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Organization and possible role of peripheral senso-efferent systems in the regulation of the behavior of aquatic mollusks *(Dreissena polymorpha, Lymnaea stagnalis)*

PhD thesis

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1. INTRODUCTION

For the coordinated operation of aquatic ecosystems, the continuous monitoring and exchange of chemical signals (communication) within a population and also among different species is inevitable (Weissburg et al. 2002, Weissburg 2010, Sata et al. 2019). Since the primary sensory modality of aquatic mollusks is the chemosensation, the proper development, organization and relationships of chemosensory structures are required to capture and process chemical information, and to elaborate suitable responses (various forms of behaviors) to external stimuli (e. g. foraging, reproduction, escaping). In bivalves, anatomical regions specialized chemical sensing are concentrated around the lip and mantle, while in gastropods, peripheral sensory neurons were identified in the lip, tentacles and foot (Zylstra 1972, Roubos and Van der Wal Divendal 1982, Dorsett 1986, Chase 2002, Wyeth and Croll 2011, Wanninger 2016). Although several sensory structures and partly their neurotransmitter content have been described in different peripheral areas of developing and adult mollusks (Voronezhskaya et al. 1999, Croll et al. 1999, Hegedűs et al. 2004, Croll 2006, Hatakeyama et al. 2007, Wanninger 2016, Battonyai et al. 2018), their functional-anatomical relationships with central efferents have not yet been revealed.

During molluscan ontogenesis, the first sensory cells appear at trochophore stage, which, in addition to the regulation of neurogenic processes, can play a key role in the analysis of environmental stimuli, the adaptation of larvae to changing conditions, and so ultimately in the survival (Kempf et al. 1997, Page 2002, Braubach et al. 2006, Voronezhskaya et al. 2008). Despite its complex function, there is little information on the formation and organization of the larval sensory and associated efferent systems in bivalves.

Investigation on chemosensation can provide a firm basis for understanding certain behaviors, elaboration of adequate responses and decision-making processes (e. g. settlement, defense), thereby exploring the chemical, ecological and neurobiological background of interactions

between both species and individuals. The identification of signaling molecules mediating chemosensory processes can be an important first step in revealing the causal relationships between environmental stimuli and ecological responses at individual, population and community levels.

2. AIMS

Based on the above, we investigated the chemical-neuroanatomical and functional morphological organization of sensory-efferent systems and their possible role in changing environmental conditions in two model animals, the great pond snail (*Lymnaea stagnalis*, Gastropoda) and the zebra mussel (*Dreissena polymorpha*, Bivalvia).

The aims of the present work were the following.

1. Chemical-neuroanatomical characterization of sensory systems, examination of the presence and spatial distribution of signaling molecules (serotonin [5-HT], dopamine [DA], histamine [HA], glutamate [Glu], acetylcholine [ACh], FMRFamid [Fa], Mytilus inhibitory peptide [MIP]) in various peripheral areas (head region, foot) of *Lymnaea stagnalis*.

2. To reveal the possible functional morphological relationships between neurochemically identified peripheral sensory structures and central efferents in *Lymnaea*.

3. Analysis of the effect of a plant infochemical (tannic acid) on the physiological function of the senso-efferent system of the head region of *Lymnaea* both at cellular and network-level, as well as on the development of different forms of behavior (feeding, locomotion).

4. Identification of neuronal elements characterizing the early development of the nervous system in *Dreissena polymorpha* larvae with special emphasis on sensory elements and their transmitter content.

5. Exploration of the functional-organizational principles of signal molecule systems and their possible role in the development of larval behaviors of *Dreissena*.

6. Chemical-neuroanatomical and ultrastructural characterization of the byssus-foot complex innervation determining the adhesion of *Dreissena*.

3. MATERIALS AND METHODS

3.1. Animals

Adult great pond snails (*Lymnaea stagnalis*) were collected from the catchment area of the Kis-Balaton and other inflows, while zebra mussels (*Dreissena polymorpha*) were obtained from Lake Balaton from late May to the end of June. The latter were propagated as described by Battonyai et al. (2018). Juvenile *Lymnaea* individuals were derived from the institutional culture. The experiments were performed according to the protocol approved by the Animal Experimental Committee of the Balaton Limnological Research Institute (VE-I-001/0189010/2013).

3.2. Methods

3.2.1. Histochemistry (hematoxylin-eosin staining)

Foot (containing the byssus retractor muscles [BRM] and the pedal ganglia) of *Dreissena polymorpha* was fixed in 4% paraformaldehyde (PFA), then 16 μm thick cryostat sections were made. The sections were stained first with 1% hematoxylin dissolved in distilled water according to the Mayer modification then stained by 10% eosin diluted in tap water (Kiszely and Barka 1965). Samples were dehydrated in graded ethanol and xylene, and finally covered with Canadian balm.

3.2.2. Immunohistochemistry

The processed *Lymnaea* tissues were fixed in 4% PFA or, in the case of anti-HA immunohistochemistry, first in a mixture of 4% 1-ethyl-3-(3-

dimethylaminopropyl) carbodiimide and 4% N-hydroxysuccinimide, followed by 2% PFA. A two-step indirect immunofluorescence technique was used on 16 μm cryostat sections. In case of *Dreissena* larvae, whole mount preparations were used. The following monoclonal (anti-5-HT, anti-α-actinin, anti-acetilated α-tubulin) and polyclonal primary antibodies (anti-TH, anti-HA, anti-Glu, anti-5-HT, anti-Fa, anti-MIP, anti-ChAT) were used in 1:500-1:3000 dilutions, while the applied secondary antibodies (anti-mouse IgG-FITC and -TRITC, anti-rabbit IgG-FITC and -TRITC, AlexaFluor488, AlexaFluor 633) were diluted to 1:200 and 1:1000, respectively. Cell nuclei were visualized by using 0.1 mg/ml 4 ', 6-diamidino-2-phenylindole (DAPI).

Samples were examined with an epifluorescence microscope (Keyence BZ-9000) or a laser scanning confocal microscope (Leica TCS SP8). We used Fiji software (Schindelin et al. 2012) for fluorescence intensity measurements and analysis, and GraphPad Prism (GraphPad Software) for statistical analysis and making graphs. The results of the control and experimental groups were compared using a non-parametric Mann-Whitney U test.

3.2.3. Electron microscopy

Fixation of the lip of *Lymnaea* and the foot-BRM pedal ganglion complex of *Dreissena* was performed in a mixture of 4% PFA and 0.1% glutaraldehyde. Tissues were post-fixed with 1% osmium tetroxide, dehydrated graded ethanol and propylene oxide, and then finally embedded in Araldite. After light microscopic examination of semi-thin sections stained with 1% toluidine blue, ultrathin sections were made from the selected area(s) and examined with a JEOL 1200EX transmission electron microscope (TEM).

In case of *Dreissena* foot immunocytochemistry, polyclonal rabbit anti-Fa primary antibody (Immunostar, 1: 1000) and goat anti-rabbit HRP-conjugated IgG (Dako, 1: 3) were used for labeling. The immunolabeling was visualized with a mixture of 0.05% 3,3 'diaminobenzidine and 0.01%

hydrogen peroxide. Following post-fixation with 0.5% osmium tetroxide, the samples were processed as described above.

3.2.4. HPLC-MS

Tissues from different peripheral areas (lip, tentacle, foot) and the CNS of *Lymnaea* were homogenized in acetonitrile (0.1 /l / mg) containing 0.1% formic acid and 0.01% dithioreitol to determine the local concentration of various signaling molecules. The samples were concentrated by sonication and centrifugation with a SpeedVac. A complex Ultimate 3000 (Dionex) micro HPLC system and a Qexactive UHR mass spectrometer (Thermo Fisher Scientific) were used for LC-MS analysis. Gradient elutions (Maász et al. 2017) were used during the separation. Mass detection was performed in positive ionic mode. Signal molecules were identified from tissue homogenates based on their molecular weight and fragments.

3.2.5. Behavior tests

3.2.5.a, b Investigation of the effect of tannic acid on the locomotion and feeding of *Lymnaea*

In order to study the locomotor activity of *Lymnaea stagnalis*, the snails were individually placed in glass cuvettes containing 10 or 100 μM TA solution. The control group was kept in filtered Balaton water. After each observation period (60 min), the time intervals spent in the active (sliding, swimming) and passive (floating, sticking) periods were summed, then the times spent in different states of locomotion were referred to 60 min in percentage in case of each animal, and averaged for each group.

To characterize the feeding behavior, stimulus generated feeding activity was induced in the individuals with sugar solution (Kemenes et al. 1986), which can be characterized by latency/delay (time range from stimulus application to the beginning of action/feeding) and feeding rate

(number of mouth opening / closing cycles). The snails were placed in Petri dishes and their feeding response was tested in 100 mM sugar, 10 μM tannic acid, and 100 μM tannic acid solution, respectively.

3.2.5.c, d Effect of increased salinity and pharmacological manipulation on serotonin metabolism on the swimming activity of *Dreissena* larvae

The upper part of the vertical two-chamber experimental set was filled with filtered Balaton water, while the lower chamber contained the incubation solution and the 48-h veligera *Dreissena* larvae (approximately 200-300 animals/ml). Following half an hour, the distribution of larvae between lower and upper chamber (vertical swimming activity) was examined. To test the effect of the 5-HT synthesis inhibitor para-chlorophenylalanine (pCPA) and the 5-HT synthesis precursor 5-hydroxytryptophan (5-HTP), the larvae were incubated with 10^{-5} mol/l pCPA and 10^{-5} mol/l 5-HTP diluted in filtered Balaton water, followed by swimming tests and finally qualitative and quantitative IHC assay. For testing the salt tolerance of the larvae, the experiments were performed under increased salinity (+2 ‰).

3.2.6. Electrophysiological analysis of the effect of tannic acid in *Lymnaea*

The effect of TA was studied using a simplified feeding model, a semi-intact preparation that included peripheral nerves connecting the CNS and head region including the lips of the snail (Kemenes et al. 1997, Staras et al. 1999a, b). By this arrangement, the effect of TA could be tested both at (feeding neuronal) network and at behavioral (feeding activity) level. As stimulus, 10 mM sugar solution was dropped on the lips, followed by the application of 10 or 100 μM TA (Magoski et al. 1995).

The direct (cellular) effect of TA on feeding neurons was examined by recording intracellular activity from identified neurons of the isolated CNS. The frequency of fictive feeding (F) was calculated by time intervals

(T) between the rhythmic synaptic inputs of buccal feeding motoneurons (F = [1 / T] * 60 [cycles / min]).

4. RESULTS

4.1. Chemical-neuroanatomical organization of peripheral senso-efferent systems in *Lymnaea*

Using fluorescence immunohistochemistry, the presence of 5-HT-IR, TH-IR, HA-IR, Glu-IR, ChAT-IR, Fa-IR, and MIP-IR neurons were demonstrated in the sensory region of the lip, tentacle and foot, moreover, further IR-nerve elements were found in their deeper subepithelial layer, too. The localization and density of their distribution showed partially different organization pattern. The 5-HT immunoreactivity was limited to the efferent axons, while in the case of other labelings, bipolar neurons, afferent fiber bundles and networks were identified. The 5-HTergic efferents ran near the axons, dendrites, or soma of the labeled sensory neurons and, in the deeper layers, along afferent bundles. Our results suggest the presence of complex local systems of sensory and efferent elements in the studied peripheral areas. The concentration of low molecular weight signal molecules detected by HPLC correlated well with the density of labeled nerve elements localized in a given area.

4.2. The effect of tannic acid on the function of the senso-efferent systems at cellular, network and behavioral level in *Lymnaea*

Tannic acid affected the locomotion and feeding of *Lymnaea* in a concentration-dependent way. At lower concentrations (10 μM) of tannic acid exposure, the locomotor activity of the animals increased, whereas at higher concentrations (100 μM), TA induced inhibition, the snails became passive. At lower concentrations, the sugar-induced feeding frequency increased significantly, while at higher concentrations, it decreased significantly, however the latency time also enhanced significantly compared to the control group. Following the application of 100 μM

tannic acid on the lips, a feeding response to sugar could not be elicited from central neurons in semi-intact preparation. TA seems to affect both afferent and efferent peripheral functions; the feeding activity is influenced primarily through the feeding associated sensory pathways, the locomotion, in turn, is affected via both sensory pathways and the inhibition of the ciliary movement of the foot.

4.3. Chemical signal systems in the trochophora and veligera larvae of zebra mussel (*Dreissena polymorpha*)

In the first 96 hours after fertilization, three anatomically well-distinguished 5-HT-IR and Fa-IR centers, the apical, posterior, gastric ones, appeared containing sensory cells, which formed the basis of the larval sensory systems of *Dreissena*. The two signal molecule systems showed a similar pattern of development and organization, however, their temporal appearance and organization of their intercellular connections were partially different. We demonstrated that altered external (increased salinity) and internal (pharmacologically modified 5-HT synthesis) conditions can influence the swimming activity of the larvae, accompanied by a significant change in the fluorescence intensity of the 5-HT-IR and Fa-IR elements.

4.4. Organization of the byssus retractor system of *Dreissena polymorpha*

We described several neurochemical features of the innervation pattern of byssus-foot complex responsible for the adhesion of *Dreissena polymorpha*. The innervation of the BRM is dominated by the extensive presence of 5-HT-IR neurons modulating muscle relaxation and Fa-IR structures acting as a ‘catch’ switch. In addition, ChAT-IR innervation was also observed but only at the origin of the byssus filaments. Several neural and glandular elements displayed MIP immunoreactivity in the pedal ganglia. The organization of the entire byssus system was also explored in detail. At ultrastructural level, the presence of non-specialized neuromuscular junctions and different vesicle and granule content of the

innervating axon profiles were demonstrated, confirming the diverse neurochemical profile of the innervation in the byssus retractor system.

5. SUMMARY

In our present study, we have revealed the formation and chemical-neuroanatomical organization of neurotransmitter systems involved in sensory processes in two different molluscan model species, the great pond snail (*Lymnaea stagnalis*; Gastropoda) and the zebra mussel (*Dreissena polymorpha*; Bivalvia). We analyzed the role of senso-efferent systems in different behaviors by changing pharmacological and chemical environmental parameters. Our results may contribute to a better understanding of the background mechanisms required for positive selection in the early stages of individual development, as well as the organization of peripheral senso-efferent networks, which may play a key role in developing a rapid adaptive capability of invertebrate species.

Our main findings are the following.

1. Peripheral sensory neurons containing six different neurotransmitters (ACh, HA, TH, Glu, Fa, MIP) were immunohistochemically distinguished in the head region and the foot of *Lymnaea stagnalis*.

2. A complex system relations of neurochemically different peripheral sensory structures and central 5-HTergic efferents was explored. Various forms of connections can occur both at the level of sensory neurons, as well as at other intercellular levels. These local networks can play a significant role in the local (peripheral) execution of rapid and adequate responses to various sensory stimuli.

3. In *Lymnaea*, we demonstrated a concentration-dependent effect of a plant infochemical (tannic acid) on feeding and locomotion. By electrophysiological experiments it was demonstrated that the application of TA on the lips inhibited the fictive feeding activity.

4. The temporal appearance, morphological characteristics, interneuronal and intercellular contact systems of 5-HT-IR and FMRFamid-IR sensory and other neural structures were described in *Dreissena polymorpha* larvae.

5. The role of the 5-HTergic and Faergic systems in the swimming activity of *Dreissena* larvae was demonstrated by the pharmacological manipulation of the 5-HT synthesis, and by changing the salinity of the aquatic environment. The larvae responded to these chemical stimuli with altered swimming activity and changed fluorescence intensity of sensory and other 5-HT-IR and FMRFamid-IR neurons that correlated well with the altered behavior. Our results may contribute to the knowledge regarding the responses of developing aquatic mollusks, in this case the zebra mussel, to external stimuli, which may play a role in selecting the appropriate site of settlement.

6. We described the chemical-neuroanatomical organization and ultrastructure of the byssus retraction muscle system responsible for the adhesion of *Dreissena*. It is assumed that 5-HT as a muscle relaxant and FMRFamid as a ‘catch’ switch may play a dual modulating role at the level of neuromuscular contacts.

6. LIST OF PUBLICATIONS AND PRESENTATIONS

6. 1. Publications

Horváth Réka, Battonyai Izabella, Maász Gábor, Schmidt János, Fekete Zsuzsanna N, Elekes Károly (2020): Chemical-neuroanatomical organization of peripheral sensory-efferent systems in the pond snail *(Lymnaea stagnalis)*, BRAIN STRUCTURE & FUNCTION 225:2563-2575. **D1; IF: 3.298**

Vehovszky Ágnes, **Horváth Réka**, Farkas Anna, Győri János, Elekes Károly (2019): The allelochemical tannic acid affects the locomotion and feeding behaviour of the pond snail, *Lymnaea stagnalis*, by inhibiting peripheral pathways, INVERTEBRATE NEUROSCIENCE 19:10. **Q4; IF: 0.556**

Battonyai Izabella, Voronezhskaya Elena E, Obukhova Alexandra, **Horváth Réka**, Nezlin Leonid P, Elekes Károly (2018): Neuronal development in the larvae of the invasive biofouler *Dreissena polymorpha* (Mollusca: Bivalvia), with special attention to sensory elements and swimming behavior, BIOLOGICAL BULLETIN 234:192-206. **Q1; IF: 1.537**

Horváth Réka, Battonyai Izabella, Elekes Károly (2021): Innervation of the byssus retractor system of the biofouling mussel, *Dreissena polymorpha*. An immunohistochemical and ultrastructural study (in prep)

6.2. Oral presentations and posters

Oral presentations

Horváth Réka: Peripheral modulation of locomotion by tannic acid in the pond snail *Lymnaea stagnalis.* 1st Symposium on Invertebrate Neuroscience, 13-17 August 2019.

Posters

Battonyai I., **Horváth R.**, N. Fekete Zs., Elekes K.: Aminergic (5-HT) and peptidergic (FMRFamide) innervation of the foot and byssus retractor muscle of *Dreissena*. ISIN Symposium, 2015. 08. 26-31., Tihany

Horváth R., Battonyai I., N. Fekete Zs., Elekes K.: Signal molecules in the senso-efferent system of the pond snail. IBRO Workshop, 2016. 01. 21-22., Budapest

Battonyai I., **Horváth R.**, Elekes K.: Signal molecules involved in the innervation of the byssus retractor muscle and foot of the zebra mussel. IBRO 2016 Workshop, 2016. 01. 21-22., Budapest

Horváth R., Battonyai I., Elekes K.: Organization of senso-efferent systems in the pond snail (*Lymnaea stagnalis*). XI. East European Conference of the ISIN, 2016. 05. 15-19., Moszkva-Zvenigorod, Oroszország

Horváth R., Battonyai I., Elekes K.: Senso-motor interactions in different peripheral organs of the pond snail, *Lymnaea stagnalis*. A chemical-neuroanatomical approach. 4th International Congress on Invertebrate Morphology, 2017. 08. 18-23., Moszkva, Oroszország

Battonyai I., **Horváth R**., N. Fekete Zs., Elekes K.: Immunohistochemical visualization of neurotransmitters in the biofouling zebra mussel byssal system. 4th International Congress on Invertebrate Morphology, 2017. 08. 18-23., Moszkva, Oroszország

Horváth R., Battonyai I., Elekes K.: Morphological basis of possible senso-motor interactions in the periphery of the pond snail, *Lymnaea stagnalis L*. FENS Regional Meeting, 2017. 09. 20-23., Pécs

Horváth R., Vehovszky Á., Farkas A., Győri J., Elekes K.: Peripheral modulation of locomotion and feeding by tannic acid in the pond snail, *Lymnaea stagnalis.* 1st Symposium on Invertebrate Neuroscience, 2019. 08. 13-17., Tihany

Vehovszky Á., **Horváth R.**, Elekes K.: Tannic acid, an allelochemical, inhibits peripheral pathways regulating the locomotion and feeding of the pond snail, *Lymnaea stagnalis*. EBPS Biennial Meeting, 2019. 08. 28-31., Braga, Portugália

6.3. Other publications

Fodor István, Zrinyi Zita, **Horváth Réka**, Urbán Péter, Herczeg Róbert, Büki Gergely, Koene Joris M., Tsai Pei-San, Pirger Zsolt (2020): Identification, presence, and possible multifunctional regulatory role of invertebrate gonadotropin-releasing hormone/corazonin molecule in the great pond snail *(Lymnaea stagnalis)*, GENERAL AND COMPARATIVE ENDOCRINOLOGY 299: 113621. **Q1; IF: 2.426**

Somogyvári Dávid, Vehovszky Ágnes, Farkas Anna, **Horváth Réka**, Győri János (2020): Multi-marker approach for the evaluation of environmental impacts of APACS 50WG on aquatic ecosystems, INVERTEBRATE NEUROSCIENCE 20:23. **Q4;**

Elekes Károly, Hiripi László, Balog Gábor, Maász Gábor, Battonyai Izabella, Khabarova Marina Yu, **Horváth Réka**, Voronezhskaya Elena E. (2018): Serotonergic regulation of the buccal (feeding) rhythm of the pond snail, *Lymnaea stagnalis.* An immunocytochemical, biochemical and pharmacological approach, ACTA BIOLOGICA HUNGARICA (1983-2018) 69:225-243. **Q3; IF: 0.707**