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The serotonergic (5-HTergic) regulation of the feeding system of the developing and adult snail (*Lymnaea stagnalis*)

Ph.D. thesis

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1. INTRODUCTION

The serotonin (5-HT) is one of the most ancient and best investigated neurotransmitters. 5-HT can be found in the central and peripheral nervous system of vertebrates and invertebrates. In most species 5-HT takes part in the regulation of important behavioral and physiological processes, including feeding and reproduction behavior. It acts as neurotransmitter, neuromodulator or neurohormon. It belongs to the group of indolamines based on its chemical structure. 5-HT is synthesized by triptophan hydroxylase and aminoacid decarboxylase during a short metabolic process. Its wide-ranging physiological effect is evolved through different 5-HT receptors. Nowdays fourteen 5-HT receptors have been known, present in seven different classes, forming the most complex family of neurotransmitter receptors.

In vertebrates 5-HT is one of the multiple neurotranmitters in the brain localized in nuclei raphe. Released 5-HT modulates the effect of different neurotransmitters with physiological functions and regulation of behavioral processes as anxiety, aggression, cycles of sleep and awakeness, pain sensation, appetite, sexual activity and locomotion.

In invertebrates the cellular localization of the 5-HT is more diverse than in vertebrates, it is distributed scattered in the different ganglions of the nervous system. 5-HTergic elements of the central neural network form an articulated system by its connection system. 5-HTergic cells in the ganglia of snails are elements of different sensory and effector neural networks and act as a neuromodulator. 5-HT plays role in the regulation of feeding and locomotion, involved in the circadian rhythm and memory formation at cellular level, synaptic plasticity and regulation of synaptic growing.

Due to the characterestics of embryonic development of *Lymnaea stagnalis,* it is suitable for developmental neurobiology research as an important model animal. The localization of the 5-HT neurons, their innervation pattern and role in the regulation of behavioral and physiological processes (respiratory, locomotion, feeding) are well-known. Howewer, much less attention has been paid to the peripheral innervation of the central 5-HTergic system including its the embryo- and postembryogenesis.

The development of the *Lymnaea* embryos (24 ^{o}C, 8 days) can be conveniently monitored through the transparent egg capsule containing yolk. After hatching from the capsule the embryos start their juvenile life as miniature snail but sexually immature adult-like snails. Two-three months later in P5 juvenile stage they become sexually mature. All stages of the embryonic development are well estabilished according to the size of embryos, appearance of different internal organs (heart, kidney), body parts, peripheral organs (eyes, skin), beginning of pigmentation, and behavioral aspects (rotation, gliding, radula movement). The embryonic developmental stages (E) were expressed as a percentage of total embryonic development, wherein 0% (E0%) corresponds to the first cleavage and 100% (E100%) to the hatching.

2. AIMS

The chemical neuroanatomy of the central 5-HTerg neurons and regulation of the feeding system is well known in snails. Howewer, much less attention has been paid to the peripheral targets of the central elements, including the morphological and physiological background of the modulation of muscle function. Therefore, the aim of the present thesis was to obtain a detailed insight into development of the 5-HTergic innervation of peripherial feeding (buccal mass) system of the developing (embryonic and juvenile) and adult pond snail, *Lymnaea stagnalis.* For this reason the combination of light- and electronmicroscopic 5-HT immunohistochemistry, as well as biochemical and physiological techniques were used. Our specific aims were:

- to characterize the organization of the 5HT-IR innervation in the developing (embryonic and postembryonic) and adult *Lymnaea* with special attention the efferentiation of the feeding system (buccal mass);
- to characterize at ultrastructural level the developing of the buccal mass and its normal and 5-HT-IR innervation during the development;
- to identify the neurochemical characteristics of the the 5-HT-IR innervation in the buccal mass;
- to investigate the physiology-pharmacology background of the 5-HTergic regulation of feeding rhythm (radulamovement) and identify the 5-HT receptors.

3. MATERIALS AND METHODS

3.1. Animals

Populations of adult *Lymnaea stagnalis* L. were collected from the area of Kis-Balaton and maintained in aquaria. Egg masses and juveniles were collected from the laboratory populations. Embryonic development was staged on the basis of a specific set of morphological, morphometric and behavioral features.

3.2. Immunhistochemistry

3.2.1. Preparation

In our investigations embryos of E60%, E80%, E90%, E100% stages, hatchlings and juveniles of P1-P3 stages and adults were used. Embryos were removed from the egg capsules. We studied all head region in cryostat sections for lightmicroscopy. For laser confocal microscopy we prepared out isolated buccal mass from head region from E80% stages.

3.2.2. Fixations

1. For cryostat sections we used 4% paraformaldehyde diluted in 0.1 M phosphate buffer (PB) (pH 7.4) for 4 hours at roomtemprature or 16 hours at 4°C.

2. For electron microscopy embryos or the isolated buccal masses from juveniles and adults were fixed overnight at 4 °C in a mixture of 4% paraformaldehyde and 0.08%-0,1% glutaraldehyde diluted in 0.01 M PB (pH 7.4) for or in 0.1 M PB (pH 7.4) for the. It was followed by postfixation with 1% OsO_4 in 0,1 M Na-cacodylat-buffer for 1 hour at 4 ºC.

3.2.3. 5-HT immunhistochemistry

3.2.3.1. Epifluorescent immunhistochemistry

Sixteen µm cryostat sections were cut, placed on chrome-alum gelatine coated slides and processed as follows: incubation overnight at 4°C with monoclonal mouse anti-5-HT antiserum (Dako) diluted 1:500 in PBS-TX-BSA, later incubation for 16 hours at 4°C in rabbit anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC, Dako) or tetraetilrodamine isothiocyanate (TRITC, Dako) 1:50 diluted in PBS-TX-BSA. After 3x10 min washing in PBS, the preparations were mounted in a 3:1 mixture of glycerol and PBS, viewed in a Zeiss Axioplan compound

microscope equipped with an appropriate filter set and photographed with a Canon PS G5 digital camera.

3.2.3.2. Laser confocal immunhistochemistry

After preparations and fixation the whole-mount specimens were incubated in polyclonal rabbit anti-5-HT antiserum (Immunostar) diluted 1:2000 for 48 hours at 10º later the preparations were incubated for 12 hours at 10°C in a mixture of goat-anti-rabbit IgG conjugated with Alexa 488 (Molecular Probes) and phalloidin conjugated with TRITC (Dako), both diluted 1:800 in PBS-TX. The specimens were examined by a Leica TCS SPE or a Leica TCS SP5 confocal laser scanning microscope. Series of 0.2-0.5 µm optical sections were projected into single images and exported as TIFF images.

3.2.3.3. Correlative light and- electronmicroscopic immunhistochemistry

After fixation the samples were embedded in a mixture of gelatin and albumin and post-fixed in 10% paraformaldehyde, then 50 µm thick slices sections were cut on a Vibratome. The Vibratome-sections were processed for immuncytochemistry using monoclonal anti-5-HT primary antibody (1:500), and rabbit anti-mouse IgG conjugated with HRP (1:200; Dako). The immuncytochemical reaction was developed in 0.05% 3,3'-diaminobenzidine-HCl (DAB, Sigma-Aldrich) diluted in 0.05 M Tris-HCl containing 0.01% H_2O_2. After postfixation with 1% OsO_4 in 0,1 M Na-cacodylat-buffer the preparations were dehydrated and block staining was performed in 70% ethanol with saturated uranyl acetate for 30 min, later embedded in and Araldite. After polymerization 1 µm serial semi-thin sections or 50-60 nm ultrathin sections were cut and semi-thin sections stained with 1% toluidine-blue. Ultrathin sections were stained with lead citrate and investigated in a JEOL 1200EX electron microscope.

3.2.3.4. Control experiments, specificity tests

Monoclonal anti-5-HT antibody

The monoclonal mouse anti-human 5-HT antibody, clone 5-HT-H209 (Dako) was produced by the antigen 5-HT and Freund's adjuvant. On the snail tissue preparations we have performed four control tests. 1. Immunostaining obtained with the polyclonal (Immunostar, 1:2000) and monoclonal (Dako, 1:500) anti-5-HT antibodies, respectively, was compared on alternate cryostat sections taken from the same buccal mass preparation. No difference was seen in the distribution of the 5-HTLIR elements. 2. Immunostaining with the monoclonal and polyclonal anti-5-HT antibodies was compared on alternate cryostat sections obtained from the adult *Lymnaea* CNS. It was established that the same neurons and nerve cell groups, including CGC, displayed 5-HT-immunoreactivity following the two labelings. 3. Double immunostaining with the mono- and polyclonal antibodies was performed on the same cryostat sections taken from the *Lymnaea* CNS and the buccal mass. An unequivocal co-localization of the immunoreactions was observed in neurons, including the CGC, and other identified 5-HT-containing cell groups, as well as in axons and axon bundles innervating the musculature. 4. In negative control experiments when the primary antibody was omitted from the diluting solution (PBS-TX-BSA) no immunoreaction could be observed either.

Western blot experiments

A Western blot experiment was also carried out on total *Lymnaea* CNS homogenate to confirm specificity of the monoclonal antibody. The membrane was reacted first for the monoclonal mouse anti-5HT antibody (1:2000), then with an anti-mouse-HRP conjugate (1:2000), and finally the immunoreaction was developed with a Ni-intensified DAB reaction. No positive bands were observed, indicating that the monoclonal anti-5-HT antibody did not recognize any proteins in the *Lymnaea* brain.

Polyclonal anti-5-HT antibody

The specifity of the polyclonal rabbit anti 5-HT antibody (Immunostar, 20080) has been previously proven by preabsorbtion tests with the antigen 5-HT creatininsulphate-BSA conjugated with paraformaldehyde both in *Lymnaea* and other gastropod species.

3. 3. Biochemical experiments

3.3.1. HPLC

For a single assay buccal masses were prepared from E90% embryos, hatchlings, P1-P5 juveniles, and adults, homogenized and then processed for high pressure liquid chromatography (HPLC, Milford) to measure the 5-HT content.

3.3.2. Determination of 5-HT uptake

Buccal masses obtained from P5 juveniles, measuered wet weight and were placed into a vial containing 1 ml *Lymnaea* saline. 5-hydroxy[^{3}H]tryptamine trifluoroacetate ([^{3}H]-5-HT 4.44 TBq/mmol, GE Healthcare UK Limited) was added to the vial in a final concentration of 0.1-50 μM, and the buccal masses were incubated for 10 min at 25 ^{o}C (total uptake) or 0 ^{o}C (nonspecific uptake). The incubation was terminated by filtration of the incubation mixture on Whatman GF/C glass fiber filters. The radioactivity was counted by a TRI-CARB 2100 TR liquid scintillation analyzer (PerkinElmer Life Science). The specific uptake was calculated as a difference of the total and nonspecific uptake.

3.3.3. Determination of 5-HT release

Three buccal masses obtained from P5 juveniles were incubated in in 1 ml *Lymnaea* saline containing 2 μCi ^{3}H-5-HT. We collected 12 Fractions contained *Lymnaea* saline, 5 fractions contained *Lymnaea* saline with elevated 100 mM K^+ concentration, and 12fractions contained again normal *Lymnaea* saline.

3.3.4. Ligand binding analysis in membrane pellets

Membrane pellet preparations from the buccal mass of P5-P6 snails were obtained and measured its wet weight, then homogenizated and processed for the binding assay. For saturation experiments the membrane pellet equivalent with 10 mg wet weight tissue was incubated in parallel with increasing concentration of [^{3}H]-5-HT trifluoroacetate (0.2 – 20 nM) for 20 min at

25 ^{o}C. Competition binding assay was performed in parallel in the presence of increasing concentration of different competition agents and 5 nM [^{3}H]-5-HT trifluoroacetate. The incubation was terminated by filtration of the incubation mixture on Whatman GF/C glass fiber filters and we measured the radioactivity. The results of kinetic and competition experiments were evaluated using the Grafit program.

3.3.5. Adenilate-cyclase activity

In the buccal mass, adenyl cyclase activity was measured as cAMP accumulation in the tissue in a response to the application of 5-HT or its agonists and antagonists. The buccal mass was prepared from young adult snails and pre-incubated in *Lymnaea* saline. 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 agonist. The agonists applied were 5-HT, 5-carboxamidotriptamin (5-CT), 5-methoxytryptamine (5-MET), 8-hidroxi-2-(di-N-propilamino) tetralin (8-OH-DPTA). The inhibitory effect of antagonists was investigated on the 100 μM of 5-HT stimulated activity of the AC. 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. To measure the ammount of cAMP by the protein binding method using the Amersham cAMP [^{3}H] assay Kit (GE Healthcare UK Limited).

3.4. Physiological-pharmacological assays

To examine the effect of 5-HT and different 5-HTergic pharmacons on the feeding activity (radula protrusion) E100% embryos were monitored inside the egg capsule. Individual embryos were separated randomly from the egg masses and placed into a small glass chamber containing FBW. After 10 min incubation in FBW at room temperature under slight light (to get accomodated to light), the embryos were recorded for an additional 10 minutes in Zeiss Axioplan compound microscope attached to a MYscope 130M Camera (Webbers). The mean frequency of mouth opening (radula protrusion) was counted and data obtained served as control. Chemical substances were added to the chamber and mixed with gentle pipetting. Following incubation with drugs for 15 and 45 minutes or sometimes 90 and 330 min, respectively, a subsequent 10 min recording was made. During each 10 minutes recording three times 1 min interval was arbitrary chosen and radula protrusions of individually selected embryos were then counted. The mean value for each treatment was calculated from a minimum of 15 embryos originating from at least two different egg masses. Effects of different pharmacological treatments on the radula movement frequency were tested with two-way ANOVA with individuals being the other predictor factor followed by Tukey post-hoc test, using Statistica 6.0 (StatSoft, Inc.)

4. RESULTS

4.1. Chemical-neuroanatomy and ultrastructure of the 5-HTergic innervation in *Lymnaea* buccal mass

4.1.1. Organization of the 5-HT-immunreactive innervation of the buccal mass in the developing and juvenile snails

According to our results, right after metamorphosis, at E60% stage, no positive 5-HT-IR elements were found in the buccal mass, howewer the developing ganglionic ring of the CNS could be clearly identified. 2 days later the first 5-HT-IR fibers in the buccal mass were seen scattered on its surface at E80-E90% stages. From this embryonic stage a fast maturation of 5-HT-IR innervation of the buccal mass can be observed. At this time of embryogenesis, the CNS and other peripheral regions (foot, bodywall) were richly supplied by 5-HT-IR processes. Few fine processes were also visible within the buccal ganglia, including a tiny fiber projecting to the surface of the buccal muscle. By E90%, the first deeper thin fibers were found to occur in the buccal mass. 5-HT-IR fibers within the buccal mass increased, but even in this relatively late stage of embryogenesis still only barely developed and poorly ramified. At E100% stage the number of labeled fibers projecting through and from the buccal ganglia grew further and formed a dense 5-HT-IR net covering the buccal muscles. The buccal mass displayed a remarkable changing in the structure and size paralell with the development of 5-HT-IR innervation. In hatchlings, the buccal mass enlarged considerably and its 5-HT-IR innervation increased dramatically. Numerous thin varicose ramifications resulted in a dense 5-HT-IR network innervating the musculature, including the deeper layers of the developing buccal muscle. The pattern of the 5-HT-IR innervation was similar in P1 and P3 postembryonic snails, but the innervation by 5-HT-IR elements became denser. The varicose ramifications containing were present in the deeper layers of the buccal mass. The buccal mass was further enlarged in P3 juveniles and muscle fibers displayed a matured symmetrically organization. Thin 5-HT fibers with numerous varicosities were seen between the muscle fibers.

4.1.2. Ultrastructural organization of the embryonic and juvenile buccal mass and its innervation

According to semi-thin sections the musclefibers appear by late embryonic development. The structure of the buccal region seen in serial 1 μm tolouidin-blue stained semi-thin sections displayed a remarkable changing in the structure and size of the buccal mass from E80% stage to E90%, due to change from a thin layered buccal musculature for a thick multilayered musculature. These changes resulted in a highly differentiated buccal mass by the very late embryonic development. By the time of E100% and early postembryonic P1stages, the buccal mass showed an increase in size, accompanied with the presence of a thick, adult-like buccal mass. The buccal mass of the embryos in the late embryonic stage (E80%) was relatively undeveloped. The structure of the thin layered buccal mass in the E90% embryos has changed and a thick multilayered buccla mass has formed around the eosephagus. Comparing with the two previous embyonic stages, the size of the buccal mass of E100% embryonic stage has enlarged. By this stage a highly differentiated multilayered parallel circular and longitudial buccal mass has formed. The buccal mass of the embryos in the late embryonic stage (E80%) was relatively undeveloped at ultrastructural level, the musclefibers contained few contractile filaments, most of the muscle elements were separetaly presented, howewer in certain cases they

were organized in smaller bundles. In the buccal mass of E80% *Lymnaea* as well as in the later embryonic stages (E90%, E100% és juveniles) in appearing nerve-muscle connections the membranes of muscle and axons are tightly (16-20 nm) and long connected but membranespecializations could not be observed. The axonprofiles and varicosities were deeply embadded either on the surface of or among the musclefibers. In most of varicosities large (80-100 nm) granular and/or small (50-60 nm) agranular vesicules appeared. The finestructure of buccal mass in E90% stage was more complex, the number of contractile elements has increased, the muscle fibers were filled with a high number of paralell arranged myofibrilla. The axonprofiles have either formed tight (16-20 nm) membraneconnections with muscle cells or they were further from them. In the buccal mass of E100% stage the muscle fibers have developed contractile apparatus. The fines tructure of the buccal mass in postembryonic *Lymnaea* was similar to the ultrastructure of late embryonic (E100%) stage. In the buccal mass of early juveniles (P1) little assymetric presynaptic vesicular and intersynaptic accumulation could be observed. The deeeply embadded axon profiles formed neuromuscular connections without membrane specializations, in certain cases axonterminals with assymetric vesicular accumulation were tightly surrounded by sarcoplasma.

4.1.3. The ultrastuctural organization of 5-HT immunreactive neuoromuscular contacts during the maturation of the buccal mass

Similar to the general ultrastructure of the muscle, the first 5-HT-IR axons and neuromuscular contacts in the buccal mass could be observed only in late E80% snails. The 5-HT-IR varicosities were deeply embedded in and among the musclefibers and were formed close membrane contacts (16-20 nm) with muscle cells. Similar to conventional electronmicroscopic investigations signs of ultrastructural membranes specialization could not be observed. Axon varicosities of different size but with mainly small diameter (0.5-1 μm) also contained agranular (50-60 nm) and granular (80-120nm) vesicules. The number of 5-HT-IR neural elements in E100% embryos, however, was higher than in the previous stage. The ultrastructure of varicosities was similar to the one in E80% embryos. The membrane contacts between muscle cells and axon profiles were without membrane specialization, varicosities were connected to the muscle cells with short membrane segments. The number of axon processes has increased in the buccal mass of postembryonic snails. The fines tructure of 5-HT-IR neuromuscular contacts observed in adult snails was similar to the varicosities in previously observed embryonic and juvenile snails. Besides 5-HT-IR varicosities of small diameter (0.5-1 μm), larger (4-5.5 μm) ones occurred as well. Apart from the close and non-membrane specialized neuromuscular connections, 5-HT-IR axon varicosities could be found located freely in the extracellular space farther (100-200 nm) from muscle bundles. Other 5-HT-IR varicosities accompanied with glia cells and processes ran among muscle cells.

4.2. Biochemical characterization of the 5-HTergic system in the buccal mass

4.2.1. 5-HT content of the buccal mass in the developing *Lymnaea*

5-HT concentration was 3.3 pmol/mg at E90% embryonic stage which thereafter showed a transient decrease to 2.2 pmol/mg by hatching, followed by a gradual increase from 3.6 pmol/mg at P1 stage (young juvenile) up to 6 pmol/mg at P6 stage (adult). It is noteworthy that while the

wet weight of the buccal muscle exhibited a 66-times enhancement (from 0.091 mg to 6 mg, not shown) in the course of P1-P6 development, meanwhile the 5-HT content of the tissue exhibited a three-fold increase only.

4.2.2. 5-HT 5-HT uptake and release in the buccal mass

5-HT uptake is an active process which has an absolute requirement for external Na^+. Na^+-dependent (Na^+ sensitive) uptake is characterized by saturation curve. The affinity of the uptake (K_M) was 13.65 μM and the rate of the uptake (Vmax) was 12.7 pmol/mg. A significant increase of 5-HT uptake was observed from hatching to P1 juvenile stage, followed by a moderate but continuous increase during further development.–When K^+ concentration in the saline was elevated to 100 mM, a stimulated 5-HT release was observed in the buccal mass of juvenile snails. [^{3}H]-5-HT release was monitored in Ca^{2+} free solution. In this case a significantly lower K^+ stimulated release occurred.

4.2.3. Characterization of the [^{3}H]-5-HT binding in the buccal mass

To determine the kinetic parameters of 5-HT receptors in the buccal mass, receptor binding was analyzed with various concentrations of [^{3}H]-5-HT in membrane pellets obtained from the buccal mass of P5 and adults. 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. Application of agonists and antagonists showed that 5-HT inhibited the binding at already lower concentration meanwhile 5-CT had low affinity to the 5-HT receptor, blocking the binding only at higher concentration. The 5-HT_6 receptor antagonists displayed a moderate affinity to the receptor, inhibiting the binding at higher concentration than 5-HT.

4.2.4. Adenylate cyclase activity in the buccal mass

Following the incubation of the buccal mass with 5-HT and its agonists (5-CT, 8-OH-DPAT, 5-Metoxytriptamine). 5-HT influenced the cAMP level more effectively than the agonists, and the rank order potency was 5-HT>5-Metoxytriptamine>5-CT>8-OH-DPAT. When 5-HT_6 receptor antagonists (SB252585, clozapine) and antidepressants (clomipramine, doxepine) were applied, the 5-HT stimulated enzyme activity decreased, and the rank order potency was SB252585> clozapine > doxepine > clomipramine.

4.3. Effect of the pharmacological manipulation of the 5-HTergic system and *in vivo* physiological-pharmacological characterization of the 5-HT receptors in the feeding activity

10 μM 5-HTP (5-HT precursor) induced a significant decrease of the radula movement by 43% after 45 min of incubation. In contrast, 100 μM 5-HTP increased the number of the rasps by 21% after 45 min. Clomipramine, the 5-HT re-uptake blocker (and 5-HT_6 receptor antagonist)

decreased the intensity of the feeding activity by 82% after 45 min. Incubation with the 5-HT synthesis blocker, pCPA (5 μM) resulted in the gradual decrease of rasps by 35% after 330 min, meanwhile 100 μM 5-HTP completly rescued this effect. 5-CT, a 5-$HT_{1,5,7}$ receptor agonist, caused a significant decrease of the frequency of rasps already at low (0.1 μM) concentration after 15 min. At higher (0.5 μM) concentration, 5-CT totally blocked (n=10) the movement of the radula after 45 min. 8-OH-DPAT (5-$HT_{1,7}$ receptor agonist) at 0.1 μM concentration decreased significantly the activity of feeding by 23% and 44% after 15 and 45 min respectively. Indorenate**,** a 5-$HT_{1,2}$ receptor agonist, significantly reduced the rasping ratio in a time-depenedent manner at 0.5 and 2 μM concentrations. Low (1μM) concentration of metergoline (5-$HT_{6,7}$ receptor agonist and a 5-HT_1 receptor antagonist) slightly increased the feeding movenments (by 7% and 15%) after both 15 min and 45 min, whereas at 5 μM concentration it increased by 7% the rasping after 15 min, and decreased it by 8% after 45 min. The combined application of S-WAY 100135 (1 μM) and metergoline (0.5 μM) led to more than a two-fold increase in the feeding activity by 116% and 238% after 15 min and 45 min. The 5-$HT_{7,\ 5,\ 1}$ receptor antagonist, SB269970 (1 μM) did not block the activatory effect of metergoline.

5. SUMMARY

We investigated the buccal mass of the embryonic, juvenile and adult pond snail, *Lymnaea stagnalis*). The combination of histology, conventional electron microscopy, light- and electronmicroscopic 5-HT immunohistochemistry, as well as biochemical and physiological-pharmacological techniques were applied to study the development of the 5-HTergic innervation, neuro-chemical characterization**,** and to identify 5-HT receptors which could ve involved in feeding. Our new results can be summarized as follows:

1. We described the spatial and temporal organization of the 5-HT-IR elements in the buccal mass during the embryo- and postembryogenesis. According to our results, the 5-HT-IR innervation is characterized by a continous and relative fast maturation. The final 5-HTergic system starts to be organized the late embryogenesis (E80%) and lasts until the early postembryogenesis (P1, P2). The 5-HT-IR innervation pattern seen in postembryogenic (P2, P3) snails resembles that found in adult snails.

2. We also investigated the ultrastructure of the buccal mass. The buccal mass in E80% embryos is characterized by a few immature muscle fibers. By the end of embryogenesis these fibers form a complex multilayered buccal musculature with mature contractile apparatus, capable of carrying out active feeding (radula movement). The size of the buccal mass continously increases during postembyogenesis.

3. At ultrastructural level, the first neuromuscular contacts were found in the buccal mass of E80% embryos. Close (16-20 nm) but mostly unspecialized neuromuscular contacts were formed by both unlabeled and 5-HT-IR axon profiles. Three types of neuromuscular contacts were found in the buccal mass of developing and adult snails. Wide (100-200 nm) and mostly close (16-20 nm) unspecialized neuromuscular contacts, as well labeled axon processes located relative far from the muscle cells in the extracellular space were found. According to these ultrastructural findings it is suggested that 5-HT plays a modulatory role in the neuromuscular contacts in

different ways. It has a fast modulatory role via close, unspecialized membrane contacts, and a slow modulatory role via the wide contacts. In addition, a neurohormonal effect can be attributed to 5-HT via the endings located far from the muscle cells in the extracellular space.

4. The biocemical data correlate well with the data obtained by 5-HT imunohistohemistry. HPLC assay showed a gradual increase of the 5-HT level in the buccal mass during development. According to our biochemical results, the 5-HTergic neurotransmission possesses a single component high affinity 5-HT uptake system, coupled with a Na^+-Ca^+ dependent release.

5. Our biochemical and pharmacological-physiological experiments refer to the presence of three types of 5-HT receptors in the buccal muscle. The inhibitory 5-HT_1-like and the stimulatory 5-$HT_{6,7}$-like receptors seem to be involved in the 5-HTergic regulation of feeding activity (radula protraction).

6. According to our results, 5-HT is suggested to play a wide neurotransmitter/modulatory role in the buccal mass (peripheral feeding system) of the developing pond snail and it may also play a role in the functional maturation of the muscle system. Our results provide new data contributing to the better understanding and interpretation of the 5-HTergic regulation of the feeding behavior of gastropod mollusks.

6. PUBLICATIONS

6.1. Publications related to thesis

Balog G, Elekes K. (2008): Functional neuroanatomy of the 5-HTergic system in the developing and adult buccal complex of the pond snail, *Lymnaea stagnalis.* Acta Biologica Hungarica, 59, 55-59. IF: 0,447

Balog G., Voronezhskaya E. E., Hiripi L., Elekes K. (2011): Organization of the serotonergic innervation of the feeding (buccal) musculature during the maturation of the pond snail *Lymnaea stagnalis*: a morphological and biochemical study Journal of Comparative Neurology, DOI 10.1002/cne.22693, IF: 3.741

Balog G., Voronezhskaya E. E., Hiripi L., Elekes K. (2011): Biochemical and pharmacological characteristics of the serotonergic innervation of the buccal muscle in the pond snail, *Lymnaea stagnalis* (kézirat előkészületben)

6.2. Posters and lectures related to the thesis

Balog G, Elekes K. (2007). Chemical-neuroanatomy of the 5-HTergic system in the buccal complex of the snail (*Helix* and *Lymnaea*). 11th Meeting Hung. Neurosci. Soc., Szeged, (poster) Abstract: Clin. Neurosci. 2007;60 (S1):1-72.

Balog G, Elekes K. (2007). Organization of the serotonergic system in the buccal region of pulmonate gastropods, *Lymnaea* and *Helix.* 31st Neurobiol. Conf. Göttingen,

(poszter) Abstract: Neurowissenschaftliche Gesellschaft e.V. p.142. Neuroforum Suppl. Februar 2007 (1). ISSN 0947-0875.

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