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A Pair of Reciprocally Inhibitory Histaminergic Sensory Neurons Are Activated within the Same Phase of Ingestive Motor Programs in *Aplysia*

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Previous studies have shown that each buccal ganglion in *Aplysia* contains two B52 neurons, one in each hemiganglion. We now show that there are two B52 neurons in a single buccal hemiganglion and four cells in an animal. We also show that the B52 neurons are histamine-immunoreactive and use reverse phase HPLC to show that the histamine-immunoreactive substance is authentic histamine. Previous studies have shown that the B52 neurons make numerous inhibitory synaptic connections with neurons active during the radula closing/retraction phase of ingestive motor programs. A computational model of the *Aplysia* feeding central pattern generator has, therefore, suggested that the B52 neurons play a role in terminating closing/retraction. Consistent with this idea we show that both B52 neurons fire at the beginning of radula opening/protraction. We also show that both B52 neurons are sensory neurons. They are depolarized when a flap of connective tissue adjacent to the buccal commissural arch is stretched. During ingestive feeding this is likely to occur at the peak of closing/retraction as opening/protraction begins. In the course of this study we compare the two ipsilateral B52 neurons and show that these cells are virtually indistinguishable; e.g., they use a common neurotransmitter, make the same synaptic connections, and are both sensory as well as premotor neurons. Nevertheless we show that the B52 neurons are reciprocally inhibitory. Our results, therefore, strikingly confirm theoretical predictions made by others that neurons that inhibit each other will not necessarily participate in antagonistic phases of behavior.

Key words: central pattern generator; feeding behavior; proprioceptive input; sensorimotor integration; half-center oscillator; histamine

A common feature in central pattern generating circuits is reciprocal inhibition (Perkel and Mulloney, 1974; Getting, 1989; Marder and Calabrese, 1996). Often reciprocally inhibitory neurons fire in alternating bursts of activity and drive antagonistic phases of behavior. Recent theoretical studies have, however, pointed out that this may not always be the case (e.g., Wang and Rinzel, 1992; Van Vreeswijk et al., 1994; Sharp et al., 1996). For example, in the most extreme case, reciprocal inhibition can synchronize neurons when the kinetics of the synaptic inhibition is slow relative to the oscillation period (Wang and Rinzel, 1992; Van Vreeswijk et al., 1994). In other cases, reciprocally inhibitory neurons may not fire in alternating bursts of activity but may spike in alternation (Sharp et al., 1996). In a number of systems single spikes may not be sufficient to drive alternating phases of behavior. Thus, theoretical studies have suggested that reciprocally inhibitory neurons will not necessarily drive antagonistic phases of behavior. Experimental data from this study strikingly confirm this prediction.

The neurons we describe are the B52 neurons in the marine mollusc *Aplysia californica*. These cells were originally described as bilaterally symmetrical buccal premotor neurons that are activated during rhythmic motor programs (Plummer and Kirk, 1990). Plummer and Kirk (1990) characterized the B52 neurons using physiological and morphological techniques. They found one neuron in each buccal hemiganglion (1) that was located on the rostral surface of the ganglion just lateral and ventral to neuron B51, (2) that showed postinhibitory rebound excitation, and (3) that had processes in a flap of connective tissue adjacent to the buccal commissural arch. Plummer and Kirk (1990) also showed that the B52 neurons make inhibitory synaptic connections with a number of neurons in the ventral motor neuron cluster and suggested that they may cause an overall shutdown of patterned activity. More recently, Baxter and coworkers have characterized additional synaptic connections of the B52 neurons (Baxter and Byrne, 1991) and generated a computational model of the *Aplysia* feeding central pattern generator (CPG) (Baxter et al., 1997). Computational results more specifically suggest that the B52 neurons may play a role in terminating the radula closing/retraction phase of ingestive motor programs.

Our experiments now show that there are actually two reciprocally inhibitory neurons in each buccal hemiganglion that have the characteristics described by Plummer and Kirk (1990). Thus there are four B52 neurons in one animal. Additionally, our data further characterize the B52 neurons in that we show that these

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cells are histaminergic mechanoafferents that are both activated at the beginning of the opening/protraction phase of ingestive motor programs. These data are consistent with the role of the B52 neurons suggested by Baxter et al. (1997). Our results, therefore, add to the growing body of data that suggest that neurons that play a prominent role in pattern generation can additionally function as sensory neurons (e.g., Pearson and Ramirez, 1997). We discuss the possible physiological significance of this arrangement and the possible physiological significance of the reciprocal inhibition between the B52 neurons.

MATERIALS AND METHODS

Animals. Experiments were conducted with 250–350 gm *Aplysia californica* that had been maintained in holding tanks containing 14–16°C artificial seawater (ASW). Animals were anesthetized by injection of isotonic $MgCl_2$ and then dissected to create reduced preparations.

Intracellular dye injection. A 3% solution of the fluorescent dye 5(6)-carboxyfluorescein in 0.1 M potassium citrate, titrated to pH 8.0, was iontophoresed into neurons from single-barreled glass microelectrodes with tips beveled to lower impedances to ~10 MΩ (Rao et al., 1986). To reduce active transport of the dye, probenecid (10 mM final concentration) was added to preparations (Steinberg et al., 1987; Rosen et al., 1991), which were kept at 4°C for 12–15 hr.

Immunocytochemistry. B52 neurons were physiologically identified and injected with carboxyfluorescein as described above. Ganglia were fixed using a procedure that was similar to one developed by Panula et al. (1988) and modified by Elste et al. (1990). Specifically, tissue was placed in a solution of 4% carbodiimide in 0.1 M phosphate buffer with 30% sucrose at room temperature for 2 hr and processed for whole-mount immunocytochemistry as has been described (Longley and Longley, 1986; Miller et al., 1994). The primary antiserum (rabbit host; Incstar, Stillwater, MN) was used at a dilution of 1:100 and was applied for 2–3 d at 4°C. The second Cy3-conjugated antibody (anti-rabbit IgG heavy and light chain; goat host; Jackson ImmunoResearch, West Grove, PA) was applied for up to 3 d (1:100 dilution; 4°C). In some cases ganglia were cleared with glycerol. In all cases tissues were viewed with a Nikon microscope equipped with epifluorescence and were photographed with Tri-X (ASA 400) film. Drawings were made with the aid of a drawing tube.

Electron microscopy. Methods used for electron microscopy were similar to those of Vilim et al. (1996). Briefly, the tissue was pinned on Sylgard in a Petri dish and fixed with 4% glutaraldehyde diluted in 0.2 M Na-HEPES buffer, pH 7.6, containing 10% sucrose and 11 mM magnesium chloride. After thorough rinsing in buffer, tissue was stained en bloc with buffer containing 1% uranyl acetate for 3 hr and was post-fixed with buffered 1% osmium tetroxide for 1 hr at room temperature. The tissue was washed with water, dehydrated in an ethanol series, washed with propylene oxide, infiltrated with EMbed 812, and polymerized at 60°C for 2 d. Ultrathin sections were cut on Quick Coat–treated 200–300 mesh hexagonal nickel grids and counterstained with 1% aqueous uranyl acetate followed by 0.25% lead citrate. Samples were examined and photographed with a Zeiss CH-10 electron microscope at 60 kV. Supplies and reagents were from Electron Microscopy Sciences (Fort Washington, PA).

In situ *radiolabeling. In situ* radiolabeling techniques were similar to those used in previous studies (e.g., see Cropper et al., 1987; Lloyd et al., 1987). Briefly, neurons were physiologically identified and were marked iontophoretically with fast green dye (Fisher Scientific, Houston, TX). Buccal ganglia were incubated for 4 hr in 1 ml of ASW, containing 1 mCi of [^{3}H]histidine (Amersham, Arlington Heights, IL). Neurons were individually dissected from labeled ganglia using a modification of a procedure developed by Ono and McCaman (1980), and radioactive histamine was extracted in the presence of unlabeled histamine (Sigma, St. Louis, MO).

Histamine and histidine were separated using HPLC as has been described by others (e.g., Sondergaard, 1982; Jensen and Marley, 1995). Specifically, we used a 4.6 × 250 mm reversed-phase column (Microsorb-MB; Rainin, Ridgefield, NJ). Solvent A was 0.1% phosphoric acid (85% H_3PO_4; Fisher Scientific), 0.006 M NaH_2P0_4 (Fisher Scientific), and 1% $C_{12}H_{25}NaO_4S$ (SDS; Life Technologies, Gaithersburg, MD); solvent B was 100% CH_3CN (Fisher Scientific). The gradient used was 40–50% solvent B in 15 min, followed by 50% solvent B for 30 min. The flow rate was 1.0 ml/min, and samples were collected every 30 sec. Radioactivity was detected by scintillation counting, and synthetic histamine and histidine were detected by absorbance measurements using a V-4 flow spectrophotometer (ISCO Inc., Lincoln, NE) at 215 nm.

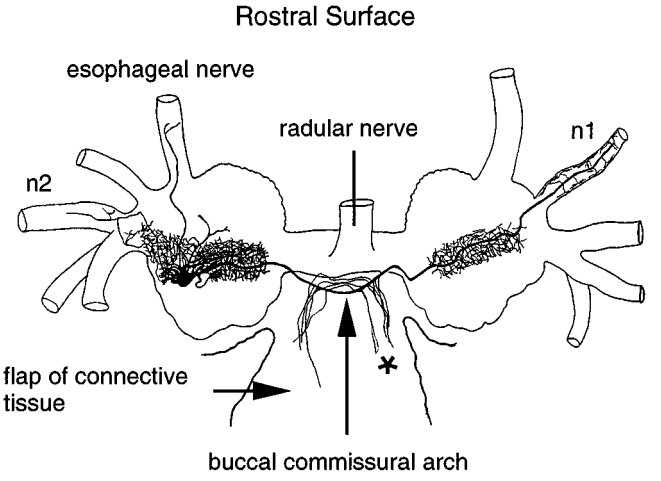

Figure 1. The anatomy of a B52 neuron injected with carboxyfluorescein dye. B52 has fine ipsilateral processes that can be observed in the esophageal nerve, buccal nerve 2 (*n2*), and buccal nerve 1 (*n1*) (processes in the ipsilateral buccal nerve 1 were not observed in this preparation). The major B52 neurite crosses to the contralateral hemiganglion through the buccal commissural arch. In the contralateral hemiganglion it gives rise to a dense dendritic field and then enters buccal nerve 1. As the B52 neurite passes through the commissural arch, it gives rise to numerous smooth, tapering dendrites that branch perpendicularly (indicated by an *asterisk*). These branches pass into an adjacent flap of tissue that emerges from the ventral surface of the buccal ganglia and anchors it to the I2 muscle (see Fig. 3).

Physiological experiments. B52 neurons were identified using the criteria of Plummer and Kirk (1990). Thus, B52 is located on the rostral surface of the buccal ganglion, makes monosynaptic inhibitory connections with B51 and a number of buccal motor neurons, and displays powerful postinhibitory rebound excitation. Additionally, it has a distinctive morphology. Its major neurite leaves the buccal ganglion, enters the buccal commissural arch (Plummer and Kirk, 1990) (Fig. 1), and then arborizes, sending branches into an adjacent flap of tissue. To characterize the function of this peripheral process, we used a reduced preparation that comprised the buccal ganglion dissected free from the buccal mass except for a portion of the I2 muscle. The I2 was sectioned to ensure that the buccal commissural arch and the adjacent flap of tissue were left intact. A silk suture (Ethicon, Somerville, NJ) was tied at one end to the portion of I2 muscle. The other end was left free so that it could be pulled to stretch the flap of tissue adjacent to the buccal commissural arch, while recording from neurons.

Experiments that characterized firing patterns of B52 neurons during motor programs were conducted in a modified version of a preparation developed by Susswein et al. (1996). This preparation has been described in detail elsewhere (Evans and Cropper, 1998). Briefly, an isolated buccal mass with the buccal and cerebral ganglia attached was pinned to a Sylgard dish. All buccal nerves were intact except for the esophageal nerve. The buccal and cerebral ganglia were pinned to a raised Sylgard platform, and the cerebral ganglion was isolated in a Lucite chamber. The esophagus was removed, and a hole was cut in the pharynx to expose the radula halves. Motor programs were elicited by applying 10^{-3} M carbachol directly to the cerebral ganglion, which was not desheathed. To monitor movements of the buccal mass, we tied a silk suture to the anterior tip of the radula. The thread was then pulled through the jaws and attached to an isotonic force transducer (Harvard Apparatus). This

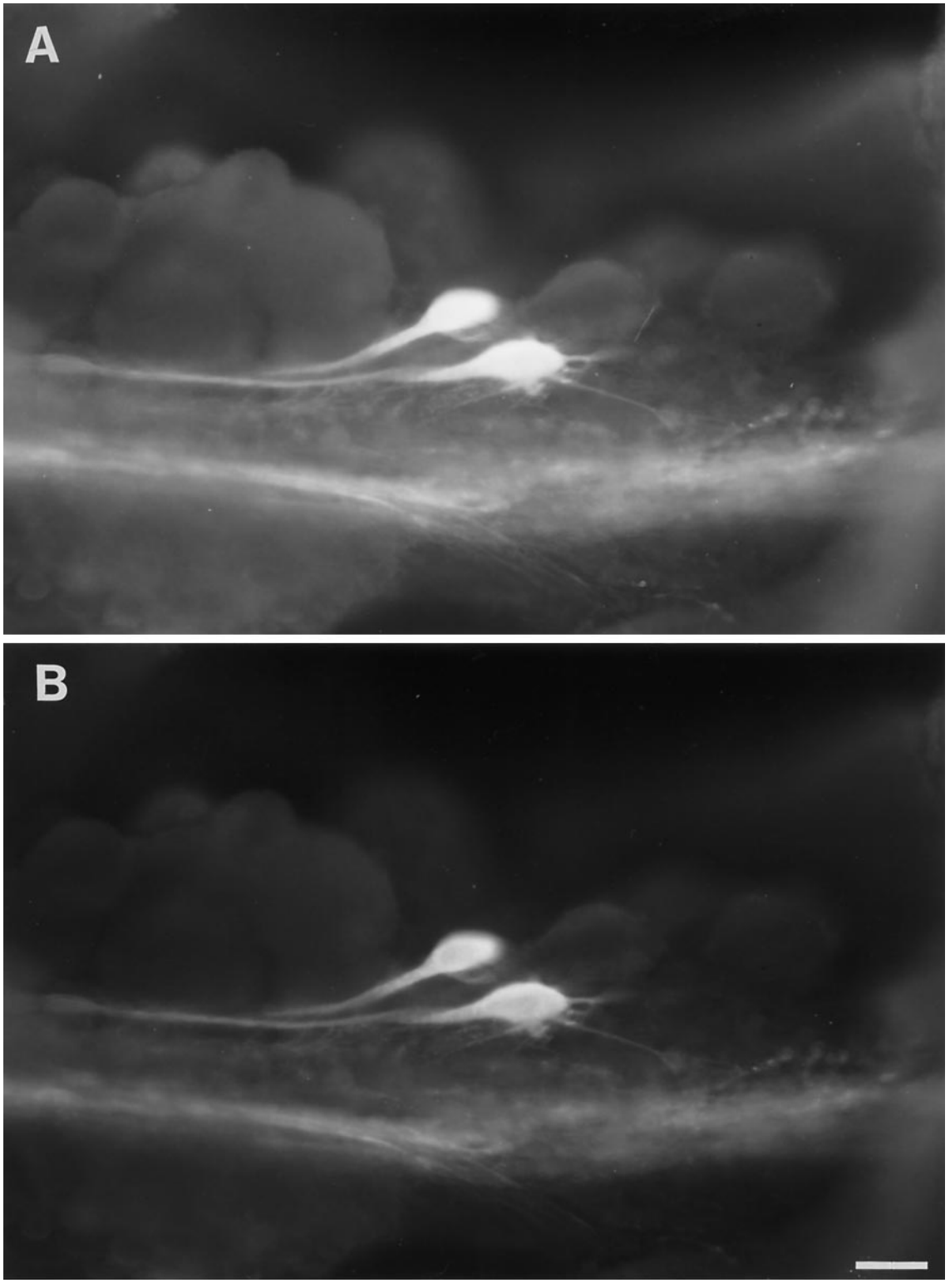

Figure 2. Physiologically identified B52 neurons are characterized by histamine-like immunoreactivity. *A*, B52 neurons were bilaterally injected with carboxyfluorescein dye, and ganglia were processed for histamine immunocytochemistry. *B*, Injected neurons did indeed stain positively. Only one buccal hemiganglion is shown in each photograph. Fluorescent processes medial to the injected neurons in the carboxyfluorescein photograph originate from the contralateral B52 neurons (see Fig. 1). Scale bar, 50 μm.

transducer detected movement of the radula toward the jaws, referred to as "protraction," and movement of the radula back toward esophageal tissue, referred to as "retraction."

Intracellular recordings were obtained with glass micropipettes filled with 3 M potassium acetate containing 30 mM KCl. For simultaneous current injection and voltage recording, we used double-barreled electrodes beveled so that they had resistances ranging from 5 to 10 MΩ.

The ASW used in these experiments had the following composition (in

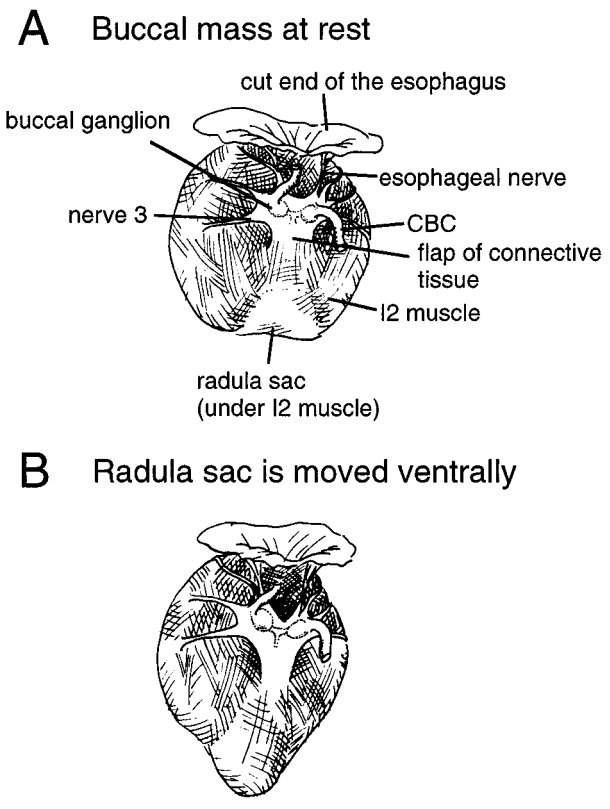

Figure 3. *A*, Drawing of the buccal mass and the attached buccal ganglion is shown. *B*, The radula sac has been moved ventrally to simulate what presumably happens during feeding behavior in intact animals (Drushel et al., 1997). This stretched the flap of tissue that contains the processes of B52 (see Fig. 1). The buccal ganglion was positioned as it is in intact animals; i.e., only the caudal surface can be seen. For the sake of simplicity the salivary ducts and cerebral ganglion are not shown. Additionally, most of the esophagus was removed. *CBC*, Cerebral buccal connective; *nerve 3*, buccal nerve 3.

mM): 460 NaCl, 10 KCl, 11 $CaCl_2$, 55 $MgCl_2$, and 5 $NaHCO_3$. All salts were obtained from Sigma.

RESULTS

The two B52 neurons are histamine-immunoreactive

Two neurons in the ventral motor neuron cluster of each buccal hemiganglion are histamine-immunoreactive (Elste et al., 1990; Soinila and Mpitsos, 1991). On the basis of their size and position, we suspected that at least one of these neurons might be B52, a premotor neuron originally described by Plummer and Kirk (1990). We, therefore, identified a B52, based on the description of Plummer and Kirk (1990), and injected it with carboxyfluorescein dye. Cells identified in this manner were characterized by histamine-like immunoreactivity. To identify the second immunoreactive cell, we sought to determine whether we could identify two neurons with B52-like characteristics in a single buccal hemiganglion. We did in fact find two such cells. We injected both cells with carboxyfluorescein to study their anatomy (as described below) and found that both cells were histamine-immunoreactive (Fig. 2).

Anatomy of the B52 cells and the flap of tissue that contains the peripheral processes of the B52 neurons

One B52 neuron is generally located immediately lateral to B51 (Plummer and Kirk, 1990). The second B52 neuron is usually lateral to the first cell and is generally partly hidden under other neurons. The two cells, however, have basically similar anatomical features, and we did not detect any consistent significant anatomical differences between them ($n = 5$). They are strikingly multipolar, extending dense dendritic branching into the neuropile (Fig. 1). Part of the dendritic field is very superficial, closely applied to the neurons on the rostral surface of the ganglion, whereas many other branches project deeply into the neuropile, toward the caudal surface of the ganglion. We found that B52 had fine ipsilateral processes that could be observed in the esophageal nerve (observed in the preparation shown in Fig. 1), in buccal nerve 2 (also observed in the preparation shown in Fig. 1), and in buccal nerve 1 (not observed in the preparation shown in Fig. 1). These fine processes were very superficial and in some cases appeared to travel in the sheath covering the nerves.

As described by Plummer and Kirk (1990), the major B52 neurite leaves the ipsilateral buccal hemiganglion and crosses to the contralateral hemiganglion through the buccal commissural arch (Fig. 1). In the contralateral hemiganglion, the neurite gives rise to a dense dendritic field and then leaves the CNS through buccal nerve 1. As the B52 neurite passes through the commissural arch, it gives rise to numerous smooth, tapering dendrites, which branch perpendicularly, i.e., along the same axis as the radular sac. These branches pass into what appears to be an adjacent flap of connective tissue, which emerges from the ventral surface of the buccal ganglion and anchors the ganglion to the I2 muscle (Fig. 3). The processes of the principal neurite are likely to terminate in this flap of tissue because they were not visualized in the I2 muscle, even when preparations were kept for 2 d in 10 mM probenecid. Additionally, fibers with histamine-like immunoreactivity were not observed on I2. (Although the flap of tissue also contains a blood vessel that runs from the I2 muscle into the buccal ganglion, B52 processes were never observed in association with this vessel.)

To define more precisely the nature of the flap of tissue that contains the peripheral processes of the B52 neurons, we examined the flap using electron microscopy. We found fibers and cell types that have been observed in the fibrous sheath that encloses the *Aplysia* nervous system (e.g., Coggeshall, 1967). For example, the bulk of fibers were cylindrical and showed cross-banding or beading (Fig. 4). These fibers are presumably collagenous connective tissue fibers (Coggeshall, 1967). Additionally, we found neural processes that contained dense core vesicles and cells that morphologically resemble the molluscan muscle fibers that are observed in the sheath of *Aplysia* (Rosenbluth, 1963; Coggeshall, 1967; Prescott and Brightman, 1976) (Fig. 4). Muscle cells were not found in bundles and generally did not make contacts with their neighbors as has been described in the *Aplysia* sheath (e.g., Prescott and Brightman, 1976).

The histamine-like–immunoreactive substance is authentic histamine

Previous investigators have demonstrated that histamine-immunoreactive neurons take up [^{3}H]histamine, whereas those that are not histamine-immunoreactive do not (Elste et al., 1990). In this study we used *in vivo* radiolabeling experiments to determine whether the histamine-like–immunoreactive substance present in the B52 neurons was in fact authentic histamine (Fig. 5*A*). Toward this end, B52 neurons were physiologically identified and injected with fast green dye. Buccal ganglia were incubated in radiolabeled histidine so that B52 neurons would take up the histidine and synthesize radiolabeled histamine. Individual cells

were then removed from the buccal ganglion and subjected to RP-HPLC to separate the radiolabeled histidine and histamine. We were not able to use this technique to detect histamine in single neurons; consequently chromatography was performed on groups of eight cells. To evaluate the specificity of this procedure, we additionally processed B4/B5 neurons from the same buccal hemiganglia. This procedure was repeated three times; i.e., a total of 24 B52 neurons were processed. In B52 neurons we found that the average conversion of histidine to histamine was 4.1% (± 1.1). B4/B5 neurons converted only 0.59% (± 0.18) of the histidine to histamine. We used a Student's two-tailed t test to determine that this difference was statistically significant ($t = 4.05$; $p < 0.01$).

The B52s as sensory neurons

We found that the two putative B52 neurons made the same monosynaptic connections. Most of these connections have been described by Plummer and Kirk (1990) and are with buccal motor neurons (i.e., neurons B3, B9, B15, B16, and B51). One additional connection we observed was with the neurons B8a and B8b, which are radula closer motor neurons (Morton and Chiel, 1993) (Fig. 6). Connections between the B52s and motor neurons are likely to be outputs of the B52s. We sought to determine, therefore, whether there were also similarities in "inputs" to the two cells. More specifically we sought to determine whether both B52 neurons are sensory neurons and, if so, whether the same type of stimulus activates both cells.

That the B52 neurons might be sensory cells was suggested by the fact that both of these neurons have peripheral processes yet do not appear to be motor neurons. Thus, the major B52 neurite has branches that appear to terminate in the connective tissue flap adjacent to the buccal commissural arch (Figs. 1, 3). When B52 neurons were stimulated, however, this piece of tissue did not contract. In contrast, excitatory responses were observed when the connective tissue was stretched ($n = 5$). If the B52 was at its resting membrane potential when the connective tissue was stretched, then action potentials were generally recorded (Fig. 7*A1*). If, however, the B52 was hyperpolarized, subthreshold depolarizing potentials became apparent (Fig. 7*A2*, *top*). Because the connective tissue flap is very close to the buccal ganglion, we simultaneously recorded from other buccal neurons to determine whether what appeared to be depolarizing potentials were actually artifacts caused by movement of the buccal ganglion. Deflections in recordings in other neurons were much smaller than were those observed in B52 neurons (e.g., Fig. 7*B*). To determine whether synaptic transmission was necessary for depolarizing responses, we replaced the normal ASW bathing preparations with a 0 Ca^{+2} and 10 mM Co^{+2} ASW (which abolishes synaptic activity in the buccal ganglion). Stretching the flap of connective tissue still evoked depolarizing responses in B52 neurons (Fig. 7*C*).

The proximity of the connective tissue flap and the buccal ganglion made it impossible for us to pull hard enough on the tissue flap to elicit large depolarizing responses in the B52. We, therefore, used tugs to perform the above experiments because small changes in membrane potential were more readily apparent as relatively rapid on–off responses. Under physiological conditions, however, the connective tissue is unlikely to be stretched in this manner. We, therefore, additionally tested slow pulls and found that they also elicited depolarizations in B52 neurons (Fig. 7*A2*, *bottom*). Interestingly, spikes triggered by peripherally generated depolarizations were effective at eliciting PSPs in B52 follower neurons (Fig. 7*B*, *left*). In fact, these spikes appeared to

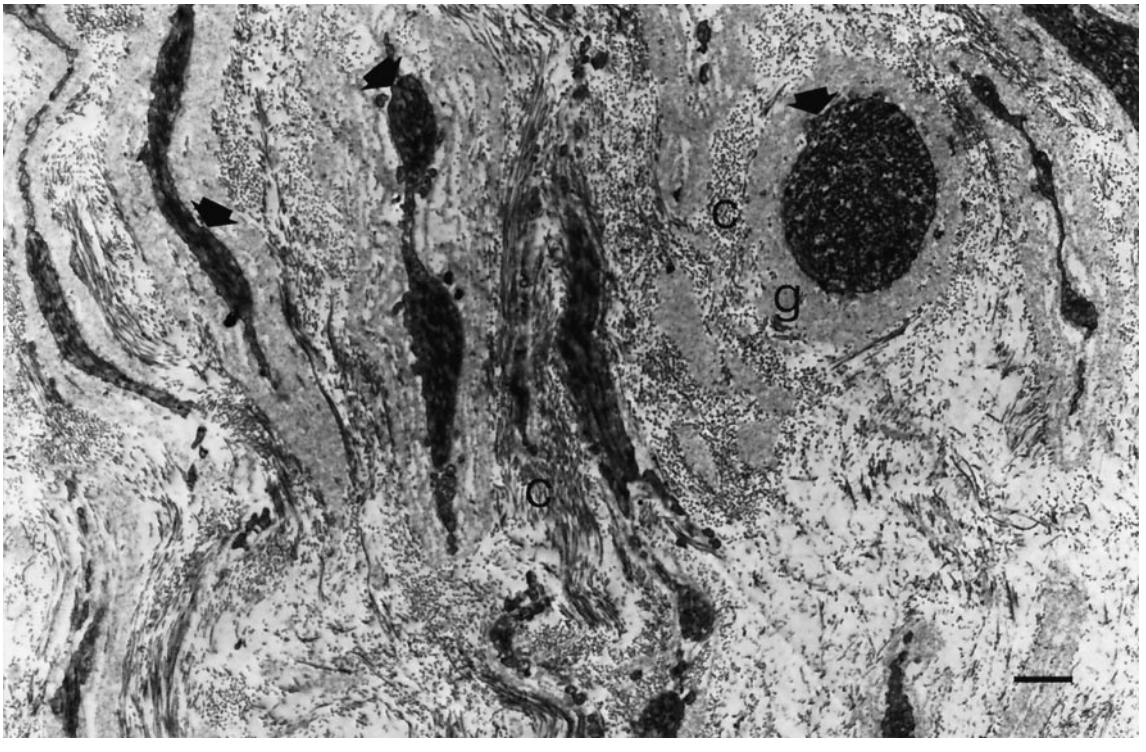

Figure 4. Electron micrograph of the connective tissue flap that connects the ventral surface of the buccal ganglion to the I2 muscle. Embedded in a collagenous matrix are several processes of smooth muscle cells (*arrows*). A wide band of ground substance (*g*) and of collagen fibers (*c*) separates the cells from each other. Scale bar, 1 μm.

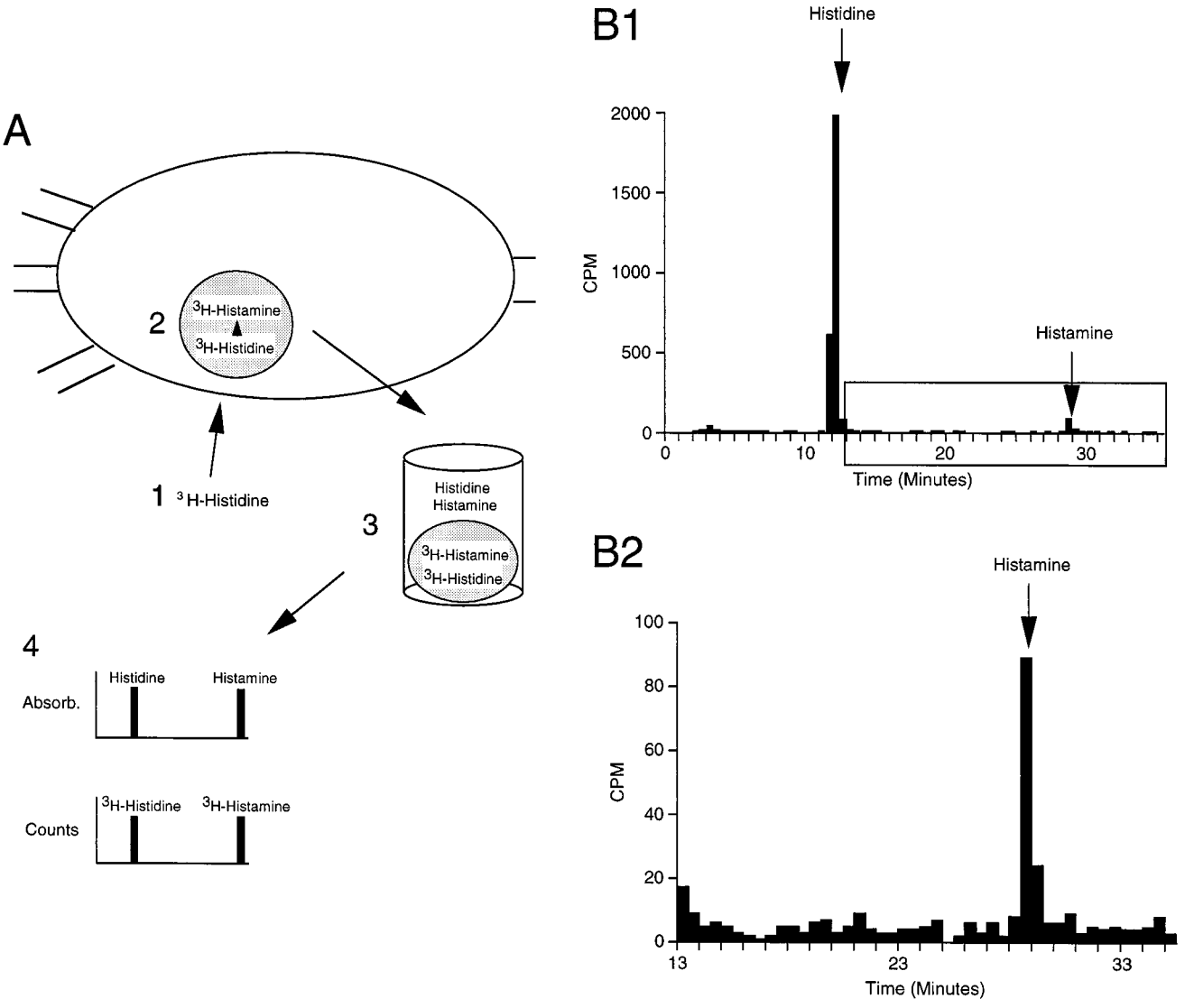

Figure 5. Biochemical confirmation that B52 neurons do in fact contain authentic histamine. *A*, The procedure used in the experiments. Briefly, B52 neurons were physiologically identified and injected with fast green dye. (1) Buccal ganglia were then incubated in a radioactive histamine precursor, i.e., [^{3}H]histidine. (2) B52 neurons took up the precursor and synthesized radiolabeled histamine. (3) Individual neurons were then removed from buccal ganglia and placed in tubes that had quantities of synthetic histamine and histidine that are easily detected with absorbance measurements. (4) Synthetic and native material was subjected to RP-HPLC. Radiolabeled material that coeluted with synthetic histidine was identified as native [^{3}H]histidine, and radiolabeled material that coeluted with synthetic histamine was identified as native [^{3}H]histamine. *B1*, *B2*, An experiment in which eight B52 neurons were chromatographed together. *B2* is an enlargement of the *boxed* region of the graph in *B1*. In this experiment the histidine to histamine conversion was 4.2%. The average conversion was 4.1% ($n = 3$).

be as effective at eliciting PSPs in B52 follower neurons as were action potentials elicited by injection of current into the B52 somata (Fig. 7*B*, *right*).

The B52 neurons reciprocally inhibit each other

In *Aplysia*, other pairs of cells that appear to be indistinguishable from one another have been described, e.g., B4/B5 (Gardner, 1971). In a number of cases these neurons are electrically coupled. This, however, is not the case for the B52 neurons. Thus, we found that the B52 neurons monosynaptically inhibited each other ($n = 6$; Fig. 8*A*).

Plummer and Kirk (1990) showed that B52 neurons generally display postinhibitory rebound excitation when they are injected with hyperpolarizing current pulses (they observed this property in 36 out of 38 preparations) (also see Fig. 8*B*). Postinhibitory rebound in the B52 neurons could at least partially account for the fact that the two cells can function as a half-center oscillator. Thus, when the two B52 neurons are at, or are close to, their normal resting potential, they can produce single alternating action potentials (Fig. 9). One B52 fires producing an IPSP in the second B52. The second B52 rebounds from the inhibition and generates an action potential that produces an IPSP in the first B52. The first B52 rebounds, and the cycle repeats. If the B52s are more depolarized, they can also fire in alternating bursts of activity (Fig. 10).

Although we cannot conclude that all of the rebound activity in the B52 neurons results from the biophysical properties of the postsynaptic cell, we did not find evidence of a slow excitatory synaptic component to the B52–B52 connection. Specifically, we

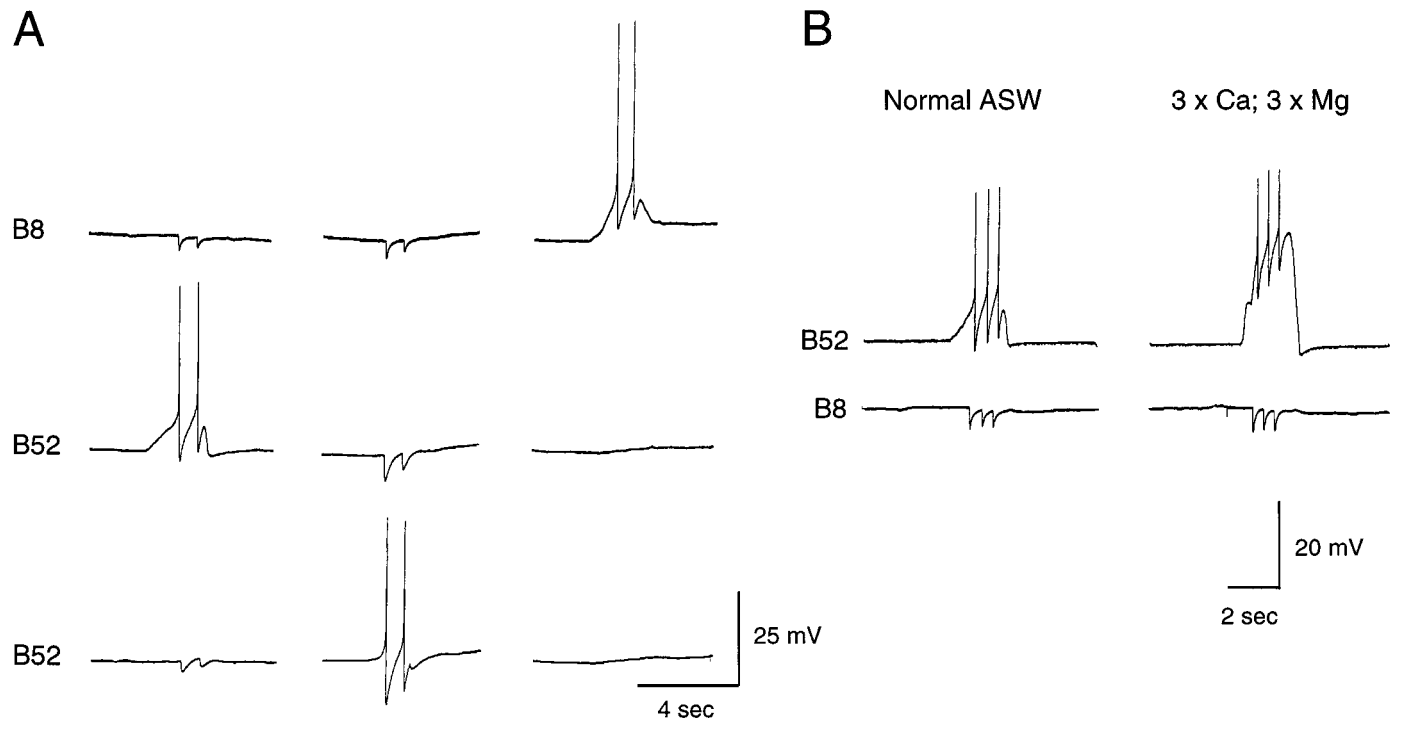

Figure 6. The B52 neurons make a synaptic connection with the B8 neurons. *A*, Both B52 neurons produce one-to-one IPSPs in the B8 neurons. The B8 neurons do not produce synaptic potentials in either B52 neuron. *B*, The B52–B8 connection is monosynaptic; i.e., it is observed when ganglia are placed in high divalent cation solutions.

sought to determine whether we could observe a slow excitatory potential attributable to a decrease in potassium conductance at the B52–B52 synapse. This type of synaptic effect has been observed previously for histamine in *Aplysia* (e.g., Weiss et al., 1986a). When postsynaptic B52 neurons were progressively hyperpolarized as presynaptic B52 neurons were stimulated, hyperpolarizations reduced the size of IPSPs in postsynaptic B52s as would be expected (Fig. 8*B*, *left* vs *right*). When postsynaptic cells were hyperpolarized in this manner, rebound excitations could obviously no longer be triggered; i.e., there was no inhibition from which to rebound (Fig. 8*B*, *right*). Under these conditions a slow excitatory component to the B52–B52 synapse could, therefore, be "unmasked." We found, however, that although we kept postsynaptic cells above the potassium equilibrium potential (approximately −80 mV), excitatory synaptic potentials did not become apparent (Fig. 8*B*, *middle* vs *right*).

Activity of B52 neurons during ingestive motor programs

Because the two B52 neurons appear to be indistinguishable yet can be made to fire out-of-phase in otherwise quiescent preparations, we sought to determine how they would fire during ingestive motor programs. Ingestive activity was elicited in a modified version of a preparation developed by Susswein et al. (1996). In these preparations rhythmic activity is elicited by applying carbachol to the cerebral ganglion.

We found that the two B52 neurons did indeed fire during the same phase of ingestive motor programs ($n = 3$; Fig. 11). Spikes evoked in the two B52 neurons generally did not occur exactly simultaneously but did occur at approximately the same time when feeding motor programs were vigorous. Specifically, in the section of the recording shown in Figure 11, the two B52 neurons began to fire before rhythmic activity was actually generated. At this point the two neurons alternated between firing more or less in synchrony and alternation (Fig. 12). When motor programs did, however, become rhythmic, the number of spikes in each burst of activity in one cell was generally the same as the number of spikes in each burst of activity in the second cell (see *numbers above* the bursts in Fig. 11). Moreover, 69% of the spikes in one cell occurred within 0.02 sec of a spike in the second cell (the average interspike interval during these bursts of activity is 0.14 sec).

To determine whether the B52 neurons are active during the opening/protraction or closing/retraction phases of ingestive motor programs, we simultaneously recorded B52 activity and monitored movements of the buccal mass (Evans and Cropper, 1998) ($n = 6$; e.g., see Figs. 11, 13, 14). We found that the B52 began to spike at the peak of radula retraction. Additionally, we obtained simultaneous recordings from B52 neurons and neurons that fire during radula closing/retraction, e.g., neuron B51 (Evans and Cropper, 1998). We found that the B52 neurons fired more or less out-of-phase with these neurons ($n = 5$; Fig. 13). When feeding motor programs cycled frequently, the B52 neurons were generally active during the first half of radula opening/protraction (Fig. 13). When feeding motor programs began to cycle more slowly, however, there was a pause between peak radula retraction and the next protraction/opening (as indicated by the *horizontal bars* in Fig. 14). Thus, the radula closed and retracted, opened, and then temporarily stopped moving before it again protracted and opened. During these pauses the B52 neurons continued to be active and again fired in alternation at least part of the time (as they did before rhythmic activity was elicited; Fig. 11). The possible functional significance of this prolonged activity in the B52 is discussed below.

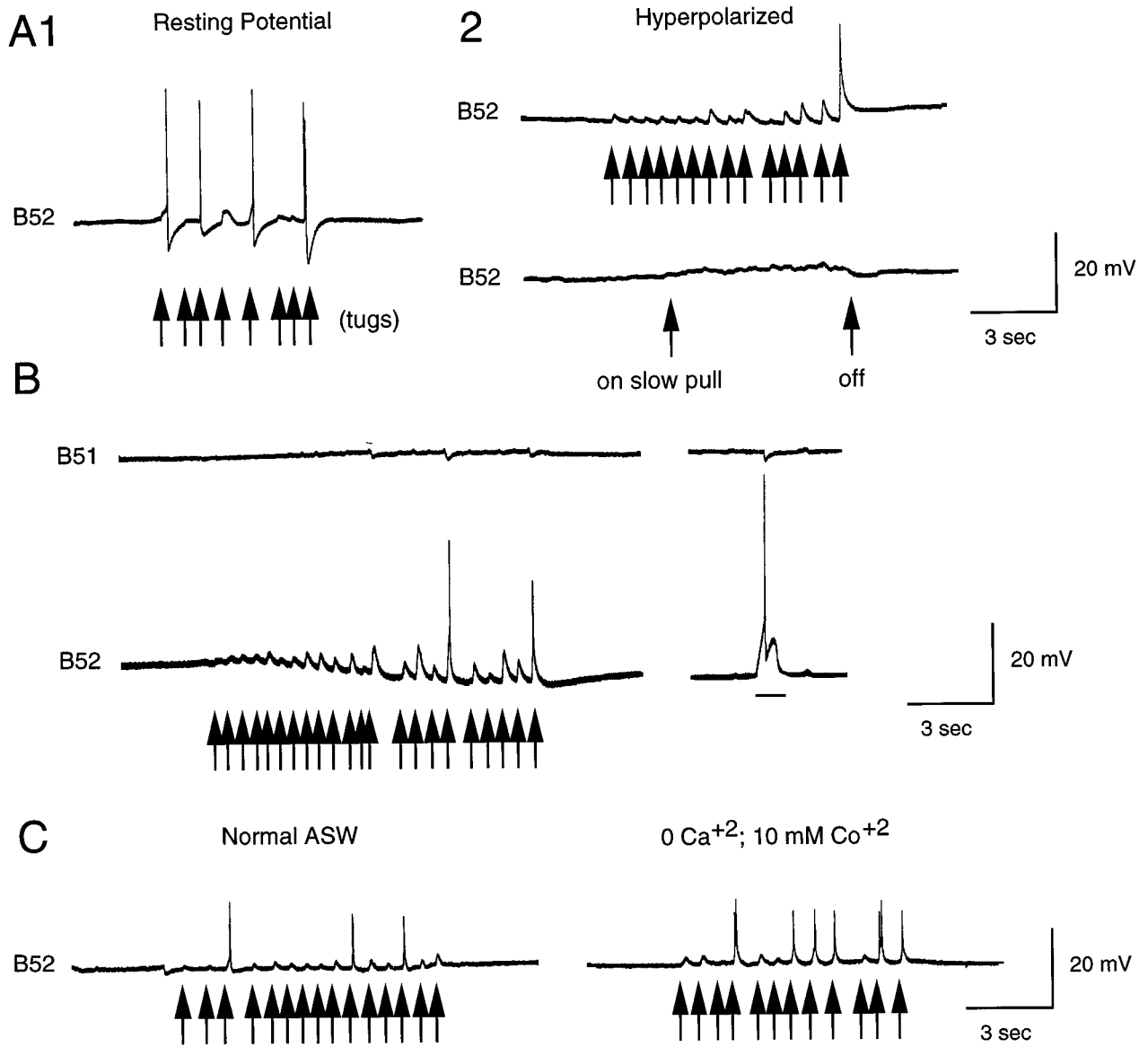

Figure 7. The B52 neurons are sensory cells. *A1*, Tugging (*vertical arrows*) on the connective tissue adjacent to the buccal commissural arch elicited action potentials in the B52 if the neuron was at its resting membrane potential. *A2*, When the B52 was hyperpolarized, underlying depolarizing potentials became apparent. Although tugs were clearly effective at evoking responses in B52 neurons (*top*), slow pulls (*single vertical arrows*) were also effective (*bottom*). *B*, Depolarizing potentials in the B52 did not appear solely to be movement artifacts because they were much larger than the deflections recorded from the B52 follower neuron B51. Also note that when responses were peripherally elicited in B52, postsynaptic potentials were recorded in B51. *The horizontal line* under the B52 trace on the *right* indicates that current was injected into the B52 neuron. *C*, Depolarizing potentials were not abolished when the normal ASW bathing preparation was replaced with a solution that abolishes synaptic transmission, i.e., a 0 Ca^{+2} and 10 mM Co^{+2} ASW.

DISCUSSION

Possible physiological significance of reciprocal inhibition between the B52 neurons

We show that there are two ipsilateral B52 neurons. In *Aplysia* other well-characterized pairs of neurons that are virtually indistinguishable are the B4/B5 (Gardner, 1971), B31/B32 (Susswein and Byrne, 1988), B61/B62 (Hurwitz et al., 1994), cerebral buccal interneuron-8/9 (Xin et al., 1996), and the B8 (Gardner, 1971; Church and Lloyd, 1991; Morton and Chiel, 1993) neurons. In most cases these pairs of neurons are electrically coupled to each other. In the case of such interneurons, therefore, this arrangement leads to feed-forward summation and amplified synaptic output from each neuron pair (Gardner, 1971).

The B52 neurons, however, are not electrically coupled; they monosynaptically inhibit each other. At this point we can only speculate as to the functional significance of this arrangement. Others have used computational techniques to show that reciprocal inhibition can synchronize neural activity (e.g., Wang and Rinzel, 1992; Van Vreeswijk et al., 1994). This is not what we observed. During motor programs that cycle vigorously, the two B52 neurons do fire at similar frequencies at the beginning of radula opening/protraction. This seems to result, however, from

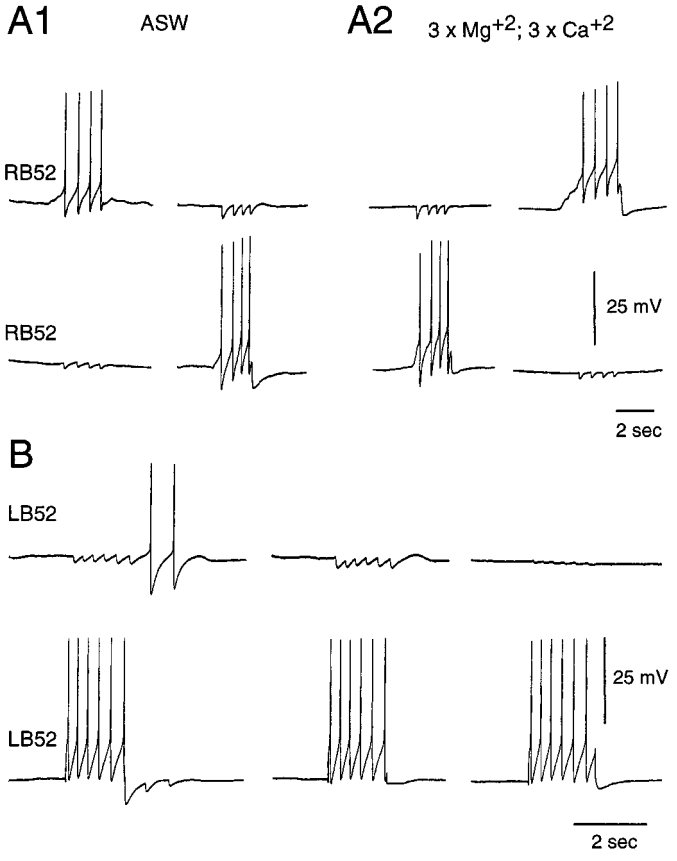

Figure 8. *A1*, The B52 neurons reciprocally inhibit each other. *A2*, The connection between the two B52 neurons appears to be monosynaptic because it is observed in high divalent cation solutions. *B*, An excitatory component to the connection between the two B52s is not revealed when the postsynaptic B52 is hyperpolarized. *Left*, The postsynaptic B52 was at its resting membrane potential. *Middle*, *right*, The postsynaptic B52 was hyperpolarized. The *middle* recordings show the postinhibitory rebound that can be seen in a typical B52 when spiking is prevented. The postsynaptic B52 on the *right* was hyperpolarized so that IPSPs were reduced in size. The postsynaptic cell has, however, not been hyperpolarized below E_K (note that the chloride IPSPs can still be observed). Under these conditions a slow synaptic component to the B52–B52 connection should be apparent if it results from a decreased potassium conductance. RB52, Right B52; LB52, left B52.

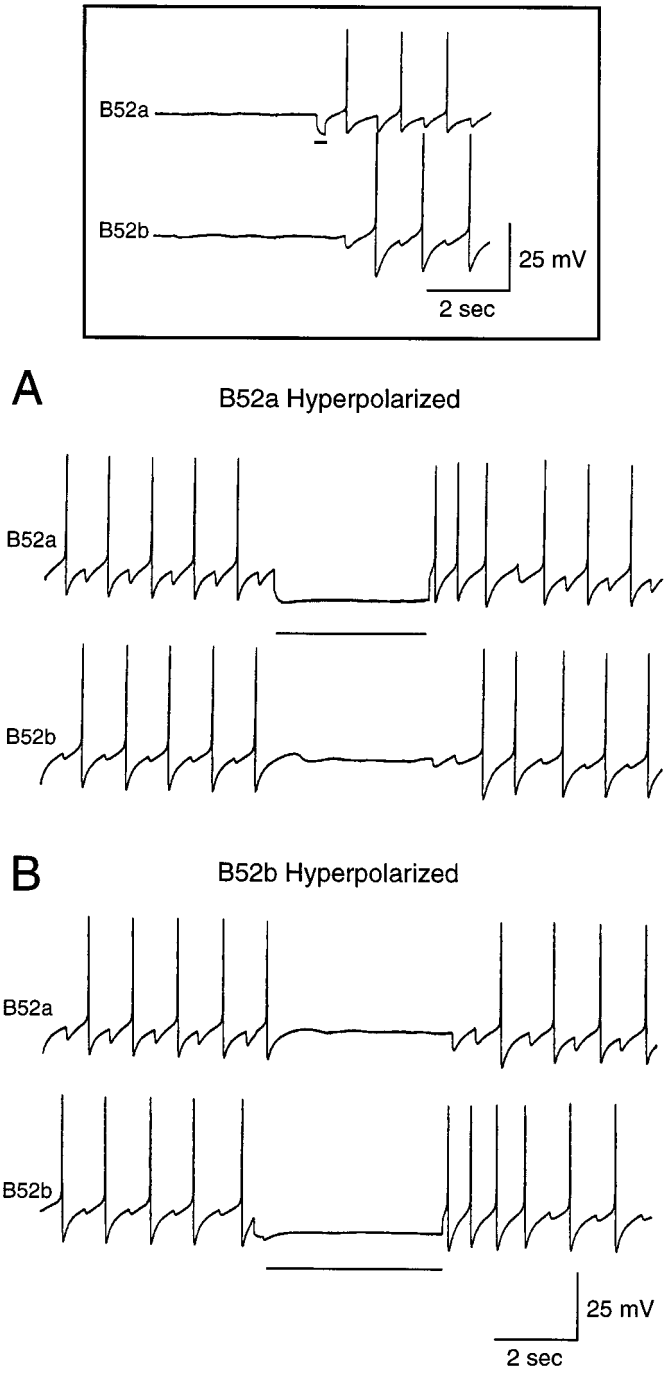

Figure 9. In otherwise quiescent preparations, the two B52 neurons can fire action potentials in alternation. *A*, That the activity of one cell is generated when it rebounds from the inhibition it receives from its partner neuron is shown when hyperpolarizing current was injected into the first neuron (indicated by the *horizontal bar*). Thus, when the first neuron was hyperpolarized and could not inhibit the second cell, the second cell no longer fired. *B*, The opposite was also true. *Inset*, That an inhibitory pulse can initiate activity in the B52 neurons is shown. At the point indicated by the *horizontal bar*, a hyperpolarizing pulse was injected into a neuron that was not firing spontaneously. The cell rebounded and generated an action potential.

the fact that the two cells are biophysically similar to each other and both receive depolarizing input at this point. IPSPs from partner neurons are presumably not sufficient to hyperpolarize cells to below-threshold membrane potentials, although they may contribute to synaptic integration in a subtle way.

Both B52 neurons display powerful postinhibitory rebound excitation. When reciprocally inhibitory neurons show this phenomenon, stable oscillatory activity can be elicited (e.g., Satterlie, 1985). This oscillatory activity can be driven solely by the autoexcitation that results from the postinhibitory rebound; i.e., oscillatory activity can occur in the absence of tonic drive from another source and can occur in the absence of intrinsic oscillating properties of member neurons (Satterlie, 1985). We show that the B52 neurons are similar in that they can fire in a reverberatory manner without additional synaptic input. Previous work has shown, however, that reverberatory activity can consist of a burst of action potentials in one neuron followed by a burst of action potentials in an antagonistic neuron. For the B52 neurons, however, reverberatory activity seems to be important within a single phase of a behavior and consists of a single spike in one cell followed by a single spike in the second cell. Thus, previous studies have demonstrated that the autoexcitation that results from postinhibitory rebound can drive groups of antagonistic

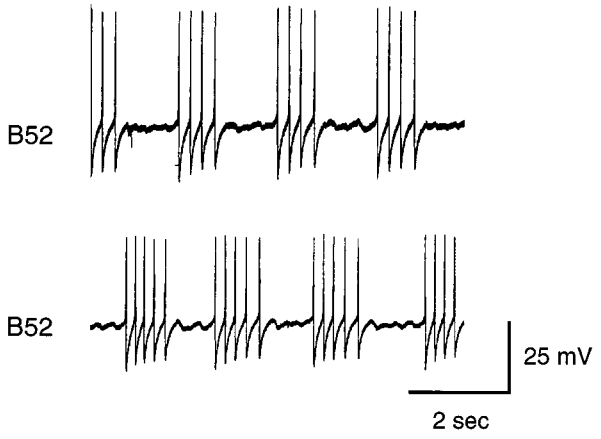

Figure 10. The B52 neurons can fire bursts of action potentials in alternation. Recordings were obtained from ipsilateral neurons.

interneurons. We are suggesting that this phenomenon may also play a role in determining the activity of functionally related neurons within a single phase of behavior.

Autoexcitation from rebound does not, however, ever seem to be the sole factor that determines B52 activity, even when motor programs begin to slow down. This is suggested by the fact that the B52 neurons never consistently fire in alternation. Instead they begin to alternate between firing more or less in synchrony and firing in alternation. Thus, it is likely that autoexcitation plays a role in maintaining B52 activity when synaptic drive is reduced but that, because the B52 neurons must fire in a manner that is appropriate for the ongoing motor program, synaptic drive can never be completely eliminated.

Physiological role of the B52 neurons

Plummer and Kirk (1990) demonstrated that B52 makes connections with many feeding neurons. Most of these connections are inhibitory. When B52 is stimulated, therefore, motor programs are not initiated. Plummer and Kirk suggested that B52 is not likely to be a cell that drives motor patterns but that it is likely to be a premotor neuron that plays an important role in terminating rhythmic activity. More recently, Baxter and Byrne (1991) have characterized additional synaptic connections of B52 and have modeled elements of the feeding CPG in *Aplysia* (Baxter et al., 1997). Baxter et al. (1997) have more specifically postulated that B52 plays a key role in terminating radula closing/retraction (Fig. 15). Our data that show that the B52 neurons make an inhibitory synaptic connection with the radula closer motor neuron B8 and begin to fire as radula opening/protraction begins are consistent with this idea.

In this study we show that centripetal activity is generated in B52 when the connective tissue adjacent to the buccal commissural arch is stretched. Will this stretch occur during normal behavior? Recent experiments suggest that it will (Drushel et al., 1997). Thus, Drushel et al. (1997) have visualized movements of

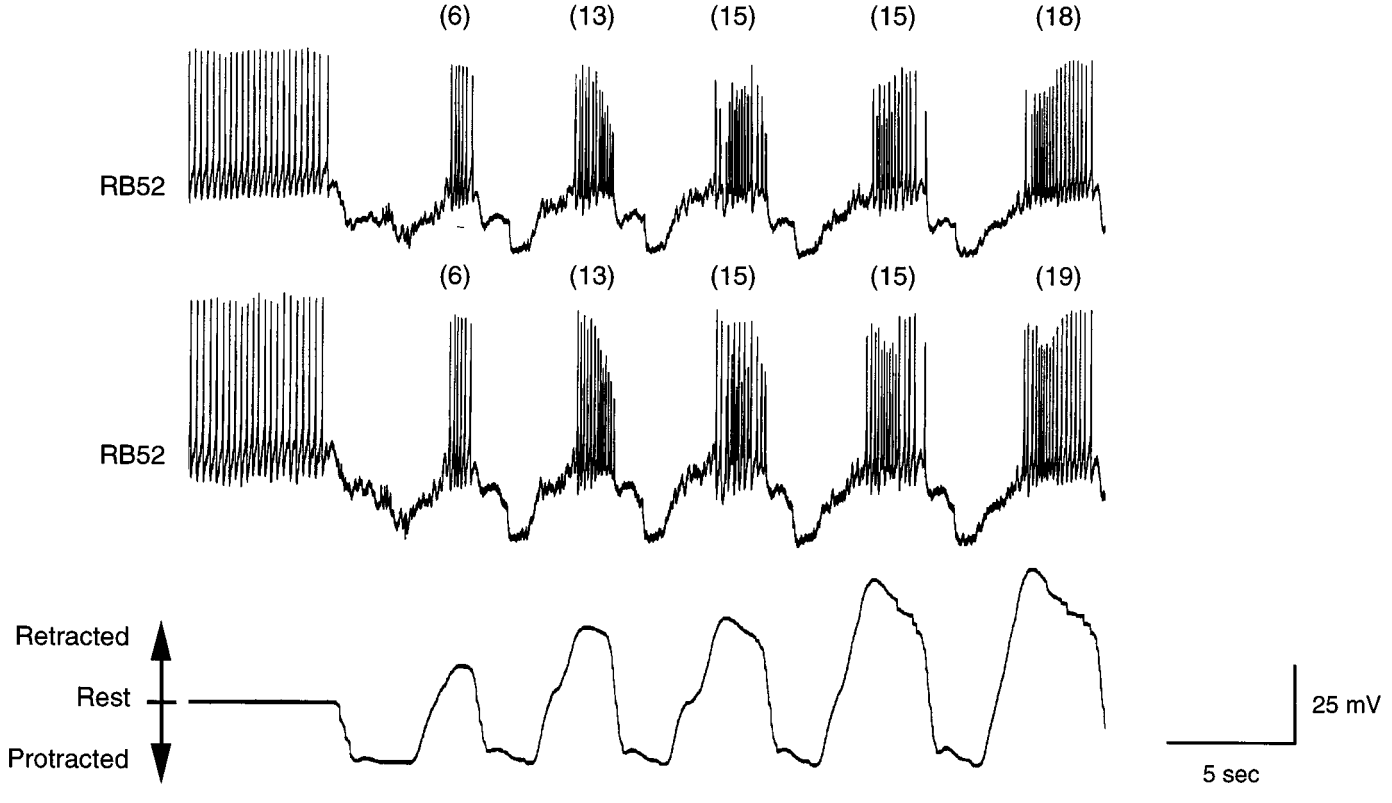

Figure 11. The B52 neurons fire during the same phase of ingestive motor programs. *Top*, *middle*, Intracellular recordings from the B52 neurons. *Bottom*, A record from a movement transducer that was attached to the anterior tip of the radula. Rhythmic activity was elicited by applying carbachol to the cerebral ganglion before the section of the record shown begins. Before rhythmic activity was generated, the two B52s alternated between firing in synchrony and firing in alternation (see Fig. 12). When rhythmic activity began, the two B52 neurons fired during the same phase of motor programs, and spikes in the two neurons occurred at approximately the same time; i.e., 69% of the spikes in one neuron occurred within 0.02 sec of a spike in the second cell (the average interspike interval during these bursts of activity was 0.14 sec). The *numbers above* the bursts of activity in the *top* and *middle traces* indicate how many action potentials were in each burst.

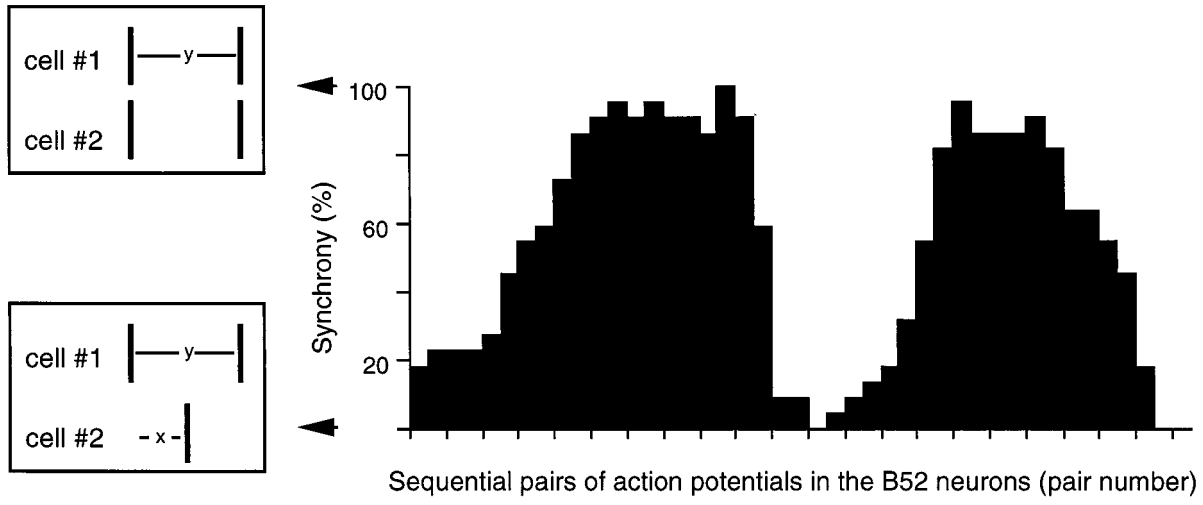

Figure 12. Data from the experiment shown in Figure 11. Before rhythmic activity began, the two B52 neurons alternated between firing in synchrony and firing in alternation. Percent synchrony was calculated as: $100 - [x/(y/2)]$. As indicated in the *insets*, y is the time between action potentials in cell #1; x is the time between an action potential in cell #1 and the next action potential in cell #2. Values were calculated every time a spike occurred. Cells were firing at ~4 Hz; therefore, a new value was calculated approximately every 0.25 sec. Forty-three values are plotted. The stretch of recording used to generate these data covers ~11 sec.

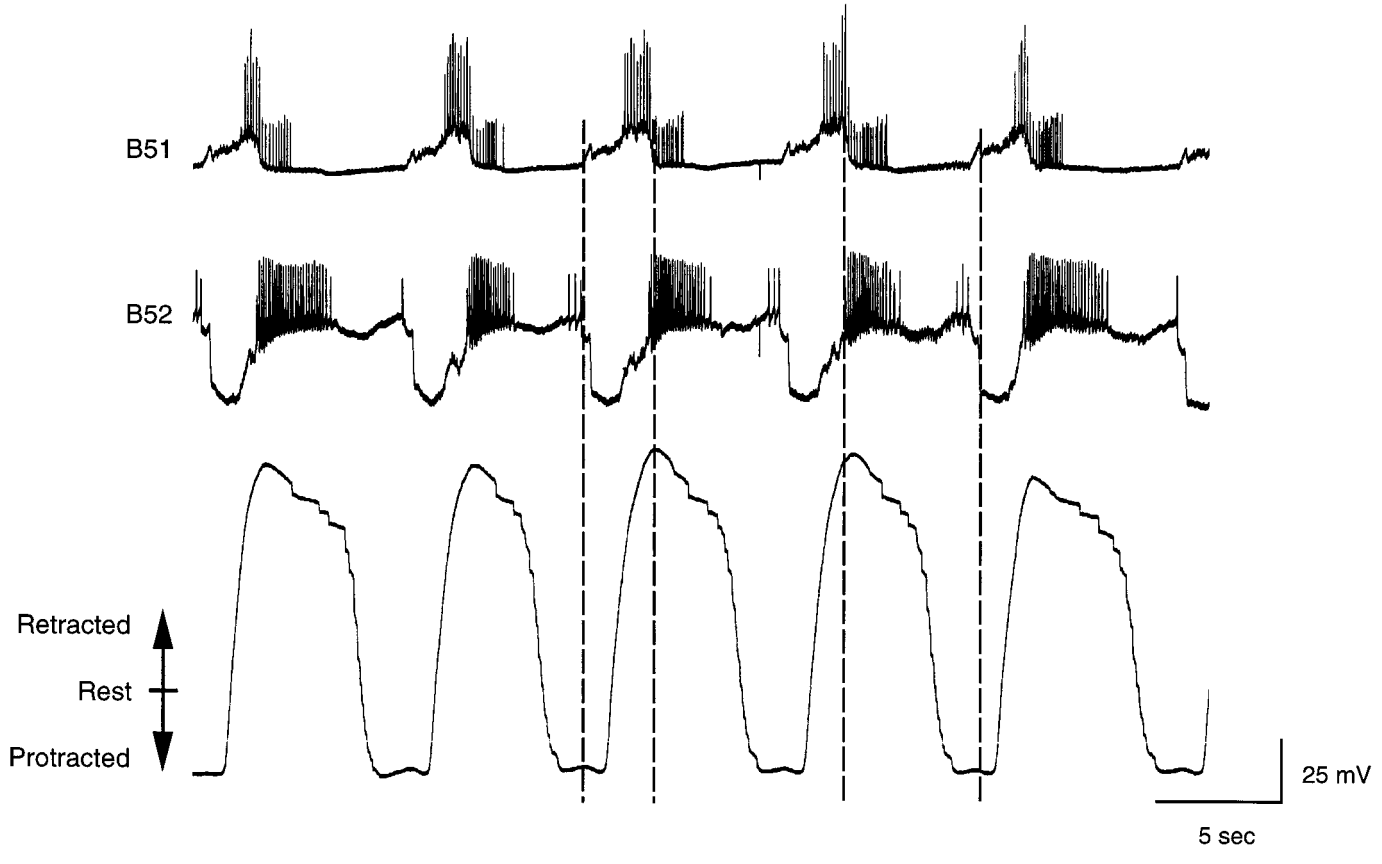

Figure 13. B52 is active during the opening/protraction phase of behavior. The *top trace* is an intracellular recording from a premotor neuron, B51, that is depolarized and fires action potentials during the closing/retraction phase of feeding motor programs (Evans and Cropper, 1998). For the most part, B51 and B52 activity was out-of-phase (see *vertical dashed lines*). Also note that depolarizations and action potentials begin to be recorded in B52 at the peak radula of retraction and continue as the radula is protracted toward the jaws.

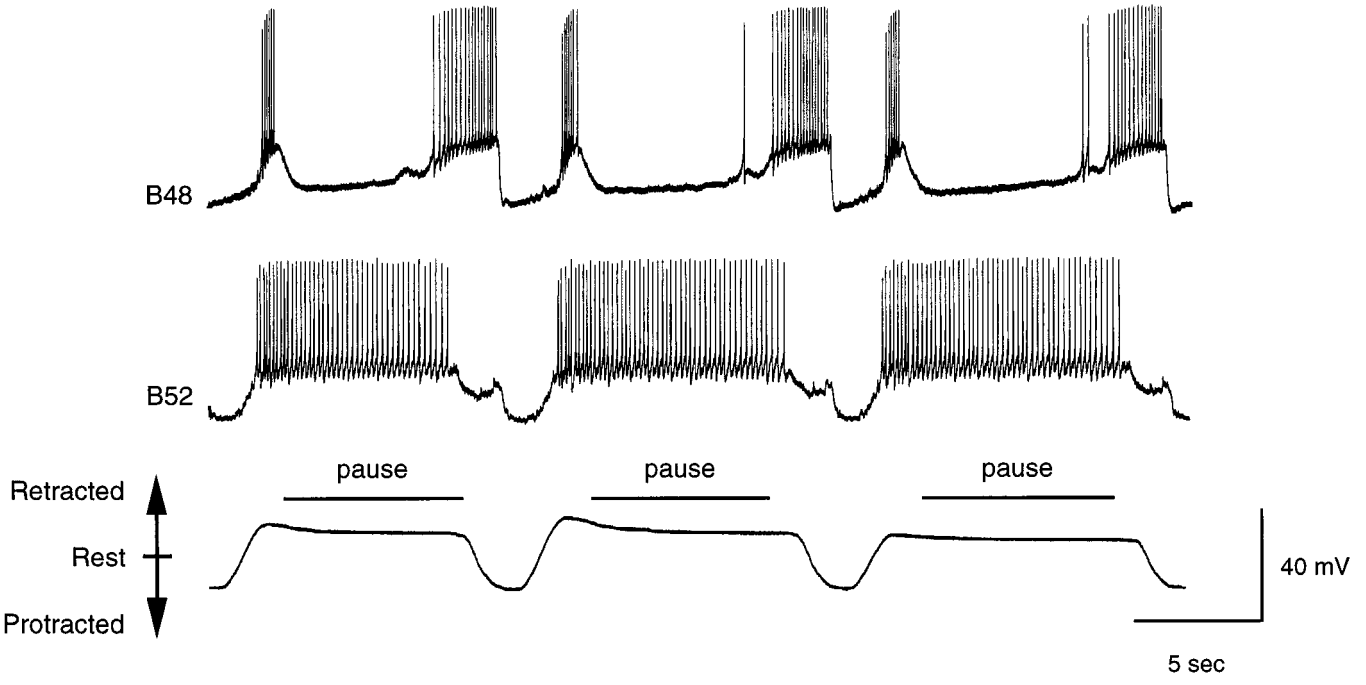

Figure 14. B52 activity during a carbachol-elicited motor program that is not cycling vigorously. *Top*, An intracellular recording from a motor neuron that elicits radula opening is shown (B48) (Evans et al., 1996). *Bottom*, Starting at the beginning of the record shown, the radula retracted, opened (see activity in B48), and paused before the next protraction and opening were elicited. *Middle*, B52 continued to fire during these pauses.

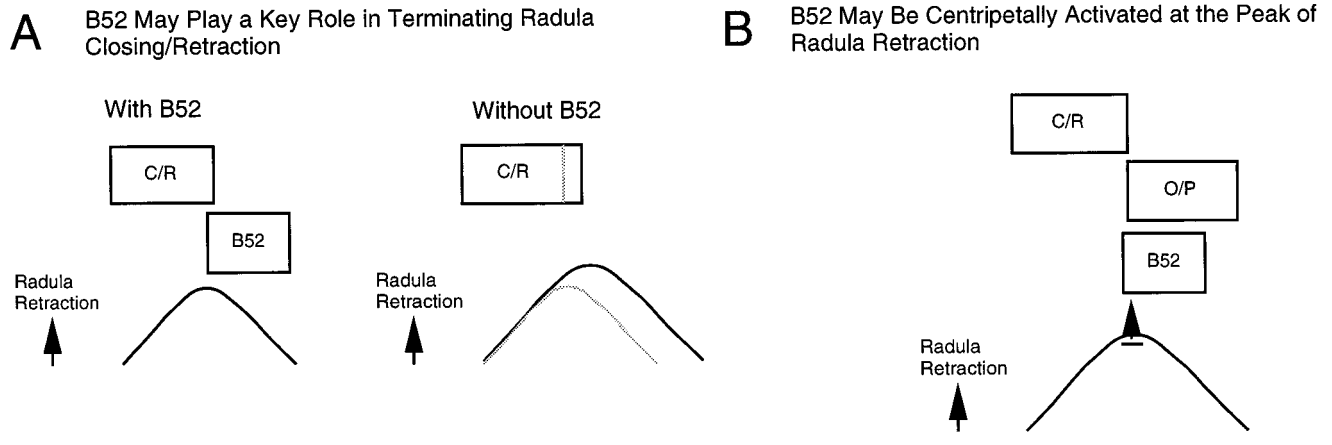

Figure 15. *A*, Computational studies (Baxter et al., 1997) have suggested that B52 may play a key role in terminating the closing/retraction phase of ingestive motor programs. *Left*, The schematic illustration shows the phase relationships between depolarization or spiking in the closing/retraction (*C*/*R*) circuitry, depolarization or spiking in B52, and retractions of the radula. Data from this study were used to illustrate radula retractions. Thus, as is shown in Figure 13, there is often some overlap between the end of activity in the C/R circuitry and the beginning of B52 activity. This overlap occurs approximately at the peak of radula retraction. Here a relatively small overlap is shown despite the fact that spiking in B51 (a retraction promoter neuron) can continue for a longer period of time. We show a relatively small overlap because recent studies have suggested that the prolonged activity in B51 is centripetally generated, and it has been demonstrated that central depolarizations can be important to "gate in" this type of activity (Evans and Cropper, 1998). Figures 11, 13, and 14 also show that B52 begins to spike when the radula is fully retracted and that B52 is at least depolarized as the radula protracts. *A*, *Right*, Computational studies indicate that activity in neurons that are part of the C/R circuitry is prolonged when simulations are run without including neuron B52 (Baxter et al., 1997). We hypothesize that radula retractions will also be enhanced. For comparison, the *gray lines* in the *diagram* on the *right* indicate the activity with B52 shown on the *left*. *B*, The schematic illustration shows the phase relationships between depolarization or spiking in the *C*/*R* circuitry, depolarization or spiking in B52, and retractions of the radula as described above. Additionally, depolarization or spiking in the radula opening/protraction (*O*/*P*) circuitry has been included. Our data and those of Drushel et al. (1997) suggest that B52 may be centripetally activated at the peak of radula retraction (at the point indicated by the *black horizontal line* and *arrow*). Thus, Drushel et al. (1997) have shown that the radular stalk moves ventrally at this point. When this occurs, the I2 muscle and the attached connective tissue that contains the terminals of B52 will be stretched. We show that stretch of this connective tissue produces depolarizations that can be recorded in the somata of B52. The schematic illustration of phase relationships shows that this occurs at a time when enhanced activity in B52 could be important for terminating enhanced activity in the C/R circuitry.

the buccal mass in strongly illuminated young animals. They have shown that the radular stalk [also known as the radular sac (e.g., Evans et al., 1996)] becomes particularly visible when animals ingest food and fully retract the radula. Under these conditions the radula moves ventrally and produces a noticeable change in the shape of the buccal mass. When the radula is in this position, the I2 muscle and the tissue containing the processes of B52 are likely to be stretched as shown in Figure 3. Data show, therefore, that the event that peripherally depolarizes B52, stretch of the connective tissue that contains its processes, is likely to occur during feeding (Drushel et al., 1997). Specifically, it is likely to occur when the radula is maximally retracted.

The B52 neurons appear, therefore, to be similar to another well-studied neuron in *Aplysia*, C2. Both cells are histaminergic sensory neurons (e.g., for data on C2, see Ono and McCaman, 1980; McCaman and Weinreich, 1982; Weiss et al., 1986c). Additionally, although C2 can function as a mechanoafferent that is activated when a mechanical stimulus is applied to the perioral zone (Weiss et al., 1986c), it can also be active in the absence of an exteroceptive stimulus. Thus, C2 will continue to fire during rhythmic buccal mass movements even when the stimulus used to trigger feeding movements has been removed (Weiss et al., 1986b). Thus both neurons appear to be capable of functioning as proprioceptors. Despite these similarities, it does not appear that all proprioceptive or mechanoafferent neurons in *Aplysia* are histaminergic. For example, neuron B21, which is a dual function radula mechanoafferent (Rosen et al., 1992)/proprioceptive (Cropper et al., 1996) neuron, is glutamatergic (Klein et al., 1998) and is not histamine-immunoreactive.

Our data indicate that peripheral stretch of the processes of the B52 is likely to contribute to the central activation of this neuron. We show that when we stretch the connective tissue adjacent to the buccal commissural arch, depolarizations are recorded from the soma of the B52 indicating that the length constant of the B52 is sufficient to conduct peripheral information passively to the CNS. These depolarizations are sufficient to trigger action potentials in the B52 when it is at its resting membrane potential. Interestingly, this centripetal activity is also effective at eliciting PSPs in the follower neurons of the B52. Thus, B52 seems to be unlike some of the other centrally located feeding sensory neurons in *Aplysia* in that afferent activity in B52 does not have to be gated in by a central depolarization to activate follower cells [compare B52 with B21 (Rosen et al., 1993, 1994) and B51 (Evans and Cropper, 1998)].

Considered in the context of the Baxter model (Baxter et al., 1997), these data suggest the following: B52 is likely to receive additional depolarizing input from the periphery at a time when it may play a role in terminating activity in the radula closing/retraction circuitry (Fig. 15). This input will be particularly pronounced when closing/retractions are enhanced and the connective tissue containing the processes of the B52 is more vigorously stretched. The more vigorous stretch of its processes will more strongly depolarize B52 and cause it to fire at an increased frequency. Thus, peripheral depolarizations of B52 may play a key role in terminating activity in the closing/retraction circuitry when this phase of behavior is enhanced.

Our data and other studies that have investigated relationships between sensory neurons and CPGs (Pearson and Ramirez, 1997) have, therefore, begun to determine how rhythmic behaviors that are generated by relatively simple CPGs are still able to adjust efficiently to changes in the external environment. Neurons that are important for basic rhythm generation can be sensory neurons. When this occurs, changes in the external environment can immediately produce changes in motor programs. Motor programs do not need to be modified by complex reflexes. This type of on-line monitor can be important for changes in motor programs that are directly elicited by the stimulus that causes the behavioral change (Pearson and Ramirez, 1997). This study demonstrates that this type of phenomenon may, however, also be important when motor programs have to be adjusted in a compensatory manner. Specifically, we suggest that food contacts the radula during the closing/retraction phase of feeding and immediately activates sensory neurons [e.g., radula mechanoafferents (Rosen et al., 1993, 1994; Miller et al., 1994)] that enhance the activity of the radula closing/retraction circuitry. Enhancements of radula closing/retraction are necessary to insure that food is deposited in the esophagus. When radula closing/retractions are more vigorous, it is important that activity in B52 is also more vigorous. This automatically occurs because enhanced radula retractions result in an increase in the peripheral depolarization of B52.

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