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Octopamine: a new feeding modulator in *Lymnaea*

Á. Vehovszky[1]*, C. J. H. Elliott[2], E. E. Voronezhskaya[3], L. Hiripi[1] and K. Elekes[1]

[1]*Balaton Limnological Research Institute of the Hungarian Academy of Sciences, Tihany H-8237, Hungary*
[2]*University of York, Department of Biology, Heslington, York YO1 5DD, UK*
[3]*Institute of Developmental Biology of the Russian Academy of Sciences, 117808 Moscow, Russia*

The role of octopamine (OA) in the feeding system of the pond snail, *Lymnaea stagnalis*, was studied by applying behavioural tests on intact animals, and a combination of electrophysiological analysis and morphological labelling in the isolated central nervous system (CNS). OA antagonists phentolamine, demethylchlordimeform (DCDM) and 2-chloro-4-methyl-2-(phenylimino)-imidazolidine (NC-7) were injected into intact snails and the sucrose-induced feeding response of animals was monitored. Snails that received 25–50 mg kg^{-1} phentolamine did not start feeding in sucrose, and the same dose of NC-7 reduced the number of feeding animals by 80–90% 1–3 hours after injection. DCDM treatment reduced feeding by 20–60%. In addition, both phentolamine and NC-7 significantly decreased the feeding rate of those animals that still accepted food after 1–6 hours of injection.

In the CNS a pair of buccal neurons was identified by electrophysiological and morphological criteria. After double labelling (intracellular staining with Lucifer yellow followed by OA-immunocytochemistry) these neurons were shown to be OA immunoreactive, and electrophysiological experiments confirmed that they are members of the buccal feeding system. Therefore the newly identified buccal neurons were called OC neurons (putative OA containing neurons or OAergic cells).

Synchronous intracellular recordings demonstrated that the OC neurons share a common rhythm with feeding neurons either appearing spontaneously or evoked by intracellularly stimulated feeding interneurons. OC neurons also have synaptic connections with identified members of the feeding network: electrical coupling was demonstrated between OC neurons and members of the B4 cluster motoneurons, furthermore, chemically transmitted synaptic responses were recorded both on feeding motoneurons (B1, B2 cells) and the SO modulatory interneuron after the stimulation of OC neurons. However, elementary synaptic potentials could not be recorded on the follower cells of OC neurons. Prolonged (20–30 s) intracellular stimulation of OC cells activated the buccal feeding neurons leading to rhythmic activity pattern (fictive feeding) in a way similar to OA applied by perfusion onto isolated CNS preparations. Our results suggest that OA acts as a modulatory substance in the feeding system of *Lymnaea stagnalis* and the newly identified pair of OC neurons belongs to the buccal feeding network.

Keywords: *Lymnaea stagnalis*; octopamine; feeding; neuromodulation

1. INTRODUCTION

The role of octopamine (OA) as a neuromodulator is widely accepted in arthropods. This substance has been proved to be involved in different behaviours, such as the crayfish posture (Kravitz 1983), insect flight (Ramirez & Pearson 1991) and jumping (Evans 1985), light emission of the firefly light organ, and feeding (Nathanson 1985). The importance of OA in this phylum is also seen from more recent papers on the modulation of the visual system (Stern *et al.* 1995), escape behaviour (Bustamante & Krasne 1991; Casagrand & Ritzmann 1992) and the stomatogastric system (Johnson *et al.* 1995). In annelids too, OA plays an important part in central and peripheral neuronal processing (Hashemzadeh-Gargari & Friesen 1990; Catarsi *et al.* 1995).

*Author and address for correspondence: University of York, Department of Biology, Heslington, York YO1 5DD, UK (agnes@tres.blki.hu).

Although OA evokes specific membrane responses on identified neurons of the central nervous system (CNS) in gastropods (Carpenter & Gaubatz 1974; Bahls 1990; Batta *et al.* 1979; Walker *et al.* 1993; Hiripi *et al.* 1998), no data are available concerning the physiological role of OA in these molluscs. The presence of OA has, however, been well demonstrated in the gastropod CNS by biochemical measurements (Robertson & Juorio 1976; Farnham *et al.* 1978; McCaman 1980; Zhou *et al.* 1993). The distribution of OA-immunoreactive neurons was described in the *Lymnaea* CNS, where they occurred in the buccal, cerebral and pedal ganglia (Elekes *et al.* 1993, 1996). The highest concentration of OA was measured in the paired buccal ganglia in *Aplysia* (McCaman 1980), *Helix* and *Lymnaea* (Elekes *et al.* 1996; Hiripi *et al.* 1998). These ganglia also contain the main elements of the feeding network: one of the most studied neuronal systems in gastropods (*Achatina*: Yoshida & Kobayashi 1992; *Aplysia*: Kirk 1989; *Helisoma*: Kater & Rowell 1973;

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Limax: Prior & Gelperin 1977; *Lymnaea* Benjamin & Elliott 1989; *Planorbis*: Arshavsky *et al.* 1988; *Tritonia*: Bulloch & Dorsett 1979).

In *Lymnaea stagnalis* the feeding network in the buccal ganglia is composed of motoneurons (B1, B2, B3, ... B10 cells; Rose & Benjamin 1979; Benjamin & Rose 1979), central pattern generator interneurons (N1, N2, N3 interneurons; Rose & Benjamin 1981*b*; Elliott & Benjamin 1985*a*; Elliott & Kemenes 1992), and higher order interneurons (SO, N1L; Elliott & Benjamin 1985*b*; Yeoman *et al.* 1995). These nerve cells act together with the cerebral interneurons (CGC, CV1; McCrohan & Benjamin 1980*a*,*b*; McCrohan 1984) to produce a rhythmic pattern (fictive feeding) which corresponds to the alternating cycles (protraction followed by retraction) of the radula movements. The motoneurons play no part in the production of the rhythm, which is generated by the central pattern generator interneurons (N1, N2 and N3). The protractor premotor cells (N1) are cholinergic (Elliott & Kemenes 1992), whereas the retractor N2 interneurons probably use glutamate (Brierley 1994; Quinlan & Murphy 1991). These cells are activated during feeding by the modulatory interneurons SO, N1L and CGC, of which the first two are cholinergic (Yeoman *et al.* 1993; Vehovszky & Elliott 1995) and the latter serotonergic (Benjamin 1983). These modulatory cells are stimulated by inputs from chemo- and mechanosensory cells, which probably use nitric oxide (NO) as a transmitter (Kemenes *et al.* 1986; Moroz *et al.* 1993; Elphick *et al.* 1995).

Particularly in feeding, serotonin (5HT) is thought to be the predominant modulator substance, released mainly by the giant neurons (CGCs) located in the paired cerebral ganglia (Pentreath *et al.* 1982; Benjamin 1983; Yeoman *et al.* 1996). Dopamine (DA) is considered to be another possible feeding modulator in gastropods as it initiates the feeding pattern in isolated CNS (Kyriakides & McCrohan 1989; Wieland & Gelperin 1983; Trimble & Barker 1984). In *Aplysia*, a DA-containing buccal neuron is identified, which is capable of initiating the feeding activity of the buccal network (Teyke *et al.* 1993). In addition, the role of modulatory peptides cannot be ruled out in feeding (Sossin *et al.* 1987; Church *et al.* 1991; Santama *et al.* 1994). In the buccal ganglia of *Lymnaea stagnalis* myomodulin, buccalin, small cardioactive peptide (SCP), and FMRFamide-related peptides were shown to co-localize with 'classical' transmitters in the cell body of both feeding motoneurons (B2, B3, B7 cells) and interneurons (N1, N2, SO, CGC; Santama *et al.* 1994).

Although OA-immunoreactive neurons have been visualized and the existence of OA receptors has also been proved both in *Lymnaea* and *Helix* CNS (Elekes *et al.* 1993, 1996; Hiripi *et al.* 1998), the possible role of OA in feeding has not been investigated. Also, and more generally, no functionally identified neurons are known to contain or use OA as a neurotransmitter or neuromodulator in *Lymnaea stagnalis* or other gastropods.

Therefore, in this paper we now address two questions: (i) does OA play any role in the feeding behaviour of *Lymnaea stagnalis*; and (ii) do OA-containing neurons in the buccal ganglia show a physiological relationship with the feeding network? To answer these questions we did behavioural tests on intact animals and a combination of electrophysiological and morphological experiments in the isolated CNS.

Our results demonstrate that feeding behaviour is inhibited or reduced by the known octopaminergic drugs phentolamine, 2-chloro-4-methyl-2-(phenylimino)-imidazolidine (NC-7), and demethylchlordimeform (DCDM). OA, applied to the isolated central nervous system, evokes the rhythmic pattern of fictive feeding from buccal feeding neurons. Furthermore, we identified a pair of nerve cells (called OC neurons) in the buccal ganglia, which participate in fictive feeding rhythms, and modulate the activity of both feeding interneurons and motoneurons. Therefore, the newly identified OC neurons can be considered as new members of the feeding network in *Lymnaea stagnalis*.

2. MATERIALS AND METHODS

Adult specimens of *Lymnaea stagnalis* were collected from the surrounding ponds of the Lake Balaton area (Hungary) or obtained from a supplier (Blades, England). The animals were kept under laboratory conditions in standard snail water (England) or filtered Balaton water (Hungary), and fed on lettuce.

(a) *Behavioural studies*

A total of three days prior to the feeding experiments, the animals were removed from their main containers, put into smaller tanks and left without food. Feeding tests were done on groups of 10–15 intact animals. A control group was injected with 100 μl of normal *Lymnaea* saline (Elliott *et al.* 1992). Treated groups were injected with the same volume of solvent in which the chosen antagonist had been dissolved, to give a final concentration from 1–50 mg kg^{-1} body weight. All snails from the control and experimental groups were tested 1, 4, 7 and 24 h after injection. Prior to the feeding tests the following drugs were injected into the experimental animals: ergotamine-D-tartrate (ROTH), ±sulpiride (SIGMA), phentolamine hydrochloride (SIGMA), DCDM (donated by Professor R. M. Hollingworth, Pesticide Research Center, Michigan State University, East Lansing), and NC-7 (donated by Professor R. G. H. Downer, Department of Biology, University of Waterloo).

Feeding tests were done by placing the snail gently into a Petri dish filled with 10^{-2} M sucrose solution, which evokes a standard feeding response, including biting movements (Kemenes *et al.* 1986). To characterize the feeding behaviour we counted: (i) the number of snails which emerged and took a bite within 2 min of placing them in sucrose; and (ii) the number of bites in the first minute after the animal started feeding.

In preliminary experiments a group of solvent-injected animals was tested before, and 1 h after the injection. No significant differences were observed in their feeding parameters, suggesting that the injection itself did not affect feeding.

The drug-injected animals were compared with a control group injected with standard saline in the same series of experiments. The χ^2 test was used to calculate the significance of the number of snails responding to sucrose. The Student's *t*-test (assuming equal variances) was used to compare the mean feeding rate.

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(b) *Morphological studies*

(i) *Intracellular staining*

After electrophysiological identification, neurons were reimpaled with a microelectrode containing 3% Lucifer yellow solution, and filled intracellularly by passing repeated hyperpolarizing current pulses (5 nA, 1 Hz, 1 s) for 10–20 min. After filling the neurons, the whole mounts of isolated buccal ganglia were fixed with 4% formaldehyde diluted in 0.1 M Na-phosphate buffer, dehydrated in ethanol and cleared in methyl salicylate (Elliott & Benjamin 1985*a*). The morphology of the neurons was reconstructed from serial micrographs of the whole-mount preparation.

(ii) *Immunocytochemical labelling*

Buccal ganglia containing the Lucifer yellow-filled neurons were processed for OA whole mount immunofluorescence. After 24 h of incubation with anti-OA antiserum (Eckert *et al.* 1992) diluted 1:1000, the ganglia were treated for 4–6 h with rhodamine-conjugated labelling described previously (Elekes *et al.* 1996), but with secondary swine anti-rabbit diluted 1:50. Both incubations were done at room temperature. The preparations were mounted in 3:1 mixture of glycerol and phosphate-buffered saline, viewed and photographed under a Zeiss Axioplan microscope, using the appropriate filter blocks to visualize either Lucifer yellow (injected dye) or rhodamine (OA antibody) labelling. The specificity of anti-OA antiserum was demonstrated previously in the CNS of *Lymnaea* (Elekes *et al.* 1993).

(c) *Electrophysiological experiments*

The CNS (including the paired buccal ganglia) was isolated and placed in a dish of standard *Lymnaea* saline (Elliott *et al.* 1992). Before impalement with microelectrodes, the connective tissue was softened with protease (Sigma, type XIV) and the outer layer was removed mechanically from the dorsal surface of the buccal ganglia. Glass microelectrodes with tip resistances from 8–30 MΩ were used to record the intracellular activity of individual buccal neurons, as described earlier (Elliott *et al.* 1992).

Known members of the buccal feeding network were identified by their position, spontaneous activity and/or the firing pattern evoked by the modulatory interneurons (Elliott & Benjamin 1985*a*,*b*; Yeoman *et al.* 1995). In addition, a pair of buccal nerve cells (later on called OC neurons) was identified by the same electrophysiological criteria and its spontaneous activity was compared with that of known members of the feeding network. To study the possible synaptic connections between the neurons, simultaneous recordings were done from one of the OC and one known buccal neuron (SO, N1M and N1L interneurons, or feeding motoneurons labelled by B1, B2, ...), passing polarizing current into the cell body through the recording electrode for intracellular stimulation.

During our experiments, the bath containing the isolated CNS was continuously perfused with standard *Lymnaea* saline (Elliott *et al.* 1992) with a flow rate of 3–5 ml min^{-1}. For testing synaptic connections between the neurons, a modified physiological solution with raised Mg^{2+} and Ca^{2+} content (Na-HiDi) was used (Vehovszky & Elliott 1995), which raises neuronal thresholds by about 10 mV, thus reducing the activity of the network. Synaptic connections which do not persist in a 1:1 manner in this saline are likely to be polysynaptic (Berry & Pentreath 1976).

3. RESULTS

(a) *Pharmacological modulation of feeding behaviour*

(i) *General behavioural effects*

The behaviour of those animals that received standard *Lymnaea* saline (members of control group) returned to normal within 1 h of injection: the snails crawled around in the water and started to feed when they were placed in sucrose.

Immediately after the drug injections of between 25 and 50 mg kg^{-1} body weight dose, the general locomotory behaviour of the treated animals was significantly affected. Those specimens that received OAergic drugs (phentolamine, NC-7 and DCDM) emerged from their shell, and their muscle tone seemed to be slightly increased compared with control animals. At the same time, local muscle contractions appeared randomly on different parts of the body. Tactile stimulation of the head region evoked short, weak contractions, but no real withdrawal of the tentacles or the body was observed. The changes of muscle tone and locomotion disappeared 2–3 h after phentolamine or DCDM treatment. A total of 4–6 h after NC-7 injection, the increased muscle tone was followed by relaxation, which lasted up to 48 h.

Animals injected with the dopamine antagonists (ergotamine and sulpiride) emerged from their shell, but their muscle tone was rather low compared with the solvent-injected snails. After 7–9 h of the injection, their spontaneous locomotory behaviour seemed to be normal in the water-filled tanks. However, after placing them into sucrose they started fast, irregular head and lip movements, usually with their mouth open.

(ii) *Effects on feeding behaviour*

The OA antagonist NC-7 significantly ($p<0.01$) reduced the feeding response even at a relatively low (10 mg kg^{-1}) dose (see figure 1). A total of two-thirds of the snails did not start feeding in sucrose, and these animals did not recover during the 48 h following the injection. With a second OAergic drug phentolamine, a higher dose (25 mg kg^{-1}) was necessary to significantly reduce the feeding response ($p<0.01$). The third OAergic drug tested, DCDM, was less effective, even at 50 mg kg^{-1}, where the number of feeding animals was 60%, but this reduction of feeding was not significant. Thus, among the OA antagonists tested, phentolamine proved the most potent feeding inhibitor, as none of the animals injected with 25–50 mg kg^{-1} phentolamine started biting in sucrose (1–4 h after injection). All drug-injected animals recovered within 24 h after injection.

After injection of 25 mg kg^{-1} dopamine antagonists (ergotamine or sulpiride), the number of feeding snails decreased by 30–40%, but in neither case was this significant at the $p=0.05$ level. Only after injecting a 50 mg kg^{-1} dose of ergotamine or sulpiride was there a significant ($p<0.01$), strong (70–90%) blocking effect on feeding (see figure 1). However, the effect of ergotamine started only 7–8 h after the injection, and the treated animals did not recover 24 h after treatment.

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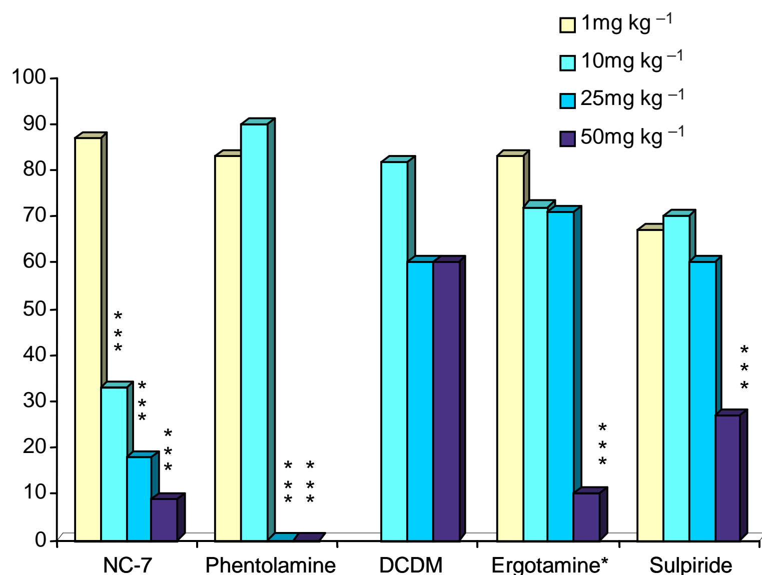

Figure 1. The feeding response of intact *Lymnaea stagnalis* specimens to sucrose (10^{-2} M) after treatment with increasing doses of putative antagonists. The *y*-axis shows the percentage of the animals responding to sucrose 1–4 h or *7–8 h after drug injection (the total number of animals in a group of 10–15 specimens was taken as 100%). The significance level of the changes compared with the vehicle injected animals was calculated using the χ^2 test ($^{***}p<0.01$).

NC-7 injection (10–50 mg kg^{-1}) significantly decreases the number of feeding snails, while phentolamine injected with a 25 or 50 mg kg^{-1} dose completely inhibits feeding of treated animals. After 50 mg kg^{-1} DCDM injection, 60% of the animals still started feeding. Ergotamine and sulpiride injected in high (50 mg kg^{-1}) doses decrease the number of feeding animals to 10–30%.

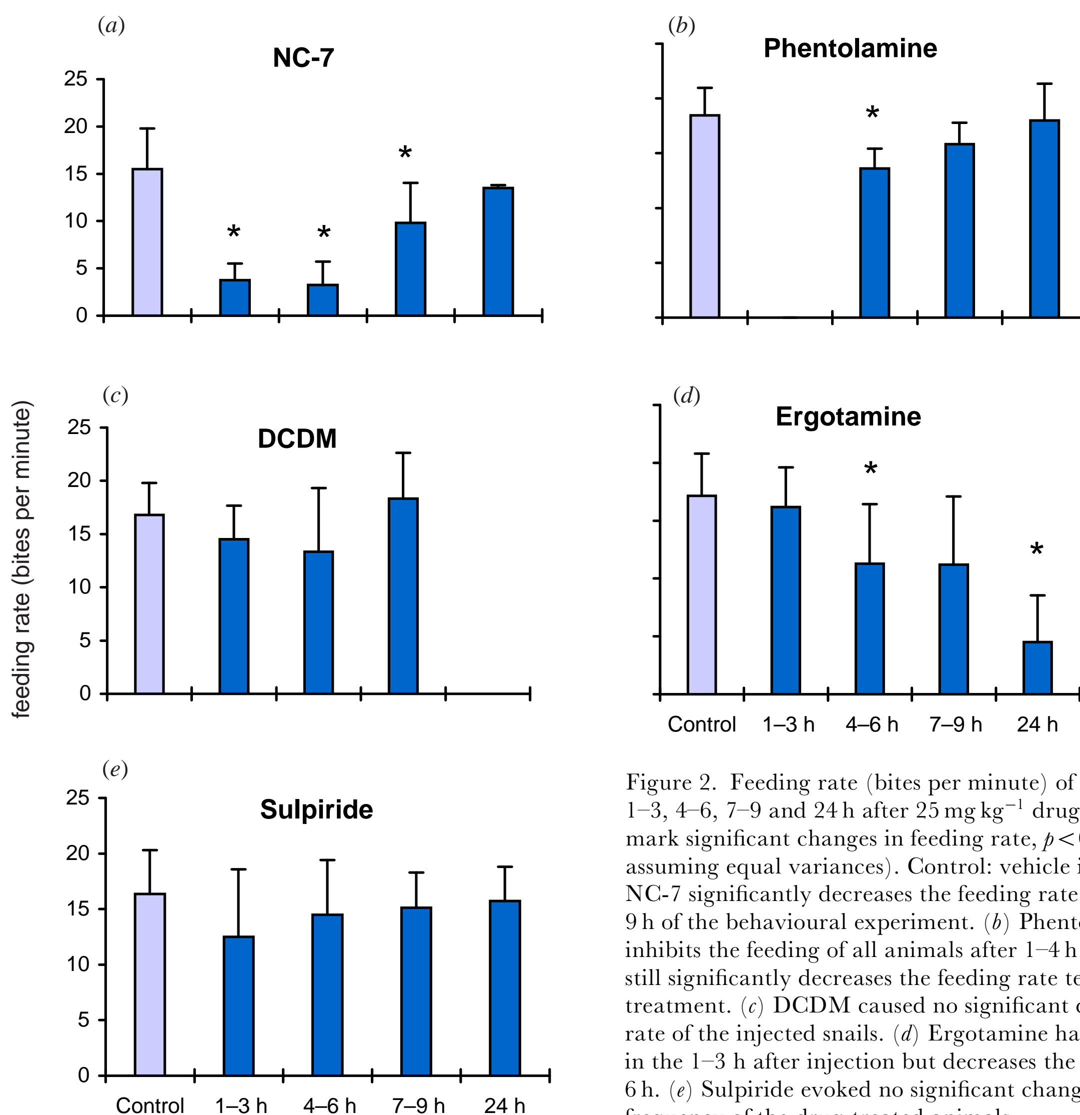

Figure 2. Feeding rate (bites per minute) of groups of animals, 1–3, 4–6, 7–9 and 24 h after 25 mg kg^{-1} drug injection. Asterisks mark significant changes in feeding rate, $p<0.05$ (Student's *t*-test assuming equal variances). Control: vehicle injected animals. (*a*) NC-7 significantly decreases the feeding rate throughout the first 9 h of the behavioural experiment. (*b*) Phentolamine completely inhibits the feeding of all animals after 1–4 h of drug injection, and still significantly decreases the feeding rate tested 4–6 h after the treatment. (*c*) DCDM caused no significant decrease in the feeding rate of the injected snails. (*d*) Ergotamine has no significant effect in the 1–3 h after injection but decreases the feeding rate after 4–6 h. (*e*) Sulpiride evoked no significant change of the biting frequency of the drug-treated animals.

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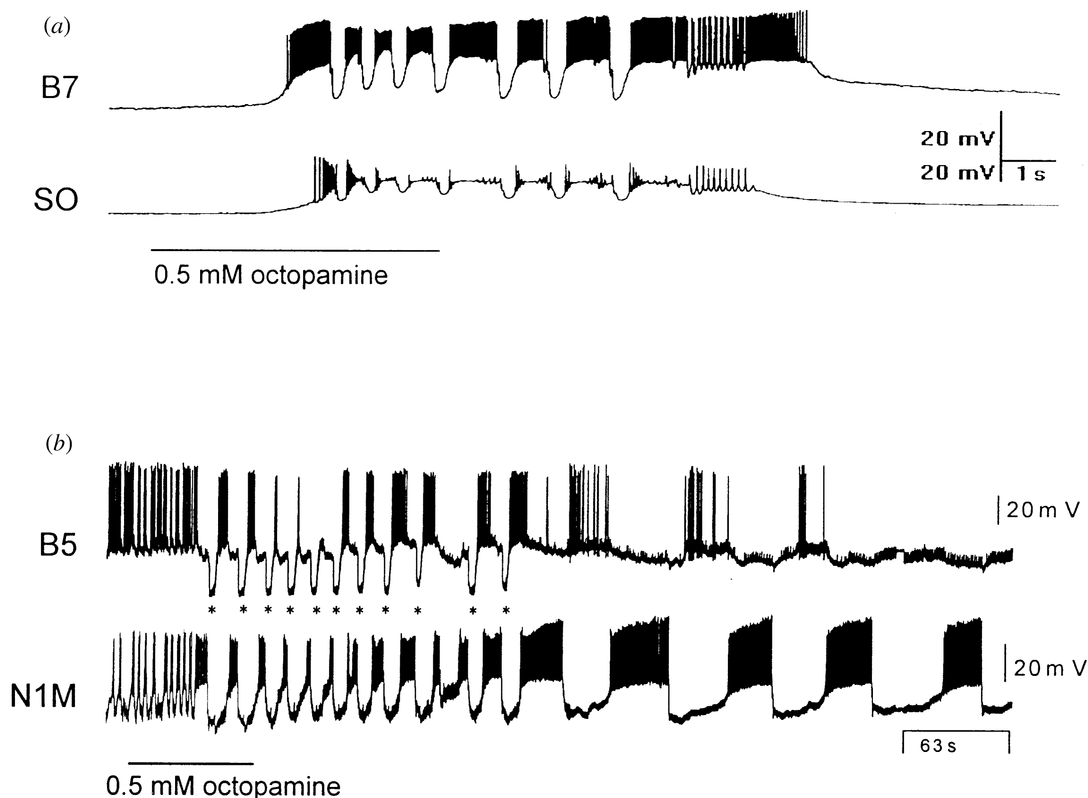

Figure 3. OA evokes feeding rhythm on buccal feeding neurons. (*a*) OA (0.5 mM concentration, 300–500 µl volume) applied by local perfusion to the bath evokes feeding pattern on both B7 motoneuron (upper trace) and SO feeding interneuron (lower trace). Note that SO firing appears after starting the feeding rhythm on B7 neuron. (*b*) OA applied to the bath evokes feeding rhythm, represented by series of inhibitory inputs (N2 phases of the feeding cycle, marked by asterisks) on both B5 motoneuron (upper trace) and N1M feeding interneuron (lower trace).

The feeding rate (measured as bites per minute) of the treated snails (those specimens, which still started feeding in sucrose) was reduced after injection of NC-7, phentolamine or ergotamine (figure 2). The animals showed a significantly lower feeding rate 1–6 h after injection of NC-7 and phentolamine, slowly recovering after 24 h (figure 2*a*,*b*). The inhibitory effect of ergotamine on the feeding rate started 4 h after the injection and lasted over 24 h (figure 2*d*). After injection of 25 mg kg^{-1} DCDM or sulpiride the feeding rate of the treated animals was not significantly different from that of the controls (figure 2*c*,*e*).

(b) *Feeding rhythm evoked by OA applied onto the isolated CNS*

After the application of 300–500 µl 0.5 mM OA by local perfusion, a highly regular activity pattern (fictive feeding) was recorded from identified feeding neurons (see figure 3). This rhythmic pattern appeared before the SO started to fire (figure 3*a*), suggesting that the feeding pattern was not generated by direct activation of the modulatory SO interneuron. Recording from a N1M interneuron, which was spontaneously firing in weak bursts, it could be demonstrated, that OA strengthened the N1M bursts and also brought in a series of strong N2 inhibitory inputs (figure 3*b*).

(c) *Identification of putative OA-containing neurons in the buccal ganglia*

We identified a pair of neurons situated on the dorsal surface of the buccal ganglia (see figure 4) in the same location, where immunocytochemical labelling visualized OA-immunoreactive cell bodies (Elekes *et al.* 1993, 1996). Assuming that our electrophysiologically described neurons are identical to those labelled by OA-immunoreactivity, we called them OC (putative OAergic cells, or OA containing) neurons. Simultaneous recordings with already-known buccal feeding neurons were done to see whether OC neurons have any relationship with the buccal feeding system in the *Lymnaea stagnalis*.

(i) *Morphological characterization of OC neurons*

OC neurons with a diameter of about 25–35 µm, are situated on the mediorostral edge of the dorsal surface of each of the buccal ganglia (figure 4). Their usually whitish appearance helps identification among neighbouring neurons of similar size.

Axonal branching pattern

A total of six OC neurons were stained intracellularly with Lucifer yellow and all of them had a characteristic morphological appearance, left and right cells having mirror-image symmetry (figure 5*a*,*b*). From the cell body two main axon processes arise, running towards the central neuropil area of each buccal ganglia. One of the axon processes can be traced throughout the buccal commissure, entering the contralateral buccal ganglion, where it forms a loop in the central neuropil area (figure 5). Turning back, the main axon process enters the buccal commissure again and reaches the neuropil of the ipsilateral ganglion. The other axon process forms a similar

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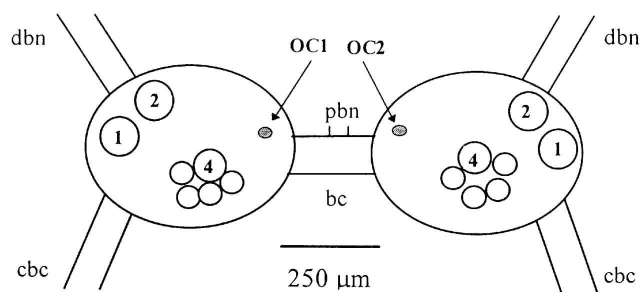

Figure 4. Location of the identified OC neurons in the buccal ganglia. Schematic representation of the dorsal surface of the paired buccal ganglia indicating the position of putative OA-containing neurons (OC1 and OC2) and previously identified feeding motoneurons (B1, B2, motoneurons, B4 cluster cells). cbc: cerebro-buccal connective; bc: buccal commissure; pbn: post-buccal nerve; dbn: dorso-buccal nerve.

loop in the ipsilateral neuropil. The other main morphological feature of the OC neurons is the extensive axonal arborization along the main neurites. In the buccal commissure, fine side-branches arise from the main axon process (figure 5*b*), whereas in the neuropil similar collaterals connect the opposite branches of the axon loop. Shorter side-branches form varicosities in the buccal neuropil and seem to terminate inside the ganglion. Occasionally (in three of six preparations) a set of fine fibres could be seen in the direction of the cerebrobuccal connective tissue (figure 5*a*), but their presence could not be traced for a longer distance within the connective tissue.

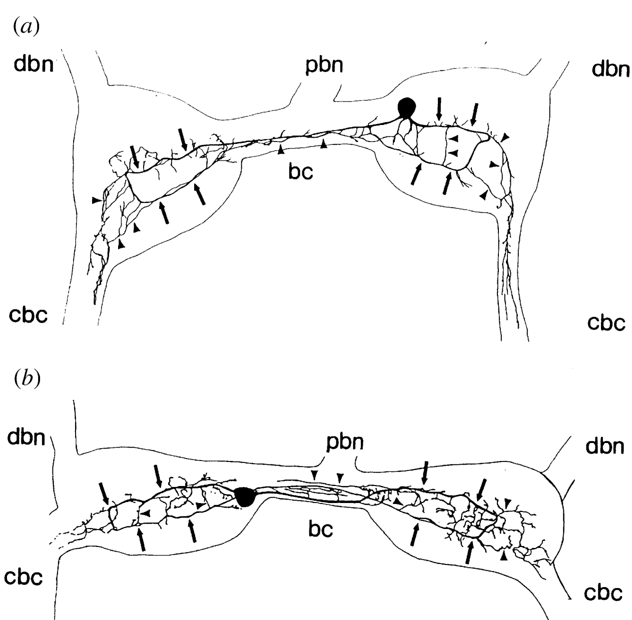

Figure 5. Morphology of OC neurons intracellularly filled with Lucifer yellow (drawings reconstructed from photomicrographs of two representative whole-mount preparations). The pair of neurons located in (*a*) the right and (*b*) the left buccal ganglion have similar morphology, characterized by two axonal loops (marked by arrows) in the central neuropil area of each buccal ganglion. Fine side branches (marked by arrowheads) arise from the axonal trunk forming parallel processes in the buccal commissure and a dense network in the buccal neuropil. cbc, cerebrobuccal connectives; bc, buccal commissure; pbn, post-buccal nerve; dbn, dorso-buccal nerve. Scale, 200 μm.

Immunocytochemistry

After intracellular filling of one of the OC neurons with Lucifer yellow, the isolated buccal ganglia were processed for OA immunohistochemistry. Using two different filter sets we compared the morphology of the Lucifer yellow-labelled OC neurons to the location and axonal branching pattern of OA-immunoreactive cells.

In the three cleared whole-mount buccal preparations studied, the cell body and the main axon branches of a Lucifer yellow-filled OC neuron with the same morphological features described (figure 5), were clearly seen (figure 6). After the filters were switched, three cell bodies appeared to be OA-immunoreactive in the same preparation (figure 6*b*). The shape and position of one of the labelled neurons corresponded well to the electrophysiologically identified, Lucifer yellow-filled cell body of the OC neuron (figure 6*a*). Furthermore, OA-immunoreactive axonal branches were clearly seen along the buccal commissure and in the neuropil of the contralateral buccal ganglion in the same pattern, as was it characterized for the Lucifer yellow-filled fibres of the OC cells (figure 5*a*,*b*).

(ii) *Electrophysiological characterization of OC neurons*

Activity pattern

In isolated CNS preparations simultaneous intracellular recordings were carried out by impaling one of the OC neurons and another, already identified member of the feeding network. When the CNS shows no regular feeding activity, OC neurons are usually characterized by series of excitatory synaptic inputs or action potentials (figure 7*a*,*b*). When the feeding rhythm starts, these inputs lead to bursts of action potentials on OC neurons (figure 7*b*,*c*). When fictive feeding is elicited by intracellular stimulation of one of the modulatory interneurons (SO, N1M, N1L), the OC neurons also participate in the rhythmic pattern of the feeding neurons (figure 7*c*,*d*). They are first inhibited in the N2 (retraction) phase, then start to depolarize and fire during N2 and in the whole of the N3 (swallowing) phases of the feeding rhythm (figure 7*d*).

Synaptic connections with feeding neurons

Intracellular injection of polarizing current into the OC neurons evoked either electrical responses (figure 8), or chemically transmitted excitatory or inhibitory postsynaptic responses (figures 9 and 10) from members of the feeding network. Furthermore, longer (15–20 s duration) bursts of the presynaptic OC neuron modified the whole firing pattern of the follower cells, most frequently leading to fictive feeding rhythm of both (presynaptic and postsynaptic) neurons recorded (figures 9*b*,*d*,*e* and 10*b*,*c*).

OC neurons form direct electrical connections with B4 cluster motoneurons and also with the contralateral OC neuron (figure 8). Whether depolarizing or hyperpolarizing current was injected into the cell body of one of the OC neurons, a similar potential change was recorded from both the contralateral OC neuron (figure 8*a*) and the four-cluster (B4) motoneurons (figure 8*b*). Although coupling coefficient was not measured, the electrical coupling formed by OC

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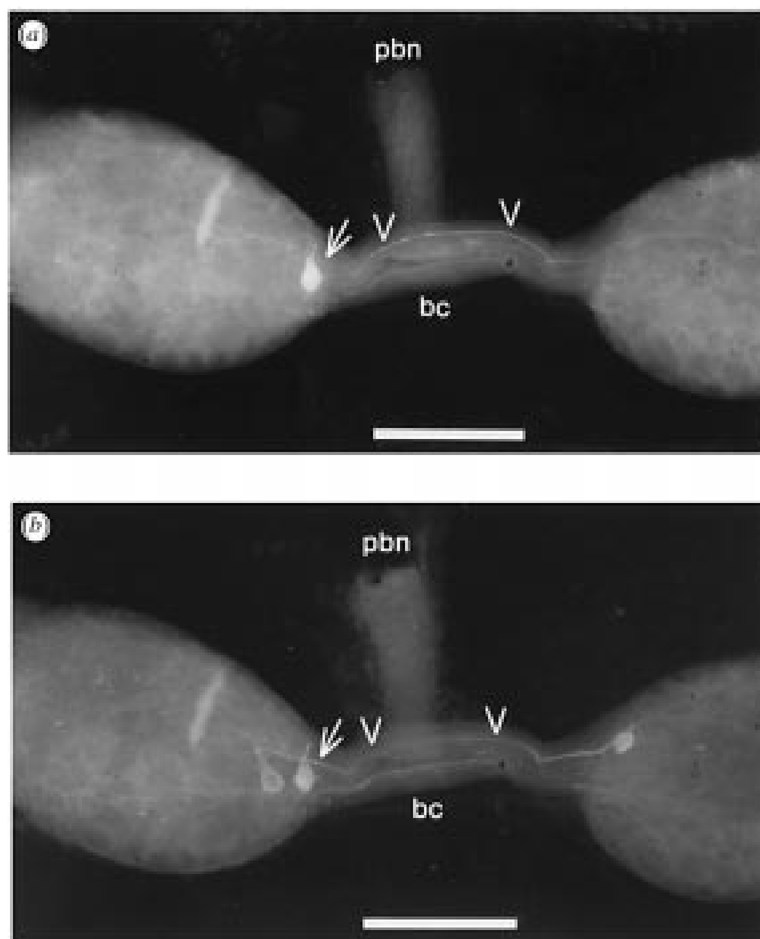

Figure 6. Double-labelling of an OC neuron (photographs taken from a cleared whole-mount preparation). (*a*) An OC neuron (arrow) intracellularly filled with Lucifer yellow in the left ganglion with one of its main axon processes (arrowheads) running contralaterally through the buccal commissure. (*b*) The same preparation as in (*a*), using a different filter set to visualize rhodamine (labelling OA antibody) in three cell bodies. The cell body of one of the OA-immunoreactive cells on the left (arrow) appears in the same position as the Lucifer yellow-labelled neuron seen in figure (*a*). Labelled axonal branches (arrowheads) in the buccal commissure correspond to the Lucifer yellow-containing main neurite of the OC neuron seen on figure (*a*). bc: buccal commissure; pbn: post-buccal nerve. Scale, 200 μm.

neurons with the four-cluster motoneurons was routinely found (nine out of ten preparations), so that these connections were used as one of the electrophysiological criteria for identifying the OC neurons.

OC neuron firing had complex effects on the activity of other feeding motoneurons studied (figure 9). OC bursting evoked by a 2–4 s current injection was followed by an excitatory postsynaptic response (depolarization or action potential generation) on B1 motoneurons (figure 9*a*). However, we did not observe elementary postsynaptic potentials following the OC stimulation, not even in Na-HiDi saline. Longer (20–30 s duration) electrical stimulation of OC neurons evoked complex changes of firing activity of B1 motoneurons (figure 9*b*). The depolarization of the B1 cell membrane was followed by rhythmic bursting on both neurons, most frequently seen on silent preparations after switching off the current injected into the OC cell body (figure 9*b*).

OC neuron stimulation first evoked hyperpolarization on B2 cell motoneurons (figure 9*c*,*d*). After injecting shorter (up to 10 s) current pulses into the OC neuron, a hyperpolarization was seen clearly on B2 neuron even after 20 min in Na-HiDi saline. The synaptic delay remained quite long (about 500 ms), furthermore, discrete 1:1 inhibitory postsynaptic potentials (IPSPs) were not recorded. The connections of the OC neurons with B1 and B2 motoneurons were not always seen clearly, in about 50% of the preparations (OC-B1: 7 out of 14, OC-B2: 5 out of 11). Longer-lasting (20–30 s) intracellular stimulation of OC neurons was followed by a general excitatory effect on the B2 cell motoneurons and similar rhythmic activity appeared on the OC neuron itself, suggesting that the whole feeding network was activated (figure 9*d*). After long stimulation of the OC neurons the activation of other buccal motoneurons (B7 neuron on figure 9*e*), was also seen, and this feeding pattern was mostly characterized by N2 (retractor phase) inputs of the feeding cycle.

OC neurons also have synaptic connections with the feeding interneurons. Following short intracellular stimulation of the OC neuron, the modulatory SO feeding interneuron responded by a complex response, in which hyperpolarization was followed by depolarization of the membrane (figure 10*a*). When the OC cells were stimulated for 20–30 s, fictive feeding (bursts of action potentials) were seen in the OC neuron itself and in the feeding interneurons (SO, N1M, figure 10*b*,*c*). This activity pattern was close to that seen when the SO feeding interneuron was intracellularly stimulated (figure 7*c*,*d*). This synaptic effect of the OC neurons leading to fictive feeding rhythm was characterized with a long (several seconds) delay and no discrete excitatory synaptic potentials were recorded on the follower cells.

4. DISCUSSION

(a) *OAergic modulation of feeding in* Lymnaea

(i) *OAergic drugs inhibit feeding of intact snails*

Immediately after injection of NC-7, phentolamine and DCDM, both locomotion and feeding of the treated animals were affected. A total of 2–3 h after phentolamine

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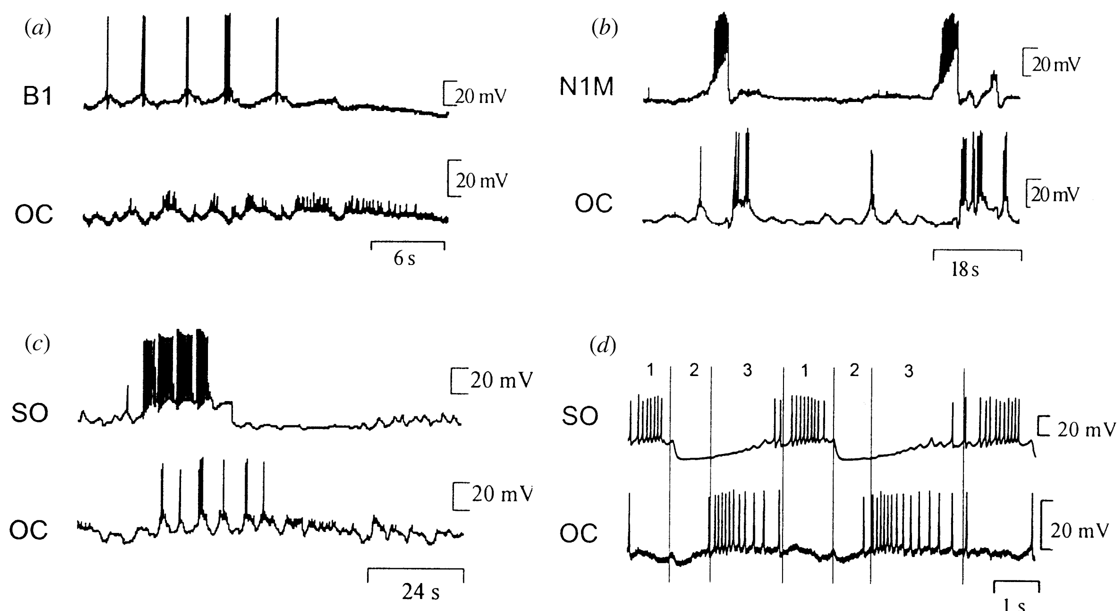

Figure 7. Activity pattern of OC neurons recorded simultaneously with feeding neurons. (*a*) Simultaneous recording from B1 motoneuron (upper trace) and OC neuron (lower trace) displays synchronous pattern of spontaneous activity. (*b*) Spontaneous bursts of N1M interneuron (upper trace) are followed by bursting activity of OC cells (lower trace). (*c*) Feeding rhythm evoked by intracellular stimulation of the SO modulatory interneuron (upper trace) is followed by bursting pattern of the OC neuron (lower trace). (*d*) The modulatory SO interneuron (upper trace) and the OC neuron (lower trace) show synchronous activity, the OC neuron first receiving inhibitory input in the N2 phase, then firing in both N2 and N3 phase of the fictive feeding (feeding cycles are marked by numbers 1, 2, and 3).

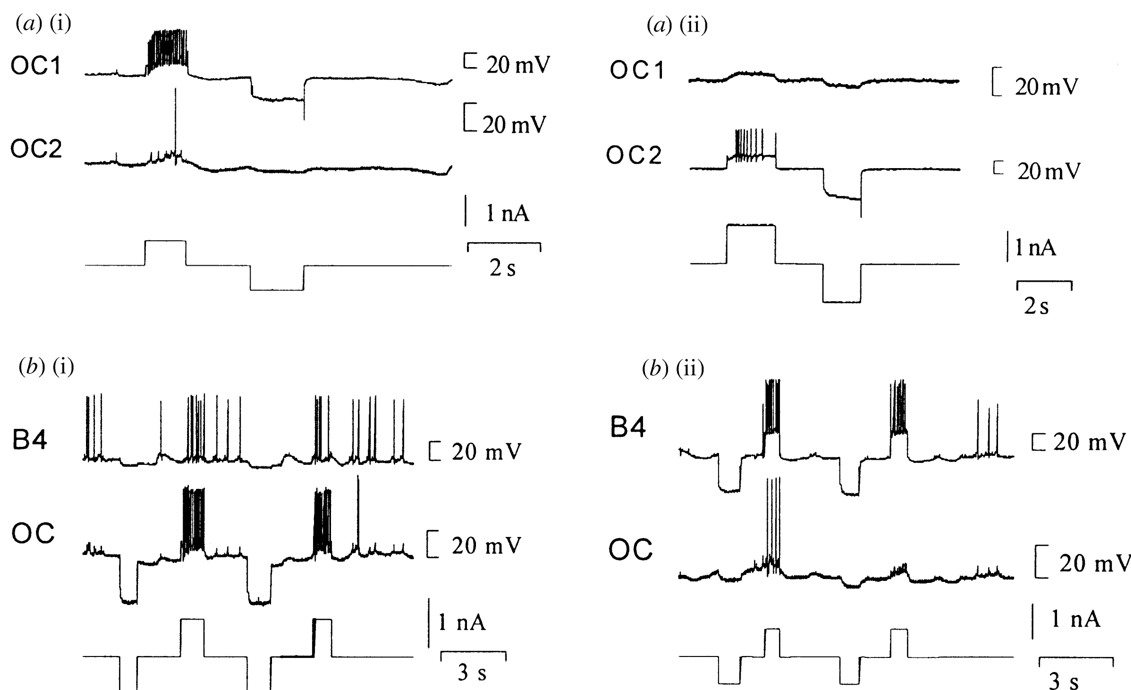

Figure 8. Electrical coupling between OC neurons and B4 motoneurons. (*a*i) Depolarizing or hyperpolarizing current (displayed on the bottom trace) injected into the cell body of one OC neuron (OC1, upper trace) evokes similar potential changes recorded on its pair (OC2 neuron) located in the contralateral buccal ganglion (middle trace). (*a*ii) Current injection into the OC2 neuron (middle trace) evokes depolarizing or hyperpolarizing responses in OC1 neuron (upper trace). (*b*i) Intracellular current injection (bottom trace) leading to hyperpolarization or burst of action potentials on OC neuron (middle trace) are accompanied with the same potential changes on B4 neuron (upper trace). (*b*ii) Current injected into the B4 motoneuron (upper trace) evokes the corresponding electrical responses (depolarization or hyperpolarization) on OC neuron (middle trace).

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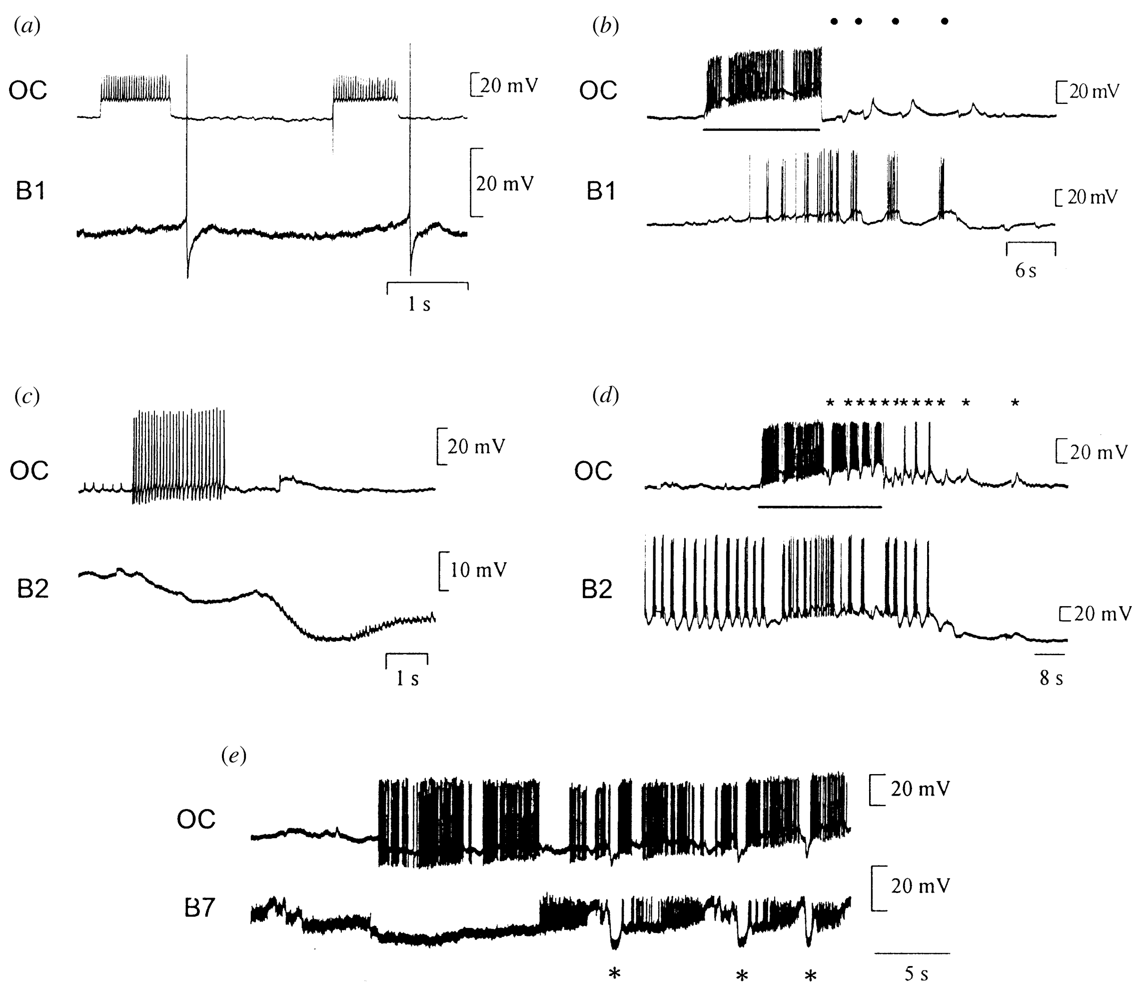

Figure 9. OC neurons modulate the activity of feeding motoneurons. (*a*) Intracellular stimulation of OC neuron (upper trace) is followed by excitatory response (action potentials) recorded on B1 motoneuron (lower trace). (*b*) OC neuron stimulation depolarizes the B1 motoneurons leading to bursting activity (lower trace) and rhythmically appearing complex synaptic inputs on the OC neuron itself (upper trace). Polarizing current injected into OC neuron is marked by a bar below the OC record. N1 phases of the feeding cycles are marked by dots. (*c*) Burst of OC neuron (upper trace) evokes hyperpolarization on B2 motoneuron (lower trace). (*d*) After longer lasting stimulation of OC neuron (upper trace) the B2 motoneuron starts to depolarize then firing with increased frequency of rhythmic activity (lower trace). Polarizing current injected into OC neuron is marked by a bar below the OC record. N2 phases of the feeding cycles are marked by asterisks. (*e*) Longer-lasting stimulation of OC neuron (upper trace) evokes hyperpolarization followed by rhythmic bursting activity on B7 motoneuron (lower trace). N2 inputs of the feeding cycles are marked by asterisks.

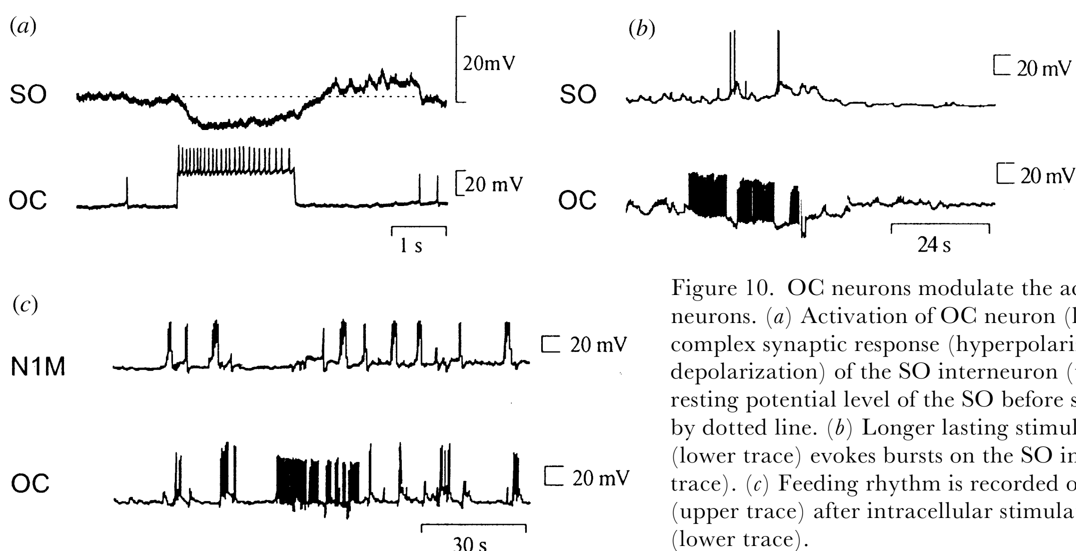

Figure 10. OC neurons modulate the activity of feeding interneurons. (*a*) Activation of OC neuron (lower trace) evokes complex synaptic response (hyperpolarization followed by depolarization) of the SO interneuron (upper trace). The resting potential level of the SO before stimulation is marked by dotted line. (*b*) Longer lasting stimulation of OC neuron (lower trace) evokes bursts on the SO interneuron (upper trace). (*c*) Feeding rhythm is recorded on N1M interneuron (upper trace) after intracellular stimulation of OC neuron (lower trace).

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and DCDM injection, however, the locomotory behaviour of the treated snails seemed to recover, but most of the phentolamine-treated animals did not start feeding in sucrose. In addition, the feeding rate of those specimens that still started biting in sucrose, was significantly decreased after injecting NC-7 or phentolamine. All three drugs (NC-7, phentolamine and DCDM) are known OAergic drugs in arthropods (Nathanson 1993; Roeder 1995; Orr *et al.* 1992) and potent inhibitors of the OA receptors in both *Lymnaea* and *Helix* CNS membrane preparations (Hiripi *et al.* 1998). Therefore, our behavioural results support the idea that OA plays a role in the feeding system. Our observations also indicate that the injected drugs probably affect the central modulatory mechanisms of feeding, in contrast to *Manduca* in which NC-7 inhibits feeding by disrupting the normal neuromuscular transmission (Nathanson 1985).

Phentolamine was the most effective substance at inhibiting feeding in the treated animals. This correlates with its highly selective antagonist action on the OA responses at many snail neurons (Batta *et al.* 1979; Bahls 1990; Gospe & Wilson 1981), including *Lymnaea* B2 buccal motoneuron (Hiripi *et al.* 1998). NC-7 was the next most potent inhibitor, as it decreased the number of animals that accepted food (started biting in sucrose), and parallel to this, it decreased the feeding rate of the responding snails. However, phentolamine and NC-7 showed clear differences in the time relationship of their effects, the phentolamine effect being reversible on both feeding parameters, whereas NC-7 had a longer-lasting effect on the behaviour of the animals. DCDM, an effective OA receptor antagonist in insects (Roeder & Gewecke 1990; Hiripi *et al.* 1994) and on *Lymnaea* neurons (Hiripi *et al.* 1998) it did not change significantly the feeding parameters of the treated animal.

Dopamine, a known feeding modulator, also evokes rhythmic patterns of the buccal neurons (Wieland & Gelperin 1983; Trimble & Barker 1984; Kyriakides & McCrohan 1989), which is similar to the OA effect demonstrated in our experiments. Furthermore, the DAergic neurotoxin, 6-hydroxydopamine (6OH-DA) has been shown to affect the feeding behaviour of *Lymnaea*, because the percentage of responding snails to sucrose decreased and the interbite intervals significantly increased after 6OH-DA injection (Kemenes *et al.* 1990). Although our previous biochemical and electrophysiological results suggested that OA and DA are acting on separate receptors (Hiripi *et al.* 1998), we had to check the possibility whether both amines are affecting the same mechanism of feeding modulation. Therefore, we tested two DAergic drugs—ergotamine and sulpiride—on feeding behaviour, as they are both effective DA antagonists on snail neurons (Bokish & Walker 1988; Nesic & Pasic 1992; Gospe & Wilson 1981; De Vlieger *et al.* 1986). Clear differences were found between the effects of DAergic ergotamine and sulpiride compared with the OAergic NC-7, phentolamine and DCDM. Although ergotamine decreased both the number and the feeding rate of the treated animals, it had a higher threshold to inhibit the feeding, and a longer latency (4–8 h) to develop its effect as opposed to the OAergic drugs NC-7 and phentolamine. The other DAergic antagonist sulpiride decreased the number of responding snails to sucrose but did not change significantly the feeding rate of injected animals. These observations suggest that both DA and OA have their own roles in feeding, and they are acting through different mechanisms in the CNS.

The initial effect of drug injections on the general locomotion and muscle tone of the animals suggests that both OA and DA participate not only in regulation of feeding but other behaviours too, related to normal muscular activity. Changes of the muscular tone and the local muscular contrations observed immediately after injection may demonstrate the peripheral effects of the treatment, when the body wall and columellar muscles, as well as the feeding muscles (muscles of the mouth and the buccal mass) are involved. DA is one of the neuromuscular transmitters in *Aplysia* gill (Swann *et al.* 1982; Ruben & Lukowiak 1983), furthermore, it is proposed to be a neuromodulator in the respiratory system of *Lymnaea* (Syed & Winlow 1991; Barnes *et al.* 1994), and locomotion of *Helix* (Sakharov & Salánki 1980, 1982). As we have no information on the cellular aspects of the OA effect in snails, we do not know where and how OA or OAergic drugs exert their action on the neuronal and/or neuromuscular system. In insects, OA is a well-known modulator of the neuromuscular system both in the peripheral and central nervous system (Kravitz 1983; Evans 1985; Ramirez & Pearson 1991). Morphological observations suggest this possibility in snails too, as the pedal ganglia, which are responsible for regulation of the locomotory behaviour in gastropods, contain high amounts of OA (McCaman 1980; Hiripi *et al.* 1998) and OA-immunoreactive cell bodies are also seen in them (Elekes *et al.* 1993, 1996).

(ii) *OA evokes feeding pattern in isolated CNS*

OA applied by perfusion onto isolated CNS preparations evokes the fictive feeding rhythm, which can be recorded from members of the buccal feeding network. Although we do not have complete evidence at the moment as to which members of the feeding network (CPG or modulatory interneurons) are the targets of OA modulation, our observations suggest that OA exerts its effect via N1 (protraction) and N2 (retraction) phase interneurons. After application of OA into the bath, the N1 and N2 inputs of the feeding cycle are evoked or increased (figure 3*b*).

The feeding pattern is evoked by food application (or sucrose) on semi-intact lip-CNS preparation (Kemenes *et al.* 1986; Yeoman *et al.* 1995), and a similar activation of the feeding network is triggered by NO-donors on semi-intact and isolated CNS preparations (Moroz *et al.* 1993; Elphick *et al.* 1995). The location of NO-producing neurons in the sensory areas of the paired buccal ganglia suggests that NO is a chemosensory transmitter in feeding (Moroz *et al.* 1993; Elphick *et al.* 1995). The similar effect of the OA on the feeding network shows that the involvement of OA in sensory processes cannot be excluded. However, when the chemosensory pathway is affected, the feeding rate of treated animals is the same as that of control animals (Elphick *et al.* 1995). In contrast to this, our behavioural results demonstrate that animals that were injected with the OA-selective antagonists phentolamine and NC-7, had a significantly lower feeding rate compared with the control group. Therefore, the

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central pattern-generating mechanisms are more likely to be affected by this treatment, than the sensory system. These results lead us to the conclusion that OA has a modulatory rather than an initiating role in feeding behaviour.

(b) *OA-containing feeding neurons in the buccal ganglia*

(i) *The pair of identified buccal neurons contains OA*

Immunocytochemical studies combined with biochemical assays (Elekes *et al.* 1996) supported the suggestion that the OA immunoreactive cells in the *Lymnaea* CNS contain OA (Elekes *et al.* 1993). Our double-labelling experiments proved that the pair of buccal nerve cells (later called OC neurons) identified by electrophysiological and morphological criteria belongs to the OA-containing neurons of the buccal ganglia. However, Elekes and co-workers (1996) visualized three OA-positive cells (an unpaired cell, and a pair of neurons) on the dorsal surface of the buccal ganglia. We did not record from all three OA-immmunoreactive neurons simultaneously, therefore we have no direct electrophysiological evidence whether the three neurons are physiologically identical or not. In the buccal feeding network, there are several examples of neurons with the same physiological role which have different locations and morphology (e.g. N1, N2, and N3 pattern-generating interneurons: Rose & Benjamin 1981*b*; Elliott & Benjamin 1985*a*).

(ii) *OC neurons are members of the feeding network*

The OC cells can participate in the feeding rhythm: in spontaneously active preparations they receive excitatory synaptic inputs in the N3 phase of the feeding rhythm, but this activation usually is not strong enough to fire action potentials. During fictive feeding evoked by stimulation of feeding interneurons the OC neurons participate in the feeding activity with regular pattern of complex discharges. Their firing is very similar to the activity pattern of B4 motoneurons, which start to depolarize, then fire in bursts in the retraction phase of the feeding cycle (Rose & Benjamin 1981*a*,*b*). It is most likely that the electrical coupling between OC neurons and B4 motoneurons provides the synchronization of the firing pattern of these neurons, but the functional significance of these connections is not yet known.

In addition, the pair of OC neurons can activate a range of feeding neurons. Among others, they make complex synaptic connections with the B1 cells (mainly excitatory) and to the B2 motoneurons (mainly inhibitory) as demonstrated in figure 9. The excitation of the B1 cells after OC stimulation could be explained by a polysynaptic pathway through the N1M interneurons, but the inhibition of B2 motoneurons cannot be explained by the activation of any known interneuron. The inhibition of the B2 cell is particularly interesting, because the B2 cell is hyperpolarized by application of OA (see Hiripi *et al.* 1998). We have not recorded, however, any discrete, chemically mediated monosynaptic potentials on B1 or B2 motoneurons after stimulating the OC cells either in normal or in Na-HiDi saline, although strong postsynaptic effects were still visible. One possible explanation for the lack of discrete excitatory postsynaptic potentials (EPSPs) or IPSPs is that the electrical coupling between OC neurons and B4 motoneurons (all the B4 cluster cells are electrically coupled; Benjamin & Rose 1979) may smooth the neurotransmission between OC neurons and their followers. Electrical coupling between the feeding neurons is a general feature of the buccal feeding system, as electrically transmitted connections were recorded between paired neurons in left and right buccal ganglia including the left and right B1 or B2 or B3 cell motoneurons (Benjamin & Rose 1979), between members of pattern-generating N1M group (Rose & Benjamin 1981*a*,*b*; Elliott & Benjamin 1995*a*), between N1M interneurons and B5 motoneurons (Elliott & Kemenes 1992), and also between the N1L and SO modulatory interneurons (Yeoman *et al.* 1995).

As OA application onto the isolated CNS activates the buccal feeding system, prolonged stimulation of OC cells may lead to a similar rhythmic pattern of many feeding neurons. Often, the feeding rhythm appears after the end of the OC stimulation like a post-inhibitory rebound, which is a principal difference from the SO or N1L modulatory interneurons, where the effect is rather fast (Elliott & Benjamin 1985*a*,*b*). Post-inhibitory rebound is considered an important factor for firing in other feeding neurons such as members of the B4 cluster motoneurons, or the pattern-generating N3 interneurons (Elliott & Benjamin 1985*a*). While this activation of the feeding rhythm can be evoked only by long-lasting (up to 20–30 s) intracellular stimulation of the OC neurons, its role in generating fictive feeding is more likely to be modulatory than initiatory.

The anatomy of OC neurons observed after intracellular labelling supports the idea that these neurons have a modulatory role in the feeding system. In contrast to the relatively simple morphology of motoneurons (Benjamin *et al.* 1979) or the central pattern-generating (CPG) interneurons (Elliott & Benjamin 1985*a*), the complex pattern of the axon processes of OC neurons is more similar to the morphological appearance of the SO modulatory interneuron (Elliott & Benjamin 1985*b*). The OC neurons have a dense network of axon branches with numerous side branches, both in the ipsilateral and contralateral neuropil of the paired buccal ganglia, suggesting that they have widely distributed connections with other neurons. This pattern of axon processes may also indicate that OA is released diffusely by a non-synaptic process.

Summarizing our results we conclude that by identifying the OC neurons, a new class of buccal feeding neurons was described. The location and anatomy of these neurons as well as their electrical properties and synaptic connections are different from any previously described feeding interneurons or motoneurons in *Lymnaea*. Therefore, OC neurons can be considered as new members of the buccal feeding network, mediating OAergic modulation of feeding in *Lymnaea stagnalis*.

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