

SEROTONERGIC REGULATION  
OF THE BUCCAL (FEEDING) RHYTHM OF THE POND  
SNAIL, *LYMNAEA STAGNALIS*.  
AN IMMUNOCYTOCHEMICAL, BIOCHEMICAL AND  
PHARMACOLOGICAL APPROACH

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Hatching is an important phase of the development of pulmonate gastropods followed by the adult-like extracapsular foraging life. Right before hatching the juveniles start to display a rhythmic radula movement, executed by the buccal complex, consisting of the buccal musculature (mass) and a pair of the buccal ganglia. In order to have a detailed insight into this process, we investigated the serotonergic regulation of the buccal (feeding) rhythm in 100% stage embryos of the pond snail, *Lymnaea stagnalis*, applying quantitative immunohistochemistry combined with the pharmacological manipulation of the serotonin (5-HT) synthesis, by either stimulating (by the 5-HT precursor 5-hydroxytryptophan, 5-HTP) or inhibiting (by the 5-HT synthesis blocker para-chlorophenylalanine, pCPA) it. Corresponding to the direction of the drug effect, significant changes of the fluorescence intensity could be detected both in the cerebral ganglia and the buccal complex. HPLC-MS assay demonstrated that 5-HTP increased meanwhile pCPA decreased the 5-HT content both of the central ganglia and the buccal complex. As to the feeding activity, 5-HTP induced only a slight (20%) increase, whereas the pCPA resulted in a 20% decrease of the radula protrusion frequency. Inhibition of 5-HT re-uptake by clomipramine reduced the frequency by 75%. The results prove the role of both central and peripheral 5-HTergic processes in the regulation of feeding activity. Application of specific receptor agonists and antagonists revealed that activation of a 5-HT<sub>1</sub>-like receptor depressed the feeding activity, meanwhile activation of a 5-HT<sub>6,7</sub>-like receptor enhanced it. Saturation binding plot of [<sup>3</sup>H]-5-HT to receptor and binding experiments performed on membrane pellets prepared from the buccal mass indicated the presence of a 5-HT<sub>6</sub>-like receptor positively coupled to cAMP. The results suggest that 5-HT influences the buccal (feeding) rhythmic activity in two ways: an inhibitory action is probably exerted via 5-HT<sub>1</sub>-like receptors, while an excitatory action is realized through 5-HT<sub>6,7</sub>-like receptors.

**Keywords:** 5-HT – feeding activity – buccal mass innervation – immunohistochemistry – pharmacology – receptors

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## INTRODUCTION

Invertebrate models have successfully been used for the elucidation of the neuronal background of behavioral phenomena. Among these models are, for example, the crustacean stomatogastric system [21, 40, 47], the insect thoracic motor system [7], the gastropod respiratory [50], locomotor [51] and feeding [6, 13, 14] networks, or the gill withdrawal reflex [20]. In gastropods, however, considerably less attention has been paid to the peripheral targets connected to these central events, such as, for example, the coordinated muscle contraction, executing the radula movement, in response to various signal molecules (transmitters, neuromodulators and neurohormones), or their receptors that mediate their effect.

Among the regulatory substances serotonin (5-HT) has been considered as one of the major neuromodulators in invertebrates. Its role in a number of physiological and behavioral processes has well been established [1, 10, 19, 23, 35, 61], including a pivotal role in the regulation of the feeding behavior of gastropods [8, 18]. In connection with a diverse set of feeding functions the role of 5-HT was addressed including the sensory initiation of feeding [41], central motor pattern generation [11, 12, 49], as well as the cellular mechanisms underlying different forms of feeding related behavior, such as arousal [29], satiety, [30] and learning [3, 26]. While it is generally accepted that 5-HT modulates the feeding system at central levels (cerebral and buccal ganglia), there is considerably less information on its presence and role at the periphery, related to the buccal mass executing the cyclic movement of the radula during feeding in *Lymnaea* [4, 15].

The key member of the central feeding network is the giant cerebral 5-HTergic neuron (CGC – *Lymnaea*, MGC – *Helix*, MCC/C1 – *Aplysia*; [44, 59; see also 8, 39]. Fibers from this cell project through the cerebro-buccal connectives to the buccal ganglia and then via the peripheral nerves to the head region, thus participating in the modulation of the entire feeding system [4, 6, 38, 60].

5-HT acts through multiple receptors to mediate a great variety of functions in vertebrates and invertebrates [5, 42, 52]. Identification of 5-HT receptor subtypes has begun over 50 years ago when Gaddum and Picarelli [16] described two types of physiological actions of 5-HT which could be blocked by different antagonists. Later a number of studies provided additional functional evidence for the existence of more than one 5-HT receptor type, and this idea was confirmed following the emergence of radio-ligand binding techniques as well. In invertebrates 5-HT was shown that a number of effects influencing different functions in the central nervous system (CNS) and periphery mediated by 5-HT were likely to be transmitted by a multitude of receptor subtypes [see e.g. 18, 43, 53, 57].

In our present investigation we analyzed the 5-HTergic modulation of the feeding activity (buccal rhythm) of the pond snail, *Lymnaea stagnalis*, with special attention to the radula movements (protraction, rasping and retraction) at the end of the embryogenesis, right before hatching (100% embryonic stage). The investigations were performed in three different directions. First, we have studied the organization of the

central and buccal 5-HT-immunoreactive (IR) elements upon the effect of different pharmacological interventions, such as the inhibition and stimulation of 5-HT synthesis, accompanied by HPLC-MS assay to determine the 5-HT level. It was followed by the *in vivo* analysis of feeding activity (radula movements) after the pharmacological manipulation of the 5-HT level and different 5-HT receptors. Finally, biochemical-binding studies were also applied to characterize the 5-HT receptor types present in the buccal mass.

## MATERIALS AND METHODS

### *Animals and staging*

Populations of adult *Lymnaea stagnalis* L. were maintained in aquaria at 20–25 °C supplied with aerated filtered water from Lake Balaton (filtered Balaton-water, FBW) and fed on lettuce. Egg masses and P5, P6 juvenile snails were collected from laboratory culture. Embryonic development (8–10 days) was staged on the basis of a specific set of morphological, morphometric and behavioral features, according to Morrill [37] and Mescheriakov [36]. Stages were expressed as a percentage of total embryonic development, according to Marois and Croll [34], wherein 0% (E0%) corresponds to the first cleavage and 100% (E100%) to embryos right before hatching. Juvenile snails were categorized according to the shell size [9].

### *Immunocytochemistry*

The immunohistochemical procedure applied for the visualization of 5-HTergic and muscle elements has been described in details elsewhere [4, 56]. Briefly, embryos of 100% stage were removed from the egg capsules, were anesthetized on ice, and then the isolated buccal complex (buccal mass, including the buccal ganglia), as well as the circumesophageal ganglionic ring (CNS) were fixed in 4% paraformaldehyde diluted in 0.1 M phosphate buffer (PB) for 4 hours at room temperature. After thorough washing in PB buffered saline (PBS), the whole mount specimens were incubated in mouse monoclonal anti-5-HT antiserum (Dako, Denmark, see also in Balog et al., 2012) diluted 1:2000 in PBS containing 2.5% normal goat serum, 0.25% bovine serum albumine and 5% Triton-X (TX) for 48 hours at 4 °C. Following 3×10 min washing in PBS the preparations were incubated in a mixture of goat-anti-rabbit Alexa 488-conjugated IgG (Molecular Probes, USA) and phalloidin conjugated with TRITC (Sigma-Aldrich), both diluted 1:800 in PBS-TX, for 12 hours at 4 °C. After 3×10 min washing in PBS, the specimens were immersed in 70% glycerol diluted in PBS, mounted on glass slides and examined by a Leica TCS SPE or a Leica TCS SP5 confocal laser scanning microscope with appropriate wavelength-filter configuration settings. The specificity of the primary antibody has been proved previously [4].

For the quantitative measurement of the immunofluorescence intensity preparations were made using the same protocol as above and then scanned under the same microscope settings as above. This allowed to measure the relative brightness of 5-HT immunostaining in the central ganglia, focusing on the cerebral ones and the buccal complex. At least 25 embryos of 100% stage were examined both for control and after 5-HTP and pCPA treatments, respectively (5-HTP: 100  $\mu$ M, 45 min, 6 h; pCPA: 5  $\mu$ M, 6 h). Series of 0.5  $\mu$ m optical sections were projected into single images and exported as TIFF images. Average brightness of corresponding areas in control and experimental preparations were measured using Image J software [43].

### *Pharmacological analysis of the feeding rhythm*

To examine the effect of 5-HT and different 5-HTergic drugs on the feeding activity, 100% embryos were monitored inside the egg capsule. According to Voronezhskaya et al. (1999), regular radula protrusions can be observed first prior to hatching in 90–100% stage embryos. Individual egg capsules with embryos were separated randomly from the egg masses and placed into a small glass chamber containing 1 ml FBW. After 10 min accommodation under shaded light the embryos were recorded for 10 minutes in a Zeiss Axioplan compound microscope attached with a MyScope 130 M Camera (Webbers). The number of the radula protrusions was counted for one minute and the mean frequency was determined thereafter. The data obtained served as control. 5-HTergic drugs in 10 or 100  $\mu$ M concentration were thereafter added to the chamber and mixed with gentle pipetting. Following incubation for 15 and 45 minutes, respectively, a subsequent 10 min recording was made. During each 10 minutes of recording three times one min intervals were arbitrary chosen when the radula protrusions of individual embryos were counted. The mean value for each treatment was calculated on the basis of data obtained from a minimum of 15 embryos originating from at least two different egg masses.

In the course of the assays the following drugs were used: 5-HTP, clomipramine, pCPA, 5-carboxamidotryptamine (5-CT), 8-OH-DPAT, indorenate, metergoline, S-WAY100135, SB269970.

Statistical evaluation of the effects of different pharmacological treatments on the radula movement frequency was performed with two-way ANOVA with individuals being the other predictor factor followed by Tukey post-hoc test, using Statistica 6.0 (StatSoft, Inc.).

### *Biochemistry*

#### *Determination of 5-HT by HPLC-MS*

*Sample preparation.* The buccal complex including the buccal ganglia and the buccal mass, and the cerebral ganglia, respectively, were prepared from both 100% control

embryos as well as from those treated previously with 5-HTP (45 min and 6 h) or pCPA (6 h).

For extraction of 5-HT acetonitrile was applied containing 0.1% formic acid and 0.01 m/v% dithiotreitol. The 200  $\mu$ l extracting solution was added to 50 embryos. Thereafter the samples were homogenized and were explored with a high energy ultrasonicator UIS250V (Hielsher Ultrasound Technology) at  $6 \times 10$  seconds, applying ice cooling between cycles. Samples were then vortex mixed and centrifuged (Heraeus Biofuge Pico, Thermo Fisher Scientific) at 10,000 rpm for 5 minutes. After it, spinning supernatants were loaded in pure tubes and the solvents were evaporated with a SpeedVac concentrator (Eppendorf Life Sciences) at room temperature. The samples were dissolved fourfold number of masses in ultra-pure water containing 0.1% formic acid and loaded into autosampler vials for HPLC-MS measurements.

**5-HT measurement.** Analyses were performed with a complex Ultimate 3000 (Dionex) micro HPLC system equipped with a quaternary pump, a degasser, and a QExactive UHR spectrometer (Thermo Fisher Scientific). Separations were performed on a Kinetex PFP column (100 mm  $\times$  2.1 mm i.d., particle size 2.6  $\mu$ m, Phenomenex). The flow rate was 200  $\mu$ l/min, the injection volume was 5  $\mu$ l, and the temperature was kept at 4  $^{\circ}$ C in the autosampler and 40  $^{\circ}$ C in the column compartment. Xcalibur (Thermo Fisher Scientific) software was used for controlling the instrument, data acquisition and spectrum evaluations. A Thermo Orbitrap mass spectrometer equipped with a HESI source was used for mass detection. The ionization source was operated with an endplate potential of 3 kV in the positive ion mode.

For quantitative analysis of 5-HT five points calibration curves were made using 122; 305; 610; 1200; 2100 pmol/ml 5-HT as standards. Correlation coefficients ( $r^2$ ) were between 0.9956 and 0.9989 for all acceptable calibration curves both in MS and MS/MS mode. The limit of detection was 2.9 pmol/ml and the limit of quantification was 9.4 pmol/ml for 5-HT. 5-HT was identified by their exact molecular weight and by their fragments according to the literature data. The quantification of the data was made parallel both in MS and MS/MS mode. 5-HT was identified at 177.10 m/z parent ion protonated form  $[M+H^+]$  and at 166.08 m/z fragment ion protonated form  $[F+H^+]$  which were characterized by 3.9 min retention time. Based on these data, the identification of 5-HT in the samples were performed.

For the measurements three parallel groups of samples (buccal mass or CNS) of embryos of 100% stage, each consisting of 50 animals, were used.

### *Ligand binding analysis*

Membrane pellet preparations from the buccal mass of P5-P6 snails were obtained and processed for the binding assay as described before [25]. Saturation and competition experiments were repeated three times. The results of kinetic and competition experiments were evaluated using the Grafit program [31]. For the inhibition of

[<sup>3</sup>H]-5-HT binding agonists (5-HT, 5-CT) and antagonists (Doxepine, SB258585) were applied in a concentration range between 1–100  $\mu$ M.

### *Cyclic adenosine monophosphate (cAMP) assay*

In the buccal mass of P5-P6 snails the adenylate cyclase activity was measured as cAMP accumulation in the tissue as a response to the application of 5-HT, or its agonists and antagonists, respectively. Following preparation, buccal masses were pre-incubated for 5 min in 250  $\mu$ l *Lymnaea* saline containing 0.5 mM isobutylmethylxanthine, 0.1 mM guanosine-5-triphosphate and 0.5 mM adenosine-5-triphosphate. 5-HT or agonists were added to the mixture and the incubation was continued for an additional 5 min. The control experiments contained no 5-HT or other agonist. The agonists applied were 5-HT, 5-CT, 5-methoxytryptamine, 8-OH-DPAT. The inhibitory effect of the antagonists was investigated on activity of the adenylate cyclase stimulated by 100  $\mu$ M 5-HT. In these experiments the incubation mixture contained also the antagonists in a concentration of  $10^{-7}$ – $10^{-4}$ . The antagonists were clozapine, SB258585, clomipramine, doxepine. The amount of cAMP was determined by the protein binding method using the Amersham cAMP[<sup>3</sup>H]assay Kit (GE Healthcare UK Limited) as described earlier [15]. Measurements were carried out four times.

### *Chemicals*

All drugs used in the experiments were purchased from Sigma.

## RESULTS

### *Effect of changes of 5-HT synthesis on the 5-HTergic innervation in the CNS and the buccal complex*

Prior to the detailed functional analysis of the role of 5-HT in the regulation of buccal (feeding) rhythm, we have investigated the organization of the 5-HT-IR elements in the cerebral ganglia and the buccal complex after 5-HTP (100  $\mu$ M, 45 min, 6 h) and pCPA (5  $\mu$ M, 6 h) administration, respectively. The investigations included, on the one hand, qualitative (Fig. 1A–F) and, on the other, quantitative immunocytochemistry (Fig. 1G). Changes evoked by the drug administration were also correlated with HPLC-MS assays (Fig. 1H, I).

The immunocytochemical studies demonstrated that before hatching (100% stage) 5-HT-IR elements were well distributed in the nervous system, including the cerebral

ganglia (Fig. 1A) and the buccal complex (Fig. 1B). The cerebral ganglia contained labeled cells and a richly innervated neuropil, and the connectives and peripheral nerves were also supplied with labeled axons. Also, 5-HT-IR processes were seen throughout innervating the buccal mass (musculature).

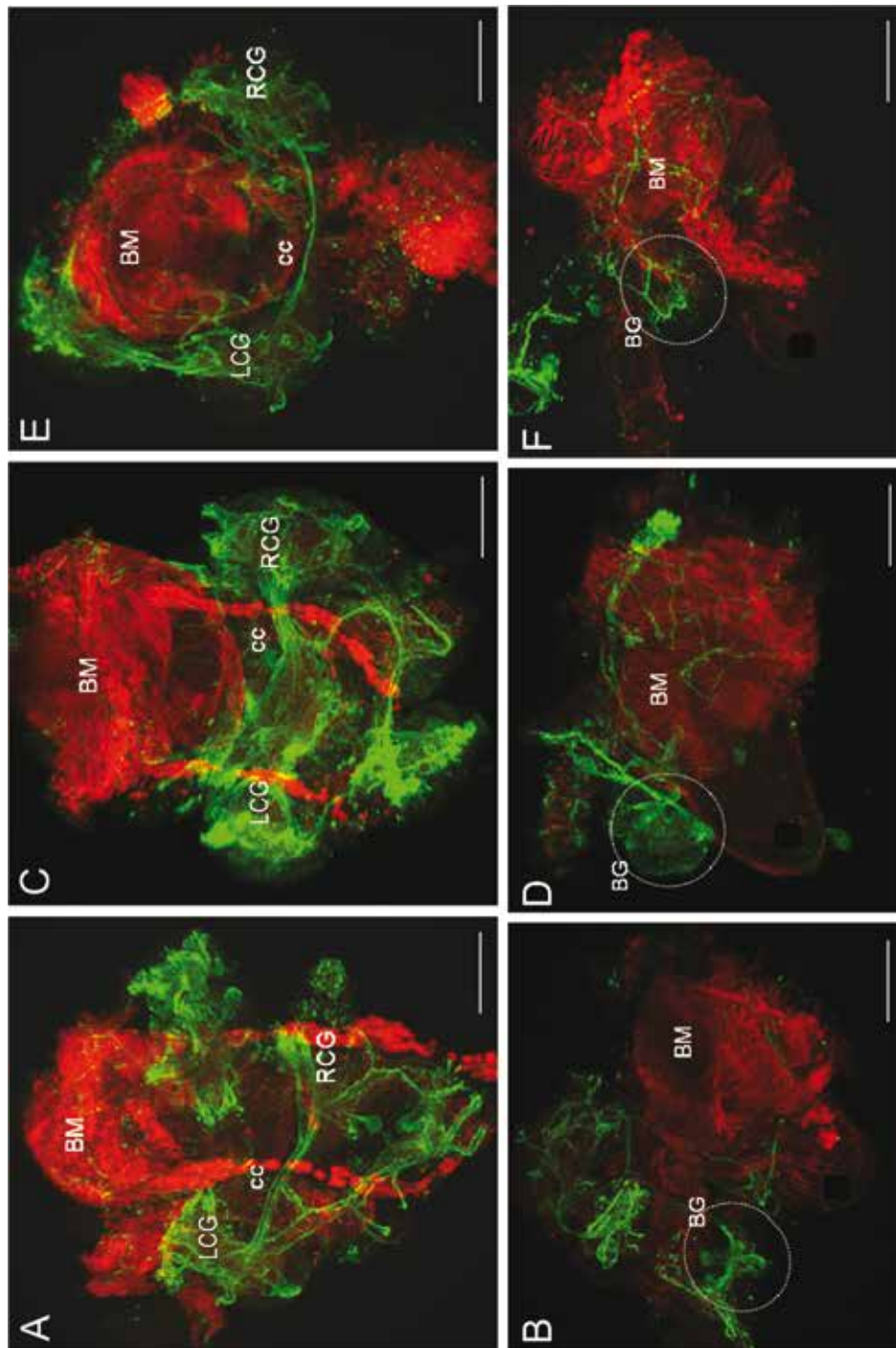
Incubation with 5-HTP resulted in a clear, significant increase of the brightness of labeled fibers in the cerebral ganglia (and the entire CNS), reflected also by quantitative measurements (Fig. 1C, G). The number of peripheral processes innervating the buccal mass, their ramification and varicosities also increased, although this was only less supported by the quantification of the 5-HT-IR intensity of this region (Fig. 1D, G). HPLC-MS assay of the 5-HT content of the buccal complex and the cerebral ganglia demonstrated a strong increase of 5-HT level both after 45 min (17-times) and after 6 h (47-times) of 5-HTP treatment, compared to the control (Fig. 1H).

Incubation with pCPA resulted in a slight (20%) but significant decrease of fluorescence intensity of the 5-HT-IR elements in the cerebral ganglia (and in the entire CNS) and resulted in a twofold reduction of brightness in the innervation of buccal complex (Fig. 1E, F). HPLC-MS assay revealed a three times reduction of 5-HT content in the buccal complex compared to the control (Fig. 1I).

### *Effect of 5-HTergic drugs on the buccal (feeding) rhythm*

In the next step we have investigated the action of selected drugs on the buccal rhythm (radula protraction), which affected either the 5-HT level or different 5-HT receptors. Figure 2A shows the time-lap frames of the various phases (resting, protraction, rasping, retraction) of the radula movement in living preparation at 100% stage. Application of 5-HT itself at 1 or 2  $\mu\text{M}$  concentrations activated the radula movement. However, it simultaneously strongly stimulated the ciliary locomotion (gliding) of the embryo on the inner surface of the egg capsule, thus making impossible to count reliably the number of the protractions. Therefore, instead of 5-HT, we used 5-HTP, its immediate precursor. Application of 5-HTP in both low (10  $\mu\text{M}$ ) or high (100  $\mu\text{M}$ ) 5-HTP concentration for 15 min or 45 min also affected the locomotion of the embryos; almost 100% of tested animals expressed gliding on the inner surface of the egg capsule accompanied with active left-right movements of the head, as well as occasional shell rotations and columellar muscle contraction. However, the intensity of these movements was never as strong as after 5-HT administration, hence the number of radula protrusions could be counted.

Application of 10  $\mu\text{M}$  5-HTP for 15 min did not influence the buccal rhythm, but after 45 min it induced a significant decrease of the radula movement (57% compared to the control,  $n = 10$ ) (Fig. 2B). To the contrary, 100  $\mu\text{M}$  5-HTP slightly increased the number of radula protrusions (21%,  $n = 21$ ) after 15 min, and this effect was the same after 45 min of incubation (Fig. 2B). Clomipramine (5  $\mu\text{M}$ ), a 5-HT re-uptake blocker and 5-HT<sub>6</sub> receptor antagonist depressed (18%,  $n = 10$ ) the frequency of the





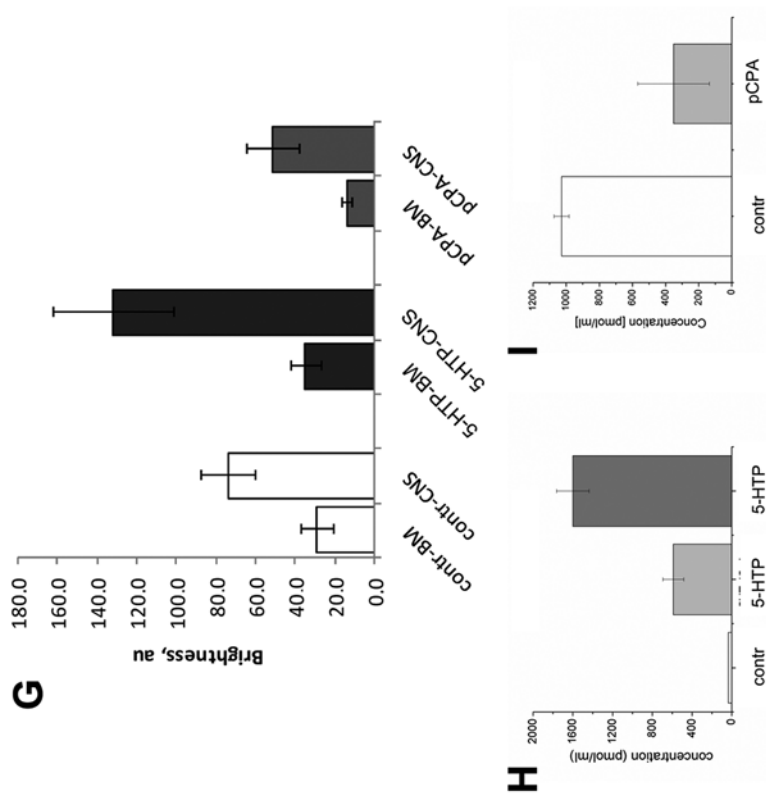
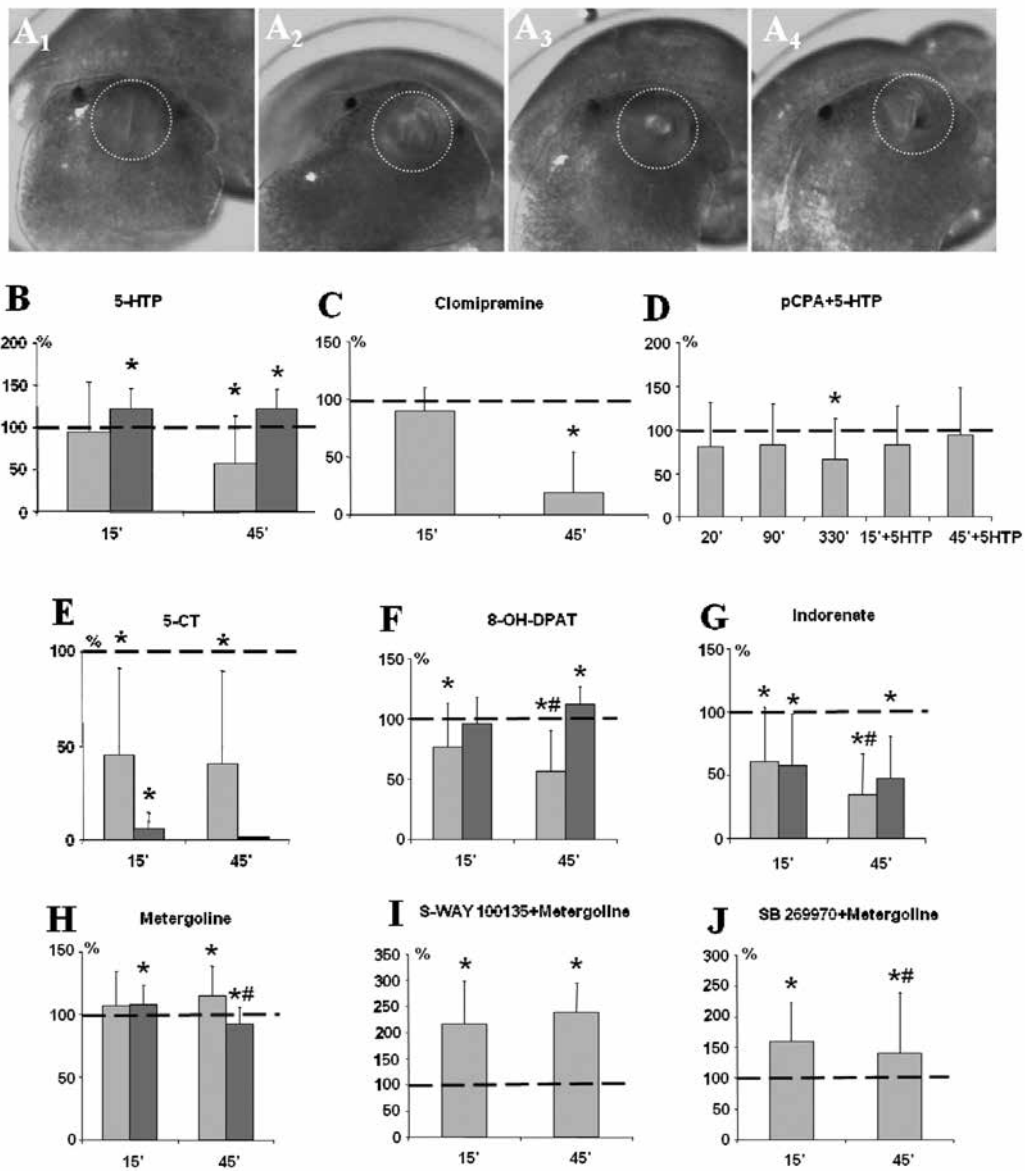


Fig. 1. A-F 5-HT-IR elements in the cerebral ganglia (A, C, E) and the buccal complex (B, D, F) of 100% *Lymnaea* embryos following pharmacological interventions. (A) control; (C) 5-HTP treatment; (E) pCPA treatment. RCG – right cerebral ganglion, LCG – left cerebral ganglion, cc – cerebral commissure. (B) control; (D) 5-HTP treatment; (F) pCPA treatment. BM – buccal mass, Encircled and BG – buccal ganglion. Note increased fluorescence intensity following 5-HTP and decreased fluorescence intensity following pCPA administration in both regions. Bars: 100  $\mu$ m. (G) Relative intensity of 5-HT immunofluorescence in the buccal complex (BM) and in the cerebral ganglia (CNS) in control embryos and following 5-HTP or pCPA administration. au – arbitrary units. (H, I) HPLC-assay of the 5-HT content in control and 5-HTP (H) or pCPA (I) treated 100% embryos



←

*Fig. 2.* Pharmacological analysis of the 5-HTergic regulation of feeding rhythm in *Lymnaea* at 100% stage of development. (A) Time-laps frames demonstrating various phases of the radula movement in living embryos. A<sub>1</sub>: mouth closed, A<sub>2</sub>: radula protraction, A<sub>3</sub>: rasping and A<sub>4</sub>: radula retraction. The mouth region is encircled. (B–J) Effects of 5-HTergic drugs on the feeding activity (frequency of the radula protrusion normalized to the control). Light grey columns represent the lower and dark grey columns represent the higher concentration of the applied drugs. (B) 5-HTP modulates the feeding activity in two different ways, depending on the applied concentration. Ten  $\mu\text{M}$  5-HTP decreases the number of radula protrusions after 45 minutes incubation, whereas 100  $\mu\text{M}$  increases it. (C) 5  $\mu\text{M}$  clomipramine decreases significantly the number of rasping after 45 min. (D) The long-lasting inhibitory effect of pCPA is rescued by the administration of 100  $\mu\text{M}$  5-HTP. (E) 5-CT has a strong concentration-dependent inhibitory effect. Note the complete block of the feeding activity after 45 minutes after 0.5  $\mu\text{M}$  application. (F) 8-OH-DPAT decreases the buccal rhythm at 0.1  $\mu\text{M}$  concentration, while at 1  $\mu\text{M}$  it has a slightly enhancing effect. (G) Indorenate applied at 0.5 and 2  $\mu\text{M}$  concentrations decreases the number of the protractions significantly both after 15 and 45 min of application. (H) 0.5  $\mu\text{M}$  metergoline slightly increases the feeding activity, meanwhile at 5  $\mu\text{M}$  concentration it induces an increase after 15 min, followed then by a slight decrease after 45 min. (I) The combined application of S-WAY 100135 (1  $\mu\text{M}$ ) and metergoline (0.5  $\mu\text{M}$ ) resulted in a more than twofold increase of the frequency of radula protractions. Note the long-lasting effect. (J) Combined application of SB269970 (1  $\mu\text{M}$ ) and metergoline (0.5  $\mu\text{M}$ ) leads to a long-lasting activating effect. \*Statistically significant difference ( $p < 0.001$ ) from the control. #Statistically significant difference from the previous time interval

radula protractions after 45 min (Fig. 2C). Incubation with the 5-HT synthesis blocker, pCPA (5  $\mu\text{M}$ ) resulted in the gradual decline of the number of protractions (up to 65% after 330 min,  $n = 11$ ), meanwhile adding 100  $\mu\text{M}$  5-HTP completely rescued this effect after 45 min (Fig. 2D).

5-CT, a 5-HT<sub>1,5,7</sub> receptor agonist, caused a significant decrease (44%,  $n = 11$ ) of the radula protrusions already at low (0.1  $\mu\text{M}$ ) concentration after 15 min. At higher (0.5  $\mu\text{M}$ ) concentration, 5-CT totally blocked ( $n = 24$ ) the buccal rhythm after 45 min (Fig. 2E). 8-OH-DPAT, a 5-HT<sub>1,7</sub> receptor agonist, induced a significant but gradual decrease of the number of rasping at 0.1  $\mu\text{M}$  concentration (up to 77% after 15 min and 56% after 45 min,  $n = 21$ ). In contrast, at 1  $\mu\text{M}$  concentration ( $n = 9$ ) this agonist had no significant effect after 15 min of incubation, but evoked a slight increase of the feeding rhythm after 45 min of application (Fig. 2F). Indorenate, a 5-HT<sub>1</sub> receptor agonist, significantly reduced the protraction rate in a time-dependent manner at both 0.5 ( $n = 5$ ) and 2  $\mu\text{M}$  concentrations ( $n = 5$ ) (Fig. 2G). Low (1  $\mu\text{M}$ ) concentration of metergoline, which is a 5-HT<sub>6,7</sub> receptor agonist and a 5-HT<sub>1</sub> receptor antagonist, resulted in a slight increase of the buccal mass activity (up to 7% after 15 min and 15% after 45 min,  $n = 17$  in both cases), whereas at 5  $\mu\text{M}$  concentration it increased (7%,  $n = 10$ ) the rasping after 15 min, but decreased (8%,  $n = 10$ ) it after 45 min (Fig. 2H). The application of 5-HT<sub>6</sub> receptor antagonist, SB258585 (1  $\mu\text{M}$ ) failed to block the activating effect of metergoline ( $n = 23$ ) (data not shown).

While the 5-HT<sub>1</sub> receptor antagonist, S-WAY 100135 or the 5-HT<sub>7</sub> receptor antagonist, SB269970 alone had no effect on the buccal activity at any concentrations tested (0.1–1  $\mu$ M) either after 15 min or 45 min (data not shown), their combined application with metergoline produced the strongest and most specific stimulatory action on the buccal rhythm of 100% stage embryos. Application of S-WAY 100135 (1  $\mu$ M) or metergoline (0.5  $\mu$ M) led to a more than a twofold increase in the radula protrusion (up to 216% and 238%) after 15 min and 45 min, respectively ( $n = 11$ ) (Fig. 2I). Application of SB269970 (1  $\mu$ M) and metergoline (0.5  $\mu$ M) increased the number of radula protrusions by 159% and 141% both after 15 min and 45 min ( $n = 23$ ) (Fig. 2J).

### *Characterization of 5-HT receptors in the buccal muscle*

#### *[<sup>3</sup>H]-5-HT binding in the buccal muscle membrane*

To determine the kinetic parameters of 5-HT receptors in the buccal mass, receptor binding was analyzed with various concentrations of [<sup>3</sup>H]-5-HT in membrane pellets obtained from the buccal mass of P5-P6 juvenile snails (Fig. 3A). The kinetic analysis of the saturation curve revealed a  $K_d$  value of 4.5 nM and a  $B_{max}$  value of 2.4 fmol/mg, respectively. The Scatchard-plot of the saturation curve indicated a single high affinity 5-HT binding site.

Inhibition of [<sup>3</sup>H]-5-HT binding by agonists (5-HT, 5-CT) and antagonists (doxepine, SB258585) showed that 5-HT inhibited the binding already at lower concentration, meanwhile 5-CT had a low affinity to the 5-HT receptor, inhibiting the binding only at higher concentration. The 5-HT<sub>6</sub> receptor antagonists displayed a moderate affinity to the receptor, inhibiting the binding at higher concentration than 5-HT (Fig. 3B).

#### *Changes of the adenylate cyclase activity after pharmacological interventions*

Following the incubation of the buccal mass with 5-HT and its agonists (5-CT, 8-OH-DPAT, 5-metoxytryptamine), the cAMP level was increased as a result of the stimulation of adenylate cyclase (Fig. 3C). 5-HT influenced the cAMP level more effectively than the agonists and the rank order potency was 5-HT > 5-metoxytryptamine > 5-CT > 8-OH-DPAT. When 5-HT<sub>6</sub> receptor antagonists (SB252585, clozapine) or re-uptake inhibitors (clomipramine, doxepine) were applied, the 5-HT stimulated adenylate cyclase activity decreased, and the rank order potency was SB252585 > clozapine > doxepine > clomipramine (Fig. 3D).

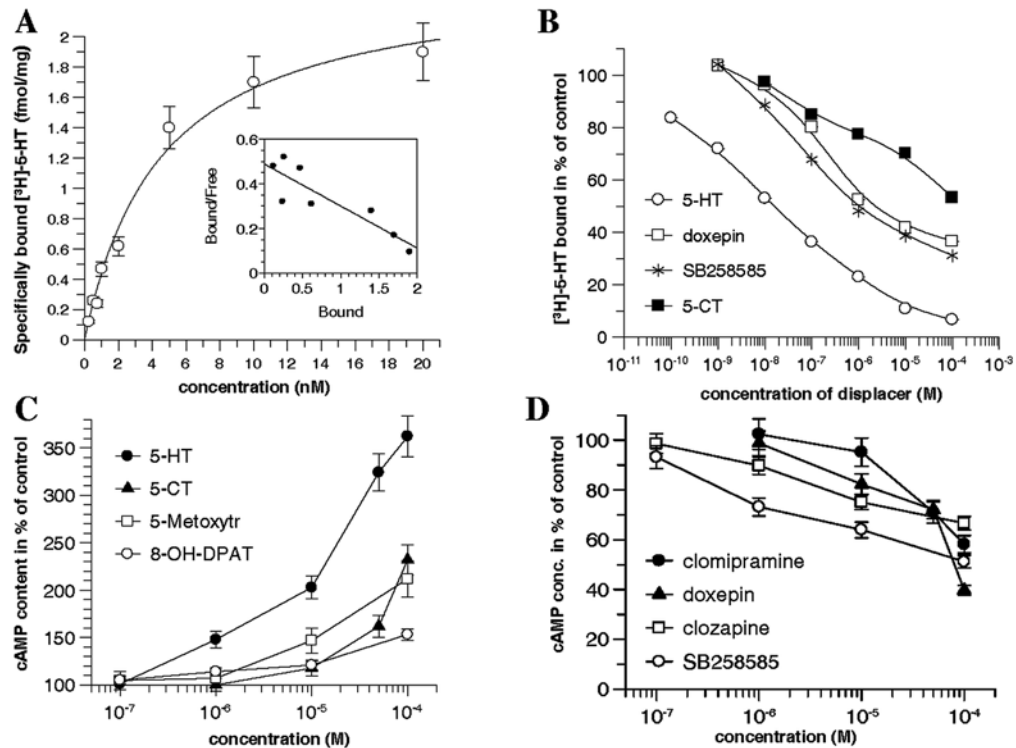


Fig. 3. Characterization of the 5-HT receptors in membrane preparations obtained from the buccal mass of P5-P6 *Lymnaea*. (A) Binding of [<sup>3</sup>H]-5-HT to 5-HT receptor. The binding shows saturation and the kinetic analysis of the curve reveals a  $K_d$  and  $B_{max}$  values of 4.5 nM and 2.4 fmol/mg, respectively. The Scatchard plot of the saturation curve (insert) indicates a single high affinity 5-HT binding site. (B) Inhibition of [<sup>3</sup>H]-5-HT binding by agonists (5-HT, 5-CT) and antagonists (Doxepine, SB258585). The data represent the mean percent of the maximum specific binding measured in parallel and repeated three times. The SEM values were less than 10%. (C) Adenylate cyclase stimulation with 5-HT or 5-HT agonists. The enhanced adenylate cyclase activity detected corresponds to the increased cAMP level. 5-HT is the most effective, followed by 5-methoxytryptamine, 5-CT and 8-OH-DPAT. The data are means  $\pm$  SEM. (D) Inhibition of 5-HT (100  $\mu$ M) stimulated adenylate cyclase activity by different 5-HT<sub>6</sub> receptor antagonists (SB252585 and clozapine) and antidepressants (clomipramine and doxepine). The data are means  $\pm$  SEM

## DISCUSSION

In the present study, with the help of combining the results of IHC, biochemical and pharmacological-physiological experiments, we have delivered data supporting, with until yet unknown details, the role of 5-HT in the regulation of feeding activity (radula protrusion) of the pond snail, *Lymnaea stagnalis*. Earlier and recent studies performed on the embryonic *Lymnaea* and related species, such as *Helisoma*, have already delivered a broad palette of results reporting on the development of the

5-HTergic system, including the distribution and sequence of appearance of 5-HT-IR elements both at central and peripheral levels [4, 34, 54], as well as the role of 5-HT in the regulation of early embryonic behaviors, such as rotation and gliding [15, 22], either by receptor characterization or pharmacological manipulation (pCPA administration) of the 5-HT level. However, an extensive analysis of the 5-HTergic regulation of the feeding behavior has not yet been performed, including the complex, quantitative immunohistochemical and pharmacological investigation of the 5-HTergic maturation of this behavior. Regarding this, the novelties of our study can be summarized as follows.

(i) Quantitative IHC findings proved that the 5-HT containing elements innervating the buccal mass (muscle system) and so being possibly involved for the execution of feeding activity are indeed 5-HTergic because they display parallel changes of fluorescence intensity with the direction of the pharmacological manipulation of their transmitter content measured by HPLC assay. Namely, following 5-HTP administration an increased whereas after pCPA treatment a decreased immunofluorescence intensity could be observed, supported also by quantitative immunohistochemical measurements. According to an earlier qualitative assay, 5-HT immunofluorescence labeling was shown to completely disappear following pCPA administration both in 60% and 85% *Lymnaea* embryos [15]. The only source of this 5-HTergic fibers in gastropods is the giant cerebral neuron (CGC/MGC) that sends peripheral processes to the head region [27, 38]; Elekes, unpublished; see also [8], about which series of earlier and recent studies reported to fulfill a key role in modulating the feeding activity [12, 62, 63]; see also [18]. In contrast, data were missing until our present study reporting about the direct peripheral effect of 5-HT on the feeding activity (buccal rhythm/radula protrusion).

(ii) Our results demonstrate that while lowering the 5-HT level evokes the reduction of the buccal rhythm, its enhancement does not result in the activation of the feeding activity, only when applying a higher concentration (100  $\mu$ M) of the precursor, 5-HTP. The results corresponds to earlier observations obtained on *Lymnaea* embryos following 5-HT application [15], and supports further the role of 5-HT in the regulation of the buccal rhythm.

(iii) Attempts to identify receptors involved in the 5-HTergic regulation of different embryonic behaviors of *Lymnaea* were already made by Filla et al. [15] and Hiripi and Elekes [25]. It was demonstrated that a 5-HT<sub>1</sub>-like receptor subtype is involved in the regulation of rotation and gliding, whereas the identification of any 5-HT-like receptor regulating radula protrusion has failed. Earlier, a 5-HT<sub>1</sub> receptor subtype was demonstrated in *Helisoma* embryos that was responsible for the ciliary (rotation) movement [22]. Early cloning experiments revealed two different 5-HT receptor types in the *Lymanea* CNS (5-HT<sub>1Lym</sub>, [48]; 5-HT<sub>2Lym</sub>, [17]). Our present pharmacological analysis to characterize 5-HT receptors contributing to the modulatory action of 5-HT suggests that 5-HT can exert an inhibitory action via 5-HT<sub>1</sub> receptors and an excitatory effect via 5-HT<sub>6,7</sub> receptors. According to the *in vivo* pharmacological-physiological experiments, effect of clomipramine confirmed the role of a 5-HT<sub>6</sub> receptor in the muscle activity. The involvement of additional 5-HT receptor types

other than the 5-HT<sub>6</sub> can be suggested in the regulation of feeding activity (radula protrusion). These receptors would be the 5-HT<sub>1</sub> and the 5-HT<sub>7</sub> receptors. The presence of a 5-HT<sub>1</sub> receptor type is supported by the effect of 5-CT. According to our findings, radula protrusion of 100% stage embryos, displaying already the adult-like feeding activity, was stimulated by 5-HT (synthesized following the application of the precursor 5-HTP) and metergoline, an antagonist of 5-HT<sub>1</sub> receptor, and the agonist of 5-HT<sub>6,7</sub> receptor, but was inhibited by the 5-HT<sub>1,5,7</sub> receptor agonist, 5-CT. Combining the application of the 5-HT<sub>1</sub> receptor antagonist, S-WAY 100135 with metergoline caused a significant (200%) increase of the number of radula protrusions already after 15 minutes, indicating the blockage of the 5-HT<sub>1</sub> receptor, and the parallel stimulation of the 5-HT<sub>6,7</sub> receptor. Indorenate, a 5-HT<sub>1</sub> receptor agonist also decreased the rate of radula protraction, indicating a negative AC coupling. The identification of the excitatory and inhibitory 5-HT receptors in the buccal rhythm regulation of *Lymnaea* seems to be an *a priori* finding, since yet data have not been reported providing evidence on the direct peripheral involvement of this monoamine in feeding. In an earlier study on the possible role of monoamines (5-HT, DA) in the regulation of different embryonic behaviors, Filla et al. [15] have shown the involvement of a 5-HT<sub>1</sub>-like receptor, similar to that of the vertebrate type, in the regulation of both rotation and gliding, whereas the identification of a 5-HTergic receptor in the radula movement remained to be cleared. Later, based on pharmacological and biochemical assays, Hiripi and Elekes [25] delivered evidences for the unequivocal role of a 5-HT<sub>1A</sub> receptor of vertebrate type in the regulation of the cilia driven rotation of early *Lymnaea* embryos. A 5-HT<sub>1</sub> receptor type was also demonstrated following different pharmacological interventions in *Helisoma* embryos by Goldberg et al. [22], coupled to early ciliary driven rotation, although Filla et al. [15] have questioned the validity of the results.

(iv) The presence of a 5-HT<sub>6</sub> type receptor in the buccal mass was also confirmed by binding experiments performed on membrane pellets, showing the displacement of the high affinity binding of [<sup>3</sup>H]-5-HT by the same 5-HTergic agonists and antagonists which were also applied in the cAMP experiments. Also, 5-HT and other agonists stimulated adenylate cyclase activity, indicating the positive coupling of the enzyme to the 5-HT<sub>6</sub> receptor. It is, however, to be noted that these experiments were carried out on intact but dissected buccal complex preparations, while the physiological experiments were performed on *in vivo* snail embryos.

(v) 5-HT was shown to stimulate adenylate cyclase activity in the buccal mass, indicating the presence of a 5-HT receptor positively coupled to cAMP. The pharmacological profile of the 5-HT receptor characterized following the cAMP experiments suggests that it is a 5-HT<sub>6</sub>-like type, since 5-HT and 5-metoxytryptamine stimulated more effectively the adenylate cyclase activity, than 5-CT and 8-OH-DPAT, and 5-HT<sub>6</sub> receptor antagonists such as SB252585, clozapine, clomipramine and doxepine decreased the adenylate cyclase activity.

In summary, the physiological-pharmacological results suggest that three 5-HT receptors, the inhibitory 5-HT<sub>1</sub>-like and the stimulatory 5-HT<sub>6,7</sub>-like receptors are involved in the 5-HTergic regulation of buccal rhythm (radula protraction). In

*Helisoma* embryos, the 5-HTergic driven ciliary rotation was regulated through a 5-HT<sub>1</sub> and a 5-HT<sub>7</sub> receptor [33], meanwhile in *Lymnaea* the 5-HT<sub>1A</sub> receptor was shown to be involved in this early form of embryonic behavior [25]. In adult gastropod mollusks, five 5-HT receptor genes have been cloned: two in the pond snail, *Lymnaea stagnalis* [17, 48] and three in *Aplysia californica* [2, 32]. When interpreting our present findings obtained on 5-HT receptors, it is to be taken into account that although the endogenous receptors are often classified according to the mammalian-based categories, in many cases the pharmacological properties of vertebrate and invertebrate receptors differ significantly and the unequivocal identity of the latter is questionable [25, 52, 58].

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