Phase-Locked Coordination Between Two Rhythmically Active Feeding Structures in the Mollusk *Clione limacina*. I. Motor Neurons

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MALYSHEV, ALEKSEY Y. AND TIGRAN P. NOREKIAN. Phase-locked coordination between two rhythmically active feeding structures in the mollusk *Clione limacina*. I. Motor neurons. *J Neurophysiol* 87: 2996–3005, 2002; 10.1152/jn.00882.2001. Coordination between different motor centers is essential for the orderly production of all complex behaviors, in both vertebrates and invertebrates. The current study revealed that rhythmic activities of two feeding structures of the pteropod mollusk *Clione limacina*, radula and hooks, which are used to extract the prey from its shell, are highly coordinated in a phase-dependent manner. Hook protraction always coincided with radula retraction, while hook retraction coincided with radula protraction. Thus hooks and radula were always moving in the opposite phases, taking turns grabbing and pulling the prey tissue out of the shell. Identified buccal ganglia motor neurons controlling radula and hooks protraction and retraction were rhythmically active in the same phase-dependent manner. Hook protractor motor neurons were active in the same phase with radula retractor motor neurons, while hook retractor motor neurons burst in phase with radula protractor motor neurons. One of the main mechanisms underlying the phase-locked coordination was electrical coupling between hook protractor and radula retractor motor neurons. In addition, reciprocal inhibitory synaptic connections were found between hook protractor and radula protractor motor neurons. These electrical and inhibitory synaptic connections ensure that rhythmically active hooks and radula controlling motor neurons are coordinated in the specific phase-dependent manner described above. The possible existence of a single multifunctional central pattern generator for both radula and hook motor centers is discussed.

INTRODUCTION

Specific coordination between activities of motor neurons controlling different aspects of the same complex behavior is vital for producing a functionally meaningful behavioral act and achieving the required goal. Such coordination ensures the orderly production of complex behaviors and represents an important and universal principle of the CNS functioning in both vertebrate and invertebrate animals. In the current study, we investigated how rhythmic activities of the two major feeding structures of the carnivorous pteropod mollusk *Clione limacina* are coordinated during complex feeding behavior and studied the neuronal mechanisms of this coordination.

*Clione* is a highly specialized carnivore that feeds on only two species of shelled pteropod mollusks of the genus *Limacina* (Lalli 1970; Lalli and Gilmer 1989; Wagner 1885). To extract the soft body of *Limacina* from its shell, *Clione* uses two specialized feeding structures, chitinous hooks and the radula, which pull the prey out of the shell to be swallowed whole (Lalli 1970; Lalli and Gilmer 1989; Wagner 1885). Chitinous hooks, the toothed radula, and muscles controlling their movements comprise the muscular buccal mass (Fig. 1). The radula is a feeding structure found in all gastropod mollusks. The functional role of its rhythmic movements, which consist of the protraction and retraction phases, is to grab the food and bring it to the opening of the esophagus. Chitinous hooks, which normally are retracted inside two symmetrical muscular hook sacs, are unique to *Clione* and other mollusks from the order Gymnosomata and reflect a high food specialization (Lalli 1970; Lalli and Gilmer 1989). The functional role of the hooks is to grab the soft tissue of *Limacina* and pull it out of the shell into the buccal cavity. Hook activity is also rhythmic and consists of protraction and retraction phases.

We report here that the rhythmic movements of the radula and hooks are highly coordinated in the phase-dependent manner. This phase-dependent coordination was observed on the behavioral level and was also always present on the motor neuronal level during both spontaneous and induced rhythmic activity. Both hooks and radula movements are controlled by neurons located in the small buccal ganglia attached to the buccal mass (Fig. 1). The first and only observation of the rhythmically active neurons in the buccal ganglia, although without a clear distinction between radula and hook rhythmic neurons, was made by Arshavsky et al. (1989). We have identified electrophysiologically and morphologically specific motor neurons from four major functional groups controlling radula and hooks protraction and retraction movements. Neurons from different groups demonstrated coordinated phase-locked rhythmic activity. Electrical and reciprocal inhibitory connections between motor neurons from different groups are suggested to underlie this coordination. The possible existence of a single multifunctional central pattern generator for both radula and hook motor centers is discussed.

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Intracellular recordings from individual neurons were made with glass microelectrodes (resistance 10–30 MΩ) filled with 2 M potassium acetate. Electrophysiological signals were amplified, displayed, and recorded using conventional electrophysiological techniques. Intracellular stimulation was achieved via an amplifier bridge circuit. Electrotonic coupling was demonstrated by applying depolarizing or hyperpolarizing square current pulses to one cell and recording similar but attenuated responses in other recorded neurons at the same time.

To test for monosynaptic connections, a high divalent cation solution was used (in mM: 110 MgCl₂, 25 CaCl₂, 400 NaCl, 10 KCl, and 3 NaHCO₃, pH 7.4). An extracellular suction electrode filled with seawater was used to stimulate the cerebro-buccal connective in reduced preparations. Each stimulus had duration of 0.5–1 s and intensity of 2–3 V. For morphological investigation of recorded neurons, a 5% solution of 5(6)-carboxyfluorescein (Sigma) prepared in 2 M potassium acetate was iontophoresed via the recording electrodes (resistances, 20–40 MΩ) with 0.3- to 2-nA negative current pulses for 5–30 min. Injected cells were observed live in the recording dish either with a Nikon (Tokyo, Japan) epifluorescence microscope equipped with filters for viewing fluorescein epifluorescence or a BioRad (Hercules, CA) MRC 600 laser scanning confocal microscope.

We used cross-correlation analysis to quantify the phase relationship between motor neurons from different functional groups. Each trace of intracellular records with rhythmic spike activity was converted into spike density function (SDF) by counting the number of spikes in each 200-ms interval. Then cross-correlation coefficient was calculated between SDF of two analyzed neurons using correlation function built in MS Excel. When one of the neurons was not spiking but still received prominent rhythmic inputs, we averaged membrane potential during each 200-ms interval and calculated cross-correlation coefficient between SDF in spiking neuron and averaged membrane potential function (AMPF) in nonspiking neuron. Cross-correlation coefficients from several pairs of neurons were presented as means ± SE.

RESULTS

Coordination between radula and hooks rhythmic movements

Spontaneous rhythmic radula protractions and retractions were frequently observed in the reduced preparations. Hooks were less active, although episodes of spontaneous rhythmic activity consisted of hooks partial protraction and retraction were sometimes seen together with radula movements. In quiescent preparations, rhythmic radula and hooks protraction and retraction was easily induced by electrical stimulation of the cerebro-buccal connective. This stimulation was very effective and induced in all preparations a train of rhythmic movements of both radula and hooks (n = 12). In all preparations, during episodes of spontaneous and induced rhythmic activities, a strict phase-dependent coordination was observed between rhythmic movements of the hooks and radula. Hook protraction always coincided with radula retraction, and hook retraction coincided with radula protraction (Fig. 2). The cross-correlation coefficient between the radula and hooks movements was −0.72 ± 0.17 (mean ± SE, n = 5). Thus hooks and radula always moved in functionally opposite directions with their rhythmic activity locked in anti-phase.

Identification of hook and radula controlling motor neurons

We identified a group of neurons in the caudal part of the buccal ganglia, on both dorsal and ventral sides, whose firing produced protraction of the ipsilateral hooks (Fig. 3). These cells were designated Buccal Hook Protractor (Bc-HP) neurons. A total of 83 Bc-HP neurons were recorded in 36 prep-
Each buccal ganglion contained between 7 and 10 bilaterally symmetrical Bc-HP neurons, which had cell bodies 30–50 μm in diameter. Carboxyfluorescein staining revealed that each Bc-HP neuron had one axon, which exited the buccal ganglia into the ipsilateral hook nerve and innervated the ipsilateral muscular hook sac (n = 11; Fig. 4A). The Bc-HP neurons were normally silent when hooks were quiescent. During induced rhythmic activity of the hooks, the Bc-HP neurons fired rhythmically in the hook protraction phase. Intracellular stimulation of a Bc-HP neuron produced protraction of the ipsilateral hooks, which persisted in high divalent solution (Fig. 5A). Even a single spike in a Bc-HP neuron induced a noticeable protraction response in the ipsilateral hooks. In addition to the prominent protraction of the ipsilateral hooks, strong induced bursts of spikes in a Bc-HP neuron also induced radula retraction and protraction of the contralateral hooks (n = 24). All recorded Bc-HP neurons were electrically coupled with coupling coefficients ranging between 0.1 and 0.2 (0.16 ± 0.03; n = 12).

Only one Buccal Hook Retractor (Bc-HR) neuron has been identified in the right buccal ganglion (n = 32 preparations). The Bc-HR neuron had a cell body 30 μm in diameter, which was located on the medial side of the ganglion under the commissure (Fig. 3). Carboxyfluorescein staining revealed that the Bc-HR neuron projected two main axons into the ipsilateral and contralateral hook nerves and produced extensive branching in the caudal part of both hook sacs (n = 9; Fig. 4B). The Bc-HR neuron rhythmic bursts coincided with hook retraction episodes before hook protraction. The Bc-HR neurons were also active when hooks were constantly retracted without demonstrating any rhythmic movements. When hooks were partially protracted, for example, after stimulation of the Bc-HP neurons, stimulation of a Bc-HR neuron always elicited complete retraction of ipsilateral and contralateral hooks, which persisted in high divalent solution (Fig. 5B).

A group of five or six Buccal Radula Protractor (Bc-RP) neurons was identified in the ventrolateral part of each buccal ganglion (Fig. 3). The neurons were small with soma diameters of 15–20 μm. Intracellular carboxyfluorescein staining revealed that each Bc-RP neuron projected a single axon into the unpaired medial radula nerve m1, which innervates the radula and muscles controlling its movements (n = 6; Fig. 4D). When the radula was quiescent, the Bc-RP neurons were silent.

During spontaneous or induced radula movements, the Bc-RP neurons fired rhythmically in the radula protraction phase. Intracellular stimulation of a single Bc-RP neuron induced radula protraction, which persisted in high divalent solution (n = 22). All Bc-RP neurons were electrically coupled with coupling coefficients ranging between 0.1 and 0.2 (0.17 ± 0.05; n = 9).

Three bilaterally symmetrical clusters of Buccal Radula Retractor (Bc-RR) neurons were identified on both dorsal and ventral surfaces of the buccal ganglia (Fig. 3). Two small clusters were located in the ventromedial area above and below the commissure, and one cluster was found in the dorsal-medial area. A total of 52 neurons were recorded in 28 preparations. All Bc-RR neurons sent their axons to the radula via the ipsilateral radula nerve m2 (n = 7; Fig. 4C). Bursting rhythmic activity of the Bc-RR neurons always correlated with the radula retraction phase of the feeding rhythm. Intracellular stimulation of the Bc-RR neurons produced radula retraction movement, which persisted in high divalent solution (n = 21). Electrical coupling was found between all Bc-RR neurons, within each cluster and between different clusters, with coupling coefficients ranging between 0.15 and 0.25 (0.21 ± 0.05; n = 9).

Rhythmic activity of the radula and hook motor neurons

During the episodes of spontaneous or induced “feeding” activity in the buccal mass, which included rhythmic protraction-retraction movements of both radula and hooks, the following phase-dependent coordination between the rhythmic activities of identified motor neurons was always observed.
The Bc-RP neurons and Bc-RR neurons burst, as expected, in opposite phases. Bursts of spikes in Bc-RP neurons always coincided with the inhibitory episodes in the Bc-RR neurons, and inhibition in Bc-RP neurons coincided with firing in Bc-RR neurons (Figs. 6 and 7). At the same time, Bc-HR neurons always burst in phase with the Bc-RP neurons (Fig. 6), and Bc-HP neurons fired in phase with Bc-RR neurons (Fig. 7). Synchronization between the rhythmic activities of the Bc-HP neurons and Bc-RR neurons was very high, with their bursts occurring simultaneously and having similar duration ($n = 11005; 32$; Fig. 8). Cross-correlation coefficient between Bc-HP and Bc-RR neurons was $0.81 \pm 0.12 (n = 7)$. Cross-correlation coefficient between Bc-HR and Bc-RP neurons was $0.54 \pm 0.17 (n = 7)$. Bursts of spikes in the Bc-HR neurons and Bc-RP neurons occurred in the same phase; however, a slight phase-shift was always observed between neurons during high-speed recording (Fig. 9A). The Bc-HP neuron bursts often ended $0.1-0.5$ s after the bursts in the Bc-RR, and Bc-HP neurons were inhibited, thus creating a brief period of coactivation of the Bc-RR and Bc-HP neurons ($n = 24$; Fig. 9B). Duration of the Bc-HP and Bc-RR neurons coactivation was $0.42 \pm 0.17$ s ($n = 8$). This coactivation may be important for producing a fast and powerful protraction movement of the hooks. Thus the four rhythmically active motor neuron groups that control radula and hook protraction and retraction burst in two phases. One phase included simultaneous activation of the Bc-RR and Bc-HP neurons, while Bc-RP and Bc-HR neurons were inhibited (Fig. 10). During the second phase, Bc-RP and Bc-HR neurons were activated, while Bc-RR and Bc-HP neurons were inhibited (Fig. 10). This phase-dependent coordination between rhythmic activities of the hook and radula controlling motor neurons was always observed during spontaneous or induced “feeding” activity that included rhythmic movements of both the hooks and radula. However, the radula was usually more active than the hooks, and there were frequent episodes of rhythmic radula movements with quiescent hooks. Intracellular recording revealed that during such episodes, Bc-HP neurons did not spike, although they still received prominent rhythmic inputs in the same phase-dependent manner (Fig. 11). These rhythmic subthreshold depolarizing inputs in the Bc-HP neurons coincided with bursts of spikes in the Bc-RR neurons ($n = 12$). The cross-correlation coefficient between Bc-HP neurons (AMPF) and Bc-RR neurons (SDF) was $0.65 \pm 0.21 (n = 5)$. The Bc-HR neurons received phase-dependent rhythmic inputs and spiked during these episodes of active radula movements and quiescent hooks ($n = 7$).

**Interactions between radula and hook motor neurons**

Strong electrical coupling was always observed between Bc-HP and Bc-RR neurons, which rhythmically fired in the same phase ($n = 21$; Fig. 8B). Coupling coefficients ranged between $0.15$ and $0.25 (0.19 \pm 0.05; n = 7)$. The Bc-HR and Bc-RP neurons, which also fired in the same phase, but had a
slight phase-shift that varied from one episode to another, were not electrically coupled \((n = 18)\).

In addition to electrical connections, buccal neurons from different functional groups produced multiple chemical inhibitory connections between each other. Induced bursts of spikes in the Bc-RP neurons produced inhibitory inputs to the Bc-RR neurons \((n = 4; \text{Fig. } 12A)\). In turn, stimulation of the Bc-RR neurons induced inhibition of the Bc-RP neurons \((n = 3; \text{Fig. } 12A)\). The Bc-HP neurons induced inhibition of the Bc-HR neurons \((n = 4; \text{Fig. } 12B)\) and Bc-RP neurons \((n = 7; \text{Fig. } 12C)\). The Bc-RP neurons, in turn, inhibited Bc-HP neurons \((n = 4)\). All these inhibitory connections were polysynaptic since they were easily blocked by high divalent solution.

**Discussion**

Functional role of a phase-locked coordination between hooks and radula movements

In *Clione limacina*, feeding behavior following prey capture includes grasping movements of specialized feeding structures, chitinous hooks, and toothed radula, whose coordinated rhythmic activities extract the prey from its shell and bring it to the gut (Lalli 1970; Lalli and Gilmer 1989; Wagner 1885). We show here that coordination between rhythmic radula and hooks movements occurs in a strict phase-dependent manner with hooks and radula moving in opposite phases, as is functionally appropriate. While the hooks are retracted, the radula is protracted attempting to seize the soft tissue of the prey inside the shell. After seizing it, the radula retracts pulling the prey from its shell, while the hooks protract and in turn grab the tissue. Then the hooks retract pulling the prey out of the shell, while the radula releases the tissue and protracts again to grasping it deeper inside. In other words, the radula and hooks take turns in pulling the prey out of the shell thus keeping a constant extracting pressure, the same way as left and right hands take turns in gripping and pulling a rope.

Mechanisms of coordination between radula and hooks rhythmic movements

During rhythmic fictive feeding, hook protraction always coincided with radula retraction and hook retraction coincided.
with radula protraction. On the neuronal level, Bc-HP and Bc-RR neurons always fired in phase with each other. The Bc-HR and Bc-RP neurons also fired in phase, although there was a slight phase shift in Bc-HR neuron bursting, which allowed brief coactivation of Bc-HR and Bc-HP neurons. Investigation of the interactions between motor neurons from different functional groups revealed that Bc-HP and Bc-RR neurons, which fire in the same phase, were electrically coupled to each other (Fig. 13). This electrical coupling is apparently one mechanism of coordination between hook protractor

**FIG. 6.** Rhythmic movements of hooks and radula induced by a stimulation of the cerebro-buccal connective and corresponding rhythmic activity of a radula protractor Bc-RP motor neuron, radula retractor Bc-RR motor neuron, and hook retractor Bc-HR motor neuron. Note that Bc-RP and Bc-HR neurons were active in the same phase, while the Bc-RR neuron burst in the opposite phase. Active retraction of the hooks (indicated by asterisks) occurred immediately before hook protraction and following passive, post protraction decay from the previous cycle, which was very prominent in the absence of a prey.

**FIG. 7.** Rhythmic movements of hooks and radula induced by a stimulation of the cerebro-buccal connective (arrow) and corresponding rhythmic activity of a radula retractor Bc-RR motor neuron, radula protractor Bc-RP motor neuron and hook protractor Bc-HP motor neuron. Note that Bc-RR and Bc-HP neurons were active in the same phase, while the Bc-RP neuron burst in the opposite phase.
and radula retractor neurons. It also explains why a strong burst of spikes in the Bc-HP neurons produced, in addition to hook protraction, a noticeable radula retraction. Electrical coupling between neurons has been identified as a mechanism of behavioral coordination in several animals (Collins 1983; Syed and Winlow 1991). The Bc-HR and Bc-RP neurons were not electrically coupled, which would allow the observed Bc-HR phase shift and coactivation of the hook retractor and protractor neurons. Brief initial coactivation of functionally opposite motor neurons followed by the inhibition of one neuron type is a well-known mechanism to produce high movement speed and power in different animals (Heitler and Burrows 1977; Norekian and Satterlie 1993).

Reciprocal inhibitory connections found between different motor neurons are a second mechanism for coordination between hook and radula controlling neurons. Bc-HP neuron activity induced inhibitory inputs to the Bc-RP neurons, while action potentials in the Bc-RP neurons, in turn, induced inhibition of the Bc-HP neurons (Fig. 12). These interactions ensure that radula protractor and hook protractor motor neurons are rhythmically active in the opposite phases. The inhibitory connections between motor neurons were polysynaptic. It

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**FIG. 8.** Hook protractor Bc-HP motor neurons and radula retractor Bc-RR motor neurons are active strictly in the same phase with similar burst duration, intervals, and even intensity (A). The mechanism underlying this synchronization is electrical coupling between these 2 types of motor neurons (B).

**FIG. 9.** A: rhythmic activity of the Bc-RP, Bc-RR, and Bc-HR motor neurons shown at high-speed resolution. Although the Bc-RP and Bc-HR motor neurons were active at the same phase, the Bc-HR neuron burst occurred a little later than the Bc-RP neuron burst. B: this slight phase shift of the Bc-HR neuron bursting allows for the brief coactivation of hook protractor Bc-HP and hook retractor Bc-HR motor neurons.
is possible that motor neurons activated interneurons of their central pattern generator (CPG) via electrical coupling, as it happens in the *Clione* swimming system in the pedal ganglia, where all motor neurons are electrically coupled to the CPG interneurons of the same phase (Arshavsky et al. 1993). Interneurons active in one phase would then produce excitatory inputs to the Bc-RP motor neurons, inhibitory inputs to the Bc-RR motor neurons, and inhibitory inputs to the Bc-HP motor neurons. Interneurons active in opposite phase would produce excitatory inputs to the Bc-HP motor neurons, inhibitory inputs to the Bc-HR motor neurons, and inhibitory inputs to the Bc-RP motor neurons. This brings us to a suggestion that the radula and hook motor neurons are driven not by two separate dedicated CPGs, but rather one multifunctional pattern-generating network controlling movements of both feeding structures. The existence of multifunctional interneurons, or “distributed” networks was demonstrated in many neural systems (Dickinson 1995; Getting and Dekin 1985; Kristan et al. 1988; Lockery and Sejnowski 1992; Oku et al. 1994; Shaw and Kristan 1997; Wu et al. 1994; Xin et al. 1996). The concept of multifunctional pattern generators was actively pursued in a well-studied crustacean stomatogastric nervous system. The gastric and pyloric networks were found to be not separate groups of neurons that independently generate two different rhythmic behaviors, but rather provide a synaptically connected pool of neurons from which many different pattern-generating circuits can be assembled (Meyrand et al. 1994; Weimann et al. 1991).

The idea that a single CPG drives both the radula and hooks movements in *Clione* is also supported by the observation that the hook-controlling motor neurons continue to receive subthreshold rhythmic inputs coordinated with radula activity even when the hooks are quiescent. The functional uncoupling of hooks and radula movement thus appears to occur simply because rhythmic depolarizing inputs to the hook protractor neurons do not reach spike threshold and do not produce firing in the Bc-HP motor neurons. Thus the mechanism of functional uncoupling of the radula and hooks systems need not be inhibition of a separate hook CPG, but could result simply from a decrease of Bc-HP motor neuron responsiveness to the incoming rhythmic inputs, or a decrease in the strength of these inputs because of a modulation. Such modulatory input from sensory afferent induces restructuring of the pyloric neural network of the lobster stomatogastric system, which results in cessation of rhythmic activity of some neurons and therefore a reduced pyloric pattern (Hooper et al. 1990).
Comparative view on the feeding system

The buccal mass in many gastropod mollusks consists of the muscles controlling rhythmic radula and jaw movements. It is important to note that jaw rhythmic activity is also coordinated with radula movements in a phase-dependent manner (Morton and Chiel 1993; Nagahama et al. 1999; Nagahama and Takata 1989; Willows 1980). In most mollusks from the order Gymnosomata, the buccal mass also includes hooks (Lalli and Gilmer 1989). In Clione, the jaws disappeared, and the buccal mass consists of only the radula and muscular hook sacs (Lalli and Gilmer 1989). These muscular hook sacs in gymnosomes presumably evolved from the ancestral buccal mass that consisted of the radula and jaws. Thus hooks and radula may represent two evolutionary very close feeding structures. This consideration again raises the question of whether separate buccal neural networks control hooks and radula movements in Clione, or whether a single multifunctional CPG drives the movements of both organs. Identification of the CPG(s) underlying hooks and radula movements will be specifically targeted by our following investigation of the coordinated rhythmic activity of these two feeding structures of Clione.

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FIG. 13. Schematic representation of the synaptic connections between the 4 different groups of radula and hooks controlling motor neurons. Hook Protractor and Radula Retractor neurons, which fire in the same phase, are electrically coupled. Identified inhibitory polysynaptic connections between neurons are shown by filled circles.


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