Conditional Rhythmicity and Synchrony in a Bilateral Pair of Bursting Motor Neurons in Aplysia

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Serrano, Geidy E. and Mark W. Miller. Conditional rhythmicity and synchrony in a bilateral pair of bursting motor neurons in Aplysia. J Neurophysiol 96: 2056–2071, 2006. First published May 31, 2006; doi:10.1152/jn.00282.2006. This investigation examined the activity of a bilateral pair of motor neurons (B67) in the feeding system of Aplysia californica. In isolated ganglia, B67 firing exhibited a highly stereotyped bursting pattern that could be attributed to an underlying TTX-resistant driver potential (DP). Under control conditions, this bursting in the two B67 neurons was infrequent, irregular, and asynchronous. However, both application of the neuromodulator dopamine (DA) increased the duration, frequency, rhythmicity, and synchrony of B67 bursts. In the absence of DA, depolarization of B67 with injected current produced rhythmic bursting. Such depolarization-induced rhythmic burst activity in one B67, however, did not entrain its contralateral counterpart. Moreover, when both B67s were depolarized to potentials that produced rhythmic bursting, their synchrony was significantly lower than that produced by DA. In TTX, dopamine increased the DP duration, enhanced the amplitude of slow signaling between the two B67s, and increased DP synchrony. A potential source of dopaminergic signaling to B67 was identified as B65, an influential interneuron with bilateral buccal projections. Firing B65 produced bursts in the ipsilateral and contralateral B67s. Under conditions that attenuated polysynaptic activity, firing B65 evoked rapid excitatory postsynaptic potentials in B67 that were blocked by sulpiride, an antagonist of synaptic DA receptors in this system. Finally, firing a single B65 was capable of producing a prolonged period of rhythmic synchronous bursting of the paired B67s. It is proposed that modulatory dopaminergic signaling originating from B65 during consummatory behaviors can promote rhythmicity and bilateral synchrony in the paired B67 motor neurons.

INTRODUCTION

Many biological systems possess extraordinary capabilities to achieve transitions from asynchronous to synchronized activity. Such transitions are frequently accompanied by, and may be contingent on, conversion from arrhythmic to rhythmic activity in the elemental components of the system (Strogatz 2003; Winfree 1980). In nervous systems, these capabilities are thought to reflect properties of populations of pulse-coupled nonlinear oscillators (Buzsáki and Draguhn 2004; Hopfield 1994; Singer 1999). To date, opportunities to examine transitions from asynchrony to synchrony in identified neurons with known functions have been limited.

Central pattern generator (CPG) circuits frequently produce alternating movements of bilateral limbs, appendages, or axial muscle systems (Cohen et al. 1988; Grillner et al. 2005; Stein et al. 1997). However, these motor networks can also generate unilateral movements and are often capable of producing synchronous activation of paired effectors (Grillner 1985; Kelso et al. 1979; Stein 2005). Considerable interest is presently focused on understanding how CPG circuits can specify such distinct classes of actions from an individual effector system (Getting 1989; Marder and Calabrese 1996; Stein et al. 1997).

One well-documented means to achieve motor system reconfiguration is via the implementation of neuromodulation (Harris-Warrick and Marder 1991; Kupfermann 1979). Acting as intercellular messengers, neuromodulators can orchestrate coherent modifications of a motor circuit by producing broad and coordinated actions on the synaptic connectivities and intrinsic membrane properties of its constituent neurons (Kiehn and Katz 1999; Marder and Weimann 1992; Parker and Grillner 1998). The actions of neuromodulators are best understood in instances where they initiate activity in CPGs or modify their frequency, phasing, or intensity. The role of modulators as specific regulators of rhythmicity and synchrony of CPG elements has received less inquiry.

Neuromodulation is prevalent in the motor circuits that control the consummatory feeding behaviors of Aplysia (Kupfermann et al. 1979, 1997). The actions of neuromodulators have been exceptionally well characterized in certain neuromuscular components of the feeding system where they typically function as cotransmitters that are released from motor neurons (Brezina et al. 2003a,b, 2005; Weiss et al. 1993; Whim et al. 1993). Although numerous observations indicate that neuromodulators also operate within the central circuits that govern feeding (e.g., Kabotyanski et al. 2000; Kirk et al. 1988; Sossin et al. 1987), our present understanding of their functional capabilities within the buccal CPG is incomplete.

This investigation examined the generation and regulation of firing patterns in B67, an identified bursting motor neuron that innervates the salivary duct and additional buccal muscles in Aplysia californica (Park et al. 1999, 2000). The bursting of B67 was found to reflect properties of an endogenous TTX-resistant sustained driver potential. Under control conditions, B67 bursting was not rhythmic and the activation of the bilateral B67 neurons was asynchronous. The neuromodulator dopamine (DA), however, conferred both rhythmicity and bilateral synchrony to the bursting of B67. A source of direct dopaminergic synaptic innervation of B67 was identified, indicating that the observed DA effects may reflect actions that occur during the physiological operation of this system.

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METHODS

Subjects
Experiments were conducted on specimens of Aplysia californica (150–250 g) that were purchased from the Aplysia Resource Facility and Experimental Hatchery (University of Miami, Coral Gables FL) or from Marinus (Long Beach CA). Animals were maintained in refrigerated aquaria (14–16°C) and fed dried seaweed twice per week.

Electrophysiology

Neurons were identified in preparations consisting of the paired buccal and cerebral ganglia. Intracellular microelectrodes filled with 2 M KCl (10–20 MΩ) were used for recording. B67 is a large superficial motor neuron located on the caudal surface of each buccal hemiganglion (Park et al. 1999, 2000). Sufficient criteria have been fulfilled to conclude that a neuron tentatively labeled B58 in a preliminary report (Serrano and Miller 2004) corresponds to B67. This cell shares several characteristics with a motor neuron previously designated the pharynx burster (PB) in A. kurodai (Nagahama and Takata 1987). Both neurons innervate multiple muscles that are thought to contribute to swallowing movements. An independent microelectrode (5–10 MΩ) was used for injecting current into B67 (Fig. 1A). B65 is an intrinsic buccal interneuron located within the confluence of the salivary nerve and buccal nerve 1 (Fig. 1A) (Kabotyanski et al. 1998). It contains markers for catecholamines (Díaz-Ríos et al. 2002; Kabotyanski et al. 1998) and GABA (Díaz-Ríos et al. 2002). Intracellular stimulation of B65 was achieved with the recording electrode across the balanced bridge circuit of the amplifier (NeuroProbe 1600, AM Systems).

Extracellular signals were recorded with polyethylene suction electrodes and AC coupled amplifiers (Model 1700, AM Systems). The typical configuration consisted of two cut-end recordings from buccal nerve 1 (Bn1; Fig. 1A). Spikes recorded intracellularly from B67 always corresponded to the largest impulses recorded from the ipsilateral buccal nerve 1 (Bn1; Fig. 1A). In the majority of preparations, B67 bursting was the only activity recorded from Bn1 that was not associated with full buccal motor programs (Fig. 1, A and B). Experiments were conducted at room temperature (19–21°C). The normal artificial seawater (ASW) contained the following (in mM): 460 NaCl, 10 KCl, 55 MgCl₂, 11 CaCl₂, and 10 HEPES. In some experiments, an ASW solution with elevated concentrations of divalent cations (2.2 × [Ca²⁺]) and 2 × [Mg²⁺)] (Liao and Walters 2002) was used to attenuate polysynaptic activity.

Pharmacology
Solutions of drugs were prepared from powder immediately before application. In one series of experiments, the concentration dependence of dopaminergic actions was determined with the antioxidant ascorbic acid (1 mM) present in the solution. Ascorbic acid alone had no detectable effects on B67. Dopamine, sulpiride, and tetrodotoxin (TTX) were obtained from Sigma Chemical (St. Louis, MO). Preparations were superfused with the ASW solution at a rate of 0.5 ml/min using a gravity-fed multi-channel system (ALA Scientific Instruments, Model VM4). Initial exposure to dopamine often initiated a period of coordinated fictive buccal motor programs (see Teyke et al. 1993). When observed, this effect was transient, subsiding within 2 min (see Kabotyanski et al. 2000). In contrast, effects on B67 were usually left at room temperature for 2–3 h to allow material to diffuse from the injection site (cell body) into small and distant processes. They were then repinned if necessary, and fixed in 4% paraformaldehyde (1–4 h). The fixed ganglia were transferred into microcentrifuge tubes, and washed five times (30 min each) with a phosphate buffer containing 1% Triton X-100 and 0.1 mM sodium azide (PTA solution). They were then incubated in Alexa Streptavidin 546 (Molecular Probes, Eugene, OR) diluted (1:800 to 1:3,000) in PTA (24–48 h, room temperature). Tissues were washed five times.

Dye injection
After neuron identification, the KCl microelectrode was withdrawn and replaced with one containing Neurobiotin. Injections were modified from the methods described by Delgado et al. (2000). The microelectrode tips were filled with 4% Neurobiotin (Vector Laboratories, Burlingame CA) dissolved in 0.5 M KCl and 50 mM Tris (pH 7.6). The electrode shafts were filled with 2 M KCl, resulting in resistances ranging from 15 to 30 MΩ. Depolarizing current pulses (1–2 nA; 0.5 s; 1 Hz; 10–30 min) were used to eject the Neurobiotin. This procedure did not appear to affect the resting potential or spontaneous electrical activity of the injected neuron. The preparations were usually left at room temperature for 2–3 h to allow material to diffuse from the injection site (cell body) into small and distant processes. They were then repinned if necessary, and fixed in 4% paraformaldehyde (1–4 h). The fixed ganglia were transferred to microcentrifuge tubes, and washed five times. The fixed ganglia were transferred to microcentrifuge tubes, and washed five times.
with PTA and viewed on a Nikon Eclipse TE200 fluorescence microscope prior to immunohistochemistry processing.

Immunohistochemistry

Standard whole mount immunohistochemical protocols were followed (see Miller et al. 1991, 1992 for details of buffer composition, incubation and wash procedures). Ganglia were washed (5 times, room temperature with agitation) in PTA. After preincubation with normal goat serum (0.8%), tissues were immersed (48 h, room temperature) in the primary antibody. A mouse monoclonal antibody (Diasorin, Stillwater MN) generated against rat tyrosine hydroxylase was used at concentrations ranging from 1:50 to 1:200 (see Díaz-Ríos et al. 2002). After repeated PTA washes (5 times, ≥30 min each, room temperature), ganglia were incubated in second antibodies conjugated to fluorescent markers (Alexa 488 goat anti-mouse IgG (H+L) conjugate; Molecular Probes: A-11029). The second antibody dilutions ranged from 1:400 to 1:2,000.

Preparations were viewed on the Nikon Eclipse or on a Zeiss Pascal laser scanning confocal microscope (LSCM). Images were captured with the Nikon ACT-1 (Version 2.10) software of the Eclipse or the Zeiss LSM 5 Image Browser (Version 3.1.0.11) program of the Pascal. They were transported as BMP files to Adobe Photoshop for adjusting overall contrast and brightness and then imported to Corel Draw 8 for addition of labels and organization of panels.

Data analysis

All results reported in this study were observed in a minimum of three specimens. Measurements are reported as the means ± SE unless noted. Statistical tests (Student’s t-test, 2-tailed) were performed by comparing measurements obtained prior to drug application to those attained at the peak of the response. Multiple group comparisons were performed with the one-way ANOVA followed by Tukey-Kramer pair-wise comparisons. A value of P < 0.05 was established as the criterion for significance. Autocorrelation and cross-correlation functions were generated from occurrences of bursts using the methods applied to spike trains by Perkel et al. (1967a,b). For our analysis, each burst was treated as a point event, the timing of which was assigned at the peak of its initial impulse. To enable quantification of synchronization, an operational index of synchrony, Is, was defined as the fraction of bursts that exhibited partial or temporal overlap of impulse firing. Correlations were displayed graphically using the NeuroExplorer (Version 3.122) software package.

RESULTS

Spontaneous activity of B67

Under control conditions, B67 produced multiple distinct burst patterns. As reported by Park et al. (1999, 2000), B67 was recruited into buccal motor programs (BMPs), where it fired during the protraction phase (Fig. 1B). During the retraction phase of BMPs, B67 received strong inhibition that was usually followed by a rebound burst of impulses.

B67 has also been reported to produce spontaneous bursting (Park et al. 1999). Such spontaneous bursts (2 marked by asterisks in Fig. 1B) were the subject of this investigation. They were highly stereotyped and were not preceded by detectable excitatory or inhibitory synaptic potentials. Each spontaneous burst consisted of 22.7 ± 0.9 action potentials that occurred within a period of 1.15 ± 0.05 s (n = 75 bursts recorded from 12 B67s in 7 preparations). In a given preparation, the burst properties of the two B67s were similar. Their durations (measured as the time from the first to the last impulse) exhibited little variation over a wide range of interburst intervals (IBIs; Fig. 2A). The number of spikes within each burst, however, was dependent on the IBI that preceded it with greater numbers of impulses occurring in bursts that followed briefer IBIs (Fig. 2A). Within the B67 burst, plots of the interspike intervals (ISIs) revealed a U-shaped form, with higher instantaneous spike frequencies (≥90/s) occurring near the midpoint of the burst (Fig. 2B). Impulse amplitudes were inversely related to the spike frequency with minimal amplitudes also occurring near the mid-point of the burst (Fig. 2B).

Intracortical current pulses introduced via an independent microelectrode were used to assess the responsiveness of B67 to depolarizing stimuli. Long (60 s) depolarizing pulses elicited repetitive bursting with greater levels of depolarization producing increased burst frequencies (Fig. 2C). With brief (0.1–1.0 s) current pulses, the capability to produce action potentials exhibited a sharp threshold below which no impulses were evoked (Fig. 2D). Above that level, the patterned burst could be evoked in its entirety. The precise threshold for eliciting a burst depended on the magnitude of the current pulse with larger pulses requiring shorter durations (Fig. 2D).

In contrast to its stereotyped intra-burst firing pattern, the intervals between B67 bursts were highly variable (Fig. 3A). In 12 B67s recorded from seven preparations for a minimum of 1 h (n = 1,116 IBIs), IBIs ranged from 12 to 1,312 s. Depicting the IBIs of a representative pair of B67s as histograms (Fig. 3B) illustrated their broad range with peaks at or near their minimum values (15–40 s). Sometimes additional minor peaks appeared to occur at multiples of the minimal value (Fig. 3B, right).

The bursting of B67 did not display a high level of rhythmicity (Fig. 3A). Auto-correlogram functions generated from the occurrences of spontaneous bursts revealed modest periodicity near the minimal IBI value, ~20 s in the example shown (Fig. 3C). No additional peaks were observed.

Paired recordings of the bilateral B67s typically revealed asynchronous bursting (Fig. 3A). Cross-correlation functions constructed from bilateral pairs disclosed a weak tendency toward synchrony (Fig. 3D). In five B67 pairs, the index of synchrony (Is, see METHODS) ranged from 0.10 to 0.37 (.19 ± 0.11, mean ± SD).

Slow potentials and signaling in B67

Signaling between the two B67s was tested to examine their possible reciprocal influence over bursting. In high-divalent solutions, large hyperpolarizing pulses (40–80 mV) injected into one B67 produced small (1–3 mV) hyperpolarizations in its contralateral counterpart (Fig. 4A). Bursting in one B67 had comparable small (1.3 ± 0.9 mV; n = 8) and long-lasting (2.4 ± 0.4 s) depolarizing effects on the contralateral B67 (Fig. 4B). Deflections corresponding to the impulses were not detected and the peak of this depolarization appeared to occur during the late phase of the contralateral burst (Fig. 4B, dotted vertical lines).

Patterned firing, such as that observed in B67, can be produced by endogenous burst-forming depolarizing responses known as driver potentials (Tazaki and Cooke 1979, 1990).
The presence of a driver potential in B67 was tested by blocking impulse activity with tetrodotoxin (TTX). Application of increasing TTX concentrations, beginning at $10^{-8}$ M, revealed that fairly high concentrations ($5 \times 10^{-6}$ to $10^{-5}$) were required to affect impulse activity in B67 (cf. Geduldig and Junge 1968; Kado 1973). This concentration blocked nearly all of the B67 spikes in somatic and nerve recordings (Fig. 4C). When B67 spikes were observed, they occurred during the onset of a prolonged TTX-resistant depolarization. In six B67s examined, the duration of this sustained depolarization, measured from the times during onset and decay at which it reached 0.1 of its maximal value, was $2.6 \pm 0.3$ s. Its peak amplitude, measured from the resting $V_m$ was $25 \pm 0.6$ mV and its time to peak was $1.28 \pm 0.3$ s. The parameters of this TTX-resistant persistent depolarization were indicative of the presence of a burst-forming driver potential in B67. Each occurrence of a driver potential was accompanied by a small depolarizing response in the contralateral B67 (Fig. 4D). As was observed with the responses produced by bursts (Fig. 4B), the peak of the contralateral depolarization corresponded to the late phase of the DP (Fig. 4D).

Effects of dopamine on B67 burst properties

The patterned bursting of B65 was modified by dopamine, a major regulator of feeding programs in Aplysia (Kabotyanski et al. 1998, 2000; Nargeot et al. 1999; Teyke et al. 1993). The concentration dependence of dopaminergic actions was tested by exposing the ganglion to increasing bath concentrations of DA with an antioxidant (ascorbic acid, 1 mM) included in all solutions (Fig. 5A). Threshold concentrations were in the micromolar range for dopamine-induced increases in the B67 burst duration and impulses per burst. The dose-response function for both parameters was rather steep with maximal effects occurring in the range of $5 \times 10^{-5}$ to $1 \times 10^{-4}$ M.

The actions of bath-applied dopamine on B67 bursting were rapid in onset, typically beginning within seconds of reaching the bath. They persisted throughout prolonged exposures and reversed on washout (Fig. 5B). The effects on burst parameters were not accompanied by detectable changes in the B67 membrane potential, measured at its most hyperpolarized interburst values, or its input resistance, measured with hyper-
FIG. 3. B67 bursting was not highly rhythmic or synchronous under control conditions. A: simultaneous recording from 2 B67 neurons (1 in each hemiganglion) in a single preparation. Bursting in the right B67 (RB67; top) and left B67 (LB67; bottom) was irregular and asynchronous. B: histograms of 42 consecutive interburst intervals from 2 B67 neurons recorded in normal artificial seawater (ASW). In each sample, maximal counts occurred near the minimal IBI values. The IBIs of each B67 varied over at least a fivefold range. C: graphing IBIs as an autocorrelogram disclosed very modest periodicity. An autocorrelogram of 40 IBIs recorded consecutively (total recording time: 39.3 min) from a single B67 exhibited a fairly even distribution over the range of ±100 s shown. Minor peaks correspond to ± the minimal IBI value of 20 s for this cell. D: bursting of the 2 B67s was weakly correlated. A cross-correlogram of 47 IBIs recorded consecutively (40 from the left B67 and 7 from the right B67; total recording time: 39.3 min) from 2 B67s over a range of ±100 s. There was a minor peak reflecting synchrony (near 0 s) and additional small peaks at ±20 s corresponding to the minimal IBI value for these cells (see C).

FIG. 4. Slow potentials and signaling in B67. A: injection of a large (−45 mV) hyperpolarizing pulse into the left B67 produced a slowly developing hyperpolarization of 1.5 mV in the right B67. When the recording electrode was positioned external to the membrane of the right B67 (0 mV), an identical pulse had no effect on the recorded potential. Experiment was performed in a raised divalent solution (see METHODS) that attenuates polysynaptic signaling. B: B67 bursting (in raised divalents) produced a small (1–3 mV) slowly developing depolarization in the contralateral B67. The peak of the contralateral depolarization corresponded to the late phase of the ipsilateral slow depolarization (dashed vertical lines). No rapid signaling corresponding to the impulses was detected. C: TTX-resistant driver potential in B67. Left: spontaneous burst (Con) recorded intracellularly from a B67 cell body (top) and extracellularly from buccal nerve 1 (bottom). Right: application of TTX (1 × 10⁻³ M) eliminated most impulses from the nerve recording. The B67 cell body produced 1 action potential (−−). This spike arose from the rising phase of a sustained depolarization that reached a peak of −25 mV depolarized from resting V_m (half-time to peak = 1.28 ± 0.29 s). D: spontaneous driver potentials in TTX (3 shown; top) produce small depolarizing responses in the contralateral B67 (bottom). The peak of each contralateral depolarization occurred during the late phase of the corresponding driver potential (dashed vertical lines).
polarizing somatic current pulses during the intervals between bursts (Fig. 5B).

The actions of dopamine on B67 bursting were examined in more detail with concentrations that produced maximal effects (Fig. 6). Application of DA at $10^{-4}$ M increased the burst duration from $0.88 \pm 0.09$ to $1.44 \pm 0.13$ s ($t = 2.57, P < 0.05, n = 6$; Fig. 6B1) and the number of impulses per burst was increased from $26.2 \pm 2.0$ to $46.2 \pm 1.3$ ($t = 2.02, P < 0.05, n = 6$; Fig. 6B2). The mean frequency of impulse firing within the bursts was not affected by dopamine (control: $31.2 \pm 3.5$ spike/s; DA: $35.7 \pm 2.8$ spike/s; $P > 0.05, n = 6$; Fig. 6B3).

Dopamine also influenced the slow signaling between the two B67's. In normal ASW, bursts that were prolonged by DA produced larger depolarizations in the contralateral B67 (control: $1.3 \pm 0.1$ mV; DA: $4.0 \pm 0.2$ mV; $t = 2.11, P < 0.05, n = 8$; Fig. 7A). In the presence of TTX, the most evident effect of dopamine was a prolongation of the driver potential duration (Fig. 7B1). DA increased the time course of the DP from control values of $2.6 \pm 0.3$ to $4.6 \pm 0.3$ s ($t = 2.92, P < 0.05, n = 6$). DA did not exert significant actions on the driver potential amplitude ($t = 2.18, P = 0.37, n = 6$). These findings are consistent with the observed effect of dopamine to increase burst duration and impulses per burst without producing significant changes in intraburst spike frequency (Fig. 6). Finally, the prolonged driver potentials observed in the presence of DA, produced enhanced depolarizing responses in the contralateral B67 (control: $1.6 \pm 0.3$ mV; DA: $5.7 \pm 0.4$ mV; $t = 2.57, P < 0.05, n = 6$; Fig. 7B2).

**Effects of dopamine on B67 rhythmicity and synchrony**

The rate of B67 bursting was also increased in the presence of dopamine (Fig. 8A). With bath application of DA ($1 \times 10^{-4}$

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**FIG. 5.** Effects of dopamine on B67 burst properties. A: application of dopamine produced concentration-dependent increases in the burst duration (●) and number of impulses per burst (■). Each pair of data points is based on 5 observations. All tests were made with 1 mM ascorbic acid present in the medium to retard oxidation. Representative recordings at $1 \times 10^{-7}$ and $5 \times 10^{-5}$ M are from the same preparation. Calibration applies to both recordings. B: effects of bath-applied DA on the B67 burst were not accompanied by detectable changes in the resting membrane potential or input resistance. B1: representative recordings (insets) of a B67 bursts prior to application of DA (Con) and at the peak of a response to $1 \times 10^{-4}$ M dopamine (DA). In both records, a dotted line is drawn at $-50$ mV. Current pulses (I) injected prior to DA application and at the peak of the response produced equivalent deflections of the membrane potential. B2: time course of dopamine actions on B67. Bath application of DA ($1 \times 10^{-4}$ M) produced an increase of nearly 200% in the number of impulses per burst (●). No effects were detected on the interburst membrane potential (●) or on the input resistance (●) measured as in B1.
M), the frequency of bursting was rapidly elevated to a higher level that was maintained throughout prolonged exposure (Fig. 8B). Dopamine reduced the mean IBI from 265.8 ± 116.5 to 16.0 ± 1.6 s (t = 2.26, P < 0.05; n = 10 B67s from 6 preparations; Fig. 8C1). The elevated burst frequency appeared to be accompanied by an increase in the regularity of B67 bursting (Fig. 8A). The degree to which DA influenced the uniformity of IBIs was therefore assessed by calculating their coefficient of variation \( C_v = \sigma / \mu \times 100 \), an attribute that normalizes their variability and expresses it as a percentage. The coefficient of variation of IBIs was decreased in the presence of DA from 90.0 ± 11.2 to 8.1 ± 2.9% (t = 2.26, P < 0.05, n = 10; Fig. 8C2).

At peak responses to dopamine, the uniformity of the IBIs produced rhythmic bursting of B67 (Figs. 8A and 9A). Plotting the IBIs as an auto-correlogram revealed such a transition from weak to strong oscillation (Fig. 9B). The frequency of the rhythmic burst activity produced in B67 by DA typically exceeded the highest burst frequency observed under control conditions.

Finally, the bursting of the paired B67s became notably more synchronous in the presence of dopamine (Fig. 9A). Plotting the timing of bursts as a cross-correlation function before DA application and during the peak DA response revealed a strong influence toward a common rhythm (Fig. 9C). In the experiment shown, 100% of the bursts recorded from the two B67s in dopamine exhibited overlap with bursting in its counterpart, compared with 18% during the hour prior to DA application. Although the burst activity in the paired B67s was synchronized, the onsets of the paired bursts were usually staggered, with one side preceding the other by 200–300 ms (Fig. 9C, inset). Moreover, the order of bursting in the two B67s reversed frequently, with one serving to “lead” the other for a series of bursts after which it became the “follower.” The index of synchrony \( I_s \) was found to be increased from control values of 0.19 ± 0.11 to 0.94 ± 0.06 in the presence of DA (t = 2.78, P < 0.05, n = 5 pairs).

Two series of tests were conducted to further clarify the relation between B67 burst rhythmicity and synchrony. As previous experiments had shown that injection of a constant depolarizing current could induce rhythmic bursting (see Fig. 2C), it was possible to test whether such rhythmic activity per se in one B67 could entrain its contralateral companion. Depolarization of either the right B67 (Fig. 10A) or the left B67 (Fig. 10A) reduced the IBI coefficient of variation of the injected cell to a level that was significantly lower than its nondepolarized mate (Fig. 10B). In neither instance, however, was the index of synchrony increased over control values (Fig. 10C). The \( I_s \) observed with depolarization of either B67 remained significantly lower than that induced by dopamine (Fig. 10C).

In a second series of experiments, both B67s were simultaneously depolarized to levels that produced rhythmic bursting (Fig. 11). In these tests, the rhythmicity of both injected neurons reached levels that were comparable to those achieved by dopamine (Fig. 11, A and B). Their synchrony, however, remained significantly lower than that produced by dopamine (Fig. 11C). Together, the experiments with current injection indicate that B67 burst synchrony is not coupled to its rhythmicity in an obligatory fashion. They further suggest that...
Finally, the effects of dopamine on B67 rhythmicity and synchrony of B67 were tested in the presence of TTX (Fig. 12). Under control conditions (10^{-5} M TTX in the bath), the driver potentials of B67 occurred spontaneously in a nonrhythmic and nonsynchronous fashion (Fig. 12A). Application of dopamine (10^{-4} M) conferred both rhythmicity and synchrony to the B67 driver potentials. As noted with the dopamine-induced burst synchrony (Fig. 9C), the onset of one driver potential preceded that of the other by several hundred milliseconds (Fig. 12B, right).

Dopaminergic synaptic innervation of B67

The limited number of catecholaminergic neurons in the buccal system (Díaz-Ríos et al. 2002; Kabotyanski et al. 1998; Teyke et al. 1993) enabled a search for possible sources of dopaminergic synaptic innervation of B67. Dye fills of one known dopaminergic interneuron, B65, revealed that it possessed suitable morphological properties for the production such signaling (Fig. 13). In agreement with previous observations (Kabotyanski et al. 1998), B65 was found to project its major process through the central core of the ganglion (Fig. 13A) to the contralateral hemiganglion. In both hemiganglia, this process passed in close proximity to B67 (Fig. 13, A and B). The soma and principal process of B65 gave rise to numerous secondary and tertiary collaterals that coursed toward the motor neurons of both hemiganglia (Fig. 13, A1 and B1) (see also Kabotyanski et al. 1998). Double-labeling experiments using an antibody to tyrosine-hydroxylase (TH), revealed TH-like immunoreactive fibers immediately adjacent to both B67s (Fig. 13, A1 and B1).

Previous studies have shown that B65 contains markers for catecholamines and GABA (Díaz-Ríos et al. 2002; Kabotyanski et al. 1998) and that its rapid excitatory signaling to several follower neurons is mediated by dopamine (Díaz-Ríos and Miller 2005; Due et al. 2004). Activation of the receptors that generate these excitatory postsynaptic potentials (EPSPs) can be accomplished with rapid focal application of high concentrations of DA (1 mM) (see Díaz-Ríos and Miller 2005; Due et al. 2004). As bath-applied DA (10^{-4} M) did not affect the membrane potential or input resistance of B67 (Fig. 5B), puffed application from a micropipette was used to probe for such receptors (Fig. 14A). A brief (200 ms) pulse of DA (1 mM in the pipette) to the soma-initial segment of B67 produced a depolarizing response that could exceed threshold (Fig. 14A, top). This depolarizing response was reversibly blocked by sulpiride (1 mM), a known antagonist of dopaminergic synaptic receptors in mollusks (Magoski et al. 1995; Quinlan et al. 1997) (Fig. 14A, middle and bottom). These findings indicate that excitatory dopaminergic synaptic receptors are present on B67.
Firing B65 produced excitatory actions on the ipsilateral and contralateral B67s (Fig. 14). In both cases, B65 caused B67 to produce its characteristic burst response. When the concentration of divalent ions was adjusted to attenuate polysynaptic activity, one-for-one EPSPs with brief and constant latencies corresponding to B65 impulses were revealed in the ipsi- and contralateral B67 (Fig. 14C). These EPSPs were blocked by 1 mM sulpiride (Fig. 14D). Together these observations demonstrate direct excitatory dopaminergic synaptic signaling from B65 to B67.

Finally, tests were conducted to assess the ability of B65 to promote rhythmicity and synchrony of B67 bursting. Bursts of B65 generated by injection of depolarizing current pulses (5 s) produced prolonged periods of bilateral bursting in B67 (Fig. 14E). The bursting during this period, which could last several minutes, was highly rhythmic. Finally, the bursting of the two B67s after firing of a single B65 was highly synchronized. It is proposed (see DISCUSSION) that modulatory dopaminergic signaling originating from B65 can produce the observed extended periods of rhythmic synchronous bursting in B67 (Fig. 14F, see DISCUSSION).

DISCUSSION

This investigation identified properties of B67 that can contribute to the generation and regulation of its signaling. Previous studies characterized B67 as a multi-target motor neuron that innervates the vicinity of the salivary duct and several intrinsic buccal muscles (Park et al. 1999; see also Nagahama and Takata 1987). We suggest that its activation by dopamine can originate from B65 during feeding and that it serves to coordinate increases in salivation and buccal movements that occur during consummatory behaviors (Fig. 14F). Synchronous salivation is postulated to reflect the adaptive value that simultaneous bilateral lubrication of the pharynx and esophagus would confer on the ability to ingest or egest large objects. Moreover, simultaneous bilateral contraction of buccal muscles could provide more effective forces for translocating objects that span the esophagus.

In agreement with previous reports (e.g., Kabotyanski et al. 2000), spontaneous buccal motor programs occurred very infrequently (once per 5–10 min) in the in vitro cerebral-buccal preparation used in this study. Although B67 was recruited into such programs (Fig. 1B) (Park et al. 1999; see also Prior and Gelperin 1977), the bursting analyzed in this investigation occurred at much higher frequencies (see Fig. 3). Although we propose that its frequency is influenced by consummatory programs (Fig. 14F), this bursting was not driven on a cycle-to-cycle basis by the buccal CPG that generates well-characterized ingestive and egestive BMPs (Cropper et al. 2004;
Elliott and Susswein 2002). This form of bursting is therefore not likely to produce movements that are readily detected with behavioral observation, such as biting and rejection (Kupfermann 1974; Morton and Chiel 1993; Rosen et al. 1989). We propose that, in common with these coordinated motor programs (Kabotyanski et al. 1998, 2000) the activation of B67-driven movements can be achieved by dopamine release from B65 (Fig. 14F).

**Endogenous properties of B67**

B67 exhibited features that contribute to pattern and rhythm generation in motor systems (Friesen 1994; Hartline et al. 1988; Pearson 1993). Several neurons in the *Aplysia* feeding network exhibit endogenous burst-forming potentials or bistable states (Perrins and Weiss 1998; Plummer and Kirk 1990; Susswein and Byrne 1988). Prolonged, regenerative, TTX-resistant potentials were originally identified in the motor neurons of the crustacean cardiac ganglion (Tazaki and Cooke 1979, 1990). They were designated “driver potentials” due to their proposed role in the generation of impulse trains. In agreement with the observations of Tazaki and Cooke (1979), the duration of the B67 driver potential in TTX was substantially longer than the slow potential that underlies normal bursting. In the case of the crustacean motor neurons, such differences in duration could be attributed, at least in part, to the relatively refractory state of the slow calcium current that underlies the DP when evoked at frequencies corresponding to the heartbeat (Tazaki and Cooke 1990). Differences in the duration of the B67 burst and its DP are unlikely to reflect refractory properties of contributing currents, as IBIs typically exceed 15 s. A complete understanding of B67 bursting will require a detailed characterization of the biophysical processes that contribute its driver potential.

Although burst-forming potentials have been described in a range of neural systems (e.g., Calabrese and Peterson 1983; Wallen and Grillner 1987; Wong and Prince 1981), their direct participation in intercellular interactions may be less prevalent. A notable instance of such signaling occurs in the crustacean stomatogastric ganglion, where complex network rhythms can...
FIG. 10. Rhythmic bursting induced by depolarization of one B67 does not entrain its contralateral counterpart. 
A: positive current (I) was injected throughout the two recordings shown (denoted by lines below the records) into the right B67 (A1: rB67) or the left B67 (A2: lB67). In both trials, the depolarization produced rhythmic bursting in the injected cell but the contralateral B67 remained arrhythmic. 
B: coefficients of variation of interburst intervals from 4 B67 pairs tested as in A. The Cv was reduced significantly in depolarized neurons (■) compared with that of their noninjected contralateral mates (□; P < 0.05). 
C: index of synchrony (see METHODS) remained at control (Con) levels when either of the B67 neurons was depolarized. Data from the same 4 preparations used in B. The values of Cv were significantly lower than observed in the presence of dopamine (DA; P < 0.05).

FIG. 11. The synchrony of B67 bursting produced by dopamine exceeded the level observed with depolarization-induced rhythmicity. 
A1: injection of constant depolarizing current into both B67s simultaneously (I, lines below each record) produced bursting that was rhythmic in each but not synchronous. 
A2: in the absence of depolarizing current, application of DA (1 × 10⁻⁴ M) to the same preparation as A1 produced bursting that was both rhythmic and synchronous. 
B: summary data (n = 4 pairs) showed that the rhythmicity (quantified as the coefficient of variation, Cv, of the IBIs) produced by simultaneous depolarization of both B67s (Depol), was comparable to that produced by dopamine (DA). Both treatments produced significant (P < 0.05) increases in rhythmicity (i.e., decreases in the Cv). The paired cells were pooled for the statistical comparisons. No differences were detected in the rhythmicity produced by the 2 treatments. 
C: synchrony (quantified as the index of synchrony, Is) produced by DA was greater than that observed in paired B67s with rhythmic bursting produced by simultaneous depolarization. These comparisons were generated with the same data used in B. Although the level of synchrony induced by simultaneous depolarization (Depol) exceeded that of the control (Con) condition (P < 0.01), it remained below the levels induced by DA (P < 0.05).
be achieved in the absence of impulses (Anderson and Barker 1981; Raper 1979). The onset of the depolarization produced by a B67 burst in its contralateral counterpart began several hundred milliseconds after the initiation of impulse firing and its peak occurred near the end of the burst. Deflections corresponding to impulses could not be distinguished. This depolarization persisted in TTX, again beginning after a delay and peaking during the late phase of the driver potential. These observations suggest the participation of the slow burst-forming potential in B67-to-B67 signaling. However, the capacity for such interactions remains enigmatic, as potential sites of apposition have not been reported for B67 or the pharynx burster of A. kurodai (Nagahama and Takata 1987; Park et al. 1999; this study).

**Modulation of B67 bursting by dopamine**

Our observations indicate that dopamine can produce coordinated modifications of several attributes of B67. The DA-induced prolongation of the B67 driver potential was consistent with its ability to increase burst duration. Also, the DA-induced enhancement of signaling between the two B67s corresponded well to its ability to increase their synchrony. The overall effects of DA on B67 are likely to reflect the concurrent and collective modulation of several properties that combine to endow B67 with increased burst duration, frequency, rhythmicity, and synchrony. Similar coordinated actions of dopamine on multiple synaptic and biophysical properties have been exceptionally well documented in the crustacean stomatogastric system where DA modifies burst rate and phase relations (Flamm and Harris-Warrick 1986; Harris-Warrick et al. 1995; Johnson and Harris-Warrick 1990).

The burst properties of B67 were modified by concentrations of dopamine ranging from $10^{-5}$ to $10^{-4}$ M. These concentrations are comparable with those that have been found to modulate fictive motor rhythms in several CPGs (Kemnitz 1997; Miller et al. 1984; Raper 1979), including the buccal CPG of Aplysia (Kabotyanski et al. 2000). They are substantially lower, however, than those ($10^{-3}$ M) that are required to activate the receptors that mediate rapid EPSPs produced by dopaminergic buccal interneurons (Díaz-Ríos and Miller 2005; Due et al. 2004). These observations indicate that multiple classes of dopaminergic receptors contribute to the operation of this circuit (see also Nargeot et al. 1999; Teyke et al. 1993). The finding that B67 receives direct rapid dopaminergic EPSPs indicates that at least some regions of its membrane, i.e., those that are subsynaptic or perisynaptic to its innervation from B65, can be exposed to high levels of DA (Fig. 14F).

**FIG. 12.** Dopamine promotes rhythmicity and synchrony of TTX-resistant driver potentials in B67. A: spontaneous driver potentials recorded simultaneously from the right B67 (RB67) and left B67 (LB67) were infrequent and asynchronous. Right: one pair of records shown at faster recording speed shows delayed and prolonged depolarization in the RB67 corresponding to the LB67 DP (see also Fig. 7). B: addition of dopamine (DA; $1 \times 10^{-4}$ M) produced rhythmic and synchronous DPs in the paired B67s. Right: paired recordings with faster time base demonstrate that although substantial portions of their DA-induced DP durations overlap, their onset and termination do not occur simultaneously. Moreover DA promotes repetitive spike-like activity during the depolarizing phase of the DP that appears to be reflected, after considerable delay, in its contralateral counterpart.

**FIG. 13.** Bilateral projections of the identified catecholaminergic neuron B65. A1: B65 and B67 in the left hemiganglion were filled with neurobiotin via current injection (see METHODS) and labeled with Alexa-546 avidin (red fluorescence). The buccal ganglion was then processed for TH-like immunoreactivity, which was detected with a 2nd antibody labeled with Alexa-488 (green fluorescence). The yellow appearance of B65 reflects double labeling. The principal B65 fiber (arrow) could be traced to the immediate proximity of B67. Finer TH-immunoreactive fibers were located in direct apposition to B67. A2: image showing dye fills only. Very fine collaterals branched from B65 in the region of the B67 cell body (arrow). Larger processes emanating directly from the B65 soma (arrowhead) also projected to a region where the axon of B67 is known to pass. B1: right buccal hemiganglion; same preparation as A. The filled fiber of B65 (arrow) projected into the contralateral buccal hemiganglion (see Kabotyanski et al. 1998). B2: image of dye fills only emphasizes the proximity of the B65 fiber to the soma and Initial segment region of B67 and enables visualization of very fine collaterals (arrow) in the region of B67.
The effects of firing a single B65 on bursting in B67 could last several minutes and were observed after detectable indications of its direct synaptic signaling had subsided. The proposed prolonged actions of DA would therefore be classified as modulatory (see Kaczmarek and Levitan 1987; Kupfermann 1979). Interestingly, although the rapid dopaminergic synaptic signaling of B65 to several follower neurons has been shown to bias motor programs toward an egestive mode (Due et al. 2004), its longer-lasting signals produce a gradual transition toward ingestive patterns (Kabotyanski et al. 1998). The prolonged duration of B65’s actions on the rhythmicity and synchrony of B67 suggests that these effects are more closely related to B65’s slower ingestive-promoting influence. If dopamine is responsible for this sustained action then the dopaminergic signaling from B65 would include both rapid EPSPs and this long-lasting modulatory component. A similar form of conjoint rapid and sustained modulatory signaling mediated by a single biogenic amine, serotonin, has been demonstrated in the escape swim CPG of the nudibranch Tritonia (Clemens and Katz 2001; Katz and Frost 1995). In the case of B65, however, it will be necessary to distinguish effects of dopamine on B67 from those of another potential modulator, GABA, as GABA-like immunoreactivity has also been localized to B65 (Díaz-Ríos et al. 2002; Jing et al. 2003) and GABA can modulate its rapid synaptic signaling (Díaz-Ríos and Miller 2005).

The effects of dopamine on B67 are consistent with its demonstrated ability to activate feeding motor programs in mollusks (Quinlan et al. 1997; Trimble and Barker 1984; Wieland and Gelperin 1983) including Aplysia (Kabotyanski et al. 2000). The capacity of additional known sources of dopamine in this system, such as the cerebral-buccal interneuron CBI-1 (Rosen et al. 1991), the buccal-cerebral interneuron B20 (Teyke et al. 1993), and esophageal afferents (Kabotyanski et al. 1998) to induce rhythmicity and synchrony in B67 remains to be explored.

**Conditional rhythmicity and synchrony**

Most models of spontaneous synchronization in biological systems have been described for large populations of oscillating elements, such as chorusing crickets, flashing fireflies, rhythmogenic myocardial cells, and circadian pacemaker neu-
rions (Kuramoto 1984; Peskin 1975; Winfree 1980). In the bag cell neurosecretory system of Aplysia, bilateral aggregates of ~400 neuroendocrine cells, intracluuster and bilateral syn-

The capability of a neuromodulator to induce bursting in otherwise quiescent motor networks (see Miller and Sullivan 1981; Ramirez and Pearson 1989; Raper 1979) has been designated “conditional bursting” (Harris-Warrick and Flamm 1987; Marder and Eisen 1984). The present observations differ fundamentally from this form of modulation, as B67 produces spontaneous bursts in the absence of dopamine. In keeping with this terminology, however, we refer to the actions of DA on B67 as promoting “conditional rhythmicity” and “conditional synchrony.”

In many synchronizing biological systems, synchronization is accompanied by, and may be contingent on, transitions to rhythmicity (Buck and Buck 1968; McClintock 1971; see Strogatz 2003). With respect to the synchrony imposed on B67 by dopamine, this also appears to be the case. Numerous additional neuromodulators are present in the buccal system (Kupfermann et al. 1979; Miller et al. 1993a,b; Whim et al. 1993), so it should be feasible to examine whether other signals can promote synchrony in the paired B67s without inducing their rhythmicity. Likewise, it should be possible to determine whether modulators can generate B67 rhythmicity without producing synchrony. The multitude of modulatory systems that are present in the feeding CPG of Aplysia should provide opportunities to explore relations between rhythmicity and synchrony in an exceptionally tractable neuronal network with well-understood behavioral functions.

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