PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

An octopaminergic system in the CNS of the snails, Lymnaea stagnalis and Helix pomatia

L. Hiripi, Á Vehovszky, S. Juhos and K. Elekes

Phil. Trans. R. Soc. Lond. B 1998 **353**, 1621-1629
doi: 10.1098/rstb.1998.0314

THE ROYAL SOCIETY

An octopaminergic system in the CNS of the snails, *Lymnaea stagnalis* and *Helix pomatia*

L. Hiripi, Á. Vehovszky*, S. Juhos and K. Elekes

Balaton Limnological Research Institute of the Hungarian Academy of Sciences, Tihany H-8237, Hungary

Octopamine (OA) levels in each ganglion of the terrestrial snail, *Helix pomatia*, and the pond snail, *Lymnaea stagnalis*, were measured by using the high pressure liquid chromatography HPLC technique. In both species an inhomogeneous distribution of OA was found in the central nervous system. The buccal ganglia contained a concentration of OA (12.6 pmol mg^{-1} and 18.8 pmol mg^{-1}) that was two to three times higher than the pedal (4.93 pmol mg^{-1} and 9.2 pmol mg^{-1}) or cerebral (4.46 pmol mg^{-1} and 4.9 pmol mg^{-1}) ganglia of *Helix* and *Lymnaea*, respectively, whereas no detectable amount of OA could be assayed in the visceroparietal complex.

In *Lymnaea* ganglia, the OA uptake into the synaptosomal fraction had a high ($K_{m1} = 4.07 \pm 0.51$ μM, $V_{max1} = 0.56 \pm 0.11$ pmol mg^{-1} per 20 min), and a low ($K_{m2} = 47.6 \pm 5.2$ μM, $V_{max2} = 4.2 \pm 0.27$ pmol mg^{-1} per 20 min), affinity component.

A specific and dissociable ^{3}H-OA binding to the membrane pellet prepared from the CNS of both *Helix* and *Lymnaea* was demonstrated. The Scatchard analysis of the ligand binding data showed a one-binding site, representing a single receptor site. The K_d and B_{max} values were found to be 33.7 ± 5.95 nM and 1678 ± 179 fmol g^{-1} tissue in *Helix* and 84.9 ± 17.4 nM and 3803 ± 515 fmol g^{-1} tissue in *Lymnaea* preparation. The pharmacological properties of the putative molluscan OA receptor were characterized in both species and it was demonstrated that the receptor resembled the insect OA_2 rather than to the cloned *Lymnaea* OA receptor. Immunocytochemical labelling demonstrated the presence of OA-immunoreactive neurons and fibres in the buccal, cerebral and pedal ganglia in the central nervous system (CNS) of both species investigated.

Electrophysiological experiments also suggested that the *Lymnaea* brain possessed specific receptors for OA. Local application of OA onto the identified buccal B2 neuron evoked a hyperpolarization which could selectively be inhibited by the OAergic agents phentolamine, demethylchlordimeform and 2-chloro-4-methyl-2-(phenylimino)-imidazolidine. Among the dopamine antagonists, ergotamine reversibly inhibited the OA response, whereas sulpiride had no effect. Based on our findings, a neurotransmitter–modulator role of OA is suggested in the gastropod CNS.

Keywords: octopamine; receptor; uptake; CNS; *Lymnaea*; *Helix*

1. INTRODUCTION

Although octopamine (OA) was identified first in a mollusc, in the salivary gland of *Octopus* (Erspamer & Boretti 1951), its physiological role was investigated first in vertebrates and in arthropods. In the vertebrate nervous system, OA is considered as a 'trace amine' or a 'false transmitter' (David & Coulon 1985). No specific OA receptor has been found in the mammalian central nervous system (CNS), though it was demonstrated to modulate the noradrenergic synaptic transmission (Axelrod & Saavedra 1977).

The role of OA as a neurotransmitter or neuromodulator has been investigated in the greatest detail in insects, and it was suggested that OA had a similar function in insects as noradrenaline (NA) in vertebrates (David & Coulon 1985). The concentration of OA in the insect CNS was found to be much higher than that of NA, and specific OA receptors were demonstrated in many insect species (Evans & Robb 1993). Using the ligand binding methods, high affinity OA receptors were identified in the *Drosophila* head (Dudai 1982), the locust brain (Hiripi 1985; Hiripi *et al.* 1994), the firefly light organ (Hasemzadeh *et al.* 1985), and the locust nerve cord (Roeder & Gewecke 1990). The pharmacological properties of these receptors were found to be similar to that of the OA_2 receptor demonstrated in the locust extensor-tibiae muscle (Evans 1981).

The presence of OA was also detected in other invertebrates such as annelids (Webb & Orchard 1980; Csoknya *et al.* 1996). Significant amounts of OA were found in the nervous system of the molluscs *Sepia officinalis*, *Loligo vulgaris* (Jourio & Molinoff 1974), *Aplysia californica* (McCaman 1980), *Helix aspersa* (Walker *et al.* 1972) and *Helix pomatia* (Guthrie *et al.* 1975).

In *Lymnaea* brain, the presence of OA-immunopositive neurons and fibres was also demonstrated (Elekes *et al.* 1993), and combining the immunocytochemical and

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

Received 30 June 1997 *Accepted* 1 October 1997

radioenzymatic methods, a correlation between the number of immunoreactive neurons and the OA content of CNS was found during the embryonic and postembryonic development (Elekes *et al.* 1996). More recently, Gerhardt and co-workers cloned two types of OA receptors from the CNS of *Lymnaea stagnalis* (Gerhardt *et al.* 1997*a*,*b*). Both receptors showed moderate affinity to OA and high affinity to rauwolscine. The OAergic stimulation of the Lym OA_1 receptor elevated the levels of both cAMP and inositolphosphates (Gerhardt *et al.* 1997*a*), whereas the Lym OA_2 receptor was involved in the activation of Cl^- channels (Gerhardt *et al.* 1997*b*), and the two receptors also showed different pharmacological properties.

Early electrophysiological studies suggested that OA might be a transmitter in the CNS of gastropods, because extracellularly applied OA evoked specific membrane responses of identified neurons of *Aplysia* (Carpenter & Gaubatz 1974), *Helisoma* (Bahls 1990), and *Helix* (Batta *et al.* 1979; Walker *et al.* 1972). These OA responses could be blocked by phentolamine, a selective OA antagonist.

The aim of the present study was to furnish further data supporting the putative transmitter or modulatory role of OA in gastropods. First, we assayed the concentration of OA in different ganglia of *Helix pomatia* and *Lymnaea stagnalis* and the OA uptake into the synaptosomal fraction of *Lymnaea* CNS. The kinetic and pharmacological properties of [^{3}H]-OA binding to the membrane preparation of the *Helix* and *Lymnaea* brains were also studied. The distribution of OA-immunoreactive neurons in *Lymnaea* and *Helix* CNS was also described briefly. In electrophysiological experiments the pharmacological characteristics of OA receptors were studied, using the giant buccal B2 neuron of *Lymnaea stagnalis* as a model for cellular membrane responses evoked by local application of OA. To study the specificity of OA effect on this neuron, characteristics of the evoked membrane responses were analysed in the presence of either octopaminergic (phentolamine, 2-chloro-4-methyl-2-(phenylimino)-imidazolidine (NC-7), and demethylchlordimeform (DCDM)) or dopaminergic (ergotamine and sulpiride) agents.

2. MATERIALS AND METHODS

Helix pomatia specimens were collected locally in the Tihany peninsula, Hungary and kept under moist conditions on a diet of lettuce. Specimens of *Lymnaea stagnalis* were collected from surrounding ponds, and were kept in running Balaton-water. Experimental drugs were purchased from Research Biochemicals Inc.

(a) *Biochemical methods*

(i) *High pressure liquid chromatography (HPLC) assay*

To determine the OA concentrations, five circumesophageal ganglion rings dissected from *Helix pomatia* were gently desheathed, then the buccal, cerebral, pedal and pleuro-visceral ganglia were separated. The same number of *Lymnaea* ganglions were dissected in a similar manner, but no desheathing was required. The ganglia were homogenized in 500 µl ice-cold 0.1 M phosphate buffer, pH 7. The homogenate was centrifuged at 10 000 *g* for 15 min. A total of 450 µl of the supernatant was used for liquid-ion extraction of OA. To the samples, 500 µl chloroform containing di-(2-ethylhexyl)-phosphoric acid (DEHPA) in 0.1 M concentration was added. The samples were vigorously shaken and centrifuged for 10 min at 10 000 *g*. The supernatant inorganic phase was discarded, and 400 µl of the organic phase was mixed with 80 µl 0.1 M HCl. After centrifugation for 10 min at 10 000 *g*, 70 µl of the acidic phase was aspirated then 50 µl was injected into the HPLC system and the separation of OA was achieved on a reverse-phase column (µBondapack C18, 10 µ, Waters). The column temperature was set at 40 °C. The eluent for chromatography was 0.05 M sodium acetate buffer pH 3.5, containing 1.5 mM octane sulphonic acid and 10% methanol. The delivery system was a Waters 510 HPLC pump, flow rate 1 ml min^{-1}. An automated Waters WISP Injector was used as a sample processor. The detector was a Waters 470 Scanning Fluorescent Detector. The excitation and emission wavelengths were set at 280 nm and 303 nm, respectively. Chromatography data were processed by a Waters 476 Integrator.

(ii) *Determination of OA-uptake*

For uptake experiments whole *Lymnaea* CNSs were used. The circumoesophageal ganglion rings were kept during dissection in ice-cold physiological solution. Tissue samples were carefully homogenized with a glass–teflon homogenizer in 0.25 M iso-osmotic sucrose, to give a 10% tissue suspension. To separate primary fractions, the nuclear (P1) fraction was sedimented by centrifuging at 500 *g* for 10 min. This pellet was resuspended, and washed with iso-osmotic sucrose. After centrifugation the supernatant fluids were combined, and the mitochondrial fraction (P2) was separated by centrifuging at 17 000 *g* for 30 min. The synaptosomal fraction was prepared by density gradient centrifugation as descibed earlier (Elekes *et al.* 1976).

To measure the total uptake, the synaptosomal fraction preparations corresponding to 10 mg wet tissue were incubated with [^{3}H]-OA in 1 ml physiological solution, containing 0.5 mg ml^{-1} ascorbic acid at 25 °C for 20 min. The non-specific uptake was measured in sodium-free physiological solution at 0 °C. The concentration of the [^{3}H]-OA ranged from 2–100 µM. After incubation the samples were filtered under vacuum through a Whatman GF–B filter, and rinsed three times with physiological solution. The radioactivity on the filter was counted after overnight extraction with a toluene-based scintillation solution. Both in uptake and binding experiments, three samples were centrifuged instead of filtration. The pellet was then extracted by methanol, centrifuged at 10 000 *g* for 10 min and an aliquot of the sample was used for measurement of the radioactivity, whereas another aliquot was co-chromatographed with 100 pmol cold OA. The eluent was collected into fractions and the radioactivity of that fraction which represented the OA peak was counted. [^{3}H]-OA (36.2 and 29.7 Ci mmol^{-1}l) was synthesized at the Isotope Research Institute of the Hungarian Academy of Sciences.

(iii) *Binding studies*

To measure the ^{3}H-OA binding to the membrane preparation the circumoesophageal ganglion rings were

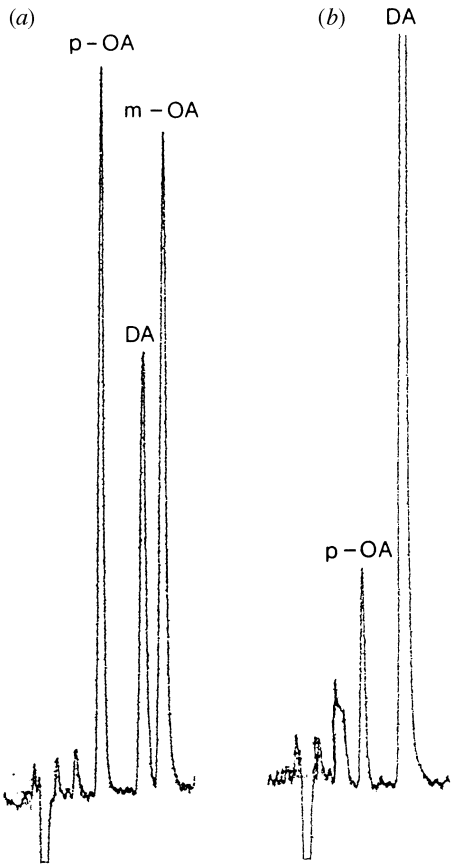

Figure 1. HPLC chromatograms of (*a*) authentic standard and (*b*) the tissue sample of *Lymnaea* CNS. Retention times for p-OA, DA, and m-OA are 5.11, 7.39, and 8.46 min respectively.

dissected from the animals and homogenized in 40 volumes of ice-cold 50 mM Tris-HCl buffer, pH 7.4 in a Polytron. The homogenate was then centrifuged three times at 50 000 *g* for 15 min. The pellet obtained after the second centrifugation was resuspended in 50 mM Tris-HCl buffer, pH 7.4 and incubated at 25 °C for 15 min to inactivate endogenous ligands. The final pellet was resuspended in cold 50 mM Tris-HCl buffer, pH 7.4. The membrane preparation corresponding to 40 mg wet tissue was incubated in 2 ml 50 mM Tris-HCl buffer, pH 7.4, containing 5 nM [^{3}H]-OA, 0.05% ascorbic acid and the experimental drugs. After incubation at 20 °C for 30 min, the contents were rapidly filtered under vacuum through a Whatman GF–B filter and rinsed three times with 5 ml 50 mM Tris-HCl buffer, pH 7.4. The radioactivity on the filter was counted after overnight extraction with a toluene-based scintillation solution. The kinetic parameters of ligand binding were determined by Scatchard analysis. These parameters and K_i values were evaluated using the GraFit computer program (Leatherbarrow 1992).

(b) *Immunocytochemistry*

The production of the anti-OA antibody and the immunocytochemical protocol have been described elsewhere in detail (Eckert *et al.* 1992; Elekes *et al.* 1993). Briefly, the CNSs of *Lymnaea* and *Helix* were fixed in GPA (a mixture of 5 ml glutaraldehyde, 15 ml saturated picric acid and 0.1 ml glacial acetic acid; Schot *et al.* 1979) and then processed as for the three-step peroxidase–anti-peroxidase method (Sternberger *et al.* 1970) on paraffin sections. The specificity of the immunostaining has also been proven earlier in *Lymnaea* (Elekes *et al.* 1993).

Table 1. *Concentration of OA in the CNS of* Helix pomatia *and* Lymnaea stagnalis

(OA measured by HPLC in different ganglia and expressed as pmol mg^{-1} ganglia.)

ganglia	*Helix pomatia*	*Lymnaea stagnalis*
whole CNS	4.05±0.33	6.01±0.59
cerebral	4.46±0.41	4.90±0.28
buccal	12.60±0.64	18.8±0.73
pedal	4.93±0.29	9.20±0.82
pleuro-visceral and parietal complex	not detected	not detected

(c) *Electrophysiology*

Electrophysiological experiments were done on the isolated nervous system of *Lymnaea stagnalis*, including the paired buccal ganglia. For recording the intracellular activity of neurons, conventional microelectrophysiological methods were used which were described earlier (Elliott *et al.* 1992). The giant B2 buccal motoneurons could easily be identified by their size, position and spontaneous activity pattern (Benjamin & Rose 1979).

For recording the membrane effect of octopamine, 0.1 M OA solution was applied locally to the surface of the B2 cell body by ionophoretic injection from a micropipette (glass microelectrode). The activity pattern of the neuron was simultaneously recorded by an intracellular microelectrode. Drug applications were repeated by 3–5 min intervals, and the bath containing the isolated CNS was continuously perfused with standard *Lymnaea* saline (Elliott *et al.* 1992) with a 3–5 ml min^{-1} flowing rate.

During the pharmacological tests a modified bathing solution (Na-HiDi) was used (Vehovszky & Elliott 1995), to reduce the indirect (synaptically activated) effects of the putative antagonists. The drugs under study were dissolved and diluted in this solution to reach the final concentration required (10^{-8} M to 10^{-4} M). The B2 neuron was impaled by two independent microelectrodes, one for recording the activity pattern of the cell and another to compensate for the possible direct membrane effect of the antagonists (to hold the membrane potential level on its control value) by injecting intracellular current into the cell.

3. RESULTS

(a) *Biochemical measurements*

In the CNS of *Helix* and *Lymnaea* the meta-isomer of OA could not be identified, but a significant amount of para-OA was detected by HPLC (see figure 1). The retention times of the OA peak in the tissue sample and in the standard solution were the same and changed identically by changing either the concentration of the octanesulphonic acid in the mobile phase or the flow rate or the column temperature. Although a 280–303 nm excitation and emission wavelength is not optimal for DA, it was also measured in the same chromatogram. The highest

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

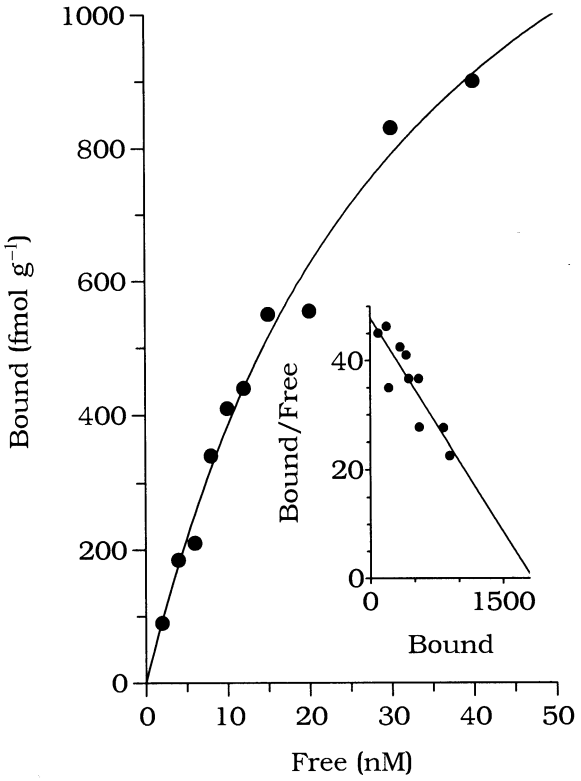

Figure 2. Saturation curve (main graph) and Scatchard analysis of binding of [^{3}H]-octopamine (insert) with *Helix pomatia* nervous tissue membrane preparation. Membranes were incubated for 30 min at 20 °C with various concentration of [^{3}H]-octopamine. The Scatchard line fitted by computer program reveals a single binding site ($K_d = 33.7 \pm 5.95$ nM, $B_{max} = 1678 \pm 179$ fmol g^{-1} tissue).

concentration of OA was measured in the buccal ganglia of both *Helix* and *Lymnaea*, and the paired cerebral and pedal ganglia also contained a considerable amount of OA. In the pleuro-visceral complex of both species OA was not detectable (table 1).

[^{3}H]-OA was taken up into the synaptosomal fraction of *Lymnaea*. The uptake process is saturable, having a high ($K_{m1} = 4.07 \pm 0.51$ μM) and a low ($K_{m2} = 47.6 \pm 5.2$ μM) affinity component. The maximum velocities for the two processes were measured as $V_{max1} = 0.56 \pm 0.11$ pmol mg^{-1} per 20 min, and $V_{max2} = 4.2 \pm 0.27$ pmol mg^{-1} per 20 min, respectively. Co-chromatography of the methanol extract showed that in both uptake and binding experiments 84–95% of the radioactivity was found in that fraction which represented the OA peak, demonstrating, that OA has not been significantly metabolized during the incubation.

[^{3}H]-OA was found to bind to the membrane preparation taken from both *Lymnaea* and *Helix* ganglia. Although the non-specific binding was rather high (50%), a specific binding of [^{3}H]-OA was also detected. The binding proved to be a saturable process, representing a single binding site. The K_d value for *Helix* brain membrane preparation was 33.7 ± 5.95 nM, and B_{max} was 1678 ± 179 fmol g^{-1} tissue (figure 2), and for the *Lymnaea* membrane preparation K_d was measured as 84.9 ± 17.4 nM, with B_{max} 3803 ± 515 fmol g^{-1} tissue. The 95% of the specifically bound [^{3}H]-OA was dissociated from the receptor when 1000 times higher concentration of cold OA was added. Many of the tested pharmacological agents inhibited the binding of OA to the membrane preparation (table 2). The most potent displacers were the para-OA itself as well as mianserin, prazosin, DCDM and NC-7. The meta-isomer of OA inhibited the OA-binding by ten times higher concentration than para-OA. The effect of some representative agonists and antagonists effects for *Lymnaea* membrane preparation are demonstrated on figure 3.

Table 2. K_i *values for different competitors* (*M*)

	Lymnaea stagnalis	*Helix pomatia*
agonists		
p-octopamine	2.12×10^{-8}	1.75×10^{-8}
synephrine	1.79×10^{-8}	1.91×10^{-8}
NC-7	9.03×10^{-9}	1.15×10^{-8}
DCDM	3.28×10^{-8}	3.77×10^{-8}
m-octopamine	2.22×10^{-7}	8.7×10^{-8}
clonidine	9.03×10^{-7}	1.06×10^{-6}
norepinephrine	7.7×10^{-5}	5.93×10^{-5}
dopamine	8.63×10^{-5}	8.76×10^{-5}
antagonists		
prazosin	3.14×10^{-8}	9.38×10^{-9}
mianserin	1.17×10^{-8}	1.04×10^{-8}
phentolamine	2.79×10^{-7}	2.15×10^{-7}
chlorpromazine	6.61×10^{-6}	4.16×10^{-6}
yohimbine	1.89×10^{-5}	2.04×10^{-5}

(b) *Immunocytochemical labelling*

OA-like immunoreactive (OALI) neurons occurred in the CNS of both species as stained cell bodies as well as varicose fibres in the neuropil were observed in different ganglia (see figure 4). The distribution of OALI neurons in the *Lymnaea* CNS has been described earlier in detail (Elekes *et al.* 1993). The number of OALI neurons was considerably less in the *Helix* CNS (14–20 labelled cells), compared with that found in *Lymnaea* (40–50 OALI neurons, Elekes *et al.* (1993)). The localization of OALI neurons in the buccal, cerebral and pedal ganglia was identical in both species, but in the cerebral ganglia of *Helix* the dorsomedial cell cluster was represented only by a few labelled neurons.

(c) *Electrophysiological experiments*

OA applied locally onto the surface of the cell body evoked large, 10–15 mV amplitude hyperpolarization lasting up to 18–20 s (figure 5*ai*), and its reversal potential was around −100 mV (mean = −103.6 mV ± 3.6, $n = 5$). Increasing the injecting current for drug application, the response displayed a saturating dose–response relation (figure 5*aii*). In the course of the pharmacological characterization of the membrane responses, OA was ionophoretically applied in the presence of putative OAergic drugs NC-7, phentolamine, DCDM or dopaminergic agents (sulpiride and ergotamine), respectively.

Phentolamine (in seven experiments) reduced the hyperpolarizing responses of the B2 neuron at a threshold concentration of 10^{-8} M (figure 5*bi*), whereas at 10^{-6} M this drug completely and reversibly blocked the OA effect. In the presence of 5×10^{-5} M DCDM in the bath ($n = 4$) the octopamine-evoked hyperpolarization decreased to 10–20% of the control amplitudes (see

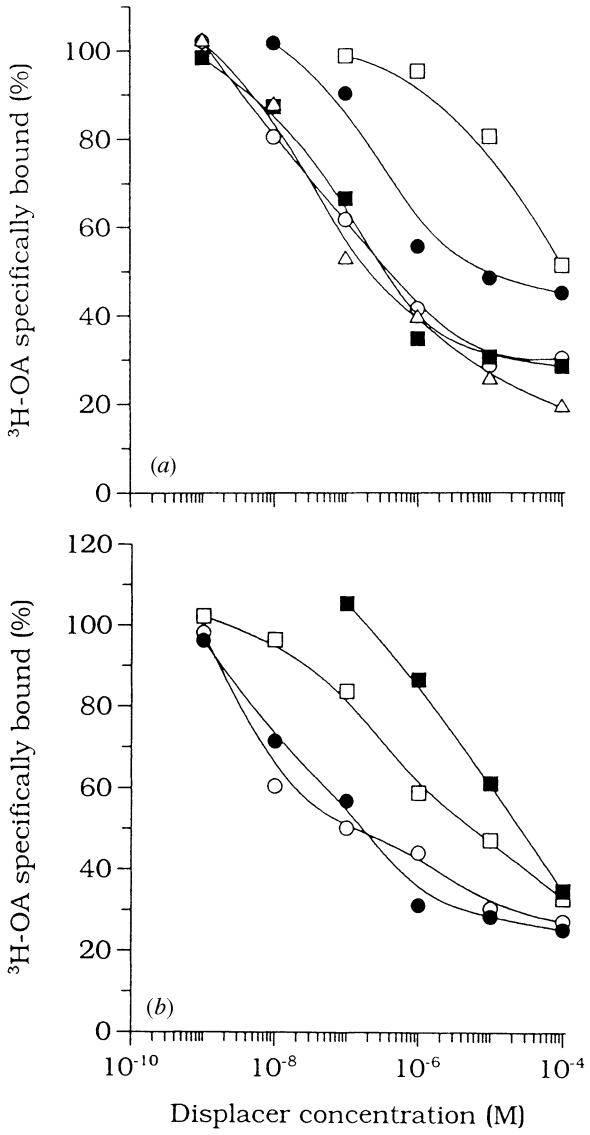

Figure 3. Representative competition curves for [^{3}H]-octopamine binding sites in *Lymnaea* CNS. (*a*) p-octopamine (empty circle), m-octopamine (filled circle), dopamine (empty square), NC-7 (filled square), DCDM (empty triangle). (*b*) phentolamine (empty circle), mianserin (filled circle), chlorpromazine (empty square), yohimbine (filled square). Data points are means of three individual experiments carried out in duplicate or triplicate. s.e.m. was less than 10% for each point.

figure 5*b*ii). A total of 10^{-4} M NC-7 completely blocked the OA responses ($n=5$), and this effect did not recover completely after washing out (figure 5*b*iii). The DA antagonist ergotamine (10^{-6} M in the bath), completely and reversibly blocked the hyperpolarization evoked by local application of OA ($n=4$, figure 5*c*i), whereas 10^{-5} M sulpiride only decreased the amplitude of the OA-evoked hyperpolarization by about 20% of the control response ($n=7$, figure 5*c*ii).

Among the substances tested phentolamine proved to be the most effective to inhibit the OA responses, whereas sulpiride applied in a concentration of more than four magnitudes higher exerted the slightest effect to the OA-evoked hyperpolarization. The other dopaminergic antagonist, ergotamine applied in the bath inhibited both the octopamine and DA responses.

4. DISCUSSION

(a) *Distribution of OA in the snail CNS*

Our results demonstrated that OA is present in the CNS of both *Lymnaea* and *Helix*. The OA concentration in the ganglionic ring of these animals (4–6 pmol mg^{-1}) is similar to that found in other molluscs (Walker *et al.* 1972; Guthrie *et al.* 1975; Jourio & Kazakoff 1984). The analysis of different ganglia shows an inhomogenous distribution of OA, with the highest concentration in the buccal ganglia, meanwhile the pleuro-visceral–parietal complex does not contain detectable amounts of OA. A very similar distribution of OA has been found in *Aplysia* (McCaman 1980). The inhomogenous distribution of OA suggests a special regulatory role of this amine localized in certain ganglia of the snail CNS.

Similarly to OA, the distribution of other biogenic amines (5HT and DA) in the different parts of the central ganglia has been shown to correlate with the locations of the histochemically labelled (5HT or DA containing) neurons throughout the snail CNS (Kemenes *et al.* 1989, 1990; Elekes *et al.* 1991; Hernádi *et al.* 1993; Hetherington *et al.* 1994).

(b) *Biochemistry of OA in the snail CNS*

[^{3}H]-OA was incorporated into *Lymnaea* synaptosomal fraction by a high and a low affinity process. The uptake process is the key inactivating mechanism for terminating the synaptic effect of the released transmitter. High affinity uptake of OA plays an important role in the inactivation of this signal molecule in gastropods, because monoamine-oxydase enzyme does not function in the nervous system of these animals (Hayashi *et al.* 1977). In an earlier investigation using whole *Helix* ganglia, Osborne and co-workers (1975) have found a high affinity uptake for DA and 5HT but failed to demonstrate a saturable accumulation for OA. In our present experiments using synaptosomal fraction the Lineweaver–Burk plot of the saturation curve of OA shows clearly the presence of a high and a low affinity component of the uptake.

Our studies demonstrated a specific and reversible binding of [^{3}H]-OA to the *Helix* and *Lymnaea* brain membrane preparation. The Scatchard plots of the saturation data show a single binding site for OA with moderate affinity in both species, with $K_d=33.7$ nM in *Helix* and $K_d=84.9$ nM in *Lymnaea*.

For characterizing the cloned *Lymnaea* OA receptors an α2-adrenoreceptor antagonist, rauwolscine (α-yohimbine), was used (Gerhardt *et al.* 1997*a*,*b*). This binds to the cloned receptors with high affinity, but OA could displace rauwolscine only at a high (0.1–1.0 μM) concentration range. For the cloned receptor the dissociation of the rauwolscine (α-yohimbine) has not been investigated (Gerhardt *et al.* 1997*a*,*b*). Furthermore, in our studies it has been found that the rauwolscine analogue ^{3}H-yohimbine did not dissociate from the receptors. Therefore, it is questionable if rauwolscine is a suitable ligand to characterize the OA receptor and fulfils all the criteria recommended for being a ligand (Laduron 1984).

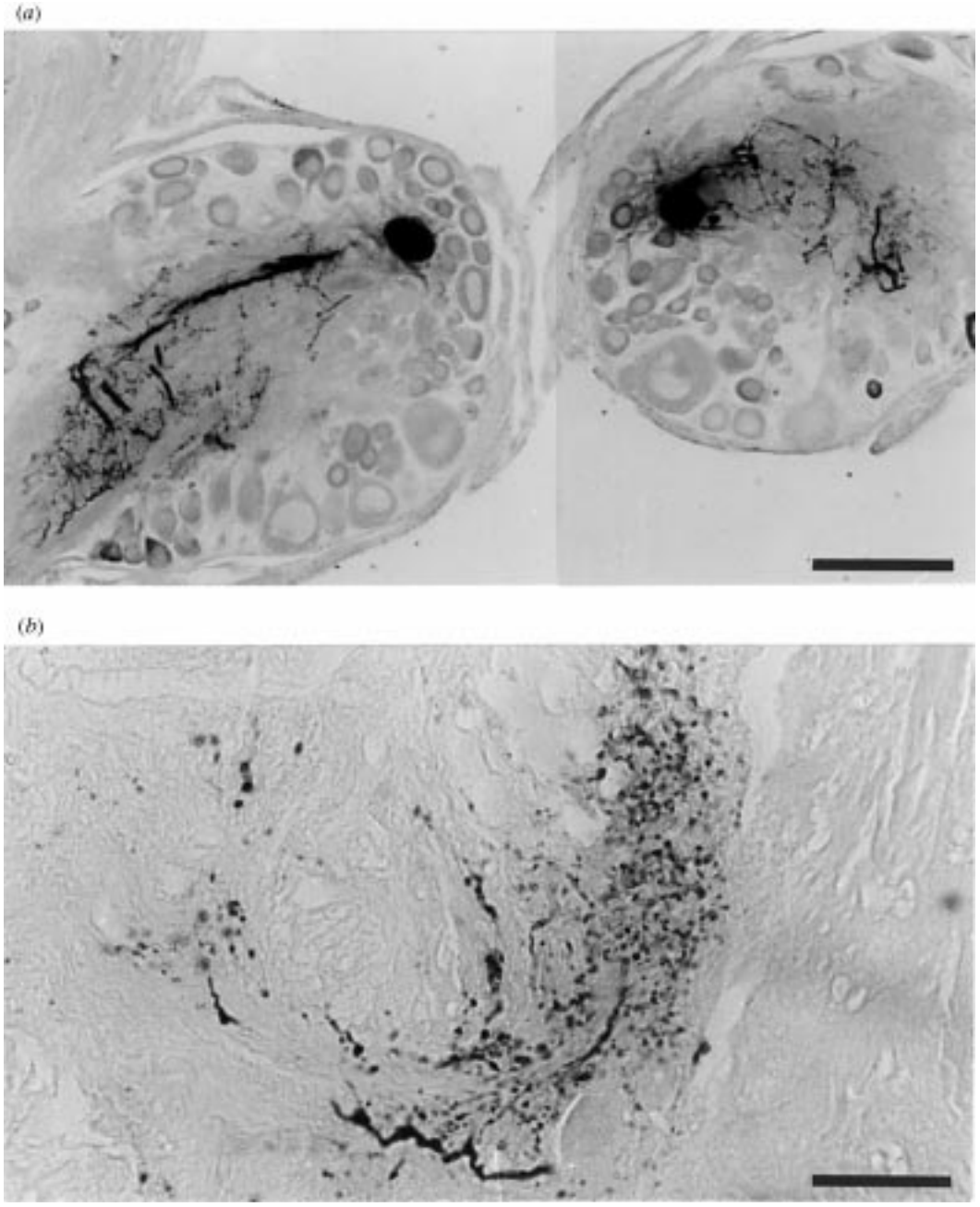

Figure 4. Octopamine-like immunoreactive (OALI) neurons in *Lymnaea* and *Helix*. (*a*) A pair of OALI neurons in the buccal ganglia of *Lymnaea*. Note the extensive arborization of axon processes in the neuropil and the extensive labelling of the cell bodies in each ganglia. Calibration bar, 100 µm. (*b*) Dense arrangement of OALI varicose processes in the neuropil of the cerebral ganglion of *Helix*. Calibration bar, 50 µm.

The pharmacological properties of the native gastropod OA receptors make them more similar to the insect receptors than to the cloned Lym OA receptors, as the phenolamine OA has a much higher affinity to the receptor than the catecholamine DA or NA. Furthermore, the activity of the meta-isomer of the OA on the snail OA receptor is lower than the activity of the para-OA. The phenylimidazolidine derivative NC-7 and the form-amidine derivative DCDM which were characterized as highly potent and specific agonists to OA receptors in insects (Evans 1981, 1987; Nathanson 1985; Roeder & Gewecke 1990; Hiripi *et al.* 1994), also have a high affinity to *Lymnaea* OA receptors. The antagonists mianserin and phentolamine have a higher affinity than chlorpromazine and yohimbine.

(c) *Electrophysiological characteristics of OA membrane responses*

OA applied locally to the membrane of the identified buccal B2 neuron of *Lymnaea stagnalis* had a clear membrane effect, evoking hyperpolarization. The reversal potential of the response suggests increased K^+-permeability of the membrane, similar to the K^+-dependent hyperpolarization evoked either by DA or OA on many other identified snail neurons (*Aplysia*: Carpenter & Gaubatz 1974; Gospe & Wilson 1981; *Helix*: Walker *et al.*

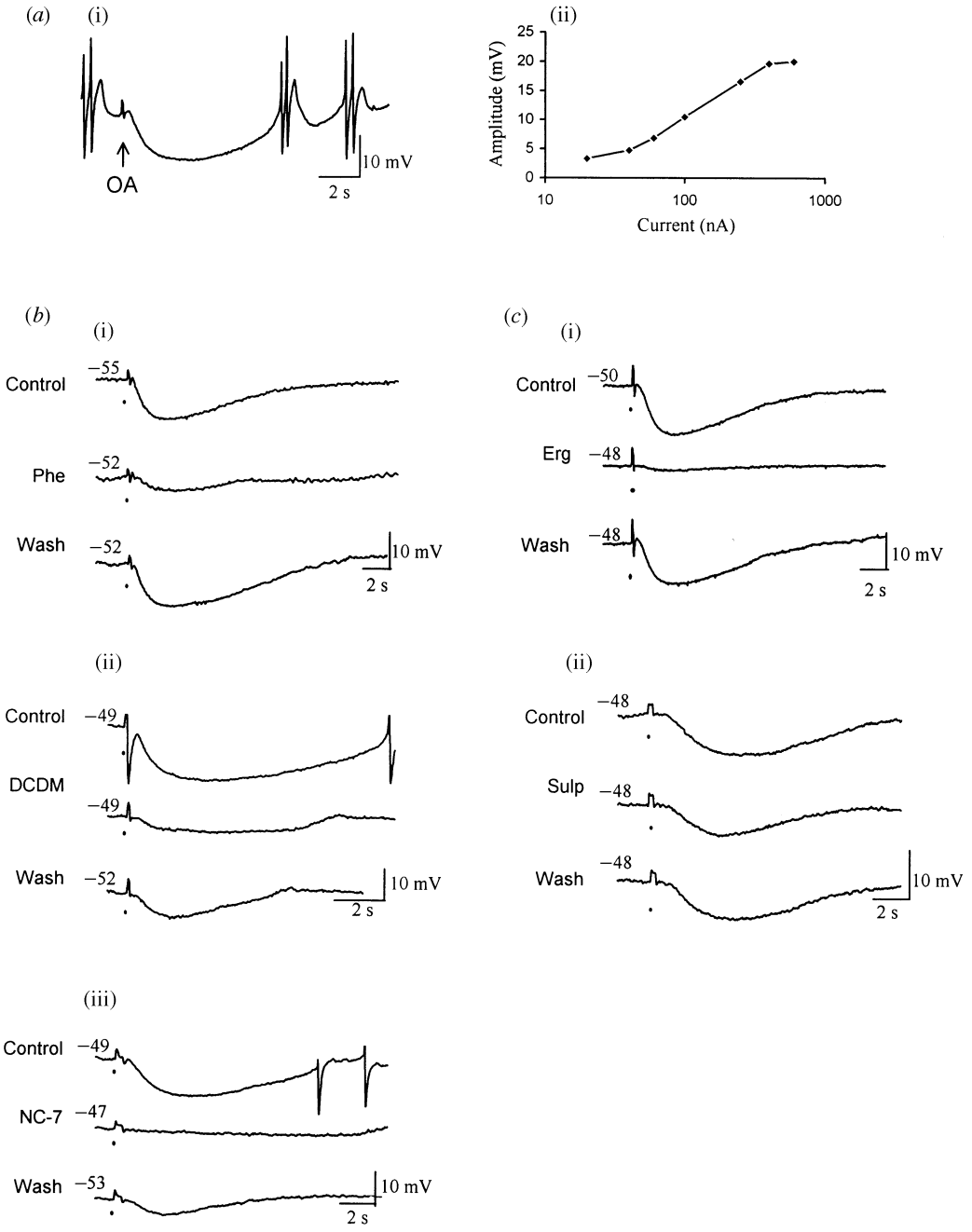

Figure 5. Membrane responses of B2 buccal neuron evoked by locally applied octopamine. (*a*)(i) Hyperpolarization recorded on B2 neuron in normal saline after application of octopamine (OA, marked by an arrow) at −44 mV membrane potential level. (*a*)(ii) Dose–response relation of the membrane voltage changes evoked by increasing ionophoretic currents for octopamine application onto the neuronal membrane. (*b*)(i) The octopamine response of B2 buccal neuron (control in Na-HiDi saline) is reversibly inhibited in the presence of 0.01 μM phentolamine (Phe). The start of the current pulses for ionophoretic application is marked by a dot below each individual record. Numbers at the beginning of each record indicate the membrane potential levels of the neurons before the octopamine application. (*b*)(ii) The hyperpolarization evoked by octopamine is decreased in the presence of 0.05 mM DCDM. (*b*)(iii) Octopamine response is inhibited in the presence of 0.1 mM NC-7 applied to the bath. (*c*)(i) Hyperpolarization evoked by octopamine is reversibly inhibited by 1 μM ergotamine (Erg) in the bath. (*c*)(ii) Membrane response evoked by octopamine is reversibly decreased in 0.01 mM sulpiride (Sulp).

1972; Batta *et al.* 1979; *Helisoma*: Bahls 1990; *Lymnaea*: Audesirk 1989). Using *Helix* and *Aplysia* neurons, additional pharmacological experiments suggested that OA and DA responses could be separated by selective antagonists as phentolamine is suitable for inhibiting selectively the OA responses (Batta *et al.* 1979; Gospe & Wilson 1981; Bahls 1990). In insects, the OAergic NC-7 and DCDM have both agonistic and antagonistic effects (Nathanson 1985). In our pharmacological tests NC-7 and DCDM proved to be OA antagonists, effectively and selectively blocking the OA responses evoked on the *Lymnaea* B2 buccal neuron.

The DA antagonist ergotamine, however, did not prove to be a selective antagonist as it was effective for

inhibiting both the OA and DA responses. This finding correlates well with other results obtained in *Drosophila* (Dudai & Zvi 1982) and *Helix* (Drummond *et al*. 1980) as ergot alkaloids have a broad specificity and act on the 5HTergic, DAergic and OAergic receptors as well. Nesic & Pasic (1992), working on *Helix* neurons, suggested that ergot alkaloids (in contrast to the DA-selective sulpiride) would act on the common ionophore involved in both DA- and OA-evoked membrane responses.

Our present results demonstrate that both the *Helix* and *Lymnaea* CNS possess OA receptors, which are biochemically and pharmacologically different from the receptors of other monoamines. These findings combined with the immunocytochemical results confirm the previous suggestion that OA acts as a neurotransmitter or neuromodulator in the gastropod CNS. A possible role of OA as a feeding modulator is described in the following paper in this issue (Vehovszky *et al*. 1998).

We thank Dr A. Szücs whose VCL computer program allowed us to present the intracellular records from our electrophysiological experiments. We also thank K. Komáromi, É. L. Karácsonyi and B. Balázs for their skilful technical assistance. The octopamine antiserum was donated by Professor M. Eckert (Jena, Germany). This work was supported by Hungarian Scientific Research Program (OTKA) grants, number 2477 and number 016015 to K.E and number 5422 to L.H.

REFERENCES

Audesirk, T. E 1989 Characterization of pre- and postsynaptic dopamine receptors in *Lymnaea*. *Comp. Biochem. Physiol.* C **93**, 115–119.

Axelrod, J. & Saavedra, J. M. 1977 Octopamine. *Nature* **256**, 501–504.

Bahls, F. H. 1990 Analysis of a long-duration hyperpolarization produced by octopamine in an identified effector neuron of *Helisoma*. *Neurosci. Lett.* **120**, 131–133.

Batta, S., Walker, R. J. & Woodruff, G. N. 1979 Pharmacological studies on *Helix* neuron octopamine receptors. *Comp. Biochem. Physiol.* C **64**, 43–51.

Benjamin, P. R. & Rose, R. M. 1979 Central generation of bursting in the feeding system of the snail, *Lymnaea stagnalis*. *J. Exp. Biol.* **80**, 119–135.

Carpenter, D. O. & Gaubatz, G. L. 1974 Octopamine receptors on *Aplysia* neurons mediates hyperpolarization by increasing membrane conductance. *Nature* **252**, 483–485.

Csoknya, M., Lengvári, I., Hiripi, L., Eckert, M., Rapus, J. & Elekes, K. 1996 Octopamine in the central nervous system of *Oligochaeta*: an immunocytochemical and biochemical study. *Cell Tissue Res.* **285**, 27–37.

David, J. C. & Coulon, J.-F. 1985 Octopamine in invertebrates and vertebrates. A review. *Prog. Neurobiol.* **24**, 141–185.

Drummond, A. H., Bucher, F. & Levitan, I. B. 1980 Distribution of serotonin and dopamine receptors in *Aplysia* tisssues: analysis by [^{3}H]LSD binding and adenylate cyclase stimulation. *Brain Res.* **184**, 163–167.

Dudai, Y. 1982 High-affinity octopamine receptors revealed in *Drosophila* by binding of ^{3}H-octopamine. *Neurosci. Lett.* **28**, 163–178.

Dudai, Y. & Zvi, Y. S. 1982 Aminergic receptors in *Drosophila melanogaster*: properties of [^{3}H]dihydroergocriptine binding sites. *J. Neurochem.* **38**, 1151–1158.

Eckert, M., Rapus, J., Nürengerger, A. & Penzlin, H. 1992 A new specific antibody reveals octopamine-like immunoreactivity in cockroach ventral nerve cord. *J. Comp. Neurol.* **322**, 1–15.

Elekes, K., Hiripi, L., S.-Rózsa, K. & Nemcsók, J. 1976 Subcellular localization of biogenic monoamines in the central nervous system and heart of the snail *Helix pomatia* L. as revealed by density gradient centrifugation. In *Neurobiology of invertebrates. Gastropoda brain* (ed. J. Salánki), pp. 207–217. Budapest: Akadémiai Kiadó.

Elekes, K., Kemenes, G., Hiripi, L., Geffard, M. & Benjamin, P. R. 1991 Dopamine-immunorecative neurons in the central nervous system of the pond snail *Lymnaea stagnalis*. *J. Comp. Neurol.* **307**, 214–224.

Elekes, K., Eckert, M. & Rapus, J. 1993 Small sets of putative interneurons are octopamine-immunoreactive in the central nervous system of the pond snail *Lymnaea stagnalis*. *Brain Res.* **608**, 191–197.

Elekes, K., Voronezhskaya, E. E., Hiripi, L., Eckert, M. & Rapus J. 1996 Octopamine in the developing nervous system of the pond snail, *Lymnaea stagnalis* L. *Acta Biol. Hung.* **47**, 73–87.

Elliott, C. J. H., Stow, R. A. & Hastwell, C. 1992 Cholinergic interneurons in the feeding system of the pond snail *Lymnaea stagnalis*. I. Cholinergic receptors on feeding neurons. *Phil. Trans. R. Soc. Lond.* B **336**, 157–166.

Erspamer, V. & Boretti, G. 1951 Identification and characterization by paper chromatography of enteramine, octopamine, tyramine, histamine, and allied substances in extracts of posterior salivary glands of *Octopoda* and in other tissue extracts of vertebrates and invertebrates. *Arch. Int. Pharmacodyn.* **88**, 296–332.

Evans, P. D. 1981 Multiple receptor types for octopamine in the locust. *J. Physiol.* **318**, 99–122.

Evans, P. D. 1987 Phenyliminoimidazoline derivatives activate both octopaminel and octopamine2 receptor subtypes in locust skeletal muscle. *J. Exp. Biol.* **129**, 239–250.

Evans, P. D. & Robb, S. 1993 Octopamine receptor subtypes and their modes of action. *Neurochem. Res.* **18**, 869–874.

Gerhardt, C. C., Bakkers, R. A., Piek, G. J., Planta, R. J., Vreugdenhil, E., Leysen, J. E. & van Heerikhuizen, H. 1997*a* Molecular cloning and pharmacological characterization of molluscan octopamine receptor. *Molec. Pharmacol.* **51**, 293–300.

Gerhardt, C. C., Lodder, H. C., Vincent, M., Bakker, R. A., Planta, R. J., Vreugdenhil, E., Kits, K. S. & van Heerikhuizen, H. 1997*b* Cloning and expression of a cDNA encoding a molluscan octopamine receptor that couples to chloride channels in HEK293 cells. *J. Biol. Chem.* **272**, 6201–6207.

Gospe, S. M. & Wilson, W. A. 1981 Pharmacological studies of a novel dopamine-sensitive receptor mediating burst-firing inhibition of neurosecretory cell R15 in *Aplysia californica*. *J. Pharmacol. Exp. Ther.* **216**, 368–377.

Guthrie, P. B., Neuhoff, V. & Osborne, N. N. 1975 Dopamine, noradrenaline, octopamine and tyrosine-hydroxylase in the gastropod *Helix pomatia*. *Comp. Biochem. Physiol.* C **52**, 109–111.

Hasemzadeh, H., Hollingworth, R. M. & Voliva, A. 1985 Receptors for 3H-octopamine in the adult firefly light organ. *Life Sci.* **37**, 433–440.

Hayashi, S., Murdock, L. L. & Florey, E. 1977 Octopamine metabolism in invertebrates (*Locusta*, *Astacus*, *Helix*): evidence for N-acetylation in arthropod tissues. *Comp. Biochem. Physiol.* C **58**, 183–191.

Hernádi, L., Juhos, S. & Elekes, K. 1993 Distribution of tyrosine-hydroxylase-immunoreactive and dopamine-immunoreactive neurons in the central nervous system of the snail *Helix pomatia*. *Cell Tissue Res.* **274**, 503–513.

Hetherington, M. S., Mckenzie, J. D., Dean, H. G. & Winlow, W. 1994 A quantitative analysis of the biogenic amines in the central ganglia of the pond snail, *Lymnaea stagnalis* (L.). *Comp. Biochem. Physiol.* C **107**, 83–93.

Hiripi, L. 1985 Biochemical characterization of the octopaminergic and serotonergic system in the CNS of *Locusta migratoria*. *Pest. Sci.* **16**, 441–442.

Hiripi, L., Juhos, S. & Downer, R. G. H. 1994 Characterization of tyramine and octopamine receptors in the insect (*Locusta migratoria migratorioides*) brain. *Brain Res.* **663**, 119–126.

Jourio, A. V. & Kazakoff, C. V. 1984 The presence of β-phenylalanine, p-tyramine, m-tyramine and tryptamine in ganglia and foot muscle of the garden snail (*Helix aspersa*). *Experientia* **40**, 549–551.

Jourio, A. V. & Molinoff, P. B. 1974 The normal occurrence of octopamine in neural tissues of the octopus and other cephalopods. *J. Neurochem.* **22**, 271–280.

Kemenes, G., Elekes, K., Hiripi, L. & Benjamin, P. R. 1989 A comparison of four techniques for mapping of serotonin and serotonin-containing neurons in fixed and living ganglia of the snail, *Lymnaea*. *J. Neurocytol.* **18**, 193–208.

Kemenes, G., Hiripi, L. & Benjamin, P. R. 1990 Behavioural and biochemical changes in the feeding system of the *Lymnaea* induced by the dopamine and serotonin neurotoxins 6-hydroxydopamine and 5,6-dihydroxytryptamine. *Phil. Trans. R. Soc. Lond.* B **329**, 243–255.

Laduron, P. M. 1984 Criteria for receptor sites in binding studies. *Biochem. Pharmacol.* **33**, 833–839.

Leatherbarrow, R. J. 1992 *GraFit* Version 3.0, Erithacus Software Ltd, Staines, UK.

McCaman, M. W. 1980 Octopamine and phenylethanolamine in the central nervous system of *Aplysia*. In *Noncatecholic phenylethylamines*, part 2 (ed. A. D. Mosnaim & M. A. Wolf), pp. 193–200. New York and Basel: Marcel Dekker.

Nathanson, J. A. 1985 Phenyliminoimidazolidines. Characterization of a class of potent agonists of octopamine-sensitive adenylate cyclase and their use in understanding the pharmacology of octopamine receptors. *Molec. Pharmacol.* **35**, 34–43.

Nesic, O. & Pasic, M. 1992 Characteristics of outward current induced by application of dopamine on a snail neuron. *Comp. Biochem. Physiol.* C **103**, 597–606.

Osborne, N. N., Hiripi, L. & Neuhoff, V. 1975 The *in vitro* uptake of biogenic amines by the snail (*Helix pomatia*) nervous tissue. *Biochem. Pharmacol.* **24**, 2141–2148.

Roeder, T. & Gewecke, M. 1990 Octopamine receptors in locust nervous tissue. *Biochem. Pharmacol.* **39**, 1793–1797.

Schot, L. P. C., Boer, H. H., Swaab, D. F. & van Norden, S. 1979 Immunocytochemical demonstration of peptidergic neurons in central nervous system of the pond snail *Lymnaea stagnalis* with antisera raised to biologically active peptides of vertebrates. *Cell Tissue Res.* **216**, 273–291.

Sternberger, L. A., Hardy, P. H., Cuculis, J. J. & Meyer, H. G. 1970 The unlabeled antibody enzyme method of immunocytochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase–antihorseradish peroxidase) and its use in identification of spirochaetes. *J. Histochem. Cytochem.* **18**, 315–333.

Vehovszky, Á. & Elliott, C. J. H. 1995 The hybrid modulatory/pattern generating N1L interneuron in the buccal feeding system of *Lymnaea* is cholinergic. *Invert. Neurosci.* **1**, 67–74.

Vehovszky, Á., Elliott, C. J. H, Voronezhskaya, E. E., Hiripi, L. & Elekes, K. 1998 Octopamine: a new feeding modulator in *Lymnaea*. *Phil. Trans. R. Soc. Lond.* B **353**, 1631–1643.

Walker, R. J., Ralph, K. L. & Woodruff, G. N. 1972 The presence of octopamine in the brain of *Helix aspersa* and its action on specific snail neurons. *Experientia* **28**, 1173–1174.

Webb, R. A. & Orchard, J. 1980 Octopamine in leeches. I. Distribution of octopamine in *Macrobdella decora* and *Erpobdella octoculata*. *Comp. Biochem. Physiol.* C **67**, 135–140.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES