**PH.D. THESIS**

# LOCALIZATION OF NITRIC OXIDE SYNTHASE AND THE POSSIBLE ROLE OF NITRIC OXIDE IN THE DEVELOPMENT OF THE NERVOUS SYSTEM OF THE POND SNAIL, *LYMNAEA STAGNALIS* L.

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# 1. INTRODUCTION

## 1.1. Nitric oxide (NO) in living organisms

NO is a unique molecule in animals, regulating physiological and pathophysiological processes by forming complexes with metal, or metal-oxo groups of proteins, by nitrosating or nitrosilating thiol residues and forming nitro-tyrosine. NO can exist in different forms which are NO radical (NO•), nitrosonium-cation ($NO^+$), nitroxyl-anion ($NO^-$), depending on the pH and electrolite properties of the enviroment, which determining also its chemical reactions. According to its physico-chemical properties and „multiface" behavior, the synthesis of NO is precisely regulated. NO derives from the oxidative desamination of arginine in cells, where one of the isoform of nitric oxide synthases (neuronal [n]NOS, endothelial [e]NOS, inducible [i]NOS) can be found. The nNOS and eNOS are constitutively expressed in the cells producing nanomolar amount of NO, whereas the iNOS is exclusively expressed due to external signals and produces micromolar amount of NO. The most important role of NO is the regulation of the hem-containing guanylyl-cyclase (GC), therefore it increases the synthesis of the second messenger, cyclic-guanosine-monophosphate (cGMP). The cGMP-dependent kinase (PKG), phosphodiesterases, cyclic nucleotide dependent ion channels (CNIC) are activated through the NO-cGMP pathway which contributes such fundamental physiological processes as vascular smooth muscle relaxation (regulating the peripheral blood supply) and transmission of the effect of the excitatory neurotransmitter glutamate in the central nervous system (CNS) (involving neurogenesis and long-term memory). On the other hand, the extraordinary increased level of NO observed in injured tissues, diseases, and in result of the cellular immune defense, produces $NO^+$, $NO^-$ forms and secondary products of reactive nitrogen and oxygen radicals (RNR, ROR) which have destructive effect on either the host or the alien cells.

## 1.2. NO as a signal molecule regulating neurogenesis and synaptic plasticity

During neurogenesis continuous communication between neurons is essential in building up the neuronal network and stabilising synapses, the connectivities of the network. The ability of synapses to change during postnatal life is also of great importance in memory formation and learning. NO influences distinct processes of neurogenesis and synaptic plasticity in experimental species of both low and high order animal groups. NO regulates the length and number of the filopodia of the growth cone in mammalian and snail neuronal cell cultures. The presence of NO is needed for the maturation of motoneurons in the spinal cord. Peripheral visual, olfactory and tasting receptors of insects and vertebrates (amphibians and mammals) can form the proper structural pattern with their central connections if the NO synthesis is intact. NO is also required for the formation of the thalamic connections of the leader projections of early cortical neurons. NO is involved in the stabilisation of the neuromuscular junctions of insects and amphibians. In most of these processes NO acts as an intercellular messenger increasing the cGMP level.

## 1.3. The snail *Lymnaea stagnalis* L. as a model in neuroscience

The microscopic structure and neurochemical characteristics of the nervous system of the pond snail, *Lymnaea stagnalis* L. are one of the best studied ones among molluscan species. In the past years, the examination of the nervous system of *Lymnaea* furnished new information about the molecular background of learning and memory and has provided new experimental approaches to understand the formation and function

of different neurotransmitter- and neuromodulator-specific neural networks. The members of the neural network regulating feeding in *Lymnaea* as well as their structural and functional connections were described, serving a basis to study feeding based learning and memory formation. Applying *in vivo* an antisense oligonucleotide of the *Lymnaea* (*Lym*)-NOS mRNA designed on the known sequence of the *Lym*-NOS gene, and by evidences obtained from pharmacological studies, it was established that the NO-cGMP system contributes to the formation of long-term memory. In other series of studies, including the description of the ultrastructure of the developing *Lymnaea* nervous system and the mapping neuronal signal molecules of invertebrate origin (members of the FMRF-amide family, octopamine), as well as classical, widely distributed neurotransmitters (serotonin, cathecolamines, histamine, GABA) in the embryonic and juvenile CNS provide growing number of data for detailed neurochemical characterization of the developing nervous system.

From the zygote stage to hatching, reaching the adult-like form, the embryogenesis of *Lymnaea* lasts 8 days at 24 °C in a closed yolk-filled glycoproteid capsule. The body pattern of the hatching juvenile is similar to the adult, however, some of the inner organs, including the sexual organs are not fully developed. The *Lymnaea* juveniles sexually develop 3 weeks after hatching, until adulthood reaching finally 1.5 cm shell size. Because of the translucent embryonic capsule, the easy culturing conditions and high reproduction, *Lymnaea* is an ideal species for developmental studies. The time of appearance, and the change of the location (e.a.: heart) or the pigmentation (eye, epidermis) of different organs and structures make it easy to separate and identify the different stages of the embryonic development. Embryonic stages (E) were given as a percentage of development, where 0% of development corresponded to the first cell division and 100% to hatching. Postembryonic juveniles were classified into five stages (P1-P5) on the basis of the size of the shell.

## 2. AIMS OF THE STUDY

In the recent few years, a growing number of evidences have been collected on the role of NO in the nervous system of *Lymnaea* and other gastropods, however, only little is known about the appearance, location and function of NO-synthetizing cells during development. Therefore, in this study, we investigated the distribution and possible function of the NO-cGMP signal pathway during the ontogenesis of *Lymnaea* with special attention to the nervous system. In the course of our experiments the main points were the following:

1. Mapping of the distribution of the *Lym*-NOS mRNA and NOS enzyme.
2. Localization of the NO-sensitive cGMP accumulation, and draw a comparison between the distribution of NOS and cGMP.
3. Investigation of the effect of NO-donors and NOS inhibitors on the ultrastructural, physiological and behavioral characteristics of the embryogenesis.

# 3. MATERIAL AND METHODS

## 3.1. Animals and tissue preparation

*Lymnaea stagnalis* L. egg masses were collected from laboratory aquaria, supplied with aerated water from the Lake Balaton. E15%-E100% developed embryos and P1-P5 juveniles were used. For histological investigations, the whole body of embryos and P1, P2 juveniles previously removed from the egg, and separated from the shell were cut into serial sections, whereas only the CNS of animals older than P3 were used either in serial sections or as total preparations. For pharmacological manipulations embryos within the capsule were investigated.

## 3.2. Light microscopical techniques

### *3.2.1. Lym-NOS in situ hybridization*

*Lymnaea* embryos, young juveniles (P1, P2), and the CNS of older juveniles and adults were fixed in a 4% formaldehyde (PFA) containing solution, cryoprotected by keeping the samples in 30% sacharose solution, embedded in mounting medium (Tissue-Tek), and cut into 15 μm thick slices in a cryostat. Sections were dryed onto slides coated with Cr-Al-gelatine, digested with pepsin, then hybridized with a digoxigenin-labeled antisense oligonucleotide probe (5'-CACAGGA(AC)GGTATGGTGTTCT-3') that recognizes the repetitive sequence of the *Lym*-NOS mRNA coding the ENTMPSC-peptide specific for the *Lym*-NOS protein. Probe was labeled by the enzyme, terminal transferase of the DIG oligonukleotide-end labeling Kit (Boehringer Mannheim). Hybridization with a sense oligonucleotide probe was performed on alternating sections as a control. For the development of the reaction, the DIG Nucleic Acid Detection Kit (Boehringer Mannheim) was applied.

### *3.2.2. NADPH-diaphorase histochemistry*

For the histochemical detection of NOS, the NADPH-diaphorase (NADPH-d) reaction was used, which is based on the ability of NOS to oxidize NADPH sustaining after aldehyde fixation. After fixation, the reaction was carried out on total preparations or 10 μm cryostat serial sections. The samples were incubated in a 1 mM β-NADPH (substrate), 0.2 mM nitro-blue-tetrasolium (NBT, electron acceptor dye), 0.3% Triton-X-100 (TX) containing 0.1 M TRIS-HCl (pH 8.1) buffer at 22 °C in dark wet chamber until the blue formazan precipitate appears. The specificity of the enzime-histochemical reaction was checked by performing the following control rections: i) β-NADPH coenzime was omitted from the solution or changed to α-NADPH and β-NADH, ii) enzymes having low oxidation stability and using also β-NADPH were inhibited by 1 μl/ml 3% $H_2O_2$, non-specific alkaline-phosphatase was blocked by addition 5 mg/ml levamisole, and 0.5mM cytochrome-c used for inhibition the cytochrome P-450-reductase.

### *3.2.3. NOS immunohistochemistry*

For the immunohistochemical detection of *Lym*-NOS, a polyclonal rabbit anibody (Calbiochem) was used that raised against a peptide found in the conservative C-terminal of the mammalian NOS (1414-1434). A sequence located in the C-terminal of the *Lym*-NOS showed approximately 82% homology with this mammalian peptide. The immunoreaction was performed on fixed cryosections (similar as in NADPH-d reaction), using the

3 step avidin-biotin-complex (ABC) technique (Universal ABC Kit, Vector). At the final step, the labeling was carried out with peroxidase or fluorescence-iso-thiocianate (FITC) conjugated to avidin. In the case of peroxidase labeling, the samples were treated with 1% $H_2O_2$, then, in every case, the non-specific binding sites were blocked using a horse serum. Sections were incubated in the following solutions: i) anti-NOS antibody (1:1000), ii) biotinilated anti-rabbit horse antibody (1:200), iii) avidin-peroxidase (1:200) or avidin-FITC (1:200). The peroxidase reaction was developed in the presence of 0.05% 3,3'-diaminobenzidin (DAB) dye and addition of 0.005% $H_2O_2$. The anti-NOS serum was omitted from the incubation step, as the secondary antibody control, whereas the NOS antibody was preincubated with its synthetic peptide (250 μg/ml) as the specificity control of the primary antibody.

#### *3.2.4. cGMP immunohistochemistry*

In normal conditions, the majority of neural tissues do not contain enough cGMP to be detected with immunohistochemistry. Therefore to sensitize the detection, stimulation with an NO-donor, Na-nitroprusside (SNP) was performed before fixation. Fifteen μm serial sections were cut from rapidly frozen total embryos and dissected CNS, then placed, and dried onto coated slides. Sections were incubated in the physiological saline (59 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 38 mM $CaCl_2$, 0.1 mM $Na_2HPO_4.12H_2O$, 5 mM HEPES buffer, pH 7.4) containing 1 mM 3-izobutyl-1-methyl-xantine (IBMX) and 10 mM SNP. After formaldehyde fixation, a cGMP-bovin serum albumin (BSA) conjugated sheep polyclonal antibody (1:500, gift of J. De Vente) was used for the detection of cGMP. The immunoreaction was developed with the ABC technique (see also in 3.2.3). In order to decide whether the cGMP-IR material is the result of NO stimulation, in control experiments, the SNP and/or the IBMX was omitted from the incubation medium. The specificity of the primary antibody labeling was previously described by J. de Vente, whereas the method control was performed by the incubation without the primary antibody.

#### *3.2.5. Double labeling experiments*

To compare results of the two techniques used for detecting NOS and to determine the relationship between the distribution of NOS-IR and cGMP-IR cells, double labeling experiments were carried out on alternative or the same sections. In the latter case, according to the cGMP detection method, samples were fixed only on slides after NO-stimulation, or (in the case of detection NOS on alternate slide) without any treatment. cGMP-IR and NOS-IR elements were developed with peroxidase-DAB on alternative sections, whereas with peroxidase-DAB and FITC labeling on the same sections.

### 3.3. NO pharmacology – Experiments on cultured embryos

Twenty intracapsular embryos were separated randomly from egg masses and transfered into small Petri-dishes, containing one of the following chemicals dissolved in filtered Balaton water: NO donors: SNP, S-Nitroso-N-acethyl-penicillamine (SNAP), NOS substrate analogue inhibitors: $N^G$-Nitro-L-Arginine (L-NOARG), $N^G$-Nitro-L-Arginine Methyl-Ester (L-NAME). The long term effects of these chemicals on the structure of the embryonic tissues, focusing on the CNS, were investigated within the $10^{-2}$ M-$10^{-5}$ M concentration range. The change of the development, physiological and behavioral characteristics were also tested. Embryos were continuously treated from the stage of E25-30% and E45-50% till the E70% or hatching

(E90%) stage. Incubation media were changed together with the culturing medium three times a day in the case of NOS inhibitors, and in every two hours in the case of NO donors. In control experiments, the D-enantiomer of L-NAME ($N^G$-Nitro-D-Arginine Methyl-Ester [D-NAME]), and the 1:1 mixture of L-NOARG and L-Arginine (L-ARG) were used, respectively.

#### *3.3.1. Electronmicroscopy*

Treated as well as untreated control embryos at the E90% stage without the shell were fixed in 3% glutaraldehyde, then postfixed in 1% osmium-tetroxide. After dehydration, the samples were embedded in Araldite (Polysciences). Semi-thin (1 μm) sections were cut on an ultramicrotome and stained with 1% toluidine-blue for light-microscopic investigation and orientation. Light microscopic images of the secretion epithelium of the foot and mantle were digitalized, and secretion material was analyzed by the *analysis 2.11* software developed by Olympus (Tokyo, Japan). Thereafter, 50 nm ultrathin sections were cut, stained with uranyl acetate and lead citrate, and viewed as well as taken photographs in a Tesla BS540 electron microscope. Ultrastructural changes were quantified, by counting lysosomes, lipid droplets in neural perikarya, and myelin-like configurations in the axon bunches and neuropil of the pedal ganglia.

#### *3.3.2. Methods for investigating physiological and behavioral characteristics*

The effect of treatments was tested on the survival ($LD_{50}$ score) and the time of hatching (duration of the embryonic life). The rate of the heartbeat, the gliding on the inner surface of the egg (the number of circles performed by embryos around the egg per minute) and the feeding activity based on the frequency of mouth opening (radula protrusion and retraction) were monitored and quantified. Five animals randomly selected out of 20 embryos were tested four times for each treatment. Hence, the total number of observations was 20 in each experiment.

### 3.4. Statistics

Quantitative data are presented as mean ± SEM. The significance of the results was determined by the unpaired Student's *t*-test.

## 4. RESULTS

### 4.1. Distribution of *Lym*-NOS mRNA in the nervous system

Sections taken from juvenile and adult snails, hybridized with the *Lym*-NOS oligonucleotide probe, displayed the hybridization signal in the perikarya and initial axon segments of some large size neurons. Samples hybridized with the sense probe did not show any specific signal. Neither the CNS nor the PNS displayed any hybridization signal during embryonic development. At the P1 juvenile stage, a pair of symmetrically located *Lym*-NOS mRNA positive perikarya appeared laterally in the ventro-caudal region of the pedal ganglia. From the stage P1 to the P3, a small number (less than 10) of neurons became additionally *Lym*-NOS mRNA positive in the pedal, cerebral, and buccal ganglia. From the P4 juvenile to the adult stage, the number of perikarya displaying the *Lym*-NOS hybridization signal steadily increased in the cerebral, pedal, parietal, and visceral ganglia (adult cerebral ganglion: 92±28 cells, pedal ganglion: 81±14 cells, parietal ganglion: 60±2 cells, visceral

ganglion: 68±10 cells). The cerebral giant cell (CGC) exhibited the *Lym*-NOS hybridization signal from P3, whereas the B2 giant motor neuron in the buccal ganglia from P4 juvenile stage.

**3.2 Appearance and distribution of NADPH-d reactivity and NOS immunoreactivity**

From the E18%-20% to the E90%-E100% embryonic stages, the larval kidney and later its disintegrated residue displayed NADPH-d reactivity. The first neuronal localization of NADPH-d reactive and NOS-IR material was seen in the axon bundles running from the periphery to the cerebral and pedal ganglia at the late stage of embryogenesis (E80%). In the CNS, the majority of these NADPH-d reactive and NOS-IR bundles arborized in the neuropil of the cerebral and pedal ganglia, and some of them ran in the cerebro-pedal connective. At the same time, a small number of bipolar cells projecting with 7-8 μm long apical dendrites to the surface, and sending distal projections to axon bundles, were found to exhibit NADPH-d reactivity in the mantle and foot. The epithelium of the mouth, pharynx and esophagus became NADPH-d reactive before hatching (E90%-E100%).

After hatching, the connectives and commissures connecting the ganglia of the CNS were found to NADPH-d reactive and NOS-IR. The NADPH-d reactive and NOS-IR axon bundles terminated mainly in the neuropil of the cerebral, pedal and buccal ganglia. Untill the P3 juvenile stage, the number of NADPH-d reactive and NOS-IR sensory cells in the mantle and the epithelium of the rostral part of the disgestive tract increased. From the P3 juvenile stage, the number of NADPH-d reactive and NOS-IR perikarya steadily increased in all ganglia of the CNS, and this was most prominently visible in the anterior lobe of the cerebral (adult cerebral ganglion: 52±8 NOS-IR cells), latero-caudal part of the pedal (adult pedal ganglion: 70±9 NOS-IR cells), and the medial and inner cell groups of the buccal ganglia (adult buccal ganglion: 77±10 NOS-IR cells). Cells showing transient NADPH-d reactivity and NOS immunoreactivity in the medial surface of the pleural, parietal, and visceral ganglia could also be observed at P3-P4 juvenile stages. In the epithelium of the mantle edge and the tentacles, exocrine gland cells became NADPH-d reactive and NOS-IR from P3 stage.

**4.3. Relationship between the localization of *Lym*-NOS mRNA and NOS-IR elements**

Beside the central and peripheral NADPH-d reactive and NOS-IR elements, *Lym*-NOS mRNA positive structures failed to detect during embryonic development. Although the majority of the *Lym*-NOS mRNA positive cell groups also exhibited NOS immunoreactivity in the CNS at juvenile stages, the following spatial, temporal, and quantitative differences were found between the two labelings. i) The first *Lym*-NOS mRNA positive cell in the pedal ganglia (at P1 juvenile stage) became NOS-IR only at P4 stage. ii) There were no significant differences between the number of *Lym*-NOS mRNA positive neurons and NOS-IR cells until reaching stages P3-P4, however, thereafter the number of *Lym*-NOS mRNA containing cells exceeded that of the NOS-IR ones. iii) NOS-IR neurons without *Lym*-NOS mRNA expression could be found in the buccal ganglia at stage P4, and in the pedal ganglia at stages P3 and P5. iv) The CGC contained only *Lym*-NOS mRNA and did not display NOS immunoreactivity. NADPH-d reactive and NOS-IR materials colocalized in axons of the nerves running to the CNS, the nerve trunks connecting the ganglia and the neuropils. NADPH-d reactive and NOS-IR signals were detected in the same cell groups of the CNS, as well as epithelium and gland cells at the periphery.

**4.4. Distribution of NO-induced cGMP accumulation**

The ciliary epithelium of the body surface exhibited cGMP immunoreactivity first at the onset of the metamorphosis, E45% embryonic stage. From the E55% embryonic (metamorphic) stage, NO-sensitive cGMP accumulation could be observed in a number of the epithelial cells of the mantle edge, and also in the epithelium of the tentacles and mouth. By the end of metamorphosis (E65%), the larval kidney with their tubes, the epithelium of the pharynx and a number of unidentified cells surrounding the developing ganglia displayed cGMP immunoreactivity. In a number of these cells, cGMP immunoreactivity was localized in the nucleus and along the cell membrane, but no staining could be observed in their cytoplasm. The number and intensity of cGMP immunolabeling of these extraganglionic cells increased until the 85% embryonic stage, then the staining rapidly declined, and became invisible after hatching. From stage E75%, longitudinal and circular smooth muscle cells located in the body wall displayed cGMP immunoreactivity. At the same time, a densely arranged, dot-like cGMP-IR material appeared in the neuropil of the central ganglia, which could be detected until the P2 juvenile stage. From E85% embryonic stage, the epithelium of parts of the digestive tract (pharynx, gizzard), and putative gland cells in the foot became cGMP-IR.

From P3 juvenile stage, tracts in the lip and pedal nerves, an axon bundle interconnecting the different ganglia of the CNS, and spot-like materials in the neuropils exhibited cGMP immunoreactivity. cGMP-IR perikarya appeared also at P3 juvenile stage in almost every ganglia of the CNS. Immunostaining was characterized by small dense dots in the cytoplasm near to the nuclear membrane. At later stages (from P4 to adult), the number of cGMP-IR cells significantly increased in all ganglia (most extensively in the cerebral, pedal, buccal and parietal ganglia), and at the same time, their immunolabeling became homogenous (adult cerebral ganglion: 157±25 cells, pedal ganglion: 131±15 cells, buccal ganglion: 88±5 cells, parietal ganglion: 58±8 cells). Some of the perikarya in the pedal, visceral, right parietal ganglia showed transient cGMP immunoreactivity at P3 juvenile stage.

### 4.5. Relationship between the localization of NOS-IR and NADPH-d reactive elements with cGMP-IR structures

Dot-like cGMP-IR material appeared in the ganglion neuropils at the late, E85% stage of embryonic development, when NOS immunoreactivity and NADPH-d reactivity were detected in the axon bundle running around in the ganglionic ring. In juveniles older than P3 stage, the same structures showed both NOS and cGMP immunoreactivity, and also NADPH-d reactivity in the axon bundles and their arborizations in the neuropils. During the embryonic development, the NADPH-d reactivity and cGMP immunoreactivity were also found in the larval kidney, ciliar epithelium located up to the mouth, and in the unknown type of cells around the ganglia, where, however, NOS-IR elements could not be detected. From late embryonic stages, in the epithelium of the anterior part (pharynx) of the digestive tract, cGMP immunolabeling was found to colocalize with both NOS immunoreactivity and NADPH-d activity. In general, the number of cGMP-IR perikarya was higher than that of the NOS-IR ones in the CNS at all juvenile stages. The spatial and temporal distribution of NOS-IR cell groups showed a good correlation with that of the cGMP immunoreactivity in all lobes of the cerebral ganglia, as well as in the buccal and visceral ganglia, but significant differences could be observed in the rest of the subesophageal ganglia. Colocalization of NOS-IR and cGMP-IR materials in perikarya was mainly found in the buccal and cerebral ganglia.

### 4.6. Effects of the NO level change on the structure of the developing embryo

Applying toluidine-blue staining, a secretory material could be visualized in the epithelial cells of the mantle edge and foot. Inhibition of NOS by $10^{-4}$ M L-NOARG resulted in a threefold increase in the mucus content of these epithelial secretory cells, whereas the application of $10^{-4}$ M SNP diminished the amount of secretory material. Neither NO donors, nor NOS inhibitors influenced the structural development of the CNS at the light microscopic level. According to the electron microscopical observations, the continuous administration of $10^{-4}$ M NO donors (SNP, SNAP) decreased the number of mithocondria and increased the number of lysosomes. Some of the axon profiles and neuropils displayed an increased number of myelin-like membrane configurations. Application of $10^{-4}$ M NOS inhibitors (L-NOARG, L-NAME) resulted in the increase number of lysosomes and accumulation of lipid droplets in some perikarya. In a part of the axon profiles of the neuropil, large pleiomorph vesicular elements and myelin-like configurations were observed.

### 4.7. Effects of the NO on the physiological and behavioral characteristics of the embryonic develoment

No effect of any of the substances applied could be observed under $10^{-5}$ M concentration, however, at higher concentrations, both NO donors (SNP, SNAP) decreased the survival of the embryos in a concentration dependent manner. The $LD_{50}$ score was $6x10^{-4}$ M.

Following treatments with NOS inhibitors between a concentration range of $10^{-4}$-$10^{-2}$ M from the E25-30% embryonic stage, the duration of the embryonic life was significantly prolonged. Application of $10^{-3}$ M L-NAME or L-NOARG prolonged the intracapsular embryonic life by about one week. Ceasing the treatment two days after the onset of metamorphosis (E70%), the prolonged hatching time was reduced, and with an inverse relation to the concentration of L-NOARG, tended to the control level. Embryonic development was diminished about 1.5 days using SNP, and 1 day using SNAP. Application of L-ARG under $10^{-3}$ M concentration failed to change the duration of hatching, however, higher doses of L-ARG increased the period of the development by some days. The simultaneous administration of $10^{-3}$ M - $10^{-4}$ M L-NOARG and L-ARG reduced the inhibitory effect of L-NOARG by 1-3 days. D-NAME decreased the time of development only at higher concentrations than $5x10^{-3}$ M; its effect proved to be negligible at lower concentrations.

NO donors slightly increased the locomotion (gliding) and the frequency of heartbeat, opposite to NOS inhibitors at $10^{-4}$ M concentration. The effect of D-NAME on both physiological properties was not significant. Application of L-NOARG together with L-ARG reduced the effect of L-NOARG, but did not abolish it completely. At the E90% embryonic stage, a new behavior appears, namely the radula movement connected to rasping which is the basic process of the feeding activity of the free-living animals. The number of radula protrusions decreased by 25-40% in the presence of the NOS inhibitors, whereas the application of NO donors strongly augmented the feeding activity by 75-100%. D-NAME, and the mixture of L-ARG - L-NOARG did not alter significantly the feeding activity.

## 5. DISCUSSION

### 5.1. Relevance of the NOS detecting techniques applied in this study

Although the different techniques used for localization of NO synthesis often resulted in similar findings, the differences observed, however, draw the attention to that these methods alone are not suitable to detect all NOS

isoforms, and/or can refer to the special, posttranscriptional control of NOS expression in *Lymnaea*. Nevertheless, the close connection observed in the spatial and temporal distribution of the NOS-IR and cGMP-IR materials provides evidence to the presence of the NO-cGMP system in the developing *Lymnaea*. At the moment, it appears that for a comprehensive description of the intracellular localization of NO in the *Lymnaea* nervous system different histochemical, immunohistochemical and *in situ* hybridization techniques need to be applied parallel and compared.

**5.2. Distribution of the NO-cGMP system and effect of NO on the embryonic development**

During the embryonic development of *Lymnaea* the NO-cGMP system appears at late stages in the CNS. At stage E85%, fibers of the interganglionic axon bundles originating from the peripheral sensory cells, and arborizing in neuropil areas become NADPH-d reactive and NOS-IR, when the neuropil displays cGMP immunoreactivity. In other invertebrate larvae, the NO synthesis of certain epithelial and neural cells is essential for the establishment of connections of these structures with the neighbouring GC-containing neural projections. Therefore, the role of the NO-cGMP system in the communication of developing neurons may also have a pivotal function in *Lymnaea*.

The number of mitochondria decrease, a large amount of lysosomes and myelin-like configurations appears in the neurons of the pedal ganglia after long-term NO exposure, suggesting the toxic effect of NO on the mitochondria. These ultrastructural changes can only be observed in a part of the neurons, which can be explained by the different sensitivity of neurons to NO. In the presence of NOS inhibitors, accumulation of lipid droplets, increased number of lysosomes are observed in the perikarya, whereas the occurance of pleomorphic synaptic vesicle-containing, swollen axon profiles are characteristic in the neuropil. According to the ultrastructural results, NO is seems to play a role in neuronal processes of *Lymnaea*, also observed in other snail species, such as axon growth and regulation of transmitter release.

Opposite to NO donors, NOS inhibitors result in a slower embryonic development, , that can be balanced by the simultaneous application of L-ARG and the interruption of the treatment at half time. NO is known to inhibit cell proliferation and accelerate differentiation in *Drosophila* embryos at low oxygen concentration. Hypoxic conditions may often occur during the embryonic development of *Lymnaea*, because these animals live in pond water and the egg clusters are bound to a given place until hatching. Hence it is possible that the change of the NO level during the development of *Lymnaea* embryos can alter the proliferation/differentiation processes in a similar way as in *Drosophila*. From the end of metamorphosis to hatching, *Lymnaea* embryos develop behaviors they need for the free-living juvenile and adult life. In this period, the main neuronal networks underlying behaviors will be organized both structurally and functionally. According to our findings, NO increases the locomotor activity, frequency of heartbeat and feeding activity. Since each physiological function is regulated by different networks in adult *Lymnaea*, NO may influence either each process separately or affect the central pattern generators regulating different motor activities. As we know from other studies, the NO-cGMP system contributes in transmitting the taste stimulus to the CNS, modifying the firing pattern of feeding motoneurons, and feeding based learning in adult *Lymnaea*. At the same time (E75-80%) when NOS-IR and cGMP-IR materials are detected in the CNS, the acquisition of conditioned taste aversion behavior is also established.

NO donors decrease, whereas NOS inhibitors increase the number of secretion granules in the epithelium of the mantle and foot containing NOS-IR, NADPH-d reactive gland cells, as well as NO-sensitive cGMP-IR cells. Therefore, the NO-cGMP system possibly regulates mucus secretion and release in *Lymnaea*.

By the end of the gangliogenesis, from E80% stage to hatching, unidentified cells around the ganglia exhibit NADPH-d reactivity and cGMP immunoreactivity. Others found that glial elements accumulated around the ganglia at the same time. Our investigations on *Helix* revealed that NO evoked cGMP production in glia cells located around intestinal neurons. The cells surrounding the ganglia showing transient NADPH-d reactivity and cGMP immunoreactivity, could be early glial cells, later entering the CNS. The NO-cGMP system may play some role in the movement of these putative glial cells as it was described in other studies.

**5.2. Distribution and possible function of the NO-cGMP system in the postembryonic development**

In the CNS of the young free-living larva (P1), the appearance of the *Lym*-NOS mRNA signal is first detected, then, at P3, NOS-IR, NADPH-d reactive and cGMP-IR perikarya are observed. The number of *Lym*-NOS mRNA positive, NOS-IR, NADPH-d reactive and cGMP-IR perikarya continuously increase from P3 stage to the adulthood. Transient NOS and cGMP immunoreactivities are found in some cells of the parietal and visceral ganglia at P3. Antisense regulation occurs in *Lym*-NOS mRNA containing neurons, being probably responsible for the low number of NOS-IR related to *Lym*-NOS mRNA positive cells, the late manifestation of NOS enzyme (P4) and the transient NOS and cGMP immunoreactivity during the postembryonic development of the CNS. P3 is an important developing stage of *Lymnaea*, because the volume of the ganglion increases due to the enhanced protein synthesis, and the long-term memory formation could be detected from this age. The presence of NO is essential in the memory storage in the adult *Helix* and *Lymnaea*, therefore, the appearance of the NO-cGMP system in the neural perikarya of the developing CNS may be connected to the formation of long term-memory.

In general, the number of cGMP-IR perikarya exceeds the number of NOS-IR cells in the developing CNS. NOS and cGMP immunoreactivities can often be found in the same perikarya or cell groups in the cerebral and buccal ganglia, whereas sometimes they can be observed in different cells or cell groups in the subesophageal ganglia. Therefore, both the intra- and intercellular regulation of the cGMP synthesis by NO is suggested in nerve cells of the developing CNS of *Lymnaea*.

## 6. LIST OF ORIGINAL COMMUNICATIONS

### 6.1. Publications providing the basis of the thesis

4. **Serfőző Z**, Elekes K, Varga V
   NADPH-diaphorase activity in the nervous system of the embryonic and juvenile pond snail, *Lymnaea stagnalis* L.
   *Cell and Tissue Research* 292:579-586 (1998) IF: 2.492

5. **Serfőző Z**, Elekes K
   Nitric oxide level regulates the embryonic development of the pond snail *Lymnaea stagnalis* L.: pharmacological, behavioral, and ultrastructural studies.
   *Cell and Tissue Research* 310:119-130 (2002) IF: 2.492

6. **Serfőző Z**, Veréb Z, Rőszer T, Kemenes Gy, Elekes K
   Development of the nitric oxide/cGMP system in the embryonic and juvenile pond snail, *Lymnaea stagnalis* L. A comparative *in situ* hybridization, histochemical and immunohistochemical study.
   *Journal of Neurocytology* 31 (in press) (2002) IF: 1.776

### 6.2. Oral and poster presentations connected to the thesis

1. **Serfőző Z**, Elekes K, Varga V
   Localization of NADPH-diaphorase activity during the embriogenesis of the pond snail, *Lymnaea stagnalis.* IV. Conference of the Hungarian Neuroscience Society, Gödöllő, 1997, abstr. no. 67. *Neurobiology* 5:208-209

2. **Serfőző Z**, Elekes K, Varga V
   Embryogenesis of the nitric oxiderg system in *Lymnaea stagnalis*: Histochemical and pharmacological studies.
   2nd Forum of the European Neuroscience, Berlin, 1998, abstr. no. 15.04. *Suppl. European Journal of Neuroscience* 10:27

4. **Serfőző Z**, Serfőző J, Varga V, Elekes K
   Effects of nitric oxide (NO) on the development of the nervous system of the pond snail, *Lymnaea stagnalis.* VI. Conference of the Hungarian Neuroscience Society, Pécs-Harkány, 1999, abstr. no. 36. *Neurobiology* 7:380

4. **Serfőző Z**, Elekes K

Development of putative NOergic neurones and their possible role in early behaviours and physiological processes of *Lymnaea stagnalis*, L.
9th Symposium on Invertebrate Neurobiology, Tihany, 1999.

5. **Serfőző Z**, Rőszer T, Kemenes Gy, Elekes K
Distribution of nitric oxide synthase mRNA in the CNS of the developing snail, *Lymnaea stagnalis* L.
IBRO-MITT International Workshop on "Signaling mechanisms in the central nervous system". Debrecen, 2002, abstr. no. . *Neurobiology* 10:.

6. **Serfőző Z**, Rőszer T, Kemenes Gy, Elekes K
Development of the NO-cGMP system in the embryonic and juvenile pond snail, *Lymnaea stagnalis* L.
3rd Forum of the European Neuroscience, Paris, 2002, abstr. no. 177.10. *Suppl. European Journal of Neuroscience* 12.

**6.3. Other publications**

1. Janáky R, Varga V, Hermann A, **Serfőző Z,** Dohovics R, Saransaari P, Oja SS
Effect of glutathione on [3H]dopamine release from the mouse striatum evoked by glutamate receptor agonists
In: *Neurochemistry* (Eds.): A. Teelken és J. Korf, Plenum Press, New York, 733-736. (1997)

6. Rőszer T, **Serfőző Z**, Elekes K
Neurochemical characterization of the enteric nervous system in some freshwater and terrestrial snails: Nitrergic and peptidergic networks
*Acta Biologica Debrecina* 23. 71-73. (2001)

7. Bánfalvi G, Szepessy E, Jenei Zs, **Serfőző Z**, Csuka I, James J
Multiple subphases of DNA repair and poly(ADP-ribose) synthesis in Chinese hamster ovary (CHO-K1) cells.
*European Journal of Cell Biology* (2003) (IF: 2.244)

8. **Serfőző Z**, Rőszer T, Serfőző J, Tóth-Jakab Á, Palatka K, Elekes K
Organization of NADPH-diaphorase/nitric oxide synthase containing neurons in the digestive tract of the snail, *Helix pomatia*, L. A light-, and electronmicroscopic study
*Journal of Comparative Neurology* (submitted for publication, under improvement) (2003) (IF: 3.515)

9. **Serfőző Z**, Rőszer T, Szentmiklósi J, de Vente J, Elekes K
Characterization of NADPH-diaphorase/nitric oxide synthase containing neurons in the digestive tract of the snail, *Helix pomatia*, L. An immunohistochemical and pharmacological study
*Journal of Experimental Zoology* (submitted for publication, under improvement) (2003) (IF: 1.488)