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The organization of the sensory system of *Oligochaeta* worms

PhD Thesis

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INTRODUCTION

In multicellular animals special cells were differentiated to detect various stimuli and to convert these into electrical signals. The oldest form of these cells is the so-called primary sensory cell with central process, which occurs mainly in invertebrates. The secondary sensory cells have no process they connect to the peripheral processes of neurons with synapses and the electric signal is transported to the central nervous system by neurons. The third type is the so-called sensory neuron, its peripheral process can form free nerve endings or sensory corpuscle in the epithelium, connective and muscle tissues.

In the nervous system the interneurons decode the electrical signals (action potentials) induced by the stimuli from sensory cells or sensory neurons; they also evaluate and form a suitable response, which comes to the effector cells and tissues mediated by the so-called motoneurons. This process is an adequate stimulus-induced response, i.e. the reflex. The investigations of the reflex arc and the examinations of anatomical and mechanical features of each component promote a better understanding of the integrative neurological processes.

In *Oligochaetes* the epithelium of the body wall consists of column-shaped supporting cells, mucinous and serous gland cells, moreover primary sensory cells are represented in a large number. They were classified based on their localization and the morphology of the soma. Their types, numbers and distribution pattern were described on various body segments based on the investigation of the imprint pattern of sensory cells on cuticle further serial sections of the body wall. The histological investigation revealed that the majority of the primary sensory cells were grouped into sensory organs (sensillae), solitary cells were seldom found. Large sensillae are located at the chaetae row encircling the segments. This arrangement is typical most of the body segments excepting the prostomium and the last segments.

Based on histological and ultrastructural descriptions of the sensory cells, five distinct groups have been identified in earthworms: phaosomal photoreceptors, penetrative uni- and multiciliate sensory cells, nonpenetrative multiciliate sensory cells and basal ciliate sensory cells. The sensory cells are heterogeneous in neurochemical characteristics. Transmitter-specific cells have been identified in several species, thus peptidergic, aminergic, GABAergic and putative nitric oxide producing sensory cells. However, the possible functions of various transmitter-specific cells remained unknown.

The primary sensory cells have one central process, from which more peripheral processes are branching. The central processes without any ramification enter the central

nervous system (CNS) via three pairs of segmental nerves. Their T- or Y-shape rami form five pairs of longitudinal sensory axon bundles, namely the intermediolateral, intermediomedial, ventrolateral, ventromedial and the dorsolateral ones. The peripheral processes of sensory cells ramifying at the border of epithelium and muscle layer produce the subepidermal plexus. This plexus contains sensory and motor fibres as well.

The physiological experiments suggested that the sensory fibres of the segmental nerves could come from not only one, but three neighbouring segments. However, the earlier morphological studies have not discussed the size of the peripheral area, whence the sensory fibres running to each ganglion could be collected, and neither the pattern of the transmitter specific sensory cells nor their central representation have been revealed.

AIMS

According to the previous experimental results it might be concluded, that a detailed anatomical and immuncitochemical description of the connections of the CNS and the peripheral nervous system (PNS) could contribute to understand thoroughly the neuronal mechanisms of the earthworms.

According to the literature data we considered it necessary to carry out the following tasks:

1. to determine the distribution patterns of sense organs and sensory cells at different body regions with examinations of whole-mount samples;
2. to characterize the morphology of the sensory cells and to describe exactly their process system;
3. to reveal the representation of the sensory cells in the CNS;
4. to identify the GABA-immunoreactive (GABA-IR) elements of the sensory system and to determine their distribution pattern as well;
5. to describe the representation of the central processes of the labelled cells at the central region.

These investigations could help to understand thoroughly the organization and the function of the sensory system of the *Oligochaetes*.

MATERIALS AND METHODS

Experiments were carried out on adult specimens of *Eisenia fetida, Lumbricus terrestris* and *Limnodrilus hoffmeisteri* (*Annelida, Oligochaeta*), which were collected from their natural habitat, or were bred at standard laboratory conditions. The model animals were selected according to the current investigation. Thus for the electronmicroscopical and immunocytochemical researches *E. fetida* and *L. hoffmeisteri* were used because of their small size; otherwise the neuronal tracing were carried out on *L. terrestris* because their axon could be easily isolated and manipulated.

Neural tracing

We used carbocyanine dye (DiI, 1,1'-Dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine iodide) as a red colour, lipophilic, fluorescent tracer and Lucifer yellow (LY, 6-amino-2-(hydrazinecarbonyl)-1,3-dioxobenzo[de]isoquinoline-5,8-disulfonate de dilithium) as a water-soluble stain with relative low molecular weight.

DiI was applied to trace both peripheral and central neuronal pathways in postmortem fixed tissue. At peripheral labelling after removing the cuticle, DiI crystal was placed directly to the epithel surface. Thus we could investigate the central representation labelling the afferent branches. Central labelling was carried out by notching the ganglion sheath and after putting DiI microcrystal to the lateral part of the ganglion. Applying this technique we could record the sensory structure at the periphery.

LY stain was used in surviving samples. The dye was injected into the intact nerves using iontophoresis technique for minimum 3 hours.

Imaging was carried out with an Olympus FV1000 confocal microscope equipped with mixed krypton argon laser, or with a Nikon Eclipse 80i epifluorescence microscope.

Light-microscopic immunocytochemistry

The samples were fixed during 3 hours in freshly prepared Boer-fixatives (3 ml saturated picric acid, 1 ml 25% glutaraldehyde and 40 µl concentrated acetic acid) at room temperature and in dark.

The specificity of the applied primary antibody was controlled by GABA preabsorption test. Samples were labelled with avidin-biotin-horseradish complex (ExtrAvidin kit, Sigma Co., Budapest) and visualised by 3,3'-diaminobenzidine (DAB). Most of the samples were used like whole-mount, others were embedded into epoxy resin and were

sectioned in serial sections. Using DePeX conversation the samples were investigated with Nikon Eclipse 80i microscope system. Multispectral images were recorded using a Zeiss Axio Imager Z1 with a CRi Nuance^{TM} Multispectral Imaging System.

Electron microscopic immunocytochemistry

For electronmicroscopical investigation the samples were fixed during 3 hours into a mixture of 4% paraformaldehyd and 2,5% glutaraldehyd at room temperature and in dark. After washing sacharose contained buffer solution the samples were embedded into Durcupan ACM resin. The ultrathin serial sections were cut and placed on nickel grids. The samples were placed to drops of goat anti-rabbit IgG secondary antibody conjugated with 10 nm colloidal gold particles and incubated for 2 hours in room temperature. The reaction was visualized using DAB or immunogold (18 nm), contrasted and observed by JEOL 1200 EX electron microscope system.

Quantification

Whole-mount and serial sections were also used for quantitative microscopic investigation. The typical GABA-IR cell number of one segment was calculated based on the set which contained 4-12 specimens. Analysis of variance (ANOVA) was used for data analysis using SPSS 17.0 statistic software.

RESULTS AND DISCUSSION

The distribution pattern of the primary sensory cells and running of their processes

By means of scanning electron microscopical investigation of *E. fetida* and *L. hoffmeisteri* we recognized that the distribution pattern of sensory cells in the prostomium and the first 3-5 segments, furthermore in the last 3-5 segments and the pygidium was identical in these species. Our calculation showed that the number of the sensory organs -which localize randomly in appearance- was the highest in the prostomium and in the pygidium. Thus it can be presumed that these body regions have an important role in sensory function, as in the initiation of the withdrawal and the escaping reflexes.

The sensory organs of the investigated postclitellar segments were different from the anterior and the posterior segments in size and also in distribution pattern. The large sized sensillae were concentrated into the chaetae row, occurred rare in the anterior and posterior

third part of the segments in scattered distribution pattern. This distribution pattern suggests that the sensory organs of the chaetae row have an important role in the sensory mechanism.

We compared the numbers of the sensory organs in anterior, midbody and posterior segments of the two investigated species. We found that the numbers of the sensillae per unit area were much higher in *L. hoffmeisteri*. Presumably these anatomical differences can be the consequence the way of life: from the water *L. hoffmeisteri* receives the chemical stimuli in dissolved form and in low concentration and this requires more sensitive detection.

In the second part of our investigations we combined a fluorescent tracing method with confocal laser scanning microscopy to reveal the organization of the sensory system of the earthworm *L. terrestris*. In this study we firstly revealed the three-dimensional structures of the primary sensory cells and their processes. We also determined the receptive field of the prostomial nerves, which was concentrated -according to the typical bilateral symmetry relation- in the ipsilateral side of the prostomium and the first two anterior segments, where the traced nerve was located.

After tracing an isolated postclitellar ventral nerve cord (VNC) ganglion, two types of peripheral sensory structures were identified in the body wall epithelium. Among the primary sensory cells free nerve endings were also found, which were identified as the peripheral processes of the central sensory neurons.

The sensory cells had 8-10 μm diameter and most of them grouped into sensory organs in the chaetae row, in the middle line of a segment. The central processes of sensory cells situated at the anterior part of a segment entered via the 1st segmental nerve to the VNC, otherwise the processes of sensory cells located the chaetae row entered via the 2nd one. The 3rd segmental nerve collected the processes of the sensory cells from the caudal part of a segment. This distribution pattern was typical of the most segments of the earthworm. Along the central axis of the animal the density of the stained cells increased in caudal direction and parallel of this the dominance of the chaetae row started to break up, and in the last 3-4 segments it was difficult to identify any characteristic pattern.

We determined the sensory and motor fields of a midbody segment after tracing one isolated ganglion. The stained cell bodies were concentrated in one segment, but we found stained cells and processes in the neighbouring segments as well. Therefore it seems that the reflex centre function of one ganglion is not restricted to one segment. Based on the results of the applied pathway tracing investigations we could assume that the sensory structures of the neighbouring segments modulate directly the interneuronal system of the VNC and might play a role in the formation of motor responses. However, we can not rule out that the sensory

structures of the neighbouring segments connect with electrical synapses. Using DiI tracing method we showed five pairs of axon bundles in the VNC ganglion.

GABA immunoreactivity in the sensory system

After immunostain clearly labelled structures were identified in different level of the sensory system of *E. fetida* and *L. hoffmeisteri*. The stained cells of the body wall were identified as primary sensory cells based on their morphological features. The photoreceptors were not labelled.

The distribution pattern of the GABA-IR primary sensory cells followed the previously described pattern using neuronal tracers. We revealed a typical pattern in the clitellum, where the stained cells concentrated into two parallel lines in the area of the adolescent bump. Presumably these GABA-IR cells, represented in high number with central processes which run directly to the VNC have a role in shaping of the mating behaviour to perceive the body surface of the partner. In the sensillae of the postclitellar segments the labelled cells were heterogeneous but we could define some characteristic cell types.

Solitary GABA-IR cell types were also detected. Their function could be relatively simply explained. According to the results of anatomical and physiological experiments they were supposed to be stretch receptors, because they prevented the strain of the epithelium of the body wall. Our results partly corroborate with this hypothesis, since the central processes of the majority of solitary sensory cells directly project to the ventral ganglia without making any synapses with the subepidermal plexus.

The GABA-IR central processes and their lateral branches with varicosities entering the subepidermal plexus were easily identified in serial sections of the body wall. The branches connecting two individual sense organs were observed in many cases. The central processes of the GABA-IR sensory cells give inputs to the non immunoreactive sensory cells localized into one sensilla, but there were GABA-IR profiles under the totally non immunoreactive sensory organs.

The running of the labeled sensory fibers in the segmental nerves and in the CNS was followed both in whole-mount preparations and in its semi-thin serial sections. It was established that only two pairs of the five longitudinal sensory axon bundles, namely the ventrolateral and the ventromedial axon bundles, contained GABA-IR fibers. The ventrolateral sensory bundles formed a thick sheath around the ventral giant axons and gave synapses to them. These ventral giant axons take a prominent role in the movement coordination. The ventrolateral giant axons make synapses with the giant motoneurons, and

affect the activity of the longitudinal muscles in the body wall. Presumably, GABA inputs influence directly the activity of the giant motoneurons through inhibiting the ventrolateral giant axons, and thus the contraction of the body.

SUMMARY

The anatomical organisation of the sensory system of three *Olygochaetes* (*Lumbricus terrestris* L., *Eisenia fetida* Sav., *Limnodrilus hoffmeisteri* Clap.) was observed by means of light and electron microscopic methods, histochemical and neural tracing processes.

We investigated the distribution pattern of sensory organs of the body wall of *E. fetida* and *L. hoffmeisteri* with scanning electronmicroscopical techniques. The first and the last 3-5 body segments contained sensory organs in high numbers, these could play role in the escape and withdrawal reflexes.

Neuronal tracers (DiI and LY) and confocal laserscanning microscopy were applied to determine primary sensory cells and their processes in *L. terrestris*. For the first time in this study the three-dimensional organization of the sensory system of an *Oligochaeta* species was reconstructed. We showed that the peripheral sensory system was not segmental in its arrangement, because between the neighbouring segments intersegmental connections were found and sensory fibres from several segments run into one ventral ganglion. Presumably the ventral ganglia have integrative functions regulating the activity of more segments.

By means of light- and electronmicroscopical immunocytochemistry the distribution pattern of GABA-IR structures of the sensory system of *E. fetida* and *L. hoffmeisteri* was observed in different body regions (prostomium, midbody and caudal segments). The labelled primary sensory cells were classified according to morphological features (the size and shape of the soma) and the running of their processes. We identified intersegmental GABA-IR fibres in the subepidermal plexus. Using ultrastructural methods we showed that the GABA-IR fibres gave inputs to the non GABA-IR fibres in the subepidermal plexus suggesting the sensory fibres modulate one another activity in the plexus. From the intersegmental sensory axonbundles of the ventral ganglia there were GABA_IR fibres only in the ventrolateral and in the ventromedial bundles. The ventrolateral sensory bundle is located around the ventral giant axon and gives inputs as well. The ventromedial axon bundle has synaptic connections with the collaterals of soma of the dorsal giant axon and influences its activity.

According to our results we can assume the GABA-IR structures of the sensory system mediated in the motor reactions and segment synchronization by modulating the activity of giant interneurons (ventral and dorsal giant axons) of the central nervous system.

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