Feeding Stimulants Activate an Identified Dopaminergic Interneuron That Induces the Feeding Motor Program in Helisoma

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Quinlan, E. M., B. C. Arnett, and A. D. Murphy. Feeding stimulants activate an identified dopaminergic interneuron that induces the feeding motor program in Helisoma. J. Neurophysiol. 78: 812–824, 1997. The neurotransmitter dopamine is shown to play a fundamental role in the generation of the feeding motor pattern and resultant feeding behavior in Helisoma. Application of exogenous dopamine triggered the fictive feeding motor pattern in the isolated CNS and triggered feeding movements in semi-intact preparations. Application of feeding stimulants to the oral cavity excited the putatively dopaminergic buccal interneuron N1a, and depolarization of interneuron N1a triggered the production of the fictive feeding motor pattern. The ability of dopamine superfusion and of interneuron N1a stimulation to activate the fictive feeding motor pattern was blocked by the dopamine antagonist sulpiride. The phase of the fictive feeding motor pattern was reset by brief hyperpolarization of interneuron N1a, demonstrating that interneuron N1a is an integral component of the buccal central pattern generator (CPG). During spontaneous fictive feeding patterns, prolonged hyperpolarizations of interneuron N1a inhibited the production of patterned activity. Exogenous dopamine maintained the fictive feeding motor pattern in the absence of interneuron N1a activity. Interneuron N1a was labeled by the formaldehyde-glutaraldehyde histochemical technique, which is indicative of the presence of dopamine in mollusks. These data suggest that interneuron N1a is an endogenous source of the neuromodulator dopamine, intrinsic to the buccal CPG, and that interneuron N1a has a prominent role in the sensory-motor integration triggering the consummatory response.

INTRODUCTION

Central pattern generators (CPGs) are neuronal ensembles that produce the motor neuron activity patterns responsible for many life-sustaining rhythmic behaviors. Although capable of producing rhythmic motor patterns in the absence of afferent input, most CPGs are highly regulated by sensory and modulatory influences. Such modulatory pathways must transduce information about the animal’s external environment and internal physiological state and transmit relevant information to the interneurons of the CPG, adapting patterned motor activity to suit immediate demands. Monoaminergic pathways that initiate and/or modulate CPG activity have been demonstrated in many species. For example, the initiation of alternating activity in the flexor and extensor nerves of curarized spinal rabbits is induced by injection of the catecholamine presurser L-dopa (Viala and Buser 1969). Similarly, bath application of L-dopa initiates patterned motor activity in the locomotory CPGs of the cat (Grillner 1986) and lamprey (Poon 1980). Applications of the monoamines dopamine and serotonin evoke distinct motor patterns in CPGs controlling the hindlimbs of neonate rats (Kiehn and Kjaerulff 1996) and controlling the stomatogastric system of the lobster (for reviews see Harris-Warrick 1988; Selverston 1995). Serotonin application can evoke feeding (Lent 1985; but see also Wilson et al. 1996) or swimming (Brodfuehrer et al. 1995) in the leech, and aspects of these behaviors can be triggered by stimulation of identified serotonergic neurons (Lent 1985; Nusbaum and Kristan 1986). In the snail Helisoma, superfusion of the buccal ganglia with serotonin evokes a biphasic motor pattern (Gran-}

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ganglia, in the presence or absence of sensory afferents or connections with the rest of the CNS (but see Kabotyanski et al. 1994). Second, dopaminergic neurons, whose activation mimics the effects of dopamine superfusion, have rarely been identified. Third, there is diversity of dopamine receptors both within and across species (Ascher 1972; Berry and Cottrell; 1975; Green et al. 1996; Lo and Weiss 1994; Magoski et al. 1995). Thus comparative analyses of dopaminergic modulation and of the roles of putatively dopaminergic neurons in feeding in mollusks remain problematic.

In *Helisoma trivolvis*, we have previously analyzed feeding behavior in intact semitransparent newly hatched snails by video microscopy. Similar video microscopic analyses of feeding in semi-intact animals were made simultaneously with intracellular recordings from identified buccal neurons and with extracellular myograms. These multidisciplinary analyses have demonstrated that the standard triphasic pattern of activity in buccal motor neurons (Quinlan and Murphy 1991, 1996; Quinlan et al. 1995) mediates the typical feeding behavior (Arnett 1996; Arnett and Murphy, unpublished data). Here we demonstrate that dopamine plays a fundamental role in the generation of the feeding motor pattern and consequent feeding behavior. We identify and characterize a dopaminergic interneuron, named N1a, that is stimulated by natural stimulants that evoke feeding. Depolarization of interneuron N1a in quiescent preparations is sufficient to trigger the generation of the feeding motor program. In addition, brief hyperpolarization of interneuron N1a resets the phase of ongoing feeding motor patterns. This indicates that interneuron N1a is an intrinsic part of the buccal CPG, and suggests that monoaminergic modulation of CPGs by interneurons intrinsic to the CPGs may be a common regulatory mechanism (cf. Katz and Frost 1995; Katz et al. 1994).

Such dopaminergic modulation of the buccal CPG is fundamental to the sensory-motor integration that selects typical feeding behavior from the behavioral repertoire of the multifunctional buccal CPG.

A preliminary report of some of these data was presented previously in abstract form (McLean et al. 1989).

**METHODS**

**Animals and experimental preparations**

All experiments were performed on an albino strain of the planorbid pond snail, *H. trivolvis*, descended from stocks originally established in the laboratory of S. B. Kater. Adult snails with a vertical shell diameter of 10–12 mm were used. The general dissection has been previously described (Kater and Kaneko 1972). Three types of experimental preparations were used. Most experiments employed an isolated CNS preparation, in which the CNS (paired buccal, cerebral, pedal, pleural, and parietal ganglia, and the visceral ganglion) and the salivary glands were removed and stabilized in a recording dish with a silicone rubber base (GE:RTV616).

Simultaneous analyses of feeding movements and their underlying neural patterns employed semi-intact preparations. A midsgittal incision was made in the dorsal body wall, from the mantle to the outer lip, to expose the CNS. Part of the lateral body wall was cut away to facilitate visualization of the buccal mass. The esophagus was severed and deflected forward to raise the buccal ganglia (attached to the caudal surface of the buccal mass) into a dorsal position. A spear-shaped microplatform, attached to the recording dish with a ball-and-socket joint, was used to stabilize the buccal ganglia for intracellular recordings during feeding movements. The microplatform was insulated with Paraplastic embedding medium and dipped in black ink to enhance visibility.

A more reduced preparation was required to determine the effects of food stimuli on interneuron N1a, because of the relatively small size (<40 μm) and lateral position of its soma. The buccal mass, salivary glands, and esophagus were extracted while innervation from the buccal ganglia was preserved. The buccal mass was split ventrally, from the radular sac to the mouth, and reflected outward. A stabilizing silicone rubber platform was placed beneath the buccal ganglia. The esophagus was cannulated with polyethylene tubing (<1 mm OD) attached to a tuberculin syringe filled with watermelon that had been homogenized and strained through cheesecloth. Approximately 0.1 ml of watermelon extract, delivered to the anterior proesophagus, spread over the surface of the buccal cavity in each experiment.

**Electrophysiology, intracellular staining, and video microscopy**

Standard electrophysiological techniques were used. Glass microelectrodes with internal fibers were filled with 3 M potassium acetate (DC resistance 20–40 MΩ) or 3% Lucifer yellow CH (in distilled and filtered water, DC resistance 100–200 MΩ). The Lucifer yellow staining procedures have been previously described (Murphy et al. 1983). Reactive red No. 4 was pressure injected into interneuron N1a via a pneumatic injection system (Picospritzer, General Valve) under visual control.

For simultaneous video microscopic and electrophysiological analyses, movements of the buccal mass were recorded with a Hitachi KP-140 solid-state video camera attached to a Wild M5a dissection microscope with a trinocular head. A Panasonic WV-CD20 closed-circuit TV camera simultaneously recorded oscilloscope traces of intracellular electrophysiological neuronal activity. The two camera signals were digitally mixed via a Panasonic AV mixer, and the neural activity was placed as an inset into the images of the buccal mass. The combined images were sent to a Panasonic AG-7300 video recorder and stored on video cassettes for subsequent analyses. Photographs were taken of individual video frames (33 ms) with a Polaroid freeze-frame video recorder.

Unless otherwise noted, intracellular recordings were made in normal physiological saline containing (in mM) 5.13 NaCl, 1.7 KCl, 1.5 MgCl₂, 4.1 CaCl₂, and 5.0 N-2-hydroxymethylporazine-N’-2-ethanesulfonic acid buffer, pH 7.3. All chemicals, including neurotransmitters and antagonists, were obtained from Sigma.

**Formaldehyde-glutaraldehyde histochemistry**

The formaldehyde-glutaraldehyde (FaGlu) histochemical procedure was performed on acutely dissected CNS following the methods of Goldstein and Schwartz (1989). Briefly, preparations were incubated in FaGlu mixture (4% formaldehyde/0.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4) for 24 h at 4°C. Preparations were dehydrated via an ascending ethanol sequence, rehydrated in distilled and filtered water, DC resistance 100 ± 200 MΩ, and then cleared and mounted for microscopy in methyl salicylate.

**RESULTS**

**Dopamine elicits the fictive feeding motor pattern from the buccal CPG in the isolated CNS**

Multiple patterns of motor neuron activity responsible for the expression of distinct rhythmic oral behaviors are produced by the buccal CPG in *Helisoma* (Arnett 1996; Quinlan and Murphy 1996). The CPG is composed of three interactive interneuronal subunits, named S1, S2, and S3, that...
There are several mechanisms by which plasticity of motor output of the CPG can arise. Each CPG subunit is a conditional neuronal oscillator that can be independently rhythmically active. The subunits also can be functionally linked in different combinations and in different temporal patterns. Additional motor plasticity can arise from variability in the rate of rhythmic activity (i.e., cycle period) and in the intensity of action potential bursts (i.e., graded changes in intraburst action potential number and frequency) in subunits 1 and 3 (Quinlan and Murphy 1991, 1996; Quinlan et al. 1995) (see below).

A triphasic buccal motor pattern, with the CPG subunits activated in the sequence S1-S2-S3, was previously described (Quinlan and Murphy 1991, 1996; Quinlan et al. 1995) and has been shown to mediate functional feeding movements (Arnett 1996; Arnett and Murphy 1991; Arnett and Murphy, unpublished data). When the CPG is active in this feeding mode, bursts of action potentials in S1 interneurons simultaneously evoke excitatory postsynaptic potentials (EPSPs) in phase 1 motor neurons and S2 interneurons. Depolarization of S2 interneurons, beyond the threshold for the production of plateau potentials, evokes excitation in phase 2 motor neurons and inhibition in both S1 and S3 interneurons. The inhibitory feedback from S2 terminates the activity of S1, and postinhibitory rebound, following the termination of S2 inhibition, activates S3 (Quinlan and Murphy 1996). S1 interneurons slowly repolarize on termination of S2 inhibition. In the presence of continuous sensory stimulation or endogenous modulation, a subsequent burst of action potentials will be generated in S1 interneurons to initiate a new feeding cycle.

Dopamine superfusion (1–10 μM) of the isolated CNS triggered the production of the triphasic fictive feeding motor pattern (Fig. 1B). During phase 1 of each cycle, motor neuron B6, which is involved in protraction of the odontophore, generated a burst of action potentials. Both neuron B6 and phase 3 motor neuron B19 were inhibited during phase 2, whereas motor neuron B27, which is involved in retraction of the odontophore, was excited and generated a burst of action potentials. Motor neuron B19, which is involved in hyperretraction of the odontophore, generated bursts of action potentials during phase 3.

**Dopamine triggers the feeding motor pattern and consequent feeding behavior in semi-intact snails**

Dopamine previously has been shown to evoke buccal motor patterns, thought to represent fictive feeding, in a number of gastropods (e.g., Kabotyanski et al. 1994; Kyракидеς and McCrohan 1989; Teyke et al. 1993; Wieland and Gelperin 1983). To determine whether dopamine triggers feeding behavior, the effects of dopamine were examined in semi-intact preparations capable of generating feeding movements while intracellular recordings were made from buccal neurons. Movements of the pharyngeal buccal mass, which contains the muscles responsible for feeding movements of the odontophore, were videotaped with a camera mounted on the dissecting microscope. Simultaneously, the activity of each CPG subunit was ascertained by recording from selected pairs of identified motor neurons. A second camera, focused on the oscilloscope screen, videotaped the

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**FIG. 1.** Dopamine (DA) modulates multifunctional buccal central pattern generator (CPG) to generate feeding motor pattern. A: schematic diagram of buccal CPG. Each of the 3 CPG subunits (S1, S2, and S3) is an independent conditional oscillator comprising ±1 pairs of interneurons. Membrane properties of individual CPG interneurons, and interactions between CPG subunits, can be modulated (diagonal arrows) to permit production of multiple motor neuron activity patterns. Chemosensory afferents stimulated by food in buccal cavity, and DA, either extrinsic to CPG or released by S1 interneurons, induce CPG to be active in feeding mode. Depolarization of S1 interneurons stimulates S1 follower motor neurons (MNs) and S2 interneurons. Depolarization of S2 interneurons stimulates S2 follower neurons and simultaneously inhibits S1 and S3 activity. S3 activity is generated by postinhibitory rebound following termination of S2 inhibition. Lines ending in horizontal bars: excitatory pathways. Lines ending in filled circles: inhibitory pathways. B: bath application of DA (10 μM) triggered production of triphasic (S1-S2-S3) feeding motor pattern. No buccal CPG activity was observed in physiological saline before application of DA. Phase 1 motor neuron B6 is excited by S1 and inhibited by S2. Phase 2 motor neuron B27 is excited by S2. Phase 3 motor neuron B19 is inhibited by S2 and excited by S3.
intracellular recordings. Superfusion of semi-intact preparations with dopamine elicited functional feeding movements similar to those evoked by application of food to the oral cavity.

The external contours of the buccal mass can be used to identify each phase of the feeding cycle (Arnett 1996; Arnett and Murphy 1991; Smith 1988, 1991). During phase 1, the odontophore is rotated forward and protracted downward through the mouth. These odontophore movements result largely from contractions of the posterior jugalis (pj) muscle, and cause a lateral bulging of the buccal mass (Fig. 2A). The pj is innervated by motor neurons B6 and B8, and bursts of action potentials in these neurons trigger pj contractions during phase 1 of the feeding motor pattern (Arnett 1996). During phase 2 of the feeding cycle, the odontophore is retracted back to the rest position, in part because of contractions of the anterior jugalis (aj) muscle. This movement causes a narrowing of the buccal mass as the aj contracts and the pj relaxes. A more pronounced narrowing of the buccal mass is seen during phase 3 as the odontophore is hyperretracted toward the esophageal opening (Fig. 2B). This movement is due largely to intense contractions of the aj and supralateral radular tensor muscles. The aj is innervated by both phase 2 motor neuron B27 and phase 3 motor neuron B19. Neuron B19 also innervates the supralateral radular tensor. Thus dopamine superfusion of semi-intact preparations configures the CPG to generate the triphasic feeding motor pattern and movements of the buccal mass diagnostic for feeding. This suggested that dopaminergic modulation may mediate the feeding response induced by chemosensory stimulation.

**Dopamine induces multiple patterns of buccal neuronal activity in a concentration-dependent manner**

During initial examinations of the effects of dopamine concentrations on feeding behavior in semi-intact preparations, we observed that 10 μM dopamine routinely evoked feeding movements, 1 μM dopamine had variable behavioral effects, and <1 μM dopamine did not induce feeding movements. Therefore the effects of various dopamine concentrations on the activities of the three CPG subunits were investigated in previously quiescent preparations (i.e., no CPG activity was observed ≥30 s before application). Simultaneous recordings were made from phase 1 motor neurons that are excited by S1 and inhibited by S2, and from phase 3 motor neurons that are inhibited by S2 and excited by S3 (Fig. 3). A dopamine concentration of 0.1 μM was sub-threshold for activation of the buccal CPG (Fig. 3A). At 0.5 μM, dopamine application stimulated activity in all three CPG subunits (Fig. 3B). However, S1 and S3 were coactivated, as indicated by simultaneous bursts of action potentials in the phase 1 and phase 3 motor neurons. S2 was also activated, indicated by the S2-induced inhibitory postsynaptic potentials (IPSPs) observed in both motor neurons. Thus an S1/S3-S2 motor pattern was produced by 0.5 μM dopamine. We have previously observed similar ‘spontaneous’ buccal motor patterns (i.e., in physiological saline) with S1 and S3 interneurons coactive (Quinlan and Murphy 1996). Applications of dopamine ranging from 1 to 3 μM induced variable effects. The S1-S2-S3 fictive feeding motor pattern (Fig. 3C) was induced in 20% of the experiments (n = 15) and an S1/S3-S2-S3 pattern (i.e., simultaneous bursts in S1 and S3, followed by S2 activation and a subsequent S3 burst) was observed in 40%. A mixture of S1-S2-S3 cycles interspersed with S1/S3-S2-S3 cycles occurred in 40% of these experiments. Dopamine concentrations of 5–10 μM evoked...
the S1-S2-S3 fictive feeding pattern in 100% \((n = 18)\) of the experiments \((Fig. 3D)\). Thus the higher concentrations of dopamine entrained S3 activity to follow S2 inhibition, apparently because of a dopamine-induced enhancement of postinhibitory rebound in S3 interneurons \((Quinlan and Murphy 1996; M. Zoran, personal communication)\).

Increasing dopamine concentrations not only changed the qualitative nature of the patterns but also increased the frequency of cyclic activity and the intensity of burst generation. Dopaminergic effects on the cycle frequency were quantified as the number of cycles of S2 activity evoked in the first 30 s after application of dopamine to quiescent preparations. 0.1 μM dopamine evoked 0.60 ± 0.89 (SD) cycles \((n = 5)\); 0.3–0.5 μM dopamine evoked 1.75 ± 1.71 cycles \((n = 4)\); 1.0 μM dopamine evoked 6.37 ± 5.71 cycles \((n = 19)\); and 5–10 μM dopamine \((n = 18)\) evoked 9.39 ± 5.23 cycles of S2 activity during the first 30 s after application. The cycle period observed in 5–10 μM dopamine was similar to feeding rates observed in intact snails \((Arnett 1996)\).

Dopamine antagonist sulpiride specifically blocks the dopamine-induced feeding pattern

In the basommatophoran snail, *Lymnaea*, the dopamine receptor antagonist sulpiride blocked both EPSPs and IPSPs at identified dopaminergic synapses, and blocked the effects of exogenous dopamine \((Magoski et al. 1995)\). Application of sulpiride also blocked the effects of dopamine superfusion on the buccal CPG of *Helisoma* \((Fig. 4)\). A feeding motor pattern, evidenced by phase 1 bursts of action potentials and phase 2 IPSPs in motor neuron B7, was activated by application of dopamine. The addition of 100 μM sulpiride \((Fig. 4A)\), in the continuous presence of dopamine, inhibited activity in both S1 and S2 of the CPG. Motor neuron B7 generated action potentials tonically, and neither S1-induced bursts of action potentials nor S2-induced IPSPs were observed. Sulpiride also blocked the dopamine-induced activity in phase 3 motor neuron B19 \((Fig. 4B)\). In the presence of dopamine, neuron B19 displayed S3-driven bursts of action potentials alternating with S2-induced IPSPs. The addition of 100 μM sulpiride, in the continuous presence of dopamine, first eliminated S2 inhibition, and then S3 excitation, in motor neuron B19.

To determine whether sulpiride was specifically antagonizing the effects of dopamine on the CPG, rather than causing a general disruption of CPG function, we tested the effects of sulpiride on the response to the neuromodulator serotonin. Serotonin application initiates an S2-S3 motor pattern by activating rhythmic plateau potentials in S2 interneurons and by enhancing postinhibitory rebound in phase 3 interneurons \((Quinlan and Murphy 1996; Quinlan et al. 1995)\). Application of serotonin to preparations made quiescent by the continuous presence of sulpiride initiated S2-evoked IPSPs followed by S3 excitation in buccal motor neuron B19. This suggests that the inhibitory effects of sulpiride on buccal CPG activity are due to the specific antagonism of modulatory dopamine receptors.

Identification and morphological characterization of ‘‘dopaminergic’’ neuron NIa

Dopamine initiates the feeding response in *Helisoma*. Putatively dopaminergic neurons were localized in the buccal ganglia to identify candidate feeding modulatory neurons. Immunocytochemistry employing antibodies to dopamine, or to enzymes involved in dopamine synthesis, has had mixed efficacy in molluscan preparations, and often fails to label identified dopaminergic neurons \((Croll and Chiasson 1990)\) (see Discussion). Therefore dopaminergic buccal neurons were localized with a fluorescence histochemical method that employs a mixture of 4% FaGlu to induce fluorophore production in catecholaminergic neurons \((Furness et al. 1977; Goldstein and Schwartz 1989)\). FaGlu histochemistry consistently induced yellow-green fluorescence in ~50 pairs of neurons in the buccal ganglia \((Fig. 5A, n = 10)\). Yellow-green fluorescence also was observed in the giant pedal dopaminergic neuron, but no fluorescence was...
seen in identified serotonergic neurons of *Helisoma* (e.g., neuron C1) (Granzow and Kater 1977). It has been reported previously that epinephrine and norepinephrine levels are low or insignificant in *Helisoma* ganglia (Trimble et al. 1984). Therefore most, if not all, of the neurons exhibiting FaGlu-induced fluorescence in the buccal ganglia of *Helisoma* are hypothesized to be dopaminergic.

FaGlu histochemistry consistently induced yellow-green fluorescence in a moderately sized (~40 μm diam) bilaterally symmetrical pair of neuronal somata on the lateral edges of the dorsal surface of the buccal ganglia. A mirror-image pair of neurons similar in size, shape, and relative soma positions was morphologically and electrophysiologically characterized and named neuron N1a. (Fig. 5, A and B). Iontophoretic injection of the fluorescent dye Lucifer yellow CH revealed the unique morphology of neuron N1a (Fig. 5, C and D). Neuron N1a is a true buccal interneuron with extensive neuritic arbors in both of the paired buccal ganglia but no processes traversing buccal nerve roots or connectives. It has a single unipolar axon that crosses the buccal commissure and terminates in the contralateral buccal ganglion. All physiological studies of neuron N1a were accompanied with dye injections to confirm morphological identity (n > 100).

**Feeding stimulants evoke the feeding motor pattern in part by activating phase 1 interneuron N1a**

Watermelon extract is a potent feeding stimulant in both intact and semi-intact snails (Arnett 1996). In reduced preparations, lacking the circumesophageal ring ganglia, superfusion of the anterior esophagus and the surface of the buccal cavity with watermelon extract initiated the feeding motor pattern (Fig. 6). Neuron N1a generated bursts of action potentials during phase 1 that were terminated by inhibition during phase 2. Thus chemosensory stimulation of the buccal cavity can activate neuron N1a and trigger feeding, via afferents in buccal nerves, in the absence of descending afferents or neuromodulatory inputs from the cerebral ganglia. Chemosensory afferents may have multiple targets in the buccal CPG. In the example shown in Fig. 6, watermelon extract triggered a large IPSP, similar to those evoked by S2, before the first burst of action potentials in neuron N1a, suggesting a direct chemosensory activation of S2. This observation is consistent with the fact that semitransparent juvenile snails, observed and videotaped feeding sporadically, typically retract the odontophore before the first protraction at the beginning of a feeding bout (Arnett 1996).

**Neuron N1a can evoke the feeding motor pattern**

To determine whether depolarization of neuron N1a is sufficient to induce the production of the fictive feeding motor pattern, the electrophysiological activity of identified buccal motor neurons was monitored simultaneously with that of interneuron N1a in quiescent preparations. Depolarization of interneuron N1a excited phase 1 motor neurons and triggered recurrent inhibition by S2 (Fig. 7, n > 10). Therefore activation of interneuron N1a is sufficient to evoke an S1-S2 pattern of CPG subunit activity. To determine whether interneuron N1a could evoke the full triphasic fictive feeding pattern, it was necessary to monitor S3 of the CPG while depolarizing interneuron N1a. Neuron N1a often accommodates during depolarizing current injection before an S1-S2-S3 fictive feeding pattern can be initiated. However, penetration of interneuron N1a with a microelectrode often triggered the fictive feeding motor pattern in previously quiescent preparations (Fig. 8). This was confirmed by phase 1 action potential bursts in neuron N1a, phase 2 IPSPs in both neuron N1a and phase 3 motor neuron B19, and phase 3 bursts of action potentials in neuron B19. Hyperpolarization of interneuron N1a eliminated rhythmic activity in the CPG. The left and right homologues of neuron N1a are electrotonically coupled (data not shown) and thus the activity of both neurons N1a can be inhibited by hyperpolarizing current injected into either the left or right soma. On termination of the hyperpolarizing current, the fictive feeding pattern was reinitiated. Rhythmic activity in S1 and S2 resumed first, and S3 activity appeared after several cycles of S1-S2 activity. This sequence of activation of CPG subunits by interneuron N1a is similar to the sequence observed with increasing concentrations of exogenous dopamine. This sug-
suggests that multiple bursts of action potentials in interneuron N1a may be required to raise dopamine levels sufficiently to entrain S3 activity and evoke the fictive feeding motor pattern. The observation that interneuron N1a stimulates phase 1 motor neurons and activates S2 of the CPG demonstrates that interneuron N1a fulfills the major physiological criteria for an S1 interneuron.

**Neuron N1a is an integral component of the buccal CPG and dopamine can substitute for the effects of N1a activity**

Modulatory neurons that influence the motor pattern produced by a CPG can be extrinsic to the CPG (e.g., Granzow and Kater 1977) or can be an intrinsic component of the CPG (e.g., Katz and Frost 1995; Katz et al. 1994). To assess whether neuron N1a is a component of the buccal CPG, the ability of neuron N1a to reset the phase of ongoing rhythmic buccal motor neuron patterns was examined. Spontaneous rhythmic activity in normal physiological saline was observed in neuron N1a and an S1 motor neuron, with a regular cycle frequency of 0.2 Hz (Fig. 9A). Inhibition of the activity of neurons N1a, via a short-duration hyperpolarizing current pulse injected into the soma of a single neuron N1a, reset the phase of the ongoing rhythmic activity (n = 4). The interburst interval exhibited by the S1 motor neuron was similar before and after the current injection.

Dopamine, however, can compensate for the absence of neuron N1a activity in maintaining a fictive feeding motor pattern. Dopamine superfusion of a rhythmically active preparation produced an increase in the frequency of ongoing activity recorded from neuron N1a and an S1 motor neuron (Fig. 9B). Hyperpolarization of interneuron N1a in the presence of exogenous dopamine had little, if any, effect on the pattern of rhythmic activity. The ability of hyperpolarizations of neuron N1a to reset the phase of ongoing buccal motor neuron activity in the absence, but not the presence, of dopamine suggests that neuron N1a is an integral component of S1 of the buccal CPG, and that the modulatory effects of neuron N1a on the buccal CPG are mediated by dopamine.

**Dopamine antagonist sulpiride blocks the neuron N1a-induced activation of S2**

To test the hypothesis that interneuron N1a is dopaminergic, the efficacy of the dopamine antagonist sulpiride for blocking the effects of interneuron N1a was tested. These studies were complicated by the accommodation of interneuron N1a during prolonged depolarization. For example, in Fig. 7 the action potential frequency during the first 0.5-s interval of the depolarization was 24 Hz. During the last 0.5-s interval of the depolarization the action potential frequency had fallen to 16 Hz. To avoid the complication caused by accommodation to depolarizing current, trains of hyperpolarizing current pulses were used to generate bursts of action potentials in neuron N1a on anode break, in the absence and presence of the dopamine antagonist sulpiride (Fig. 10). The activation of interneuron N1a in physiological saline triggered activity in S2 of the CPG, as indicated by the recurrent S2-evoked IPSPs in neuron N1a. Application of sulpiride blocked the ability of interneuron N1a to activate S2 (n = 5 preparations). In an exemplary preparation (Fig.
DOPAMINERGIC INTERNEURON MODULATES FEEDING CPG

FIG. 6. Chemosensory stimulation of oral cavity triggered rhythmic activity in interneuron N1a and activated feeding motor program. Watermelon extract, a potent feeding stimulant, was superfused over epithelial lining of buccal cavity of quiescent reduced preparation (arrowhead). Feeding motor pattern was induced, as evidenced by production of rhythmic bursts of action potentials in interneuron N1a during phase 1, phase 2 IPSPs in both neurons N1a and B19, and bursts of action potentials in neuron B19 during phase 3. Note that S2-evoked IPSPs occurred before 1st burst of action potentials in interneuron N1a, indicating multiple sites of chemosensory input to CPG.

10), in physiological saline anode-break-induced bursts of action potentials in neuron N1a activated S2 in 89% of the trials (n = 9). Application of sulpiride (100 μM) completely blocked the ability of anode-break-induced action potential bursts in neuron N1a to activate S2 following termination of identical current pulses (n = 12). On return to physiological saline, the ability of interneuron N1a to activate S2 was immediately restored, demonstrating that the activation of S2 by interneuron N1a is dependent, either directly or indirectly, on the activation of dopamine receptors.

FaGlu-induced fluorescence characteristic of dopamine in the physiologically characterized interneurons N1a

To further demonstrate that buccal interneuron N1a is dopaminergic, the FaGlu histochemical staining procedure for catecholamines was combined with intracellular dye injections into electrophysiologically characterized interneurons N1a (n = 4). Following intracellular recordings, the dye reactive red No. 4 was pressure injected into the somata of interneurons N1a, and buccal ganglia were subjected to the FaGlu histochemical staining procedure (Fig. 11). FaGlu-induced fluorescence, indicative of the presence of dopamine, was revealed in the dye-injected interneurons N1a.

DISCUSSION

These data demonstrate that dopamine plays a major role in the organization of the consummatory feeding response in Helisoma. The dopaminergic interneuron N1a is stimulated by chemosensory afferents arriving from the oral cavity and anterior esophagus via buccal nerve roots. Interneuron N1a is a component of S1 of the buccal CPG and modulates the multifunctional CPG to generate the fictive feeding motor pattern. A comparative analysis of the literature on gastropod feeding suggests that a homologous dopaminergic modulatory pathway may be a general feature in the organization of the consummatory feeding response.

Dopamine may be a common regulator of gastropod feeding

Dopamine has been implicated in the control of feeding in basommatophoran and stylomatophoran pulmonates, as

FIG. 7. Depolarization of interneuron N1a excited phase 1 motor neurons and activated subunit 2 of CPG. In quiescent preparation, depolarizing current pulse injected into interneuron N1a evoked bursts of action potentials that excited S1 follower motor neuron (B6 or B7, morphology not confirmed). Stimulation of interneuron N1a also triggered rhythmic S2 activity, as evidenced by phase 2 IPSPs observed in both neuron N1a and phase 1 motor neuron. Vertical calibration bar: 20 mV (top trace); 40 mV (bottom trace).

FIG. 8. Interneuron N1a activated feeding motor pattern. Activity of buccal CPG was inhibited by injection of hyperpolarizing DC (1 nA) into interneuron N1a, before onset of record shown. On release from current injection, interneuron N1a generated rhythmic bursts of action potentials during phase 1, which were terminated by phase 2 IPSPs. Simultaneous S2-evoked IPSPs also were observed in phase 3 motor neuron B19. Generation of triphasic fictive feeding pattern, with distinct burst of action potentials in motor neuron B19 during phase 3, occurred only following sustained activity in interneuron N1a. Injection of hyperpolarizing current (H) into interneuron N1a again eliminated rhythmic CPG activity. Burst of action potentials in neuron B19 at beginning of record was initiated before neuron N1a was released from hyperpolarization and was similar to other spontaneous bursts of unknown origin that occurred in this neuron B19 with intervals of 18–25 s. One minute was excised from record (- -).

FIG. 9. Interneuron N1a activated feeding motor pattern. Activity of buccal CPG was inhibited by injection of hyperpolarizing DC (1 nA) into interneuron N1a, before onset of record shown. On release from current injection, interneuron N1a generated rhythmic bursts of action potentials during phase 1, which were terminated by phase 2 IPSPs. Simultaneous S2-evoked IPSPs also were observed in phase 3 motor neuron B19. Generation of triphasic fictive feeding pattern, with distinct burst of action potentials in motor neuron B19 during phase 3, occurred only following sustained activity in interneuron N1a. Injection of hyperpolarizing current (H) into interneuron N1a again eliminated rhythmic CPG activity. Burst of action potentials in neuron B19 at beginning of record was initiated before neuron N1a was released from hyperpolarization and was similar to other spontaneous bursts of unknown origin that occurred in this neuron B19 with intervals of 18–25 s. One minute was excised from record (- -).
Dopamine application evokes the feeding motor pattern and consequent feeding behavior in Helisoma

The large repertoire of motor neuron activity patterns produced by the multifunctional buccal CPG of Helisoma is mirrored by the diversity in observed oral behaviors (Arnett 1996; Quinlan and Murphy 1996). Functional feeding movements (i.e., the cyclic protraction, retraction, and hyperretraction of the buccal odontophore) are produced when the CPG subunits are active in the S1-S2-S3 sequence (Arnett 1996). Dopamine initiates the fictive feeding motor pattern in the isolated CNS and evokes feeding behavior in semi-intact preparations. Previous reports had demonstrated that dopamine increased the firing rate of a phase 3 motor neuron, and increased the frequency of spontaneously occurring rhythmic activity in isolated buccal ganglia of Helisoma (Trimble and Barker 1984; Trimble et al. 1984). At that time, phase 1 protractor motor neurons had not been identified, and the feeding motor pattern was only partially characterized.

Localization of dopamine and putatively dopaminergic neurons in the CNS of Helisoma and other gastropods

Several techniques have been used to localize catecholamines to molluscan ganglia and to specific gastropod neu-

![Image of interneuron N1a](http://jn.physiology.org/)

**FIG. 9.** Interneuron N1a is component of buccal CPG and its effects are mimicked by exogenous DA. A: spontaneous rhythmic phase 1 action potentials and phase 2 IPSPs were observed in interneuron N1a and S1 motor neuron (B6/7/8, morphology not confirmed) in normal physiological saline (NS). Injection of brief hyperpolarizing current (0.3 nA) into interneuron N1a reset phase of ongoing rhythmic activity. B: exogenous DA substituted for effects of interneuron N1a on buccal CPG. Following DA superfusion (10 μM), injection of same hyperpolarizing current into same interneuron N1a had little effect on rhythmic activity observed in S1 motor neuron. Vertical calibration bar: 20 mV (top traces); 40 mV (bottom traces).

well as opisthobranchs. In addition to its effects in Helisoma, dopamine application reversibly stimulated rhythmic activity in buccal motor neurons, or increased the rate of spontaneous activity in the buccal ganglia, in the related basommatophoran snail Lymnaea stagnalis (Kyriakides and McCrohan 1989). The pattern of motor neuron activity induced by dopamine appeared similar to the triphasic buccal motor pattern reported to be associated with feeding in Lymnaea. However, some motor neurons active during the feeding motor pattern were inhibited by dopamine application. In the stylommatophoran terrestrial slug, Limax maximus, dopamine applied to the cerebral and buccal ganglia triggered a fictive feeding motor program measured by extracellular suction electrodes on peripheral buccal nerve roots (Wieland and Gelperin 1983). The motor program elicited by superfusion of dopamine was indistinguishable from the pattern elicited by chemostimulation of the lips in a semi-intact preparation (intact afferent neural connections between the lips and the CNS, but no buccal mass). The effects of dopamine in Limax maximus were mimicked by the vertebrate D1 dopamine receptor agonist 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydrodronaphthalene and blocked by the dopamine antagonist ergovine maleate. In the opisthobranch Aplysia, application of dopamine or the metabolic precursor L-DOPA to the cerebral (Rosen et al. 1991; Teyke et al. 1993) or buccal ganglia (Kabotyanski et al. 1994) activated patterned buccal motor activity. It is unclear whether these dopamine-induced motor patterns mediate feeding, but semi-intact preparations perfused with L-DOPA produced rhythmic radular movements suggestive of feeding (Kabotyanski et al. 1994).

**FIG. 10.** The DA antagonist sulpiride blocked ability of interneuron N1a to stimulate activity in S2 of buccal CPG. In quiescent preparations in normal physiological saline, trains of hyperpolarizing current pulses (2.5 s, 2 nA) were used to generate bursts of action potentials in interneuron N1a via postinhibitory rebound (i.e., anode break). Bursts of action potentials generated in neuron N1a stimulated S2 activity, evidenced by recurrent S2-evoked IPSPs (2) observed in interneuron N1a. After superfusion with 100 μM sulpiride (bottom trace), identical hyperpolarizing current pulses continued to trigger similar bursts of action potentials but these bursts no longer activated S2. In this preparation action potential frequency (measured during 1.0-s interval on termination of hyperpolarizing pulse) ranged from 26 to 30 Hz in physiological saline and from 23 to 27 Hz in presence of sulpiride.
rons. Monoamines were first identified in molluscan CNS by Sweeney (1963) with the use of fluorimetric and paper chromatographic techniques. Substantial amounts of dopamine, but little or no trace of norepinephrine or epinephrine, were found in ganglia of 10 different molluscan species. More recently, high-performance liquid chromatographic analysis yielded measurements of 8 pmol dopamine per paired buccal ganglia and 90 pmol dopamine per circumsophageal ring ganglia of Helisoma (Gadotti et al. 1986), 10 pmol dopamine/buccal ganglia in Limax (Wieland and Gelperin 1983), and 25 pmol dopamine/buccal ganglia in Lymnaea (Elekes et al. 1991). Radioactive precursors used to examine the synthesis of dopamine in the CNS of Helisoma demonstrated that neurons of the buccal, cerebral, and pedal ganglia synthesized \(^{3}H\)-dopamine but not \(^{3}H\)-norepinephrine after incubation in \(^{3}H\)-tyrosine (Trimble et al. 1984). Tritiated dopamine synthesis was revealed in all buccal nerve roots except the cerebrobuccal connectives, the only connections between the bucail ganglia and the central circumsophageal ganglia. This suggested the possibility of a dopaminergic system intrinsic to the buccal ganglia.

Immunocytochemistry (e.g., Elekes et al. 1991), as well as the Falck-Hillarp (Falck et al. 1962; Trimble et al. 1984), glyoxylylate (DeLaTorre and Surgeon 1976; Kabotyanski et al. 1994; Trimble et al. 1984; Wieland and Gelperin 1983), and FaGlu (Croll and Chiasson 1990; Furness et al. 1977; Goldstein and Schwartz 1989; Teyke et al. 1993) histochemical techniques, has indicated the presence of catecholaminergic neurons in the buccal ganglia of several gastropods. We employed the FaGlu histochemical technique, which induces autofluorescence in catecholaminergic neurons after incubation in a combination of 4% paraformaldehyde and 0.5% glutaraldehyde, because it is both highly sensitive and quite specific for dopamine in Helisoma. Because the tissue is fixed by the same mixture that produces the fluorescence, there is good preservation of both the aldehyde-induced fluorescence and of staining produced by procedures preceding exposure to the FaGlu mixture.

In an early study in which FaGlu histochemistry was used in whole mounts of guinea pig myenteric and submucosal plexuses, relatively nonspecific fluorescence was found in adrenergic, noradrenergic, dopaminergic, and serotonergic neurons, but not in neurons known to contain histamine (Furness et al. 1977). In Lymnaea, FaGlu histochemistry produced green-yellow fluorescence in the same cells that fluoresced when the glyoxylic acid technique was used to localize catecholaminergic neurons. Occasionally, FaGlu induced fluorescence in known serotonergic neurons (identified by immunocytochemical or other histochemical methods), but the yellow-brown fluorescence induced in serotonergic neurons was easily distinguished from the green-yellow fluorescence induced in catecholaminergic neurons (R. Croll, personal communication).

In Helisoma, the FaGlu histochemical method did not label identified serotonergic neurons (e.g., the giant cerebral neuron C1) but did label the giant pedal dopaminergic neuron. Because epinephrine and norepinephrine levels have been reported to be low or insignificant (Trimble et al. 1984), it is presumed that most, if not all, of the buccal neurons that exhibit FaGlu-induced fluorescence are dopaminergic. In addition, FaGlu histochemistry was more sensitive than other methods in Helisoma. FaGlu induced fluorescence in ~50 pairs of buccal neurons, more than were localized when the glyoxylic acid procedure (Trimble et al. 1984) or antidopamine immunocytochemistry (Murphy, unpublished data) was used. In addition, FaGlu induced fluorescence in axons in the cerebrobuccal connectives that had not been detected with the glyoxylyte staining procedure.

In contrast, FaGlu histochemistry induced autofluorescence in only four pairs of neurons on the dorsal surface of the buccal ganglia in Lymnaea (Croll and Chiasson 1990). A similar staining pattern was obtained with the glyoxylic acid histochemical procedure and with antidopamine immunocytochemistry (Elekes et al. 1991). In our hands, the FaGlu histochemical technique labeled a similarly small number of cells of the buccal ganglia of Lymnaea in comparison with Helisoma. FaGlu also stained relatively few neurons in the buccal ganglia of Aplysia (Teyke et al. 1993). In Helisoma a number of small, FaGlu histofluorescent somata often were observed in buccal nerve roots, especially the esophageal nerve trunk that innervates the foregut (data not shown). Ganglionic neurons migrate into the ganglia from the periphery during gastropod development. Thus Helisoma buccal ganglia may contain a population of small dopaminergic neurons whose homologues in Lymnaea and Aplysia may remain in the peripheral nerve plexus. Consistent with this suggestion, it has been demonstrated that the buccal ganglia of Aplysia receive an abundant catecholaminergic input from the foregut (Susswein et al. 1993).
Evidence that interneuron N1a in Helisoma is dopaminergic

Although it is difficult to rigorously demonstrate all the original criteria for the identification of a neurotransmitter at a specific synapse (Paton 1958), we present the following substantial evidence that interneuron N1a is dopaminergic. 1) The presence of dopamine in interneuron N1a was indicated by observing FaGlu-induced fluorescence in electrophysiologically characterized, dye-injected interneurons N1a. 2) The effects of depolarization of interneuron N1a on the buccal CPG were mirrored by exogenous dopamine. 3) The effects both of depolarization of interneuron N1a and of exogenous dopamine were blocked by the dopamine antagonist sulpiride. Together these data suggest that interneuron N1a is dopaminergic.

Comparison of interneuron N1a with potential homologues in other gastropod buccal CPGs

We have previously suggested that the neural basis of feeding is fundamentally similar in gastropods that feed by grasping or rasping movements of a radula (Quinlan and Murphy 1996). In support of this hypothesis, a buccal interneuron similar in many respects to Helisoma interneuron N1a has been described in Lymnaea (Yeoman et al. 1995). Depolarization of this neuron, called neuron N1L, stimulated the production of the triphasic feeding motor pattern in Lymnaea. Interneuron N1L also excited some identified phase 1 interneurons and motor neurons in the Lymnaea buccal ganglion. Neuron N1L exists as a bilateral pair of mirror-image homologues with somata near the lateral edges of the buccal ganglia. They project a single unipolar axon to the contralateral buccal ganglion. Thus neurons N1a of Helisoma and N1L of Lymnaea are very similar in morphology and physiology.

Despite these similarities, it is still unclear whether neuron N1L in Lymnaea and neuron N1a in Helisoma are true homologues, in part because of differences in their reported neurotransmitters. Action potentials in neuron N1L in Lymnaea evoked one-for-one fast EPSPs superimposed on a slow depolarization in follower cells. The fast EPSPs were inhibited by the “cholinergic antagonists” d-tubocurarine and hexamethonium (Vehovszky and Elliott 1995). However, d-tubocurarine also blocks some identified dopaminergic synapses in gastropods (Ascher 1972; Berry and Cottrell 1975). No effective antagonists for the slow depolarization were reported, but two dopamine antagonists, fluphenazine and ergometrine (i.e., ergonovine), were ineffective at blocking the slow depolarization. This observation prompted the authors to conclude that neuron N1L in Lymnaea was not dopaminergic. However, there is considerable diversity in the pharmacology of molluscans dopamine receptors, and neither ergometrine nor fluphenazine were tested as antagonists against the effects of exogenous dopamine in the buccal ganglia of Lymnaea. Ergometrine blocks dopaminergic effects in the buccal ganglia of Limax (Wieland and Gelperin 1983) and Aplysia (Teyke et al. 1993), but is less effective than sulpiride in Helisoma (data not shown). Sulpiride, but not ergometrine or fluphenazine, was an effective antagonist at identified synapses of the giant pedal dopamine cell (RpeD1) in Lymnaea (Magoski et al. 1995).

We have shown that sulpiride blocks the effects both of exogenous dopamine and of depolarization of interneuron N1a on the buccal CPG of Helisoma. In addition, electrophysiologically characterized neurons N1a exhibited yellow-green fluorescence following FaGlu histochemistry, indicative of the presence of dopamine. Dopamine-immunoreactive somata have also been located in the position of interneuron N1L in the Lymnaea buccal ganglia (Elekes et al. 1991). It remains possible that interneuron N1a in Helisoma and interneuron N1L in Lymnaea are true homologues that utilize both dopamine and acetylcholine as neurotransmitters. Analysis of cholinergic antagonists in Helisoma and of other dopaminergic antagonists (i.e., sulpiride) in Lymnaea are necessary to resolve this issue.

Interneurons involved in feeding that are potentially homologous to interneuron N1a of Helisoma also have been identified in opisthobranchs. In Aplysia, two putatively dopaminergic buccal interneurons (B20 and B65) can evoke buccal motor patterns (Kabotyanski et al. 1994; Teyke et al. 1993). Neuron B20 seems unlikely to be homologous to Helisoma neuron N1a on the basis of its morphology and soma position (Teyke et al. 1993). The soma of neuron B20 is adjacent to the buccal commissure and it projects an axon into the contralateral cerebrobuccal connective. On the other hand, the somata of interneurons B65 are on the lateral edges of the buccal ganglia, and they project axons that arborize and terminate in the contralateral buccal ganglion (Kabotyanski et al. 1994). Stimulation of interneuron B65 evoked patterned buccal neuronal activity similar to that evoked by dopamine application. Thus the morphology, relative soma position, and physiological effects of Aplysia interneuron B65 appear similar to those of interneuron N1a in Helisoma. A buccal protractor interneuron with similar morphology to Helisoma neuron N1a, Lymnaea neuron N1L, and Aplysia neuron B65 also has been identified in Clione (Arshavsky et al. 1989). We hypothesize that these buccal interneurons are homologous, and that similar dopaminergic neurons with major roles in organizing the consummatory response will be found in other gastropods.

Roles of interneuron N1a in mediation of feeding in Helisoma

Our data suggest that interneuron N1a couples sensory information provided by feeding stimulants in the buccal cavity to the generation of the feeding consummatory response. Food applied to the oral cavity and esophagus in semi-intact preparations activates neuron N1a and the feeding response. Activation of interneuron N1a by food in the buccal cavity must occur via afferents in buccal nerves, because the response is seen in the absence of the remainder of the CNS. Because the lip afferents indirectly affect the buccal CPG via pathways through the cerebral ganglia, sensory inputs from the external lips are not required for the consummatory response. Sensory afferents from the external lips stimulate identified giant serotonergic cerebral neurons that are apparently homologous in many gastropods (e.g., Croll 1986; Granzow and Rowell 1981). Activation of these serotonergic neurons has been associated with behavioral arousal and an enhancement of the feeding neuromuscular system at many levels (e.g., Kupfermann and Weiss 1982;
McCrohan and Audesirk 1987). In Lymnaea, stimulation of the external lip afferents with sucrose in lip-brain preparations activated a nitric-oxide-dependent pathway that evoked variable buccal motor patterns, in part by stimulating the large serotoninergic cerebral neuron C1 (Elphick et al. 1995). This suggests that in rasping and grasping radular-feeding gastropods, external lip afferents trigger "feeding arousal" and some components of the feeding response via nitric-oxide- and serotonin-dependent pathways through the cerebral ganglia. Subsequently, food stimuli in the buccal cavity activate the full consummatory response via afferents reaching the buccal ganglia through buccal nerve roots and acting, in part, by stimulating dopaminergic interneurons, such as interneuron N1a in Helisoma and homologous neurons in other gastropods.

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