

RESEARCH REPORT

GABA immunoreactive elements in the sensory system of the earthworm *Eisenia fetida* (Annelida, Clitellata)**G Kiszler^{1*}, E Várhalmi¹, L Krecsák², Z Solt¹, E Pollák¹, L Molnár¹**¹*Department of Comparative Anatomy and Developmental Biology, University of Pécs, Ifjúság u. 6, H-7624 Pécs, Hungary*²*Department of Systematic Zoology and Ecology, Eötvös Loránd University, Pázmány Péter u. 1. 1/C, H-1117 Budapest, Hungary***recent address: 3DHISTECH Ltd. Konkoly-Thege u. 29-33. H-1121 Budapest, Hungary**Accepted May 20, 2016***Abstract**

The presence of γ -aminobutyric acid (GABA) immunoreactive elements was observed in the sensory system of *Eisenia fetida* (Annelida, Clitellata). Among the primary sensory cells a high number of labelled cells was found in the epithelium. Using whole-mount preparations and multispectral recording, the number and the distribution pattern of the immunopositive cells were determined in different body segments. Our morphological analysis revealed four typical types of stained primary sensory cells, which could be responsible for the different role of the GABA mediated sensory functions. The peripheral processes of the primary sensory cells ramifying at the border of epithelium and muscular layer produced a basiepidermal network where GABA-positive fibres and their connections were observed. The central processes of the labelled cells projected directly to the ventral nerve cord (VNC) through the segmental nerves to form the ventrolateral and ventromedial sensory longitudinal axon bundles (SLABs). Inhibitory GABA sensory inputs could influence indirectly the activity of the giant motoneurons through the ventrolateral giant axons, and thus the contraction of the body organizing withdrawal and escape reflexes. Applying ultrastructural investigations the synaptic connections of GABA-immunoreactive structures were identified both in the basiepidermal plexus, in the segmental nerves, and in the SLABs of VNC suggesting multistep regulatory effect of GABA in sensory processing of earthworms.

Key Words: primary sensory cells; sensilla; basiepidermal plexus; sensory longitudinal axon bundles; multispectral imaging

Introduction

Primary sensory cells are distributed in the body wall of the Clitellata between the supporting and glandular cells (Coonfield, 1932; Mill, 1978). These cells send sensory information to the VNC and influence the dorsal giant fibres and motoneurons, controlling rapid reflexes like withdrawal and escape (Drewes, 1984). Their anatomical features were described by Langdon (1895) in *Lumbricus agricola* (Annelida, Clitellata), who noted that most of the sensory cells are grouped (forming a sensillum), solitary cells being rare. The location of the sensilla follows a well

identifiable pattern. Most of the sensilla are found in a middle line of segments so named chaetae-row; however, there is a seemingly random pattern on both the anterior and posterior parts of segments. Langdon counted 1,900 sensilla on the first segment and prostomium, 1,200 on the 10th segment and 700 on the 56th segment. Knapp and Mill (1971) estimated that the larger sensilla contain 35 - 45 sensory cells. These data were confirmed by many later workers and they described that distribution pattern and the numbers of the sensilla are similar in the sister species (Myhrberg, 1979; Aros *et al.*, 1978; Moment and Johnson, 1979; Jamieson, 1981; Spöhrhase-Eichmann, 1998; Molnár *et al.*, 2006; Kiszler *et al.*, 2012).

Based on their morphological characteristics the primary sensory cells were classified (Retzius, 1892). Five distinct types were detected by ultrastructural investigations (for review see Mill, 1978, 1982; Jamieson, 1981): penetrative uniciliate

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and penetrative multiciliate sensory cells (Aros *et al.*, 1971b; Knapp and Mill, 1971) nonpenetrative multiciliate cells (Aros *et al.*, 1971a), phaosomal photoreceptors (Hess, 1925a; Röhlich *et al.*, 1970) and basal ciliated sensory cells (Myhrberg, 1979). The functions of the different types are partly known. Results of morphological and electrophysiological investigations suggest that nonpenetrative multiciliate cells are mechanoreceptors, penetrative uni- and multiciliar cells are chemoreceptors (Mill, 1978). Electrophysiological investigations showed that the sensitivity of sensilla is different in each part of the body, although the sensory cells have similar morphology (Laverack, 1963; Mill, 1978, 1982). This suggests that the morphology of the sensory cells does not determine precisely their function; the location is at least as important.

The synchronization of sensory inputs and motor outputs of the nervous system is mediated by transmitter-specific neural structures (Dorsett, 1978; Mill, 1982). Distinct groups of the primary sensory cells were determined by their neurochemical features (Schpörhase-Eichmann, 1997). Transmitter-specific sensory cells have been described by many workers in several species, thus peptidergic (Gesser and Larsson, 1986; Renda *et al.*, 1987; Curry *et al.*, 1989; Reglödi *et al.*, 1999, 2000), aminergic (Myhrberg, 1967, 1971; Ehinger *et al.*, 1971; Spörhase-Eichmann, 1998; Csoknya *et al.*, 2005) and GABAergic (Telkes *et al.*, 1996; Spörhase-Eichmann *et al.*, 1997; Koza and Csoknya, 2004; Molnár *et al.*, 2006) cells however, the possible functions of the transmitter-specific cells remained unknown. Electrophysiological observations were used to find connections between the different types of sensory cells and their function. The receptive fields of the segmental nerves were determined by Knapp and Mill (1967), who described that these are not concentrated in one segment but overlap to the adjacent segments.

The neural processes of the sensory cells form a neural plexus at the border of the epidermis and the muscle layer called basiepidermal plexus (Rude, 1966; Myhrberg, 1967). The plexus includes both efferent fibres and sensory axons (Langdon, 1895; Hess, 1925; Ogawa, 1939; Mill, 1978), and the most probable role of this network is controlling the contraction of the longitudinal muscle and acting as a reinforcing agent in the peristaltic waves (Miller and Ting, 1949). However, there are conflicting opinions that this system is segmental or continuous in its organization (Retzius, 1892; Langdon, 1895; Smallwood, 1923, 1926; Hess, 1925; Coonfield, 1932; Ogawa, 1939; Rude, 1966; Myhrberg, 1967; Mill, 1978).

The central processes of the sensory cells, without branching enter the VNC via three segmental nerves. After entering they divide in a T-shaped manner and form five longitudinal sensory axon bundles. These are the intermediolateral, intermediomedian, ventrolateral, ventromedian and the dorsal bundle (Günther, 1971; Dorsett, 1978). From calculations of Knapp and Mill (1971) it was concluded that the relationship between the sensory cells and number of sensory axons in the segmental nerves are 1:1 (Mill, 1975, 1978, 1982). Also, their

investigations stated that chemoreceptive and tactile inputs enter the central nervous system (CNS) primarily via the first and the third segmental nerves and from proprioceptors via the second nerve (Knapp and Mill, 1967). Because of the close locations of the second and the third segmental nerves, later studies suggested that all three kinds of sensory input enter the CNS via all three segmental nerves (Mill, 1982).

Based on the results of previous studies we can conclude that the identification of transmitter specific sensory structures and their exact anatomical characterization -localization, distribution density- are necessary to reveal the possible mechanism of the sensory system. Therefore, the aim of present work has been to identify and map GABA immunoreactive (GABA-IR) sensory elements in peripheral (PNS) and CNS of the earthworm *Eisenia fetida*, including detailed morphological description of the labelled structures.

Materials and Methods

Animals

Experiments were carried out on 15 sexually matured *Eisenia fetida* (Annelida, Clitellata) specimens. The animals were kept in plastic boxes containing soil under standard laboratory conditions (20 - 22 °C, 50 - 60 % humidity). Specimens were anaesthetized in carbonated water and cut into pieces of several body segments and their guts were removed. After the preparation segments were fixed in dark at 4 °C in a freshly prepared mixture of saturated picric acid (3 ml), 25 % glutaraldehyde (1 ml) concentrated acetic acid (40 µl). Most of the specimens were used as whole-mounts (n = 50) but the CNS and various body regions were dissected out from a few specimens (n = 18).

Light-microscopic immunocytochemistry

The following protocol was used in the case of the whole-mount and the dissected specimens as well. After several rinses in 70 % ethanol, fixed samples were washed in phosphate-buffered saline (PBS), then treated with 2 % Triton-X-100 diluted in PBS for two nights at 4 °C. Samples were incubated for 2 h in 10 % normal goat serum in 2 % Triton-X-100 to decrease background staining, and were afterwards incubated in 1:500 dilution of anti-GABA primary antibody (Sigma Chemical Company, Budapest, Hungary) in 2 % Triton X-100 buffer for 4 days at 4 - 6 °C.

The specificity of the antibody to GABA was confirmed by the preincubation of the antibody together with GABA-glutaraldehyde-bovine serum albumin complex (1 mM of GABA). After preincubation, the immunohistochemical protocol resulted in no staining. By means of stereomicroscopy labelled structures were visualized using diaminobensidine (DAB), Avidin-biotin-horseradish peroxidase method, Extravidin kit, (Sigma Chemical Company, Budapest, Hungary). Control samples (n = 15) were processed utilizing the above immunohistochemical staining protocol except for the omission of the primary antibody.

Stained samples were prepared in two different ways: 1) whole-mounts were cleared with buffered

glycerol (50 % PBS and 50 % glycerol) for 2 - 3 days then mounted between cover-slips in pure glycerol for microscopy, or 2) dehydrated in graded ethanol series and embedded into epoxyresin (Durcupan ACM) and were after sectioned in 1.5 μm thin serial sections using a Reichert Supernova ultramicrotome, attached to gelatine-chrome-alum covered slides and stained with toluidine blue.

Electron microscopic immunocytochemistry

For electron microscopy the samples ($n = 12$) were fixed in a mixture of 4 % freshly prepared paraformaldehyde and 2.5 % glutaraldehyde for 2 h at 4 °C. After thorough washing with several changes of 0,1 M PBS (pH 7) they were post-fixed in ice-cold 1 % OsO_4 for 60 min. Samples were dehydrated and embedded into Durcupan ACM resin. Ultrathin serial sections were cut by Reichert Supernova ultramicrotome and collected on nickel grids. Sections were etched in 1 % HIO_4 , deosmicated with 1 % NaIO_4 and then were transferred to several drops of distilled water. The preincubation was carried out applying normal goat serum for 30 min and then samples were incubated overnight with a polyclonal rabbit anti-GABA serum (Sigma Chemical Company, Budapest, Hungary) diluted 1:300 in TRIS-buffered saline (TBS, pH 7.4) at 4 - 6 °C. After incubation process the samples were washed in several changes of 0.01 M TBS and placed to drops of goat anti-rabbit IgG secondary antibody conjugated with 10nm colloidal gold particles (Sigma) diluted 1:50 in TBS, for 2 - 4 h. Finally the samples were rinsed in distilled water and dried at 35 °C.

Imaging

Nikon Eclipse 80i microscope equipped with Nomarsky optic was used to visualize the samples. At the level of light microscopy the detection of the DAB signal was difficult due to the relative high pigment content of the body wall, thus a multispectral imaging technique was applied. Multispectral images were recorded using a Zeiss Axio Imager Z1 with a CRi Nuance™ Multispectral Imaging System. The spectral recording of the whole-mount samples were in the spectra interval from 400 nm to 750 nm. The digitalization of serial sections was performed using a *Pannoramic™* SCAN digital slide scanner (3DHISTECH Ltd, Budapest, Hungary) with Zeiss plan-apochromat objective (magnification: 20X, Numerical aperture: 0,8) and Hitachi (HV-F22CL) 3CCD progressive scan color camera (resolution: 0,2325 $\mu\text{m}/\text{pixel}$). 3DView scientific software application (version: 1.5, 3DHISTECH Ltd, Budapest, Hungary) was used to make three-dimensional reconstructions from the digitalized serial tissue sections. Ultrastructural examinations were carried out with JEOL 1200 EX electron microscope system.

Cell counts

After binarization of the DAB spectral images the number of GABA-IR cells for relevant body segments was counted using particle analysis application of Image J software (ImageJ 1.32j, Wayne Rasband, NIH, USA). Used samples were: 4 prostomium, 5 first segments, 12 postclitellar

segments, and 5 posterior segments. Eight clitella were measured separately because of the special cell pattern. As the body areas used for counting differed between samples, cell counts were standardized to a unit area of 5,000 μm^2 . The distribution of the cells was additionally investigated within four different midbody segments of similar size (216,500 μm^2), the number of cells being counted in the dorsal, ventro-lateral and ventral part of the segments. Analysis of variance (ANOVA) was used for data analysis using the SPSS 17.0 for Windows (SPSS Inc., Chicago, USA).

Results

The applied antibody and the immunocytochemical process revealed a specific and reproducible staining of sensory structures in the PNS and the CNS of *E. fetida*.

Immunoreactivity in the body wall

Based on the investigations of whole-mount samples the distribution pattern of GABA-IR primary sensory cells were described in relevant body segments. Stained cells were represented consequently in all segments of the animal, the highest density being detected in the first five and the last three posterior segments.

Applying multispectral recording the spectra of the DAB were identified on each the recorded image (Figs 1a - e). The maximum density of DAB measured was 430 nm. The spectra line of DAB was separated from the pigment spectra (Figs 1a, b). Further the distribution pattern of the stained sensory cells was described based on normal photomicrograph and spectrum separated images.

The prostomium and oral cavity (segments 1 to 5)

In the anterior part of the animal, the prostomium and the oral cavity was studied in detailed. The labelled primary sensory cells in the prostomium have small sized circular soma (approximately 8 - 10 μm in diameter) and stand typically alone in the sensilla (Fig. 2a). Three dimensional reconstruction of the prostomium allowed more precise morphological analysis and showed that the most of the stained cells were located near the surface of the epithelial layer with short processes to the cuticle (Figs 3a, c). Others reached through the epithelium or were located at the base of it on the basal layer (Fig. 3b). Equal distribution was typical, the distance between the stained cells was in average 20 μm (min = 12, max = 25) in the investigated samples (Fig. 2a).

In the epithelium of the buccal cavity clearly labelled cell bodies were detected. The cells had typically small soma and their processes created a dense plexus (Fig. 2b). In the dorsal and the lateral side of the pharynx most of the fibres were linear and run in parallel however, in the ventral region the dense network with crossings were typical (Fig. 2c).

Midbody and anterior segments

In the midbody segments most of the GABA-IR cells were concentrated into a lane which was extending in the middle of the segments, along the line of the chaetae (Fig. 2d). These cells were

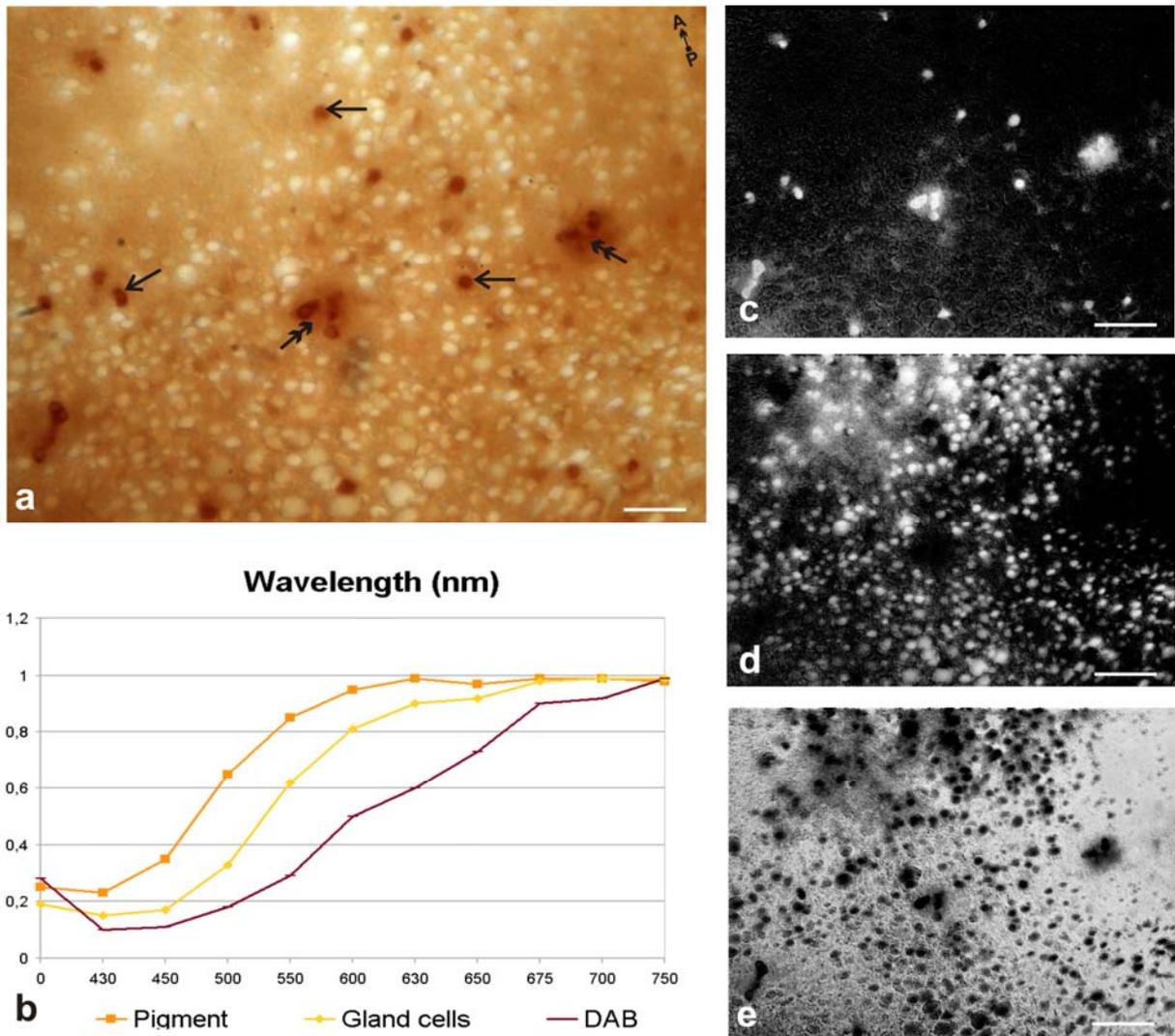


Fig. 1 Multispectral recording of the body wall epidermis of *Eisenia fetida* after GABA immunostain. a) Solitary and grouped sensory cell were also represented in the epithelial layer. b) Spectra of the immunostain (DAB), pigment and gland cells based on a Cri multispectral recording (c). Stained cells were detected on the DAB spectral image (d, e). In the spectral segmentation process the gland cells (d) and the pigment (e) spectra were also defined and these images were used as negative control. Sharp arrow: solitary sensory cell, double arrow: sensory cells into sensilla, DAB: diaminobenzidine. Direction A - P anterior-posterior. Bar: 50 μ m

generally grouped in sensilla which contain 2 - 8 stained cells and additionally up to 20 other unlabeled sensory cells. In the large sensilla (average 80 μ m diameters) we counted about 20 - 40 sensory cells and GABA-IR ones were more than a half of these. However there were sensilla with high number of stained cells close around the chaetae, in the marginal part of the chaetae sac (Fig. 2e). The observation of the deeper regions of the chaetae row revealed stained transversal fibres between the sensilla (Fig. 2f). In the anterior and the posterior part of the segments, solitary sensory cells were generally, following random distribution pattern. The localization of the sensory structures of the chaetae row coincided with the running of the second segmental nerve (Fig. 4a). This distribution

pattern was typical for all midbody segments, except for the clitellar segments. Here low number of the GABA-IR cells was found in dispersed location. The solitary cell type occurred generally, sensilla with stained cells were rarely detectable. However, high numbers of stained cells were concentrated in two thin lanes which were parallel to the long axis of the animal and localized the ventrolateral side of the segments. These cells were round, stained intensively and had very small (2 - 3 μ m) soma. The lane was narrow in its anterior and posterior end (Fig. 4b).

The pattern described in the midbody segments was found in most segments of the animal. The labelled cells in the posterior segments had similar distribution pattern as in the anterior segments.

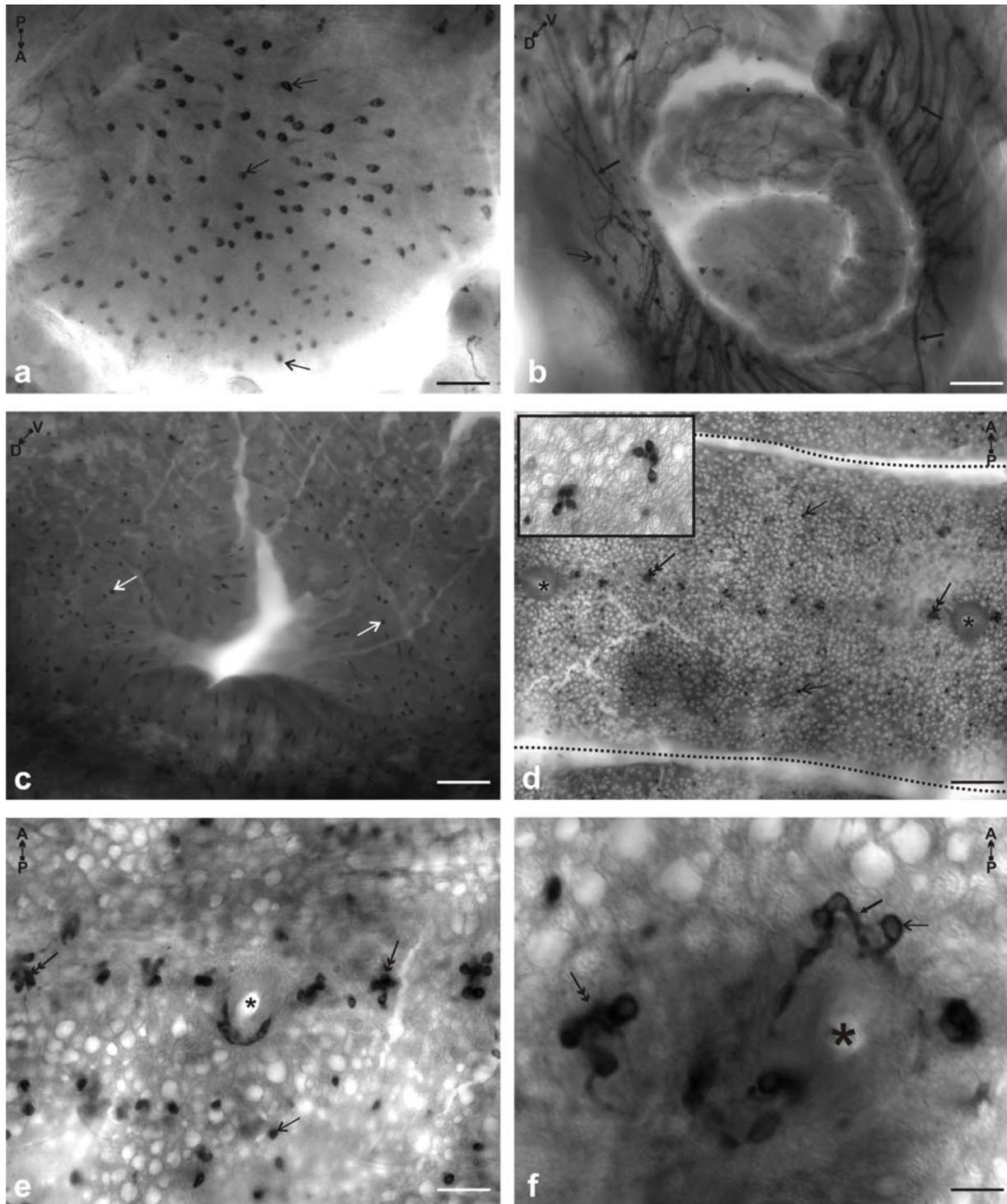


Fig. 2 GABA-IR sensory elements in different body regions of *E. fetida*. a) Labelled primary sensory cells in the epithelial surface of prostomium. b, c) Stained structures in the epithelium of the oral cavity. d) The localization and the distribution of labelled cells and sense organs in midbody segment. Thumbnail: two sensilla. e, f) Sensilla with GABA-IR sensory cells in the chaetae row. Stars: chaetae sac, sharp arrow: solitary sensory cells, arrows with double arrowhead: grouped sensory cells, arrows: the processes of the sensory cells. Direction A - P: anterior-posterior, D - V: dorsal-ventral. Bar: a - e: 100 μ m; f: 10 μ m.

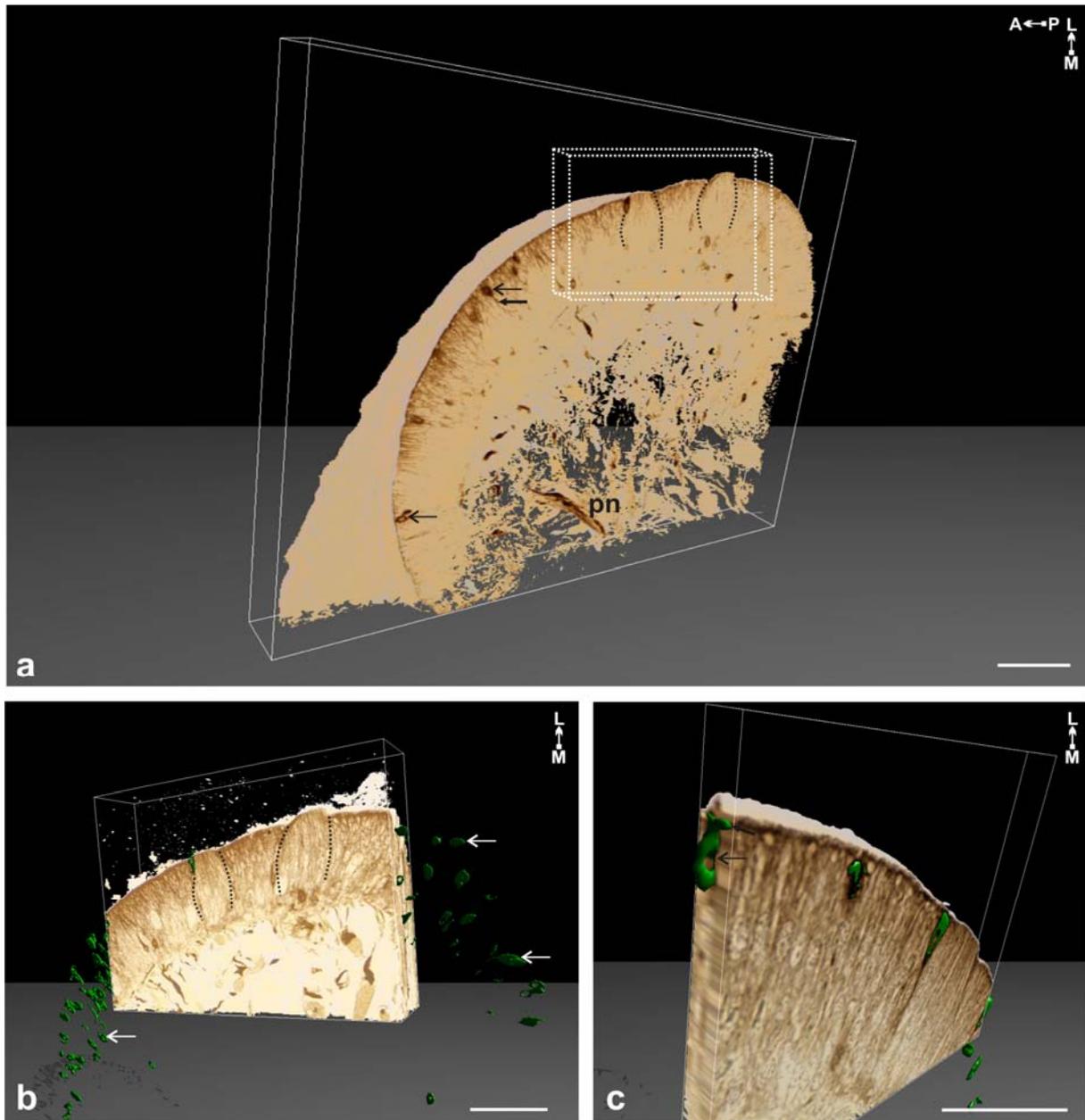


Fig. 3 Three-dimensional micrographs of stained GABA-IR structures in the prostomium. a) The whole reconstruction of the prostomium with labelled primary sensory cells and their central processes which run into the prostomial nerve. b) Virtual tissue segment at high magnification with the detected GABA-IR sensory cells which localized in different part of the epithelial layer. c) Stained primary sensory cells with short apical processes to the cuticle. Dotted line: sensilla, sharp arrow: solitary sensory cells, arrows: processes of the sensory cells, pn: prostomial nerve. Direction A - P: anterior-posterior, L - M: lateral - medial. Bar: a: 10 μ m; b, c: 50 μ m.

The average number of GABA-IR cells varies between the analyzed body segments as follows: 600 \pm 52 cells in the prostomium, 530 \pm 40 cells in the first segment, 800 \pm 65 cells in a postclitellar segment and 300 \pm 17 cells in the posterior segments. Most of the cells was identified in the clitellum (*i.e.*, 1,300 \pm 72, $n = 8$), where they were mostly distributed in one line. ANOVA revealed significant difference between the cell numbers of different body parts

($F(2, 10) = 78.45$, $p < 0.001$). The prostomium and the body end segments bore the highest number of GABA-IR cells (Fig. 5). Within an average midbody segment, ca. 40 % of the cells are located in the dorsal side, ca. 25 %, in the ventral side and 35 %, in the ventrolateral side. The investigations on serial sections confirmed the result of the whole-mount observations: only the subpopulation of the primary sensory cells show GABA immunoreactivity.

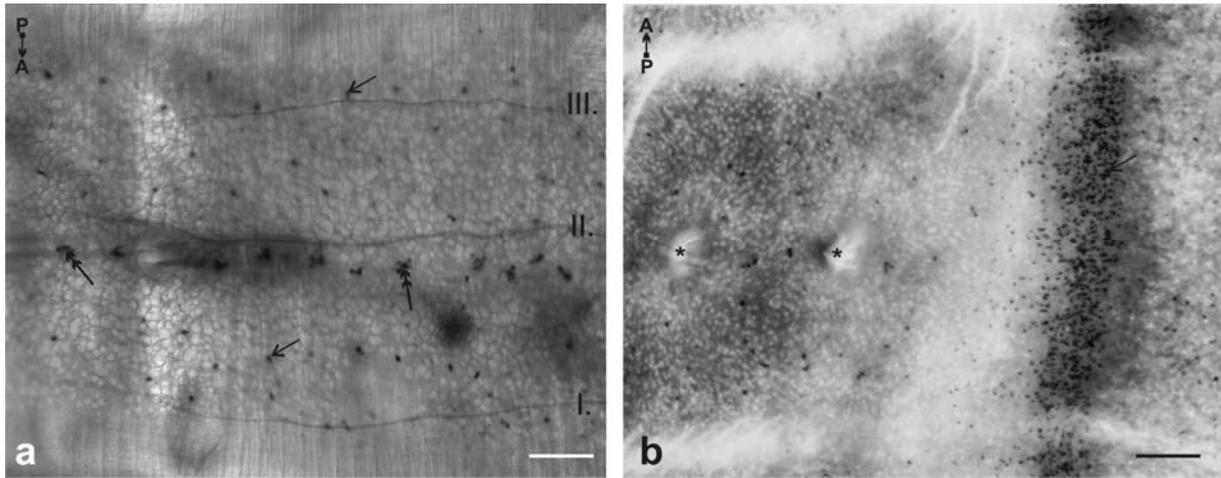


Fig. 4 Distribution pattern of GABA-IR primary sensory cells in various segments. a) The position of the stained cells coincided with the position of the segmental nerves which were also stained. b) Specific distribution pattern were revealed in the clitellum, where the stained cells were localized in the area of the adolescent bump. Stars: chaetae sacs, sharp arrows: solitary sensory cells, arrows with double arrowhead: grouped sensory cells, I., II., III.: position of the segmental nerves. Direction A - P: anterior-posterior. Bar: 100 μ m.

There were labelled cells represented together with non-labelled ones inside one sensillum, whereas the phaosomal cells (*i.e.*, photoreceptors) were not stained as shown in Figure 6a. We could not distinguish areas inside the sensilla, where the GABA expression was preferred, the stained cells localized randomly in the investigated sensilla. From morphological aspect the labelled cells were heterogeneous even in one sensillum, but particularly significant differences could be observed in the anatomical attributes of the labelled cells in the different body regions.

The morphology of GABA-IR primary sensory cells

In the sensilla of the prostomium long, extended GABA-IR cells with well-developed central processes were found in high numbers. The structures of the labeled cells in the sensilla of the postclitellar segments were more heterogeneous, although a few basic types could be identified:

I. The characteristic features of the first form were: relatively small sized, nearly globular soma localized in the apical part of the epidermis, some ramifications of their central processes entered in the basiepidermal plexus, whereas the thickest branch runs into the segmental nerve (Figs 6b, g).

II. The second type resembled bipolar cells, had a short peripheral and a long central process. The latter was essentially thicker, than in the other sensory cells (Figs 6c, d) and entered directly, without any ramifications, into the segmental nerve.

III. The third type was mainly detected in the centre of sensilla, reached across the whole thickness of the epithelium and its process coming out from the soma sprouted and broke up into several fibres ended in the plexus (Figs 6e, h).

The solitary sensory cells were represented in low number, had thick central branches which without ramification run directly to the basiepidermal

plexus (Fig. 6f). Small dendritic processes were detected in the apical part of these cells, which run in the top of the cuticle layer. These cell types were typically in the anterior and the posterior parts of the midbody segments (Figs 6a, f, i)

GABA-IR branches in the basiepidermal plexus

The varicose GABA-IR central processes entered the basiepidermal plexus (Figs 6h; 7a, b). Putatively in the motor axons, dense core and clear vesicles with large diameter were located, whereas in the sensory axons, 0.5 - 1 μ m in diameter, small clear vesicles could be located (Fig. 7c).

By means of immunocytochemical observations it could be well detected that the central process of GABA-IR sensory cells frequently gave inputs to processes of the non-GABAergic sensory cells in the same sensilla (Figs 2f; 6b).

The GABA-IR central processes and their lateral branches with varicosities entered to the basiepidermal plexus which could be clearly identified in serial sections (Figs 6c, e, h). The branches connecting two individual sense organs were observed in many cases (Fig. 2f). The stained processes of the GABA-IR sensory cells frequently gave inputs to the non immunoreactive sensory cells localized into one sensillum (Fig. 7d).

The central representation of the GABA-IR sensory branches

The ascending labelled sensory fibres in the segmental nerves and in the CNS were 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 fibres. The intensive labelling of the ventrolateral axon bundles surrounding the ventral giant axons was conspicuous (Figs 8a, b).

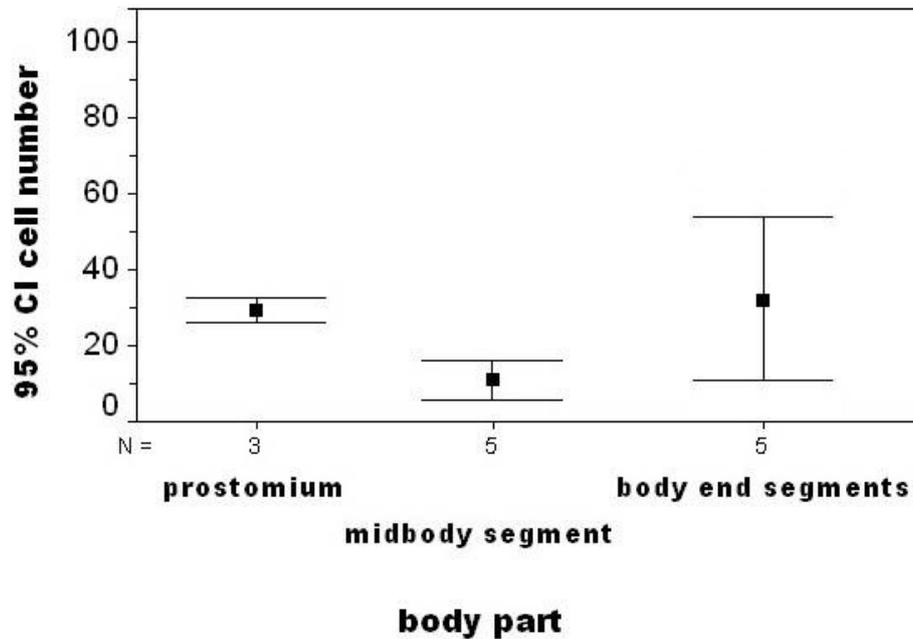


Fig. 5 The distribution of GABA-IR sensory cells in the investigated body segments.

The ultrastructural features of the GABAergic axon profiles were investigated in the neuropil of the ventral ganglion. Selective stained branches could be observed in the ventrolateral (Figs 8c, d) and the ventromedial (Figs 8e, f) longitudinal axon bundles. The gold particles were uniformly distributed over the GABA-IR varicosities; vesicles and the cytoplasm were also labelled. The GABAergic axon profiles mainly contained pleomorphic agranular synaptic vesicles with an average diameter of 25 nm. Moreover dense-core vesicles of about 50 nm in diameter were additionally seen in the GABA-IR profiles.

Discussion

In present study we applied a controlled GABA immunohistochemical protocol to identify distinct sets of inhibitory -GABAergic- structures in the sensory system of the earthworm *E. fetida*. We showed that a subpopulation of the primary sensory cells was GABA immunoreactive. Based on the result of the preabsorption tests and the negative control where we never received staining, we could assume that indeed GABA-containing cells and fibres were stained. The specificity of the applied serum was tested in several laboratories in invertebrates (Spöhrhase-Eichmann, 1997) and vertebrates (Meza, 1998) as well and specific staining was achieved with it. The presence of GABA, known as an inhibitory neurotransmitter, is not customary in receptor cells (Sepherd, 1994), though according to many earlier studies there are GABAergic sensory cells in vertebrates as well, which might play a role in fine modulation of the sensation (Meza, 1998).

Our result demonstrates that GABAergic primary sensory cells are represented in all segments. The distribution of the stained cells was not randomly in the body surface, but there were well defined, constant patterns in relevant segments of body regions. The distribution pattern followed consequently the pattern of sensory cells which was described earlier by Langdon (1895). This showed that the majority of the primary sensory cells are GABA-IR, so GABA could play an important role in the sensory regulation.

Stained sensory cells were found in high number and high density in the prostomium, moreover, these patterns were typical the first and the second anterior segments and the last three posterior segments. We applied a pixel based tissue reconstruction of digitalized serial sections to reveal the three-dimensional organization of the GABA-IR elements in the prostomium. Based on this method we described the exact localization and the morphological features of the stained primary sensory cells. Measurements showed that the number of the sensory cells per area is the maximum in the prostomium and in the anal segment. Because of these results it could be presumed that these body regions had an important role in the GABA-mediated sensory function.

In the midbody segments we revealed a typical pattern. Most of the GABAergic cells grouped into sensilla and were concentrated in the chaetae rows. In the anterior and the posterior part of segments solitary sensory cells were randomly distributed and less number of sensilla were also located. Observing whole-mount preparations we found that the localization of the stained cells of the chaetae rows was coincided with the running of the 2nd and

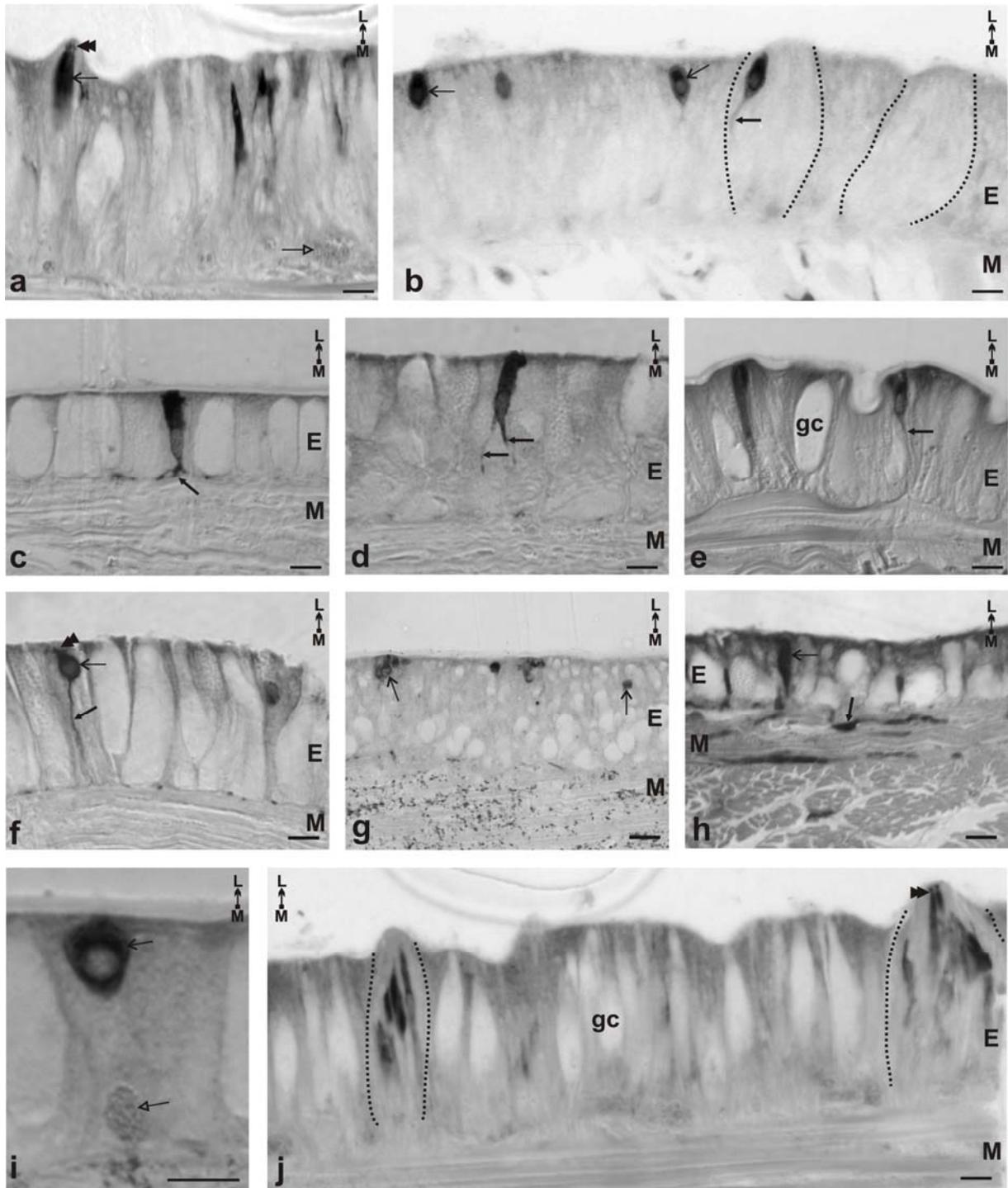


Fig. 6 Types of GABA-IR primary sensory cells in the epidermis of *Eisenia fetida*. a) Cells with oval somata in the epithelium of the prostomium. The basal localized phaosomal photoreceptors were not stained. b) Type I cells in small size, localized at the top of the apical surface occurred frequently in sensilla. c, d) Stained type II sensory cell with branched processes. e - h) Labelled type III sensory cell with elongated soma. f - i) Apically localised sensory cells which stand alone. g) Stained cells grouped into sensilla. h) GABA-IR processes in the level of the basiepidermal and muscular plexus. GABA immunoreactive processes at the level of the basiepidermal and muscular plexus. j) Stained cells with heterogeneous morphology grouped in sensilla. Sharp arrows: cell bodies, double arrowheads: dendritic processes of sensory cells, arrows: central processes of sensory cells, arrows with empty arrowhead: phaosomal photoreceptors, dotted line: border of sense organs, E: epidermis, M: muscle layer, gc: gland cell- Direction L - M: lateral-medial. Bar: 10 μ m.

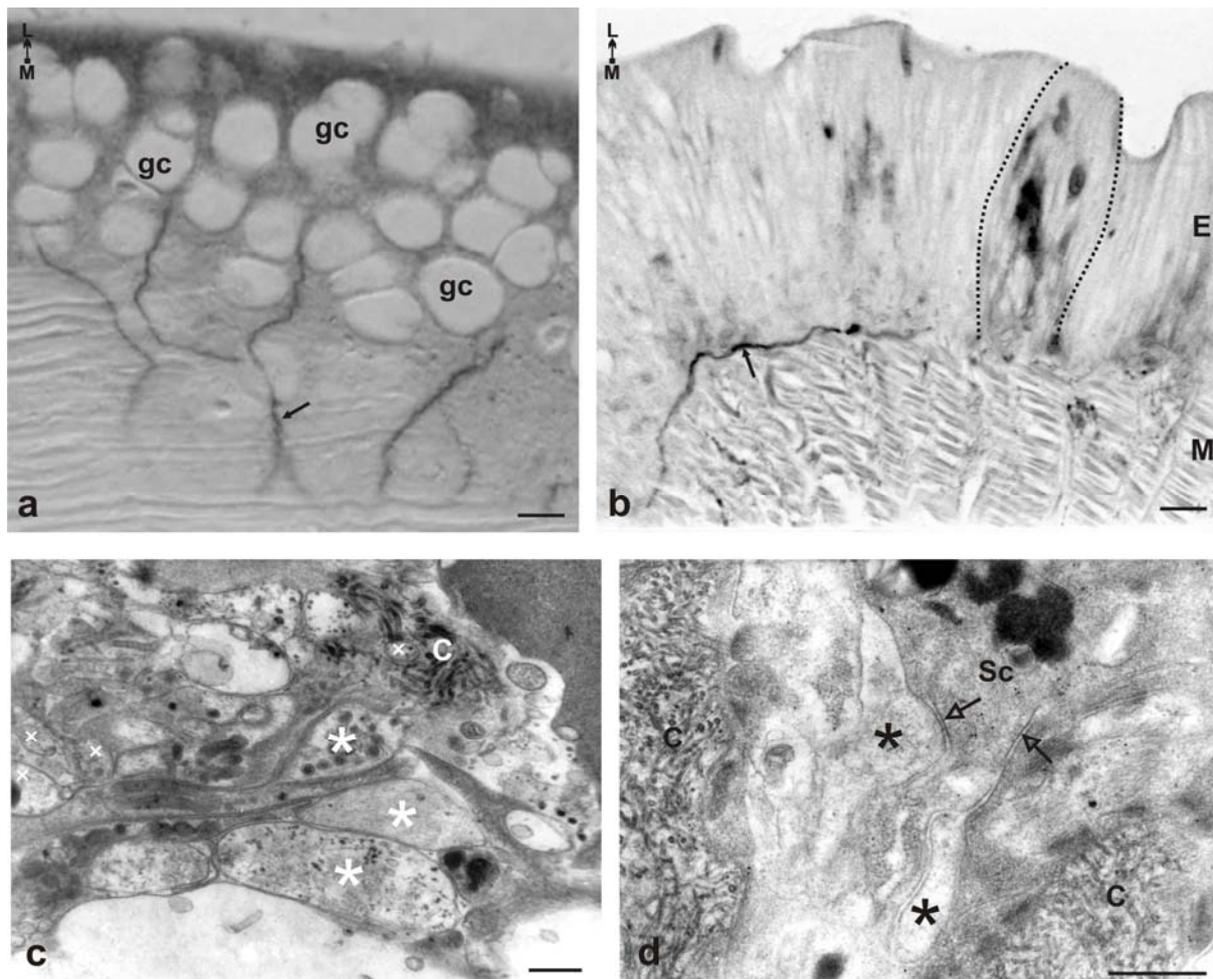


Fig. 7 GABA-IR processes in the basiepidermal nerve plexus. a, b) Stained processes in whole mount sample. c) The ultrastructure of the basiepidermal plexus. d) GABA-IR central processes of the primary sensory cell in the plexus. Arrow: stained processes, gc: gland cells, dotted line: border of sensilla, stars: motor axons, cross: sensory axons, Sc: sensory cell, C: collagen fibres, arrows with empty arrowhead: possible synaptic connections. Directions L - M: lateral-medial. Bar: a, b: 10 μ m; c, d: 500 nm.

3rd segmental nerves showing this is the most important region of the GABAergic sensory mechanism.

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 sensilla of the postclitellar segments the labelled cells were heterogeneous but we could define some characteristic cell types, which could be responsible for different GABA mediated sensory functions.

Based on the characteristic location of the type I cells (they were situated at the top of the epithelial layer close to the body surface) we can conclude that they play a role in the direct sensory perception and the fast transmission of the primary stimulus.

The type II which resembled bipolar cells, because of the reach arborisation of their processes possibly has a role to support an integrative function in the peripheral sensory system. The type III was mainly located in the centre of sensilla have probably function in the synchronization of the intersensillar sensory function. Moreover, the processes of these cells make dense arborisation in the level of the plexus and because of this possibly make role to the intersensillar communication.

The function of solitary GABA-IR sensory cells could be relatively simply interpret. According to the results of anatomical and physiological experiments they were supposed to be stretch receptors, because they inhibit to overstrain the epithelium of the body wall (Edwards and Kuffler, 1959; Craelius and Fricke, 1981). Our results partly corroborate 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 basiepidermal plexus. Similar cells were

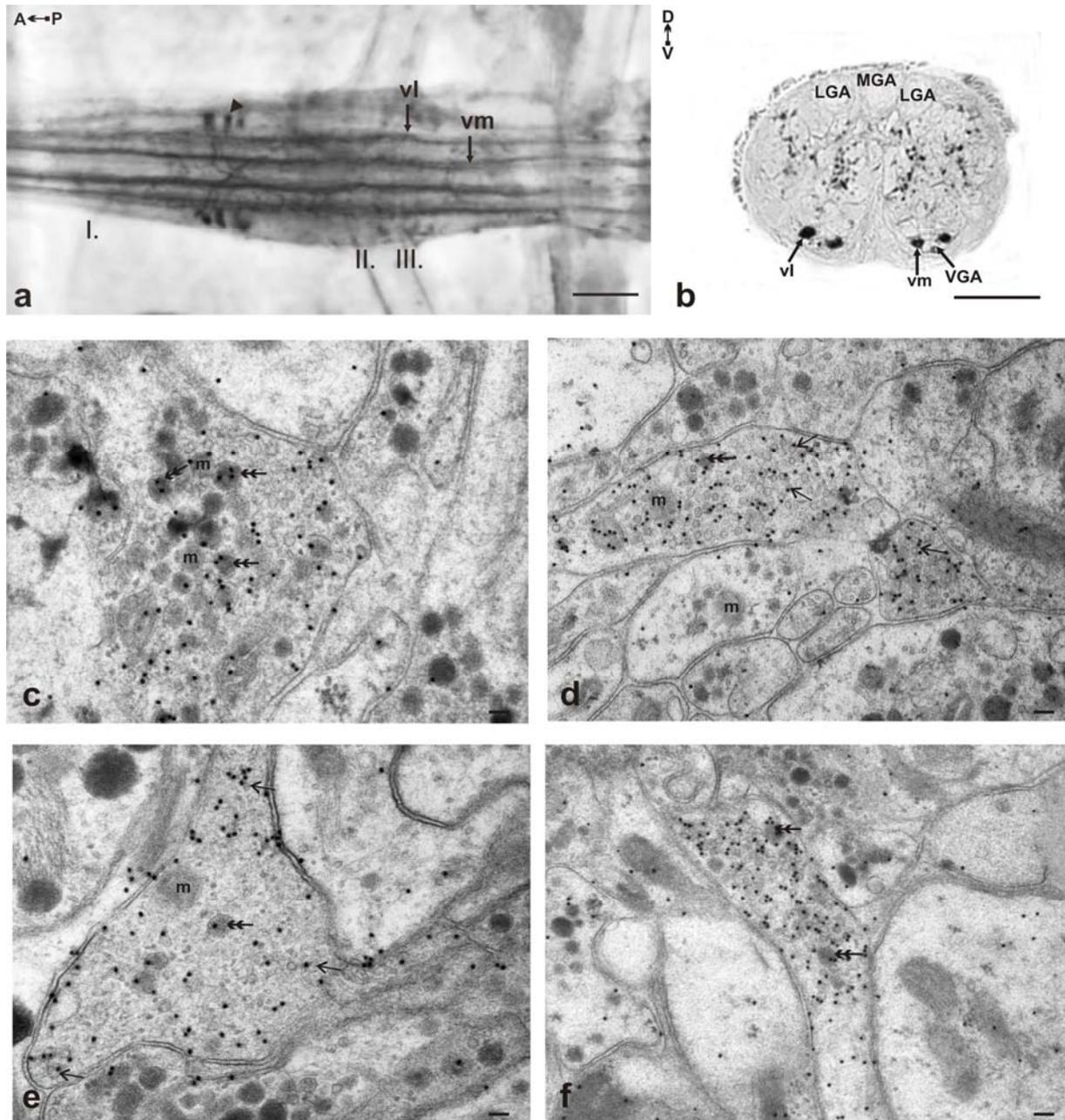


Fig. 8 Location of GABAergic in the VNC. a, b) Stained axon bundles and sensory cells in a whole-mount preparation (a) and in its cross sections (b). Ultrastructure of GABA-IR axon profiles in the ventrolateral (c, d) and the ventromedial (e, f) sensory longitudinal axon bundles after postembedding immunogold staining. Arrowhead: stained central sensory cell, I., II., III.: the position of the segmental nerves, MGA: medial giant axon, LGA: lateral giant axon, vl: ventrolateral axon bundle, vm: ventromedial axon bundle, sharp arrow: agranular vesicle, arrow with double arrowhead: granular vesicle, m: mitochondria. Direction A - P: anterior-posterior; D - V: dorsal-ventral. Bar: a, b: 100 μ m; c - e: 100 nm.

found in some sensilla. Moreover, most of the stained cells had fibres running to the ganglia (Fig. 7). In the ventral ganglia the GABA-IR fibres were only concentrated into two sensory bundles (ventrolateral and ventromedial), and it is obvious that they have synaptic connections with the determined branches of the CNS.

The ventrolateral sensory longitudinal axon bundles formed a thick sheath around the ventral giant axons, which take a prominent role in the movement coordination and give a synapsis to them (Bullock, 1945; Coggeshall, 1965; Mill, 1982; Büschges and El Manira, 1998). The ventral giant axons make synaptic contacts with the so-called

giant motoneurons, and affect the activity of the longitudinal muscles in the body wall (Mill, 1978; Jamieson, 1981). Presumably GABA inputs influence indirectly the activity of the giant motoneurons through inhibiting the ventral giant axons, and thus the contraction of the longitudinal muscles of the body wall.

It is more difficult to interpret the function of the GABAergic endings of the basiepidermal plexus. Based on our current knowledge, sensilla contain mainly uni- and multiciliated cells, which mostly take part in chemoreception (Laverack, 1963; Mill, 1978, 1982). The GABA-IR cells of the sensilla presumably inhibit not only the function of the certain structures of the CNS, but they could modify and thus influence the activity of a part of the sensory cells. Consequently they could play a role in the delicate modulation of the processes of the perceptibility, or in the formation of the receptive fields of the body wall. Laverack (1963) reported on the excitatory and inhibitory structures of the plexus. On the evidence of electrophysiological observations isolated receptive fields could be identified in the epithelia of the body wall (Mill, 1982). These structures support the presumption of the integrative function of the basiepidermal plexus. GABA, like a modulatory transmitter could be in the background of this function. Based on ultrastructural investigation it was revealed that the labelled presynaptic profiles in high numbers contained small, pleomorphic, agranular vesicles. This morphological statement correlates with the GABA profiles described already in the CNS in the vertebrates (Somogyi and Soltész, 1986; Ottersen *et al.*, 1988; Gábel and Straznický, 1995), and also in the invertebrates (Wattson, 1988; Telkes *et al.*, 1996; Ganeshina and Menzel, 2001).

Summing up these immunohistochemical investigations where the majority of the sensory structures were GABA positive, showing that GABA has an important role in the sensory mechanism. Based on the anatomical organization of the GABAergic neurons, presumably these cells have a modulator role in the intercellular and intersensillar communication at the level of the basiepidermal plexus. 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 (both ventral and dorsal giant axons) of CNS.

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