Title:

Transforming Tonic Firing into a Rhythmic Output in the *Aplysia* Feeding System: Presynaptic Inhibition of a Command-like Neuron by a CPG Element

Authors:

Itay Hurwitz\textsuperscript{1,2,3}, Abraham J. Susswein\textsuperscript{1,2} and Klaudiusz R. Weiss\textsuperscript{3}

Department and Institution:

\textsuperscript{1}The Leslie and Susan Gonda (Goldschmied) Multidisciplinary Brain Research Center, and \textsuperscript{2}Faculty of Life Sciences, Bar Ilan University, Ramat Gan, 52900, Israel, and \textsuperscript{3}Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, NY, 10029, USA,

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TRANSFORMING TONIC INPUT TO RHYTHMIC OUTPUT

Address for Correspondence:

Itay Hurwitz
Interdisciplinary Program in the Brain Sciences
Bar-Ilan University
Ramat Gan 52900
Israel
Phone: 972 3 531 8388
FAX: 972 3 736 9231
E-mail: hurvitz3000@hotmail.com
SUMMARY

Tonic stimuli can elicit rhythmic responses. The neural circuit underlying *Aplysia californica* consummatory feeding was used to examine how a maintained stimulus elicits repetitive, rhythmic movements. The command-like cerebral-buccal interneuron 2 (CBI-2) is excited by tonic food stimuli, but initiates rhythmic consummatory responses by exciting only protraction-phase neurons, which then excite retraction-phase neurons after a delay. CBI-2 is inhibited during retraction, generally preventing it from exciting protraction phase neurons during retraction. We have found that depolarizing CBI-2 during retraction overcomes the inhibition and causes CBI-2 to fire, potentially leading CBI-2 to excite protraction phase neurons during retraction. However, CBI-2 synaptic outputs to protraction phase neurons were blocked during retraction, thereby preventing excitation during retraction. The block was caused by presynaptic inhibition of CBI-2 by a key buccal ganglion retraction phase interneuron, B64, which also causes post-synaptic inhibition of protraction-phase neurons. Presynaptic and post-synaptic inhibition could be separated. First, only presynaptic inhibition affected facilitation of EPSPs from CBI-2 to its followers. Second, a newly identified neuron, B54, produced post-synaptic inhibition similar to that of B64, but did not cause pre-synaptic inhibition. Third, in some target neurons B64 produced only presynaptic, but not post-synaptic inhibition. Blocking CBI-2 transmitter release in the buccal ganglia during retraction functions to prevent CBI-2 from driving protraction phase neurons during retraction, and regulates the facilitation of the CBI-2 induced EPSPs in protraction phase neurons.
INTRODUCTION

A tonic stimulus can elicit rhythmic movements (Marder and Calabrese, 1996; Pearson and Gordon, 200). Neural circuits giving rise to such movements must contain mechanisms for transforming a tonic stimulus into a rhythmic response. The present report uses the feeding behavior in the marine gastropod mollusc *Aplysia* to examine some of the mechanisms that transform a tonic stimulus into rhythmic, repetitive responses.

*Aplysia* consummatory feeding behaviors are elicited by food touched to the lips or stimulating the interior of the mouth (Kupfermann, 1974). Tonically maintained stimuli elicit repeated, rhythmic protraction and retraction movements of the toothed radula (Kupfermann, 1974; Morton and Chiel, 1993a,b). Buccal motor programs corresponding to protraction and retraction movements in the intact animal can be monitored from many neurons within the buccal ganglia, as well as via extracellular recordings from buccal ganglia nerves (Morton and Chiel, 1993a,b; Church and Lloyd, 1994; Hurwitz and Susswein, 1996). Buccal motor programs are organized by a central pattern generator (CPG) containing separate interneurons that drive the protraction and retraction phases (Hurwitz and Susswein, 1996; Hurwitz et al., 1997; Plummer and Kirk, 1990). CPG activity is initiated via command-like neurons. The most prominent such neuron, CBI-2, is excited by food stimulating the lips, and monosynaptically excites protraction-phase interneurons, thereby initiating their activity (Hurwitz et al., 2003). Similar to the effects of tonic food stimulation on behavior, tonic depolarization of CBI-2 causes repeated, rhythmic buccal motor programs that cease when CBI-2 stimulation is terminated.

How are tonic stimuli to the lips or to CBI-2 converted into rhythmic buccal motor programs? Previous studies have identified two such mechanisms. One arises from reciprocal inhibition within the CPG. Activity in protraction phase neurons initiates activity, after a delay, in retraction phase neurons, which in turn inhibit protraction phase neurons, and
thereby turn off protraction while retraction progresses. A second mechanism arises from a feedback loop from the CPG to the command-like CBI-2 neuron. CBI-2 is inhibited during the retraction phase of a buccal motor program, thereby reducing its likelihood to fire during the retraction phase (Rosen et al., 1991; Hurwitz et al., 1999b). Because of the recurrent inhibition from retraction-phase neurons, activity of CBI-2 in response to a tonic stimulus is itself phasic, and CBI-2 generally fires and excites protraction-phase interneurons only during the protraction-phase. However, these two mechanisms are unlikely to be sufficient to ensure that tonic stimuli elicit a rhythmic buccal motor program. Strong inputs to CBI-2 that depolarize it sufficiently during the retraction phase will override the inhibition and elicit firing. CBI-2 firing during retraction would then excite and perhaps cause firing in protraction phase neurons during the retraction phase.

This study examined the consequences of driving CBI-2 to fire during the retraction phase. We found that firing CBI-2 during retraction had no effects on buccal motor programs, because a third mechanism is present that contributes to the conversion of a tonic stimulus to CBI-2 into a phasic response. In addition to inhibiting all of the protraction-phase neurons, the major retraction phase interneuron B64 also causes powerful presynaptic inhibition of CBI-2, thereby blocking its effects in the buccal ganglia, and preventing it from driving protraction phase neurons during the retraction phase.

MATERIALS AND METHODS

The experimental subjects were *Aplysia californica* weighing 150-300 grams provided by Marinus, Inc., (Long Beach, CA) and by the National Resource for *Aplysia* at the University of Miami. Animals were maintained at 14-16 °C in holding tanks containing aerated, filtered seawater. Before being dissected, animals were anesthetized and immobilized by injection with isotonic MgCl₂ (50% of body weight). The buccal and cerebral ganglia were removed with the cerebral-buccal connectives (CBCs) intact. The cerebral and buccal ganglia were
pinned to the floor of a recording chamber with the ventral surface of the cerebral ganglion and the caudal surface of the buccal ganglia facing up. The sheath overlying the surface of the ganglia was removed using ultrafine scissors.

*Cerebral and buccal ganglia circuitry*

Tonic depolarization of the command-like CBI-2 neurons induces repeated buccal motor programs (BMPs) that are organized by the buccal ganglion CPG (Rosen et al., 1991; Church and Lloyd, 1994; Perrins and Weiss, 1998; Hurwitz et al., 1999a, b). The ability of CBI-2 to elicit BMPs arises in part from the large, facilitating fast excitatory post-synaptic potentials (EPSPs) that CBI-2 firing induces in the protraction-phase interneurons that are part of the CPG (Hurwitz et al., 2003; Sanchez and Kirk, 2000). CBI-2 also induces slow EPSPs in the protraction-phase interneurons (Hurwitz et al., 1999a). Buccal ganglia protraction-phase neurons are excited by CBI-2. The present study examined a number of protraction-phase neurons that are excited by CBI-2, such as B31/B32, B34, B61/B62 and B63 (Fig. 1A). Previous studies have indicated that activity in most of the protraction phase neurons is not necessary to elicit a buccal motor program, since preventing them from firing does not abolish the ability of the ganglion to express a program (Hurwitz et al., 2003). Exceptions are the electrically coupled B31/B32 and B63 neurons, which are always active during buccal motor programs. In addition, hyperpolarizing them can block the expression of a program. The ability of CBI-2 to initiate buccal motor programs is largely explained by its strong, facilitating excitation of B63 (Hurwitz et al., 2003).

The retraction phase of a buccal motor program is triggered by firing in neuron B64 (Hurwitz and Susswein, 1996). Additional interneurons have important functions in retraction (Plummer and Kirk, 1990), but they are not always active during the expression of a buccal motor program (Nargeot et al., 2002), whereas B64 firing is an invariant feature of a buccal motor program. B64 activity is initiated as a result of activity in the protraction phase
neurons, via their effects on an unidentified element of the CPG that has been called the “z” cell (Hurwitz and Susswein, 1996; Baxter et al., 1997). The existence of the “z” cell has been inferred by modeling studies (Baxter et al., 1997), as well as via its effects on B64. B64 displays a prominent, endogenous plateau potential: a brief depolarization induces a burst of spikes that long outlasts the stimulus. Firing in B64 produces a large IPSP in protraction phase neurons, and depolarizes and caused firing in other retraction phase neurons (Hurwitz and Susswein, 1996).

Recording apparatus and bathing solutions.

Preparations were bathed in artificial seawater (ASW: NaCl 460 mM, KCl 10 mM, CaCl2 11 mM, MgCl2 55 mM and NaHCO3 5 mM) at pH = 7.64. In some experiments the buccal and cerebral ganglia were placed in a solution containing an increased concentration of divalent cations (HiDi saline: NaCl 311 mM, KCl 9 mM, CaCl2 33 mM, MgCl2 132 mM and NaHCO3 5 mM), in order to reduce polysynaptic activity of coupled neurons and follower neurons (Hurwitz et al., 2000). Except for experiments using voltage clamping, intracellular recordings were obtained from isolated ganglia preparations maintained at room temperature (18-22°C). Most experiments were performed under continuous fluid exchange, using a peristaltic pump at a rate of 10% volume per minute.

Intracellular recordings were made from CBI-2 as well as from buccal interneurons B64, B54, B63 and B34, and the buccal protraction-muscle (I2) motoneurons B31/B32 and B61/B62. Other neurons that were often recorded include radula closure motor neuron B8 and B4/B5. The neurons were identified by previously established morphological and/or physiological criteria (Gardner and Kandel, 1977; Jahan-Parwar et al., 1983; Susswein and Byrne, 1988; Rosen et al., 1991; Morton and Chiel, 1993a,b; Hurwitz et al., 1994, 1996, 1999b; Hurwitz and Susswein, 1996).
To generate an action potential in a neuron, 20 millisecond depolarizing current pulses were injected, and the appearance of one-for-one action potentials was monitored. CBI-2 was tonically fired at 7 to 20 Hz, for a period that did not exceed 3 minutes. Repeated stimulus sets to CBI-2 were separated by 10 minutes intervals.

**Intracellular recording and stimulation**

For intracellular recording and stimulation, neurons were impaled with single-barreled microelectrodes that were made of thin-walled glass tubing that was filled with 1.9 M potassium acetate and 0.1 M potassium chloride. The electrodes were pulled so that their impedances ranged from 10 to 15 MΩ, and following beveling they had final resistances of 6 to 10 MΩ. The activity of up to four neurons was monitored via intracellular recording using conventional electrometers. The extracellular activity in up to two nerves was also monitored. A Grass stimulator (S88) controlled the intracellular stimuli delivered to the neurons.

**Voltage clamping**

Voltage clamping was performed at 17º C using 1-5 MΩ electrodes filled with 1M KCl. Experiments were performed in an 0.5 ml chamber. The currents and voltages were recorded with an Axoclamp 2 (Axon Instruments) current/voltage clamp that was controlled by a computer running the Clampex component of pClamp 8.0 (Axon Instruments). Data were digitized and recorded using this program, via a Digidata 1200A digitizer (Axon Instruments). Preliminary data analyses used the Clampfit component of pClamp 8.0.

**RESULTS**

*In Aplysia*, a central pattern generator (CPG) in the buccal ganglia drives repeated cycles of radula protraction and retraction (Figs. 1B, C, 2A). The buccal CPG is composed of mutually inhibitory protraction and retraction phase interneurons (Figs. 1B, 1C, 2A). There is
little or no overlap in the firing of protraction phase (e.g., B31/B32, B34, B61/B62, and B63) and retraction phase (e.g., B64) interneurons and motor neurons (Hurwitz et al., 1997). A small population of additional neurons (e.g., B4/B5, the B8s) may shift the degree to which they are active in the two phases (Figs. 1D1, 1D2). In the isolated buccal ganglia, activation of the CPG causes organized buccal motor programs (BMPs), which correspond to protraction and retraction movements in the intact animal (Hurwitz et al., 1996, 1997). CBI-2 elicits monosynaptic facilitating EPSPs in protraction phase neurons, thereby initiating the protraction phase of a buccal motor program (BMP) (Hurwitz et al., 2003). Following a delay of several seconds, retraction-phase interneurons are activated (Hurwitz et al., 1997). The firing pattern of CBI-2 is similar to that of the protraction-phase interneurons: it fires in phase with buccal ganglia protraction phase neurons (although CBI-2 activity slightly precedes buccal ganglia activity), and the firing is suppressed during retraction phase of a BMP, in part via buccal to cerebral interneurons (Hurwitz et al., 1999b; Rosen et al., 1991). The inhibition of CBI-2 during retraction would be functionally adaptive, since firing of CBI-2 during retraction might cause protraction-phase interneurons and motor neurons to fire during the retraction phase. The simultaneous firing of the protraction and retraction muscles would interfere with the retraction movement.

**Similar motor programs are generated by phasic and tonic firing of CBI-2**

Although CBI-2 is inhibited during the retraction phase, the inhibition can be overcome by depolarizing stimuli. We examined the possible consequences on BMPs of firing CBI-2 during the retraction phase (Fig. 2). Firing in CBI-2 was regulated so that it fired at a constant rate during both the protraction and retraction phases. In this experiment (N=5), the protraction phase was monitored by intracellular recordings from protraction-phase interneuron B63, as well as by extracellular recordings from the I2 nerve, which contains axons of the protraction phase neurons B31/B32 and B61/B62 that drive contractions of the
major protraction muscle, I2 (Hurwitz et al., 1994, 1996, 2000). The retraction phase was monitored via intracellular recordings from neuron B4, which fires during retraction, as well as via intracellular recordings from motor neuron B8, and extracellular recordings from the radula nerve. B8 and radula nerve activity are seen during both protraction and retraction. The activity that is maintained after the end of protraction was utilized for monitoring retraction phases. BMPs were initiated via continuous stimulation of CBI-2 at 17 Hz with brief current pulses that evoked only a single spike in CBI-2.

When CBI-2 was injected with 18 nA current pulses (Fig. 2A1), spikes were initiated in CBI-2. The spikes elicited summating and facilitating monosynaptic EPSPs in B63. When the amplitude of the EPSPs exceeded threshold, B63 began to fire and BMPs were elicited, as has been described previously (Hurwitz et al., 2003). The 18 nA current pulses that were sufficient to initiate spikes in CBI-2 prior to and during the protraction phase did not initiate spikes during the retraction phase (Fig. 2A2), presumably because spikes were blocked by the phasic inhibitory input that CBI-2 received during retraction.

Raising the amplitude of the current pulses in CBI-2 from 18 to 25 nA (Fig. 2B1) overcame the inhibition that CBI-2 received during retraction, and elicited spikes during both phases of the BMP (Fig. 2B2). However, the patterns of activity in the buccal ganglia were remarkably similar to those elicited when CBI-2 was fired only during protraction (compare Figs. 2A1 and 2B1), in spite of the additional firing of CBI-2 during retraction. In addition, no EPSPs were observed in B63 when CBI-2 was fired during the retraction phase.

Previous experiments have shown that CBI-2 elicits facilitating EPSPs in a number of protraction phase neurons (see figure 1C), including B34, B63, B31 and B61 (Hurwitz et al., 2003; Sanchez and Kirk, 2000). We examined the ability of CBI-2 to elicit EPSPs in a variety of protraction phase neurons at different phases of a BMP (n=26). Figure 3 illustrates that a train of spikes in CBI-2 elicited facilitating EPSPs in B63, B34 and B31 (see fast sweep in
Fig. 3B). These eventually summated, causing a BMP (Fig. 3A). However, a train at the same frequency (14 Hz) delivered during the retraction phase completely failed to elicit detectable EPSPs (see the fast sweep in Fig. 3C). Later trains, which occurred during the late portion of the retraction phase, or following the end of the retraction phase, elicited EPSPs of progressively increasing amplitude within the bursts and between them (Fig. 3C).

These data indicate that when CBI-2 is fired early in the retraction phase, some mechanism completely suppresses CBI-2 elicited EPSPs in protraction phase neurons. The suppression gradually declines through the later portion of the retraction phase, and subsequent to retraction. The suppression of CBI-2 elicited EPSPs is likely to explain why CBI-2 firing during retraction has little or no effect on BMPs. This suppression could be caused by either presynaptic or postsynaptic inhibition.

*Postsynaptic inhibition cannot suppress CBI-2 elicited EPSPs*

Protraction phase neurons, including B34, B31/B32, B63 and B61/B62, are strongly inhibited during the retraction phase by a large inhibitory post-synaptic potential (IPSP) mediated via a conductance increase (Hurwitz et al., 1994, 1997). In principle, this IPSP could account for the suppression of EPSPs from CBI-2 to protraction phase neurons during retraction. If the conductance increase were sufficiently large, it could shunt completely the CBI-2 elicited EPSP. We tested this possibility by examining the conductance increases elicited in the protraction phase neurons during the retraction phase. In this experiment, EPSPs in protraction neurons were simulated by intracellular depolarizing current pulses.

Firing CBI-2 was used to elicit BMPs (N=11). CBI-2 stimulation was terminated just after the onset of the retraction phase, and sometime later two identical currents pulses were injected into a variety of protraction phase neurons. One pulse was delivered during the retraction phase, and the other was delivered a number of seconds after the retraction phase had ended. The voltage change during the retraction phase was decreased by somewhat less
than 50% with respect to the voltage change after the retraction phase (Fig. 4). These data indicate that the conductance change caused by the IPSP that underlies retraction would shunt a CBI-2 elicited EPSP in protraction-phase neurons, thereby lowering its amplitude, but the shunting would be insufficient to block the EPSP completely.

To examine quantitatively whether the retraction causes a conductance change that is sufficient to shunt CBI-2 induced EPSPs, protraction-phase neuron B31/B32 was voltage clamped (Fig. 5). The neuron was held at -60 mV, its resting potential. Either 4 or 8 second sweeps were observed at a frequency of once per 30 seconds. Voltage steps were applied in +10 mV increments, until 0 mV, and the steps were maintained for most of the sweep. Many records showed spontaneous intermittent slow inward deflections, followed by outward deflections. In current clamp conditions, the inward currents would underlie depolarizations of B31/B32 during protraction, and the outward currents would underlie the retraction phase. The currents underlying the BMPs were seen at a variety of voltages, allowing us to determine the voltage dependence of the currents underlying the retraction phase. Currents recorded in traces showing a BMP were subtracted from currents measured in the same cell, at the same voltage, in the absence of a BMP (BMPs were prevented by treating ganglia with tetrodotoxin), allowing us to estimate the currents that are exclusively attributable to the presence of a BMP. The current-voltage relationship for the retraction phase was then plotted, and a linear regression best fit was calculated. The best fit linear regression showed that the currents underlying the retraction phase have a net reversal potential of -61 mV, and the change in input resistance caused by the retraction phase is 1.01 MΩ (Fig. 5B). The input resistance of B31/B32 at rest was calculated from conductances recorded in response to voltage steps in B31/B32, at voltages in which the current-voltage relationship is ohmic. The measured passive input resistance was 2.71 MΩ. The 1.01 MΩ change in input resistance during the retraction phase in B31/B32 would have reduced the input resistance to 1.70,
which is a 37.25% reduction in the input resistance. Thus, current adequate to cause a 20 mV voltage change at rest would cause a 13 mV voltage change during the retraction phase of a buccal motor program. These data are consistent with those in Fig. 4, and indicate that the shunting of the CBI-2 induced EPSP by the increase in conductance during retraction would be adequate to reduce the amplitude of an EPSP from CBI-2, but would be too small to block completely the CBI-2 induced EPSP. Thus, another mechanism must account for the complete block of the EPSP.

*Complete block of CBI-2 elicited EPSPs is related to firing of B64.*

A number of retraction-phase interneurons have been identified (Plummer and Kirk, 1990; Hurwitz and Susswein, 1996; Nargeot et al., 1999). The most prominent of these, B64, is sufficient to induce the retraction phase (Hurwitz and Susswein, 1996). In spontaneous or elicited BMPs a burst of spikes in B64 signals the start of the retraction phase. In addition, if B64 is stimulated and begins to fire prematurely this firing leads to the termination of protraction, and an immediate, premature onset of the retraction phase (Hurwitz and Susswein, 1996). Since firing in B64 is a fixed marker of the start of retraction, we examined whether the firing of B64 is temporally related to the block of the CBI-2 elicited EPSPs in protraction phase neurons.

During CBI-2 initiated BMPs the termination of the protraction phase was correlated with firing in B64, as well as with the block of CBI-2 elicited EPSPs in the protraction phase neurons (Fig. 6A). In one experiment, stimulating B64 to fire during the protraction phase caused a premature termination of the protraction phase (Fig. 6B), as was described previously (Hurwitz and Susswein, 1996). This stimulus also caused a premature initiation of the retraction phase (see the first and the second cycles of BMP in Fig. 6B). Furthermore, firing B64 during the protraction, before it would have been activated without experimenter intervention, led to three cycles of BMP in the recorded period instead of two (CBI-2 firing
was maintained for 50 seconds, compare Figs. 6A and 6B). In addition, the premature onset of firing in B64 also blocked the CBI-2 elicited EPSPs in the protraction phase neurons (N=7). This experiment indicates that firing of B64 causes the block of the CBI-2 elicited EPSPs in protraction-phase neurons, in addition to initiating the retraction phase.

The block of CBI-2 initiated EPSPs caused by firing B64 could be mediated via direct effects of B64, or via indirect effects, by B64 recruiting other retraction phase neurons, which in turn could block the EPSPs. If the effect of firing B64 were via intervening neurons, the block of CBI-2 induced EPSPs should be eliminated, and the EPSPs should be restored when the buccal ganglia are bathed in HiDi saline (see Methods), which elevates the firing threshold of neurons, and therefore makes it more difficult to recruit potential followers of B64.

To test whether the inhibitory effects of B64 stimulation may be mediated via intervening neurons, various protraction phase neurons were examined during the combined firing of B64 and CBI-2, or during alternated firing of B64 and CBI-2, while both the buccal and cerebral ganglia were bathed in HiDi saline. To increase the size of the EPSPs, the various protraction phase neurons were held at –80 mV via injection of constant hyperpolarizing current. To control the firing frequency of B64, the neuron was stimulated with brief depolarizing pulses, which initiated individual spikes.

CBI-2 elicited EPSPs in protraction phase neuron B34 were blocked by firing B64 in the presence of HiDi saline only when B64 and CBI-2 fired simultaneously (Fig. 7). Driving CBI-2 with trains of spikes that are followed by a number of seconds of rest elicited trains of facilitating EPSPs in B34. During the first minute of stimulation, the facilitation of the EPSPs grows from burst to burst (not shown). Following the first minute, facilitation from burst to burst is constant (Fig. 7a). Firing of B64 2 seconds before firing CBI-2 did not affect the CBI-2 elicited EPSPs (Fig. 7B). By contrast, firing of B64 and CBI-2 simultaneously completely blocked CBI-2 elicited EPSPs (Fig. 7C). This experiment indicates that B64 probably inhibits
CBI-2 elicited EPSPs directly, since the inhibition is seen in HiDi saline. It also suggests that B64 may produce presynaptic inhibition of the CBI-2 processes, since the block is critically dependent on the simultaneous activity of CBI-2 and B64. Data similar to those in Fig. 6 were also obtained when recordings were made from additional protraction phase neurons, such as B31/B32, B61 and B63 (N=11).

Block of CBI-2 induced EPSPs is caused by pre-synaptic inhibition

A conclusive demonstration that B64 acts on CBI-2 processes would require recordings from the CBI-2 processes within the neuropile of the buccal ganglia. Attempts to perform such recordings were not successful in our hands (N=11), (but see Sanchez and Kirk, 2001). We therefore gathered indirect evidence supporting the hypothesis that B64 directly inhibits CBI-2 neurites, and thereby prevents them from releasing transmitter.

Simultaneous firing of B64 and CBI-2 blocks facilitation buildup of CBI-2 elicited EPSPs.

Previous data indicated that the facilitation of the EPSPs induced in protraction phase neurons by CBI-2 is a presynaptic process (Sanchez and Kirk, 2000). This facilitation is strongly affected by small changes in the firing frequency of CBI-2 (Hurwitz et al., 2003). If the block of the EPSPs induced by CBI-2 arises via presynaptic inhibition, which causes a complete block of transmitter release, the facilitation should be affected by the block, in a manner similar to that caused by a complete interruption of CBI-2 firing. By contrast, if the block of the EPSPs arises as a result of a post-synaptic mechanism, the facilitation should be unaffected by the block.

To examine the effects of firing B64 on the facilitation, CBI-2 was stimulated in bursts so that the bursts of EPSPs recorded in B34 reached a steady-state facilitation (N=3). These experiments were performed in a HiDi solution, and B34 was held at –80 mV. In these conditions, firing CBI-2 in trains drives repeated bursts of EPSPs with summated amplitudes that reach ~35 mV at the end of a burst (Fig. 8A).
On the background of trains of current pulses applied to CBI-2 alone, B64 and CBI-2 were stimulated in tandem 4 times. The simultaneous stimulation of B64 and of CBI-2 blocked the EPSPs in B34 (Fig. 8B). Following the 4 stimuli with combined trains of stimuli to both CBI-2 and B64, firing of CBI-2 alone again elicited EPSPs in B34. However, the amplitude of these EPSPs was decreased, with respect to that seen prior to the firing of B64. The amplitude of the EPSPs returned to the values seen before the B64 stimulation only after 3 bursts in which CBI-2 was stimulated alone (18 seconds after the end of the B64 stimulus).

Skipping four trains of CBI-2 spikes produced effects that were similar to those produced by the simultaneous stimulation of B64 and CBI-2. When CBI-2 stimulation was resumed the amplitude of the summated EPSPs was reduced, with full recovery of summated EPSPs amplitudes being observed only by the third train after the pause (Fig. 8C). These results indicate that the simultaneous firing of B64 and CBI-2 affects the decay of facilitation in a manner similar to that caused by a complete lack of CBI-2 stimulation.

*Dissociation of inhibition and block of EPSPs.* B64 produces postsynaptic inhibition of the protraction phase neurons, as well as a block of the EPSPs from CBI-2. The data above suggest that these are separable processes. Additional evidence in support of this suggestion could be obtained if a second neuron could be found that causes postsynaptic inhibition of the protraction phase neurons, similar to that caused by B64, but which does not also block the CBI-2 induced EPSPs.

We have found such a neuron, which was not previously identified, and which we now name B54 (see fig. 1A). B54 has a soma approximately 150% larger than that of B64. It is located medial to B64 (n=9). We have not examined systematically the role of B54 in CBI-2 generated BMPs. B54 is similar to B64 in its ability to hyperpolarize protraction phase neurons, including B34 (Fig. 9). In addition, the increase in the conductance of protraction phase neurons elicited by firing B54 is similar to that caused by B64 (not shown, N=5). B54
can be distinguished from B64, since a brief depolarization of B64 elicits a plateau potential that long outlasts the stimulus (Hurwitz and Susswein 1996), whereas B54 does not display this property. By contrast to B64, firing in B54 did not block CBI-2 elicited EPSP (Fig. 9C), although it did cause a 50% decrease in the amplitude of the EPSPs, as would be predicted if it shunted the EPSPs via a conductance increase IPSPs. This inhibition can be seen by comparing the EPSP amplitudes resulting from combined bursts of CBI-2 and B54 (Fig. 9C) to the EPSPs amplitudes resulting from driving CBI-2 alone (Fig. 9B). These data are consistent with the interpretation that postsynaptic inhibition is separable from the block of CBI-2 induced EPSPs, and provide further evidence that the EPSP block arises from presynaptic inhibition.

Since B54 appears to exert only postsynaptic actions, one would predict that the simultaneous firing of CBI-2 and B54 would affect the size of summatting EPSPs but not the facilitation of the CBI-2 elicited EPSPs, as does the simultaneous firing of CBI-2 and B64. This prediction was confirmed in 5 experiments (Fig. 10). In the absence of either B54 or B64 stimulation, three trains of stimuli to CBI-2 elicited a gradual buildup in the amplitude of the EPSPs recorded in B34 (Fig. 10A). When CBI-2 and B54 were stimulated together the amplitude of the EPSPs elicited by stimulating CBI-2 was decreased (compare the first and the second trains in Figs. 10B to the first and the second trains in 10A). However, when B54 was not stimulated during the third train, the EPSPs elicited by CBI-2 were fully facilitated, as they would have been if there had been no B54 stimulation in the previous trains (compare last trains of EPSPs in Fig. 10A and 10B). By contrast, when CBI-2 and B64 were stimulated in tandem during the first two trains, the CBI-2 elicited EPSPs were not seen, and in their place the characteristic B64 elicited IPSPs were observed (Fig. 10C, first 2 trains). When B64 was not stimulated during the third train, the EPSPs elicited by CBI-2 were expressed. However, in place of full recovery of facilitation during the third train, like the one observed
when B54 was not stimulated, skipping the third train of firing in B64 caused a reduction in the amplitude of CBI-2 elicited EPSPs with respect to the amplitude seen when B64 was not stimulated. Furthermore, during the third train of EPSPs, when B64 was not stimulated, the EPSPs elicited by CBI-2 were comparable to those seen during the first train of CBI-2 stimulation alone (compare the last train of EPSPs in Fig. 10C to the first train in Fig. 10A). Similar results were also obtained when another protraction phase neuron, B63, was monitored in place of B34 (not shown, N=2). These data indicate that the post-synaptic inhibition of protraction phase neurons by B54 and B64 reduces the amplitude of the EPSPs elicited by CBI-2. However, only the presynaptic inhibition elicited by B64 blocks synaptic release from CBI-2 terminals, and thereby prevents the gradual buildup of EPSP facilitation.

B64 can also elicit purely presynaptic effects. The data above provide strong evidence that the presynaptic and postsynaptic inhibitions produced by B64 are separable processes, and the effects of each can be characterized. Further evidence that B64 can produce both presynaptic and postsynaptic inhibition could be marshaled if an additional neuron were found that displayed only presynaptic inhibition when B64 is fired, with no concomitant postsynaptic inhibition.

B8 was found to be such a neuron (N=9). CBI-2 elicited a slow EPSP in B8. The slow EPSP was also present in HiDi saline, indicating that the connection is likely to be monosynaptic (Fig. 11A). In addition, firing of B64 did not inhibit B8, as it did the protraction phase neurons, but rather elicited a weak conductance decrease (Fig. 11B – compare the responses to current injections just before and after firing of B64 with those during B64 firing, which also elicits IPSPs in B61).

A conductance decrease should increase the amplitude of CBI-2 elicited slow EPSP in B8, if firing of CBI-2 precedes the firing in B64. Such an increase was seen when B64 was fired during the slow EPSPs elicited by CBI-2 (Fig. 11C1). In this experiment, a series of 5
CBI-2 bursts were elicited, and B64 was fired during the slow EPSPs elicited by the second and third burst. In the absence of B64 firing, CBI-2 elicited 6 spikes. By contrast, when B64 was also fired, CBI-2 elicited 8 spikes. There is a significant delay between firing of CBI-2 and the expression of the slow EPSP in B8, which permits the expression of B64 effects on the ongoing EPSP (i.e., B64 was fired during the slow EPSP, rather than during CBI-2 firing). However, when B64 was fired simultaneously with CBI-2, it completely blocked the slow EPSP in B8 (Fig. 11C2). Thus, firing CBI-2 alone evoked 5-6 action potentials in B8. When CBI-2 and B64 were fired together, no spikes were seen in B8 (compare the responses to the first and the last trains of CBI-2 firing with that of the second train). These data conclusively illustrate that the presynaptic and postsynaptic effects of firing B64 are separable. Firing B64 in tandem with CBI-2 causes presynaptic inhibition, whereas firing of B64 in tandem with the slow EPSP elicited by CBI-2 causes postsynaptic excitation.

DISCUSSION

Neural circuits in many animals are designed to transform a tonic input into rhythmic output. Investigating the mechanisms underlying the transformation of a tonic input into a rhythmic output in the *Aplysia* buccal motor system provides insight into the general question of how to design a circuit to convert a tonic input into a rhythmic output.

The data presented in this paper, in addition to those shown previously, indicate that at least three mechanisms contribute in parallel to the transformation of a tonic input to CBI-2 into a phasic motor output. *First*, the buccal CPG that is responsible for generating protraction-retraction sequences consists of separate, mutually inhibitory protraction-phase and retraction-phase interneurons (Hurwitz and Susswein, 1996; Hurwitz et al., 1997). CBI-2 monosynaptically recruits the protraction-phase neurons, but not the retraction-phase neurons (Hurwitz et al., 2003). The protraction-phase neurons in turn recruit retraction-phase neurons, with a delay, via a presently unidentified element that has tentatively been named the z cell.
(Baxter et al., 1997). Activation of the retraction-phase neurons leads to inhibition of protraction-phase neurons (Hurwitz and Susswein, 1996; Hurwitz et al., 1997). Thus, CBI-2 induces biphasic activity by activating protraction, which itself then activates retraction, and thereby shuts itself off. **Second**, phasic feedback from the buccal CPG to the cerebral ganglia post-synaptically inhibits CBI-2 (Hurwitz et al., 1999b; Rosen et al., 1991), leading to a rhythmic pattern of CBI-2 firing (Rosen et al., 1991). CBI-2 will then fire and excite the protraction phase neurons in the buccal ganglia during one phase of a cycle. Constraining the firing of CBI-2 to the protraction phase restricts the recruitment of buccal ganglion protraction-phase neurons by CBI-2 to the appropriate phase of a motor program, and inhibits the firing of CBI-2 during the inappropriate phase. **Last**, we have now shown that if the CBI-2 neuron is sufficiently excited, and therefore fires during the retraction phase in spite of the inhibition seen during this period, such firing does not elicit excitation of protraction-phase neurons in the buccal ganglia. The lack of excitation is a result of pre-synaptic inhibition from the major retraction element of the CPG, B64, onto the output of CBI-2 within the buccal ganglia.

**Evidence in favor of presynaptic inhibition**

Our evidence that B64 blocks CBI-2 output via pre-synaptic inhibition is indirect. Pre-synaptic inhibition is most directly demonstrated via intracellular recordings from the presynaptic terminals (e.g., Baxter and Bittner, 1991), and via quantal analysis (see Boyd and Martin, 1956), which compares the quantal number and the quantal content of a synapse while it is inhibited to the values before inhibition, and then shows that the quantal number, rather than quantal content, is reduced by pre-synaptic inhibition (e.g., Boyd and Martin, 1956). In our system a quantal analysis would be unlikely to provide useful information, since the synapse from CBI-2 to protraction phase neurons is completely blocked during the retraction, rather than merely being reduced. When a synaptic connection is completely absent, it would
unrealistic to estimate changes in the quantum number or the quantal content. In place of a quantal analysis, we have marshaled a great deal of indirect support for the contention that B64 pre-synaptically inhibits the terminals of CBI-2 on protraction phase. The indirect measures of presynaptic inhibition are similar to those used previously in other systems that display presynaptic inhibition (Dudel and Kuffler, 1961; Peng and Frank, 1989). First, we have shown that overriding the inhibition of CBI-2 during retraction and causing it to fire does not cause excitation of the protraction phase neurons during the retraction phase, and therefore does not affect the BMPs recorded in the buccal ganglia (Figs. 2 and 3). Although retraction is characterized by post-synaptic inhibition of protraction-phase neurons, particularly by B64, the lack of any excitation during retraction could not be explained by post-synaptic inhibition of the protraction phase neurons, since such inhibition would attenuate the amplitude of the EPSP caused by CBI-2 by 37% (Fig. 5), but would not eliminate it completely (Fig. 4), as was observed. In other systems, a reduction of a synaptic potential beyond that which can be explained by post-synaptic inhibition has been used as evidence of pre-synaptic inhibition (Frost et al., 2003). Second, we have shown that the block of excitation from CBI-2 to protraction phase neurons is correlated precisely with firing in B64, as would be expected if the block were exerted via a phasic pre-synaptic inhibition (Fig. 7). The need for a precisely timed inhibitory input immediately preceding the input that is inhibited is a widely seen feature of presynaptic inhibition that is caused by directly-gated receptors (for review, see MacDermott et al., 1999). Third, we have shown that block of excitation from CBI-2 to protraction phase neurons causes changes in synaptic facilitation (a presynaptic process) identical to those seen when CBI-2 does not fire, whereas post-synaptic inhibition does not affect synaptic facilitation in this synapse (Fig. 8). Modulation of facilitation has been widely used as a monitor of presynaptic inhibition (Baxter and Bittner, 1991). Fourth, we have identified a neuron, B54 (Figs. 1, 9), that produces post-synaptic
inhibition of the CBI-2 initiated EPSPs similar to that produced by B64, but does not produce
presynaptic inhibition, thereby demonstrating the separate effects of pre-synaptic and post-
synaptic inhibition of the connection from CBI-2 to protraction phase neurons (Figs 9, 10).

Last, we have found that for some neurons B64 produces only a pre-synaptic inhibition of the
EPSP from CBI-2, with no post-synaptic inhibition at all (Fig. 11), further emphasizing that
pre-synaptic and post-synaptic inhibition are separable processes.

Mechanism of pre-synaptic inhibition. Presynaptic inhibition can be caused via directly-gated
channels (for review, see MacDermott et al., 1999), as well as via second-messenger mediated
channels (Fossier et al., 1994). A number of cellular mechanisms have been proposed to
explain pre-synaptic inhibition. These mechanisms lead to a reduction in Ca\(^{+2}\) entry into the
presynaptic terminal, or a decrease in the efficacy of Ca\(^{+2}\) in producing transmitter release.
Presynaptic inhibition is often associated with a reduction in the size of the pre-synaptic
action potentials, which may be correlated with depolarization of the pre-synaptic terminals
(e.g., Pearson and Goodman, 1981), or with hyperpolarization of the terminals (Dudel and
Kuffler, 1961; Kretz et al., 1986b). An increase in conductance in the terminal, independent of
whether it causes a depolarization or hyperpolarization, would shunt the currents in the
terminal, and thereby reduces the amplitude of presynaptic spikes (Baxter and Bittner, 1991),
or perhaps blocks their invasion into the terminal. In addition, depolarization itself reduces the
size of action potentials, and also causes partial inactivation of the presynaptic terminal
(Burrows and Matheson, 1994). Presynaptic inhibition may also be caused by an inhibition of
the voltage-dependent Ca\(^{+2}\) currents (Kretz et al., 1986b; Wu and Saggau, 1997) by which
Ca\(^{+2}\) for synaptic release enters the cell, or by a direct inhibition of transmitter release (Parnas
et al., 2000).

Presynaptic inhibition of the CBI-2 synaptic output by B64 leads to a complete block
of the EPSP, rather than to its reduction. Mechanisms that reduce the size of the presynaptic
action potential, without eliminating it, could not account for a complete block of synaptic transmission. Depolarization of the terminal large enough to inactivate it completely, and thereby block spikes would probably also affect facilitation of the synapse differently from that observed, and are therefore unlikely to explain presynaptic inhibition in our system. Blocking Ca\textsuperscript{+2} entry via the regulation of Ca\textsuperscript{+2} channels is likely to be mediated via second-messengers, whose operation would be too slow to account for the precise timing that is seen in the effects of B64 on CBI-2 induced EPSPs, and are therefore also unlikely to operate in our system. The most likely hypothesis to explain presynaptic inhibition in our system is block of spike invasion into the terminals.

Transmitter identification of B64. Additional evidence that B64 causes presynaptic inhibition of CBI-2 could be gathered by showing that exogenous application of the B64 transmitter produces inhibition of CBI-2 similar to that produced by firing B64. However, the B64 transmitter has not yet been identified. In Aplysia, histamine release by identified neurons has been shown to cause presynaptic inhibition in both the cerebral (Chiel et al., 1988) and the abdominal ganglion (Kretz et al., 1986a). A number of buccal ganglia neurons use histamine as their transmitter (Evans et al., 1999). Studies that have localized transmitters to neurons in the buccal ganglia effectively eliminate histamine, GABA, dopamine, serotonin and NO as transmitters used by B64 (Evans et al., 1999; Diaz-Rios et al., 1999, 2002; Kabotyansky et al., 1998; Jacklet and Koh, 2001). Although, glutamate and Ach have been demonstrated to affect network properties of the feeding CPG in Lymnaea (Straub et al., 2002), their ability to mimic B64 effects has not been tested.

Function of presynaptic inhibition

Many neural systems are characterized by multiple, parallel mechanisms that act to achieve a common design feature, similar to the multiple, parallel mechanisms that act to transform a tonic input to CBI-2 into a rhythmic motor output. Finding this feature in the buccal motor
system, therefore, is not surprising. Nonetheless, the existence of parallel means to achieve the same goal raises the question of what the presence of pre-synaptic inhibition contributes to a system that already has at least two additional methods of converting a tonic input to CBI-2 into a rhythmic motor output.

Differential regulation of CBI-2 effects in different ganglia. In the stomatogastric nervous system of the crab a single modulatory interneuron projects to two different ganglia. In one, its terminal are presynaptically inhibited, leading to phasic modulation, whereas in the other ganglion tonic modulation is seen (Coleman and Nusbaum, 1994). Presynaptic inhibition of CBI-2 by B64 could also cause different patterns of activity in the cerebral and buccal ganglia of *Aplysia*. B64 neurites are restricted to the buccal ganglia (Hurwitz and Susswein, 1996), thereby limiting presynaptic inhibition of CBI-2 to these ganglia. However, firing CBI-2 excites neurons in both the cerebral (Rosen et al., 1991; Morgan et al., 2002) and the buccal ganglia (Hurwitz et al., 2003; Sanchez and Kirk, 2000; Jing and Weiss, 2001). Excitation of CBI-2 that causes firing during retraction could therefore drive CBI-2 followers in the cerebral ganglion while CBI-2 followers in the buccal ganglion are blocked. Depolarization of CBI-2 has been shown to cause firing in other CBIs (Rosen et al., 1991). Since many of these CBIs fire during both protraction and retraction, excitation from CBI-2 during retraction would not interfere with the patterning and timing of firing of these neurons.

Differential control of facilitation. A second possible function of the pre-synaptic inhibition produced by B64 may be to regulate the strong facilitation of the synapses from CBI-2 to the protraction phase neurons. Previous data (Hurwitz et al., 2003; Sanchez and Kirk, 2000), as well as data in this paper (Figs. 7 and 8), have shown that these synapses undergo frequency-dependent changes in amplitude, leading to a large facilitation. The facilitation in the CBI-2 induced EPSP is a central feature in the ability of CBI-2 to induce a buccal motor program, since the amplitude of the EPSPs preceding the protraction phase may grow from 0 mV to
over 10 mV (Hurwitz et al., 2003). A decrease in the facilitation may significantly delay or block a buccal motor program (Hurwitz et al., 2003). In addition, small changes in the background firing frequency of CBI-2 can profoundly affect the amplitude of the PSPs during a subsequent burst of activity in CBI-2 (Hurwitz et al., 2003). As in most systems (Katz and Miledi, 1967), facilitation of the CBI-2 to protraction neuron synapses is a pre-synaptic process (Sanchez and Kirk, 2000). Pre-synaptic inhibition assures that the possible occasional firing of CBI-2 during the retraction phase will not cause an undesired modification in the amplitude of the PSPs during the subsequent protraction phase. Our data support the possibility that pre-synaptic inhibition has this function, since the effect of pre-synaptic inhibition on facilitation is equivalent to that of a complete cessation of CBI-2 firing, whereas post-synaptic inhibition does not affect facilitation (Fig. 9).

Regulating peptide release. CBI-2 contains a number of peptide co-transmitters that can initiate or modulate buccal motor programs (Morgan et al., 2000). The release of peptide co-transmitters, as well as their effects, is strongly affected by variations in firing pattern and frequency (Vilim et al., 2000). The presynaptic inhibition of CBI-2 may contribute to achieving a functionally appropriate release of peptide co-transmitters.

Developmental constraints. Biological features can be explained by ontogenic or phylogenic constraints of a system (Gould, 2002). Pre-synaptic inhibition of CBI-2 by B64 could also be explained in this way. B64 post-synaptically inhibits protraction phase neurons in the buccal ganglia, while exciting retraction phase neurons (Hurwitz and Susswein, 1997). During development, appropriate synapses must be made between B64 and the protraction and retraction phase neurons. Since CBI-2 fires during protraction, its neurites within the buccal ganglia may share molecular markers with protraction phase neurons. Such markers may lead to the development of inhibitory connections from B64.
Comparison to other systems

In most systems, presynaptic inhibition reduces the amplitude of synaptic potentials, rather than blocking them completely. By contrast, B64 blocks the synaptic output. Presynaptic block of a synapse is an effective mechanism for preventing a synapse from functioning at an inappropriate time, or to an inappropriate stimulus. In the crayfish lateral giant initiated tail flip, presynaptic inhibition blocks the sensory inputs that initiate the response, so that a tail flip will not itself initiate a second tail flip, leading to habituation of the response (Krasne and Bryan, 1973) Presynaptic inhibition also phasically blocks the output of crab neuron MCN1 in the stomatogastric ganglion, but not in the commissural ganglion, thereby creating phasic firing in one ganglion, and tonic firing in the other. In addition, MCN1 activity initiates both the gastric mill and pyloric rhythms. However, MCN1 is presynaptically inhibited during one phase of the gastric mill rhythm, thereby decreasing the recruitment of the pyloric rhythm. Thus, presynaptic inhibition participates in the coordination of two rhythmic behaviors (Bartos and Nusbaum, 1997)

Cerebral to buccal interneurons similar to the CBIs in *Aplysia* have been described in a number of different gastropods (Elliott and Susswein, 2002). Similar to the CBIs, these neurons also receive tonic input, and act on a buccal ganglion CPG that generates a phasic response. Both tonically and phasically firing neurons have been identified in *Pleurobranchaea* (Gillette et al., 1982; Kovac et al., 1982, 1983a,b, 1986), as well as in *Limax* (Delaney and Gelperin, 1990a,b). In *Lymnaea*, a single phasically firing CBI-like neuron has been identified (McCrohan and Kyriakides, 1989). The possibility that presynaptic inhibition contributes to the conversion of a tonic input to a phasic output in these systems has not been examined.
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FIGURE LEGEND

**Fig. 1.** **CBI-2 drives a central pattern generator organizing repetitive protraction-retraction movements located in the buccal ganglia.** (A) A map of the rostral (right) and caudal (left) surfaces of the buccal ganglia indicating the positions of the neurons discussed in this paper. (B) A schematic diagram showing the relationships between command-like neuron CBI-2 and the protraction and retraction phase neurons in the buccal ganglia. CBI-2 drives the buccal ganglia CPG by exciting protraction-phase neurons. The protraction phase neurons in turn drive retraction phase neurons after a delay, via a polysynaptic pathway whose elements are incompletely characterized. Protraction and retraction phase neurons are mutually inhibitory. Retraction phase neurons also inhibit CBI-2. In this and in part C, triangles represent excitation and circles represent inhibition. The size of the triangles and circles are roughly proportional to the amplitude of the synaptic connections. A resistor represents an electrical synapse, and a dashed line represents a polysynaptic connection. (C) A diagram showing the synaptic relationships among the neurons discussed in this paper. CBI-2 monosynaptically excites protraction phase CPG elements B63, B34 and B31/B32. The protraction phase neurons are mutually excitatory. B64 is a major interneuron driving retraction. It strongly inhibits all of the protraction phase interneurons, and is itself weakly inhibited by them. (D) Firing patterns during a buccal motor program of the various neurons discussed in the paper. A darker area represents an increased firing. Asterisks mark motor neurons.

**Fig. 2.** **Tonic and phasic firing of CBI-2 generate similar cycles of biphasic motor program.** The protraction phase was monitored by an intracellular recording from neuron B63, and by an extracellular recording from the I2 nerve (I2N). The retraction phase was monitored by intracellular recordings from neurons B4 and B8, and by an
extracellular recording from the radula nerve (RN). The activity that outlasts the protraction phase denotes retraction. (A1) Intracellular stimulation of CBI-2 with brief 18 nA current pulses drives a repetitive motor program. During the retraction phase of the motor program CBI-2 received inhibitory input that blocked the initiation of action potentials. (B1) Intracellular stimulation of CBI-2 with larger (25 nA) current pulses drives a similar BMP, though the neuron was spiking continuously. The lack of firing during the retraction phase (A2), in contrast to the continuous firing (B2) is clearly seen in the fast sweep recordings of CBI-2 during the protraction and retraction phases of the motor program. The facilitated EPSPs develop to an amplitude of about 10 mV (see arrow in A1), and no EPSPs were detected during the retraction phase (see arrow in B1).

Fig. 3. CBI-2 elicited EPSPs are not detected during retraction phase of CBI-2 generated BMP. (A) In protraction phase neurons B34, B63 and B31 firing of CBI-2 at a fixed rate for 10 seconds elicited facilitating fast EPSPs that generate a biphasic single cycle of BMP. By contrast, shorter trains of CBI-2 firing during the retraction phase do not cause detectable EPSPs. (B) A fast sweep of the first part of the train marked as B in part A demonstrates a train of facilitated EPSPs that increase their amplitude from ~1 mV to more than 5 mV in B34 and B63. (C) In contrast, a train of similar duration and rate failed to elicit detectable EPSPs during retraction phase. The amplitude of EPSPs progressively increased during the repeated trains.

Fig. 4. The postsynaptic change in resistance during retraction does not block depolarization in response to a current pulse. Injection of two depolarizing current pulses into (A) B34 and (B) B63 during and following the retraction phase demonstrates changes in resistance. During retraction, B31 shows the same
hyperpolarization seen in B34 and B63. The input resistance of B34 and B63 both were decreased by approximately 50% during the retraction phase.

Fig. 5. Change in membrane resistance during the retraction phase in B31/B32. (A) Difference currents at the transition from protraction to retraction during spontaneous buccal motor programs recorded at different voltages in a single B31/B32 neuron. In this experiment, currents underlying spontaneous BMPs were recorded in voltage clamp conditions at different voltages. The currents recorded were subtracted from those seen in the absence of a BMP at the same voltage, yielding the difference currents: that is, currents that are attributed only to the BMP. The vertical dashed line shows the point of transition from protraction (which causes inward currents) to retraction (which causes outward currents). The horizontal dotted line marks zero current. (B) A linear best fit line was drawn for all values of the peak outward current recorded at different voltages (22 current measurements from 8 B31/B32 neurons). The graph shows that there is a 1.01 MΩ change in resistance during the retraction phase.

Fig. 6. B64 firing is temporally related to block of the CBI-2 elicited EPSPs. (A) Tonic firing of CBI-2 for 50 seconds generated two cycles of biphasic BMP. The protraction phase was monitored via extracellular recording from I2n and via intracellular recording from B61. The retraction phase was monitored via the Rn activity that outlast the I2n activity, and via intracellular recording from both B64 neurons. The termination of the protraction phase was correlated with firing in B64, as well as with the block of CBI-2 elicited EPSPs in the protraction phase neurons. (B) Prematurely firing a single B64 neuron initiated a plateau depolarization in both B64 neurons, and also led to three cycles of BMP in the recorded period instead of two. During the retraction phases induced by premature firing of B64, there was a block of CBI-2
elicited EPSPs identical to that seen when B64 is recruited without depolarizing it. The arrow in A marks the termination of the retraction phase. The arrow in B marks CBI-2 elicited EPSPs that follow termination of the retraction phase.

Fig. 7. Combined, but not alternated firing of B64 and CBI-2 eliminates CBI-2 elicited EPSPs. CBI-2 was stimulated at a rate of 10 trains per minutes, each train with a 1-second duration at 16 Hz. This protocol leads to a progressive increase in the amplitude of the summated EPSPs over a minute or two, until reaching steady state values. The figures shown are after the EPSPs in B34 have reached steady-state values. The bars indicate the maximum amplitudes of the summated EPSP. B34 was hyperpolarized to ~-80 mV throughout the experiment via constant current injection. HiDi saline was replacing ASW. (A) The EPSP produced in B34 by stimulating CBI-2, in the absence of activity in B64. (B) When B64 was stimulated several seconds preceding CBI-2 stimulation, CBI-2 elicited EPSPs were unaffected. (C) By contrast, when B64 fired simultaneously with CBI-2, the EPSPs were blocked. The recording of CBI-2 is also indicative of the timings of CBI-2 activity in parts A and B.

Fig. 8. Effects of B64 activity are equivalent to those produced by rest in CBI-2. (A) CBI-2 was repetitively stimulated so those bursts of EPSPs in B34 were fully potentiated. The bars indicate the maximum amplitudes of the summated EPSP. (B) When B64 and CBI-2 were simultaneously activated during the third, fourth, fifth, and sixth bursts of stimulation, the EPSPs in B34 were eliminated completely. After the simultaneous stimulation of CBI-2 and B64, the first bursts of EPSPs recorded in B34 were decreased in size, indicating a loss of potentiation. (C) When CBI-2 stimulation was skipped for 4 trains, 3 consecutive bursts of spikes in CBI-2 were required until the amplitude of the facilitated EPSPs was restored to the amplitude before the interruption in CBI-2 stimulation. The rate at which EPSP amplitude was restored was
similar when B64 was stimulated with CBI-2 and when CBI-2 stimulation was interrupted. This experiment was performed in HiDi saline, and facilitation may differ from that seen in ASW (Hurwitz et al., 2003; Sanchez and Kirk, 2001). B34 was held at ~80 mV.

**Fig. 9. B54 causes IPSPs in B34 that decrease the amplitude, but do not block CBI-2 elicited EPSPs.** (A) Brief depolarization of B54 evoked spikes, which elicited a train of IPSPs that hyperpolarized B34 by several milivolts. (B) When CBI-2 spikes where triggered during firing of B54, they elicited facilitating EPSPs in B34. (C) Firing a train of spikes in CBI-2 in the absence of B54 spikes evoked facilitating EPSPs in B34 that were larger in amplitude by 50%. This experiment was performed in HiDi saline while the neurons were held at their membrane potential (~ -60 mV).

**Fig. 10. Coactivation of CBI-2 and B64, but not of CBI-2 and B54, blocks buildup of facilitation of CBI-2 elicited EPSPs.** (A) Three bursts of action potentials in CBI-2 elicited three trains of monosynaptic EPSPs. The trains showed inter-train and intra-train facilitation of both fast and slow EPSPs. (B) Coactivation of CBI-2 and B54 for two bursts was followed by single train in which CBI-2 firing was triggered alone. Co-activation of B54 and CBI-2 caused a decrease in EPSPs amplitude, but the third train, in which the EPSPs were elicited with B54 stimulation, was the same as that seen when B54 was not fired in any of the 3 bursts (compare the last burst in B to the last burst in A). (C) By contrast, when B64 and CBI-2 are coactivated during the first two trains, the last amplitude of the EPSPs elicited during the third train, when CBI-2 alone is stimulated, is similar to that seen during first train when CBI-2 alone is active (compare the last EPSP of the last train in C to the last EPSP of the first train in A). The ganglia were bathed in HiDi saline and the membrane potential of the neurons was left at rest (~55 mV).
Fig. 11. **B64 block CBI-2 excitation of B8, but does not cause postsynaptic inhibition of B8.** (A) CBI-2 causes a slow EPSP in B8. (B) B64 causes a conductance decrease in B8. The bar indicates the voltage change caused by a depolarizing pulse in the absence of B64 activity. (C1) During repeated trains of CBI-2 spikes, B64 was triggered to fire subsequent to CBI-2, in phase with the excitation and firing of B8. In response to B64 activity, the firing of B8 increased, *i.e.*, instead of 6 spikes 8 spikes were evoked. This reflected the decrease in conductance induced by B64, which amplified the effect of the CBI-2 induced slow EPSP. (C2) By contrast to the amplification of the CBI-2 induced EPSP when B64 fired after CBI-2, simultaneous firing of B64 and CBI-2 blocked the CBI-2 elicited EPSPs.
FIGURE # 03

A

B

C

I2N
RN
B31
B34
B63
CBI-2

15 mV
5 sec

1 sec 20

1 sec 20

1 sec 20
FIGURE # 04

A
B34L
B31R
CBI-2 R

B
B63L
B31R
CBI-2 R

20 mV
2 sec
FIGURE # 05

A                                     B

Protraction         Retraction

Volta
ge
Current (nA)
FIGURE # 11

A
B8

CBI-2

B
B8
B61
B64

C1
B8
B64
CBI-2

C2

10 mV
1 sec
20 mV

10 mV
1 sec
20 mV

10 mV
2 sec
50 mV