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Biological and Sportbiological Doctoral School

Functional anatomy of transmitter specific neural structures in *Lumbricid* earthworms

PhD Thesis

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PÉCS, 2016
INTRODUCTION

Lumbricid species are widely used to monitor the environmental pollution and model animals of toxicological experiments carried out at laboratory conditions and developmental and regeneration biological experiments. Because of their simple body plan (homonymous metamerism) the organization of their central and peripheral nervous systems have some ancient characteristics thus their investigation can contribute to understand the functional characteristics and phylogeny of the ganglionated nervous system.

Central and peripheral nervous system of earthworms (according to their body plan) are segmented. Except for the anteriormost 30 segments (containing differentiated parts of the alimentary canal and genital organs) and the posteriormost 15-20 (less differentiated) segments all other body segments have ventral nerve cord ganglia characterized by the same morphological and physiological properties. The centralization level is relatively low in their central nervous system, however the suprpharyngeal ganglion located in the third segment is thought to be the sensory centre while the subesophageal ganglion located in the fourth segment is the motor centre.

Every ventral nerve cord ganglia located in midbody segments is a local reflex centre and regulates the functioning of muscles and organs of each segment. The synchronization of the functioning of the ventral nerve cord ganglia is regulated by polysegmental neural structures (like sensory longitudinal axon bundles organized from central processes of primary sensory epithelial cells, dorsal giant axons), further ventral giant axons and giant interneurons that connect to each other adjacent segmental ganglia as well as interganglionic fibre bundles that are nominated by their position, however neither the origin nor the destination of fibres are known. A typical ganglion contains much more than thousand cells from which four pairs of giant motoneurons that innervate the longitudinal muscles of the body wall, 26-30 small motoneurons and a few central sensory cells (touch sensitive T-cells, pressure sensitive P-cells) were identified by histological and physiological methods.

Characteristic structures of the ventral nerve cord ganglia are (i) fibre cross-bridges where processes of dorsal giant axons and giant interneurons form synapses with crossing processes of giant motoneurons, (ii) sensory longitudinal axon bundles which form synapses with ventral giant axons, dendrites and somata of lateral dorsal giant axons further processes of giant motoneurons. The giant axon systems synchronising the activity of giant motoneurons play a role in the mediation of escape and withdraw reflexes. In the central
neuropile, where fibre cross-bridges are located, no other structures from the above mentioned are identified.

In the central and peripheral nervous systems of Lumbricid species the occurrence of more than 30 neurotransmitters (acetylcholine, monoamines, amino acid transmitters, neuropeptides and nitric oxide) was described based on the results of enzyme- and immunohistochemical stainings of sections. Identification of the exact anatomical location and possible functions of transmitter specific structures was only partially revealed. Currently it is not known what neurotransmitters can mediate the activity of dorsal and ventral giant axons and giant motoneurons.

AIMS

Based on the literary data we concluded that the identification and description of the three dimensional structure and pattern of neural structures and their possible functions could be carried out by the investigation of the good quality total preparations and their serial sections.

Therefore our aims were the development of those enzyme- and immunohistochemical methods that support to establish three dimensional structures of neural ones in whole mount preparations. Our investigations focus on the organization of those transmitter specific systems from which experimental data are already available from our or other laboratories.

Our aims were the detailed investigation of those transmitter specific systems of Lumbricid worms that were identified earlier, namely identification of

1. putative nitric oxide (NO) producing structures (since NOerg structures are involved in chemosensation), to reveal connections and possible functions of central processes of the primary sensory epithelial cells applying standard NADPH-diaphorase enzyme histochemistry known to be the histochemical marker of NOS (nitric oxide synthase) activity; and neural NOS immunohistochemistry;

2. distribution pattern of GABA immunoreactivity in primary sensory epithelial cells of the body wall and neurons of the ventral nerve cord ganglia to establish central connections of sensory structures and stained neurons of the ventral nerve cord ganglia; at last

3. PACAP 27 immunoreactive structures in both the peripheral and central nervous systems to reveal the possible functions of this conservative peptide.
MATERIALS AND METHODS

Experimental animals

Sexually matured (clitellated) specimens of *Eisenia fetida*, *Eisenia andrei* and *Lumbricus terrestris* (Annelida, Clitellata, Oligochaeta) were used in all experiments. Specimens of *Eisenia* species were selected from standard laboratory breeding stocks while specimens of *L. terrestris* were collected from their natural habitats and maintained at standard laboratory conditions for acclimatization then used for experimental investigations. After anaesthesia postclitellar segments and their organs were dissected by the aid of a stereomicroscope in earthworm-Ringer at pH 7.4.

Light microscopic observations

For light microscopic observation stained whole mount preparations and their serial sections made from resin (1.5-2 μm) or paraffin (10 μm) sections were used. Microphotos were taken by a Nikon Optiphot-2 microscope.

Identification of the neural NOS activity with β-NADPH-diaphorase histochemistry

Dissected body wall and ventral nerve cord parts (10-12 segments long) were fixed in freshly prepared 4% paraformaldehyde dissolved in phosphate buffer (pH 7.4) then washed in the same buffer. After permeabilization in 1% Triton X-100 tissues were stained with a standard incubating solution (10 mg Nitro Blue Tetrazolium, 5 mg β-NADPH in 10 ml phosphate buffer (pH 7.4) at room temperature). Prevention of aspecific formazan production was blocked by specific enzyme inhibitors and reactive group blockers (Dicumarol, Levamisol, Miconazol, Sodium azide, Pyruvate, N-ethylmaleimid, dichlorophenolindophenol). Substrate soecifiity of the enzyme was tested by the application of false substrates like (α-NADPH, β-NADH). After visualization of the enzyme activity samples were thoroughly washed in phosphate buffer (to remove aspecific colorization) and postfixed in 4% paraformaldehyde. After thorough washing in phosphate buffer whole mount preparation was cleared in glycerol, or samples were dehydrated and embedded into Durcupan ACM resin and sectioned at 2 μm.

Immunohistochemical investigations

Dissected body parts were fixed either in 4% paraformaldehyde (dissolved in phosphate buffer, pH 7.4) for neural NOS and PACAP 27 immunohistochemistry or in freshly
prepared Boer-solution (3 ml concentrated picric acid, 1 ml 25% glutaraldehyde, 40 µl glacial acetic acid) for GABA immunohistochemistry. Before the incubation with primary antibodies tissue samples were treated with conventional histochemical protocols, as usual. For optimal staining of whole mount preparations the duration of the permeabilization with 1% Triton X-100 was used from 12 hours to 2 weeks. For immunohistochemical staining distinct antibodies against neural NOS (Becton Dickinson, N3103), PACAP 27 (product number 88120-5 and produced by Akira Arimura) and GABA (Sigma, A2052) were used. Labelling of immunoreactive structures was carried out with avidin-biotin horse radish peroxidase complex method (ExtrAvidin kit, Sigma) and visualized in 3,3’-diaminobenzidine (DAB) solution. A part of whole mount preparations was cleared in glycerol for microscopic observation, while the rest was embedded into paraffin or resin, then sections cut from paraffin blocks at 10µm, or from resin blocks at 1-2µm.

**Identification of neural structures with extracellular backfilling**

Via the cut segmental nerves tracer molecules (Lucifer yellow, Sigma) were pumped into neural structures by iontophoresis (negative current, 1 Hz, 600 nA, 500 msec). Anatomical position of the central processes of primary sensory cells and some projecting neurons were revealed with this method. After thorough washing in phosphate buffer backfilled samples were fixed in 4% paraformaldehyde for NADPH-d histochemistry.

**RESULTS AND DISCUSSION**

**NADPH-d positive structures in the postclitellar segments of Lumbricid species**

Specific staining was observed in the body wall and ventral nerve cord of both investigated species and the pattern of the labelled structures proved to be the same.

**1. NADPH-d positive structures of the peripheral nervous system and the body wall**

In the body wall epithelium free nerve endings (probably peripheral processes of the central sensory cells), solitary and grouped primary sensory cells in sensilla were labelled by NADPH-d staining. Stained cells were heterogeneous in morphology, both uni- and multiciliate sensory cells (thought to be chemoreceptors) were labelled.

Larger sensilla were located in the chaetae row and smaller ones were situated in anterior or posterior hemisegments. Axons of the primary sensory cells either attached to the basiepidermal plexus or entered into segmental nerves forming easily identifiable axon
bundles that enter into the ganglionic neuropile. No NADPH-d activity was found in muscular plexuses.

All of the three segmental nerves of the ventral nerve cord ganglia contained NADPH-d stained axon bundles characterised by distinct distribution pattern. Labelled axons formed a large and a smaller bundle at the ventral part of the 1st segmental nerve. The 2nd segmental nerve contained 5, the 3rd one only 1 thin axon bundle. Motor axons did not stain in any segmental nerve but close to them thin labelled (probably sensory) axons ran.

2. NADPH-d positive structures of the ventral nerve cord ganglia

Most characteristic structures of the ventral nerve cord ganglia were the highly stained sensory longitudinal axon bundles (intermediomedian, ventromedian, ventrolateral, intermediolateral and dorsolateral) formed by the central processes of the primary sensory cells of the body wall. The ventrolateral sensory longitudinal axon bundles mainly received axons from the 1st and 3rd segmental nerves and smaller number of axons from the 2nd ones. The ventromedian and intermediomedian sensory longitudinal axon bundles characterized by weaker staining received axons from the 2nd segmental nerves only. The dorsolateral sensory longitudinal axon bundles built up from sensory axons of the 1st and 3rd segmental nerves while the intermediomedian sensory longitudinal axon bundles received axons from the 2nd segmental nerves only.

In the ventral nerve cord ganglia occurrence of three pairs of strongly stained neurons (diameter: 20-25 µm) was characteristic to both species investigated. The 1st pair of neurons located behind the 1st segmental nerves and having ipsilateral processes ran in the dorsolateral sensory longitudinal axon bundles, so they were identified as putative T-cells. The pathway of the neural processes of the 2nd pair of neurons cannot be followed. The processes of the 3rd pair of neurons run to the axons of the giant interneurons. Neurons located close to the 3rd segmental nerves send their ipsilateral processes to dorsomedial sensory longitudinal axon bundles so they were identified as putative central sensory cells.

All of the other stained perikarya located in the middle region of ganglia and at the level of the 2nd and 3rd segmental nerves. Perikarya of some small motoneurons were NADPH-d positive as well.

Results of the NADPH-d histochemistry suggest that the central processes of the putative NO producing primary sensory cells (chemoreceptors) are located in all sensory longitudinal axon bundles. These structures forming synapses with ventral giant axons, dendrites of the lateral giant axons, and axons of the giant motoneurons mediate their
functioning so can play a role in the regulation of motor commands that initiate the contraction of the longitudinal muscles of the body wall. Based on the results of double labelling (extracellular filling with Lucifer yellow and NADPH-d histochemistry) experiments we can conclude that NO production partially contributes to the mediation of the tactile and pressure information in Lumbricid earthworms.

**Neural NOS immunohistochemistry**

The applied histochemical staining resulted elective staining of some neural structures in the ventral nerve cord ganglia, however labelled structures were not identifiable with any NADPH-d staining ones. Based on these results we can conclude that labelled structures are not identical with neural NOS expressing ones, instead of them a peptide in which NOS epitope occurs was stained.

**GABA immunoreactive structures in Lumbricid species**

Sensory and interneuronal structures were identified with the applied immunostaining protocol in the peripheral and central nervous system of Lumbricid species.

1. **GABA-IR structures of the body wall and peripheral nervous system**

   Based on the investigation of whole mount preparations and their serial sections the distribution pattern of GABA-IR primary sensory cells was described in postclitellar segment. Most of the stained cells were solitary ones (probably mechanoreceptors) showing seemingly random distribution in the anterior and posterior halves of segments. Grouped sensory cells (uni- and multiciliate cells known to be chemoreceptors) were located in sensilla situated in the chaetae rows. Morphology of the labelled cells was extremely heterogeneous even if located in the same sensillum, however they have some common characteristics, namely their shorter processes run to the basiepidermal plexus while the thicker central processes run to the ventral nerve cord via segmental nerves.

2. **GABA-IR structures of the ventral nerve cord ganglia**

   Light microscopic observations revealed that central processes of the GABA-IR primary sensory cells enter into the ventrolateral sensory longitudinal axon bundles that surround ventral giant axons, and ventromedian ones. No other sensory longitudinal axon bundles contained GABA-IR processes in detectable amounts.
Distribution pattern of GABA-IR neurons seemed to be identical in investigated species. Pathway of processes of labelled neurons could not be followed in some cases so we have not got any chance to conclude their functions. Characteristic structures of the ventral nerve cord ganglia were the four polysegmental interneural axon bundles located close to dorsal giant axons and organized from the processes of those 4 pairs of neurons situated behind the first segmental nerves and those ten-twelve pairs of neurons located near the 3rd segmental nerves. The former group of neurons have two distinct process groups, the shorter ones run anteriorly and enter into the ipsilateral axon bundles while the thicker and longer ones run posteriorly and after crossing, enter contralateral axon bundles. At the level of crossing axons are located in (?) an anatomical landmark named crossing fibre-bridge formed by the processes of dorsal giant axons, giant interneurons and giant motoneurons where several synapses are located. Thin processes of those neurons located near the 3rd segmental nerves enter into ipsilateral polysegmental interneuronal axon bundles.

GABA is a well-known inhibitory transmitter and its occurrence in certain primary sensory cells and their processes enter into basiepidermal plexus, which suggests that GABA plays a role in the peripheral regulation of sensory procession.

Central processes of the labelled primary sensory cells were only collected into ventrolateral (located around the ventral giant axons) and ventromedian sensory longitudinal axon bundles, suggesting transmitter specific sensory pathways are characteristic of Lumbricid earthworms. Since both sensory longitudinal axon bundles form synapses with processes of both ventral and lateral dorsal giant axons that mediate the activity of giant motoneurons GABAerg sensory axons can have an indirect influence on the contraction of longitudinal body wall muscles innervated by giant motoneurons.

The polysegmental interneuronal axon bundles attaching to processes of dorsal giant axons and crossing fibre-bridge (consisting of processes of giant interneuron, axons of giant motoneurons) can modify their activity so they can play a role in the coordination muscle functioning that are needed for escape and withdraw reflexes.

**PACAP 27-like immunoreactive structures in the peripheral and central nervous system of Lumbricid earthworms**

PACAP 27-like immunoreactivity was found in both the body wall epithelium and ventral nerve cord ganglia of *Eisenia andrei*. Besides of solitary sensory cells and those ones located in sensilla high number of free nerve endings were found in the body wall epithelium. No stained central process of primary sensory cells was seen in the body wall, segmental
nerves or in the ventral nerve cord ganglia. Based on these findings we can conclude that only small amounts, if any, of labelled central processes enter the central nervous system. Most of them form synapses with the subepidermal plexus and influencing its activity can play a role in the local sensory processes. Based on the anatomical location of PACAP 27-like immunoreactive neurons in the ventral nerve cord ganglia we can conclude that some of them can be central sensory cells and their peripheral processes are located in the body wall epithelium as free nerve endings. Our results suggest that those structures showing PACAP 27-like immunoreactivity have a special role in the sensory processing of Lumbricid earthworms.

SUMMARY

The functional anatomy of the transmitter specific (putative NO synthesizing NADPH-diaphorase positive, GABAerg and a peptidergic, namely PACAP-immunoreactive) neural structures of the Lumbricid worms was investigated by means of whole mount preparations and their serial sections.

NADPH-diaphorase activity was found in the primary sensory cells and their central processes (named sensory longitudinal axon bundles, SLABs) moreover on certain central sensory cells of all species investigated and their detailed descriptions were shown. All SLABs were strongly stained suggesting that NO is a key player in the sensory processing in Lumbricid worms. Combining retrograde labelling and NADPH-diaphorase staining it was shown that a pair of central sensory cells (so-called T-cells) are probably NOergic ones.

The presence of GABA was found in the sensory system, several interneuronal structures, and some putative motoneurons (identified by their anatomical location). Most of the labelled primary sensory cells were solitary ones and grouped cells were only found in sensillas located in the chaetae rows. Their central processes enter the ventolateral and ventromedial sensory longitudinal axon bundles only. The former ones surrounded the ventral giant axons that thought to be a regulatory structure of giant motoneurons, suggesting that GABAergic sensory processes play a central role in the coordination of locomotion.

This is the first report on the identification of the fine fibred polysegmental interneuronal system. Its cell bodies are located at the lateral and ventrolateral part of the ganglia situated behind the first nerve roots. The ipsi- and contralateral nerve fibres run close to the dorsal giant axons and connect to their collaterals, to the giant interneurons and to the fibre cross-bridge where the processes of giant motoneurons are located, too. This part of the
ganglion is known as a regulatory structure of locomotion because of the high number of synaptic connections between various axons. Based on our present results we have to suggest the revision of earlier studies on the organization of the GABAergic system of Lumbricid worms.

Strongly stained PACAP-immunoreactive solitary sensory cells and sensillas were located in the body wall epithelium. Pathways of their central processes could only be followed to the basiepidermal plexus, suggesting that PACAP as neurotransmitter is present in the sensory system and it regulates the activity of other sensory axons located in the plexus.

**PUBLICATIONS**

*Publications related to the thesis*


4. **Solt Zs.**, Pollák E., Molnár L., (2016) PACAP-like peptides can modulate the sensory processing in earthworms. Invertebrate Survival Journal. Accepted. IF: 0,929

*Conference abstracts to the thesis*


Other publications
