A Newly Identified Buccal Interneuron Initiates and Modulates Feeding Motor Programs in Aplysia


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Dembrow, N. C., J. Jing, A. Proekt, A. Romero, F. S. Vilim, E. C. Cropper, and K. R. Weiss. A newly identified buccal interneuron initiates and modulates feeding motor programs in Aplysia. J Neurophysiol 90: 2190–2204, 2003. First published June 11, 2003; 10.1152/jn.00173.2003. Despite considerable progress in characterizing the feeding central pattern generator (CPG) in Aplysia, the full complement of neurons that generate feeding motor programs has not yet been identified. The distribution of neuropeptide-containing neurons in the buccal and cerebral ganglia can be used as a tool to identify additional elements of the feeding circuitry by providing distinctions between otherwise morphologically indistinct neurons. For example, our recent study revealed a unique and potentially interesting unpaired PRQFamide (PRQFVa)-containing neuron in the buccal ganglion. In this study, we describe the morphological and electrophysiological characterization of this novel neuron, which we designate as B50. We found that activation of B50 is capable of producing organized rhythmic output of the feeding CPG. The motor programs elicited by B50 exhibit some similarities as well as differences to motor programs elicited by the command-like cerebral-to-buccal interneuron CBI-2. In addition to activating the feeding CPG, B50 may act as a program modulator.

INTRODUCTION

The feeding motor system of Aplysia is a useful preparation for the study of both the central and the peripheral mechanisms underlying control of rhythmic motor behavior. This system produces a number of ingestive or egestive feeding behaviors, which can be distinguished by phasing and duration of protraction–retraction and opening–closing movements of the radula, the main feeding apparatus of Aplysia (Kupfermann 1974; Morton and Chiel 1993a,b). Although many of the neurons involved in central control of the feeding system have been identified (see following text), the full complement of neurons involved in the generation of feeding motor programs has not yet been identified.

The output of feeding motor programs is organized by a central pattern generator (CPG) that is located in the buccal ganglion (e.g., Hurwitz and Susswein 1996; Hurwitz et al. 1997, 2003; Susswein and Byrne 1988). Activity in the buccal CPG is initiated and modulated by higher-order command-like neurons located in the cerebral ganglion (Hurwitz et al. 2003; Jing and Weiss 2001; Morgan et al. 2000, 2002; Rosen et al. 1991; Xin et al. 1999). A combination of semi-intact preparations and chronic nerve recordings in behaving animals has enabled correlation of patterned outputs (motor programs) generated in the isolated CNS with several of the feeding-related behaviors in the intact animal (Cropper et al. 1990; Kupfermann and Weiss 1982; Morgan et al. 2002; Morton and Chiel 1993a,b; Rosen et al. 1991). To understand how the buccal CPG generates such coordinated and yet flexible motor programs, the role of the various buccal CPG elements must be elucidated. To this end, the contributions of several identified interneurons to the output of the feeding CPG have been characterized (e.g., Brembs et al. 2002; Hurwitz and Susswein 1996; Hurwitz et al. 1997, 2003; Jing and Weiss 2001, 2002; Jing et al. 2003; Kabotyanski et al. 1998; Susswein and Byrne 1988; Teyke et al. 1993). These interneurons contribute to the organization and flexibility of motor programs. For example, some interneurons can modify the ingestiveness or egestiveness of motor programs by altering the coupling of the opening–closing motor neurons’ activities to motor neurons mediating protraction–retraction (Jing and Weiss 2001, 2002; Jing et al. 2003). In addition, some interneurons can modify the duration of specific phases in the motor programs (Evans and Cropper 1998; Hurwitz and Susswein 1996; Hurwitz et al. 2003; Jing et al. 2003). Despite the progress made toward characterizing the functions of identified CPG elements, the current description of the buccal CPG is incomplete and important interneurons remain to be identified.

A recent study mapping the distribution of a newly isolated and cloned neuropeptide PRQFamide (PRQFVa) revealed an unpaired neuron on the caudal surface of the buccal ganglion (Furukawa et al. 2003). Because no unpaired neurons had been identified in that region of the buccal ganglion (Church and Lloyd 1994; Evans and Cropper 1998; Goldstein and Schwartz 1989; Kabotyanski et al. 1998), we hypothesized that the PRQFVa-positive neuron might be a heretofore-unidentified CPG element.

In this study, we have identified and characterized the unpaired PRQFVa-positive neuron, which we designated B50. We describe B50’s morphology, electrophysiological properties, and its connections within the feeding circuitry. These properties identify B50 as a novel protraction-phase interneuron, distinct from the other neurons located within its vicinity. We found that B50 was capable of initiating patterned activity in the CPG and was recruited during multicyle programs driven by the higher-order cerebral-to-buccal interneuron 2 (CBI-2). We then found that when B50 was activated during...
CBI-2–elicited programs it shortened the duration of their protraction phase. The QRQFVa neuropeptide localized to B50 accounted for some, but not all, of its effects on the CPG output. B50’s other effects on the CPG appear to be mediated in part by its fast synaptic outputs.

**METHODS**

Experiments were performed on *Aplysia californica* weighing 50–250 g, obtained from Marinus (Long Beach, CA) and the Aplysia Research Facility (Miami, FL). All animals were maintained for 2–7 days in holding tanks at 14–16°C. Animals were anesthetized by injection of 50% of their body weight of isotonic MgCl₂ into the body cavity.

Depending on the experiment, different dissections were performed. For the semi-intact preparation the buccal and cerebral ganglia were removed with the buccal mass attached by buccal nerves 1–3. The nerves connecting the cerebral ganglion to the buccal mass were cut. The ganglia and buccal mass were transferred to a dissecting dish bottomed with a silicone elastomer (Sylgard; Dow Corning) formed. For the semi-intact preparation the buccal and cerebral ganglia were then transferred to a recording dish filled with a 50:50 mixture of ASW:isotonic MgCl₂. The connective tissue sheath covering the buccal ganglion neurons was surgically removed. The ganglia and buccal mass were then transferred to a recording dish filled with ASW. The preparation was then pinned on an elevated portion of silicone elastomer such that contractions and movements of the buccal mass did not transfer to the ganglia. Seaweed was inserted into the buccal mass with forceps to initiate patterned motor activity.

For studies on the isolated CNS, buccal ganglia, and the buccal ganglion with the cerebral ganglion attached (the cerebral-buccal connectives intact) were removed from the animal and transferred to a dissecting dish coated with a silicone elastomer and filled with a 50:50 mixture of ASW:isotonic MgCl₂. The connective tissue sheath covering the neurons of the buccal and cerebral ganglia was then surgically removed. The ganglia were then pinned to the correct orientation for recording from neurons. Unless otherwise mentioned, the cerebral ganglion was pinned ventral side up, and the buccal ganglion pinned caudal surface up. Fresh ASW was perfused into the dish at a rate of 375 μl/min using a peristaltic pump (Dynamax, Rainin) and removed using vacuum suction. Using the cooling plate, the preparation was cooled to 14–16°C. In cases in which we sought to determine whether connections were monosynaptic, we substituted ASW containing a high-concentration divalent cation solution (HiDi, in mM: 312 NaCl, 10 KCl, 33 CaCl₂, 132 MgCl₂, and 20 HEPES, at pH 7.5):isotonic MgCl₂. The connective tissue sheath of a ganglion was surgically removed. The ganglia were then pinned to the correct orientation for recording from neurons. Unless otherwise mentioned, the cerebral ganglion was pinned ventral side up, and the buccal ganglion pinned caudal surface up. Fresh ASW was perfused into the dish at a rate of 375 μl/min using a peristaltic pump (Dynamax, Rainin) and removed using vacuum suction. Using the cooling plate, the preparation was cooled to 14–16°C. In cases in which we sought to determine whether connections were monosynaptic, we substituted ASW containing a high-concentration divalent cation solution (HiDi, in mM: 312 NaCl, 10 KCl, 33 CaCl₂, 132 MgCl₂, and 20 HEPES, at pH 7.5).

Hexamethonium and PRQFVa neuropeptide (Synpep, Dublin, CA) were applied by replacing the ASW perfusate with ASW containing freshly dissolved hexamethonium or PRQFVa at the concentrations indicated. Unless otherwise mentioned, all chemicals were obtained from Sigma (St. Louis, MO).

**Dye injections**

To mark identified neurons, dye injections were performed with intracellular recording electrodes (see above) that had a tip filled with 3% 5(6)-carboxyfluorescein in 0.1 M potassium citrate titrated to pH 8.0 with potassium hydroxide (Rao et al. 1986). The dye was injected iontophoretically by either applying a constant hyperpolarizing current (~1 to ~5 nA) or brief pulses (500 ms) of hyperpolarizing current (~1 to ~10 nA) for 10 to 15 min. In cases in which the morphology of neurons was examined, the ganglion was transferred to a new dish after injection and incubated at 16°C for 3–12 h with probenecid (10 mM), a compound that reduces the active transport of the dye out of the cells. Ganglia were then visualized using a Nikon microscope with epifluorescence and photographed using a Nikon CoolPix 990 digital camera attached through a standard C-mount. Digital images were compiled using Adobe Photoshop 5.0.

**Immunocytochemistry**

Immunocytochemistry was performed using the previously characterized rat anti-PRQFVa antibody (Furukawa et al. 2003), following the previously described protocol (Vilim et al. 1996). Ganglia were fixed (4% paraformaldehyde, 0.2% picric acid, 25% sucrose, 0.1 M NaH₂PO₄, pH 7.6) overnight at 4°C. After several washes with PBS to remove the fixative, the 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 Na₂HPO₄, 50 mM EDTA, and 0.01% thimerosal, pH 7.4). Primary antibody was diluted 1:250 in BB and incubated with the tissue for 4–7 days. The tissue was then washed twice a day for 2–3 days with washing buffer (WB: 2% Triton X-100, 1% BSA, 154 mM NaCl, 10 mM Na₂HPO₄, 50 mM EDTA, and 0.01% thimerosal, pH 7.4). After the washes, the tissue was incubated with 1:500 dilution of secondary antibody (lis-samine–rhodamine donkey anti-rat: Jackson ImmunoResearch, West Grove, PA) for 2–3 days. The tissue was then washed twice with WB, and 4 times with storage buffer (SB: 1% BSA, 154 mM NaCl, 10 mM Na₂HPO₄, 50 mM EDTA, and 0.01% thimerosal, pH 7.4). After unbound secondary antibody was washed out, ganglia were visualized and photographed using a Nikon microscope as described above.

In situ hybridization

Whole mount in situ hybridization was performed in the same manner as described previously (Fujisawa et al. 1999). Briefly, desheathed buccal ganglia were fixed as described for immunocytochemistry, washed 3 × 10 min at room temperature (RT) in PBT (0.8% NaCl, 0.02% KCl, 0.3% Na₂HPO₄, 12H₂O, 0.02% KH₂PO₄, and 0.1% Tween 20, pH 7.4). The ganglion was then digested with 50 mg/ml of proteinase K in PBT for 30 min at 37°C, and then washed again with PBT 3 × 10 min at RT. The ganglion was then postfixed with 4% paraformaldehyde in PBT for 1 h at RT, and then washed once more for 3 × 10 min at RT with PBT. The ganglion was prehybridized for 1 h at 42°C in hybridization buffer (5× SSC, 1% blocking reagent, 50 mg/ml salmon sperm DNA, 0.1% sarkosyl, and 0.02% SDS) and then hybridized overnight at 42°C in hybridization buffer containing 1 mg/ml of the labeled antisense oligo probe designed to bind to the PRQFVa mRNA precursor sequence at many different locations (PR-QISH: CCC AAC AAA TTG ACG AGG CC). The oligo was labeled by tailing with digoxigenin (DIG)—dUTP/dATP according to the manufacturer’s instructions (Boehringer Mannheim, Indianapolis, IN). Unbound probe was washed out with 2× SSC and 0.01% SDS for 3 × 1 h at 42°C then with PBT for 2 × 10 min at RT. The ganglion was then blocked with 1% blocking reagent (Boehringer, catalog no.1096176) in 0.15 M NaCl and 0.1 M maleic acid, pH 7.5, for 3 h at RT and then incubated in 1:200 dilution of anti-DIG antibody labeled with alkaline phosphatase (Boehringer, catalog no.1093274) in blocking solution for 24 h at 4°C. Unbound antibody was washed out with PBT for 5 × 1 h at RT, then washed with detection buffer (0.1 M Tris, 0.1 M NaCl, 5 mM MgCl₂, and 10 mM levamisole) for 2 × 30 min at RT. The signal was developed for 30 min at RT with detection buffer containing 350 mg/ml nitroblue tetrazolium, 175 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, and 0.1% Tween 20, and the reaction was then stopped by washing the ganglion with PBT containing 1 mM EDTA (PBTE). The ganglia were postfixed with 4% paraformaldehyde in PBT for overnight at 4°C. After washing with PBT, they were stored protected from light in 50% glycerol and PBTE at 4°C. All reagents and solutions used were made with diethyl pyrocarbonate-treated MilliQ water, and care was taken to avoid contamination with RNAses.
**Electrophysiological recordings**

Intracellular recordings were made using single-barreled glass microelectrodes filled with 2 M potassium acetate and 30 mM potassium chloride. Electrodes were beveled to a resistance of 5–8 MΩ. Recordings were obtained using either an AxoClamp 2A or 2B (Axon Instruments, Union City, CA), or a Getting 5A amplifier (Getting Instruments, Iowa City, IA). Neurons were stimulated with repeated short pulses using a model S88 stimulator (Grass Medical Instruments), whereas the Getting or AxoClamp amplifiers were used to perform longer DC injections.

Extracellular nerve recordings were obtained using polyethylene suction electrodes connected to a differential AC amplifier (M1700; A-M Systems, Carlsborg, WA). The S88 stimulator was used for nerve stimulation.

All electrophysiological data were recorded both on a chart recorder (MT9500; Astro-Med, West Warwick, RI) as well as on a modulator recorder (VDAT8; Vetron Technology). Data were digitized using a DigiData 1322A with the AxoScope software (Axon Instruments, Burlingame, CA).

**RESULTS**

**Identification of the PRQFVa-containing neuron B50**

To examine which hemiganglion the unpaired neuron was located in, we processed buccal ganglia with either in situ hybridization to the mRNA of the PRQFVa precursor or immunohistochemistry to the neuropeptide itself. In situ hybridization showed that the unpaired neuron was located in the left hemiganglion of the animal (5 out of 5 experiments). Immunohistochemistry also localized the PRQFVa-positive neuron primarily to the left hemiganglion (10 out of 12 experiments). This hemiganglion was therefore targeted for further characterization.

We used a double-labeling strategy to characterize identifying characteristic features of the PRQFVa-positive neuron. Once the electrophysiological features and connectivity of a putatively PRQFVa-positive neuron were determined, we injected it with carboxyfluorescein dye. The ganglion was then processed for immunohistochemistry to PRQFVa with a rhodamine-conjugated secondary antibody. We found that the PRQFVa-positive neuron, which we named B50, has morphological and electrophysiological properties (see following text) that distinguish it from other neurons in its vicinity.

Figure 1A illustrates B50’s position relative to the identified protraction interneuron B34 (Hurwitz et al. 1997; Susswein and Byrne 1988). Both neurons were injected with carboxyfluorescein dye (Fig. 1, A1 and A2); but only B50 was PRQFVa immunopositive (Fig. 1, A3 and A4). B50 (black arrow) was located lateral relative to B34 (white arrow) and had a slightly larger soma.

**MORPHOLOGY OF B50.** To ascertain B50’s morphology, carboxyfluorescein dye injected into B50 was allowed to spread through the cell’s processes overnight. None of B50’s processes exited the buccal ganglion, indicating that B50 was a buccal interneuron. B50’s main process projected to the contralateral hemiganglion and then looped back, returning to the hemiganglion containing B50’s soma (n = 5, Fig. 1B1). Both the turning point and the terminal portion of the main process were located in the region containing several protraction neurons: B31/B32, B33, B34, and B63 (Hurwitz et al. 1994, 1997; Susswein and Byrne 1988). Higher-magnification views of the terminal portion (Fig. 1B2) and the turning point (Fig. 1B3) of B50’s main process are shown. Several smaller processes branching from the main process were visible at its terminal portion.

**ELECTROPHYSIOLOGICAL PROPERTIES OF B50.** The resting membrane potential of B50 ranged from -66 to -39 mV with a mean resting membrane potential of -52.44 ± 2.1 mV (mean ± SE, n = 18). Unlike several identified neurons in the vicinity (e.g., B33, B63, B65) (Hurwitz et al. 1994, 1997; Kabotyanski et al. 1998; Susswein and Byrne 1988) large (i.e., >5 nA) current injections were required to drive action potentials in B50 (Fig. 1C1). When B50 did fire action potentials in response to DC injection, they had a characteristically large afterhyperpolarization (Fig. 1, C1 and C2). B50 was distinguishable from another high-threshold protraction interneuron in its vicinity (B34; Hurwitz et al. 1997) by the spike frequency adaptation B50 displayed when depolarized above threshold by current injection (Fig. 1, C1 and C2). B50 did not display plateau properties, nor did it display postinhibitory rebound in response to brief hyperpolarizing current injections (Fig. 1C3). During spontaneous rhythmic activity in the buccal ganglion (Fig. 1D), B50 did not spike, although it became slightly depolarized during the protraction phase (open bar, Fig. 1D) as monitored by 12 nerve (12N) and B31/B32 activity (Hurwitz et al. 1994). B50 became hyperpolarized during the retraction phase (black bar) as monitored buccal nerve 2 activity (BN2: Morton and Chiel 1993a).

**B50’s activity during evoked motor programs**

*Aplysia* feeding circuitry is normally quiescent (spontaneous programs do occur, however; e.g., Fig. 1D). Once activated, the feeding circuitry can generate several categories of patterned activity: ingestive, egestive, or intermediate motor programs (Morgan et al. 2002; Morton and Chiel 1993a,b). Each category of motor program recruits some neurons to different degrees (Jing and Weiss 2001, 2002). Therefore to characterize the role that B50 plays in the feeding circuitry we examined B50’s activity during several types of motor programs.

Of the various means of eliciting motor programs, only CBI-2 stimulation recruited B50 (Fig. 2). CBI-2 is a command-like higher-order neuron that can initiate biting-like motor programs in the CPG. In response to seaweed application to the inner lips of the animal, CBI-2 can fire >10 Hz during each protraction phase, for several cycles of activity (Rosen et al. 1991). When CBI-2 is stimulated at similar frequencies (8–15 Hz) during the protraction phase, a single cycle of patterned activity (single-cycle motor program) is elicited (Jing and Weiss 2001, 2002; Li et al. 2001). Stimulating CBI-2 for longer periods (beyond a single cycle of patterned activity) triggers multiple motor program cycles (Church and Lloyd 1994; Hurwitz et al. 2003; Morgan et al. 2000; Rosen et al. 1991). B50’s recruitment into CBI-2–elicited programs depended on whether CBI-2 was stimulated to elicit single- or multicycle motor programs. CBI-2–elicited single-cycle motor programs usually failed to recruit B50 to fire (Fig. 2A), although in a few cases, B50 was recruited (2 out of 6 preparations, Fig. 2B). CBI-2–elicited multicycle programs recruited B50 more reliably (5 out of 6 preparations, Fig. 2C).

When B50 was recruited during CBI-2–elicited programs, it fired a burst of action potentials (0.5–10 Hz) at the beginning...
of the protraction phase (Fig. 2, B and C). B50 displayed similar spike frequency adaptation when recruited during CBI-2–elicited motor programs (Fig. 2) as it had when injected with depolarizing current (Fig. 1C). In some cases, the frequency of action potentials decreased until B50 activity ceased fully before the termination of the protraction phase (Fig. 2B). In other cases, the active termination of the protraction phase occurred before B50 activity fully ceased, although spike frequency accommodation was still present (Fig. 2C). B50’s action potentials during CBI-2–elicited programs were slightly different from those observed during DC injections. The large afterhyperpolarizations observed in B50’s action potentials in response to DC injection were far less pronounced during motor programs. It is therefore important to note that although the large afterhyperpolarization in B50’s action potentials is useful for identifying B50, it is not as pronounced when B50 is activated by synaptic input. This difference between B50’s properties is perhaps not surprising given that during motor programs many neurons receive modulatory inputs that alter their properties.

Similarly, although B50 displayed no postinhibitory rebound in response to hyperpolarizing current injections (Fig. 1C3), at the end of the retraction phase of a motor program, when B50 was relieved from inhibitory synaptic input, B50 was far more likely to fire action potentials. We cannot exclude the possibility that B50 displays postinhibitory rebound that is conditional on network activity. B50’s recruitment in CBI-2–elicited multicycle motor programs was more robust during the second cycle of the protraction phase than during the first (Fig. 2, B and C). When CBI-2–elicited single-cycle motor programs recruited B50, B50 also fired a second burst of action potentials after the end of the first motor program (Fig. 2B) even in the absence of continued CBI-2 stimulation. This second burst of B50 activity always corresponded with an additional motor program cycle.

Motor programs elicited by a number of methods besides CBI-2 stimulation did not recruit B50 activity. In all of these motor programs, B50’s membrane potential did depolarize at the onset of the protraction phase but this was insufficient to drive the cell to fire. This was the case whether the motor programs were elicited by stimulating key elements of the CPG (B63: Fig. 3A, n = 4), pharmacologically activating the CPG by the muscarinic agonist oxotremorine (Fig. 3B, n = 3), or by stimulating the esophageal nerve (EN: Fig. 3C, n = 6).

FIG. 1. Identifying features of PRQFVa positive-neuron (B50). A: double labeling of PRQFVa-positive neuron. A1: both previously identified neuron B34 (white arrowhead) and newly identified neuron B50 (black arrowhead) were identified electrophysiologically and injected with 3% 5(6)-carboxyfluorescein. All immunohistochemistry and dye injection images are of caudal surface of buccal ganglion, with ventral clusters and cerebro-buccal connec-tives facing down. A2: same dye injection shown at higher magnification. A3: same ganglion, examined for PRQFVa immunoreactivity using primary anti-serum for PRQFVa and rhodamine-conjugated goat, anti-rabbit secondary antiserum (Jackson Immunoresearch Laboratories). B50 was immunopositive, B34 was not. Scale bar: 500 μm. A4: same immunohistochemistry, shown at higher magnification. Scale bar: 80 μm. B: morphology of PRQFVa-positive neuron, B50. B1: B50’s processes are limited to buccal ganglion. 5(6)-Carboxyfluorescein dye (3%) was injected into B50, and allowed to spread into B50’s processes overnight in artificial seawater (ASW) containing 10 mM probenecid. Main process of B50 projected from left buccal hemiganglion to right buccal hemiganglion, and then looped back into left hemiganglion. Scale bar: 500 μm. B2: photograph focused on the terminal of B50’s process, shown at higher magnification. Terminal was split into several smaller processes. Scale bar: 50 μm. B3: turn of loop in contralateral ganglion shown at higher magnification. Scale: same as for B2. C: intrinsic electrophysiological properties of PRQFVa-positive neuron B50. C1: B50 has high action potential threshold. Depolarizing DC steps of 2, 4, 6, 8, and 10 nA (left traces) were applied for 5 s to B50 (marked by thin bar underneath trace). Depolarizing current step of 11 nA for 35 s (right trace) demonstrated both characteristic spike frequency buildup and subsequent adaptation such that B50 fired burst of action potentials only when depolarized for a sustained period. For all intracellular recordings, membrane potential of neuron at start of each recording is listed beneath trace in mV. C2: expanded trace of action potentials in 11-nA, 35-s (C1, right trace) current injection reveals distinctly large afterhyperpo-larization of action potentials. Membrane potential listed (−28 mV) at start of trace has had bridge balance subtracted from it. C3: hyperpolarizing pulses of 2, 4, and 6 nA injected into B50 (thin bars above voltage trace) produced no postinhibitory rebound in B50. D: B50 activity during spontaneous motor program. Protraction phase (open bar) was monitored by both I2 nerve activity (I2N) and activity in protraction motor neuron B31/B32. Retraction phase (filled bar) was monitored by activity in buccal nerve 2 (BN2).
The touch of seaweed inside the buccal cavity (marked by black arrow) did elicit a brief depolarization in B50 during the protraction phase, but this was also insufficient to drive the cell to fire (Fig. 3D, n = 3).

Synaptic connections

INPUTS TO B50. B50 received inhibitory input from the retraction interneuron B64 (Hurwitz and Susswein 1996). Fast, hyperpolarizing postsynaptic potentials (PSPs) in B50 corresponded one-for-one to spikes in B64 (n = 3, Fig. 4A). The one-for-one PSPs were present in a solution containing a high concentration of divalent cations (HiDi), indicating that the connection was monosynaptic. In addition to receiving fast inhibitory input from the retraction interneuron B64, B50 also received a slow hyperpolarizing input from the protraction interneuron B34 (n = 5, Fig. 4B). The hyperpolarizing input from B34 was very small and required several seconds of high-frequency firing in B34 (about 15 Hz) before it became visible. Nonetheless, this connection persisted in HiDi (data not shown). Of the identified protraction neurons examined (B31/B32, B63, B65), only B34 elicited this inhibitory input to B50.

CBI-2–elicited PSPs in B50 were one-for-one with CBI-2 action potentials (n = 4, Fig. 4C) both in normal saline and HiDi. These PSPs were relatively small compared with CBI-2–elicited PSPs in other protraction interneurons (B34/B63; Hurwitz et al. 2003). We found that the modulatory serotoninergic neuron, metacerebral cell (MCC) (Weiss et al. 1978), also elicited a slow depolarizing input to B50 (Fig. 4D). The slow PSP elicited in B50 during MCC stimulation persisted for several seconds after the end of MCC stimulation (n = 5). Consistent with B50 morphology (i.e., the lack of projections to the cerebral ganglion), stimulation of B50 did not elicit synaptic potentials in CBI-2 or MCC.

B50 Excites Key Elements of the Protraction Circuitry. B50 made connections to both the protraction motor neurons B31/B32 and B61/B62 and the protraction interneurons B34 and B63 (Fig. 5). B50-elicited PSPs in B31/B32 (n = 21, Fig. 5A), B63 (n = 9, Fig. 5A), and B34 (n = 10, Fig. 5B) corresponded one-for-one to B50 action potentials. The B50-elicited PSPs in B61/B62 could not be unequivocally established as following B50 action potentials in a one-for-one manner (but see following text). B50 drove the protraction neurons B34, B63, and B61/B62 to fire with a relatively brief period of stimulation (B63, Fig. 5A; B61/B62, Fig. 5B; B34, Fig. 5B). Because the B31/B32 neurons must be driven to a plateau potential to elicit action potentials, and the B31/B32 plateau requires more prolonged stimulation, the brief B50 stimulation that was used in this experiment was insufficient to drive the B31/B32 neurons to fire. With a longer stimulation, B50 did drive the B31/B32 neurons into a plateau potential (e.g., see Figs. 8 and 11). All the B50-elicited PSPs in B31/B32, B34, and B63 persisted in a HiDi solution, indicating that they were monosynaptic (B31/B32, n = 8; B34, n = 7; B63, n = 3; Fig. 5). When the B50 to B61/B62 synaptic connections were examined in a HiDi solution, the PSPs now followed one-for-one B50 action potentials (n = 3, Fig. 5B). Although the connection from B50 to B61/B62 was small, it appeared to be monosynaptic. It is possible that the small amplitude of B50-elicited PSPs in B61/B62 made them difficult to discern among the various synaptic inputs B61/B62 receives. In a HiDi solution, the presence of a higher concentration of calcium (therefore more transmitter release) and lower polysynaptic activity enhances fast PSP size (Trudeau and Castellucci 1992), thus revealing the one-for-one correspondence between B50 action potentials and PSPs elicited in B61/B62. All of the connections B50 made to B31/B32, B34, and B63 were bilateral. The ipsilateral B50 to B61/B62 connection was tested only once, however, because of difficulties in recording from preparations with the buccal mass attached, seaweed application to the inside of the buccal cavity also did not recruit B50.
both B50 and the ipsilateral B61/B62 simultaneously. No appreciable differences in PSP sizes were detected between B50’s contralateral and ipsilateral followers. In cases where ipsilateral and contralateral followers of B50 were recorded simultaneously, the contralateral follower received the PSP first. The ipsilateral PSP occurred, on average 7.2 ± 0.8 ms (n = 6), after the contralateral one.

B50-elicited PSPs in the protraction-phase neurons displayed synaptic facilitation. PSPs both facilitated within a train of B50 stimulation and potentiated between trains (Fig. 6). This was examined by stimulating B50 three times with short (3-s) trains of depolarizing pulses (5–15 Hz) every 10 s. PSPs elicited by B50 in B31/B32, B34, and B63 all displayed similar forms of plasticity (Figs. 5 and 6). This facilitation was most pronounced at the B50 to B31/B32 synapse (Fig. 6B). Increases in PSP amplitude were significant within trains (ANOVA, F = 36.82, P < 0.05) and between trains (ANOVA, F = 3.284, P < 0.05).

Post-tetanic potentiation (PTP) of synaptic transmission within the feeding circuit of *Aplysia* contributes to the plasticity of motor programs (Hurwitz et al. 2003; Sanchez and Kirk 2000). We therefore examined whether the B50-elicited PSPs in B31/B32 demonstrated PTP. B50 was stimulated with single pulses (30 ms) at a low frequency (0.25 Hz) for 2 min, then stimulated at a high frequency (10–20 Hz) for 30–60 s, and then at a low frequency (0.25 Hz) again for several minutes. The amplitudes of the B50-elicited PSPs in B31/B32 before and after the high-frequency stimulation were then compared. B50-elicited fast PSP amplitude was increased several-fold after the high-frequency stimulation (Fig. 6C). The amplitude of subsequent B50-elicited PSPs decayed to prestimulation values within a few minutes. Overall, there was an eight-fold increase in the PSP amplitude that returned to control values within 160 s (Fig. 6C). The increase in the PSP amplitude was statistically significant for each of the groups ≤160 s (Fig. 6D, ANOVA, F = 58.02, P < 0.001, Student’s t-test P < 0.01, n = 5).

B50-ELICITED FAST PSPS ARE HEXAMETHONIUM SENSITIVE. Previous studies have shown that the fast EPSPs elicited by CBI-2 and some of the protraction interneurons are blocked by the cholinergic antagonist hexamethonium (Hurwitz et al. 2003). We examined whether the B50-elicited fast PSPs were also hexamethonium sensitive. B50 was stimulated using 15-ms pulses of suprathreshold depolarizing current, in 3- to 5-s bursts at 5–10 Hz, with an interval of 30 s while perfusing hexamethonium into the bath solution. We found that the B50-elicited fast PSPs in B63 (n = 3, Fig. 7A), B31/B32 (n = 6, Fig. 7A), and B34 (n = 4, Fig. 7B) were blocked by 100 μM hexamethonium. The application of hexamethonium also revealed a hexamethonium-insensitive slow PSP elicited by B50 stimulation in each of the protraction neurons (Fig. 7). Because of the fact that the fast PSPs from B50 to B61/B62 were very small, their hexamethonium sensitivity was not examined.

**FIG. 3.** B50 was not recruited in motor programs elicited by a variety of means. A: during B31-elicited motor programs, B50 was not recruited. Protraction interneuron B63 was stimulated by DC injection (5 nA) to drive several cycles of patterned activity. Protraction phase (open bar) was monitored by activity in II nerve (I2N). Retraction phase (filled bar) was monitored indirectly by hyperpolarization in protraction neuron B31. Radula closure was monitored by activity in radula closure motor neuron B8. For all intracellular recordings, initial membrane potentials of neurons are listed below each trace in mV. B: perfusing oxotremorine (10−5 M) onto buccal and cerebral ganglia elicited patterned activity in feeding circuitry, but did not recruit B50. Protraction phase was monitored by I2N and B63 activity. Retraction phase was monitored indirectly by hyperpolarization in protraction neurons. Closure phase was monitored by activity in B8. C: egestive motor programs elicited by esophageal nerve (EN) stimulation failed to recruit B50. EN was stimulated using 3-ms 5-V pulses at 2 Hz. I2N activity was used to monitor protraction; buccal nerve 2 (BN2) to monitor retraction, and the RN was used to monitor radula closure. D: activation of patterned activity using food stimulus in preparation with buccal mass attached did not recruit B50 activity. Application of seaweed into buccal cavity (black arrow) elicited two cycles of activity in semi-intact preparation. Protraction phase was monitored by activity in I2 nerve (I2N). Retraction phase (open bar) was monitored by activity in I2 nerve (I2N). Retraction phase (open bar) was monitored by activity in I2 nerve (I2N).
B50 stimulation initiates patterned activity in the buccal ganglion

To test whether B50 itself was capable of driving single cycles of patterned activity we examined activity in the monitors of protraction, retraction, and closure in response to stimulating B50 with 15-ms suprathreshold depolarizing current pulses at set frequencies (5–15 Hz) for the duration of the protraction phase (Fig. 8). This occurred whether the cerebral ganglion was attached (Fig. 8A) or not attached (Fig. 8B and C). B50-elicited motor programs did not require B50 stimulation for the entire duration of protraction phase. When B50 was stimulated briefly (Fig. 8C), a full cycle of protraction–retraction could be elicited. Similar to CBI-2–elicited programs, B50 stimulation elicited activity in the protraction phase neurons (B31/B32, B61/B62, B63) followed by the retraction phase neurons (B64). Recruitment of the radula closure neuron B8 [as monitored by activity in B8 directly or the radula nerve (RN) it projects to] occurred as well, although to variable degrees (analyzed further below).

B50 elicited multicycle motor programs when its stimulation was maintained beyond the initial protraction phase (Fig. 9). CBI-2 was not recruited during these B50 motor programs (Fig. 9A), nor was the cerebral ganglion required to elicit them (Fig. 9B). Similar to single-cycle motor programs, B50-elicited multicycle programs varied in the degree of radula closure recruitment. In some programs, robust B8 or RN activity was observed in retraction (Fig. 9A), whereas in others only weak activity was observed (Fig. 9B). In some cases, the radula coupling to a specific phase would shift during an ongoing motor program, apparently switching from egestive-like to ingestive-like motor programs (Fig. 9C).

To further characterize B50-elicited single-cycle motor programs, we compared them to the well-characterized single-cycle CBI-2–elicited motor programs (Hurwitz et al. 2003; Morgan et al. 2002). Several parametric features of CBI-2–elicited programs vary as a function of CBI-2 stimulation frequency (e.g., the total cycle duration, the protraction phase duration, and the ratio of the protraction and retraction phase durations) (Morgan et al. 2000). We sought to characterize the effects of B50 stimulation frequency on these basic parameters of the B50-elicited motor programs. We found that total cycle

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**FIG. 5.** B50 spikes elicit one-for-one fast PSPs in protraction neurons. A: stimulating B50 with 4-s, 10-Hz pulse resulted in one-for-one PSPs in both B63 and B31/B32 in normal saline (ASW). These one-for-one PSPs persisted in HiDi solution. B: stimulation of B50 with a 4-s pulse at 10 Hz elicited one-for-one fast PSPs in B34 but not B61/B62 in normal saline. Although B50-elicited PSPs in B61/B62 did not correspond one-for-one with B50’s action potentials, they did summate, thus driving B61/B62 past threshold. In HiDi solution B50-elicited PSPs in B61/B62 and B34 correspond one-for-one with B50 action potentials. Initial membrane potential of cell at start of each trace is listed in mV.
duration (Fig. 10A; ANOVA, $F = 9.33$, $P < 0.01$), protraction phase duration (Fig. 10B; ANOVA, $F = 8.55$, $P < 0.01$), and retraction phase duration (Fig. 10C; ANOVA, $F = 2.94$, $P < 0.05$) all decreased as a function of B50 stimulation frequency. Because both protraction and retraction durations were shorter as B50 stimulation frequency increased, the ratio of protraction

**FIG. 6.** Facilitation and potentiation of B50’s connections. A1–A3: stimulating B50 at 10 Hz for 3 s every 10 s revealed that postsynaptic potentials (PSPs) elicited by B50 facilitated both within a train and also showed potentiation between trains of PSP sizes elicited in ipsilateral protraction interneuron (B63L) and motor neurons (B31/B32L), as well as contralateral motor neuron (B31/B32R). For each trace, initial membrane potential of neuron is listed in mV. B: grouped data demonstrated significant increase in B50-elicited B31/B32 PSP within a train (first PSP vs. last PSP), as well as potentiation between trains (Trains 1–3). Significant increases in PSP amplitude within trains are marked by single asterisk (*) and between trains marked by double asterisk (**) (ANOVA, $F_{5.72} = 4.87$, Student’s $t$-test with Bonferroni corrections, $P < 0.05$). C: post-tetanic potentiation (PTP) at B50 to B31/B32 synapse. Data from single experiment, demonstrating PTP at B50 to B31/B32 synapse. PSPs in B31/B32 were elicited by stimulating B50 once every 4 s (0.25 Hz) for 2 min. B50 was then stimulated at 10 Hz for 30 s (shaded bar). Immediately after 30 s of high-frequency stimulation, B50 was stimulated once again every 4 s for another 3 min. D: grouped data ($n = 5$). All B50-elicited PSPs in B31/B32 for 2 min before high-frequency stimulation were grouped together (PRE). B50-elicited PSPs after high-frequency stimulation were grouped into 20-s bins. Values statistically significantly different from pretetanic stimulation are marked by asterisk (ANOVA, $F = 58.02$, $P < 0.001$).

**FIG. 7.** Hexamethonium sensitivity of B50-elicited fast PSPs. A: B50-elicited fast PSPs in B63 and B31/B32 were hexamethonium sensitive. ASW: stimulation of B50 in normal saline at 7.5 Hz for 5 s elicited both fast and slow PSPs in both B31 and B63 neurons. HEX: in presence of hexamethonium ($10^{-4}$ M) fast PSPs are blocked and slow PSPs are revealed. WASH: washing out hexamethonium with normal saline resulted in recovery of fast PSPs. In all traces, initial membrane potential of neuron is listed in mV.
to retraction did not change significantly (Fig. 10D; ANOVA, $F = 0.94$, $P = 0.42$).

Another well-characterized feature of CBI-2–elicited programs is that they can be segregated into distinct program types, depending on the firing frequency of the radula closure neuron B8 in protraction versus retraction phase (Morgan et al. 2002; Morton and Chiel 1993a). This is illustrated by plotting the firing frequencies of the closure neuron B8 during the protraction versus during the retraction phase. Plotting CBI-2–elicited programs in this manner reveals two distinct clusters: one ingestive-like and one egestive-like. B50-elicited programs were distinct from CBI-2 programs in this feature. B50-elicited programs did not cluster depending on B8 firing frequencies, as did CBI-2–elicited programs. B8 was recruited to fire less in B50-elicited motor programs than in CBI-2–elicited ones, regardless of which phase it was more strongly coupled to (Fig. 11, A vs. B). Occasionally, B50-elicited programs drove B8 to fire at frequencies that were within the cluster boundaries of CBI-2 ingestive-like or egestive-like motor programs, but most B50-elicited motor programs had B8 firing frequencies that were too low in either phase to be classified as either ingestive-

\[ F_{/}H1005 \ 0.94, \ P_{/}H1005 \ 0.42). \]

B50-elicited motor programs were also distinct from CBI-2–elicited motor programs in the timing of the recruitment of the B31/B32 projection motor neurons (Fig. 11). In CBI-2–elicited programs, B31/B32 recruitment occurred a few seconds after activity in the I2N had commenced. In B50-elicited programs, B31/B32 was recruited much earlier in the protraction phase.

**B50 shortens CBI-2–driven motor programs**

B50 was recruited during certain cycles but not others of CBI-2–elicited multicycle programs. We observed that the

\[ B50 \text{ driven motor programs} \]

B50 was recruited during certain cycles but not others of CBI-2–elicited multicycle programs. We observed that the
individual cycles during which B50 was recruited correlated with shortened protraction phase durations (Fig. 2C). To test whether B50 was capable of causing this shortening of protraction duration, we activated B50 during CBI-2–elicited single-cycle programs, during which it was not normally recruited (Fig. 12). We triggered single-cycle motor programs by stimulating CBI-2 with 10-ms depolarizing pulses administered at 9 Hz for the duration of the protraction phase with an interstimulation interval of 2 min. B50 was coactivated during a CBI-2–elicited motor program either by stimulating it with 15-ms suprathreshold depolarizing pulses at a frequency of 4–10 Hz (n = 2), or by simply depolarizing it just below its action potential threshold (e.g., Fig. 12A, n = 4). Although CBI-2 stimulation usually requires multiple cycles of activity to recruit B50 (Fig. 12A1), when depolarizing current was injected into B50 (thin bar, Fig. 12A2), CBI-2 stimulation recruited B50 during the first cycle of activity. B50 coactivation during CBI-2–driven motor programs shortened the protraction duration from motor programs elicited before (Pre) and after (Post) the B50 stimulation (Fig. 12, t-test with Bonferroni corrections, P < 0.01, n = 6). Retraction phase duration was...
not significantly affected by B50 coactivation (Fig. 12B). In addition to shortening the protraction phase of the first CBI-2–elicited programs, B50 also elicited a cycle of protraction–retraction (Fig. 12A2). Consistent with its activity patterns when recruited during CBI-2–elicited programs without the added depolarization, B50 fired primarily toward the early part of protraction (Fig. 12A2).

In addition to shortening the protraction phase duration, B50 also advanced the recruitment of the protraction motor neuron B31/B32. CBI-2–elicited programs recruit B31/B32 with some delay relative to other protraction neurons. To characterize this, we examined the latency until B31/B32 entered a plateau potential as well as the time period that B31/B32 plateau potential persisted. B50 coactivation significantly reduced the duration of both of these parts of protraction phase (Fig. 12C; t-test with Bonferroni corrections, \( P < 0.01, n = 6 \)). As seen in the representative trace (Fig. 12A2), B50 activity caused a much faster rise in the depolarization of B31/B32’s membrane potential and shortened the plateau duration as well. By shortening both parts of the protraction phase B50 coactivation maintained the relative recruitment of the 2 sets of protraction-phase motor neurons while shortening the protraction duration.

**Modulatory effects of PRQFVa neuropeptide on CBI-2–elicited programs**

It was of interest to examine whether the neuropeptide PRQFVa localized to B50 might mimic any of B50’s modulatory actions on CBI-2–elicited motor programs. To examine this, we stimulated CBI-2 to elicit single-cycle motor programs while perfusing increasing concentrations of PRQFVa. CBI-2 was stimulated using 10-ms suprathreshold pulses at a frequency of 9 Hz for the duration of the protraction phase, with an interstimulus interval of 1 min. As shown in both the representative traces (Fig. 13A) and the grouped data (Fig. 13B, \( n = 3 \)), perfusion of the PRQFVa neuropeptide profoundly shortened the total cycle duration of CBI-2–elicited motor programs by shortening the protraction phase duration. The degree of shortening increased at higher concentrations of PRQFVa. Grouped data are presented as a percentage of the mean total duration of programs elicited without the neuropeptide added. PRQFVa significantly shortened protraction-phase duration [ANOVA, \( F_{(4,48)} = 44.14, P < 0.01 \)]. At all concentrations the PRQFVa neuropeptide significantly reduced protraction duration com-
pared with that of control and wash (t-test with Bonferroni corrections, P < 0.01). PRQFVa did not significantly affect the duration of the retraction phase of CBI-2–driven motor programs. There was, however, a small nonsignificant reduction in the retraction phase duration.

Interestingly, the protraction duration shortening caused by PRQFVa was attributed only to the shortening of the period in which B31/B32 was generating its plateau potential, not before it. As Fig. 13 illustrates, the onset of B31/B32 recruitment was not significantly different [ANOVA, $F_{(4,48)} = 0.59, P = 0.565$] from that of control. By contrast, the plateau duration of B31/B32 was significantly different when neuropeptide was present [ANOVA $F_{(4,48)} = 33.38, P < 0.01$] and all individual comparisons between control and peptide groups were significantly different (t-test with Bonferroni correction, P < 0.001). PRQFVa shortened plateau duration in a concentration-dependent manner, and thus appeared to account for most of the shortening of protraction duration.

**DISCUSSION**

A study mapping the distribution of the PRQFVa neuropeptide in the *Aplysia* CNS had revealed a previously unidentified,
unpaired neuron in the buccal ganglion (Furukawa et al. 2003). Here, we have characterized this neuron’s morphology, electrophysiological properties, and connections to the feeding circuitry. We have designated this newly identified interneuron B50. Our data have shown that B50 was both capable of eliciting motor programs on its own and modulating motor programs elicited by the higher-order neuron CBI-2. B50 appears to modulate CBI-2–elicited motor programs using both the modulatory actions of the PRQFVa neuropeptide and its fast excitatory synaptic outputs.

B50, a pattern-initiating neuron within the feeding circuitry

We found that B50 was capable of eliciting patterned activity in the buccal CPG. The means by which B50 elicited patterned activity was very similar to that of the command-like neuron CBI-2. As is the case with CBI-2, brief B50 stimulation was sufficient to elicit a single-cycle motor program (B50: Fig. 8C; CBI-2: Hurwitz et al. 2003), whereas prolonged stimulation triggered several cycles. Both B50 and CBI-2 elicited motor programs by making excitatory synaptic connections to the same set of key protraction neurons (B31/B32, B34, B61/B62, and B63). The fast PSPs elicited by both neurons displayed similar facilitation and post-tetanic potentiation (PSTP; Fig. 6), and were hexamethonium sensitive (Fig. 7; Hurwitz et al. 2003; Sanchez and Kirk 2002).

CBI-2–and B50-elicited programs shared several similarities. The same CPG neurons were recruited (e.g., B4/5, B8, B31/B32, B34, B61/B62, B63, and B64; for B50: see Figs. 8, 9, and 11; for CBI-2: Church and Lloyd 1994; Hurwitz et al. 2003; Rosen et al. 1991; Sanchez and Kirk 2002). The durations of the single-cycle motor programs elicited by CBI-2 and B50 were similar across several stimulation frequencies (means ± SE): 5 Hz (B50: 41.12 ± 4.90; CBI-2: 44.7 ± 5.1 s, from Morgan et al. 2000), 10 Hz (B50: 25.27 ± 1.58, CBI-2: 25.3 ± 2.3 s, from Morgan et al. 2000), or 15 Hz (B50: 19.31 ± 1.45, CBI-2: 18.7 ± 2.9, from Hurwitz et al. 2003).

Despite the fact that B50- and CBI-2–elicited programs were similar in some features, they differed in others. CBI-2– and B50-elicited motor programs were distinct in two important features: the degree of radula closure motor neuron recruitment and the timing of the protraction motor neuron recruitment. B50-elicited programs could not be clustered into the same set of key protraction neurons (B31/B32, B34, B61/B62, and B63). The fast PSPs elicited by both neurons displayed similar facilitation and post-tetanic potentiation (PSTP; Fig. 6), and were hexamethonium sensitive (Fig. 7; Hurwitz et al. 2003; Sanchez and Kirk 2002).

The differences between CBI-2– and B50-elicited programs in B8 and B31/B32 recruitment are likely to be important for the expression of feeding behavior in the intact animal because activity of these neurons correlates well with the expression of important actions of the radula: closure and protraction (Hurwitz et al. 1996; Morton and Chiel 1993a,b).

Modulatory actions of B50 in CBI-2–elicited programs

Typically, B50 was not recruited during a CBI-2–elicited single-cycle motor program. However, if CBI-2 was stimulated for longer periods, B50 was reliably recruited during the protraction phase of subsequent cycles of activity. The fact that the initial CBI-2–elicited motor program occurred in the absence of B50 activity suggested that B50 was not a required constituent of a CBI-2 motor program. We hypothesize that rather than acting as a fixed CPG element, B50 may conditionally modify/modulate CBI-2–elicited motor program cycles during which it was recruited. Several lines of evidence support this idea. First, individual program cycles that recruited B50 activity in CBI-2–elicited multicycle programs displayed shorter protraction phase duration and recruited B31/B32 sooner than those that did not. Second, coactivating B50 during a CBI-2–elicited single-cycle program also shortened the protraction phase and advanced B31/B32 recruitment. Finally, B50 contains the neuropeptide PRQFVa, which shortened the protraction phase duration of CBI-2–elicited programs in a dose-dependent manner.

B50 affected CBI-2–elicited programs in two ways: 1) it advanced the recruitment of the protraction phase neurons B31/B32 and 2) it shortened protraction phase duration. The effects of the PRQFVa neuropeptide can readily account for the shortening of the protraction phase duration in CBI-2–elicited motor programs (Fig. 13). Although it remains to be
tested whether PRQFVa also shortens the protraction phase durations of B50-elicited programs, this perhaps would not be surprising given that the protraction phase durations in B50-elicited programs are shortened by higher frequencies of B50 stimulation (Fig. 10), and peptide release in B50-elicited programs are shortened by higher frequencies tested whether PRQFVa also shortens the protraction phase durations of B50 stimulation (Fig. 10), and peptide release in B50-elicited programs are shortened by higher frequencies of B50 stimulation. In turn, this contributes to the lack of change in the ratio of protraction to retraction duration at higher B50 stimulation frequencies.

Although PRQFVa shortened protraction phase duration, it did not advance the recruitment of B31/B32 in CBI-2–elicited motor programs (Fig. 13). It is likely that B50 advanced B31/B32 recruitment using its putatively cholinergic fast excitatory synaptic connections to B31/B32 (Fig. 14). Convergence of B50 and CBI-2’s excitatory inputs to the protraction-phase neurons may increase the protraction neurons’ rate of depolarization and thereby advance their recruitment to fire. The effects of B50 are further accentuated because B50 elicits large PSPs in B31/B32, whereas CBI-2 excites these neurons weakly (Hurwitz et al. 2003).

By appearing to act on two different aspects of B50-induced effects, the actions of PRQFVa and the fast PSPs seem to complement each other. Many neurons that both initiate and modulate CPG activity have been shown to possess cotransmitters that work in concert to modify CPG output (Nusbaum et al. 2001). In Aplysia, for example, both CBI-2 and CBI-3 have been shown to possess a combination of classical and neuropeptide neurotransmitters that all contribute to the higher-order neurons’ modification of buccal motor programs (Hurwitz et al. 2003; Jing et al. 2003; Morgan et al. 2002). Similarly, neurons in the commissural ganglia of the crustacean feeding system use different combinations of classical and neuropeptide transmitters to modulate the gastric and pyloric rhythms in the stomatogastric ganglion (for review see Nusbaum and Beenhakker 2002).

Although the behavioral role of B50-induced shortening of the protraction phase remains to be investigated, several studies have suggested that protraction phase shortening plays a major role within the feeding plasticity exhibited by Aplysia (Morgan et al. 2000; Rosen et al. 1991). Repetitive activation of biting in the intact animal with food has been shown to decrease the latency to the peak of the protraction phase of the subsequent bite (Kupfermann 1974; Susswein et al. 1986). Interestingly, B50’s recruitment, and therefore actions, depends on the repeated occurrence of CBI-2–elicited programs. Because CBI-2 has been shown to drive biting behavior in the semi-intact preparation (Rosen et al. 1991), it is possible that B50-induced protraction shortening observed in vitro may have parallels in plasticity observed in intact animals. In this context, it is also interesting that B50 receives an excitatory input from the serotoninergic MCCs, shown to play an important role in the generation of food-induced arousal state that, among other features, displays a shortened peak to protraction phase (Kupfermann and Weiss 1982).

**Comparisons of B50 and the slow oscillator in Lymnaea**

An evident feature of B50 was its similarity to a modulatory buccal interneuron in the mollusc Lymnaea stagnalis, called the slow oscillator (SO) neuron. B50 shared a number of characteristics with SO. They are both unpaired neurons possessing “loop” morphologies (Elliott and Benjamin 1985). When stimulated, they both elicit motor programs in the buccal ganglion by exciting protraction (N1 phase in Lymnaea) interneurons and motor neurons (Elliott and Benjamin 1985; Yeoman et al. 1993) with hexamethonium-sensitive PSPs. In both systems, these excitatory connections converge onto the same protraction (N1 phase) neurons as the higher-order cerebral- buccal interneurons (CBI-2 in Aplysia; CVn1 in Lymnaea: Elliott and Benjamin 1985; Elliott and Susswein 2002; McCrohan 1984; McCrohan and Kyriakides 1989). Also, both B50 and SO receive depolarizing input from the cephalic giant modulatory serotonergic neurons (MCC in Aplysia, Fig. 4; CGC in Lymnaea, Yeoman et al. 1996).

In one fundamental feature B50 was different from SO, however. B50 was excited by CBI-2 and recruited during CBI-2 multicycle motor programs, whereas CVn1 does not recruit SO, even during multicycle programs (Kemenes et al. 2001). This is important because it suggests that B50 and SO might fulfill distinct functions in the feeding systems of these two species. This fact is perhaps not surprising, given that the mode of feeding of the two animals is distinct in several features (Elliott and Susswein 2002).

In conclusion, in this study we have identified a novel modulatory interneuron, designated B50, which may act as a conditional program modifier/modulator when recruited during CBI-2–elicited multicycle programs. Similar to other neurons that modify CPG outputs, B50 is likely to use a combination of putatively cholinergic fast synaptic outputs and the modulatory actions of the PRQFVa neuromodulator to implement its actions. Further studies of B50’s role in the feeding circuitry and its ability to modify CBI-2–elicited programs may provide insight into plasticity in the feeding CPG of Aplysia.

**DISCLOSURES**

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