J. exp. Biol. 155, 415–433 (1991)
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NEURAL CONTROL OF THE BUCCAL MUSCLE MOVEMENT IN THE AFRICAN GIANT SNAIL *ACHATINA FULICA*

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Accepted 31 July 1990

Summary

Five pairs of neurones, R(L)-B1, R(L)-B2, R(L)-B3, R(L)-B4 and R(L)-B5, controlling buccal muscle movement, were identified in the buccal ganglia of the African giant snail *Achatina fulica* Férussac. All these neurones fired during the radula retraction phase of rhythmic buccal activity. Neurones B1, B2, B4 and B5 made direct excitatory connections to the radula retractor, and B1, B2, B3 and B5 also made direct excitatory connections with the outer muscle of the buccal mass. Of these neurones, B4 had the most potent effect on contraction of the ipsilateral radula retractor. Physiological and pharmacological analyses suggested that the principal excitatory transmitter of B4 at the neuromuscular junctions was acetylcholine (ACh), although glutamate and aspartate also elicited the contraction. A pair of cerebral ganglion cells, v-RCDN and v-LCDN, was found to have modulatory effects on the muscle contraction evoked by B4 firing and ACh application. Morphological, physiological and immunohistochemical analyses suggested that the modulatory actions of v-CDN on muscle contraction are mediated by serotonin, which may be released from nerve terminals of v-CDN and act directly on the muscle. v-CDN also increased the activity of motoneurone B4. v-CDN shared several common features with serotonergic cerebral cells in other gastropod molluscs.

Introduction

In some gastropods, such as *Helisoma trivolvis*, *Pleurobranchaea californica*, *Aplysia californica*, *Tritonia hombergi* and *Lymnaea stagnalis*, neurones controlling the buccal muscle movement have been functionally identified (Kater, 1974; Siegler *et al.* 1974; Cohen *et al.* 1978; Bulloch and Dorsett, 1979; Rose and Benjamin, 1979). These systems provide us with a great deal of information about neuronal coordination in generating cyclic feeding activity, which has been well investigated, especially in *L. stagnalis* (Rose and Benjamin, 1981*a*,*b*; Kyriakides and McCrohan, 1989) and *P. californica* (Siegler, 1977; Gillette *et al.* 1978). In

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Key words: identified neurones, buccal muscle, serotonin, acetylcholine, African giant snail, *Achatina fulica*.

Aplysia californica, the feeding system has been utilized for investigating the mechanisms of modulatory effects of neurones on muscle contraction (Weiss *et al.* 1978; Cropper *et al.* 1987, 1988).

Like other gastropods, the pulmonate *Achatina fulica* Férussac shows rhythmic feeding activity, but little work has been carried out on the mechanisms of the feeding movement. In the present study, we analyse the neural control mechanisms of feeding in *Achatina fulica* after identifying several neurones controlling the buccal muscle movement. The investigation then focuses on the regulation of muscle contraction by one buccal ganglion neurone, B4, identified in the present study, together with a cerebral ganglion neurone, v-CDN, identified by Ku *et al.* (1985). In addition, the modulatory effects of v-CDN on muscle contraction are compared with those of the serotonergic cerebral cells (SCCs) (Granzow and Rowell, 1981) of other gastropods.

Materials and methods

Preparation and physiology

The African giant snails, *Achatina fulica* Férussac, used in the present experiments were captured in Okinawa and reared in our laboratory at 24 °C. For identification of neurones and investigation of electrical couplings between buccal ganglion neurones, a preparation consisting of buccal ganglia with half the buccal mass attached *via* two buccal nerves (buccal nerves 1 and 2) was used. Anatomically, buccal nerve 1 (n1) innervates the radula retractor and buccal nerve 2 (n2) the outer muscle. In the other experiments, the cerebral ganglia were attached to the buccal ganglia *via* bilateral cerebrobuccal connectives (CBCs), while the other nerves, with the exception of n2, were cut off. The experimental arrangement is shown in Fig. 1. The ganglia and basal tissue of the muscle were pinned onto the bottom of the chamber, which was covered with silicone resin. The recording chamber was separated into two compartments so that the ganglia and muscle could be perfused independently. The outer thick connective tissue and inner thin sheath were usually surgically removed to facilitate penetration of electrodes.

Intracellular recording from neurones and current injection into neurones were performed using either one or two glass microelectrodes filled with 3 mol l^{-1} potassium acetate and having a resistance ranging from 8 to 20 MΩ. Extracellular recording of electrical activity from the muscles was carried out using glass suction electrodes. Muscle tension was monitored by a force transducer connected to the muscle by a fine thread. In some cases, the cerebrobuccal connective was stimulated using a suction electrode placed over the cut end to initiate cyclic motor activity in the buccal ganglia. Acetylcholine (ACh) was applied to the muscle through a small pipette placed adjacent to the muscle (Fig. 1). An electromagnetic valve was connected to the pipette, which enabled application of ACh solution as reproducible pulses. ACh, once applied, was washed away by continuous

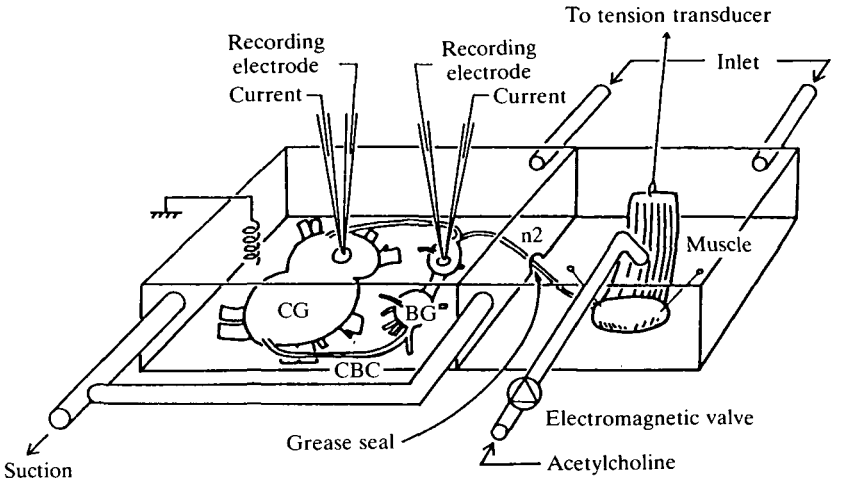

Fig. 1. Diagram of the experimental arrangement used for simultaneous recordings of electrical activity of neurones and muscle tension. CG, cerebral ganglia; BG, buccal ganglia; CBC, cerebrobuccal connective; n2, buccal nerve 2.

Table 1. *Composition of physiological solutions used in experiments*

	Concentration (mmol l^{-1})					
Solution	NaCl	KCl	$CaCl_2$	$MgCl_2$	Glucose	Hepes
Normal	61	3.3	10.7	13	5	10
High-Mg^{2+}, Ca^{2+}-free	38	3.3	–	39	5	10
High-Mg^{2+}, low-Ca^{2+}	33	3.3	3.56	39	5	10

All solutions were at pH 7.5.

perfusion with saline. The data were stored on tape for later analysis and permanent records were made using a pen recorder.

The composition of physiological solutions is shown in Table 1. To block the intraganglionic chemical synapses, the ganglia compartment was perfused with high-Mg^{2+}, Ca^{2+}-free solution or high-Mg^{2+}, low-Ca^{2+} solution.

All physiological experiments were performed at room temperature (20–25°C). Each experiment was done at least in triplicate.

Morphology of neurones

Intracellular staining with Lucifer Yellow CH was carried out to examine the axonal pathways of neurones. The fluorescent dye (4–6% w/v) was injected into the cell body of neurones through a microelectrode by pressure or ionophoresis. After incubation for several hours, the preparation was fixed in 4% formal-

dehyde, dehydrated, cleared in methylbenzoate, and then viewed with a fluorescence microscope. The stained neurone was photographed and reconstructed.

Immunohistochemistry

Ganglia

The dissected tissues, consisting of cerebral and buccal ganglia, were incubated in saline containing protease ($12.5\,mg\,ml^{-1}$; type XV, Sigma) for 1 h at room temperature and then fixed in 4% paraformaldehyde in $0.1\,mol\,l^{-1}$ phosphate buffer (pH 7.4) for 12–18 h at 4°C. All subsequent steps were performed at room temperature (20–25°C). The tissues were washed in phosphate buffer solution (PBS) for 24 h and incubated in blocking solution (1% v/v normal goat serum, 1% Triton X-100, 0.1% sodium azide in PBS). Then, the ganglia were incubated for 78 h in a 1:50 dilution of primary antibody (rabbit polyclonal antiserum to serotonin obtained from INCSTAR). They were washed in PBS for 48 h and then incubated in a 1:25 dilution of secondary antibody (goat anti-rabbit IgG rhodamine obtained from Biomedical Tec.) for 42 h. The ganglia were washed in PBS for 12 h and then viewed and photographed through a fluorescence microscope.

Muscle

The radula retractor was dissected from the animal and fixed in Zamboni's solution overnight at 4°C. The tissue was dehydrated and embedded in polyester wax. Serial sections each 10 μm thick were made. The sections were plated on gelatin-coated slides and, after dissolution of wax, incubated in a 1:1000 dilution of primary antibody to serotonin for 3 h at room temperature. They were washed in PBS and then processed with a Vectastain ABC kit. The sections were viewed under a microscope and photographed.

Results

Identification of neurones

Muscles in the buccal mass usually showed rhythmic contractions when they were dissected with the buccal ganglia attached, although such contractions were not seen in the buccal mass alone. Thus, rhythmic feeding activity of the buccal mass could be generated at least partially by the buccal ganglia. Fig. 2 shows simultaneous recordings of electrical activities from three ipsilateral buccal muscles; radula protractor, radula retractor and the outer muscle of the buccal mass. During the existing rhythmic buccal activity, the radula protractor first contracts to lengthen the radula (radula protraction phase) and then the radula retractor is activated to shorten the radula (radula retraction phase). This is followed by an inactive phase. In the latter half of the radula retraction phase, the outer muscle, which is located at the side of the buccal mass, contracts the buccal mass longitudinally. All the neurones identified in the present experiment fired during the radula retraction phase.

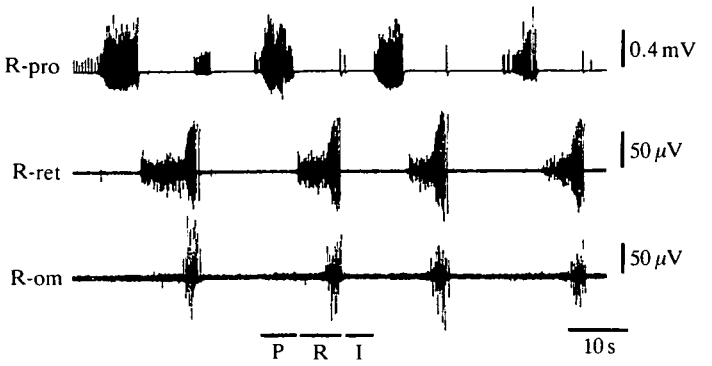

Fig. 2. Simultaneous recordings of electrical activities from three ipsilateral buccal muscles showing rhythmic activity. Recordings were made using glass suction electrodes. R-pro, right radula protractor; R-ret, right radula retractor; R-om, right outer muscle; P, radula protraction phase; R, radula retraction phase; I, inactive phase.

Four neurones have been identified morphologically in the buccal ganglion of *Achatina fulica*, and their pharmacological characteristics have been studied (Matsuoka *et al.* 1987). In the present study, five pairs of neurones controlling the buccal muscle movement were identified in the buccal ganglia of *Achatina fulica*, both by examining the neuronal morphology and by means of simultaneous recordings of neurones and buccal muscles. All these neurones were located symmetrically on the caudal surface of each buccal ganglion; they were named R(L)-B1, R(L)-B2, R(L)-B3, R(L)-B4 and R(L)-B5 (Fig. 3). The diameter of the cell body of each of the identified neurones was about 100 μm. Judging from the

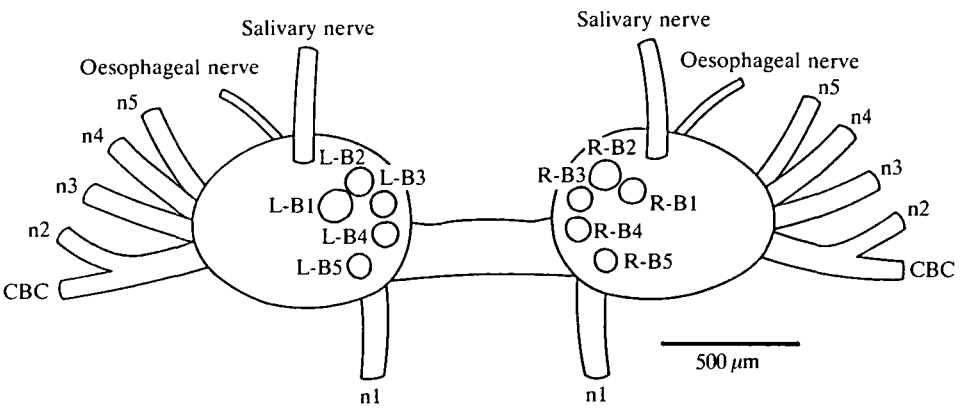

Fig. 3. Schematic drawing of the caudal view of the buccal ganglia showing the positions of neurones identified in the present study. n1–n5, buccal nerve 1–5; CBC, cerebrobuccal connective.

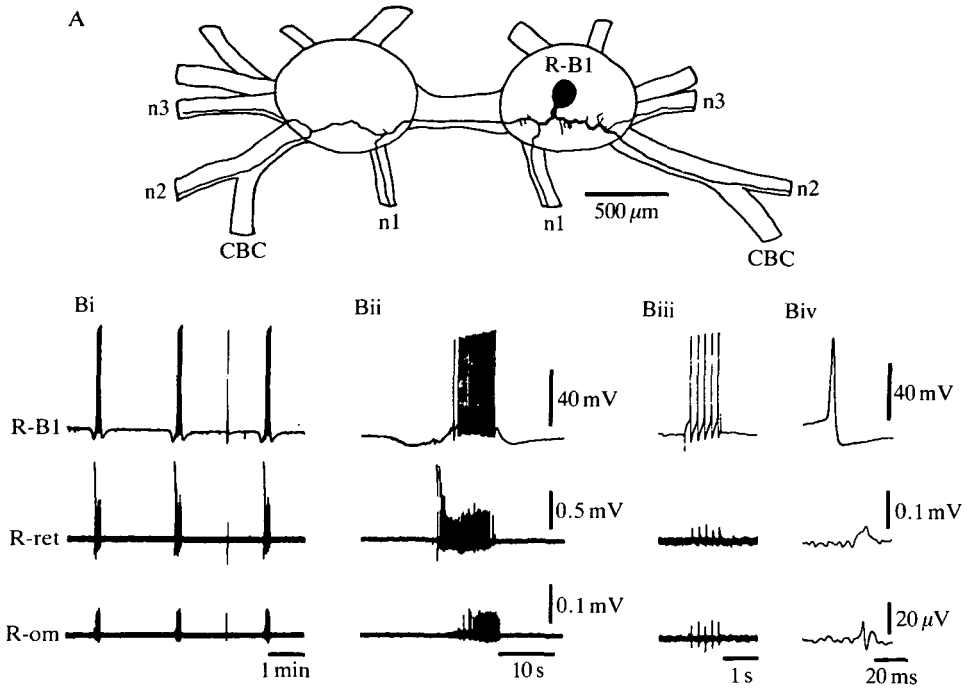

Fig. 4. (A) Morphology of R-B1 stained by the injection of Lucifer Yellow. (Bi) Simultaneous recordings of R-B1, R-ret and R-om, during rhythmic buccal activity, made intracellularly from the neurone and extracellularly from the muscles. They all fired in the radula retraction phase. (Bii) Expanded recordings of the third burst in Bi. (Biii) A 1:1 relationship between R-B1, R-ret and R-om. R-B1 was made to fire by depolarizing current injection. (Biv) Expanded recordings of the first firings in Biii.

locations of their cell bodies and axonal pathways, L-B1 and L-B2 appeared to be identical with previously identified d-LBPN and d-LBMN (Matsuoka *et al.* 1987).

Fig. 4 shows a reconstructed drawing of the axonal pathways of neurone R-B1 stained intracellularly with Lucifer Yellow, together with simultaneous recordings of the electrical activities of R-B1, the ipsilateral radula retractor and the outer muscle. R-B1 sent its axonal branches bilaterally to nerves n1, n2 and n3. During rhythmic activity, both the radula retractor and the outer muscle fired in the radula retraction phase, which synchronized with cyclical bursts of firings in R-B1. It appeared that neurones other than R-B1 also innervated these muscles, since some action potentials of the muscles preceded those of R-B1 in many cases. When neurone R-B1 was electrically stimulated to fire, a 1:1 relationship of action potentials with constant latency was observed between the neurone and these muscles. Such a relationship was also observed between R-B1 and the contralateral radula retractor and outer muscle (not shown). Similar observations were made with neurone L-B1, the radula retractors and the outer muscles. From the above results, it could be concluded that B1 made excitatory connections to the bilateral radula retractors and to the outer muscles. This study revealed no

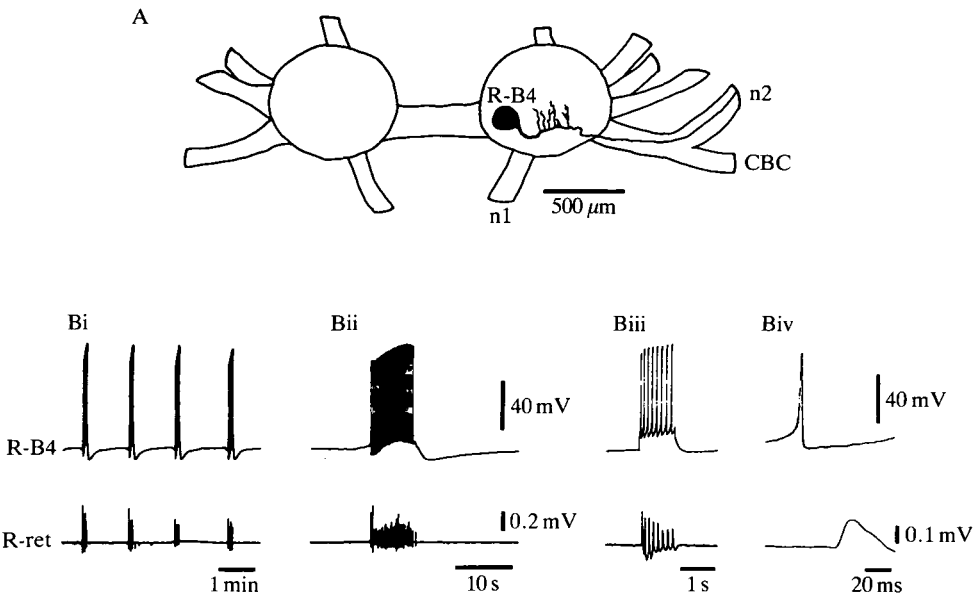

Fig. 5. (A) Morphology of R-B4 stained by the injection of Lucifer Yellow. (Bi) Simultaneous recordings of R-B4 and R-ret, during rhythmic buccal activity, made intracellularly from the neurone and extracellularly from the muscle. They both fired in the radula retraction phase. (Bii) Expanded recordings of the first burst in Bi. (Biii) A 1:1 relationship between R-B4 and R-ret. R-B4 was made to fire by depolarizing current injection. (Biv) Expanded recordings of the first firings in Biii.

differences in anatomy or function between the right and left sides of paired neurones. Thus, in the following sections of the paper, each pair of identified neurones will be described without specifying laterality.

Neurone B2 sent its axonal branches to the same nerves in which B1 had its projections. From physiological analyses, B2 appeared to exert its excitatory effects on both ipsi- and contralateral radula retractors and outer muscles.

In contrast, neurones B3, B4 and B5 sent their axonal branches only to the ipsilateral nerves. Fig. 5 shows the morphology of R-B4 and simultaneous recordings of electrical activities of R-B4 and the ipsilateral radula retractor. Spontaneous firings of B4 during the radula retraction phase were synchronized with firings of the radula retractor (Fig. 5Bi). When B4 was made to fire by depolarizing current injection, a 1:1 relationship of action potentials with constant latency was observed between B4 and the radula retractor (Fig. 5Biii). Anatomical examinations suggested that the radula retractor was only innervated by n1, and B4 was found to send its axon to n2 and not to n1. However, even when n1 was cut, leaving only n2 intact, spike potentials of B4 could produce 1:1 muscle potentials in the radula retractor. Thus, the radula retractor may be innervated by n2 as well as by n1.

By performing similar physiological experiments, it was found that B3 inner-

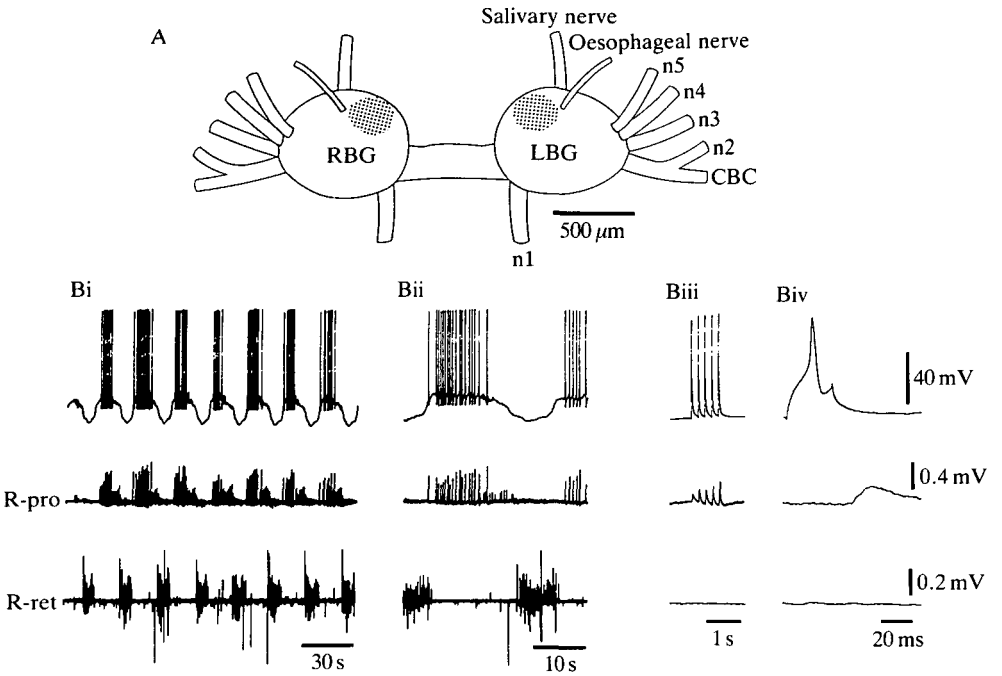

Fig. 6. (A) Schematic rostral view of the buccal ganglia. Protractor motoneurones were located within the dotted areas. (Bi) Simultaneous recordings of these neurones, R-pro and R-ret, during rhythmic buccal activity. Firing of the neurone and R-pro in the radula protraction phase alternated with R-ret firing. (Bii) Expanded recordings of the second and third bursts in Bi. (Biii) A 1:1 relationship between the neurone and R-pro. The neurone was made to fire by depolarizing current injection. (Biv) Expanded recordings of the third firings in Biii. RBG, right buccal ganglion; LBG, left buccal ganglion.

vates excitatorily the ipsilateral outer muscle of the buccal mass and B5 innervates the ipsilateral radula retractor and outer muscle.

Some neurones that fired during the radula protraction phase were found to be situated on the rostral surface of the buccal ganglia (Fig. 6A). They could not reliably be identified, since they had cell bodies of a relatively small size and were surrounded by many cells of similar size. A typical recording from one neurone which fired in the radula protraction phase is shown in Fig. 6B. During the rhythmic activity, firings of the radula protractor corresponded with firings of this neurone, and alternated with firings of the radula retractor. In addition, a 1:1 relationship with constant latency was observed between experimentally evoked spikes of this neurone and the muscle potential of the radula protractor.

Electrical coupling between identified neurones

Some of the neurones identified in the present experiment showed weak electrical coupling with each other. When the membrane potential of R-B1 was

shifted by current injection into the soma, a corresponding alteration of membrane potential was recorded in the soma of L-B1. This coupling was weak (coupling ratio, <0.06) so that firing in one neurone did not cause firing in the other. Electrical coupling between right and left B2s was similar to that between two B1 neurones. There was also electrical coupling between B4 and B5. Coupling between B4 and B5 appeared to be somewhat stronger (coupling ratio, <0.1) compared with that between B1s or B2s, although the coupling efficacy varied from preparation to preparation.

Monosynaptic connections between identified neurones and buccal muscles

To study the mode of innervation of identified neurones to buccal muscles further, neural activity and muscle tension were simultaneously recorded. Fig. 7 shows two examples suggesting monosynaptic connections between identified neurones and buccal muscles. In normal saline, when B3 was made to fire at 20 Hz for 1 s by depolarizing current injection, the ipsilateral outer muscle responded with contraction (Fig. 7A). Then, to test the monosynaptic connection, the ganglia compartment, but not the muscle compartment, was perfused with high-Mg^{2+}, Ca^{2+}-free solution, a treatment considered to block chemical synapses (Furukawa and Kobayashi, 1987). However, contraction of the outer muscle produced by B3 firing was not reduced by this treatment. This result means that B3 innervates the ipsilateral outer muscle directly and could produce its contraction directly. Similarly, the contraction of the radula retractor evoked by B4 firing was not reduced by perfusing the ganglia compartment with high-Mg^{2+}, Ca^{2+}-free solution (Fig. 7B). Thus, B4 was considered to produce the contraction of the ipsilateral radula retractor directly.

The same procedure was applied to the other neurones and it was concluded that B1 and B2 could directly evoke the contraction of the outer muscles of both

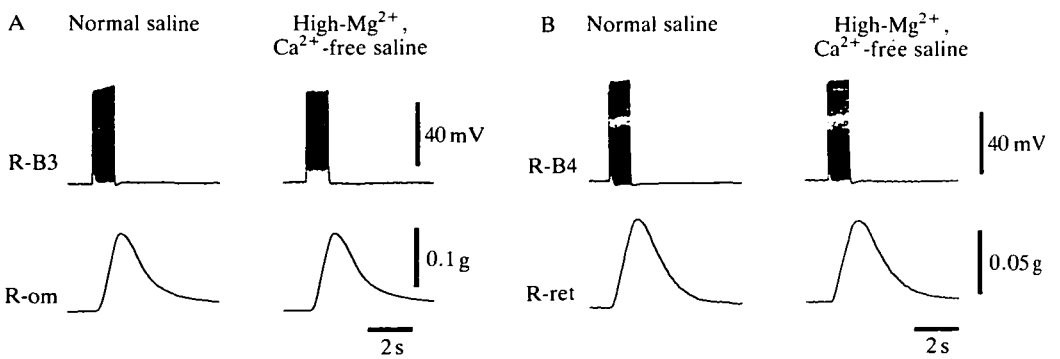

Fig. 7. Effect of perfusion of the ganglia with high-Mg^{2+}, Ca^{2+}-free solution on the muscle contractions evoked by neurones R-B3 and R-B4. Note that the muscle was constantly perfused with normal saline. Contraction of R-om evoked by R-B3 firing (20 Hz, 1 s) and that of R-ret evoked by R-B4 firing (20 Hz, 1 s) are not changed by this treatment.

sides and that B5 directly evoked the contraction of the ipsilateral outer muscle and the radula retractor. Furthermore, contraction of the radula retractor produced by firing of B1 or B2 was also observed, although a recording was not made.

Such relationships between neurones and buccal muscles are consistent with the observations described in the previous section.

Excitatory transmitter at neuromuscular junctions

Among the identified neurones, B4 exerted the most potent excitatory drive on the radula retractor. To analyse further the neural control of buccal muscle movement, the modes of action of several putative excitatory transmitters at junctions between neurone B4 and the radula retractor were investigated. Application of ACh, which has been established as, or suggested to be, an excitatory transmitter at many other gastropod neuromuscular junctions (Cohen *et al.* 1978; Kobayashi and Muneoka, 1980; Zoran *et al.* 1989), evoked a contraction of the radula retractor with a threshold concentration of 10^{-5} mol l^{-1} (Fig. 8A).

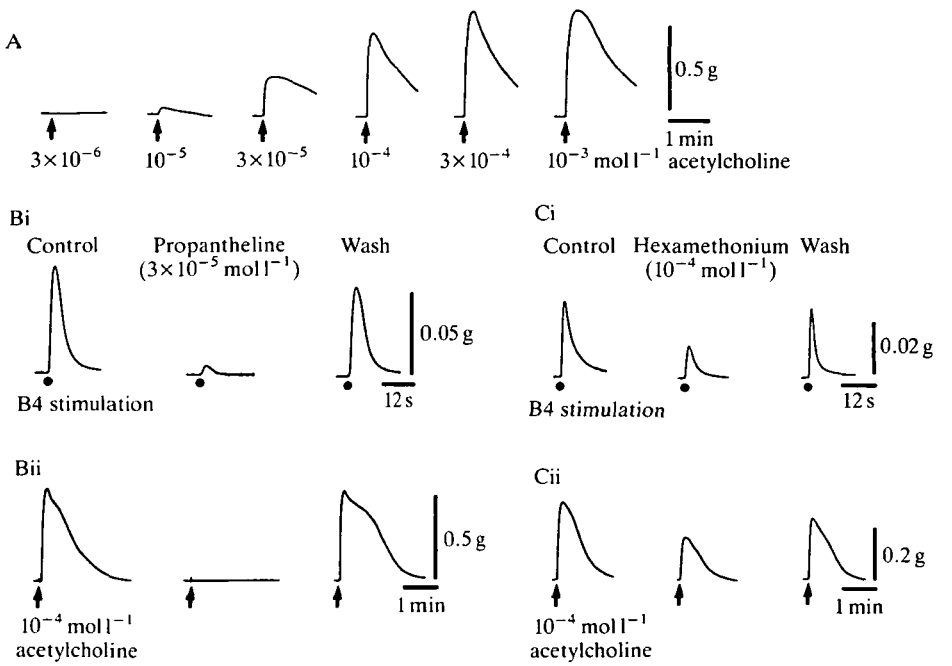

Fig. 8. (A) Contractions of the radula retractor in response to acetylcholine (ACh). ACh of various concentrations was applied as a pulse at the arrows through a small pipette placed adjacent to the muscle. Each pulse was separated by a 10 min wash. (B) Effect of propantheline on the contraction in response to B4 stimulation (i) or ACh application (ii). (C) Effect of hexamethonium on the contraction in response to B4 stimulation (i) or ACh application (ii). B4 was stimulated at 30 Hz for 1 s at the dots, and ACh was applied at the arrows. Propantheline and hexamethonium were applied to the muscle by perfusion for 10 min before the recording was made.

Although glutamate and aspartate also evoked the contraction, the peak tension of the contraction in response to these compounds was much smaller than that in response to ACh at the same concentration.

In molluscan neuromuscular junctions, propantheline (Yanagawa *et al.* 1987) and hexamethonium (Cohen *et al.* 1978) have been shown to be cholinergic blocking agents. Bath application of propantheline to the muscle compartment at a concentration of 3×10^{-5} mol l^{-1} almost completely blocked the contraction of the radula retractor produced by B4 firing, as well as that produced by ACh application. The contraction recovered after washing with saline (Fig. 8B). Hexamethonium at a concentration of 10^{-4} mol l^{-1} also reduced muscle contractions evoked by B4 stimulation and ACh application (Fig. 8C). These reductions were partially reversed after washing with saline. However, muscle contraction evoked by 3×10^{-4} mol l^{-1} glutamate and that evoked by the same concentration of aspartate were not affected by the presence of 10^{-4} mol l^{-1} propantheline. These results suggest that the transmission from neurone B4 to the radula retractor may be mediated by ACh, although the possibility of involvement of other excitatory transmitters cannot be excluded.

Ventral cerebral distinct neurones

A pair of cerebral giant neurones, the ventral right(left) cerebral distinct neurones (v-RCDN and v-LCDN) identified by Ku *et al.* (1985), are situated on the ventral surface of the cerebral ganglia, and have been shown to possess serotonin-like immunoreactivity (Croll, 1988). In the present experiments, the role of v-CDNs in the regulation of buccal muscle contraction was investigated by concentrating on the relationship between neurone B4 and the radula retractor.

Morphology of v-CDN

Fig. 9 shows the morphology of v-RCDN stained by the injection of Lucifer Yellow. v-CDN sends its axonal branches abundantly to the ipsilateral cerebrobuccal connective and to the sublabial nerve on the same side. Just before entering the buccal ganglion, some of the axons branch into n2, which innervates the outer muscle, radula protractor and radula retractor, while others enter the buccal ganglion and then branch out to n3, n4, n5 and the salivary nerve. Although other branches could not be detected by intracellular staining with Lucifer Yellow, v-CDN appeared to send its axons to all buccal nerves on the same side of the neurone, because action potentials recorded in the cell body of v-CDN were followed, with constant delay, by those recorded extracellularly from all ipsilateral buccal nerves. v-CDN may not have contralateral projections, since action potentials corresponding to firings of v-CDN were not recorded from the buccal commissure.

Effects of v-CDN on muscle contraction

To examine the effects of v-CDN on muscle contraction evoked by firing of B4, simultaneous recordings of electrical activities of v-CDN and B4 and the

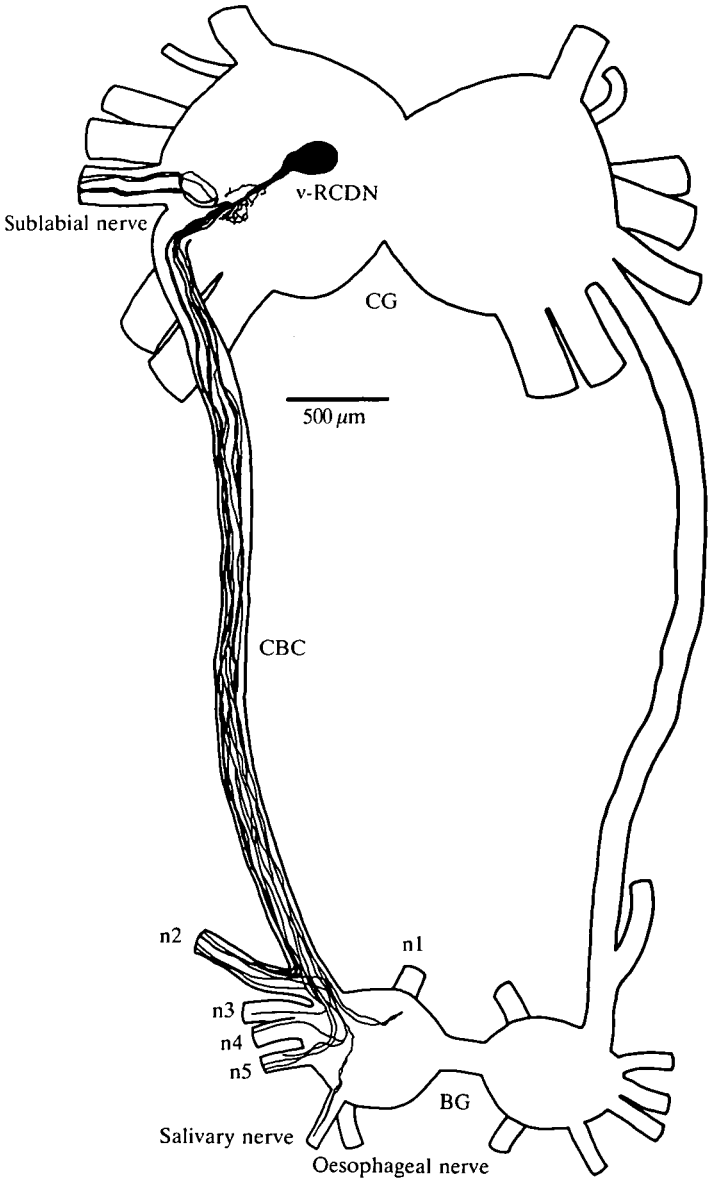

Fig. 9. Morphology of v-RCDN stained by the injection of Lucifer Yellow. Ventral view of the cerebral ganglia (CG) and caudal view of the buccal ganglia (BG) are shown. CBC, cerebrobuccal connective; n1–n5, buccal nerves 1–5.

contraction of the radula retractor were carried out. Firings of v-CDN produced by depolarizing current injection enhanced contraction of the radula retractor evoked by B4 firing (Fig. 10Ai), although firing of v-CDN did not directly cause the muscle to contract. Usually, v-CDN could enhance the muscle contraction when it fired at more than 1 Hz. Recovery from the enhancement was observed within

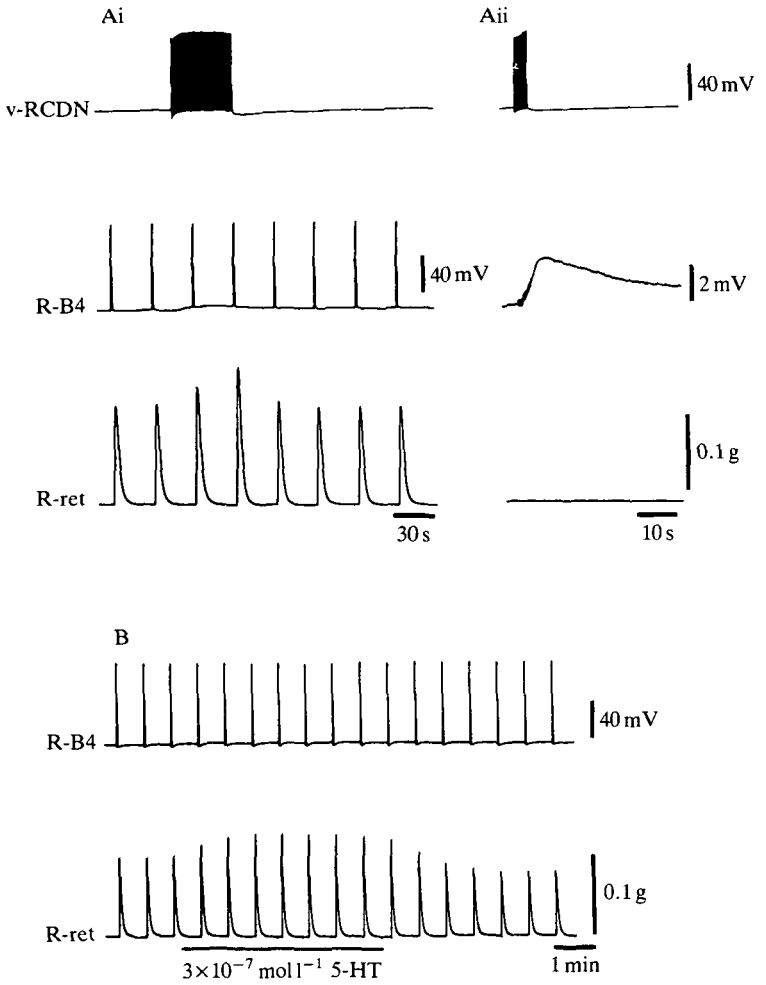

Fig. 10. Enhancing effects of v-RCDN and serotonin on contractions of the radula retractor produced by R-B4 firing. (Ai) Effect of v-RCDN, which was made to fire at 5 Hz for 45 s. R-B4 was fired at 20 Hz for 1 s at 30 s interburst intervals. (Aii) v-RCDN also makes excitatory connections to R-B4. v-RCDN was fired at 5 Hz for 4 s. (B) Effect of 3×10^{-7} mol l^{-1} serotonin (5-HT), which was applied to the muscle during the period indicated by the bar under the record. R-B4 was fired at 20 Hz for 1 s at 40 s interburst intervals.

1 min after the firing of v-CDN ceased. In addition, B4 responded to firing of v-CDN with depolarization of the membrane (Fig. 10Aii). The depolarization exhibited a gradual rise and a slow decay. In some cases, the depolarizing response led to firing of B4. Bath application of serotonin to the muscle mimicked the enhancing effect of v-CDN (Fig. 10B). The threshold concentration was about 10^{-8} mol l^{-1}

Fig. 11 shows the effects of v-CDN firing and serotonin application on the muscle contraction produced by a brief application of 10^{-4} mol l^{-1} ACh. Firing of

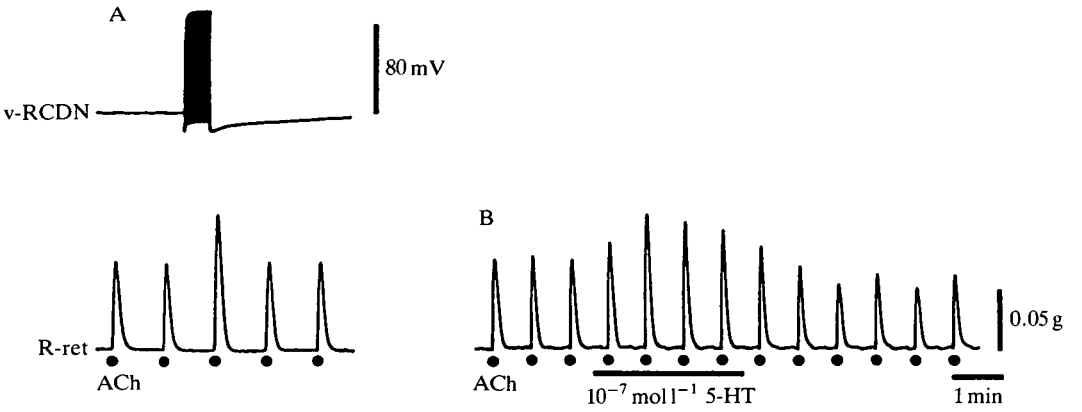

Fig. 11. Enhancing effects of v-RCDN and serotonin on contractions of the radula retractor evoked by ACh application. (A) Effect of v-RCDN, which was made to fire at 10 Hz for 30 s. ACh at 10^{-4} mol l^{-1} was applied briefly to the muscle at 1 min intervals. (B) Effect of 10^{-7} mol l^{-1} serotonin (5-HT), which was applied to the muscle during the period indicated by the bar under the record. ACh at 10^{-4} mol l^{-1} was applied at 40 s intervals shown by the dots. In this experiment, the ganglia were perfused with high-Mg^{2+}, low-Ca^{2+} solution to block intraganglionic chemical synapses.

v-CDN increased the size of the contraction (Fig. 11A). In this experiment, the ganglia were perfused with high-Mg^{2+}, low-Ca^{2+} solution to depress the activities of intraganglionic chemical synapses and, therefore, v-CDN may exert its ability to enhance muscle contraction at least partially by acting directly on the muscle. Serotonin also increased the size of the contraction when it was applied to the muscle (Fig. 11B). The contraction size after washing away the serotonin tended to settle down at a somewhat decreased level compared with that before the application and did not recover in the majority of cases. This tendency was seen when the contraction was evoked either by B4 firing or by ACh application.

Immunohistochemistry

In the cerebral ganglia, serotonin-like immunoreactivity was clearly detected in the somata of v-CDNs and in several smaller cell bodies. No somata in the buccal ganglia were labelled, but immunoreactive fibres were observed in CBCs, all buccal nerves, the buccal commissure and most cerebral nerves, including the labial nerves. These results show that nerves containing axonal projections of v-CDN possess serotonin-like immunoreactive fibres, and are consistent with the observations of Croll (1988).

Serotonin-like immunoreactivity was also detected in many neural fibres and in varicosities in the radula retractor (Fig. 12). The immunoreactive neural fibres appeared to be spread throughout the muscle.

These observations, together with the results from morphological and physiological analyses, suggest that the modulatory effect of v-CDN on the muscle

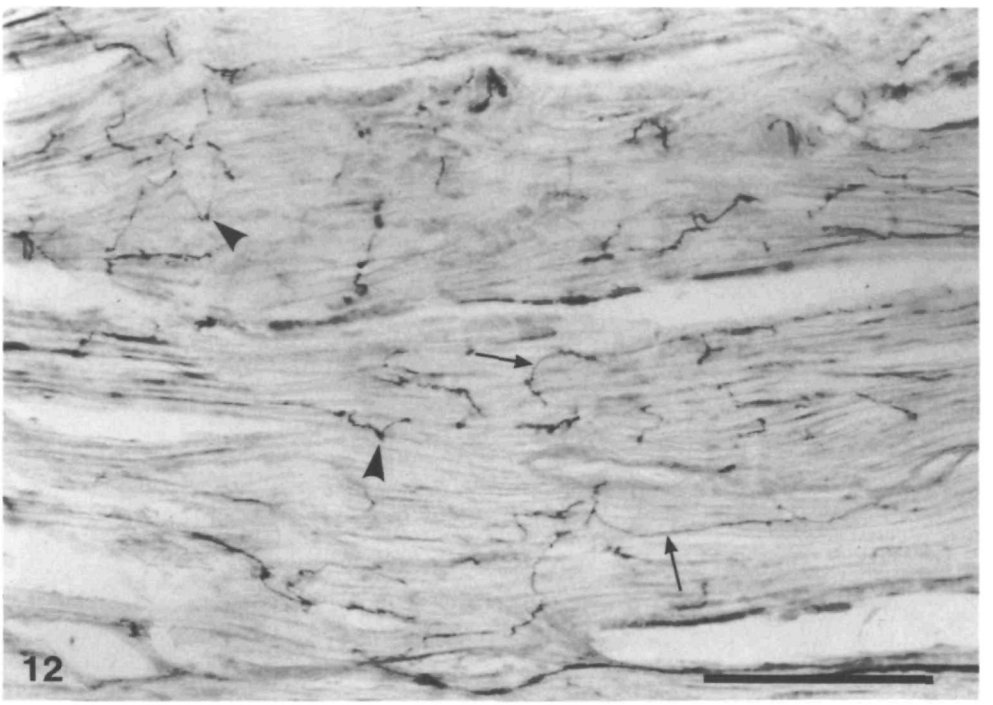

Fig. 12. Serotonin-like immunoreactivity in the radula retractor (longitudinal section). Many neural fibres (arrows) and varicosities (arrowheads) show serotonin-like immunoreactivity. Scale bar, 100 μm.

contraction due to B4 firing is mediated by serotonin, which may be released from terminals of v-CDN and act on the muscle directly.

Discussion

The innervation of the buccal muscles by five pairs of buccal ganglion neurones, B1–B5, and a pair of cerebral ganglion neurones, v-CDN, together with their interneuronal connections, are summarized in Fig. 13. As for the relationship between v-CDN and buccal ganglion neurones, only the connection to B4 is described in this diagram, as the connections of v-CDN to the other neurones are not yet clear.

Buccal muscles and neurones

In *Achatina fulica*, the buccal muscles contract cyclically according to the rhythmic motor output generated by the buccal ganglion. The cyclic contraction of the buccal muscles can be divided into three phases; the radula protraction phase, the radula retraction phase and the inactive phase. All the identified neurones make monosynaptic excitatory connections with the muscles that contract during the radula retraction phase. B1 and B2, the cell bodies of which are adjacent to

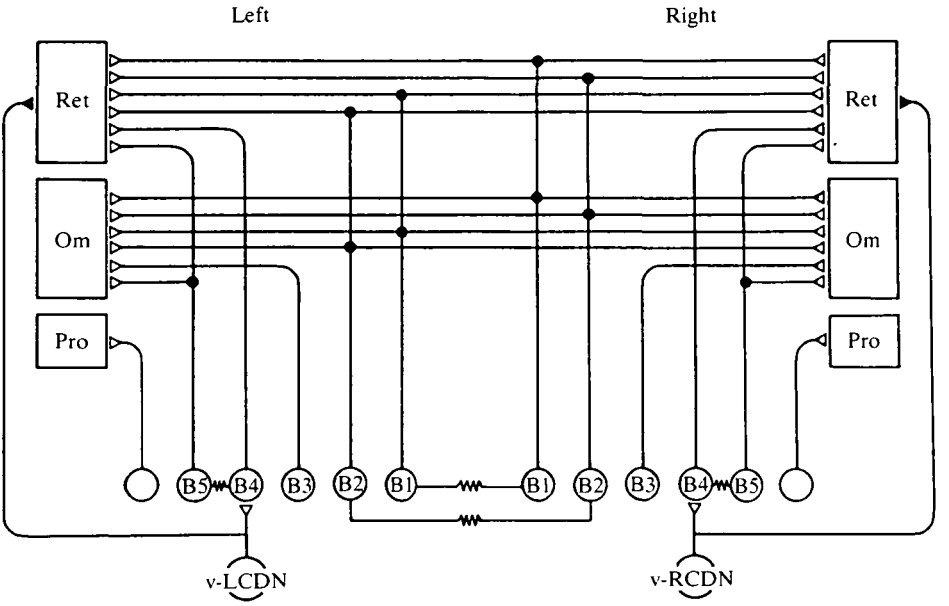

Fig. 13. Schematic diagram showing the innervation of the five pairs of buccal ganglion neurones, B1–B5, and a pair of cerebral ganglion neurones, v-CDN, to buccal muscles and their interneuronal connections. Open and closed triangles indicate excitatory and modulatory synapses, respectively. Zigzag lines indicate weak electrical couplings. Ret, radula retractor; Om, outer muscle; Pro, radula protractor.

each other, have very similar axonal projections in bilateral buccal nerves. They may play a role in coordinating the activities of both sides of the buccal muscles. If so, the electrical coupling between B1s or B2s may increase such effects.

It has been reported that in *Aplysia californica* (Cohen *et al.* 1978) and *Lymnaea stagnalis* (Rose and Benjamin, 1979) one buccal muscle is innervated by more than one excitatory neurone. In *Achatina fulica*, the radula retractor is innervated by both pairs of B1s and B2s and the ipsilateral B4 and B5. The outer muscle is innervated by both pairs of B1s and B2s and the ipsilateral B3 and B5. Although the necessity for such polyneuronal innervation is not clear, there are two possible reasons. First, the spatial summation of excitatory junction potentials (EJPs) may be required for regulation of contraction and, second, individual neurones may innervate different regions of the same muscle. It is difficult to investigate these two possibilities by extracellular recording alone; intracellular recording from a muscle fibre is also required.

In some gastropods, ACh has been suggested as an excitatory transmitter at identified neuromuscular junctions, such as those from neurones B15 and B16 to the accessory radula closer (ARC) muscle in *Aplysia californica* (Cohen *et al.* 1978), and from neurone B19 to the supralateral radular tensor (SLT) muscle in *Helisoma* (Zoran *et al.* 1989). In *Achatina fulica*, physiological and pharmacologi-

cal analyses of the transmission between B4 and the radula retractor suggest that B4 releases ACh as a principal excitatory transmitter.

The radula retractor of *Achatina fulica* also responded to glutamate and aspartate, although the contractions were smaller than those with ACh. In *Rapana thomasiana*, at the radula retractor, glutamate was suggested to be the main excitatory transmitter, although ACh also evoked the contraction (Muneoka and Kobayashi, 1980; Yanagawa *et al.* 1987). Glutamate and aspartate might be involved in the regulation of contraction of the radula retractor in *Achatina fulica*.

Modulation of muscle contraction by v-CDN

The results described in this paper show that v-CDN can modulate the muscle contraction peripherally as well as centrally. Peripherally, serotonin, which may be released from nerve terminals of v-CDN, enhanced the B4-evoked contraction of the radula retractor, possibly by acting postsynaptically. Centrally, firing of v-CDN increased the activity of retractor motoneurone B4.

It is known that in gastropods such as *Aplysia californica*, *Helix pomatia*, *Lymnaea stagnalis*, *Helisoma trivolvis* and *Pleurobranchaea californica*, a bilaterally symmetrical pair of large serotonin-containing cerebral neurones has several common features, suggesting that they are homologous (Granzow and Rowell, 1981). They are designated the serotonergic cerebral cells (SCCs) and show the following general features: (1) large size, (2) serotonin content, (3) a complex projection with axons extending down the cerebrobuccal connectives and into the buccal nerve trunks, and (4) the ability to influence neurones in the buccal ganglia (Granzow and Rowell, 1981). The v-CDNs in *Achatina fulica* may belong to the category of SCCs, since they possess these four features.

Some differences were found between the v-CDN and other SCCs. Most SCCs have their axonal projections in the contralateral buccal nerves (Pentreath and Cottrell, 1974; Weiss and Kupfermann, 1976; Gillette and Davis, 1977; Granzow and Rowell, 1981). The cerebral giant cell (CGC) of *Lymnaea* does not have contralateral projections, but the strong electrical coupling between a pair of CGCs enables them to act as a single unit (McCrohan and Benjamin, 1980). In contrast, the v-CDNs in *Achatina fulica* appear to have neither contralateral projections nor strong electrical coupling.

It has been shown that the serotonergic metacerebral cell (MCC) in *Aplysia* modulates the buccal muscle contraction by excitation of cholinergic motoneurones. The modulatory effects of v-CDN on muscle contraction appear to be similar to some extent to those of MCC. However, it should be noted that the enhancing effect of v-CDN on the muscle contraction declined quickly, whereas the effect of MCC lasted for a relatively long period. Weiss *et al.* (1978) have suggested that MCC potentiates muscle contraction in at least two different ways: by increasing the size of EJPs and by affecting the process of excitation–contraction coupling at the muscle. Further investigations of the molecular mechanisms involved in the modulation of contraction by v-CDN in *Achatina fulica* are required.

The authors thank Dr T. Shirane and Ms M. Sakata for their assistance in the morphological and immunohistochemical examinations of the preparations. This research was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan.

References

BULLOCH, A. G. M. AND DORSETT, D. A. (1979). The functional morphology and motor innervation of the buccal mass of *Tritonia hombergi*. *J. exp. Biol.* **79**, 7–22.

COHEN, J. L., WEISS, K. R. AND KUPFERMANN, I. (1978). Motor control of buccal muscles in *Aplysia*. *J. Neurophysiol.* **41**, 157–180.

CROLL, R. P. (1988). Distribution of monoamines within the central nervous system of the juvenile pulmonate snail, *Achatina fulica*. *Brain Res.* **460**, 29–49.

CROPPER, E. C., MILLER, M. W., TENENBAUM, R., KOLKS, M. A. G., KUPFERMANN, I. AND WEISS, K. R. (1988). Structure and action of buccalin: A modulatory neuropeptide localized to an identified small cardioactive peptide-containing cholinergic motor neuron of *Aplysia californica*. *Proc. natn. Acad. Sci. U.S.A.* **85**, 6177–6181.

CROPPER, E. C., TENENBAUM, R., KOLKS, M. A. G., KUPFERMANN, I. AND WEISS, K. R. (1987). Myomodulin: A bioactive neuropeptide present in an identified cholinergic buccal motor neuron of *Aplysia*. *Proc. natn. Acad. Sci. U.S.A.* **84**, 5483–5486.

FURUKAWA, Y. AND KOBAYASHI, M. (1987). Neural control of heart beat in the African giant snail, *Achatina fulica* Férussac. I. Identification of the heart regulatory neurones. *J. exp. Biol.* **129**, 279–293.

GILLETTE, R. AND DAVIS, W. J. (1977). The role of the metacerebral giant neuron in the feeding behavior of *Pleurobranchaea*. *J. comp. Physiol.* **116**A, 129–159.

GILLETTE, R., KOVAC, M. P. AND DAVIS, W. J. (1978). Command neurons in *Pleurobranchaea* receive synaptic feedback from the motor network they excite. *Science* **199**, 798–801.

GRANZOW, B. AND ROWELL, C. H. F. (1981). Further observations on the serotonergic cerebral neurones of *Helisoma* (Mollusca, Gastropoda): the case for homology with the metacerebral giant cells. *J. exp. Biol.* **90**, 283–305.

KATER, S. B. (1974). Feeding in *Helisoma trivolvis*: The morphological and physiological bases of a fixed action pattern. *Am. Zool.* **14**, 1017–1036.

KOBAYASHI, M. AND MUNEOKA, Y. (1980). Modulatory actions of octopamine and serotonin on the contraction of buccal muscles in *Rapana thomasiana*. I. Enhancement of contraction in radula protractor. *Comp. Biochem. Physiol.* **65**C, 73–79.

KU, B. S., ISOBE, K. AND TAKEUCHI, H. (1985). Pharmacological characteristics of four giant neurons identified in the cerebral ganglia of an African giant snail (*Achatina fulica* Férussac). *Comp. Biochem. Physiol.* **80**C, 123–128.

KYRIAKIDES, M. A. AND MCCROHAN, C. R. (1989). Effect of putative neuromodulators on rhythmic buccal motor output in *Lymnaea stagnalis*. *J. Neurobiol.* **20**, 635–650.

MATSUOKA, T., YONGSIRI, A. AND TAKEUCHI, H. (1987). Identification and pharmacological characteristics of giant neurones situated in the buccal ganglia of an African giant snail (*Achatina fulica* Férussac). *Comp. Biochem. Physiol.* **88**C, 35–45.

MCCROHAN, C. R. AND BENJAMIN, P. R. (1980). Patterns of activity and axonal projections of the cerebral giant cells of the snail, *Lymnaea stagnalis*. *J. exp. Biol.* **85**, 149–168.

MUNEOKA, Y. AND KOBAYASHI, M. (1980). Modulatory actions of octopamine and serotonin on the contraction of buccal muscles in *Rapana thomasiana*. II. Inhibition of contraction in radula retractor. *Comp. Biochem. Physiol.* **65**C, 81–86.

PENTREATH, V. W. AND COTTRELL, G. A. (1974). Anatomy of an identified serotonin neurone studied by means of injection of tritiated 'transmitter'. *Nature* **250**, 655–658.

ROSE, R. M. AND BENJAMIN, P. R. (1979). The relationship of the central motor pattern to the feeding cycle of *Lymnaea stagnalis*. *J. exp. Biol.* **80**, 137–163.

ROSE, R. M. AND BENJAMIN, P. R. (1981*a*). Interneuronal control of feeding in the pond snail *Lymnaea stagnalis*. I. Initiation of feeding cycles by a single buccal interneurone. *J. exp. Biol.* **92**, 187–201.

ROSE, R. M. AND BENJAMIN, P. R. (1981*b*). Interneuronal control of feeding in the pond snail *Lymnaea stagnalis*. II. The interneuronal mechanism generating feeding cycles. *J. exp. Biol.* **92**, 203–228.

SIEGLER, M. V. S. (1977). Motor neurone coordination and sensory modulation in the feeding system of the mollusc *Pleurobranchaea californica*. *J. exp. Biol.* **71**, 27–48.

SIEGLER, M. V. S., MPITSOS, G. J. AND DAVIS, W. J. (1974). Motor organization and generation of rhythmic feeding output in buccal ganglion of *Pleurobranchaea*. *J. Neurophysiol.* **37**, 1173–1196.

WEISS, K. R., COHEN, J. L. AND KUPFERMANN, I. (1978). Modulatory control of buccal musculature by a serotonergic neuron (metacerebral cell) in *Aplysia*. *J. Neurophysiol.* **41**, 181–203.

WEISS, K. R. AND KUPFERMANN, I. (1976). Homology of the giant serotonergic neurons (metacerebral cells) in *Aplysia* and pulmonate molluscs. *Brain Res.* **117**, 33–49.

YANAGAWA, M., TAKABATAKE, I., MUNEOKA, Y. AND KOBAYASHI, M. (1987). Further pharmacological study on the cholinergic and glutaminergic properties of the radula muscles of a mollusc, *Rapana thomasiana*. *Comp. Biochem. Physiol.* **88**C, 301–306.

ZORAN, M. J., HAYDON, P. G. AND MATTHEWS, P. J. (1989). Aminergic and peptidergic modulation of motor function at an identified neuromuscular junction in *Helisoma*. *J. exp. Biol.* **142**, 225–243.