

**Physiological and pharmacological properties of superior
tentacular muscles participating in olfactory orientation of the
land snail, *Helix pomatia***

PhD Thesis

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1. INTRODUCTION

Chemical perception (olfaction and sense of taste) is the most ancient sensory modality (Thar & Köhl, 2003). In order to enhance the efficacy of olfaction, animals perform special movements with their olfactory organs (Schmitt & Ache, 1979; Peteraitis, 1999). These movements promote localization of the odor source and improve the accessibility of the “new” odor molecules to the olfactory receptors by attenuating the fluid film, thus removing the “old” odor molecules (Moore *et al.*, 1991).

The main olfactory organ of terrestrial snails is located at the tip of the superior tentacles and structurally it has several similarities to the vertebrate olfactory organ. During adaptation to the environment, terrestrial snails developed various tentacle movements. For example in case of danger they retract the whole tentacle into the body cavity, which is the fastest of all of the snail’s motor behavior (Chase, 1986). Pointing the tentacles downward - called bending - can be observed, when conditioned snails move one, and then both tentacles in the direction of the odor source (Peschel *et al.*, 1996). Similarly to other animals, in order to enhance olfactory performance, fine movements of the snail tentacle can occur, like twitching and quivering described by Lemaire and Chase. A twitch is a brief retraction of the tentacle tip. A quiver is - similarly to the vibrations of the antennules in crustacea - a rapid lateral movement unaccompanied by retraction (Lemaire & Chase, 1998). Previously it was assumed that the tentacle retractor muscle (TRM) is responsible for the retraction of the tentacle into the body cavity, while bending is performed by the tegumental muscle (TM) located in the stem of tentacle. Anterograde cobalt-lysine tracing revealed that the external peritentacular nerve (ePTn) and the internal peritentacular nerve (iPTn) innervate different parts of the TM, which allows the dislocation of the tentacles into different directions, without tentacle retraction (Peschel *et al.*, 1996). In our opinion TM alone is not able to perform the movements of tentacle completely protruded by the hydrostatic pressure of the haemolymph around the longitudinal axis. Therefore the tentacle movements described above cannot be explained solely by the TRM and TM contractions.

Recently three, string-like muscles were described, located to the superior tentacles. They span along the whole tentacle from the ventral surface of the olfactory epithelia to the base of tentacle. The muscles were named as flexor muscles (FM1, FM2 and FM3) (Hernádi & Teyke, 2012). Based on their location it is suggested that during olfactory orientation these muscles participate in the execution of twitching, quivering and bending.

2. AIMS

Based on the facts outlined above, the fine movements of the superior tentacles of terrestrial snails cannot be explained solely by the TRM and TM contractions. It is assumed that the three, recently described muscles (FMs) participate in the execution of the fine tentacular movements. Until now the ultrastructure of the FMs, their innervation, the neurotransmitters and their receptors controlling muscle function were unknown. Therefore the aim of our work was an overall study of the FM, by which we wanted to have a better insight into the muscle ultrastructure, the neuromuscular contacts, the presence and possible physiological effects of the most common invertebrate neurotransmitters, and the pharmacology of neurotransmitter receptors with special attention to the nicotinic ACh receptors. In addition we intended to investigate the modulation of FM contractility by neurotransmitters (5-HT, DA) and neuropeptides (FMRFamide, PACAP).

The aims of our present work were the following:

1. To define the ultrastructural organization of the FMs providing their extreme elongation and to investigate the innervation of the muscles.
2. To characterize the contractile and pharmacological properties of the FMs; from the common invertebrate neurotransmitters to study the effect of ACh and Glu, which are excitatory transmitters in molluscan muscles.
3. To examine the distribution in the CNS and FM and to characterize the pharmacological properties of AChRs, formerly identified controversially in molluscan muscles.
4. To study the possible modulatory effect of 5-HT and DA and neuropeptides like PACAP on the FM contractility.

3. MATERIALS AND METHODS

3.1. Animals

Adult specimens of the land snail, *Helix pomatia* were used. Part of the animals were kept under laboratory conditions. Until experiments they were fed on lettuce, humidity and room temperature (RT) were provided for them.

3.2. Muscle contraction measurement

In every muscle contraction experiment the properties of FM3 were studied. Both isometric and isotonic measurements were made on innervated or denervated muscle

preparations. Innervated preparations consisted of the FM3, the innervating olfactory nerve and the CG. For electric stimulation of the innervated muscle, a pair of silver electrodes were placed under the olfactory nerve. Electrical pulses of 5–10 V for 10 ms were applied. In isometric experiments muscle contraction curves were registered and the integral and amplitude of the contractions were measured. In isotonic experiments, the change in muscle length - evoked by different solutions and/or electric nerve stimulation - was measured by an ocular micrometer.

3.3. Electronmicroscopy

The superior tentacles were pinned out and covered with a fixative containing a mixture of 4% paraformaldehyde (PFA) and 0,1 % glutaraldehyde. Preparations were washed thoroughly in PB and the FMs were cut off from the rest of the tentacle. Preparations were post-fixed for 1 h at 4°C in 1% OsO₄ diluted in 0,1 M Na-cacodylate buffer, dehydrated in graded ethanol and propylene oxide and embedded in Araldite (Durcupan ACM, Fluka). In the course of dehydration block, staining was performed in 70% ethanol saturated with uranylacetate. After polymerization, 1 µm semi-thin sections were cut, stained with 1% toluidine blue and used for orientation. For ultrastructural investigations, 50–60 nm ultra-thin sections were taken (LKB Nova), stained with lead citrate and viewed in a JEOL 1200EX electron microscope.

3.4. Retrograde and anterograde neurobiotin tracing

For retrograde tracing the end of the FMs which is near to the base of tentacle were cut off and placed in 5% neurobiotin. For anterograde tracing the olfactory nerve was dissected from the CG and placed in 5% neurobiotin. In both cases the preparations were incubated for one day at RT covered by *Helix* saline. The preparations were then fixed in 4% PFA diluted in 0,1 M PB for 6 h at 4°C. From the preparation used for retrograde tracing 30 µm cryostat sections were taken, for anterograde tracing FMs were used as whole-mount preparations. The neurobiotin-labeled structures were visualized by applying avidine-conjugated Alexa-fluor 488 (Molecular Probes, 1:1.000). Samples were mounted in PBS–glycerol (2:1) and viewed under a fluorescence microscope.

3.5. Immunohistochemistry

In our experiments we used two-step indirect (fluorescent dye or peroxidase-IgG) visualization. CNS and tentacles with the FMs were fixed in 4% PFA. CG were separated from the CNS, and the FMs were isolated from the tentacles. Fixed preparations were incubated in 20% sucrose for 1h at RT. For immunohistochemical procedure 40 µm cryostat sections made from the CG were transferred to slides covered by Cr-Al-gelatine. FMs were used as whole-mount preparations. Primary antibodies were diluted in PBS containing 0,25% BSA and 0,25% Triton-X. Preparations were incubated with the primary antibodies for 24h at 24 °C.

3.6. Western blot analysis

Samples from CNS, FM and columellar muscle (CM) were homogenized in SDS-containing lysis buffer. Homogenates were centrifuged and the supernatant was collected and diluted in sample buffer containing DTT and SDS at 1:1. Proteins of the samples were denatured with boiling at 95°C. They were separated on 8% gel by SDS-PAGE and thereafter blotted onto nitrocellulose or PVDF membrane. Membranes were blocked with 5% non-fat milk for 2h at RT. Primary antibodies were diluted in blocking solution and the membranes were incubated with them at 4°C overnight. Incubation with HRP-conjugated goat anti-rabbit secondary antibody (Sigma, 1:10.000) lasted for 1h at RT. Immunoreaction was visualized with WesternBright (Advansta) ECL substrate.

3.7. PCR

Total RNA was extracted using TRI Reagent from freshly homogenated CNS, CM and FM. Reverse transcription of the isolated total RNA was performed, and the resulting cDNA was subjected to PCR, using degenerate or non-degenerate primer pairs designed to detect nAChR subunits in *Lymnaea*. PCR reaction was performed in 41 cycles. One cycle consisted of the following steps: 95°C for 3 min, 95°C for 30 sec, 45°C for 1 min, 72°C for 1 min, 72°C for 10 min. Amplified products were run on 2% agarose gel, using ethidium bromide UV detection. For DNA sizing GeneRuler 100 bp DNA Ladder Plus ready-to-use (0.1 mg/ml) was applied (Fermentas).

4. RESULTS

4.1. *Tentacular location of the muscles and their ultrastructure*

Three, string-like muscles are located within the superior tentacle of *Helix pomatia*. At the tip of the tentacle the muscles are attached to the ventral surface, close to the sensory epithelia; FM1 in frontal, FM2 in caudal, FM3 in medial position. Other end of the muscles are attached to different points of the connective tissue at the base of the tentacle. Muscle cells are separated by large extracellular spaces, containing connective tissue, filled with collagen and fine elastic fibers. The muscle cells has long sarcoplasmic protrusions at their surface. Mitochondria are organized into large packages forming a central core of the muscle fibers. At the subsurface of the cells an array of tubular membrane profiles can be observed. The arrangement of the contractile elements displays the characteristics of smooth muscles; thin and thick filaments are not organized into sarcomeres and electron dense bodies can rarely be observed. Organized tubular or sarcoplasmic reticular system are absent in the muscle cells.

4.2. *Contractile properties of the flexor muscle*

In normal physiological saline 40 mM KCl evoked 42% contraction compared to the resting length. This effect was due to the membrane depolarizing effect of KCl. ACh (10^{-4} M) and caffeine (5 mM) induced 54% and 19% contraction. In Na^+ -free saline the effect of both KCl and ACh decreased substantially, indicating that Na^+ ions of the extracellular space play a key role in inducing muscle contraction. In Ca^{2+} -free saline, KCl-contractions were completely diminished. However, ACh and caffeine induced contractions were not attenuated, indicating the different way of action of ACh and KCl. Based on our results not only extracellular Ca^{2+} , but Ca^{2+} released from the intracellular stores also contributes to the development of FM contractions.

4.3. *Innervation and neuromuscular contacts of the flexor muscles*

Visualization of axons innervating FMs by different methods revealed similar innervation pattern, suggesting that innervation of the muscles is polyneuronal and multiterminal. The muscles receive common innervation via the olfactory nerve, while iPTn innervates only FM1 and ePTn innervates only FM2 and FM3. Neurobiotin tracing revealed that FM1 is innervated solely by the CNS neurons running through the TG, but in case of FM2 and FM3 local peripheral neurons of the TG participate in the innervation besides the central

neurons. Two types of presynaptic varicosities were distinguished based on their vesicle and granule content. One type of the axon profiles contained a large number of clear synaptic vesicles, intermingled with a few clear dense-core vesicles. The other type of varicosity contained clear synaptic vesicles mixed with a large number of electron dense granules.

4.4. Pharmacological properties of flexor muscles

Muscle contraction experiments demonstrated that acetylcholine (ACh) and glutamate (Glu) are the main excitatory neurotransmitters of the FM, released from the olfactory nerve and 5-HT, DA, Gli and His suggested to be modulators. Cholinergic and glutamatergic antagonists blocked the contractions evoked by electric nerve stimulation, ACh or Glu. Immunohistochemistry revealed the presence of ACh and Glu in the CNS and FMs. The presence of ACh was further confirmed by Western blot analysis. The evidences presented strongly supported the excitatory neurotransmitter role of both ACh and Glu.

4.5. ACh receptors in the flexor muscles

Experiments with nicotinic and muscarinic ACh receptor agonists and antagonists revealed that the ACh receptor in the FM is nicotinic. Furthermore, using specific ligands we established that the receptor contains the vertebrate neuronal, α -BgTX-sensitive $\alpha 7$ subunit. However, unlike the vertebrate receptor, it did not, or slowly desensitized. Immunohistochemistry verified the presence of $\alpha 7$ subunit in the FM, and the presence of $\alpha 4$ subunit in the axons innervating the FM and in the CNS. In the CNS the $\alpha 7$ subunit was also detected. The presence of $\alpha 7$ subunit in the CNS and FM was further revealed by PCR.

4.6. Modulatory effect of neurotransmitters and neuropeptides on the flexor muscle contractility

The monoamines 5-HT and DA effectively modulated the FM contractions induced by nerve stimulation and exogenous ACh. 10^{-5} M 5-HT oppositely acted on the integral and amplitude of contractions evoked by nerve stimulation and ACh; in case of contractions evoked by nerve stimulation the integral decreased, the amplitude increased or it did not change. Integral of ACh contractions was increased or remained unchanged, while amplitude was increased by 5-HT. This effect was observed both in case of FM1/FM2 and FM3. 10^{-6} M DA oppositely modulated the contractions of FM1/FM2 and FM3. The integral and amplitude

of FM1/FM2 contractions evoked by nerve stimulation and ACh were both attenuated. In contrast with this effect, integral and amplitude of FM3 contractions were augmented.

Immunohistochemistry demonstrated the presence of two peptides, the classical molluscan FMRFamide and the evolutionary conserved PACAP in the axons innervating the FM. Nerve stimulation and exogenous ACh evoked contractions were both decreased by FMRFamide. However, PACAP27 increased the contractions of FM induced by nerve stimulation and ACh, which effect could be blocked by PACAP receptor antagonists. Presynaptically PACAP exerted its effect by stimulating the ACh release, while the postsynaptic effect of the peptide is due to the release of Ca^{2+} ions from the intracellular stores of FM.

5. SUMMARY

The function of the muscles responsible for the retraction of the whole superior tentacles into the body cavity of *Helix pomatia* has long been known. However, we have little information about the muscles participating in the fine, spatial movements of tentacles during chemical orientation. Therefore, we investigated the tentacular localization, innervation, ultrastructure of the FM. We gave the first description of contractile and pharmacological properties of the muscles, with special attention to ACh and its receptors. Data have been inconsistent on the pharmacological properties of molluscan ACh receptors, not only between different species, but between different muscles. For this reason ACh receptors of the peripheral muscles of *Helix pomatia* were studied by pharmacological and immunohistochemical techniques. Furthermore, modulatory effect of two monoamines, 5-HT and DA and two neuropeptides, PACAP and FMRFamide on the FM contractions was also described. Our results would contribute to the better understanding of molluscan muscle regulation and the motor mechanisms behind the olfactory behavior of terrestrial snails.

Our results can be summarized as follows:

1. We have characterized in detail the special ultrastructural organization of the FMs. We identified FM as smooth muscle. Muscle cells are loosely packed and separated by large extracellular spaces. Extracellular space is filled with connective tissue containing large amount of collagen fibers. Unlike the most molluscan smooth muscle, large amount of mitochondria compose a central core in the FM cells. In absence of developed sarcoplasmic reticulum, mitochondria and subsarcolemmal vesicles serve as intracellular Ca^{2+} stores.

2. We established that innervation of the FMs is polyneuronal and multiterminal. Immunohistochemistry revealed the presence of several potential neurotransmitters and neuromodulators (Glu, ACh, DA, 5-HT, FMRFamide and PACAP) in the axons innervating the FMs. Muscle contraction studies confirmed that the three FM dispose a common innervation pathway via the olfactory nerve arose from the CNS and the axons reach the muscles travelling through the TG. On the other hand, FMs receive distinct innervation through the peritentacular nerves, reaching the muscles from the base of tentacle; FM1 is innervated by the iPTn, FM2 and FM3 are innervated by two different branches of ePTn. We revealed the presence of peripheral neurons in the TG which provide the control of FM2 and FM3 contractions without the CNS. In our ultrastructural studies we identified two types of neuromuscular contacts in the FM. In one of them the nerve terminal contained almost only clear synaptic vesicles. In the other type of neuromuscular contacts the nerve terminal contained clear and electron dense vesicles, suggesting the colocalization of neurotransmitters and neuromodulators (amino acids, DA and 5-HT). In some cases the innervating axon contacts the finger-like cytoplasmic protrusion of the muscle cell. Sometimes the axon varicosities are embedded into the distal region of the sarcoplasm.

3. Investigation of the contractile properties of the FM revealed that muscle responses to the high concentration KCl are depended on the extracellular Ca^{2+} ions, while the responses to ACh are depended mostly on the extracellular Na^+ ions. KCl contractions could be blocked using Ca^{2+} -free saline or bivalent cations, suggesting that KCl depolarizes the muscle cell membrane by opening the surface Ca^{2+} channels. Contractions induced by ACh could be blocked by Na^+ -free saline, indicating that under physiological conditions ACh depolarizes the muscle membrane mainly by increasing the membrane permeability to Na^+ ions. After a sustained treatment of the muscle in Ca^{2+} -free saline, caffeine was able to evoked muscle contraction. It suggests that caffeine release high amounts of Ca^{2+} ions from the ryanodine-sensitive intracellular stores of the FM.

4. Immunohistochemical and physiological studies demonstrated that ACh and Glu are the two excitatory neurotransmitters controlling the function of the FMs. It was also established that ACh acts through nicotinic ACh receptors. The $\alpha 4$ subunit containing nAChR is present in the axons innervating the muscles and in the CNS. In the FM cells only the α -BgTX-sensitive, non- or slowly desensitizing, $\alpha 7$ subunit containing nAChR is present, which is a neuronal

subunit in vertebrates. The presence of $\alpha 7$ subunit in the CNS and FM was further verified by PCR.

5. 5-HT and DA act as neuromodulators controlling the FM contractions. 5-HT decreases the time course of contraction of FM1, FM2 and FM3, suggesting postsynaptic effect of 5-HT. DA exerts its effect both pre- and postsynaptically. It is speculated that presynaptically it can act through two different D-receptors of cholinergic terminals, exciting or inhibiting the release of ACh. Thus DA inhibits the contractions of FM1 and FM2, but stimulates the contractions of FM3.

6. We demonstrated the presence of FMRFamide in the axons innervating the FM. FMRFamide decreases the muscle contractions evoked by both ACh and electric nerve stimulation. The presence of PACAP and PAC1 receptor were also revealed in the innervating axons. PACAP27 augmented the contractions evoked by ACh and nerve stimulation, however it did not have any effects on the contractions induced by KCl. The peptide can modulate FM contractility through pre- and postsynaptic mechanisms. Presynaptically it activates secondary signaling pathways, thus increases the release of ACh. Postsynaptically it releases Ca^{2+} ions from the intracellular stores of the muscle cells, increasing the intracellular Ca^{2+} concentration. Furthermore, PACAP may stimulate the FM contractility by increasing the ACh-sensitivity of the muscle cells.

Based on our results it is concluded that regulation of FMs by different neurotransmitters and modulators is an extremely complex mechanism, which ensures the fine movements of tentacles during olfaction.

6. LIST OF PUBLICATIONS AND PRESENTATIONS

6.1. Publications

Krajcs N., Hernádi L., Pirger Z., Reglódi D., Tóth G., Kiss T. PACAP modulates acetylcholine elicited contractions at nicotinic neuromuscular contacts of the land snail. *J Mol Neurosci* (under review)

Krajcs N., Márk L., Elekes K., Kiss T. (2012) Morphology, ultrastructure and contractile properties of muscles responsible for superior tentacle movements of the snail. *Acta Biol Hung* 63:(Suppl. 2) 129-140 **IF: 0.504**

Krajcs N., Hernádi L., Elekes K., Kimura S., Kiss T. (2014) Excitatory neurotransmitters in the tentacle flexor muscles responsible for space positioning of the snail olfactory organ. *Inv Neurosci* 14:59-69 **IF: 1.192**

Hernádi L., Kiss T., **Krajcs N.**, Teyke T. (2014) Novel peripheral motor neurons in the posterior tentacles of the snail responsible for local tentacle movements. *Inv Neurosci* 14:127-136 **IF: 1.192**

Kiss T., **Krajcs N.**, Pirger Z., Hernádi L. (2014) Nicotinic acetylcholine receptors containing the $\alpha 7$ -like subunit mediate contractions of muscles responsible for space positioning of the snail, *Helix pomatia* L. tentacle *PLoS One* 9(10):e109538, doi: 10.1371/journal.pone.0109538 **IF: 3.534**

6.2. Posters

Krajcs N., Hernádi L., Elekes K., Kiss T. Neurotransmitter candidates at an excitatory synapse of muscles in the snail tentacle. Magyar Idegtudományi Társaság XIV. Konferenciája, 2013. január 17-19, Budapest.

Krajcs N., Márk L., Elekes K., Kiss T. Éti csiga tapogatómozgásban résztvevő izomsejtek ultrastruktúrális és kontraktilis jellemzése. VII. Magyar Sejtanalitikai Konferencia, 2012. május 31-június 2, Budapest.

Krajcs N., Hernádi L., Márk L., Elekes K., Kiss T. Pharmacological and anatomical background of twitching and quivering of the superior tentacles of *Helix*. 12th Symposium of Invertebrate Neurobiology, 2011. augusztus 31-szeptember 4, Tihany.

Battonyai I, **Krajcs N.**, Kiss T, Elekes K: Serotonin is involved in both the central and peripheral regulation of snail olfaction. 3rd European Synapse Meeting, 2011. október 13-15, Balatonfüred.

Krajcs N., Battonyai I., Hernádi L., Elekes K., Kiss T. Neural control of string muscles responsible for patterned tentacle movements of the snail, *Helix pomatia*. IBRO International Workshop, 2012. január 19-21, Szeged. Az absztrakt megjelent: *Ideggyógyászati Szemle* 2012;65(S1):39.

Krajcs N., Hernádi L., Elekes K., Kiss T. Complex neural regulation of superior tentacle movements of the land snail. 8th FENS Forum of Neuroscience, 2012. július 14-18, Barcelona.

Krajcs N., Hernádi L., Elekes K., Kiss T. Complex neural regulation underlying patterned tentacle movements of the snail. 7th European Conference on Comparative Neurobiology, 2013. április 25-27, Budapest.

Krajcs N., Hernádi L., Kiss T. Pharmacology of choline receptors of the snail tentacle muscles. FENS Featured Regional Meeting, 2013. szeptember 11-14, Prága, Csehország.

Krajcs N., Hernádi L., Pirger Z., Reglődi D., Kiss T. PACAP enhances muscle contraction at an excitatory synapse of the snail. IBRO Workshop, 2014. január 16-17, Debrecen.

Krajcs N., Hernádi L., Kiss T. Alpha 7 subunit containing nicotinic acetylcholine receptors mediate contractions of muscles responsible for space positioning of the snail tentacle. 10th FENS Forum of Neuroscience, 2014. július 5-9, Milánó, Olaszország.

Krajcs N., Pirger Zs., Hernádi L., Kiss T. Nicotinic acetylcholine receptors containing the $\alpha 7$ -like subunit mediate contractions of muscles responsible for space positioning of the snail tentacle. FEPS, 2014. augusztus 27-30, Budapest.

6.3. Other publications

Pirger Z., Battonyai I., **Krajcs N.**, Elekes K., Kiss T. Voltage-gated membrane currents in neurons involved in odor information processing in snail procerebrum (2014) *Brain Struct Funct* 219:673-682 **IF: 4.567**

Battonyai I., **Krajcs N.**, Serfőző Z., Kiss T., Elekes K. Potassium channels in the central nervous system of the snail, *Helix pomatia*: localization and functional characterization *Neuroscience* 268:87-101 **IF: 3.389**