Concerted GABAergic Actions of *Aplysia* Feeding Interneurons in Motor Program Specification

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GABAergic inhibitory interneurons regulate the activity of diverse types of neural networks, but the specific roles of these interneurons in motor control are poorly understood. In the *Aplysia* feeding motor network, three interneurons, cerebral–buccal interneuron-3 (CBI-3) and buccal interneurons B40 and B34, are GABA-immunoreactive and evoke fast IPSPs in their postsynaptic followers. Using a combination of pharmacological experiments with GABA antagonists, agonists, and uptake inhibitors, we found that these fast IPSPs are likely mediated by GABA. Functionally, these fast IPSPs specify two parameters for ingestive motor programs elicited by the command-like interneuron CBI-2: (1) the appropriate phasing of activity of the radula closer motor neuron B8 relative to protraction–retraction, and (2) protraction duration. First, in ingestive programs, B8 activity is phased such that it fires minimally during protraction. CBI-3 and B40 exert fast inhibition to minimize B8 activity during protraction, by either acting directly on B8 (B40) or indirectly on B8 (CBI-3). Second, these ingestive programs are characterized by long protraction duration, which is promoted by B40 and B34 because hyperpolarization of either cell shortens protraction. Such effects of B40 and B34 are attributable, at least partly, to their inhibitory effects on the retraction-phase interneuron B64 whose activation terminates protraction. Consistent with a GABAergic contribution to both B8 phasing and protraction duration, blockade of GABAergic inhibition by picrotoxin increases B8 activity during protraction and shortens protraction, without disrupting the integrity of motor programs. Thus, the concerted actions of GABAergic inhibition from three *Aplysia* feeding interneurons contribute to the specification of multiple features that define the motor program as an ingestive one.

Key words: **GABAergic interneurons; *Aplysia;* feeding; ingestion; egestion; central pattern generator; GABA; picrotoxin; bicuculline; muscimol; cotransmission; chloride channel; mollusc; motor program; motor pattern selection**

Introduction

Many, if not all, motor circuits, such as central pattern generators (CPGs), use a variety of mechanisms to produce multiple motor outputs (Getting, 1989; Marder and Calabrese, 1996; Stein et al., 1997; Kupfermann and Weiss, 2001). One pervasive mechanism is neuromodulation that alters the cellular and synaptic properties of CPGs and may originate from extrinsic and/or intrinsic sources (Harris-Warrick and Marder, 1991; Katz and Frost, 1996). However, recent work has shown that "conventional", fast synaptic actions of higher-order interneurons, can act, either alone (Combes et al., 1999) or in conjunction with modulatory actions (Meyrand et al., 1994; Blitz and Nusbaum, 1999; Jing and Weiss, 2001), to configure CPGs for a specific type of motor program. Here, we show that concerted actions of fast GABAergic inhibition exerted by a higher-order interneuron and two CPG interneurons contribute to the expression of one specific type of motor program in *Aplysia* by influencing the phasing and timing of the pattern.

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The *Aplysia* feeding CPG generates distinct motor patterns using partially overlapping sets of motoneurons and muscles. *Aplysia* produce two broad classes of motor programs, ingestive and egestive, which involve two sets of movements of the feeding apparatus (the radula), protraction–retraction versus opening–closing (Kupfermann, 1974; Jing and Weiss, 2002). The two motor programs differ from each other in several parameters, e.g., the phasing and timing of motoneuronal activity (Morton and Chiel, 1993a,b). We undertook this study to investigate how identified CPG elements and their neurotransmitters specify the timing of radula closure and the duration of the protraction phase for ingestive motor programs elicited by a command-like interneuron CBI-2 (Rosen et al., 1991). Specifically, these ingestive programs are characterized by two major criteria. The first criterion, which distinguishes ingestive from egestive motor programs, is that radula closure motoneuron B8 is predominantly active during retraction with minimum activity during protraction. The second criterion, which further defines these programs as a subtype of ingestive programs (biting), is that protraction duration is relatively long. A long protraction duration is consistent with a role of CBI-2 in biting (Rosen et al., 1988) because during biting, protraction is extended to facilitate food grasping (Morton and Chiel, 1993a). Thus, closure activity during retraction enables the food to be grasped and moved into the esophagus, whereas long protractions ensure sufficient time for the radula to contact food during biting.

Three feeding interneurons appear to be critical for generating CBI-2-elicited ingestive programs (Jing and Weiss, 2001, 2002;

Morgan et al., 2002). First, a higher-order interneuron, CBI-3, and a CPG interneuron, B40, contribute to the phasing of B8 activity that is appropriate for ingestion. Additionally, B40 and another CPG interneuron, B34, may promote a long protraction duration. Here, we show that CBI-3, B40, and B34 are GABA-immunoreactive and that GABAergic inhibition originating from these three interneurons specifies multiple parameters of the ingestive program, such as the phasing of B8 activity and protraction duration. Previous work demonstrated that in several nonmotor systems (see Discussion), concerted GABAergic actions are critical for the expression of coordinated network activity. Our study may be the first example of network-coordinating actions by GABAergic interneurons in motor control.

Materials and Methods

Immunocytochemistry. Immunocytochemical methods were a modification of previously published procedures (Vilim et al., 1996; Diaz-Rios et al., 1999). Briefly, ganglia were fixed in freshly prepared fixative (4% paraformaldehyde, 0.1% glutaraldehyde, 0.2% picric acid, 25% sucrose, and 0.1 M NaH_2PO_4, pH 7.6) for either 3 hr at room temperature (RT), or overnight at 4°C. All staining procedures were performed at RT with rocking. After washes with PBS (0.8% NaCl, 0.02% KCl, 0.3% $Na_2HPO_4 \cdot 12\ H_2O$, 0.02% KH_2PO_4, pH 7.4) to remove the fixative, 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, 10 mM Na_2HPO_4, 50 mM EDTA, and 0.01% thimerosal, pH 7.4). After several washes with PBT (PBS with 2% Triton X-100), the tissue was incubated in primary antibody to GABA (catalog #A2052; Sigma-Aldrich, St. Louis, MO) diluted 1:250 in PBT for 4–7 d. PBT, instead of BB, was used to incubate the antibody because incubation with this GABA antibody in BB failed to produce any staining (presumably, a component of the BB interferes with the binding of this antibody). The tissue was then washed twice a day for 2–3 d with washing buffer (WB: 2% Triton X-100, 1% BSA, 154 mM NaCl, 10 mM Na_2HPO_4, 50 mM EDTA, and 0.01% thimerosal, pH 7.4). After the washes, ganglia were incubated with 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, 10 mM Na_2HPO_4, 50 mM EDTA, and 0.01% thimerosal, pH 7.4) for 1 d. The ganglia were then stored at 4°C or viewed and photographed with a Nikon Coolpix 990 digital camera mounted on a Nikon Labphot2 microscope that is equipped with epifluorescence (Morrell Instrument Company, Melville, NY). The Nikon microscope was equipped with filter sets to visualize rhodamine (Y-2E; EX 540–580/DM 590/BA 600–660) or fluorescein (B-2A; EX 450–490/DM 505/BA 520).

For double labeling of identified neurons with GABA immunocytochemistry, neurons were physiologically identified, and an electrode containing 3% 5(6)-carboxyfluorescein dye in 0.1 M potassium citrate was used to re-impale the cells. The dye, also used for revealing the morphology of the cells, was ionophoretically injected into the cells, and the ganglia were fixed and processed as described above.

Electrophysiology. Specimens of marine mollusc *Aplysia californica* were obtained from Marinus (Long Beach, CA) and from the National Resource for *Aplysia* at the University of Miami. They were maintained in circulating artificial seawater made from Instant Ocean (Aquarium Systems, Mentor, OH) at 14–15°C. Animals weighing 50–300 gm were anesthetized by injection (50% of the body weight) of isotonic $MgCl_2$ (337 mM). Cerebral ganglia together with buccal ganglia were dissected out. The connective tissue surrounding ganglia was removed. Desheathed ganglia were pinned in a chamber that had a volume of ~1.5 ml of artificial seawater [ASW, composition (in mM): 460 NaCl, 10 KCl, 55 $MgCl_2$, 11 $CaCl_2$, and 10 HEPES buffer, pH 7.6]. The preparation was continuously perfused at a rate of 0.3 ml/min and maintained at 15–18°C.

Conventional intracellular recordings were obtained using glass microelectrodes filled with 2 M potassium acetate (KAc) and beveled to 6–12 MΩ. Extracellular recordings were made using suction electrodes that were manufactured from polyethylene tubing. Data were recorded on a chart recorder (Astro-Med, MT9500, West Warwick, RI) and a PCM recorder (Vetter, 3000A, Rebersburg, PA). Data were digitized and analyzed using Axoscope/Clampfit software (Axon Instruments, Union City, CA), and plotted using Axum (Mathsoft, Cambridge, MA). Functional synaptic connections were examined in normal saline, and the ability of postsynaptic potentials (PSPs) to follow one-for-one presynaptic spikes was taken as an indication of probable monosynapticity. Assays of monosynapticity were conducted in high-divalent saline (in mM: 312 NaCl, 10 KCl, 132 $MgCl_2$, 33 $CaCl_2$, and 10 HEPES, pH 7.6), a solution that elevates spike threshold and thus curtails polysynaptic activation.

Identification of neurons and synaptic connections. Neurons were identified based on location, size, electrophysiological, and morphological (axon pathways) characteristics (Jing and Weiss, 2001).

B40 shares many features with the previously described feeding interneuron B34 (Hurwitz et al., 1997). The two neurons are morphologically similar, and both are GABA-immunoreactive (see Figs. 2, 3). Both neurons receive fast excitation from CBI-2 and CBI-3: the connections from CBI-2 and CBI-3 to B34 (Sanchez and Kirk, 2000; Hurwitz et al., 2003) and from CBI-2 to B40 (Jing and Weiss, 2002) have been described earlier; the connection from CBI-3 to B40 is shown in Figure 4*B*. Both neurons also have fast inhibitory and slow excitatory connection with B8 (Hurwitz et al., 1997; Jing and Weiss, 2002). Criteria that can be used to distinguish B40 from B34 are as follows. B34 is close to B8, whereas B40 is further away, i.e., lateral to B65 (Kabotyanski et al., 1998). Fast EPSPs from CBI-2 to B40 are much smaller than those from CBI-2 to B34 and do not facilitate as much with repetitive stimulation of CBI-2. Fast IPSPs from B40 to B8 are much larger than IPSPs from B34 to B8. Finally, their synaptic inputs to B31/B32 are different. When B31/B32 are held at resting potential, both B34 and B40 elicit depolarizing potentials in B31/B32, but the potentials are much larger from B34 than from B40 (Fig. 4*A*). Additionally, B34-elicited-potentials have a slow component. Thus, activation of B34 for more than several seconds normally leads to firing of B31/B32. This was rarely true for B40.

Because B40 and B34 are located on the opposite surface of the buccal ganglion than B64, the two buccal hemiganglia were twisted at the commissure so that the caudal surface of one hemiganglion was facing up, whereas the rostral surface of the contralateral hemiganglion was facing up to obtain recordings of these contralateral neuronal pairs.

Motor programs. To elicit single-cycle motor programs, CBI-2 was stimulated with 10 msec current pulses at 10–15 Hz, and the stimulation was stopped after termination of protraction and initiation of retraction. The intertrial interval was 1.5 min. Previous work has shown that CBI-2 is active during protraction and is inhibited during retraction (Rosen et al., 1991). Thus, our stimulation paradigm mimicked the normal CBI-2 activity pattern. Furthermore, because we terminated CBI-2 stimulation only after the protraction phase had ended, the timing of stimulation–termination could not affect the characteristics of the protraction phase (e.g., protraction duration). In fact, with this paradigm, the motor cycles are reproducible, and the details of the cycles remain constant in the absence of other manipulations (see Fig. 9A).

Drugs. GABA, ACh (acetylcholine), GABA agonist: muscimol hydrobromide, GABA antagonists: picrotoxin and 1(*S*),9(*R*)-(−)-bicuculline methobromide (a soluble form of bicuculline), and GABA uptake inhibitor: (±)-nipecotic acid, were obtained from Sigma. All other reagents were also from Sigma unless otherwise stated.

Most experiments with pharmacological reagents (e.g., GABA, GABA antagonists) were performed while preparations were perfused with high-divalent saline. In these cases, reagents were also dissolved in high-divalent saline. In addition, in desensitization-block experiments with B8 and experiments in which the reversal potential for the fast IPSPs from B40 to B8 was determined, B8 was impaled with two electrodes. One electrode was used for recording (monitoring membrane potential), the other electrode was used for current injection.

Bath applications of pharmacological reagents were done by replacing the ASW perfusate with a perfusate consisting of ASW with freshly dissolved reagents. Pressure application (Puff) of GABA was done with a Picospritzer II (General Valve, Fairfield, NJ). Pressure electrodes were pulled with a Narishige (East Meadow, NY) PP-83 puller, with tip diam-

eter of 3–6 μM, which was filled with 1 mM GABA in high-divalent saline. In a subset of experiments, GABA puff was also applied through a blunt electrode that was positioned close to the cell and immediately withdrawn out of bath.

Throughout the results, *n* refers to the number of preparations. Data are expressed as mean ± SEM.

Results

GABA immunoreactivity, morphology, and identifying features of feeding interneurons

The prominent role that synaptic inhibition plays in the generation of ingestive motor programs elicited by CBI-2 is illustrated in Figure 1, which incorporates previous work (Hurwitz and Susswein, 1996; Hurwitz et al., 1997, 2003; Sanchez and Kirk, 2000; Jing and Weiss, 2001, 2002; Morgan et al., 2002) as well as several new findings that are described in this paper. Because GABA is an ubiquitous inhibitory transmitter in vertebrates and invertebrates alike (Hosie et al., 1997; Martin and Olsen, 2000), we first performed GABA immunocytochemical experiments to determine which feeding interneurons are immunopositive (Fig. 2). We injected carboxyfluorescein dye into physiologically identified neurons and processed ganglia for GABA immunocytochemistry. We found that B40 ($n = 4$) and B34 ($n = 3$) were GABA-immunoreactive. Earlier work (Diaz-Rios et al., 1999) demonstrated that two caudal buccal neurons that backfilled from the contralateral cerebral–buccal connective (CBC), named buccal–cerebral interneurons (BCIs), are GABA-immunoreactive, but the identity of these neurons had not been determined. We show that these two unidentified BCIs are B40 and B34, because both interneurons are located on the caudal surface of the buccal ganglion and send axons to the contralateral CBC. Another caudal BCI, B63, was not GABA-immunoreactive (Fig. 2*A*). In addition, we verified (Fig. 2*A*,*B*) that a dopaminergic feeding interneuron B65 (Kabotyanski et al., 1998) was also GABA-immunopositive (Diaz-Rios et al., 2002). Because earlier work has shown that B65 typically was not active in CBI-2-elicited motor programs (Jing et al., 1999; Jing and Weiss, 2000), B65 was not studied further. Similar experiments performed on CBI-3 ($n = 3$) also showed that CBI-3 was GABA-immunopositive (Fig. 2*C*).

Morphological features of CBI-3 (Rosen et al., 1991) and B34 (Hurwitz et al., 1997) have been previously described. Morphological features of B40, however, have not been described in detail (Jing and Weiss, 2002). To reveal B40 morphology (Fig. 3), we injected it with carboxyfluorescein ($n = 14$), and to allow the dye to diffuse, we maintained the preparation at 4–8°C overnight. B40 is 40–70 μm in diameter, somewhat smaller than B34 (compare Fig. 2*B2*). B40 is located on the caudal surface of the buccal ganglion and is close to B65, typically located laterally to B65. B40 is located dorsally to B34, i.e., further away from motoneuron B8 than B34 (compare Fig. 2*B2*). B40 is similar to B63 and B34 in that its major axon courses medially to the buccal commissure where it crosses to the contralateral buccal ganglion (Fig. 3). The axon then turns ventrally to the CBC, and it projects toward the cerebral ganglion. In three preparations, we traced B40 axon to the cerebral ganglion and observed branching in the E and M clusters. These two clusters contain cerebral–buccal interneurons (Rosen et al., 1991; Xin et al., 1999) that play various roles in feeding. Within the buccal ganglion, in the area immediately adjacent to the B40 cell body, we observed multiple B40 processes that extended ventrally toward the areas that contain neurons B34/B63, B31/B32, and B8 (Fig. 3*A*). Along the route of its major axon, B40 branches extensively, mostly toward the ventral part of the ganglion (Fig. 3*B*).

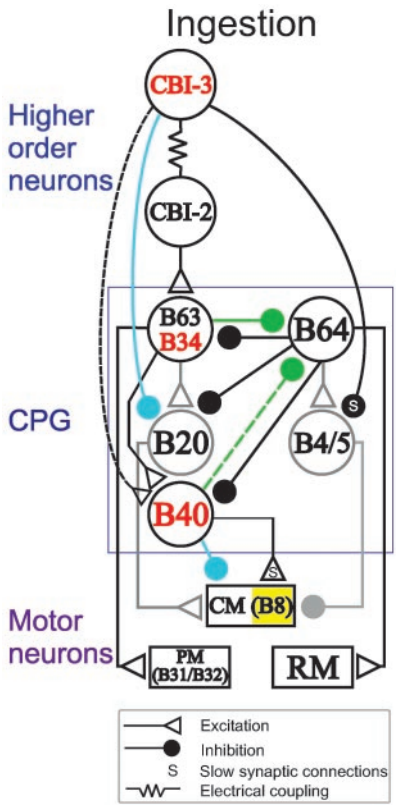

Figure 1. Schematic diagram of the connectivity of the *Aplysia* multifunctional feeding motor network that consists of the higher order interneurons (CBI-2, CBI-3) and CPG elements (B40, B34, B63, B20, B64, B4/5). Each motor program, irrespective of which type it is, consists of an alternating protraction–retraction sequence, illustrated as PM (protraction motoneurons, B31/B32) to the left, and RM (retraction motoneurons) to the right. However, depending on which type of program is expressed, CM (closure motoneurons, B8) can be active during either protraction or retraction, i.e., B8 is predominantly active during protraction in egestion, and is predominantly active during retraction in ingestion. To indicate the latter, we follow an earlier convention (Jing and Weiss, 2002) and divide the B8 box into two halves, with the yellow half (right) representing B8 activity during retraction. Opening motoneurons are active out of phase with CM, but are less well understood, and are not depicted for clarity. Most interneurons are active during protraction, except for B64 and B4/5, which are active during retraction. The diagram depicts the configuration for generation of ingestive motor programs elicited by the command-like interneuron CBI-2. Two new connections described in the present paper are shown in broken lines. Notice that the fast inhibition from B40 to B8 is effective during protraction, whereas the slow EPSPs that B40 elicits in B8 are most effective during retraction. B20 and B4/5 are shown in *gray* because they are strongly active in egestive motor programs, but are only weakly active in CBI-2-elicited ingestive motor programs, partly because of inhibition from CBI-3. CBI-2-elicited ingestive motor programs are characterized by two major parameters. First, B8 is predominantly active during retraction. Second, the protraction duration is long. The functions of inhibitory actions of three GABAergic interneurons (red), CBI-3, B40, and B34, are highlighted. The GABAergic inhibition from CBI-3 to B20 and that from B40 to B8 is relevant to B8 phasing (blue), because both synaptic actions reduce B8 activity during protraction. The GABAergic inhibition from B40 and B34 to B64 is relevant to protraction duration (green), because B40 and B34 promote long protraction duration, partly through their synaptic actions on B64 whose activation terminates protraction. Note that the IPSPs from B63 to B64 are not GABAergic.

B40 appears to share a number of characteristics with B34 (see Materials and Methods). However, we show here one major criterion that can be used to distinguish B40 from B34. Whereas B34 elicits large facilitating EPSPs in B31/B32 with a significant slow component (Hurwitz et al., 1997, 2003), B40 elicits small depolarizing potentials that have no slow component ($n = 7$) (Fig. 4*A*). In addition, the PSPs that B40 elicits in B31/B32 became hyperpolarizing when B31/B32 was depolarized as little as ~10

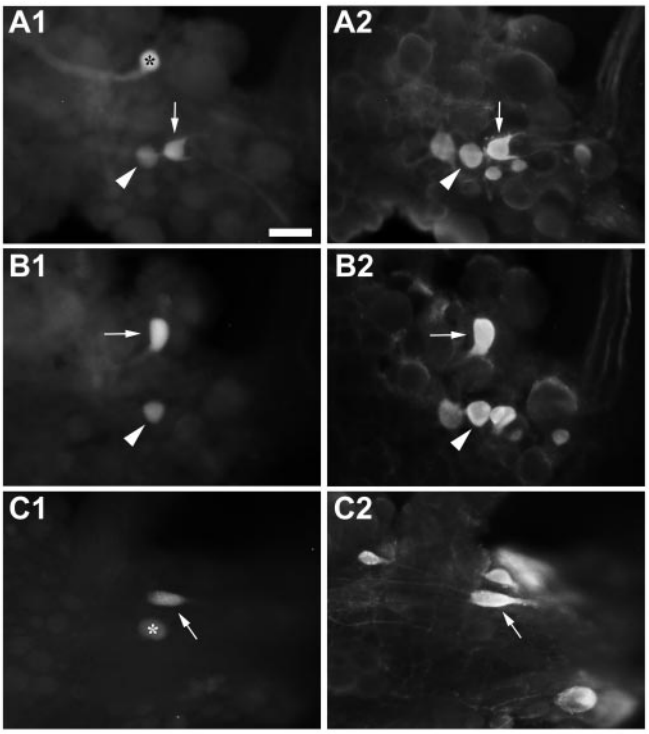

Figure 2. B40, B34 and CBI-3 are GABA immunoreactive. Left panels show cells injected with carboxyfluorescein. Right panels show GABA-immunoreactive cells. *A1, A2,* B40 (arrows, both panels) was GABA-immunoreactive, whereas B63 (star, left panel) was not. B65 (arrowheads, both panels) was also GABA-immunoreactive. Note that in the right panel, B34 (see *B1, B2*) was not present because it was removed to gain assess to B63. *B1, B2,* B34 (arrows, both panels) and B65 (arrowheads, both panels) were GABA-immunoreactive. The GABA-immunoreactive cell to the right of B65 is B40 (right panel). *C1, C2,* CBI-3 (arrows, both panels) was also GABA-immunoreactive, whereas CPR (Teyke et al., 1990) (star, left panel) was not. All panels show the left sides of the buccal (*A1, A2, B1, B2*) or cerebral (*C1, C2*) hemiganglia. Scale bar, 100 μm.

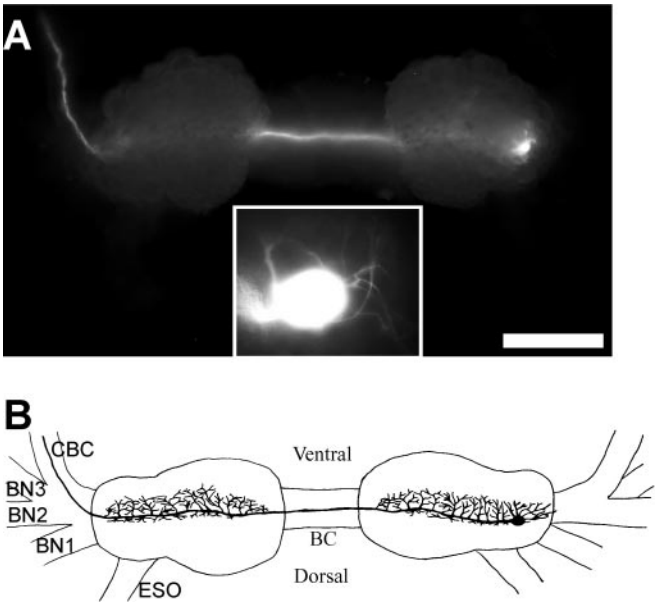

Figure 3. B40 morphology. *A,* A photograph of the caudal surface of a live buccal ganglion. B40 was injected with carboxyfluorescein dye. Note that B40 axon crosses the buccal commissure to the contralateral hemiganglion, and exits the buccal ganglion through the contralateral CBC. *Inset*, B40 cell body and adjacent fine processes at higher magnification. Scale bar: 500 μm; *inset*, 100 μm. *B,* Composite drawing of B40 morphology (caudal surface). The buccal ganglion was placed on a slide and coverslipped to reveal fine processes. BC, Buccal commissure; BN, buccal nerve; CBC, cerebral–buccal connective; ESO, esophageal nerve.

mV in high-divalent saline (Fig. 4*A3*). The reversal potential was difficult to observe in normal saline because B31/B32 received numerous spontaneous synaptic inputs, and these inputs were particularly prominent when a B31/B32 was depolarized.

Relevance of GABAergic inhibition to the control of B8 phasing

The three feeding interneurons, CBI-3, B40, B34, are active in CBI-2-elicited ingestive motor programs (Jing and Weiss, 2001, 2002; Morgan et al., 2002). Demonstration of GABA immunoreactivity in these interneurons allowed us to further examine the putative GABAergic nature of the fast inhibitory synaptic actions in their postsynaptic followers and the functional roles of these connections in defining CBI-2-elicited ingestive motor programs. In particular, we focused on the inhibitory connections from these interneurons to B8 motoneurons, and B20 and B64 interneurons (Fig. 1).

The first criterion that defines CBI-2-elicited motor programs as ingestive ones (see Introduction) is the phasing of the activity of the radula closure motor neuron B8 relative to protraction–retraction. Specifically, B8 displays a high level of activity during the retraction phase of the motor program and a low level of activity during the protraction phase. Thus, a decrease of B8 activity during protraction and an increase of B8 activity during retraction can be taken as indications that the motor programs become more ingestive. Earlier work (Jing and Weiss, 2001, 2002; Morgan et al., 2002) (compare Fig. 1) has shown that the low level of activity of B8 during protraction is promoted by fast inhibitory actions of CBI-3 and B40. Thus, CBI-3 can convert intermediate/egestive programs into ingestive ones, partly through its fast inhibition to B20 interneurons, which normally mediate egestive motor programs and provide fast excitation to B8 during protraction. Additionally, B40, which is strongly active in ingestive motor programs, directly inhibits B8 during protraction. Therefore, synaptic inhibition from both CBI-3 and B40 reduces B8 activity during protraction, enabling expression of ingestive programs. Furthermore, consistent with the fact that CBI-3 makes programs more ingestive, we found that CBI-3 monosynaptically excites the interneuron B40 ($n = 10$; Fig. 4*B*). In addition to receiving fast EPSPs from CBI-3, B40 also received a slow EPSP. It is not known how the GABAergic interneuron CBI-3 exerts fast excitation. Among other things, CBI-3 may use GABA, which has been shown to depolarize some *Aplysia* neurons (Yarowsky and Carpenter, 1978), as a fast excitatory transmitter. Consistent with this possibility, we found that GABA puffs depolarized B40 (data not shown). Alternatively, CBI-3 may contain yet another transmitter that mediates fast excitation, consistent with prominent transmitter colocalization for GABAergic interneurons (see Discussion).

The fast IPSPs from B40 to the contralateral B8

To probe the putative GABAergic nature of the inhibitory synaptic connections that CBI-3 makes with B20, and B40 makes with B8, we performed a series of pharmacological experiments. First, we performed desensitization-block experiments using bath-application of 1 mM GABA (Fig. 5*A1*). GABA at 1 mM reduced the size of B40-elicited IPSPs in B8 by 91% ($n = 3$). Perfusion of muscimol, a GABA agonist, also reduced the IPSPs that B40 elicited in B8. Muscimol reduced these IPSPs by 78% ($n = 4$) (Fig. 5*A2*). Initial perfusion of GABA and, to a lesser extent, muscimol hyperpolarized B8. The response desensitized, and B8 gradually

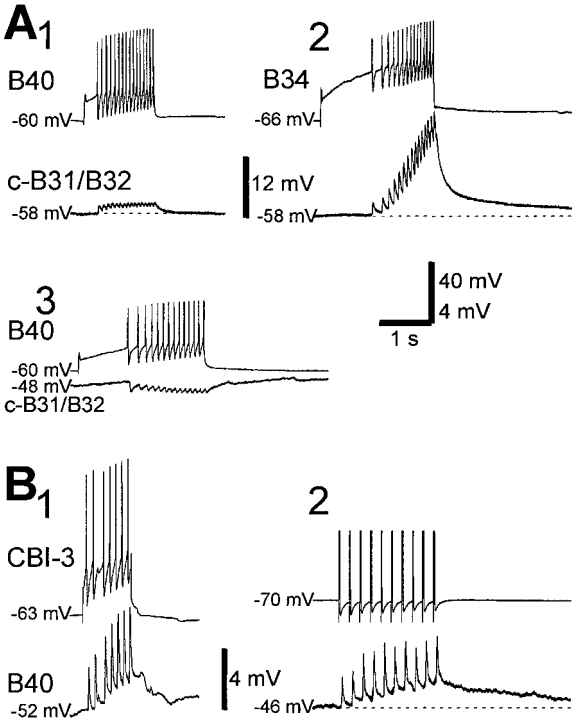

Figure 4. Synaptic connections of B40 with B31/B32 and CBI-3. *A,* Both B40 and B34 elicited depolarizing postsynaptic potentials (PSPs) in the contralateral B31/B32 (c-B31/B32) that followed presynaptic spikes one-for-one (*A1, A2*). Records in *A1* and *A2* are from the same preparation and were obtained in normal saline. *A3*, With 10 mV depolarization of B31/B32 in high divalent saline, the PSPs in B31/B32 elicited by B40 became hyperpolarizing. *B,* CBI-3 elicited one-for-one fast EPSPs in B40 in both normal saline (*B1*) and high-divalent saline (*B2*). Dotted lines represent the resting membrane potential of the postsynaptic neurons. Voltages to the left of the records of individual neurons indicate membrane potentials.

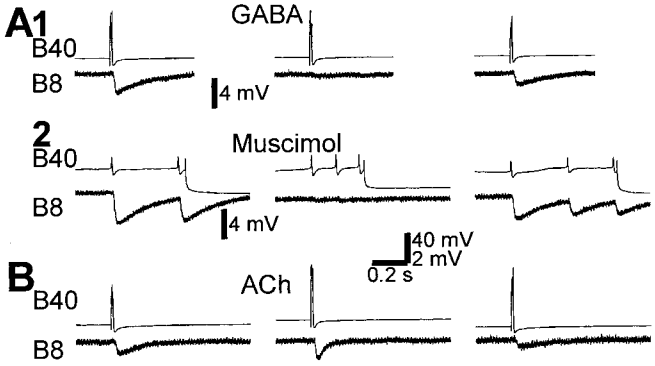

Figure 5. The amplitude of IPSPs that B40 elicits in the contralateral B8 is decreased by desensitization with GABA or the $GABA_A$ receptor agonist (muscimol). The same treatment with ACh is ineffective. Left panels, recordings made under control conditions. Middle panels, recordings made during perfusion of 1 mM reagents. Right panels, recordings made after drug washout. *A,* Both GABA (*A1*) and muscimol (*A2*) abolished the IPSPs from B40 to B8. *B*. ACh did not block the IPSPs from B40 to B8, instead it enhanced the amplitude of the IPSPs from B40 to B8. All recordings were made in high-divalent saline.

returned to its original resting membrane potential; at this time, test stimulation of B40 was performed.

To determine the specificity of GABA desensitization-block, we also tested the effect of 1 mM ACh (a common neurotransmitter in *Aplysia*, e.g., Gardner and Kandel, 1977) on B40-elicited IPSPs in B8. We found that ACh did not reduce, but instead somewhat increased IPSPs from B40 to B8 ($n = 3$) (Fig. 5*B*). ACh perfusion, as well as ACh puff, depolarized B40 neurons (data not shown), suggesting that ACh may enhance B40-elicited IPSPs in B8, at least partly, through presynaptic mechanisms.

We next examined the effects of the $GABA_A$ receptor antagonists, picrotoxin and bicuculline on the IPSPs elicited by B40 (Fig. 6*A*). Both antagonists had similar effects on the IPSPs that B40 elicited in B8. Picrotoxin and bicuculline diminished the IPSPs by 87% ($n = 3$) or 91% ($n = 3$), respectively. Furthermore, we tested the effects of the GABA uptake inhibitor, nipecotic acid, on the IPSPs from B40 to B8. As expected, nipecotic acid increased the amplitude of the fast IPSPs that B40 elicited in B8 by 64% ($n = 3$) (Fig. 6*B*). Finally, we directly pressure-ejected GABA from a pipette containing 1 mM GABA, onto the B8 soma. The GABA puff hyperpolarized B8, thus mimicking the inhibitory effects of B40 on B8. Bath application of 1 mM picrotoxin blocked these hyperpolarizing responses ($n = 3$) (Fig. 6*C*).

Fast GABAergic IPSPs are often mediated by Cl^- conductance (Martin and Olsen, 2000). Earlier, we showed that the fast IPSPs from B40 to B8 are associated with a conductance increase (Jing and Weiss, 2002). We now measured the reversal potential of the IPSPs that B40 elicits in B8 and found that it was, on average, -68.6 ± 1.17 mV ($n = 7$) (Fig. 7*A*), which is close to the equilibrium potential of Cl (Gardner and Kandel, 1977). In addition, we injected Cl ions intracellularly into B8 and found that the hyperpolarizing potential elicited by B40 became depolarizing when B8 was held at the same membrane potential (Fig. 7*B*), thus reversing the IPSPs. These data suggest that the IPSPs are at least partially mediated by Cl. In Cl injection experiments, two electrodes with different filling solutions were used to impale the postsynaptic neuron B8. The first electrode, filled with 2 M KAc, was used for recording and monitoring the membrane potential of B8. The second electrode, filled with 3 M KCl, was used for injecting Cl into the cell by passing hyperpolarizing current for ≥5 min.

The fast IPSPs from CBI-3 to B20

We tested the effects of the GABA antagonists, picrotoxin ($n = 3$) and bicuculline ($n = 4$) on the IPSPs in B20 elicited by CBI-3. Both antagonists had similar effects, i.e., they diminished the IPSPs in B20 elicited by CBI-3 (Fig. 8).

Effects of picrotoxin on CBI-2-elicited ingestive motor programs

The preceding experiments suggested that the fast IPSPs that CBI-3 and B40 elicit in their postsynaptic targets may be mediated by GABA. To further characterize the possible functions of the inhibitory synaptic actions induced by GABAergic interneurons in the generation of ingestive motor programs, we examined the effects of 1 mM picrotoxin on the rhythmic activity elicited by CBI-2. Consistent with a regulatory role of GABAergic interneurons, picrotoxin had two specific effects on motor programs, without affecting the ability of CBI-2 to evoke programs. First, picrotoxin increased the firing frequency of B8 during the protraction phase without significantly affecting B8 activity during the retraction phase ($n = 6$) (Fig. 9*A*,*B*). In control episodes, the average B8 firing rate during protraction was 2.23 ± 0.27 Hz; during picrotoxin perfusion, B8 activity increased to 4.05 ± 0.19 Hz. Paired *t* test of B8 firing in control programs and programs during picrotoxin perfusion showed that there is a significant difference between the two groups ($p < 0.01$). B8 activity during retraction in control motor programs was similar to that in the presence of picrotoxin. The picrotoxin effect on B8 activity during protraction specifically mimicked the effect of B40 hyperpolarization on B8 activity during protraction (Jing and Weiss,

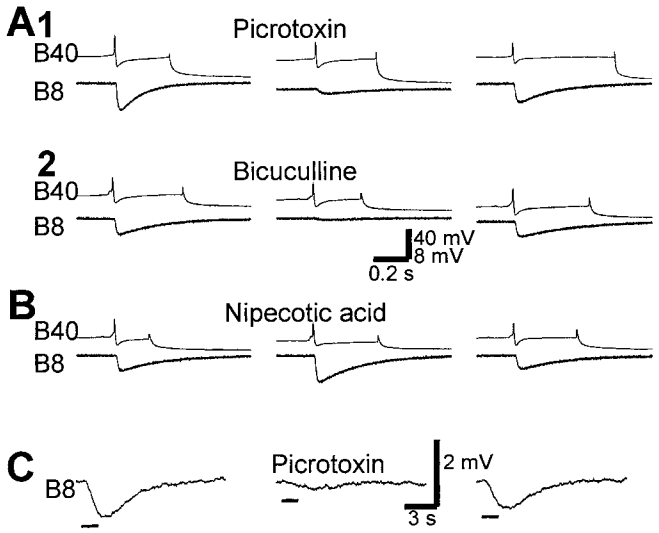

Figure 6. The fast IPSPs from B40 to the contralateral B8 are blocked by the GABA antagonists (picrotoxin and bicuculline), and enhanced by the GABA uptake inhibitor (nipecotic acid). Hyperpolarization of B8 by a GABA puff is also blocked by picrotoxin. Left panels, Recordings made in control conditions. Middle panels, recordings made during perfusion of 1 mM reagents. Right panels, recordings made after drug washout. *A,* Both picrotoxin (*A1*) and bicuculline (*A2*) diminished the IPSPs from B40 to B8. *B,* Nipecotic acid greatly enhanced the amplitude of the IPSPs from B40 to B8. *C,* A puff of 1 mM GABA (bar) applied to the B8 soma hyperpolarized B8 (left). The hyperpolarizing response of B8 to GABA puff was diminished by bath application of 1 mM picrotoxin (*middle*), which recovered after washout of picrotoxin (right). All recordings were made in high-divalent saline.

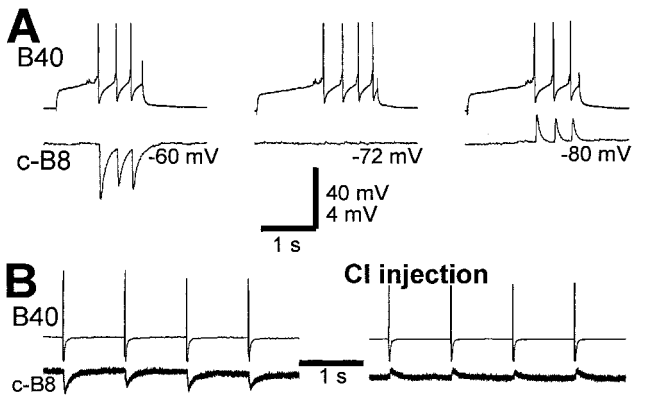

Figure 7. Reversal potential and ionic basis of the fast IPSPs from B40 to the contralateral B8 (c-B8). *A,* The IPSPs from B40 to B8 (left) became nearly invisible when B8 was hyperpolarized to −72 mV (middle), and became depolarizing when B8 was further hyperpolarized to −80 mV (right), suggesting that the reversal potential of the IPSPs was approximately −72 mV. Recordings were made in normal saline. *B,* Stimulation of B40 elicited fast IPSPs in B8 (left). After intracellular injection of Cl in B8 (right), the PSPs that B40 elicited in B8 became depolarizing while B8 membrane potential remained the same, thus suggesting that the IPSPs from B40 to B8 are mediated, at least in part, by Cl ions. Recordings were made in high-divalent saline.

2002). Thus, this picrotoxin effect confirmed the idea that fast inhibition of B40 onto B8 is indeed important in reducing B8 activity during protraction.

Second, picrotoxin also reduced the protraction duration (Fig. 9*A*,*C*). In control ingestive motor programs elicited by CBI-2, average protraction duration was 24.8 sec ($n = 7$). After >5 min of perfusion of 1 mM picrotoxin, the protraction duration on average was reduced to 9.5 sec, a 62% reduction. Repeated measures ANOVA of three groups, "control," "picrotoxin," and "wash," showed significant difference among these groups ($F_{(2,12)} = 28.68$; $p < 0.0001$), and a Bonferroni multiple comparisons

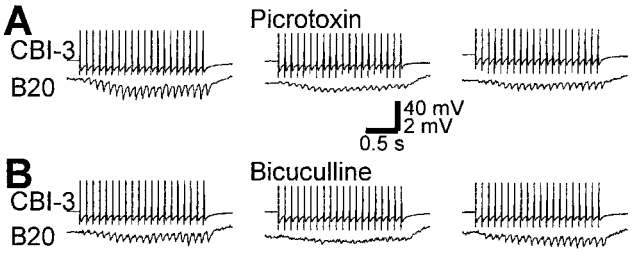

Figure 8. The fast IPSPs from CBI-3 to B20 are diminished by the GABA antagonists (picrotoxin and bicuculline). Left panels, Recordings made in control conditions. Middle panels, Recordings made during perfusion of 1 mM reagents. Right panels, Recordings made after drug washout. B20 was depolarized 10 mV above its resting potential to increase the amplitude of the IPSPs. Both picrotoxin (*A*) and bicuculline (*B*) reduced the fast IPSPs from CBI-3 to B20, with partial washout for picrotoxin and nearly complete washout for bicuculline. Recordings were made in high-divalent saline.

test of different group pairs showed that picrotoxin group was significantly different from control ($t = 6.82$; $p < 0.001$) and wash ($t = 6.27$; $p < 0.001$) groups, whereas the control group was not significantly different from wash group ($t = 0.55$; $p > 0.05$).

This effect of picrotoxin on protraction duration is interesting because earlier data (Jing and Weiss, 2002, their Fig. 3) suggested that hyperpolarization of either B40 or B34 might also shorten the duration of the protraction phase. We repeated this experiment, and we provide group data for this effect (Fig. 10). Analysis of the group data from multiple experiments, in which bilateral hyperpolarization of neurons B40 or B34 was performed, demonstrates that the protraction phase was shortened. In these experiments, the B40 or B34 neurons were hyperpolarized enough to prevent them from firing. In five experiments with B40, the average protraction duration in control motor programs induced by CBI-2 was 24.5 sec. The protraction duration was reduced to 16.5 sec when both B40 were hyperpolarized, a 33% reduction. Repeated measures ANOVA of three groups, "before," B40, and "after," showed a significant difference among these groups ($F_{(2,8)} = 15.81$; $p < 0.01$), and a Bonferroni multiple comparisons test of different group pairs showed that the B40 group was significantly different than the before ($t = 4.96$; $p < 0.01$) and after ($t = 4.77$; $p < 0.01$) groups, whereas the before group was not significantly different from the after group ($t = 0.19$; $p > 0.05$). Similarly, in four experiments with B34, the average protraction duration in control motor programs induced by CBI-2 was 27.4 sec. The protraction duration was reduced to 16.7 sec when both B34 were hyperpolarized, a 39% reduction. Repeated measures ANOVA of three groups (before, B34, and after), showed a significant difference among these groups ($F_{(2,6)} = 20.32$; $p < 0.01$), and a Bonferroni multiple comparisons test of different group pairs showed that the B34 group was significantly different than the before ($t = 5.38$; $p < 0.01$) and after ($t = 5.65$; $p < 0.01$) groups, whereas the before group was not significantly different from the after group ($t = 0.26$; $p > 0.05$). Thus, picrotoxin mimicked the effects of hyperpolarization of neurons B40 and B34 by shortening the duration of protraction, and consistently, picrotoxin effect was nearly double that of hyperpolarization of B40 or B34 alone. In the next section, we report the data that suggest that the protraction shortening effect by hyperpolarization of B40 and B34 is likely to be mediated by the lack of fast inhibition that B40 and B34 elicit in the retraction-phase interneuron B64. Thus, the stronger effect of picrotoxin on protraction duration may be attributable to the fact that it blocks the inhibitory actions of both B40 and B34.

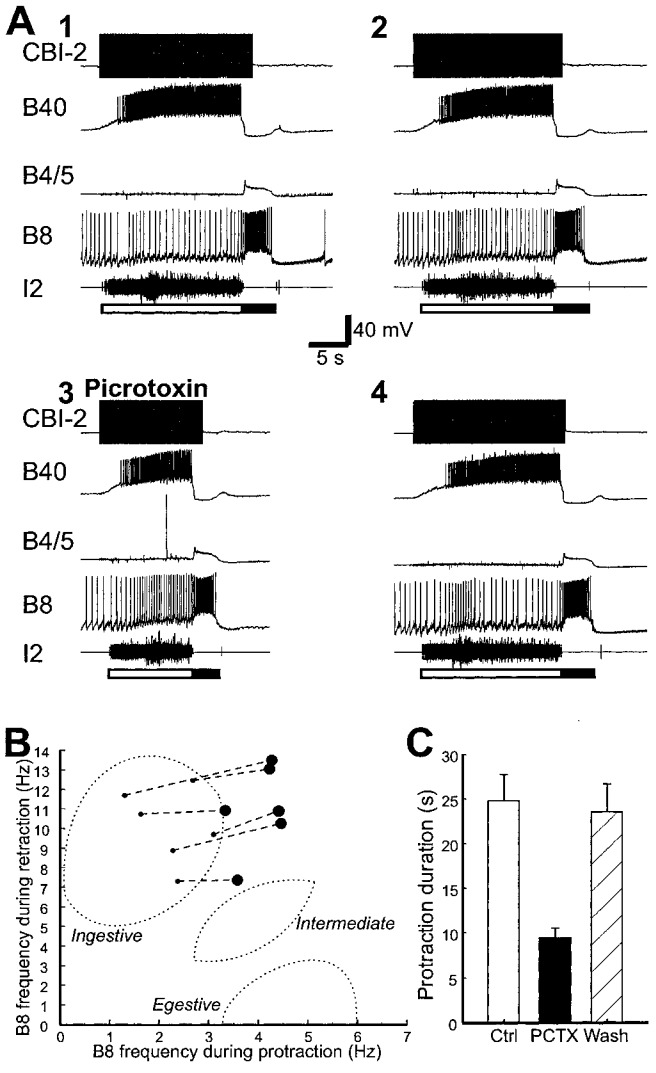

Figure 9. The effects of picrotoxin on CBI-2-elicited ingestive motor programs. Ingestive motor programs were elicited by repetitive stimulation of CBI-2 with brief current pulses at 11 Hz. Protraction (open bar) was monitored by activity in the I2 nerve. Retraction (filled bar) was monitored by sustained depolarization of the multifunctional interneuron–motor neuron B4/5 (Jing and Weiss, 2001) after protraction. *A1, A2,* Two consecutive control motor programs elicited by CBI-2 had similar characteristics. *A3,* Perfusion of 1 mM picrotoxin had two major effects on the motor program. First, B8 activity during protraction was increased. Second, protraction duration was reduced. *A4,* After washout of picrotoxin, the characteristics of the motor program were similar to the controls. *B,* Group data showing that picrotoxin increases B8 activity during protraction, but has little effect on B8 activity during retraction. Data points for B8 activity in control motor programs are shown in small symbols, whereas data points for B8 activity in motor programs during picrotoxin are shown in large symbols. Dotted lines encircling the data points show three clusters derived from cluster analysis (Morgan et al., 2002) that categorized motor programs into ingestive, intermediate, and egestive programs. *C,* Group data showing that picrotoxin (PCTX) reduces protraction duration. Ctrl, Control. Error bars indicate SEM.

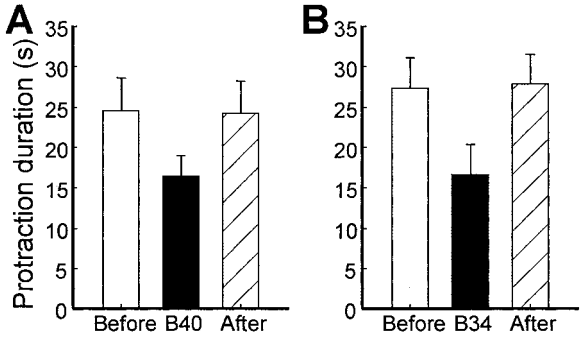

Figure 10. Hyperpolarization of B40 or B34 shortens protraction duration, suggesting that normal activity of B40 and B34 promotes long protraction duration. The two bar graphs show group data of the effects on protraction duration by hyperpolarization of B40 (*A; n* = 5) or B34 (*B; n* = 4). *Before*, Average protraction duration before B40/B34 hyperpolarization; *After*, average protraction duration after B40/B34 hyperpolarization. Error bars indicate SEM.

Relevance of GABAergic inhibition to the control of protraction duration

To study the mechanisms by which B40 and B34 may prolong the protraction phase, we examined their potential actions on the retraction-phase interneuron B64. Previously, it was shown that B64 is active during the retraction phase and that it monosynaptically inhibits protraction-phase interneurons/motoneurons, B63, B34, and B31/B32 (Hurwitz and Susswein, 1996; Hurwitz et al., 1997). Furthermore, B64 stimulation terminates the protraction phase, thereby causing a phase shift from protraction to retraction when buccal motor programs are elicited in isolated buccal ganglia. More recently, Borovikov et al. (2000) have shown that activity in a sensory neuron that excites B64 shortens protraction. Thus, B64 is a likely target interneuron on which B40 and B34 may act to prolong the protraction.

The retraction-phase interneuron B64 is important in determining protraction duration during CBI-2-elicited ingestive motor programs

First, we examined the effect of injecting a brief depolarizing current, which initiated spiking activity in B64, on termination of the protraction phase of CBI-2 elicited ingestive programs. We found that a brief (<1 sec) suprathreshold depolarization of B64 that induced B64 spiking immediately terminated protraction in CBI-2 ingestive programs (data not shown) just as B64 did for other types of buccal motor programs elicited by other means (Hurwitz and Susswein, 1996). Second, and more importantly, we injected subthreshold constant currents into B64 before and during CBI-2 stimulation. When we injected subthreshold depolarizing currents into B64, we found that B64 firing was initiated earlier during protraction, and thereby significantly shortened the duration of protraction (Fig. 11). On average ($n = 6$), protraction duration in control ingestive motor programs elicited by CBI-2 was 23.63 ± 2.03 sec. When subthreshold depolarization was applied in B64, protraction duration was 9.21 ± 0.48 sec. Subsequent motor programs without B64 depolarization had protraction duration of 22.12 ± 1.39 sec. Repeated measures ANOVA of three groups (before, B64, and after), showed a significant difference among these groups ($F_{(2,10)} = 39.82$; $p < 0.0001$), and a Bonferroni multiple comparisons test of different group pairs showed that the B64 group was significantly different than the before ($t = 8.12$; $p < 0.001$) and after ($t = 7.27$; $p < 0.001$) groups, whereas the before group was not significantly different from the after group ($t = 0.85$; $p > 0.05$). These experiments suggested that neurons that increase B64 excitability may shorten protraction duration.

To directly examine the potential role of B64 in shortening the protraction duration during application of picrotoxin, we recorded from both B64s (Fig. 12). Before picrotoxin application, the control ingestive motor program elicited by CBI-2 had a long protraction duration (Fig. 12*A*). During picrotoxin application, the protraction duration was shortened considerably (compare Fig. 9). Coincidentally, it appeared that the slow depolarization in B64, apparently unhindered by the fast IPSPs, developed more rapidly thus advancing the spiking activity of B64 (Fig. 12*B1*,*B3*).

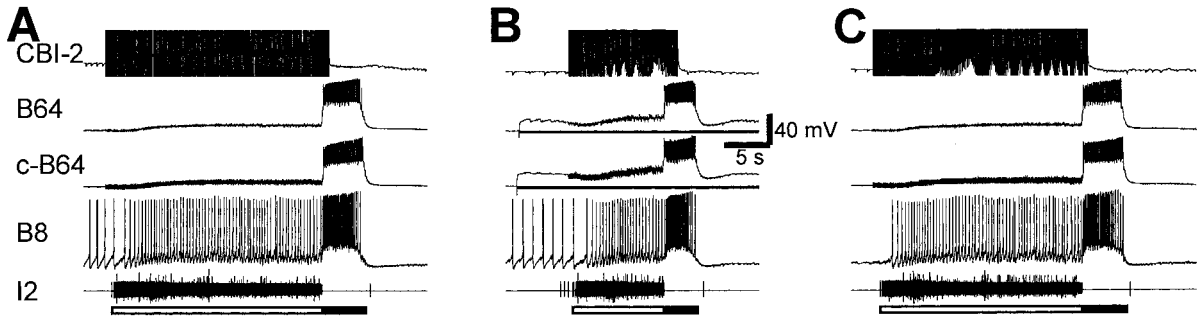

Figure 11. Subthreshold depolarization of B64 shortens the protraction phase of CBI-2 elicited ingestive motor programs. Ingestive motor programs were elicited by repetitive stimulation of CBI-2 with brief current pulses at 15 Hz. Protraction (open bar) was monitored by activity in the I2 nerve. Retraction (filled bar) was monitored by bursting in B64. *A,* A control motor program elicited by CBI-2. *B,* When subthreshold depolarizations (bars) were applied in both the ipsilateral B64 and contralateral B64 (c-B64), the protraction phase was shortened. *C,* Subsequent motor program elicited by CBI-2 without B64 depolarization had a protraction duration similar to the control program (*A*).

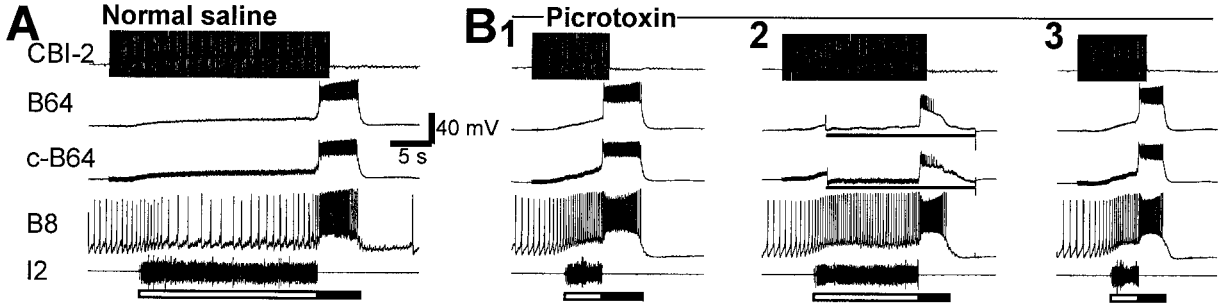

Figure 12. Hyperpolarization of B64 prolongs protraction of CBI-2 elicited ingestive motor programs during picrotoxin perfusion. Ingestive motor programs were elicited by repetitive stimulation of CBI-2 with brief current pulses at 14 Hz. Protraction (open bar) was monitored by activity in I2 nerve. Retraction (filled bar) was monitored by bursting or sustained depolarization in B64 after protraction. *A,* A control motor program elicited by CBI-2. *B,* Recordings made during perfusion of 1 mM picrotoxin. *B1,* The motor program elicited by CBI-2 during picrotoxin had a shorter protraction duration than the control (*A*). *B2,* When both the ipsilateral B64 and contralateral B64 (c-B64) were hyperpolarized (*bars*), protraction duration was lengthened. *B3*. Subsequent motor program elicited by CBI-2 without B64 hyperpolarization had a protraction duration similar to that in *B1*.

In the presence of picrotoxin, hyperpolarization of both B64s lengthened the protraction phase (Fig. 12*B2*). During picrotoxin perfusion, on average ($n = 4$), the protraction duration before B64 hyperpolarization was 9.63 ± 2.62 sec, during B64 hyperpolarization it increased to 13.75 ± 2.14 sec, and after B64 hyperpolarization it was 9.4 ± 2.73 sec. Repeated measures ANOVA of three groups (before, B64, and after), showed a significant difference among these groups ($F_{(2,6)} = 23.99$; $p < 0.01$), and a Bonferroni multiple comparisons test of different group pairs showed that the B64 group was significantly different than the before ($t = 5.83$; $p < 0.01$) and after ($t = 6.15$; $p < 0.01$) groups, whereas the before group was not significantly different from the after group ($t = 0.32$; $p > 0.05$). These data suggest that enhancement of B64 excitability caused by block of inhibitory inputs to B64 by picrotoxin is at least partly responsible for protraction shortening during picrotoxin perfusion.

The ability of B40/B34 to lengthen protraction may be affected by their inhibitory actions on B64

From the above and earlier experiments, it appears that B64 is indeed an important CPG element that regulates the protraction duration. Therefore, we reasoned that prolongation of protraction by B40 and B34 may be affected, at least in part, through their inhibitory actions on B64. Indeed, we found that B40 elicited fast IPSPs in B64, which followed presynaptic spikes one-for-one (Fig. 13*A*, left panel). Furthermore, the recordings in Figure 13*A* were made in high-divalent saline, suggesting that the IPSPs are monosynaptic. Previously, it was reported that B34 also elicits one-for-one IPSPs in B64 (Hurwitz et al., 1997), which we have

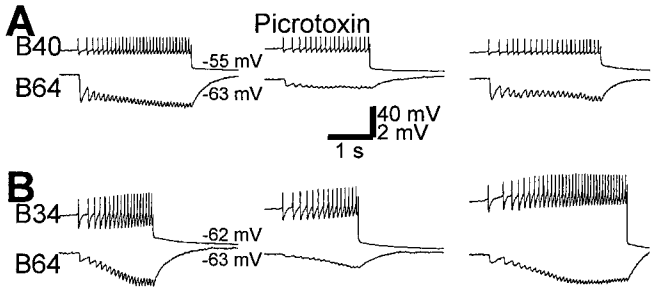

Figure 13. Both B40 and B34 elicit fast IPSPs in the contralateral B64 that are diminished by picrotoxin. Left panels, Recordings made in control conditions. Middle panels, Recordings made during perfusion of 1 mM picrotoxin. Right panels, Recordings made after picrotoxin washout. B64 was depolarized 15 mV above its resting potential to enhance the amplitude of the IPSPs. The IPSPs in B64 elicited by B40 (*A*) and B34 (*B*) were reduced by picrotoxin. *A* and *B* were from the same preparation, and the B64 was maintained at the same membrane potential when either B40 or B34 was stimulated. Voltages to the right of the records of individual neurons indicate membrane potentials. All recordings were made in high-divalent saline.

confirmed. To investigate the possibility that the IPSPs that B40 and B34 elicit in B64 may be mediated by GABA, we examined the effects of picrotoxin on these IPSPs. Bath application of 1 mM picrotoxin reduced the size of the IPSPs from both B40 and B34 to B64 ($n = 3$) (Fig. 13). Note that in addition to the fast component, the IPSPs in B64 elicited by B40 or B34 also appeared to have a slow component, and the slow component was still present in picrotoxin.

Furthermore, to determine if the IPSPs from B40/B34 to B64 are indeed functional, we examined the effect of B40/B34 activity

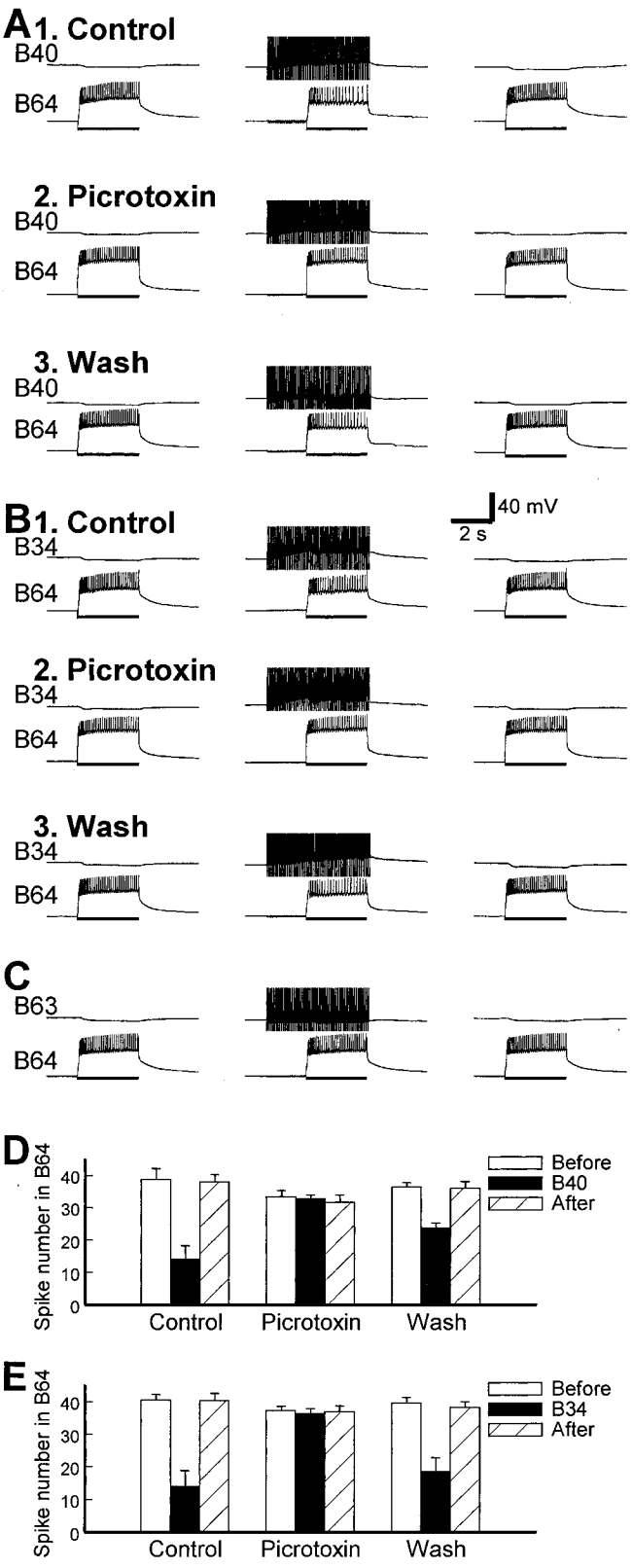

Figure 14. Both B40 and B34 reduce excitability of the contralateral B64, and the effects can be blocked by picrotoxin. *A–C*, B64 excitability was tested with 3 sec current pulses (bars). Left and right panels show B64 excitability in control conditions, i.e., before (left) and after the experimental tests (right). Middle panels (the experimental tests) show the effects of B40, B34, or B63 activity (20 Hz through brief current pulses) on B64 excitability. B40, B34, or B63 was fired for 2 sec before and throughout the current pulses (bars) injected into B64. *A1*, *B1*, *C*, recordings made in control condition. B64 excitability was reduced by B40 (*A1*) or B34 (*B1*) activity (spike number in B64, left to right, *A1*, 35, 21, 35; *B1*, 36, 26, 35), but only slightly reduced by B63 (*C*) activity (*left to right*, 35, 33, 34). *A2* and *B2*, recordings made during perfusion of 1 mM picrotoxin. Picrotoxin abolished the suppressive effects of B40 (*A2*) or B34 (*B2*) on B64 excitability (*left to right*, both *A2* and *B2*, 31, 30, 32). *A3*, *B3*, Recordings made after picrotoxin washout. The suppressive effects of B40 (*A3*) or B34 (*B3*) on B64 excitability recovered (*left to right*, *A3*, 34, 23, 34; *B3*, 35, 24, 35). *D*, *E*, Two bar graphs showing group data for the effect of B40 (*D*) and B34 (*E*) on B64 excitability and its blockade by picrotoxin. *D* corresponds to examples shown in *A* (B40), and *E* corresponds to examples shown in *B* (B34). Before, Left panels in *A* or *B*; B40 or B34, middle panels; after, right panels. Error bars indicate SEM.

on B64 excitability (Fig. 14*A,B,D,E*). In these experiments, B64 excitability was tested with 3 sec current pulses every 30 sec. B40/B34 was stimulated at 20 Hz with brief current pulses, 2 sec before and throughout the injection of constant DC current pulses into B64. Indeed, both B40 ($n = 3$) and B34 ($n = 4$) reduced B64 excitability by 64 and 65%, respectively. For B40 experiments, repeated measures ANOVA of three groups (before, B40, and after), showed a significant difference among these groups ($F_{(2,4)} = 12.62$; $p < 0.05$), and a Bonferroni multiple comparisons test of different group pairs showed that the B40 group was significantly different than the before ($t = 4.41$; $p < 0.05$) and after ($t = 4.29$; $p < 0.05$) groups, whereas the before group was not significantly different from the after group ($t = 0.12$; $p > 0.05$). For B34 experiments, repeated measures ANOVA of three groups (before, B34, and after), showed a significant difference among these groups ($F_{(2,6)} = 17.17$; $p < 0.01$), and a Bonferroni multiple comparisons test of different group pairs showed that the B34 group was significantly different than the before ($t = 5.09$; $p < 0.01$) and after ($t = 5.05$; $p < 0.01$) groups, whereas the before group was not significantly different from the after group ($t = 0.05$; $p > 0.05$).

Previously, it was shown that another protraction interneuron B63, which is not GABA-immunoreactive (Fig. 3*A*), also elicited fast IPSPs in B64 (Hurwitz et al., 1997). We found that the IPSPs from B63 to B64 were very small compared with the IPSPs elicited by B40 or B34 (data not shown). Consistently, when we tested the effect of B63 on B64 excitability, we found that unlike B34 and B40, B63 did not produce a strong suppression of B64 firing (Fig. 14*C*). In four experiments, B64 on average fired 36.5 ± 2.33 spikes with 3 sec current pulses; during B63 activity, B64 fired 34 ± 2.08 spikes, only 7% reduction.

Because picrotoxin blocked the fast IPSPs from B40/B34 to B64, we determined whether picrotoxin could block the inhibitory effects of B40/B34 on B64 excitability (Fig. 14*A,B,D,E*). Indeed, when 1 mM picrotoxin was bath-applied, B40/B34 no longer reduced B64 excitability. For B40 experiments, repeated measures ANOVA of three groups (before, B40, and after), showed no significant differences among these groups ($F_{(2,4)} = 1.90$; $p > 0.05$). For B34 experiments, repeated measures ANOVA of three groups (before, B34, and after), showed no significant differences among these groups ($F_{(2,6)} = 2.05$; $p > 0.05$). Note that although picrotoxin block of the IPSPs from B40 and B34 to B64 was incomplete (Fig. 13), picrotoxin was able to eliminate the suppressive effects of B40/B34 on B64 excitability.

After washout of picrotoxin, the suppressive effects of B40/B34 on B64 excitability recovered. For B40 experiments, repeated measures ANOVA of three groups (before, B40, and after), showed a significant difference among these groups ($F_{(2,4)} = 16.17$; $p < 0.05$), and a Bonferroni multiple comparisons test of different group pairs showed that the B40 group was significantly different than the before ($t = 4.99$; $p < 0.05$) and after ($t = 4.86$; $p < 0.05$) groups, whereas the before group was not significantly different from the after group ($t = 0.13$; $p > 0.05$). For B34

experiments, repeated measures ANOVA of three groups (before, B34, and after), showed a significant difference among these groups ($F_{(2,6)} = 13.62$; $p < 0.01$), and a Bonferroni multiple comparisons test of different group pairs showed that the B34 group was significantly different than the before ($t = 4.65$; $p < 0.05$) and after ($t = 4.38$; $p < 0.05$) groups, whereas the before group was not significantly different from the after group ($t = 0.28$; $p > 0.05$).

Discussion

Multiple GABAergic actions in motor program specification

We studied functions of GABAergic interneurons in motor control. In *Aplysia* feeding motor network (Fig. 1), three feeding interneurons (CBI-2, B63, B64) are critical for initiation and triggering of the radula protraction–retraction sequence of motor programs (Rosen et al., 1991; Church and Lloyd, 1994; Hurwitz and Susswein, 1996; Hurwitz et al., 1997, 2003; Jing and Weiss, 2001) and can be considered as principal elements of the network. Three GABAergic interneurons (CBI-3, B40, and B34) can be distinguished from the principal interneurons in that they play *regulatory* roles. They regulate two parameters of CBI-2-elicited ingestive motor programs, the phasing of activity of the radula closure motoneuron B8, and the protraction duration. Consistent with this notion, blockade of GABAergic inhibition by picrotoxin causes specific changes in B8 activity and protraction duration without disrupting activation of motor programs. Furthermore, GABAergic interneurons use multiple mechanisms to specify ingestive motor programs.

The phasing of B8 activity is implemented by a CPG interneuron, B40, and a higher-order interneuron, CBI-3. In earlier studies (Jing and Weiss, 2001, 2002), we showed that B40 and CBI-3 are dual-function interneurons that reduce B8 activity during protraction and increase B8 activity during retraction. B40 does this directly through fast inhibition of B8 during protraction and slow excitation of B8 during retraction. CBI-3 does this indirectly through fast inhibition of B20, which excites B8 during protraction; and slow inhibition of B4/5 which inhibits B8 during retraction. Here, we show that the fast inhibitory synaptic potentials elicited by B40 and CBI-3 are likely to be mediated by GABA. Furthermore, CBI-3 excites B40, providing feedforward inhibition to B8 during protraction. Thus, concerted mechanisms operating at multiple sites within the network ensure the phasing of B8 activity that is appropriate for ingestive motor programs.

The lengthening of protraction duration is implemented by two CPG interneurons, B40 and B34. Previously it was shown that B40 and B34 also have other functions (Hurwitz et al., 1997; Jing and Weiss, 2002). Specifically, B40 and B34 preferentially control radula closure and protraction motor neurons, respectively. Based on these actions, B40 and B34 have been classified as a closer controller and protraction controller, respectively. We now show that the functions of these interneurons may be more complex, because of the fact that they are an integral part of the CPG and as such they also control the timing of the motor pattern. In this case, B40 has a similar, rather than dissimilar, function as B34, i.e., lengthening protraction duration. Indeed, it appears that such effects are partly accounted for by similar, fast GABAergic inhibitory actions that B40 and B34 exert on the retraction-phase interneuron B64. Supporting evidence includes protraction shortening by subthreshold depolarization of B64, and protraction lengthening by hyperpolarization of B64 during picrotoxin perfusion.

Notably, although actions of B40/B34 on B64 provide a partial explanation of their effects on protraction duration, there appears to be an additional, unidentified interneuron (compare Baxter et al., 1997) that is also important for the retraction phase. Indeed, the existence of such a neuron was previously suggested because hyperpolarization of B64 did not prevent retraction (Hurwitz and Susswein, 1996). Similarly, during picrotoxin application, although hyperpolarization of B64 lengthened protraction, retraction still occurred (Fig. 12). This is evident both in the abrupt depolarization of B64 and in the spiking of B8 after protraction. Furthermore, protraction duration, although longer, was still shorter than that before picrotoxin perfusion. We speculate that B40/B34 inhibit both B64 and the unidentified neuron to promote a long protraction in ingestive motor programs elicited by CBI-2. After blockade of GABAergic inhibition, excitability of both postsynaptic neurons is increased, resulting in shorter protraction. Possibly, the increase in B64 excitability was somewhat larger than the increase in excitability of the unknown neuron, which may account for the partial lengthening of protraction by hyperpolarization of B64 during picrotoxin.

In brief, concerted GABAergic actions of three interneurons in the *Aplysia* feeding network function to bring about the coordinated expression of multiple parameters that define CBI-2-elicited ingestive motor programs in a behaviorally relevant manner. GABAergic actions of CBI-3 and B40 on two targets (B20, B8), allow coordination of control at different levels, interneuronal and motoneuronal. GABAergic actions of B40 and B34 on the same target (B64) produce cumulative effects. These findings complement earlier studies demonstrating that extrinsic sources of neurotransmitters and/or neuromodulators induce changes in multiple parameters of a motor pattern in crustacean stomatogastic nervous system (STNS) (Hooper and Marder, 1987; Turrigiano and Selverston, 1989; Harris-Warrick et al., 1992; Ayali and Harris-Warrick, 1999; Wood et al., 2000).

Implications for cotransmission

One common way to achieve multifunctionality in a given network is transmitter colocalization in single neurons (Brezina and Weiss, 1997; Nusbaum et al., 2001). There are two basic colocalization schemes: (1) a classical transmitter (e.g., ACh, glutamate or GABA), colocalized with one or more peptide transmitters, or (2) colocalization of two classical transmitters. By combining present findings with earlier ones, we can identify both schemes in the *Aplysia* feeding circuit. First, in addition to GABA, which mediates fast inhibition to B20, CBI-3 also contains a neuropeptide, APGWamide, which mediates slow inhibition of B4/5 (Jing and Weiss, 2001; Morgan et al., 2002). In this case, both fast and slow synapses contribute to motor program switching by acting in two different phases, thereby solving a temporal problem. This contrasts with the STNS where two cotransmitters of the modulatory proctolin neuron (MPN) act at different levels: GABA acts on another higher-order interneuron, whereas proctolin acts on the pyloric CPG (Blitz and Nusbaum, 1999).

Second, B34 may contain two classical transmitters: GABA and ACh. ACh appears to mediate B34 excitatory actions on protraction motoneurons (Hurwitz et al., 2003). We now show that inhibitory actions on B64 by B34 may be mediated by GABA. One implication of this type of a colocalization scheme is that a CPG element can use two classical transmitters to mediate, simultaneously, fast excitatory and inhibitory actions on different targets. Although such a scheme has been previously described (see below), its exact functions are not well understood. Here, the two synaptic actions serve distinct functions. Excitation mediates firing of in-phase motoneurons, and inhibition controls protraction duration through actions on an out-of-phase interneuron.

Although relatively few examples in the CNS are documented, colocalization of more than one small-molecule transmitters has been described in spinal cord (GABA and glycine, Jonas et al., 1998; GABA and ATP, Jo and Schlichter, 1999; 5-HT and dopamine, Svensson et al., 2001), substantia nigra (dopamine and glutamate, Sulzer et al., 1998), dentate gyrus granule cells (GABA and glutamate, Walker et al., 2001), and *Aplysia* CNS (GABA and dopamine, Diaz-Rios et al., 2002). It is striking that GABA is involved in many of these colocalization schemes. Colocalization of GABA and ACh in B34 resembles that of GABA and ATP in spinal cord, and GABA and glutamate in dentate gyrus, where GABA exerts fast inhibitory actions, whereas the other cotransmitter exerts fast excitatory actions.

Regulatory functions of GABAergic inhibitory interneurons

We obtained evidence for the presence of inhibitory GABAergic interneurons and synaptic actions in *Aplysia*. Previously, potential transmitter roles of GABA in molluscan nervous systems have been suggested by extensive studies of GABA actions on molluscan neurons (Yarowsky and Carpenter, 1978; Kehoe and Vulfius, 2000), and GABA immunoreactivity and biochemistry in a number of molluscan species (Cottrell, 1974; McCaman et al., 1979; Cooke and Gelperin, 1988; Richmond et al., 1991; Arshavsky et al., 1993; Diaz-Rios et al., 1999; Norekian, 1999; Hatakeyama and Ito, 2000). Thus, it may appear surprising that no molluscan GABAergic inhibitory interneuronal synapses have been identified, although inhibitory synapses from GABAergic vestibular hair cells to photoreceptors in *Hermissenda* (Alkon et al., 1993) have been reported.

In several systems where the functions of GABAergic inhibitory interneurons have been studied in great detail (Traub et al., 1999; McBain and Fisahn, 2001; Laurent, 2002), one emerging thread appears to be the regulatory functions of the GABAergic interneurons. Specifically, in each neural network, there are principal elements that mediate the normal activity of the network. GABAergic interneurons come into play to regulate activity of these principal neurons. In insects, local GABAergic interneurons cause picrotoxin-sensitive oscillatory synchronization in antennal lobe projection neurons (MacLeod and Laurent, 1996), that is functionally relevant for tasks that require fine, but not coarse, odor discrimination (Stopfer et al., 1997). In vertebrates, a variety of GABAergic inhibitory interneurons coordinate activity of principal neurons and promote network oscillation in hippocampus (McBain and Fisahn, 2001) and cortex (Galarreta and Hestrin, 2001). Interestingly, based on the diversity of GABAergic interneurons and their synapses, it was hypothesized (Gupta et al., 2000) that a control scheme that uses different combinations of GABAergic interneurons, similar to the scheme described here, may also operate in the neocortex.

As outlined in the beginning of the discussion, coordinating roles of GABAergic inhibitory interneurons can also be extended to *Aplysia* feeding motor network. Here, the GABAergic actions of CBI-3, B40, and B34 regulate the activity of principal neurons (e.g., B64) as well as motoneurons to produce a specific type of ingestive motor program (biting).

In summary, integrative functions of GABAergic inhibitory interneurons in motor control of *Aplysia* feeding parallel the coordinating roles found for GABAergic interneurons in several nonmotor systems. Because of advantageous features of the *Aplysia* network, we elucidated in detail mechanisms of the concerted actions of these GABAergic interneurons and prominent roles of cotransmission of GABA with other neurotransmitters in motor program specification.

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