

Galanin-like immunoreactivity in the nervous system of *Oligochaeta*

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SUMMARY

The distribution of galanin-like immunoreactivity in the nervous system of an annelid worm, *Eisenia fetida*, was studied by means of immunohistochemistry. The cerebral ganglion contains 14-45 immunoreactive cells, organized mainly into dorsomedial and dorsolateral cell groups. The number of immunopositive cells in the subesophageal and ventral cord ganglia varies between 1 and 5. Immunostained fibers have been found in the stomatogastric ganglia and the wall of the gastrointestinal tract. The subepithelial and the muscular walls of the enteric system contain numerous stained cells, but no epithelial cells display immunoreactivity. Immunopositivity has also been observed in the epidermal sensory cells with a random distribution. Some cells in all ganglia show immunoreactivity using both serotonin and gastrin antisera.

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Key words: Galanin - Immunohistochemistry - Earthworm

INTRODUCTION

Galanin was originally isolated from extracts of the porcine intestinal tract (Tatemoto et al., 1983) and subsequently localized in the gastrointestinal tract of a number of mammals and non-mammalian vertebrates (rev. Vrontakis et al., 1991). Galanin consists of 29 amino acids and shares little homology with other known peptides. Therefore, it is considered to be a member of a new

peptide family. The amino acid sequence appears to be highly conserved across species. Like other peptides first discovered in the intestinal tract, galanin or galanin-like immunoreactivity (G-LI) have been shown to have a wide distribution in both the central and peripheral nervous systems of mammals (Merchenthaler et al., 1993) and other vertebrates (Batten et al., 1990; Lázár et al., 1991; Józsa and Mess, 1993). Galanin has also been shown to extensively co-localize with other substances, including peptides and amines (Merchenthaler et al., 1993). Since its discovery, numerous investigations have shown that galanin plays a role in various physiological processes, including hormone secretion, neuronal activity and smooth muscle contractility (rev. Rökaeus 1987; Vrontakis et al., 1991).

Investigation of the invertebrate nervous systems is important for elucidating evolutionary questions, but it may also provide a better understanding of basic neuronal mechanisms. One of the most widely used model animal groups is the phylum of annelida, where the nervous system is segmentally arranged and centralization first occurs in phylogenesis. A number of vertebrate neuropeptides have been demonstrated in most invertebrates (e.g. O'Shea and Schaffer, 1985; Curry et al., 1989; Nässel, 1993). G-LI has so far been reported in only three invertebrate species. The first observation was made by Roberts et al. (1989), who found a single immunoreactive cell in the cerebral ganglion of a mollusc, *Bulla gouldiana*. A wider distribution of G-LI was found by Lundquist et al. (1991) in the blowfly nervous system and by Díaz-Miranda et al. (1996) in the sea cucumber. No data are available on the occurrence of G-LI in annelida.

The aim of the present study was to describe the distribution pattern and morphological cha-

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characteristics of galanin-like immunoreactive elements in an annelid species, *Eisenia fetida*. The co-localization of G-LI with serotonin immunoreactivity was also studied.

MATERIALS AND METHODS

Adult specimens of the earthworm, *Eisenia fetida* were purchased from a local supplier. For fixation, the segments anterior to the clitellum were removed and fixed in Zamboni's fixative (Zamboni and de Martino, 1967). Penetration of the fixative was promoted by microwave irradiation as described previously (Lubics et al., 1997). The specimens were embedded in paraffin, and serial frontal and horizontal 10 or 20 μm thick sections were cut.

Sections were pre-treated with trypsin solution (0.12%, Réanal, Budapest). Non-specific background staining was inhibited by incubation in 1% normal sheep serum (20 min). Immunohistochemistry was performed using the peroxidase-antiperoxidase (PAP) method (Sternberger et al., 1970). Anti-galanin was raised against synthetic chicken galanin in our laboratory (Balásperi et al., under publication). Incubation with the antiserum, diluted 1:1000, was performed for 48 h at 4°C. The rest of the staining procedure was carried out at room temperature. The sections were then incubated with anti-rabbit-gamma globulin (ARGG, sheep, 1:300) for 10 min, and PAP (1:600) for 10 min. ARGG was raised in our laboratory and PAP was obtained from Arnel (New York, NY, USA). The staining procedure beginning with treatment with ARGG was carried out twice (double PAP method). A thorough washing in phosphate-buffered saline solution was performed after each step. Bound PAP was visualized by 0.25% 3,3-diaminobenzidine (DAB) in 0.05M Tris-HCl buffer, 7.5 pH, containing 0.01% H_2O_2 (20 min). The DAB end product was intensified with osmium tetroxide vapor. After completing the DAB reaction, some of the sections were intensified by the silver-intensification method as described by Gallyas et al. (1982).

For the co-localization studies, serial frontal cryostat sections (10 μm) were alternatively mounted on separate slides coated with 3-aminopropyl triethoxysilan (Sigma-Aldrich, Budapest) for better adhesion. Immunohistochemistry was performed on the two sets of alternate sections. The anti-serotonin antiserum was generously provided by Dr. T. Görcs (Görcs et al., 1985).

A method control was performed by omitting the primary antiserum. Anti-serotonin is a well defined antiserum and does not show cross-reactivity with any known antigen (Görcs et al.,

1985). A specificity control was carried out by incubating the primary antibody with 1 g/ml of galanin antigen. No staining was obtained following these tests, except in some blood vessels which displayed endogenous peroxidase activity. This could be blocked by 0.3% H_2O_2 in saline, which was applied in control sections.

Sections were examined and then photographed using a Nikon photo microscope, Microphot-FXA. Comparison of digitized pictures of consecutive sections was performed using the NIH image program, as described previously (Lubics et al., 1997). Briefly, parallel projections of adjacent sections of ganglia enabled the localization of double-stained cells. Using the "AND" image processing operation on two consecutive sections, only those immunostained cells could be seen which had the same position in both sections. Visualization of the vessels running in close association with the ganglia support matching tissue landmarks of the two neighboring sections by rendering a contour to the ganglia.

Neurolucida for Windows (Version 1.5) was used to map the immunostained cell groups by drawing the boundaries of the ganglia and the outlines of the immunoreactive cells. Co-localization could be demonstrated by simultaneous projection of two consecutive sections stained with different antisera, confirming that the outlines of the stained profiles belong to the same cells.

RESULTS

In annelida, the central nervous system consists of the cerebral ganglion, which is connected to the subesophageal ganglion by the circumpharyngeal connectives, and the segmental ventral ganglia (Bullock and Horridge, 1965).

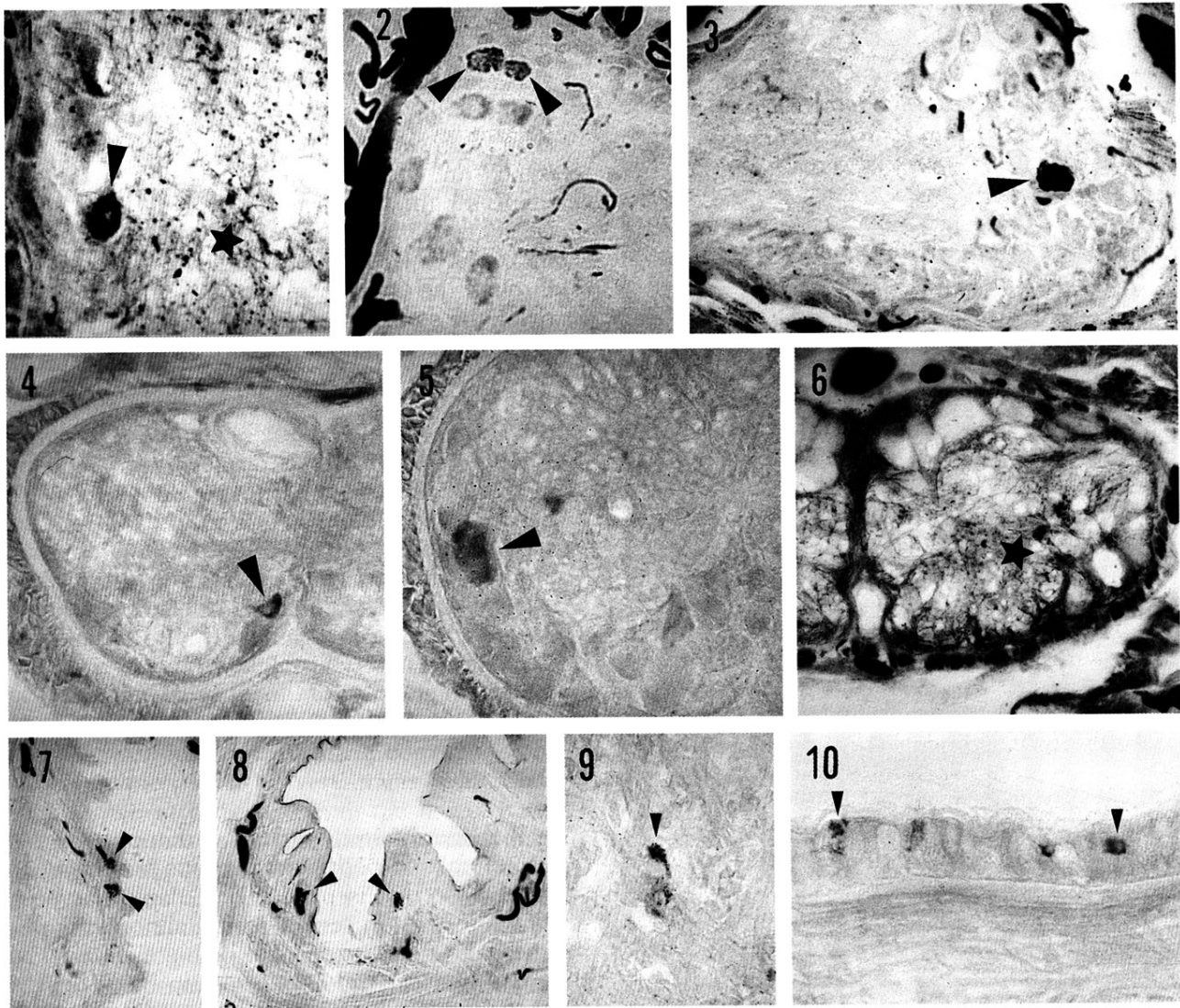
G-LI is found in all parts of the central nervous system of *Eisenia fetida*, and some parts of the peripheral nervous system also contain immunoreactive cells and fibers. The cerebral ganglion contains numerous immunopositive cells, which are situated in 4 major groups of the dorsal cell mantle. Most immunopositive cells are found in the dorsolateral (Fig. 1) and dorsomedial cell groups. The average number of immunoreactive cells observed is 4-14 in the dorsolateral-, 5-17 in the dorsomedial-, 3-8 in the medial- (Fig. 2) and 2-6 in the lateral cell groups. The immunoreactive cells represent 0.6-2% of the 2170 total cells (Bánvölgyi et al., 1994) of the cerebral ganglion. The size and shape of these cells are variable. The staining of the neuropil is faint and expression of G-LI is mostly limited to the central longitudinally-running fibers (Fig. 1).

The number of stained cells in the subesophageal and ventral cord ganglia is lower than in

the cerebral ganglion, but staining is found in all animals. Most of the immunoreactive cells belong to the lateral and medial cell groups (Figs. 3, 4, 5), where the number of perikarya varies between 1-5 per ganglion. This represents only 0.07-0.3% of the total cell number. There are only a few immunostained cells in the ventromedial and ventrolateral cell groups. The neuropil and the segmental nerves originating from the ganglia contain a few immunoreactive fibers, whose destination cannot be traced (Fig. 6).

The most consistent staining in the peripheral nervous system is found in the enteric nervous system. The stomatogastric ganglia contain

immunoreactive fibers, but no cells displaying G-LI are observed. The muscular wall of the buccal cavity contains fibers running towards the epithelium. In the digestive canal from the pharynx downwards many immunopositive cells are visible beneath the epithelium in the subepithelial connective tissue (Figs. 7, 8). The fibers of these cells run towards the epithelium or parallel with its surface to other parts of the gastrointestinal tract. The pharyngeal wall contains the most cells, and the number of immunoreactive perikarya diminishes in the caudal direction. The muscle layer of the intestinal tract also contains a large number of cells staining for G-LI (Fig. 9).



- Fig. 1.**— A galanin immunoreactive perikaryon in the dorsolateral part of the cerebral ganglion (*arrowhead*). Fibers of the neuropil also display immunoreactivity (*star*). Silver intensification. x400.
- Fig. 2.**— Medial cells displaying G-LI in the posterior part of the cerebral ganglion (*arrowheads*). Silver intensification. x200.
- Fig. 3.**— Lateral immunopositive cell in the subesophageal ganglion. Silver intensification (*arrowhead*). x200.
- Fig. 4.**— A medial pyriform cell in a ventralcord ganglion (*arrowhead*). x200.
- Fig. 5.**— A lateral cell displaying immunoreactivity in a ventral cord ganglion (*arrowhead*). x300.
- Fig. 6.**— Immunoreactive fibers in the ventral cord neuropil with silver intensification (*star*). x200.
- Fig. 7.**— Immunostained cells beneath the pharyngeal wall (*arrowheads*). Silver intensification. x300.
- Fig. 8.**— Subepithelial immunopositive cells in the anterior gut wall (*arrowheads*). Silver intensification. x300.
- Fig. 9.**— Immunostained cell with a process running among the muscle fibers in the wall of the pharynx (*arrowhead*). Silver intensification. x200.
- Fig. 10.**— Sensory epithelial cells in the epidermis of the earthworm (*arrowheads*). x200.



Fig. 11.— Immunostained ventromedial neurons in the ventral ganglia. Fig. a and b show photomicrographs of adjacent transverse sections, stained with anti-serotonin (a) and antigalanin (b) antisera. *Arrowheads* indicate the co-labeled neurons. x200. The drawing (c) represents simultaneous projection of the NeuroLucida drawings of the above sections. Anti-serotonin-stained cell is shown in black and anti-galanin-stained cell is in light grey. In the resulting image the double-stained cell is shown.

These are small, elongated cells, and their fibers run among the interlacing muscle fibers of the alimentary wall with no specific direction. Such immunoreactive cells are not observed in adjacent sections stained with anti-serotonin. No epithelial cells display G-LI.

Immunoreactivity is also observed with a random distribution in surface epidermal cells, which are most numerous in the cranial segments of the animals (Fig. 10). Most of these cells are small and are found in the basal part of the epidermis, although some of them extend through the entire height of the epithelium and reach the surface. No epidermal cells express G-LI in adjacent sections stained with the anti-serotonin antiserum.

Co-localization with serotonin immunoreactivity

The number of cells displaying immunostaining for both galanin and serotonin is relatively low. In the cerebral ganglion, 1-2 dorsomedial and 2-3 lateral double-stained cells are found. In the subesophageal and ventral cord ganglia, an average of one ventromedial cell is observed per ganglion. No double staining can be observed in the peripheral nervous system.

DISCUSSION

In the present study G-LI was demonstrated in the central and peripheral nervous systems of an annelid worm, *Eisenia fetida*. Galanin has been shown to exist in several forms, although the N-terminal sequence seems to be highly conserved across species, and is believed to be of special importance for biological actions (Rökæus 1987; Vrontakis et al., 1991). In invertebrates, galanin has been reported in *Bulla gouldiana* (Roberts et al., 1989), in the blowfly (Lundquist et al., 1991) and in the sea cucumber (Diaz-Miranda et al., 1996). Diaz-Miranda et al. (1996) provide several lines of evidence suggesting the presence of a galanin-like peptide in the sea cucumber. Four different antisera have shown a similar pattern of immunoreactivity, and RIA results have also shown marked similarities with porcine galanin.

The substances responsible for the immunoreactivity in the above-mentioned species have not been identified. According to the immunoreactive peaks obtained in HPLC analysis, one or more galanin-like peptides have been shown in the blowfly that are more basic than mammalian galanins (Lundquist et al., 1991). In various tissues of the sea cucumber, the estimated levels of G-LI are within the range found in other species (Diaz-Miranda et al., 1996). Since galanin does not appear to share homology with other known peptide families, the immunoreactivity found in our study is most probably due to galanin-like substances. As in other species more peptides may be present. Thus it cannot be excluded that by using other antisera more immunoreactive cells and fibers could be found.

According to the distribution of galanin-like immunoreactivity and functional studies performed by other researchers, it is suggested that galanin-like peptides may be involved in the digestive regulation, sensory processes and central integratory processes of the earthworm. One of the best documented functions of galanin is the modulation of gastrointestinal function in vertebrates: the peptide appears to coordinate gastrointestinal motility and to participate in regulating absorption, secretion and blood flow (Rökæus, 1987).

In higher invertebrates the stomatogastric nervous system is homologous with the vertebrate autonomic nervous system (Hartenstein, 1997). In the annelids, the stomatogastric system is composed of a chain of stomatogastric ganglia situated parallel to the circumpharyngeal connectives and the nerves linking these ganglia to the connectives on one side and to the digestive tract on the other side, where the enteric nervous plexus is formed. Fibers found in the enteric plexus may also be of intrinsic origin (Csoknya et al., 1992). It is generally agreed that the enteric plexus of the earthworm corresponds to the myenteric plexus of vertebrates, which has been shown to project to both the mucosa and the external muscles of the gastrointestinal tract (Furness et al., 1991).

The effects of galanin in the invertebrate digestive tract are not known. Lundquist et al. (1987) found galanin-like immunoreactive cells in parts of the nervous system which are involved in feeding behavior. In the earthworm, consistent staining was shown in nerve cells in the subepithelial and muscle layer of the digestive tract, with neurites running towards the epithelium, or among the muscle fibers. No epithelial cells displayed immunoreactivity. Our findings indicate a correlation with the distribution of G-LI found in vertebrates, where it has been shown to be present in nerves innervating all layers of the gut from the esophagus to the rectum, but not in enteroendocrine cells (Rökæus, 1987;

Furness, 1991). Our results suggest that galanin-like peptides are involved in the modulation of gastrointestinal motility and the activity of the mucous layer of the digestive tract, mainly in the proximal part, where most immunoreactive cells are found. Further functional studies are needed to elucidate the roles of galanin in the annelids.

Another role of galanin in sensory inputs has been postulated (Vrontakis et al., 1991). Earthworms possess a variety of epidermal sensory cells that are involved in the reception of different tactile, chemical and light stimuli (Mill, 1978). The fact that anti-galanin antiserum stains a number of sensory epidermal cells in the earthworm implies that galanin-like peptides also play a sensory role in the earthworm. The morphology of these cells is variable, but a large number of small stained cells is situated in the basal part of the epithelium. These cells are believed to be mainly of the photo-receptor type (Jamieson, 1981). A role of galanin in visual processes in vertebrates has been proposed (Strömberg et al., 1987) and similar suggestions have been made for the blowfly (Lundquist et al., 1991). Apart from the suggested involvement of galanin-like peptides in photo-sensation in the earthworm, our observations indicate that other sensory inputs may also be influenced by these peptides.

The cellular morphology of the central nervous system in Oligochaeta has been well documented by a number of investigators (Ogawa, 1939; Bullock and Horridge, 1965; Günther, 1971), although functionally identified nerve cells are not as well known as in other invertebrates. The functional identification of the immunostained cells is therefore difficult, and is further hindered by the paucity or absence of staining of the neurites. The number of galanin-immunoreactive cells in the central nervous system represents a very low percentage of the total cell number and is also very low compared to the number of immunopositive cells stained with antisera against other neuropeptides (Curry et al., 1989; Reglódi et al., 1997a, b). This cannot be due solely to the limitations of the method or the antiserum, because the enteric nervous system and the epidermal cells showed very intensive staining. In spite of the low number of neurons, it is suggested that galanin-like peptides may also play a modulatory role in central integratory processes, as has been proposed for other invertebrates (Lundquist et al., 1991; Diaz-Miranda et al., 1996). The importance of the co-localization of serotonin and galanin-like immunoreactivities in some cells in the central nervous system remains unclear. Further investigation with other antisera as well as functional studies are needed to obtain a better understanding of the role of galanin in the annelid nervous system.

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