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Role of serotonin and dopamine in the embryogenesis and in the regulation of embryonic behaviors of the pond snail (*Lymnaea stagnalis*)

PhD Thesis

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1. INTRODUCTION

Although serotonin (5-HT) was discovered in the middle of the 19th century, the exclusive investigation on its role has begun in the 1930's, and in 1951 serotonin was first synthesized. The dopamine (DA) was synthesized in 1910 early than 5-HT, but the importance of his physiological effect started being recognized only in 1950's. Both monoamines are ancient and widespread molecule. Their presence has been detected almost in all vertebrate and invertebrate species. In the living organization they can be found both in the central nervous system (CNS) and in the periphery. They exert their effect as neurotransmitters, neuromodulators or neurohormone. On the basis of their structure and biosynthesis both belong to the group of biogenic amines. 5-HT is synthesized from tryptophan by a short metabolic pathway consisting of tryptophan hydroxylase and amino acid decarboxylase, whilst DA is produced from tyrosine by tyrosine-hydroxylase and amino acid-decarboxylase. These monoamines have physiological effects realized through membrane-bound receptors. Currently fourteen 5-HT receptor subtypes are distinguished which are in seven distinct classes. Five types of DA receptors have been recognized that are categorized into two classes. With the exception of 5-HT_3, all DA and 5-HT receptor types are G protein-coupled.

In the central nervous system of vertebrates, 5-HT neurones occur exclusively in the raphe nuclei of the brainstem, their projections innervate different part of forebrain as well as their descending fibers terminate on the sensory and motor neurons of spinal cord. 5-HT released from nerve terminals meanly modifies the effect of other neurotransmitters and in this way 5-HT is involved in the control of many physiological functions and behaviors, such as body temperature, sleep, emotional state, feeding, aggression, memory and learning. In contrast to 5-HT, DA is produced in several areas of the vertebrate brain. The axons of DAergic neurons project to a large area of the brain through four major pathways. The DAergic system has many functions, including important role in cognition, motor activity, motivation and reward.

In contrast to vertebrates, in higher invertebrates 5-HT containing neurons are distributed throughout the nervous system, located in the different ganglia and their axons innervate densely the entire CNS. 5-HT acts mainly as a neuromodulator and plays prominent role in the formation of arousal. 5-HT has functions in various behaviors and physiological processes, including feeding, locomotion, circadian rhythms, learning and memory, synaptic plasticity and synaptic growth. DAergic neurons are also dispersed throughout the CNS of invertebrates, and the majority of them is interneuron. All central ganglia contain DAergic fibers. The DAergic system plays a role in the control of feeding, locomotion and respiration, as well as in learning.

Among of Gastropod species, *Lymnaea stagnalis* is one of the significant model animals for comparative neurobiology. The location of 5-HTergic and DAergic neurons in this gastropod has been mapped in details and their projection systems have been well characterized. The embryogenesis of DAergic and 5-HTergic neurons has been also studied. The role of both monoaminergic systems has been demonstrated in several behaviors and physiological functions (respiration, circulation, locomotion, feeding). Some 5-HTergic and DAergic interneurons are intrinsic elements of behavioral networks and full fill an important role in control of these functions. For example, the central pattern generator (CPG) element of the respiration, DAergic RPeD1, whereas in the feeding behavior 5-HTergic giant cerebral cell is the key CPG member. These neurons participate also directly in the regulation of behaviors via their efferent axons. In contrast, there is very little information on the possible regulatory role played by both monoaminergic systems in the development of *Lymnaea* embryo, although the distribution of monoaminergic neurons and their neuronal development has been extensively examined by immunohistochemical method. Few experiments performed on other gastropods indicated their possible function in the control of embryogenesis and embryonic behaviors.

Lymnaea embryos develop inside transparent and nutritional fluid filled egg capsules (25˚C, 8 days), hence they are well-suited for studies on embryogenesis and related processes. The hatched animals resemble miniature adult snails, but are yet not capable for reproduction. The embryonic development can be staged on the basis of specific set of morphological, morphometric and behavioral features, in the course of which the stages are expressed as a percentage of total embryonic development, wherein 0% corresponds to the first cleavage and 100% to hatching.

2. AIMS

Although the 5-HTergic and DAergic system of adult *Lymnaea* and their role in the regulation of physiological processes and behaviors has been extensively examined, their function in the course of embryogenesis is very little known. Therefore, we performed a detailed biochemical and pharmacological analysis on *Lymnaea* embryos to have a clear view on the 5-HTergic and DAergic regulatory processes during embryonic development. The aims of our present work were the following:

- to determine the changes of 5-HT and DA levels during embryogenesis;
- to define the synthetic pathway of both monoamines in embryos and characterize their synthesizing enzymes;
- to reveal the possible role of 5-HT and DA in the regulation of development;

- to describe the effects of both monoamines on embryonic behaviors, such as rotation, gliding, radula movement and on physiological processes, such as heartbeat;
- make an attempt for the pharmacological characterization of the types of 5-HTergic and DAergic receptors involved in the aminergic regulation

3. MATERIALS AND METHODS

3.1. Animals

Adult pond snails (*Lymnaea stagnalis*) were collected from canals of Kis-Balaton and they were maintained and raised in aquaria. Egg masses were collected from these populations. Stages of embryos were established on the basis of their morphological, morphometric and behavioral features.

3.2. HPLC assays

3.2.1. Monoamines quantification

Twenty-five specimens of each embryonic stage between 12%-100% (12, 25, 30, 35, 40, 45, 55, 60, 75, 80, 85, and 100) were removed randomly from egg mass. The embryos were homogenized and centrifuged, then the supernatant was directly injected into HPLC system and the level of DA and 5-HT was measured. We used Waters 460 electrochemical detector (+0,65 V) to detect monoamines. Waters HPLC system (Milford, USA) consisted of pump (1500 model), injector (717 plus Auto sampler) and column heater (Waters), and all systems were operated by a Millenium 4 software.

3.2.2. Tyrosine hydroxylase activity determination

Seventy embryos of 90% stage were homogenized in *Lymnaea* physiological solution (40 mM NaCl, 1,7 mM KCl, 1,5 mM Mg^{2+}, 4 mM Ca^{2+} and 10mM Tris-HCl; pH 7,4). The homogenates were used as the source of the enzyme. The kinetic parameters of enzyme were determined by changing the concentration of substrate in present of constant enzyme concentration and measuring the amount of product. The incubation mixture contained homogenate, 1 mM 6,7-dimethyl-5,6,7,8-tetra-hydropterine, 1 mM ferrous ammonium sulphate, 0.15 M potassium phosphate buffer (pH 6) and tyrosine as substrate. The reaction was stopped by adding perchloric acid containing inner standard. Before HPLC analysis, aluminium-oxide selective purification was used.

3.2.3. Decarboxylase activity determination

Decarboxylase activity was measured continually from embryos of 35% to 95% stages. During development removed embryos were homogenized in 0.1 M phosphate buffer (pH 7.0). The homogenate was used as enzyme source. Since this enzyme is involved in the synthesis of both monoamines, both L-DOPA and 5-HTP were used as substrate in experiments. The incubation mixture contained 0.02 mM pargyline, 0.3 mM pyridoxal-5 phosphate, 2.0 mM L-DOPA or 0.1 mM 5-HTP tissue homogenate. In analysis for determine the kinetic parameters of enzymes, the concentration of substrates were changed between 0,0025-0,1 mM. Liquid ion-change purification was used before injection into HPLC. The enzyme

activity was measured in same way, when it was inhibited *in vitro* by carbidopa (0.0001-100 μM), Nsd-1015 (0.001-200 μM) and alpha-methyl-DOPA (0.001-1000 μM) for 20 min.

3.3. Pharmacological treatments

3.3.1. Analysis of embryonic development

Twenty-twenty five specimens of veliger larvae were isolated and placed into Petri dishes containing filtered Balaton water. The long-lasting effects of monoamine precursors (tryptophan and tyrosine), tryptophan hydroxylase inhibitor (p-chlorphenylalanine [pCPA]), aromatic-L-amino acid decarboxylase inhibitor (3-hydroxybenzylhydrazine [Nsd-1015]), tyrosine hydroxylase inhibitor (α-methyl-p-tyrosine [αMPT] and neurotoxins (5,7-dihydroxytryptamine [5,7-DHT], 6-hydroxy-dopamine [6-OH-DA], N,methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP] and 1-methyl-4-phenylpyridinium iodide [MPP^+ iodide]) on the embryonic development were examined between concentration ranges of 0,001-1 mM. The treatment lasted to hatching and the solutions were changed daily. The alteration of 5-HT and DA contents was measured in embryos of 90% following treatments with the same drugs at threshold concentration. Effect of the pharmacons was expressed as percentage of the change of monoamines, where 100% is corresponds to the monoamine contents determined in control embryos.

3.3.2. Analysis of embryonic behaviors

The effects of 5-HT, DA, 5-HTergic agonists (5-carboxyamidotryptamine [5-CT], 2,5-dimethoxy-4-iodoamphetamine [DOI], N,N-dimethyltryptamine [DMT], 8-hydroxydipropylaminotetralin [8-OH-DPAT], D-lysergic acid diethylamide [LSD], 1-(2-methoxyphenyl) piperazine [2-MPP], 2-methylserotonin and tryptamine), 5-HTergic antagonists (ketanserin, 7-methyltryptamine, methysergid, mianserin and bromo-lysergic acid diethylamide [BOL]), DAergic agonists (apomorfin, bromocriptin, m-tyramine, p-tyramine and SKF-38393) and DAergic antagonists (flupenthixol, chlorpromazine, SCH-23390 and sulpiride) were examined on the intensity of rotation and gliding, feeding activity (radula protrusion) as well as frequency of heartbeat. In the experiments, five embryos were separated randomly from the egg masses and their physiological feature and behaviors were analysed four times in few minute intervals. The mean value for each treatment was obtained from a minimum of 20 embryos.

3.4. Immunohistochemistry

Veliger larvae were raised in 0,01 mM pCPA or 0,1 mM Nsd-1015 containing solutions. In the case of pCPA treatment embryos of 60% and 85% stages were used, whereas in the case of Nsd-1015 treatment embryos of 75% stage were used for 5-HT immunohistochemistry. The egg capsule and shell were removed then embryos fixed in 4% paraformaldehyde solution. To detect 5-HT immunoreactivity, the preparations were incubated with monoclonal mouse anti-5-

HT primary antibody (1:500), followed by incubation in anti-mouse IgG (1:50) conjugated with fluorescein isothiocyanate (FITC, Dako) to visualize the immunoreaction.

3.5. Cyclic adenosine monophosphate (cAMP) assay

Fifty embryos of 90% stage were incubated in physiological solution containing 0,5 mM izobuthylmethylxanthine (IBMX), 0,1 mM guanosine-5-triphosphate (GTP), 0,1 mM 5-HT or LSD and 0,5 mM adenosine-5-triphosphate (ATP). After centrifugation and removal of fluid, the embryos were homogenized in acidified ethanol. The homogenate was centrifuged then the supernatant was lyophilized. After resuspendation, cAMP concentration was measured by Amersham Kit.

3.6. ^{3}H-5-HT binding assay

Membrane pellet was prepared from embryos before metamorphosis by homogenization in 0,05 M Tris-HCl buffer (pH 7,4), several times centrifugation and resuspendation. To examine the binding of ^{3}H-ligand, the pellets were incubated in 1 ml Tris-HCl buffer (pH 7,4) containing ^{3}H-5HT (0,25-10 nM). In competitive binding experiments, the binding of 0,5 nM ^{3}H-5HT was measured in the presence of following pharmacons: 5-CT, spiperon, WAY100635 and 8-OH-DPAT. The incubation was stopped by fast vacuum filtration through GF/C fibreglass filter then the radioactivity of pellet was detected. The nonspecific binding was measured in the presence of 10 nM unlabeled 5-HT.

3.7. Statistical analysis

The enzyme kinetic parameters and IC_{50} values were counted by GraFit program, while pharmacological results were analyzed and graphically illustrated by Origin program. The results are presented as mean ± standard error of means (S.E.M.). Statistical significance was determined using Student's t test (paired). During the analysis of embryonic behaviors, ANOVA and Dunnet were used as post-hoc tests.

4. RESULTS AND CONCLUSIONS

4.1. Change of monoamine concentrations and 5-HTP/DOPA decarboxylase enzyme activity during the development of *Lymnaea* embryo

The presence of monoamines was detectable at very early (12%) embryonic stage by biochemical (HPLC) assay. At this time, 5-HT content was 47 fmol/embryo and DA level was only 15 fmol/embryo. 5-HT content did not change largely until 75% stage, while DA level increased continuous until the beginning of metamorphosis (40%). Thereafter DA concentration remained at a constant level (150 fmol/embryo) during metamorphosis till 80% stage. At 12% stage 5-HT level was higher than that of DA. During metamorphosis 5-HT/DA ratio changed in favour of DA. In the last two days of development (80%-100%) the concentration

of both monoamines increased striking, at the hatching 5-HT content reached a concentration of 490 fmol/ embryo and DA concentration was 400 fmol/embryo. In the end of embryogenesis the 5-HT concentration was again higher than that of DA.

Tyrosine hydroxylase, tryptophan hydroxylase and 5-HTP/DOPA decarboxylase were also demonstrated in *Lymnaea* embryos. From embryos of 35% to 80% stages, 5-HTP/DOPA decarboxylase activity showed a threefold increase, which then increased further by an additional 100-150% during the last two days (80%-100%) of embryogenesis. The monoamine levels and the decarboxylase activities displayed a similar course of alteration during embryonic development. It suggests that the elevation of monoamine level can be due to the increase of enzyme activity.

The reaction of tyrosine hydroxylase and 5-HTP/DOPA decarboxylase with their substrates displays simple saturation kinetics, Lineweaver-Burk plot of kinetic data shows linear relationship. V_{max} and K_M values for tyrosine hydroxylase were 1,3 ± 0,21 pmol/embryo/min and was 3,7 ± 0,2 mM, respectively. 5-HTP/DOPA decarboxylase had a higher activity in the decarboxylation of DOPA substrate than in that of 5-HTP (3.52 and 0.4 pmol synthesized amines/embryos/min, respectively), whereas the enzyme showed a higher affinity towards 5-HTP than DOPA (0.045 mM and 1.98 mM respectively).

In *in vitro* experiments, it was demonstrated that carbidopa inhibited the most efficiently (IC_{50} = 0,2 μM) the activity of 5-HTP/DOPA decarboxylase. IC_{50} value for Nsd-1015 was 41,6 μM and for α-methyl-DOPA was 630 μM. Nsd-1015 at a concentration of 2,9 μM inhibited the decarboxylase activity for 5-HTP by 50%. The increase of activity in the decarboxylation of 5-HTP and DOPA showed a parallel course during embryonic life and Nsd-1015 inhibited both decarboxylations. These results indicate that both 5-HTP and DOPA can be decarboxylated by the same enzyme in embryos.

4.2. Effect of pharmacological manipulation on the duration of embryonic development and on the 5-HT and DA levels

L-tryptophan (100 μM) altered significantly the duration of embryogenesis and prolonged the intracapsular life by 7 days (78%). It enhanced both 5-HT content by 189,5 ± 40,6% and DA level by 123,6 ± 49,4%. The embryos treated by 50 μM tyrosine hatched by 7 days (78%) later than control embryos, while their DA level increased moderately (24%). Higher concentration of the precursors resulted in a distortion of body pattern of embryos or killed them. pCPA (10 μM) extended the embryonic life by 7 days (78%) and decreased both 5-HT (65,5 ± 19%) and DA (43,45 ± 14%) content. At the concentration of 300 μM, αMPT failed to alter the duration of intracapsular life, whereas higher concentrations were applied the embryos exhibited first morphological distortions, then they died. Nsd-1015 (10 μM) slowed down the development, the hatching of embryos delayed by 7 days. MPTP (10 μM) prolonged embryonic life by 6days (67%) and caused a significant decrease in the DA concentration (46 ± 5%). MPP^+ (100 μM) induced a

4 days (44%) delay and decreased the DA content by 37 ± 6%. Our results demonstrated that both increased and decreased monoamine levels resulted in prolonged embryogenesis. It seems that the actual concentration of 5-HT regulates the embryonic DA level. Consequently, an optimal 5-HT/DA ratio is needed for the normal course of embryonic development.

4.3. Effect of DAergic and 5-HTergic pharmacons on different embryonic behaviors

4.3.1. Rotation

The applied pharmacons were effective on the rotation of veliger larvae (35%) at different concentration, ranging between 1-1000 μM. Among the 5-HTergic agonists LSD (105%), 5-HT (50,5%), 8-OH-DPAT (56%) and DMT (40%) at 1 μM concentration stimulated the rotation. Other 5-HT receptor agonists acted at a higher concentration (10-1000 μM) and the rank order of their effect was as follows: tryptamine > 2-methyl-serotonin > DOI > 5-CT. In contrast, 2-MPP decreased the rotation by about 75%. Among the 5-HTergic antagonists mianserin (10 μM) caused a 35% inhibition, whereas methysergide had a 169% stimulatory effect. The 7 methyl-tryptamine (1000 μM) also acted as an agonist. Ketanserin proved to be inactive. DAergic agonists also enhanced the rotation rate of embryos, although they proved to be less effective than the majority of the 5-HTergic drugs. DA was the most potent agent (138%) following by apomorphine (66%). At the higher concentration (1000 μM) m-tyramine and p-tyramine were able to stimulate the rotation (65-119%). Both bromocriptine and SKF38393 were inactive. Chlorpromazine as a DAergic antagonist (1000 μM) decreased the rotation rate (82%). According to our findings, both 5-HT and DA play a role in the regulation of embryonic rotation. The 5-HTergic system proved to be more effective than the DAergic system in stimulating rotation.

4.3.2. Gliding

From embryos of 85% stage, the embryos perform adult-like locomotion by gliding over the inner surface of egg capsule. In the course of the pharmacological manipulation of movement, LSD (2254%,), methysergide (1550%) and 8-OH-DPAT (787,5%) increased the rate of gliding at 1 μM concentration. 5-HT (10 μM) accelerated gliding by 265%. 5-CT (1 mM) induced an increase (3323%) in the rate of this movement. Mianserin (100 μM) as a 5-HTergic antagonist, inhibited gliding by 74%. DA did not influence gliding activity.

4.3.3. Radula protrusion

The feeding activity of embryos of 95% stage was the most effectively stimulated by LSD (140%) at 1 μM concentration among the 5-HTergic drugs. DMT (10 μM) increased the rate of radula movement by 74%. 5-HT caused a 78% increase of radula protrusion. At a higher concentration (1 mM), tryptamine and 2-methyl-serotonin stimulated it by 77-145%. DOI (100 μM) and 8-OH-DPAT (1

mM) both had the opposite effect to that expected, slowing down the radula movement by 64% and 39%, respectively. Among the 5-HTergic antagonists applied, only mianserin (100 µM) was an effective inhibitor (54%), while methysergide and 7-methyltryptamine (10-100 µM) accelerated the radula protrusion (70-35%). Similarly to 5-HT, DA accelerated the feeding activity by 50%, at 1mM concentration. Apomorphine, chlorpromazine, flupenthixol (10-1000 µM) inhibited the radula movement (50-80%). On the basis of our results, both 5-HT and DA had a stimulatory effect on radula protrusion and 5-HT proved to be more effective than DA.

4.3.4. Heartbeat

All pharmacons applied inhibited the heartbeat of embryo except LSD, tryptamine and 5-HT. LSD (100 µM) accelerated the beating by 10%, while the stimulating effect of 5-HT and tryptamine was near same (20%) at 1 mM concentration. The rank order of inhibitor effect of other 5-HTergic drugs was as follows: DOI (10 µM, 15%) > 7-methyltryptamine (100 µM, 25%) > 8-OH-DPAT (200 µM, 23%) > mianserin (500 µM, 246%) > DMT (1 mM, 41%) > 2-methyl-5-HT (1 mM, 16%). Methysergide and ketanserin did not influence on the heartbeat. Among DAergic agonists only p-tyramine, m-tyramine and DA had inhibitor effect (15-32%) at concentration of 100-1000 µM, while bromocriptine and SKF-38393 proved to be inactive. DAerg antagonists such as chlorpromazine, SCH-23390 and sulpiride were also ineffective but flupenthixol (1 mM) decreased the frequency of beating by 95%. According to our experiments, 5-HT is the stimulatory, while DA is the inhibitory substance of the embryonic *Lymnaea* heart.

4.4. Effect of pharmacological treatments on the embryonic appearance of 5-HT immunoreactive elements

Following indirect immunofluorescence labeling with anti-5-HT antibody, *Lymnaea* embryos of 60-85% stages displayed distinct labeling within the developing central and peripheral nervous system. In postmetamorphotic, adult-like embryos 5-HT immunoreactive (IR) cell bodies located mainly in the cerebral and pedal ganglia were seen. 5HT-immunoreactive (IR) processes running in the cerebral and pedal commissures and connectives delineated the developing circumpharyngeal ganglion ring. Also, varicose arborizations within the central ganglia and along peripheral projections towards the foot were present. In contrast, no 5-HT immunoreactivity could be detected either at central or peripheral level in embryos pretreated with 0,01 mM pCPA or with 0,1 mM Nsd-1015. The decrease of 5-HT level was also detected by immunohistochemical method in pCPA or Nsd-1015 treated *Lymnaea* embryos. These results confirm the presence of tryptophan hydroxylase and decarboxylase in the embryos.

4.5. Effect of 5-HT and LSD on cAMP concentration in *Lymnaea* embryos

In embryos of 90% stage, cAMP concentration was 0.12 pmol/embryo. After pharmacological treatment, 5-HT and LSD at concentration of 0,1mM decreased cAMP content by 62,7 % and 54,4 %, respectively.

4.6. Analysis of ^{3}H-5-HT binding

According to our results, the binding of the radioactive 5-HT to the receptor is specific. This specific binding can be determined by saturation curve. 5-HT binding is high affinity (K_d 7,36 nM, B_{max} 221 fmol/mg pellet). The Scatchard plot shows single binding site. The binding of ^{3}H-5-HT was inhibited by applied pharmacons in the following rank order: 5-HT > 8-OH-DPAT > WAY100635 > 5-CT = spiperon.

The results from pharmacological experiments of rotation, cAMP and 5-HT binding suggested that the 5-HT receptor controlling the embryonic rotation is similar to vertebrate 5-HT_{1A} receptor type. In the case other behaviors, we failed to determine the type of 5-HT and DA receptor which may play role in the regulation.

SUMMARY

We analysed the role of 5-HTerg and DAerg systems in the regulation of the development, embryonic behaviors (rotation, gliding and radula movement) and heartbeat in the embryos of pond snail, *Lymnaea stagnalis*. In experiments, we used biochemical, pharmacological and immunohistochemical methods. Our results can be summarized as follows:

1. Both 5-HT and DA are present in *Lymnaea* embryos at very an early stage of development (12%), when the DA content increases gradually until metamorphosis, whereas the 5-HT level remains unchanged. During metamorphosis, the concentration of both monoamines remains constant, but in the last two days of embryogenesis 5-HT and DA level displays a striking increase.
2. In *Lymnaea* embryos the presence of monoaminergic synthesizing enzymes, such as tyrosine hydroxylase, tryptophan hydroxylase and 5-HTP/DOPA decarboxylase can be detected. In the case of decarboxylase, it was found that the same enzyme is responsible for the decarboxylation of both monoamines. This assumption is confirmed by that that the vertebrate decarboxylase inhibitor, Nsd-1015 inhibits the decarboxylation of both 5-HT and DA as well as the activity of 5-HTP and DOPA decarboxylation increase parallel during embryogenesis. The activity of this enzyme and the monoamine levels also display a similar course of alteration during development.
3. Our pharmacological experiments demonstrated that both increased and decreased monoamine levels effected unfavourably the development of embryo and resulted in their prolongation. The alteration of 5-HT level

affected that of DA. It seems that an optimal 5-HT/DA ratio is needed for the normal course of embryonic development.

4. The monoamines affected not only the embryogenesis but several embryonic behaviors and the heartbeat. The rotation was stimulated by both 5-HT and DA, while gliding was accelerated by only 5-HT; DA had no effect on it. Both monoamines stimulated also the radula movement. In the case of rotation and radula protrusion, 5-HT was more effective than DA. The beating was altered by both monoamines at same concentration; 5-HT stimulated the heartbeat, while DA inhibited it.
5. An embryonic monoamine receptor type could be identified only in the case of rotational movement. Our results revealed that the 5-HT effect on the rotation of veliger larvae is mediated by a 5-HT_{1A}–like receptor type. Our results suggest that a 5-HT_1 receptor type plays also a role in the regulation of gliding but further biochemical analysis is required to confirm this hypothesis. In the experiments analyzing radula protrusion and heartbeat, the monoaminergic drugs had to be applied at high concentrations therefore their effects were not specific.
6. Our results clearly indicate that both 5-HT and DA play an essential role in the regulation and maturation of early and adult behaviors of *Lymnaea stagnalis*.