Frequency-Dependent Regulation of Afferent Transmission in the Feeding Circuitry of Aplysia

Colin G. Evans, Jian Jing, Alex Proekt, Steven C. Rosen, and Elizabeth C. Cropper

Evans, Colin G., Jian Jing, Alex Proekt, Steven C. Rosen, and Elizabeth C. Cropper. Frequency-dependent regulation of afferent transmission in the feeding circuitry of Aplysia. J Neurophysiol 90: 3967–3977, 2003. First published September 24, 2003; 10.1152/jn.00786.2003. During rhythmic behaviors, sensory-motor transmission is often regulated so that there are phasic changes in afferent input to follower neurons. We study this type of regulation in the feeding circuit of Aplysia. We characterize effects of the B4/5 interneurons on transmission from the mechanoefferent B21 to the radula closer motor neuron B8. In quiescent preparations, B4/5-induced postsynaptic potentials (PSPs) can block spike propagation in the lateral process of B21 and inhibit afferent transmission. B4/5 are, however, active during the retraction phase of motor programs, i.e., when mechanoefferent transmission to B8 presumably occurs. To determine whether mechanoefferent transmission is necessarily inhibited when B4/5 are active, we characterize the B4/5 firing frequency during retraction and show that, for the most part, it is low (below 15 Hz). There is, therefore, a low probability that spike propagation will be inhibited. The relative ineffectiveness of low frequency activity is not simply a consequence of insufficient PSP magnitude, because a single PSP can block spike propagation. Instead, it is related to the fact that PSPs have a short duration. When B4/5 fire at a low frequency, there is therefore a low probability that afferent transmission in the lateral process of B21 can be inhibited. In conclusion, we demonstrate that afferent transmission will not always be affected when a neuron that exerts inhibitory effects is active. Although a cell may be ineffective when it fires at a low frequency, ineffectiveness is not necessarily a consequence of spike frequency per se. Instead it may be due to spike timing.

INTRODUCTION

Motor behaviors are often mediated by central pattern generators (CPGs) that can generate rhythmic output without afferent input (e.g., Delcomyn 1980; Marder 2001). Physiologically, however, CPGs often receive sensory information so that activity is adjusted to compensate for changes in the periphery (Marder 2001; McCrea 2001; Pearson 1993; Rossignol et al. 1988; Suster and Bate 2002). When this occurs, changes in motor output are not always solely determined by stimulus properties. Instead, peripherally generated and centrally generated activity can be integrated so that stimulus-induced changes in motor output depend on the state of the ongoing motor program (e.g., McCrea 2001; Pearson and Ramirez 1997). Thus afferent transmission can be regulated during rhythmic activity. For example, centrally induced depolarizations can reduce or eliminate afferent transmission (e.g., Cattaert et al. 2001; Clarac and Cattaert 1996; Rudomin 1999).

In this report, we study afferent transmission from a buccal sensory neuron (the radula mechanoafferent, B21; Rosen et al. 2000b) to a follower, the radula closer motor neuron B8 (Fig. 1) (Gardner 1971). We have shown that afferent transmission in this pathway is somewhat unusual in that it does not occur when B21 is at its resting membrane potential (Evans et al. 2003). Spikes fail to actively propagate to the lateral process of B21, which is the primary point of contact with B8 (Evans et al. 2003). When B21 is centrally depolarized via current injection, the propagation failure is eliminated and afferent transmission occurs (Evans et al. 2003). During feeding motor programs, B21 is phasically depolarized via its electrical connections with the retraction circuitry (Rosen et al. 2000a,b). Motor program–induced changes in membrane potential are sufficient to affect spike propagation (Evans et al. 2003). In previous work, we have therefore shown that B21 mechanoafferent input to B8 is gated-in during the retraction phase of ingestive-like motor programs.

In this study, we show that B21 mechanoafferent transmission to B8 can also be regulated by synaptic input from identified neurons (the B4/5 cells; Fig. 1). The B4/5 cells are interneurons that make inhibitory synaptic connections with a number of cells in the feeding network and are thought to be important for determining characteristics of feeding motor programs (e.g., whether programs are egestive-like or ingestive-like) (Jing and Weiss 2001). We show that the B4/5 cells produce hyperpolarizing postsynaptic potentials (PSPs) in B21 that have an inhibitory effect on spike propagation. Specifically, the B4/5 neurons inhibit spike propagation to the region of B21 (the lateral process) that contacts B8. During rhythmic activity, B4/5 are active when B21 afferent input to B8 is gated-in, i.e., during the radula retraction phase of motor programs (e.g., Jing and Weiss 2001; Rosen et al. 1991). This could suggest that two opposing processes are operative during radula retraction; centrally induced depolarizations, which would tend to gate-in mechanoefferent input to B8, and synaptic input from the B4/5 neurons, which would tend to inhibit afferent transmission. A goal of this study was to determine how these two processes interact during a physiologically characterized motor program. In particular, we concentrated on...
in B21 are characterized. Mechanoafferent transmission to B8, i.e., effects of B4/5 on spike propagation

3968 EVANS ET AL.

To record from the somata of neurons, we used single barrel electrodes fabricated from thin walled capillary tubing and filled with 3 M potassium acetate and 30 mM potassium chloride. Electrodes were beveled so that their impedances were generally just below 10 MΩ. To record from the lateral process of B21, microelectrodes were high resistance (generally about 50 MΩ) and contained a drop of 5(6)-carboxyfluorescein dye in 0.1 M potassium citrate with 10 mM probenecid (to verify recording sites). The viability of neurons throughout an experiment was primarily determined by monitoring the resting membrane potential. In all experiments where electrical coupling was measured, presynaptic cells were penetrated with two electrodes so that one electrode could be used for passing current, and the other electrode could be used for recording the corresponding change in membrane potential.

Recordings from the lateral process of B21

Recordings from the lateral process were obtained as described (Evans et al. 2003). Briefly, we injected Fast Green dye into the B21 soma for about 5–10 min to inject the smallest amount of dye that would permit visualization of the lateral process. After approximately 15–30 min, the lateral and medial processes could be visualized. To facilitate penetration of the lateral process, we often removed some of the overlying connective tissue and small cells, using a glass micropipette. Physiological experiments were initiated by placing a microelectrode in the soma of B21. We then attempted to penetrate the lateral processes. We assumed that we were successful if we saw a simultaneous disturbance in the soma recording. Additionally, we attempted to gate-in responses to peripheral stimulation (Evans et al. 2003). At the conclusion of experiments, we verified recording sites by injecting carboxyfluorescein dye. Dye-filled cells were viewed with a Nikon Labphot2 microscope equipped with a filter set to visualize fluorescein (B-2A; EX 450–490/DM 505/BA 520).

Identification of neurons B4/5

Gardner (1971) described two neurons with similar properties that have been given the designations B4 and B5. Both cells are bilaterally symmetrical; thus there are two B4 neurons and two B5 neurons. Studies that have described characteristics of B4 and B5 have failed to notice any differences between the two cells (e.g., Gardner 1971, 1977; Hurwitz and Susswein 1996; Jing and Weiss 2001; Plummer and Kirk 1990; Rosen et al. 2000b). In this study, we confirmed that B4 and B5 are both electrically coupled to B21 (Fig. 2, A1–A3) and that both cells make synaptic connections with B21 (Fig. 2B). Consequently, B4 and B5 were used interchangeably throughout this study. In most cases we use the designation B4/5 to indicate that we did not specifically distinguish between the two cells.

Buccal motor programs

Motor programs were induced by stimulation of cerebral buccal interneuron-2 (CBI-2) (e.g., Rosen et al. 1991). When rhythmic activity is referred to as ingestive-like, cycles of motor programs were classified using cluster analysis (Morgan et al. 2002). Specifically, the average firing frequency of the radula closer motor neuron B8 was determined during both the protraction and retraction phases of motor programs. The protraction phase was monitored via the I2 nerve, which contains the axons of the protraction motor neurons B61 and B62 (Hurwitz et al. 1996). The retraction phase of the motor program was monitored via buccal nerve 2 (Morton and Chiel 1993). If B8 fired at ≥6 Hz during retraction and ≤3.5 Hz during protraction, the cycle of the motor program was classified as ingestive-like. We estimated the instantaneous B4/5 firing frequency by dividing the retraction phase of ingestive cycles of activity into 100-ms bins. The average firing frequency over each 100-ms period of time was computed, and data were pooled from 37 cycles of activity recorded in 11 preparations. Plotted are means ± SE.

Experiments studying afferent transmission during the retraction phase of motor programs were conducted in preparations that consisted of the buccal and cerebral ganglia and the tissue innervated by B21, the SRT. Programs were triggered via CBI-2, and B21 was peripherally activated when a probe contacted the SRT (see Periph-

![Figure 1](image1.png)

**FIG. 1.** B21 is a bipolar neuron with major medial and lateral processes. The medial process innervates the periphery, i.e., the subradula tissue (SRT). In this study, B21 was peripherally activated when a probe contacted the SRT. B21’s lateral process is the primary point of contact with the follower neuron B8. B4/5 neurons make monosynaptic chemical connections with both B21 and B8. Additionally, B4/5 are weakly electrically coupled to B21 (not shown). These experiments study “presynaptic” effects of the B4/5 neurons on B21 mechanoafferent transmission to B8, i.e., effects of B4/5 on spike propagation in B21 are characterized.

Ingestive-like activity since it has been hypothesized that afferent transmission does occur during this type of motor program (Evans et al. 2003). We sought to determine whether afferent transmission from B21 to B8 could occur despite activity in the B4/5 neurons.

**METHODS**

**Animals**

Experiments were conducted in 200–300 g *Aplysia californica* (Marinus) that had been maintained in 14–16°C holding tanks. Animals were anesthetized by injection of isotonic MgCl₂ and dissected to create the reduced preparations described below. The nomenclature used in this study follows that of Gardner (1971).

**Preparations**

Most experiments were conducted in preparations that consisted of the buccal ganglion and the isolated subradula tissue (SRT), i.e., the buccal mass was dissected so that the SRT could be removed from the radula, which it underlies. The sensory innervation of the SRT passes through the radula nerve; consequently this nerve was left intact. All other buccal nerves were severed. In motor program experiments, preparations additionally included the cerebral ganglion and the isolated subradula tissue (SRT). In general, experiments were conducted at approximately 16°C.

**Electrophysiology**

Standard-current clamp intracellular techniques were used to obtain up to four recordings simultaneously. Equipment used included the following; Getting Model 5A amplifiers modified for 100-nA current injection (Getting Instruments, Iowa City, IA), Tektronix AM 502 amplifiers (Tektronix, Wilsonville, OR), a Tektronix storage oscilloscope (model 5111), and an Astro-Med Chart Recorder (model 9500, Grass Instruments, Quincy, MA). Some data were digitized (Digidata, Axon Instruments, Foster City, CA) and were acquired and analyzed using Axograph software (Axon Instruments) and a Macintosh G3 or G4 computer. Spike amplitude was determined by measuring the difference between the peak level of depolarization and the preceding baseline potential, which was not always resting membrane potential. To record from the somata of neurons, we used single barrel electrodes fabricated from thin walled capillary tubing and filled with 3 M potassium acetate and 30 mM potassium chloride. Electrodes were beveled so that their impedances were generally just below 10 MΩ. To record from the lateral process of B21, microelectrodes were high resistance (generally about 50 MΩ) and contained a drop of 5(6)-carboxyfluorescein dye in 0.1 M potassium citrate with 10 mM probenecid (to verify recording sites). The viability of neurons throughout an experiment was primarily determined by monitoring the resting membrane potential. In all experiments where electrical coupling was measured, presynaptic cells were penetrated with two electrodes so that one electrode could be used for passing current, and the other electrode could be used for recording the corresponding change in membrane potential.

Recordings from the lateral process of B21

Recordings from the lateral process were obtained as described (Evans et al. 2003). Briefly, we injected Fast Green dye into the B21 soma for about 5–10 min to inject the smallest amount of dye that would permit visualization of the lateral process. After approximately 15–30 min, the lateral and medial processes could be visualized. To facilitate penetration of the lateral process, we often removed some of the overlying connective tissue and small cells, using a glass micropipette. Physiological experiments were initiated by placing a microelectrode in the soma of B21. We then attempted to penetrate the lateral processes. We assumed that we were successful if we saw a simultaneous disturbance in the soma recording. Additionally, we attempted to gate-in responses to peripheral stimulation (Evans et al. 2003). At the conclusion of experiments, we verified recording sites by injecting carboxyfluorescein dye. Dye-filled cells were viewed with a Nikon Labphot2 microscope equipped with a filter set to visualize fluorescein (B-2A; EX 450–490/DM 505/BA 520).

Identification of neurons B4/5

Gardner (1971) described two neurons with similar properties that have been given the designations B4 and B5. Both cells are bilaterally symmetrical; thus there are two B4 neurons and two B5 neurons. Studies that have described characteristics of B4 and B5 have failed to notice any differences between the two cells (e.g., Gardner 1971, 1977; Hurwitz and Susswein 1996; Jing and Weiss 2001; Plummer and Kirk 1990; Rosen et al. 2000b). In this study, we confirmed that B4 and B5 are both electrically coupled to B21 (Fig. 2, A1–A3) and that both cells make synaptic connections with B21 (Fig. 2B). Consequently, B4 and B5 were used interchangeably throughout this study. In most cases we use the designation B4/5 to indicate that we did not specifically distinguish between the two cells.

Buccal motor programs

Motor programs were induced by stimulation of cerebral buccal interneuron-2 (CBI-2) (e.g., Rosen et al. 1991). When rhythmic activity is referred to as ingestive-like, cycles of motor programs were classified using cluster analysis (Morgan et al. 2002). Specifically, the average firing frequency of the radula closer motor neuron B8 was determined during both the protraction and retraction phases of motor programs. The protraction phase was monitored via the I2 nerve, which contains the axons of the protraction motor neurons B61 and B62 (Hurwitz et al. 1996). The retraction phase of the motor program was monitored via buccal nerve 2 (Morton and Chiel 1993). If B8 fired at ≥6 Hz during retraction and ≤3.5 Hz during protraction, the cycle of the motor program was classified as ingestive-like. We estimated the instantaneous B4/5 firing frequency by dividing the retraction phase of ingestive cycles of activity into 100-ms bins. The average firing frequency over each 100-ms period of time was computed, and data were pooled from 37 cycles of activity recorded in 11 preparations. Plotted are means ± SE.

Experiments studying afferent transmission during the retraction phase of motor programs were conducted in preparations that consisted of the buccal and cerebral ganglia and the tissue innervated by B21, the SRT. Programs were triggered via CBI-2, and B21 was peripherally activated when a probe contacted the SRT (see Periph-
erated by B4 were 1.6 when B21 was at its resting membrane potential, IPSPs generated by B4 and B5. The coupling between B21 and both B4 and B5 (Fig. 2A). When hyperpolarizing current is injected into B21, the coupling ratio is 0.045 ± 0.002 (n = 6). When current is injected into B4 or B5, the coupling ratio is 0.054 ± 0.004 (n = 7).

Peripheral stimulation of the SRT

The SRT was periaperally stimulated as has been described (Crompton et al. 1996). Briefly, mechanical stimuli were delivered by means of a mini-speaker (Quam) that had a wooden stick (tip diameter 1 mm) that was perpendicularly attached to the speaker membrane. Reproducible movements of the membrane were regularly elicited by driving the speaker with a stimulator (Grass Instruments, S48).

RESULTS

Connection between B21 and B4/5

The B4/5 neurons make a monosynaptic inhibitory chemical connection with B21 (Rosen et al. 2000). In most cases, B4/5-induced potentials are hyperpolarizing (although see following text). There was no significant difference in the amplitude of the inhibitory PSPs (IPSPs) generated by B4 and B5. When B21 was at its resting membrane potential, IPSPs generated by B4 were 1.6 ± 0.4 mV. IPSPs generated by B5 were 1.7 ± 0.3 mV (Fig. 2B; n = 4). Additionally there is electrical coupling between B21 and both B4 and B5 (Fig. 2A). When hyperpolarizing current is injected into B21, the coupling ratio is 0.045 ± 0.002 (n = 6). When current is injected into B4 or B5, the coupling ratio is 0.054 ± 0.004 (n = 7).

B4/5 are cholinergic interneurons that produce chloride-mediated IPSPs in other buccal interneurons and motor neurons (e.g., Gardner 1971, 1977; Gardner and Kandel 1972, 1977). As might be expected, therefore, B4/5 stimulation produced a conductance increase in B21 (Fig. 2C; n = 5), and B4/5-induced PSPs changed sign when we increased the intracellular chloride concentration in B21 (Fig. 2D; n = 5).

B4/5 neurons inhibit spike propagation in B21

The primary region of contact between B21 and B8 is B21’s lateral process (Fig. 1) (Borovikov et al. 2000). Consequently, B21 afferent input to B8 is only gated-in if spikes are actively propagated to the lateral process (Evans et al. 2003). To determine whether stimulation of B4/5 could affect spike propagation in B21, we peripherally activated B21 (Fig. 3A) and centrally depolarized it to the point where full size spikes were recorded in the lateral process every time a peripheral stimulus was applied, i.e., afferent activity was gated-in (Fig. 3A). When B4/5 was stimulated, pulse amplitude was decreased (e.g., see Fig. 3C vs. 2 in insert). D: when chloride is iontophoresed into B21, the B4/5-induced IPSP is reversed (middle and right). The experiment shown in D was conducted in a solution with a higher than normal concentration of divalent cations (i.e., 3 × Mg2+ and 3 × Ca2+).
potential amplitude decreased from 52.7 ± 2.5 to 13.7 ± 1.4 mV; paired t-test, P < 0.0001; Fig. 3B). Thus B4/5 stimulation can cause spike propagation to fail when B21 is peripherally activated.

In seven of nine preparations, B4/5 stimulation induced hyperpolarizing potentials in B21. In two of nine preparations, however, B21 was relatively hyperpolarized (i.e., was below the reversal potential for the B4/5-induced PSP, which was approximately −74 mV). In these cases, B4/5-evoked potentials were depolarizing. In these preparations, B4/5 stimulation still had an inhibitory effect on peripherally triggered activity (Fig. 3C).

Are effects of B4/5 on spike propagation frequency-dependent?

To determine whether effects of B4/5 on spike propagation in B21 are frequency-dependent, we stimulated B4/5 at different frequencies and monitored spike amplitude in the lateral process. Results of these experiments differed and depended on whether the B4/5 firing frequency was above or below approximately 15 Hz.

Below 15 Hz. We found that when the B4/5 firing frequency was below 15 Hz, effects on spike propagation were variable, i.e., in some cases low-frequency activity in B4/5 inhibited spike propagation in B21, in other cases it did not (left of the dashed line in Fig. 3B; note that there are 2 points plotted for many frequencies). This was apparent even when trials within the same preparation were compared. When we compared some of the trials where B4/5 stimulation was effective, peripherally triggered spikes in B21 occurred relatively early during a B4/5-induced IPSP (i.e., a and b). In contrast, when B4/5 stimulation was not effective, peripheral spikes in B21 were triggered late during B4/5-induced IPSPs, or after the IPSP had ended (i.e., c and d).
between the B4/5 spike and the peripheral activation of B21 spike is relatively short. If spike timing is not controlled (as it was not in these experiments), effects of B4/5 stimulation will be variable since in some cases B21 will be peripherally activated at a time when inhibition can occur and in other cases it will not.

To further characterize temporal requirements of B4/5-B21 interactions, we performed additional experiments in which we controlled the delay between spiking in B4/5 and peripheral activation of B21. With this paradigm we were able to confirm that a single B4/5-induced IPSP can affect afferent transmission if the delay between the B4/5 spike and the peripherally activated B21 spike is less than approximately 50 ms (Fig. 4, A, B, and D; n = 4). The mean spike amplitude in the lateral process was 47.5 ± 3.1 mV when B4/5 was not stimulated. When B4/5 stimulation was effective, potentials in the lateral process were decreased in amplitude to a mean of 17.3 ± 2.1 mV (paired t-test P = 0.0007).

In the experiments described above, B21 was always centrally depolarized so that spikes would be actively propagated to the lateral process prior to activation of B4/5. The depolarization in B21 was just sufficient to reliably permit active spike propagation. We sought to determine whether temporal requirements of B4/5 effects on B21 spike propagation are membrane potential–dependent. We found that a B4/5-B21 synaptic delay interval that affected spike propagation at one B21 membrane potential could become ineffective if the depolarization in B21 was increased (e.g., Fig. 4D). With more extreme depolarization, we found that a single spike in B4/5 could become ineffective, even with a minimal delay (Fig. 4C; n = 4). Our data indicate therefore that when B4/5 fire at low frequencies, spike propagation in B21 will not be affected by

**FIG. 4.** Effect of a single B4/5 action potential on spike propagation in B21. **A:** a single spike in B4/5 can inhibit spike propagation. **Left:** B21 was peripherally activated so that a single spike was triggered every time a probe contacted the SRT and was depolarized so that the spike in the lateral process was full size. **Right:** when B4/5 was stimulated (bar under bottom trace) and the same peripheral stimulus applied, the lateral process recording was attenuated. **B:** group data showing temporal effects of B4/5 on spike propagation in B21. Data from 4 preparations are each plotted with a different symbol. Note that a log scale is used on the x axis. B4/5 and B21 were activated as shown in A, and the interval between the B4/5 spike and the spike in the lateral process of B21 was progressively increased. Note that as the interval increased, B4/5 no longer affected spike propagation. **C:** group data showing the effect of membrane potential on gating. Data from 4 preparations are each plotted with a different symbol. B4/5 and B21 were activated as shown in A. The interval between the B4/5 spike and the peripheral spike in B21 was kept constant at a minimal value (i.e., the shortest interval that would not cause the peripheral spike to be triggered before initiation of the IPSP). Depolarization in B21 was, however, increased via current injection into the soma. Note that when B21 was sufficiently depolarized, a single B4/5 spike no longer inhibited afferent transmission. **D:** data from a single preparation showing that effects of B4/5 on spike propagation in B21 depend on both membrane potential and relative timing. Experiment as in A; spikes in B4/5 are not shown. Top numbers indicate the delay between the spike in B4/5 and the spike in B21’s lateral process. Initially B21 was 15 mV above resting membrane potential and with a 30-ms interval between the B4/5 and B21 spikes, action potential propagation in B21 was affected, i.e., the spike in the lateral process was attenuated. When the B4/5-B21 delay was kept constant (at 30 ms) and B21 was further depolarized (to 16 and then 20 mV above resting potential) B4/5 was no longer effective. When the B4/5-B21 delay was decreased to 15 ms, however, B4/5 again became effective.
B4/5 activity if B21 is highly depolarized. At least depolarized membrane potentials, B4/5 can inhibit spike propagation, but only if a peripheral spike in B21 is triggered relatively soon after a spike in B4/5.

ABOVE 15 HZ. When B4/5 was stimulated at frequencies that were $>15$ Hz, results were not variable, i.e., we consistently found that efferent transmission was inhibited (Fig. 3B, right). In general, this result is not surprising given the fact that effects of B4/5 do persist for some time (i.e., about 50 ms). Results such as those shown in Fig. 4B would, however, seem to suggest that B4/5 effects on spike propagation in B21 would only reliably be seen when a B4/5 neuron fires at about 20 Hz, i.e., at 20 Hz B4/5 would spike about every 50 ms. Since reliable inhibition is seen at a lower frequency, we sought to determine whether temporal summation could have affected results when B4/5 was stimulated with DC current injection. The mean amplitude of B4/5-induced IPSPs in B21 was $5.0 \pm 1.1$ mV in these experiments. (They are larger than IPSPs at rest because B21 was depolarized so that spikes would be actively propagated to the lateral process before B4/5 was stimulated.) IPSPs of this magnitude do summate, even at frequencies below 15 Hz (e.g., summation at 10 Hz is shown in Fig. 5A). This suggests that a delay between a B4/5 spike and a B21 spike that was ineffective early within a burst of B4/5 activity could become effective later. Recordings from experiments with the paradigm shown in Fig. 3A demonstrate this (Fig. 5B). In this comparison, mean spike amplitude in the lateral process was $50.2 \pm 14.4$ mV early within the burst of activity (when the delay examined was ineffective) and was decreased to $14.4 \pm 1.9$ mV later in the burst ($n = 4$, paired $t$-test, $P = 0.0015$).

Taken together, these results indicate that when the B4/5 neurons fire at relatively low frequencies, effects will be variable, i.e., they will depend on specific temporal relationships between spiking in B4/5 and peripheral activation of B21. In contrast, at high frequencies, there is a high probability that spike propagation in B21 will be affected. Specific B4/5 firing frequencies that will produce reliable gating will in part be determined by the amount of temporal summation that occurs, i.e., when during a burst of B4/5 activity B21 is peripherally activated.

Do the B4/5 neurons inhibit afferent transmission to the postsynaptic follower B8?

To confirm that effects of B4/5 on afferent transmission from B21 to B8 are at least qualitatively similar to B4/5 effects on spike propagation in B21, we performed experiments in which we peripherally activated B21, recorded from the soma of B21 to insure that peripheral stimuli were effective, and recorded PSPs from B8. B21 was centrally depolarized before peripheral stimulation so that afferent input would be gated-in before B4/5 was stimulated. DC stimulation of B4/5 reduced the amplitude of B21-induced PSPs in B8 (Fig. 6A). The mean PSP amplitude before B4/5 stimulation was $3.8 \pm 0.3$ mV. The mean amplitude after stimulation was $0.9 \pm 0.5$ mV (paired $t$-test; $P = 0.004$). Thus effects of B4/5 stimulation on B21-induced PSPs in B8 are inhibitory as are B4/5 effects on spike propagation in B21.

In relating spike propagation in B21 to PSP amplitude in B8, the most straightforward relationship appears to be that PSPs are virtually nonexistent in B8 when spike propagation fails (Evans et al. 2003). Since a single spike in B4/5 can inhibit spike propagation in B21, we hypothesized that a single B4/5 spike could also eliminate, or virtually eliminate, B21-induced PSPs in B8. In all preparations tested, we found that this was the case (Fig. 6B; $n = 4$). As with effects on spike propagation, when the interval between the B4/5 spike and the peripherally triggered spike in B21 was increased, B4/5 less effectively decreased spike amplitude (Fig. 6B). Also similar to effects on spike propagation, effects of B4/5 on efferent transmission were membrane potential-dependent, i.e., B4/5 was most effective when B21 was less depolarized (Fig. 6C). Thus effects of B4/5 stimulation on B21-induced PSPs in B8 are like effects of B4/5 on spike propagation in that temporal charac-

---

**FIG. 5.** A: temporal summation of B4/5-induced IPSPs in B21. The black line is a typical B4/5-induced IPSP in B21 when B21 was depolarized to insure active spike propagation to the lateral process. Membrane potential is indicated with respect to the membrane potential prior to the initiation of the IPSP (dark gray line). Dashed lines: when this IPSP is repeated every 100 ms (i.e., at a frequency of 10 Hz) successive IPSPs are triggered at more hyperpolarized membrane potentials. B: B4/5 is more effective at inhibiting spike propagation when B21 is peripherally activated relatively late during a burst of activity. Experiment as shown in Fig. 3A. Inset: responses labeled a and b at a faster sweep speed. Note that B4/5 did not initially inhibit spike propagation when the delay between the spike in B4/5 and spike initiation in B21 was relatively long (i.e., 100 ms; response a). As B4/5 activity continued, B21 progressively hyperpolarized (the dotted lines indicate the membrane potential before B4/5 stimulation). When B21 was peripherally activated relatively late during the burst of B4/5 activity, B4/5 now effectively inhibited spike propagation, even with a 100-ms delay (response b).
teristics of B4/5-B21 interactions are important, as is the membrane potential of B21.

Role of B4/5 in inhibiting B21 afferent activity during motor programs

During motor programs, B4/5 are active when B21 afferent input to B8 is gated-in, i.e., during radula retraction (e.g., Jing and Weiss 2001; Rosen et al. 1991). This could suggest that two opposing processes are operative; centrally induced depolarizations, which would tend to gate-in mechanoafferent input to B8, and synaptic input from the B4/5 neurons, which would tend to inhibit afferent transmission. A goal of this study was to determine how these two processes interact during ingestive-like activity, which is a time when it has been hypothesized that mechanoafferent transmission to B8 does occur (Evans et al. 2003). We sought to determine whether afferent transmission from B21 to B8 could occur despite activity in the B4/5 neurons.

In theory, presynaptic inhibition of B4/5 could prevent it from affecting afferent transmission in B21 during the retraction phase of ingestive motor programs (e.g., Nusbaum 1994). To determine whether this is the case, we triggered motor programs in preparations in which the peripheral tissue innervated by B21 (the SRT tissue) was intact. Rhythmic activity was triggered via stimulation of CBI-2 (Rosen et al. 1991), and B21 was repeatedly activated when a probe contacted the SRT. PSPs were observed as B1 and the interval between the spike in B4/5 and the peripheral spike in B21 was progressively increased. Note that as the interval increased, B4/5 became ineffective. C: effects of B4/5 on B21-induced PSPs in B8 are membrane potential dependent. C1: B4/5 and B21 were activated so that B4/5 produced a 61% decrease in PSP amplitude (right). C2: central depolarization in B21 was increased by 5 mV, and B4/5 and B21 were activated as shown in C1. B21-induced PSPs in B8 are increased in amplitude as has been previously shown (Evans et al. 2003). Note, however, that B4/5 stimulation is less effective in that B4/5 only produced a 22% decrease in PSP amplitude (left).
with stimulation (paired t-test, \( P = 0.0002 \)). These data indicate that presynaptic inhibition does not occur at the B4/5-B21 synaptic junction during the retraction phase of CBI-2-elicited motor programs.

In the experiments conducted in otherwise quiescent preparations, we show there is a low probability that inhibition of afferent transmission will occur when the B4/5 neurons fire at relatively low frequencies. To determine how firing frequency is likely to affect gating during motor programs, we measured the firing frequencies of single B4/5 neurons during ingestive-like cycles of CBI-2 elicited motor programs (see METHODS for a description of the classification of rhythmic activity). We found that the B4/5 firing frequency reached a maximum value (of about 13 Hz) relatively early during retraction. Firing frequency decreased as retraction progressed (Fig. 8A). From these data, it would be predicted that the probability that B4/5 would inhibit afferent transmission in B21 would initially be relatively high but would decrease and presumably be relatively low for most of the duration of retraction. For example, for most of the retraction phase, the firing frequency of a single B4/5 neuron was well below 15 Hz, which was the frequency that reliably inhibited afferent activity in otherwise quiescent preparations (Fig. 3B).

Mechanism by which B4/5 inhibit spike propagation in B21

B4/5 are weakly electrically coupled to B21 and make a direct chemical connection. It is likely that most of the inhibition of spike propagation occurs as a result of the chemical input. This is suggested by the fact that the electrical connection between B4/5 and B21 will tend to produce depolarization in B21 when B4/5 are activated. Direct depolarization of B21 promotes rather than inhibits spike propagation (Evans et al. 2003). It should be noted, however, that conductance increases in B4/5 that occur during spiking could have an inhibitory effect that could tend to counteract the effect of transmitted depolarization. We currently have no data that specifically indicate whether this would be relevant.

The mechanism whereby chemical input from B4/5 inhibits spike propagation is likely to be functionally similar to mechanisms that have been described in other invertebrates (e.g., Cattaert et al. 2001). Thus the B4/5 neurons produce fast PSPs when the B4/5 neuron is depolarized every time a probe contacted the SRT. Left: single cycle of the motor program with no current injection in B4/5 or B21. Note that B21 was depolarized during the retraction (Retract) phase of the motor program, and full size spikes were recorded in the lateral process. Right: subsequent cycle of the motor program, with current injected into a B4/5 neuron (bar under bottom trace). When the B4/5 neuron was stimulated, attenuated potentials were recorded in the lateral process.

**Discussion**

In this report, we study effects of two retraction interneurons, the B4/5 cells, on the transmission of B21 mechanoefferent input to a follower, the radula closer motor neuron B8. Previous experiments had demonstrated that B21 is rhythmically depolarized via central input during the retraction phase of ingestive-like motor programs and that this depolarization tends to gate-in afferent input to B8. In this study, we show that mechanoefferent transmission can also be inhibited by synaptic input from the B4/5 cells. We characterize a mechanism for this inhibition and show that it occurs, in part, because B4/5 inhibit spike propagation in B21. Below we discuss 1) the presumed mechanism for the inhibition of spike propagation and 2) the probable functional significance of the regulation of mechanoefferent transmission during the retraction phase of ingestive motor programs.
FIG. 8. A: instantaneous B4/5 firing frequency during the retraction phase of an ingestive motor program. Motor programs were triggered via CB1-2 and cycles of activity that were classiﬁed as ingestive-like were used for analysis. To quantify B4/5 activity, the retraction phase of the motor program was divided into 100-ms bins, and the average B4/5 firing frequency during each 100-ms bin of activity was determined. We pooled and plotted data from 37 cycles of activity taken from 11 preparations. Error bars represent SE. The beginning of the retraction phase of the motor program is indicated by time 0 on the x axis (which is the bottom line in the ﬁgure). Retraction is also indicated by the bar directly above the x axis. Note that the B4/5 firing frequency peaks early during retraction and progressively decreases. B: spike propagation in B21 during the retraction phase of a motor program. B1: data from 7 preparations. B2: typical recording from a single preparation. Motor programs were triggered via CB1-2 (data not shown), which induces very little centrally generated activity in B21 (Evans et al. 2003). In the recording shown in B2, 3 centrally generated spikes were observed. The 1st is labeled as such (above the top trace in B2). Additionally, centrally triggered spikes are shown in gray in the top and middle traces of B2. Peripherally generated spikes are shown in black in the top and middle traces in B2 and were triggered when a probe contacted the SRT. We recorded intracellularly from both the lateral and soma of B21 during the retraction phase of a motor program. We recorded intracellularly from both the lateral and soma of B21 during the retraction phase of a motor program. The beginning of the retraction phase of the motor program is indicated by time 0 on the x axis (which is the bottom line in the figure). Retraction is also indicated by the bar directly above the x axis. Note that the B4/5 firing frequency peaks early during retraction and progressively decreases.

Therefore likely to be observed for a signiﬁcant portion of the time that B4/5 input inhibits afferent transmission (which is approximately 50 ms). We expect therefore that a conductance increase (i.e., shunting) is a factor when afferent transmission is inhibited (as it is in other invertebrate systems) (e.g., Cattaert et al. 2001).

Inhibition in our system is, however, not likely to be solely due to shunting. Our system differs from other systems where afferent transmission is regulated by chloride potentials in that the increase in chloride conductance in B21 generally results in hyperpolarization instead of the depolarization that is generally seen with primary afferent depolarization (PAD). We observe hyperpolarization when B21 is at its resting potential, because resting potential is about −64 mV (Evans et al. 2003). (The reversal potential for B4/5-induced IPSPs is approximately −74 mV; Gardner and Kandel 1972, 1977). When B4/5-induced gating occurs during motor programs, B21 will be even further from the chloride reversal potential. The B4/5 neurons are active during the radula retraction phase of motor programs (Church and Lloyd 1994; Jing and Weiss 2001; Rosen et al. 1991). During retraction, B21 is depolarized via central input (Evans et al. 2003; Rosen et al. 2000a). Therefore B21 will be hyperpolarized rather than depolarized by synaptic input from B4/5.

Because we see hyperpolarization, some phenomena that can affect afferent transmission with PAD will not be observed as a result of synaptic input from B4/5. Namely, sodium channels that are important for spike generation can be partially inactivated by depolarization, which can decrease action potential amplitude (Lamotte D’Incamps et al. 1998). Additionally, if PADs are of sufﬁcient magnitude, they can trigger action potentials that can negatively inﬂuence incoming afferent activity (e.g., Cattaert and Bevengut 2002). Neither of these phenomena is observed with B4/5 input. B4/5-induced hyperpolarizations are, however, likely to negatively impact afferent transmission in a way that PAD will not. Hyperpolarizing input moves B21 away from the threshold for spike initiation. This will increase the likelihood that there will be a spike propagation failure (Evans et al. 2003). To summarize, mechanisms by which B4/5 activity inhibits afferent transmission are likely to be similar to mechanisms operative during PAD in other invertebrates in that inhibition is likely to be at least partially mediated via shunting. The B4/5 mechanism may differ, however, in that hyperpolarization is likely to be important when afferent transmission is inhibited during ingestive motor programs.

Signiﬁcance of mechanoaﬀerent transmission to B8

A putative role for the transmission of mechanoeﬀerent input to B8 has been most clearly described when bites are converted to bite-swallows (e.g., Evans et al. 2003). When Aplysia make ingestive responses but are not able to grasp food, responses are referred to as bites (Kupfermann 1974). When food is grasped, a bite is converted to a bite-swallow, and radula closing and retraction are enhanced so that food will be deposited in the esophagus (Kupfermann 1974). Radula mechanoeﬀerents have receptive ﬁelds on the biting surface of the radula and make excitatory connections with the radula closing and retraction circuitry (Rosen et al. 2000b). It has therefore been hypothesized that radula mechanoeﬀerent activity plays a role in bite to bite-swallow conversions (Evans et al. 2003; Klein et al. 2000). Consistent with this idea, B21 is centrally depolarized during the retraction phase of ingestive-l
like motor programs, which will tend to gate-in afferent input to B8 (Evans et al. 2003; Rosen et al. 2000a). However, the B4/5 neurons, which make inhibitory synaptic connections with much of the closing/retraction circuitry, tend to inhibit afferent transmission during retraction (Church and Lloyd 1994; Jing and Weiss 2001). We therefore sought to determine whether B4/5 activity necessarily indicates that afferent transmission will be inhibited.

During CBI-2–induced motor programs, the firing frequency of the B4/5 neurons progressively decreases as retraction progresses, i.e., it is below 10 Hz for most of the retraction phase. A possibility that we explored in this study therefore was that effects of the B4/5 neurons might be frequency-dependent, and low-frequency activity might be relatively ineffective at regulating afferent transmission. To take this a step further, we also expected that frequency dependence might result from the fact that a single B4/5 PSP might be insufficient to inhibit afferent transmission. Temporal summation (i.e., relatively high-frequency B4/5 activity) might be necessary for inhibition to occur.

Our data support the hypothesis that the B4/5 neurons will be relatively ineffective when they fire at low frequencies. The mechanism for this frequency dependence is, however, not what was expected (i.e., it is not due to the fact that a single PSP does not inhibit afferent transmission). We found that a single B4/5-induced PSP in B21 can be as effective as summated PSPs. Thus low-frequency activity is not ineffective because temporal summation is necessary. Instead, the ineffectiveness of low-frequency activity is, in part, a consequence of the fact that there will be relatively few B4/5-induced PSPs in B21 per unit of time. Additionally, effects of the B4/5 neurons on spike propagation in B21 are relatively short-lived (i.e., PSPs have a short duration and afferent transmission is only inhibited during the PSP). When B4/5 fire at a low frequency, there are relatively few brief periods of time when spike propagation can be inhibited. It is not very likely that B21 will be peripherally activated during these periods of time if some mechanism does not specifically coordinate activity in B21 and B4/5.

When animals bite, it is very unlikely that activity in B4/5 and B21 will be coordinated. During the retraction phase of motor programs, spikes in B21 are likely to be primarily triggered peripherally via food contact to the radula (Rosen et al. 2000b). Spikes in B4/5 are likely to be triggered via central input (Church and Lloyd 1994; Jing and Weiss 2001) and directly or indirectly via input from the periphery (Fiore and Geppetti 1981; Fiore and Muenier 1979; Jahan-Parwar et al. 1983). As afferents, the B4/5 neurons are likely to act as proprioceptors rather than detectors of food contact. When animals bite, it is therefore very unlikely that spiking in B21 and B4/5 will be so tightly phase-locked that B21 will be peripherally activated within the period of time that spike propagation can be inhibited (i.e., within 50 ms of each B4/5 spike). Consequently, low-frequency activity in B4/5 is likely to be relatively ineffective and radula mecanoafferent transmission to B8 is likely to occur for most of the duration of retraction. A paradoxical finding of this study is that the B4/5 firing frequency early in retraction is high enough to significantly affect afferent transmission. In our motor program experiments in the SRT preparation, approximately 60% of the peripherally triggered spikes failed to propagate during the first 2 s of retraction. The functional significance of this early inhibition is not yet clear. It may, however, help ensure that radula mecanoafferent output to B8 does not produce premature closing of the radula, i.e., induce closing very early with respect to radula retraction (Rosen et al. 2000a).

**General relevance**

Afferent transmission is most commonly studied as a result of the application of an exogenous transmitter, a motor program, or a task-induced change in function. This study differs in that we studied effects of synaptic input from identified cells by making simultaneous intracellular recordings from neuronal somata and their processes. We were therefore able to manipulate neurons and analyze the mechanism for frequency dependence in our system. We show that it is determined by spike timing rather than firing frequency per se. That frequency dependence can be determined this way is likely to have specific consequences. For example, if frequency dependence is a consequence of the fact that a single PSP is insufficient to affect transmission, neural activity at sufficiently low frequencies will presumably always be ineffective, i.e., the probability that afferent transmission will be inhibited will be zero. In contrast, in our system, the probability that afferent transmission will be inhibited will never be zero as long as an inhibitory neuron is active. We show therefore that the type of analysis we perform can provide important insights into the physiological consequences of a specific pattern of neural activity.

We thank K. R. Weiss for valuable comments on an earlier version of this manuscript.

**DISCLOSURES**

This work was supported by National Institute of Mental Health K02 Award MH-01267 and by MH-51393 and MH-35564. Some of the Aplysia used in this study were provided by the National Resource for Aplysia of the University of Miami under Grant RR-10294 from the National Center for Research Resources.

**REFERENCES**


Hurwitz I and Susswein AJ. B64, a newly identified central pattern generator element producing a phase switch from protraction to retraction in buccal motor programs of Aplysia californica. J Neurophysiol 75: 1327–1344, 1996.


Morton DW and Chiel HJ. In vivo buccal nerve activity that distinguishes ingestion from rejection can be used to predict behavioral transitions in Aplysia. J Comp Physiol 172: 17–32, 1993.


