for B16). Of the 243 test cycles, 163 were ingestive-like, 51 cycles were egestive-like, and 10 cycles could not be placed into either group. The ingestive-like cycles had an average protraction phase firing frequency of 1.9 (range 0–3.1) and an average retraction phase firing frequency of 6.0 (range 4.6–7.7) for B8 and 3.6 (range 2.0–4.5) and 7.6 (range 5.6–10.1), respectively, for B16. The egestive-like cycles had an average protraction phase firing frequency of 4.5 (range 3.5–6.2) and an average retraction phase firing frequency of 0.98 (range 0.36–2.5) for B8 and 6.0 (range 5.1–7.0) and 1.8 (range 1.3–2.4), respectively, for B16. The cycles that did not fit either the ingestive-like or egestive-like criteria either had sustained high-frequency activity of B8 or B16 throughout protraction and retraction or had moderate activity of B8 or B16 throughout protraction and retraction. There were no cycles that met the criteria for placement based on absolute frequencies but failed based on the protraction to retraction firing frequency ratio. These criteria were used to define the three types of motor patterns in the present study and are used throughout this manuscript to describe our findings. In the figures, ingestive programs are indicated by the open circle within the bar that marks protraction, and egestive programs are indicated by the closed circle within the protraction marking bar.

Immunostaining of neuron CBI-3

The cerebral ganglion of *Aplysia* contains a number of interneurons (the CBIs) that project to the buccal ganglia that contain the essential elements of the feeding CPG and generate feeding-related motor outputs. Similar interneurons have been described in related species of mollusks (e.g., Delaney and Gelperin 1990a–c; Gillette et al. 1982; Kemenes et al. 2001; Kovac et al. 1983a,b; McCrohan and Kyriakides 1989). Many of the *Aplysia* CBIs receive sensory inputs known to initiate feeding behavior, and stimulation of several of these neurons can induce or modify the feeding motor patterns of the buccal ganglia (Church and Lloyd 1994; Morgan et al. 2000; Perrins and Weiss 1998; Rosen et al. 1991; Sanchez and Kirk 2000; Xin et al. 1999). However, it is not known whether distinct CBIs activate different motor patterns or whether some CBIs participate in the initiation of more than one motor pattern. In view of extensive evidence that motor pattern selection is often accomplished through neuromodulation, we reasoned that the CBIs involved in pattern selection may contain neuromodulators. Previous studies demonstrated the presence of neuromodulators in CBI-1, CBI-2, CBI-8/9, and CBI-12 (Hurwitz et al. 1999; Morgan et al. 2000; Rosen et al. 1991; Xin et al. 1999). Although some of these modulators could modify the

characteristics of motor programs, the modulators did not appear to be involved in motor pattern selection. Previous studies (Phares and Lloyd 1996) reported immunostaining for neuropeptide CP-1 in the CBI-containing M cluster of the cerebral ganglion. This raised the question of whether motor pattern selection is mediated by CP-1 or perhaps by the neuropeptide APGWamide, which is coded on the same precursor as CP-1 (Fan et al. 1997). Our interest in APGWamide was further stimulated by the observation that in *Lymnaea* this peptide is present in one of the *Lymnaea* CBIs, cell CBWC (McCrohan and Croll 1997). Unfortunately, in *Lymnaea* the effects of APGWamide were complex and could not be clearly matched with the effects of CBWC stimulation.

To determine whether the immunostaining reported in the M cluster is localized to the CBIs, we first ($n = 4$) combined backfills of the cerebro-buccal connectives with immunostaining for CP-1 and APGWamide, two products of the same gene (Fan et al. 1997). Figure 2 illustrates that in each M cluster only one of the backfilled neurons showed APGWamide-like immunoreactivity (Fig. 2, *A* and *B*), and only one showed CP-1-like immunoreactivity (Fig. 2, *C* and *D*).

The position of the immunostained neurons suggested that the immunostained CBI may be CBI-3. To determine whether CBI-3 is the immunoreactive CBI, we injected CBI-3 with carboxyfluorescein and stained the ganglia for APGWamide and CP-1. All of the injected CBI-3s, which were stained for APGWamide, showed immunoreactivity (6 of 6 experiments). Also, all of the injected CBI-3s, which were stained for CP-1 (4 of 4 experiments), showed immunoreactivity (Fig. 3). The staining was specific in that C11 and C12, which are adjacent to CBI-3, was not APGWamide or CP-1 immunoreactive in any of the experiments. Furthermore, when CP-1 antibody was preabsorbed with synthetic CP-1, staining with CP-1 antibody
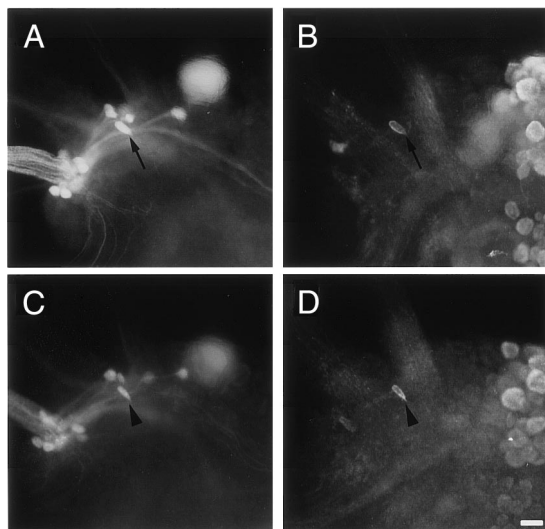

FIG. 2. A CBI in the M cluster of the cerebral ganglion immunostains for APGWamide and CP-1. *A*: a unilateral backfill of a cerebro-buccal connective (CBC) toward the cerebral ganglion (anterior ventral surface of the cerebral ganglion is shown) visualized with fluorescein. *B*: the same preparation as *A* stained with a rat antibody to APGWamide visualized with lissamine rhodamine. Arrows in *A* and *B* point to a backfilled neuron in the cerebral ganglion M-cluster that immunostains for APGWamide. *C*: another CBC backfill of the cerebral ganglion (anterior ventral surface) visualized with fluorescein. *D*: the same preparation as *C* stained with a rabbit antibody to CP-1 visualized with lissamine rhodamine. Arrowhead in *C* and *D* point to a backfilled neuron in the cerebral ganglion M-cluster that immunostains for CP-1. Scale bar is 100 μM.
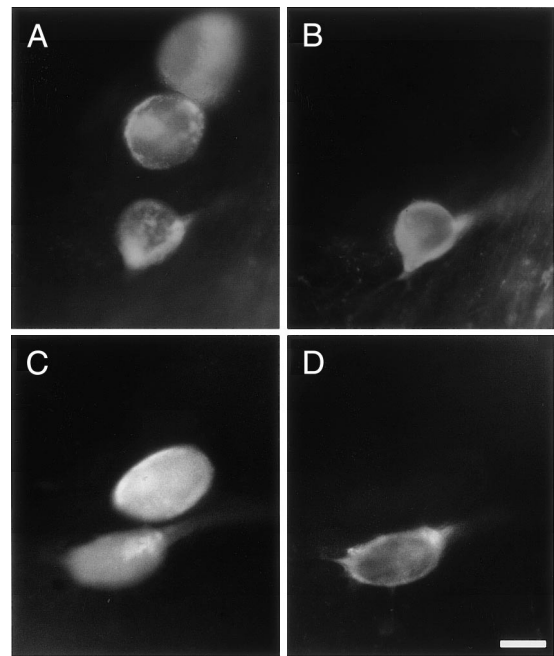

FIG. 3. CBI-3 is APGWamide and CP-1 immunoreactive. *A*: photograph showing the soma of 2 motor neurons, C11 and C12 (*top* and *middle*), and CBI-3 (*bottom*), which have been injected with carboxyfluorescein. *B*: same preparation as in *A* but with CBI-3 recognized by an antibody for APGWamide and visualized with rhodamine conjugated secondary antibody. *C*: photograph showing the soma of C12 (*above*), and CBI-3 (*below*), which have been injected with carboxyfluorescein. *D*: same preparation as in *C* but with CBI-3 recognized by an antibody for CP-1 and visualized with rhodamine conjugated secondary antibody. Scale bar is 50 μM.

was abolished ($n = 2$). Similarly, when APGWamide antibody was preabsorbed with synthetic APGWamide, staining with APGWamide was abolished ($n = 2$). The fact that antibodies directed against two structurally unrelated peptides derived from the same precursor both stained CBI-3 provides additional evidence that the antibodies are specific, and that CBI-3 expresses both CP-1 and APGWamide.

CBI-2 and CBI-3 are electrically coupled

To examine the possible role of CBI-3 in *Aplysia*'s feeding pattern selection, we first examined its relationship to CBI-2. Of the 12 identified CBIs, CBI-2 is by far the most studied (Church and Lloyd 1994; Morgan et al. 2000; Rosen et al. 1991; Sanchez and Kirk 2000). CBI-2 is involved in the generation of patterned motor neuron activity that correlates with biting and actual feeding movements (Church and Lloyd 1994; Rosen et al. 1991). The ability of CBI-2 to induce fictive biting is not diminished when chemical synaptic activity in the cerebral ganglion is blocked (Rosen et al. 1988). Thus CBI-2 activity alone or together with other CBIs that are electrically coupled to CBI-2 is sufficient to induce fictive biting.

To study the role of CBI-3 in *Aplysia* feeding, we first sought to determine whether CBI-2 and CBI-3 are coupled either chemically or electrically. No apparent chemical synaptic connections between CBI-2 and CBI-3 were observed. However, electrical coupling between CBI-2 and the ipsilateral CBI-3 was routinely observed (Fig. 4*A*). The average coupling ratio was 5.3 $\pm$ 0.5 (mean $\pm$ SE; $n = 13$) in the CBI-2 to CBI-3 direction (i.e., a current pulse in CBI-2 that produced a
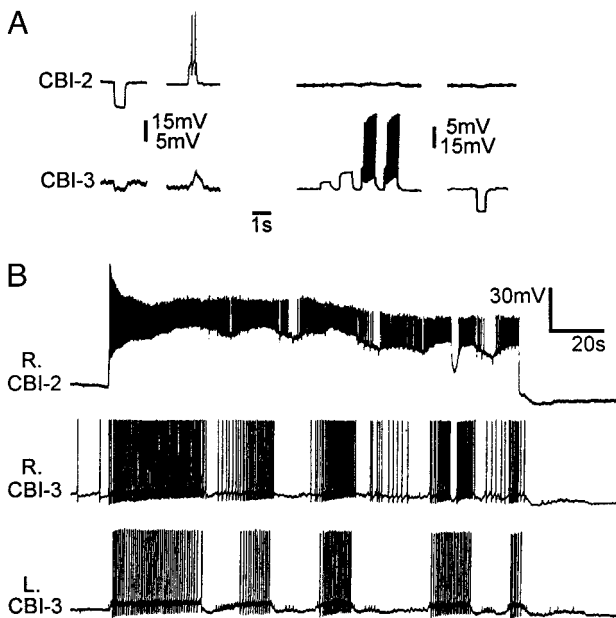
FIG. 4.   Coupling of CBI-2 and CBI-3. *A*: typical record showing the electrical coupling between CBI-2 and CBI-3. Hyperpolarizing current injected into CBI-2 (*1st record*) results in a hyperpolarization of the ipsilateral CBI-3. Depolarizing current injected into CBI-2 (*2nd record*) results in a depolarization of CBI-3. Injecting current into CBI-3 (*3rd* and *4th records*) results in small changes in CBI-2 membrane potential. *B*: typical record showing the activity in both CBI-3 s during a CBI-2 (9 nA) DC stimulation induced rhythm. Both CBI-3s are active with CBI-2 stimulation, but the ipsilateral CBI-3 shows more activity and shows a quicker response to changes in CBI-2 membrane potential.

the possibility that latent chemical connections could be upregulated during feeding. Consistent with the lack of processes to the contralateral cerebral ganglion from either neuron (Rosen et al. 1991), no electrical coupling was detected between CBI-2 and the contralateral CBI-3 ($n = 8$). Figure 4*A* shows the effect on CBI-3 of injecting hyperpolarizing or depolarizing current into the ipsilateral CBI-2.

Electrical coupling between CBI-2 and CBI-3 is not the only mechanism that is responsible for activation of CBI-3 in response to stimulation of CBI-2. When cyclic activity was induced by stimulation of CBI-2 (Fig. 4*B*), both the ipsilateral and contralateral CBI-3s were active along with CBI-2. The ipsilateral CBI-3 was invariably more active than the contralateral CBI-3 ($n = 8$). The resting potential of the two CBI-3 was similar and therefore cannot explain the differences in the firing of the two CBIs. Hence the electrical coupling between CBI-2 and the ipsilateral CBI-3 and feedback from the induced motor program onto both CBI-3s may influence the activity of CBI-3s during sustained CBI-2 activity.

Stimulation of CBI-3 modifies CBI-2–elicited motor program

As CBI-2 and CBI-3 are both activated by food stimuli that elicit ingestive feeding behavior (Rosen et al. 1991) and as CBI-3 is normally activated by stimulation of CBI-2, the effect of CBI-3 stimulation on CBI-2–induced motor programs was tested. Typically, motor programs induced by strong stimulation of CBI-2 (action potentials in CBI-2 over 10 Hz elicited by constant current injections into CBI-2) are primarily ingestivelike (Church and Lloyd 1994; Rosen et al. 1991). Furthermore, CBI-3 is active during an ingestive-like CBI-2–elicited motor program (Rosen et al. 1991). We found that CBI-2 stimulation at approximately 10 Hz by short current pulses rather than by constant current injection produced less activation of CBI-3 and produced a mixture of buccal motor programs, most of which were egestive-like or were ambiguous (i.e., neither ingestive-like nor egestive-like by the criteria set in the methods section). However, co-stimulation of CBI-3 with CBI-2 altered egestive-like or ambiguous CBI-2 motor programs to become ingestive-like.

Figure 5 illustrates a CBI-3–elicited conversion of an egestive-like program to an ingestive-like one. In these experiments, motor programs were elicited by stimulating CBI-2

15-mV depolarization would produce a depolarization in CBI-3 of approximately 2.8 mV) but was weaker, although not absent, in the CBI-3 to CBI-2 direction. This asymmetry could be partly due to differences in input resistances of these two CBIs, but this possibility was not investigated as the mechanisms underlying the asymmetrical coupling are not germane to the present study. The electrical coupling between CBI-2 and CBI-3 persisted when Ca^{2+} in the bathing solution was replaced with Co^{2+}, and synaptic potentials elicited by CBI-2 in CBI-3 were not significantly reduced, indicating that these connections are primarily electrical. Since these experiments were performed in quiescent preparations, we cannot exclude
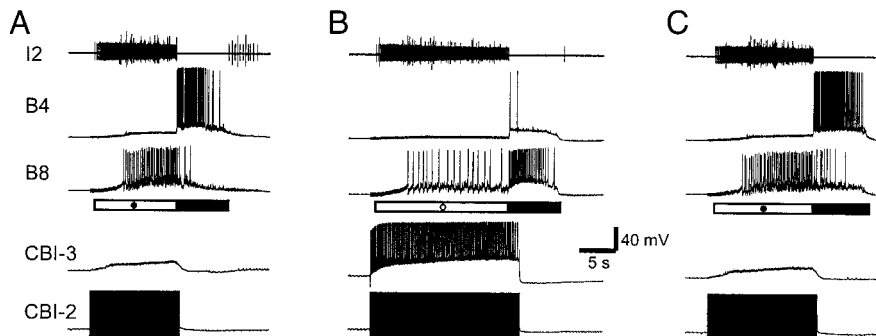

FIG. 5.   CBI-3 converts CBI-2–elicited egestive-like motor programs to ingestive-like programs. *A*: stimulation of CBI-2 with brief current pulses (10 ms; 12 nA) elicits an egestive-like motor program, i.e., the radula closure motor neuron B8 fires at high-frequency during the protraction phase (open bar) that was monitored using the bursting activity of protraction-phase nerve I2. *B*: co-stimulation of CBI-2 and CBI-3 elicits ingestive motor programs, i.e., as B8 now fires at high frequency during retraction (filled bar), as defined by the sustained depolarization of the retraction-phase interneuron B4. Note the pronounced reduction of B4 firing. *C*: stimulation of CBI-2 alone after that shown in *B,* again elicits an egestive-like program. Open circles within the protraction bar indicate ingestive-like cycles, while closed circles within the protraction bar indicate egestive-like cycles.

every 90 s. Since during normal motor programs CBI-2 fires only during the protraction phase and is inhibited during the retraction phase, stimulation of CBI-2 was terminated at the onset of the retraction phase. In the example shown, when CBI-3 was not stimulated, the motor pattern generated in response to stimulation of CBI-2 was egestive (Fig. 5, *A* and *C*), i.e., the radula closure motor neuron B8 fired at high-frequency during the protraction phase (open bar) that was monitored using neural activity in the protraction nerve I2. However, when CBI-3 was co-stimulated with CBI-2, the motor pattern became ingestive-like (Fig. 5*B*), as B8 now fired weakly during protraction but strongly during retraction (filled bar) that was monitored by the sustained depolarization of the retraction phase interneuron B4. Also notice that B4 firing was markedly reduced. Stimulation of CBI-3 during an ambiguous or egestive-like CBI-2 motor program caused an increase in the frequency of action potentials in B8 (average increase was $242 \pm 24\%$; $n = 5$) during the retraction phase. There was also a decrease in the frequency of action potentials in B8 during the protraction phase, although this effect was less dramatic than the effect on retraction phase. In addition to affecting the frequency of firing of B8, stimulation of CBI-3 also produced a decrease in the frequency of action potentials in B4 (average decrease was $76 \pm 16\%$; $n = 5$) during the retraction phase. This is consistent with the lower level of B4 activity that was observed during ingestive behavior relative to egestive behavior (Church and Lloyd 1994; Warman and Chiel 1995).

APGWamide mimics the effects of CBI-3 on the CBI-2–elicited motor program

Because APGWamide immunoreactivity is present in CBI-3, we assessed what role, if any, APGWamide might play in the action of CBI-3. To this end, motor programs were elicited by stimulation of CBI-2 before and after exogenous application of APGWamide to the buccal ganglion. Preliminary experiments in which 1 μM APGWamide and 0.1 μM APGWamide were used gave inconsistent results. In one of four experiments with 1 μM APGWamide, there were no observable changes; in two of three experiments with 0.1 μM APGWamide, there were no observable changes. However, in five experiments in which 10 μM APGWamide was used, egestive-like or ambiguous programs elicited by stimulation of CBI-2 were converted to ingestive-like programs.

Figure 6 shows that when CBI-2 and the two CBI-3 s were co-stimulated, the resulting motor program was ingestive, as the high-frequency activity of the radula closure motor neuron B16 occurred during the retraction phase, i.e., after the termination of activity in nerve I2 and while CBI-2 received an inhibitory input (Fig. 6*A*). However, when CBI-2 was stimulated but the two CBI-3 were hyperpolarized to prevent firing, the resulting motor program was egestive-like (Fig. 6*B*), i.e., the radula closure motor neuron B16 fired at high frequency during the protraction phase that was monitored using neural activity in the protraction-phase nerve I2. The effects of CBI-3 stimulation were mimicked by application of APGWamide. In the presence (after 5 min) of APGWamide (Fig. 6*C*), even though the two CBI-3s were hyperpolarized, stimulation of CBI-2 produced a motor program that was ingestive-like, as the radula closure motor neuron B16 fired at high frequency during the retraction phase, i.e., after the termination of activity in nerve I2 and while CBI-2 received an inhibitory input.

Bilateral manipulations of CBI-3s activity produce a motor program switch

The observations that stimulation of CBI-3 during an egestive-like or ambiguous CBI-2–induced motor program can convert the programs to ingestive-like led us to examine the contribution that CBI-3 activity makes to producing ingestive
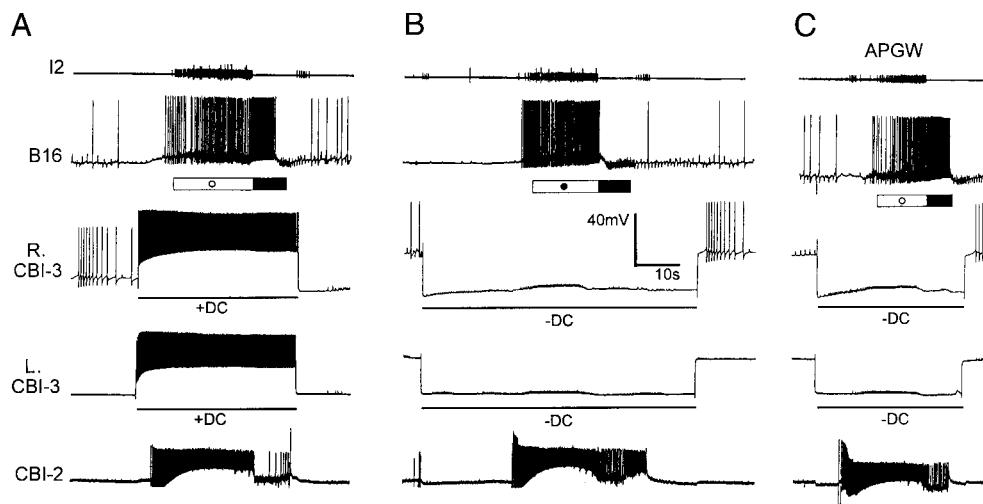

FIG. 6. The effect of APGWamide and CBI-3 stimulation on the CBI-2–induced motor programs. The activity in the I2 nerve indicates the protraction phase; the inhibition in CBI-2 after the protraction phase indicates the retraction phase (Church and Lloyd 1994). *A*: co-stimulation of CBI-2 (8 nA; DC) and 2 CBI-3s (4 nA; DC) elicits a motor program that is ingestive-like. Notice that the radula closure motor neuron B16 fires at lower frequency during protraction (open bar) than during retraction (filled bar). *B*: the 2 CBI-3s were hyperpolarized (8 nA; DC) prior to CBI-2 stimulation, and the resulting rhythm is egestive-like as indicated by the high-frequency firing of B16 activity during protraction. *C*: in the presence of 10 μM APGWamide, when the 2 CBI-3 s were hyperpolarized (8 nA; DC) prior to CBI-2 stimulation, the resulting rhythm is ingestive-like, i.e., the high-frequency firing of B16 occurs during retraction. Open circles within the protraction bar indicate ingestive-like cycles, while closed circles within the protraction phase indicate egestive-like cycles.

CBI-2 programs. However, unilateral inhibition of CBI-3 during what had been an ingestive-like CBI-2 motor program only occasionally caused a switch in the program to become egestive-like (2 of 6 experiments). Because the contralateral CBI-3 was not being monitored in these experiments, and stimulation of CBI-2 can recruit activity of the contralateral CBI-3 (see Fig. 4), it was possible that activity of the contralateral CBI-3 was sufficient to maintain the ingestive nature of the motor program. Therefore to assess the role of CBI-3 activity in producing ingestive-like CBI-2 motor programs, it was necessary to monitor and control the membrane potential of both CBI-3s simultaneously.

In nine experiments, the effects of bilateral hyperpolarization and stimulation of CBI-3 on the CBI-2–elicited motor program were assessed. In six of the nine experiments, radula closure was monitored using motor neuron B8, and in the remaining 3 experiments, radula closure motor neuron B16 was used. In all of these experiments, CBI-2 was stimulated strongly by the injection of constant current, a procedure that elicited mostly ingestive motor programs. All of the noningestive programs that we observed in this series of experiments occurred either at the beginning of CBI-2 stimulation or immediately after CBI-3s were returned to resting potential from a hypepolarization produced by intracelluar current injection.

Figure 7 illustrates an experiment in which membrane potentials of both CBI-3s were manipulated. Figure 7*A* shows a low-speed recording in which three cycles of buccal motor programs were elicited by continuous stimulation of CBI-2. *B* shows the same three cycles but with an expanded time base. *B1* corresponds to the first cycle shown in *A, B2* corresponds to the second cycle from *A,* and *B3* corresponds to the third cycle from *A*. In this experiment, the onset of small unit activity in buccal nerve 2 and concurrent depolarization of B8 were used to define the onset of protraction. Also, two criteria were used to define the onset of retraction. They were the onset of multiunit activity in buccal nerve 2 and a concurrent inhibition of CBI-2. Under control conditions (Fig. 7*B1*), when no current was injected into the two CBI-3s, CBI-2 stimulation elicited ingestive motor programs, i.e., high-frequency activity of radula closure motor neuron B8 occurred during the retraction phase (filled bar). When both of the CBI-3s were prevented from generating action potentials by injections of hyperpolarizing currents, the programs became egestive (Fig. 7*B2*); i.e., the radula closer motor neuron B8 fired at high frequency during the protraction phase. When both CBI-3s were depolarized and therefore generated additional action potentials, the program remained ingestive and the duration of protraction phase was extended (Fig. 7*B3;* see also Fig. 5).

Of the 46 control cycles in which CBI-3 membrane potential was not manipulated, 38 were ingestive-like, 5 were ambiguous, and 3 were egestive-like. Of the 28 cycles produced while sufficient hyperpolarizing current was injected into both CBI-3s to eliminate action potentials in them, 20 were egestive-like, 4 were ambiguous, and 4 were ingestive-like. Hyperpolarization of both CBI-3s that was sufficient to eliminate action potentials during a CBI-2–induced motor program caused a switch from ingestive patterns to egestive ones in seven of nine experiments. All of the 14 cycles that were produced during bilateral CBI-3 stimulation were ingestive.
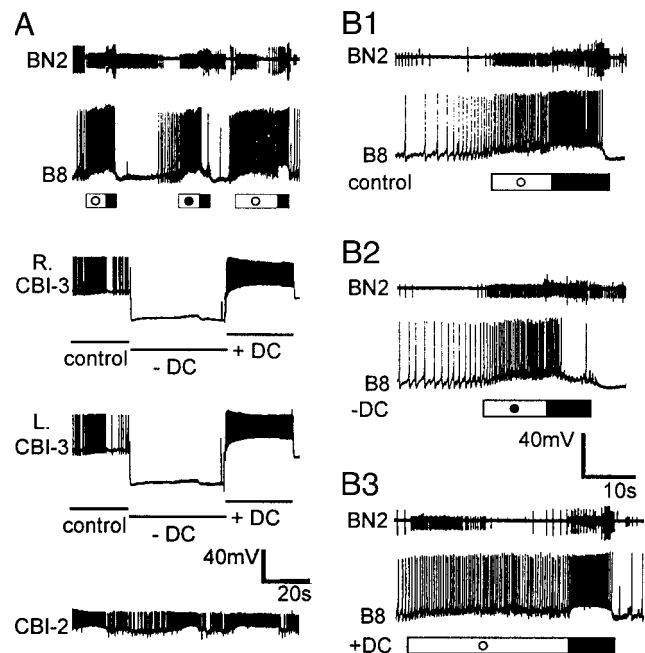

FIG. 7. The effect of bilateral manipulations of the membrane potential of CBI-3 during a CBI-2 induced motor program. *A*: continuous record of an experiment in which both the right and left CBI-3s were simultaneously hyperpolarized (8 nA), depolarized (8 nA), or left at their resting membrane potentials during an ongoing CBI-2 stimulation (9 nA; DC) elicited motor program ("control" indicates that the membrane potentials of the CBI-3s were not altered; "−DC" indicates hyperpolarization; "+DC" indicates depolarization). Manipulations of the membrane potentials of CBI-3s affected the nature of motor programs elicited by CBI-2 stimulation. *B*: expanded sample records of B8 and BN2 activity from *A*. *B1*: "Control" cycle from *A* (1st cycle) shows that when the membrane potential of CBI-3s was not manipulated, stimulation of CBI-2 elicited a motor program that was ingestive as the high-frequency firing of B8 occurred during the retraction phase (filled bar). *B2*: "−DC" cycle from *A* (2nd cycle) shows that when the 2 CBI-3s were bilaterally hyperpolarized, most of the high-frequency firing of B8 occurred during the protraction phase (open bar), and therefore the program was egestive. *B3*: "+DC" cycle from *A* (3rd cycle) shows that when the 2 CBI-3s were stimulated, the motor program remained ingestive, but the duration of protraction phase increased (see also Fig. 5). Open circles within the protraction bar indicate ingestive-like cycles, while closed circles within the protraction bar indicate egestive-like cycles.

CBI-3 stimulation and/or application of APGWamide changes activity elicited by esophageal nerve stimulation from egestive- to ingestive-like; esophageal nerve stimulation during an ingestive-like CBI-2 motor program inhibits CBI-3 and converts the program to egestive-like

Because switching from ingestive activity to egestive activity and back (or vice versa) is part of *Aplysia* behavior (Morton and Chiel 1993a,b), we examined the effect of CBI-3 activity on an egestive motor program induced by stimulation of the esophageal nerve (Chiel et al. 1986) and the effect of esophageal nerve stimulation on CBI-3 activity.

In six preparations, egestive-like motor programs were elicited by 1-Hz stimulation of the esophageal nerve (Chiel et al. 1986). Stimulation of CBI-3 during these programs caused a switch from egestive-like to ingestive-like cycles. One such experiment is illustrated in Fig. 8, which shows that when CBI-3 was not stimulated, stimulation of the esophageal nerve elicited egestive program (Fig. 8*A*); i.e., activity of the radula closure motor neuron B16 was out of phase with activity of the retraction phase interneuron B4. However, when CBI-3 was
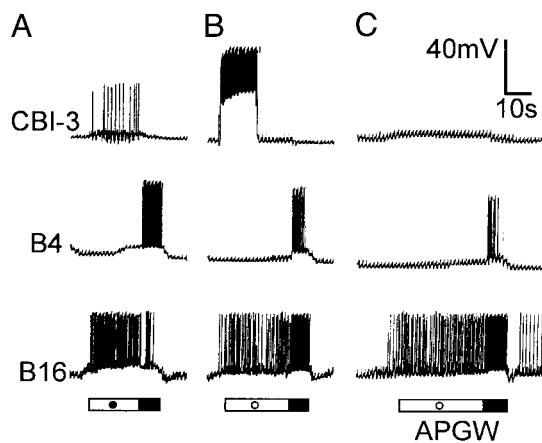
FIG. 8. The effect of CBI-3 stimulation and APGWamide on the esophageal nerve stimulation-induced egestive motor patterns. *A*: typical cycle of a motor program that was elicited by stimulation of the esophageal nerve (continuous stimulation with 3-ms, 1-V, 1-Hz current pulses) without any other manipulations showed a weak CBI-3 activity, and the motor program was egestive-like as B16 was strongly active during protraction (open bar). *B*: initiation of CBI-3 stimulation (7 nA DC) at or before the beginning of the protraction phase converted the cycle to an ingestive-like program as B16 was strongly active during retraction (filled bar) and B4 spiking was reduced. *C*: application of APGWamide converted this pattern to ingestive-like even in the absence of CBI-3 activity.

stimulated during the protraction phase (Fig. 8*B*), the program became ingestive; i.e., high-frequency firing of B16 was in phase with B4 firing. Exogenous application of APGWamide also caused a switch to ingestive-like motor programs regardless of CBI-3 activity (Fig. 8*C*).

In a converse experiment, 1-Hz stimulation of the esophageal nerve during an ingestive-like CBI-2 motor program reduced the activity of CBI-3 and produced egestive-like cycles. Figure 9 illustrates such an experiment. Continuous recordings are shown in Fig. 9*A,* and expanded records are shown in *B.* In the absence of esophageal nerve stimulation (Fig. 9*B1*), strong stimulation of CBI-2 with constant current recruited firing of CBI-3. The resulting motor program was ingestive, i.e., the radula closure motor neuron B16 fired at high-frequency during the retraction phase (filled bar), which was monitored using the retraction phase interneuron B4. Stimulation of the esophageal nerve elicited a transient burst of B4 and then converted the motor program to an egestive one; i.e., the high-frequency firing of B16 occurred during the protraction phase (open bar) and was out of phase with firing of B4. Characteristic of egestive programs, the activity of B4 was increased during the esophageal nerve stimulation. On cessation of esophageal nerve stimulation, the motor program returned to the ingestive mode (Fig. 9*B3*). It was possible to stimulate the esophageal nerve for short or long durations and produce one or more egestive patterned cycles in the midst of ingestive ones.

CBI-2 and CBI-3 respond similarly to a food stimulus but dissimilarly to a stretch of the esophagus

Previously, it has been shown that CBI-2 and CBI-3, which do not have peripheral processes, were both activated strongly by light tactile and food stimuli presented to the lips or tentacles (Rosen et al. 1991). However, the response of both of these two neurons to stimuli that likely produce egestive behavior has not been described. In view of the role that CBI-3

may play in the selection of ingestive versus egestive behavior, and CBI-3's ability to override the egestive nature of the esophageal nerve stimulation induced pattern, we investigated the sensory responses of CBI-2 and CBI-3 to stimuli that lead to ingestive feeding behavior and to stimuli that lead to egestive feeding behavior.

As previously reported (Rosen et al. 1991), on presentation of seaweed to the mouth in the periphery-attached preparation, CBI-2 and CBI-3 were strongly and simultaneously activated (Fig. 10*A*). Presentation of a small piece of seaweed to the perioral area produced similar results. However, a circumferential stretch of the esophagus (a string was tied through the wall of the esophagus and pulled perpendicular to the axis of the esophagus) produced a markedly different result (Fig. 10*B*). This action may simulate ingestion of an inappropriate (large and/or incompressible) substance and resulted in a brief activation of CBI-3 followed by a strong inhibition of CBI-3 during a strong activation of CBI-2. Esophageal stretch produced these effects reliably, as long as the preparation was allowed to recover for at least 15 min between trials.

DISCUSSION

We used the isolated nervous system of *Aplysia* to gain insights into the organization and functioning of circuits that select and implement multiple stimulus-dependent behaviors. Specifically, we studied fictive ingestion and egestion, two motor programs that correspond to two forms of stimulus-dependent behaviors. Previous work suggested that the command-like neuron CBI-2 is responsible for initiation of ingestive behaviors of *Aplysia*. This view was supported by the observation that CBI-2 is activated by food stimuli and that strong DC stimulation of CBI-2 initiates a motor program that produces ingestive behavior in semi-intact preparations (Church and Lloyd 1994; Rosen et al. 1991, 1998). We confirmed that when CBI-2 was stimulated by injecting a sustained DC current, which fired CBI-2 at frequencies higher than 10 Hz, the resulting motor patterns were ingestive. Surprisingly, lower frequency stimulation with brief current pulses produced mostly egestive motor patterns. Since this type of stimulation reduces the ability of CBI-2 to activate CBI-3 via electrical coupling, and since bilateral hyperpolarization of CBI-3 converted ingestive patterns into egestive ones (in 7 of 9 preparations in which ingestive patterns were produced by strong stimulation CBI-2), it appears that the capacity of CBI-2 to evoke ingestive motor programs in part depends on CBI-2's ability to activate CBI-3. However, despite a bilateral hyperpolarization of CBI-3s, CBI-2 still elicited ingestive programs in two of nine preparations. These results suggest that other factors or neurons may also contribute to CBI-2's ability to elicit ingestive motor programs.

How do our observations concerning CBI-2 and CBI-3 fit into the two schemes that have been proposed to describe the organization of neural circuits responsible for selection of different motor patterns (Kristan and Shaw 1997)? One scheme postulates that dedicated command elements control individual behaviors and through cross-inhibition may assure that only one behavior is selected (e.g., Edwards 1991; Jing and Gillette 1995; Kovac and Davis 1980a; Krasne and Lee 1988; Kupfermann and Weiss 1978; Norekian and Satterlie 1996). The alternative scheme (combinatorial command) postulates that
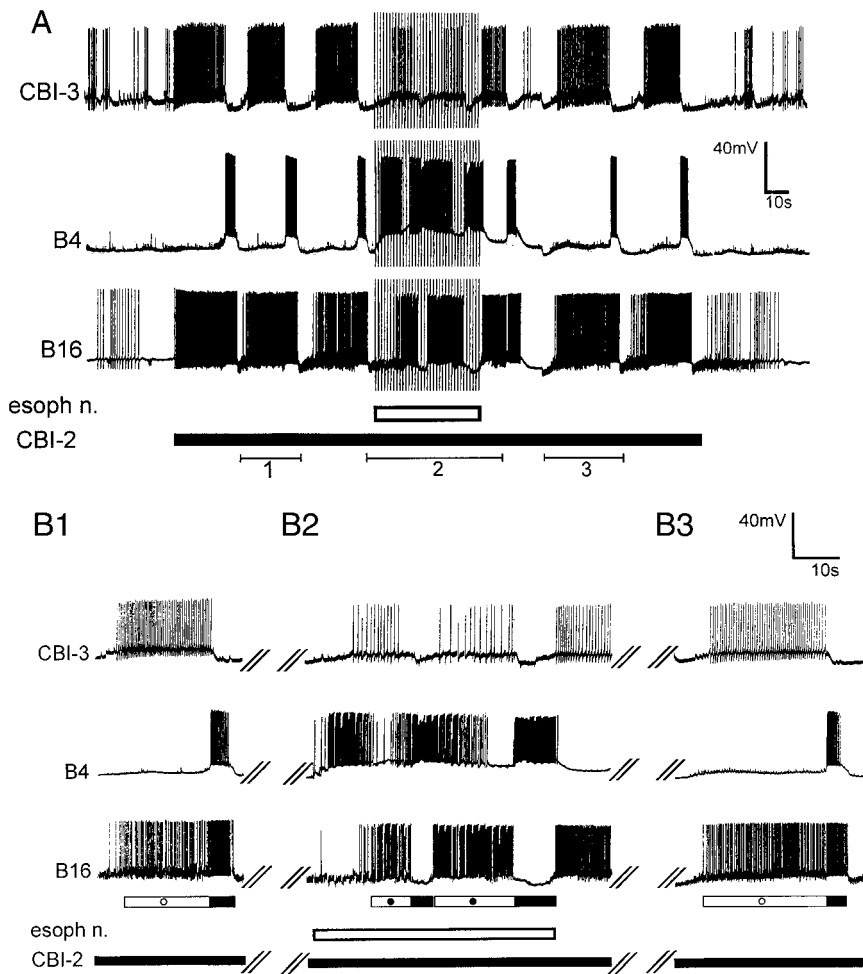
FIG. 9. Esophageal nerve stimulation alters the CBI-2–elicited motor rhythm and CBI-3 activity. *A*: stimulation of the esophageal nerve during a CBI-2–induced rhythm decreased CBI-3 spiking and converted the cycles from ingestive-like to egestive-like. Filled bar at *bottom* indicates CBI-2 stimulation; open bar above it indicates esophageal nerve stimulation. *B*: expanded sample records of parts labeled 1, 2, and 3 in *A*. *B1*: typical cycle from the CBI-2 rhythm prior to esophageal nerve stimulation was ingestive-like with strong B16 activity coincident with B4 depolarization during retraction. *B2*: with the onset of esophageal nerve stimulation, strong B16 activity occurred out-of-phase with B4 firing while CBI-3 activity was diminished. *B3*: typical cycle from the CBI-2 rhythm after esophageal nerve stimulation reverted to an ingestive-like pattern.

distinct combinations of partially overlapping populations of higher order neurons (command neurons, trigger neurons, gating neurons) contribute to multiple behaviors (Shaw and Kristan 1997; Xin et al. 1996). Support (e.g., Jing and Gillette 1995; Kovac and Davis 1980b; Krasne and Lee 1988; Norekian and Satterlie 1996) for the dedicated-command scheme comes
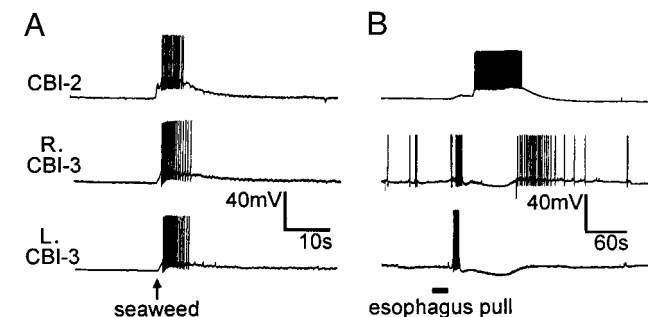

FIG. 10. Responses of CBI-2 and CBI-3 to food and esophageal stretch. *A*: the response of one CBI-2 and both CBI-3s to the brief presentation of a food stimulus (seaweed) to the periphery attached preparation. All 3 neurons were quickly, strongly, and coincidentally activated. The fact that the CBI-3 activity was initially stronger than the CBI-2 activity suggests that the CBI-3 response is not exclusively a byproduct of the electrical coupling between CBI-2 and CBI-3. *B*: the response of one CBI-2 and both CBI-3s to a pull on the esophagus in the periphery attached preparation. After a brief activation of both CBI-3s, CBI-2 showed a strong and sustained activation during which the CBI-3s were inhibited. The strong activation of CBI-2 was accompanied by a visible contraction of the buccal musculature.

from studies of behaviors that are to a large extent implemented by separate pools of motor neurons and muscles, e.g., feeding versus withdrawal. In contrast, the major support (Combes et al. 1999a,b; Shaw and Kristan 1997; Xin et al. 1996) for the combinatorial-command scheme comes from studies of behaviors that share the motor neurons and muscles (but see Croll et al. 1985). It is thus attractive to hypothesize that the dedicated-command scheme preferentially operates when there is little overlap between motor neurons and muscles used in different behaviors, while the combinatorial-command scheme may be operational in cases of a significant overlap.

Our study has probed the generality of this hypothesis by investigating a circuit that differs from those that were previously investigated. Specifically, the circuit that we investigated mediates stimulus-dependent behaviors rather than autoactive or stimulus-autonomous behaviors. Furthermore, the selection of motor outputs that correspond to two patterned behaviors (implemented by heavily overlapping sets of motor neurons and muscles) represents a re-arrangement of the CPG output that is much more complex than those seen in most of the previously studied cases where the selection was made between a patterned behavior and a reflex (e.g., Getting and Dekin 1985; Shaw and Kristan 1997; but see Combes et al. 1999a,b). Despite the differences in the types of behavior and details of architecture of circuits in which it operates, the combinatorial coding scheme appears to be the method of

choice for generating multiple motor patterns for behaviors that share motor neurons and muscles.

The combinatorial-command scheme implies that some of the neurons responsible for initiation of multiple programs are multifunctional. Of the two neurons that we have identified as crucial for pattern selection, CBI-2 belongs to the class of multifunctional higher order neurons (e.g., Combes et al. 1999a,b; Ritzmann et al. 1980; Shaw and Kristan 1997; Xin et al. 1996), whereas CBI-3 may be a dedicated switch cell. Our study extends examples of multifunctionality to circuits that mediate stimulus-dependent behaviors. The giant interneuron of the cockroach is similar to CBI-2 in that it can produce different motor patterns (running or flying) depending on the presence or absence of leg contact with a substrate (Ritzmann et al. 1980). However, CBI-2 and the cockroach interneuron differ in that, unlike the cockroach interneuron, CBI-2 evokes distinct programs in an experimental preparation without additional sensory inputs. However, in intact animals the difference between the behavioral role of cockroach interneurons and CBI-2 may not be so profound. Additional sensory inputs, especially esophageal stretch acting via inhibition of CBI-3, may modify the program much as substrate contact modifies motor programs in the cockroach.

Irrespective of the details of neuronal connectivity that are responsible for channeling sensory inputs to various CPGs, the maintenance of a specific motor pattern must rely on some mechanisms that coordinately affect the activity of the CPG elements that implement the motor pattern. Numerous studies have demonstrated that modulators exert complex effects on a variety of CPGs (e.g., Dickinson et al. 1990; Flamm and Harris-Warrick 1986a,b; Hooper and Marder 1987; Katz and Frost 1995, 1997; Morgan et al. 2000; Nusbaum and Marder 1989; Sherff and Mulloney 1991). Indeed, modulators are thought to best suit the need to coordinate complex circuit rearrangements such as those in CPG pattern selection (Harris-Warrick and Marder 1991), although nonmodulation-dependent modes of rearrangement are also possible (Combes et al. 1999a,b).

To demonstrate that a neuron or neurons can modify motor programs through the release of modulators, one first needs to demonstrate that the action of a neuron is mimicked by a modulator contained in that neuron. There are a number of examples in which the actions of a neuron are mimicked by its transmitter/modulator. However, these examples of matching are mostly limited to modulatory actions that alter specific features of the motor program, such as rhythm frequency or probability. Although it has been more difficult to find such a match for the more dramatic case in which a switch or selection involves two behaviors generated by a single network, previous examples of such matching have been described. For instance, such matching has been reported in *Tritonia* where the actions of a group of serotonergic neurons and serotonin have been analyzed and the role that these serotonergic neurons and serotonin play in selecting a patterned behavior versus a reflex is well understood (Katz and Frost 1995, 1997). Another example of matched neuron/modulator action can be found in the stomatogastric system, but the behavioral meaning of the observed pattern modification remains to be elucidated (Wood et al. 2000). The discovery of APGWamide in CBI-3 created an opportunity for studying the actions of modulatory neuropeptides in a system in which the neuron that contains this peptide subserves a well-characterized pattern-switching function.

The actions of APGWamide and CBI-3 share a number of features. First, neither CBI-3 nor APGWamide can initiate a motor program on their own and at the network level the actions of CBI-3 and APGWamide could be observed only when the network was activated by other means (stimulation of CBI-2 or esophageal nerves). Second, APGWamide mimics the effects of CBI-3 on the frequency and duration of firing of several neurons (B4, B8, B16). Third, APGWamide mimics CBI-3's ability to switch into ingestive programs the egestive programs that were elicited by stimulation of CBI-2 or the esophageal nerve. The fact that APGWamide mimics several of the actions of CBI-3, a neuron that contains APGWamide, strongly suggests that this peptide may be mediating the pattern-switching actions of CBI-3. These findings, however, do not prove that APGWamide is the sole mediator of the effects of CBI-3. Since the complement of neurotransmitters that are present in CBI-3 has not been yet been characterized, it is possible that other transmitters/modulators contained in CBI-3 may also contribute to the effects of CBI-3. In the absence of APGWamide antagonists, we cannot exclude this possibility. It may soon be feasible, however, to define the contribution of APGWamide to the effects of CBI-3 by studying the effects of APGWamide and CBI-3 at a cellular level. This should become possible once the role of individual buccal CPG neurons that have been implicated in the selection of ingestive versus egestive programs (e.g., Jing and Weiss 2000, 2001; Nargeot et al. 1999) is elucidated.

In conclusion, our findings suggest that the combinatorial-command scheme may underlie the selection of different motor programs from a single CPG that generates fictive motor patterns corresponding to two distinct stimulus-dependent behaviors. Since ingestion and egestion use overlapping populations of motor neurons and muscles (Church and Lloyd 1994; Cropper et al. 1990; Morton and Chiel 1993a,b), our findings are consistent with the hypothesis that combinatorial command may be the dominant mode of pattern selection when such an overlap exists. Furthermore, our findings support the view that combinatorial coding operates not only in the choice between reflex and patterned behavior but also in the choice between two patterned, albeit fictive, behaviors. Finally, by demonstrating that the pattern switching function of a neuron is mimicked by its transmitter modulator, we provide support for the hypothesis that higher order neurons, at least in part, may select different motor patterns by releasing specific modulators.

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