ACTA BIOL. SZEGED. 39. pp. 39-49. (1993)

SEROTONIN CONTENT DURING THE REGENERATION OF NERVOUS SYSTEM IN EARTHWORM (*LUMBRICUS TERRESTRIS* L., OLIGOCHAETA) *

M. CSOKNYA+, I. LENGVÁRI++, L. HIRIPI+++, K. ELEKES+++, J. VINCZE+, M. SZELIER++ and J. HÁMORI+

+Department of Zoology, Janus Pannonius University, Pécs, H-7604

++Department of Anatomy, University Medical School, Pécs, H-7643

+++Department of Experimental Zoology, Balaton Limnological Research Institute, Hungarian Academy of Science, Tihany, H-8237, Hungary

(Received: June 15, 1993)

Abstract

Serotoninergic nerve cells were found in both the cerebral as well as the segmental ganglia of the earthworm (*Lumbricus terrestris* L.) by immunohistochemistry, and a high level of the amine by HPLC. Comparing the intact ganglia to that of the regenerating nervous system it was found that

1. in the regenerating tissue at first serotoninergic fibers, later serotoninergic cells were detected by immunostaining;

2. during the first 3 days of the regeneration the level of serotonin is highly decreased in the all intact ganglia;

3. after day 3 the content of serotonin gradually increases and maximal level is reached by day 17;

According to our data it seems probable that there are some interrelationships between the regeneration process and the serotonin content of both the intact and regenerating nervous tissue.

Key words: nervous system, regeneration, serotonin, immunohistochemistry, serotonin assay, earthworm

Introduction

The regeneration of the central nervous system in the earthworm has been studied by many authors (CHAPRON, 1970; HERLANT-MEEWIS, 1962, 1964; ZHINKIN, 1936).

If the ventral cord of an intact earthworm is transected, or if pieces of the nerve cord are removed, or the cerebral ganglia is extirpated (without removing the head) complete morphological as well as functional regeneration takes place. FRIEDLÄNDER

* This paper is dedicated to the centennial anniversary of Prof. AMBRUS ÁBRAHÁM's birth.

(1888) regarded the regenerated ganglia as derived mainly from the remaining nervous tissue, and perhaps partly from the regeneration tissue. It is supposed, that the new nerve cells originated from small indifferent cells which retained their embryonic character in the ganglia. CORNEC et al. (1987) hypothesized that the migration of these cells is limited. In this regard, it is interesting to relate the regenerative of process of earthworm to the information we have about regeneration of nervous system in vertebrates. Migrating chicken neural crest cells were seen to accumulate and according to their location, to from organs including spinal ganglia, medulla-suprarenal glands (THIERY et al., 1977).

Since SCHARRER (1937) first described neurosecretory cells in the central nervous system of the earthworm, many investigators have studied neurosecretion of these animals. It has been suggested that some types of neurosecretory cells in the cerebral ganglion are involved in the growth and regeneration of the nervous system. Numerous questions are arise concerning the mechanisms by which molecules participate in the growing or regenerating process of the nervous system (BERG, 1984; JESSEL, 1988).

Information on the distribution and function of neuroactive compounds in the nervous system of annelids are still limited. The occurence of some neuroactive materials (serotonin, dopamine, noradrenalin, octopamine) has been established in the ventral cord of the earthworm (LENGVÁRI et al., 1992; MYHRBERG, 1967; 1972; RUDE, 1966; SPÖRHASE-EICHMANN et al., 1987a; 1987b).

Additional studies (KORITSÁNSZKY and HARTWIG, 1974; STEPHAN-DUBOIS, 1956; WELSH and MOORHEAD, 1960) dealt with he distribution and production of monoaminergic cells. The aim of the present investigation was to clarify the possible role of monoamine serotonin in the regeneration process of the nervous tissue in Annelids.

Material and methods

Adult specimens of earthworms (*Lumbricus terrestris* L., *Oligochaeta*) were collected locally (April and May) and were kept at 4 °C in moistened soil supplemented with leaves until required. The animals were sacrified either by decapitation or by anesthesia in carbonic acid solution. The ganglia were dissected out for immunohistology, electron microscopy as well as chromatography.

For immunohistological purposes 3-5 mm long pieces of whole animals were embedded into paraffin after fixation in ZAMBONI'S fixative (ZAMBONI and DE MARTINO, 1967) and 10 µm thick serial sections were cut. The sections were immunostained according to the method of STERNBERGER (STERNBERGER et al., 1970) utilizing serotonin primary antiserum developed in rabbit (1:4000; GÖRCS et al., 1985). Sheep anti-rabbit-gammaglobulin (1:300) and rabbit peroxidase-antiperoxidase complex (1:600) were obtained from Amel.

For electron microscopic studies the ganglia were prefixed in KARNOVSKY'S fixative (1965) at 4 °C for 2 h followed by fixation in 2% osmiumtetroxide and they were embedded in Durcupan (Fluka). The materials were post-stained in blocks with uranil-acetate and on sections with lead-nitrate (REYNOLDS, 1963). The sections were examined under JEOL 100B electron microscope.

Serotonin assay: earthworms were narcotized as above and isolated ganglia were dissected out. The frozen samples were homogenized ice-cold in 200 µl 0.1 n $HClO_4$ containing isoproterenol as internal standard. The homogenate were centrifuged at 10,000xg for 20 min. at 4 °C. Aliquot of the supernatant was transferred into the LCEC with a Wisp automatic injector (Waters). Tissue serotonin content was measured using a reverse-phase chromatography procedure coupled with an electrochemical detection. The LCEC system consisted of a solvent delivery system (Waters 510), electrochemical detector (Waters 460) and 745B integrator (Waters). The column, Nucleosil C18 5 µm (Macherey Nagel) were kept at 40 °C. The flow rate was 1 ml/min. The mobil phase consisted of 0.1 M sodium phosphate buffer pH 1.1 mM octane sulfonic acid, 10% methanol.

Results and discussion

A detailed description of the distribution of the monoamines in central and peripheral nervous system of *Lumbricus terrestris* has previously been given by EHINGER et al. (1971), LENGVÁRI et al. (1992), MYHRBERG (1967), RUDE (1966), SPÖRHASE-EICHMANN (1987a; b). Serotonin immunoreactive cell bodies as well as fibers occur in all parts of the central nervous system. In the present study, we determined the distribution of serotoninerg neurons of the ganglia relative to their position to the clitellum, as well as that of sub- and supraesophageal ganglia. The ganglia situated anterior to clitellum have been regarded as "typical" ganglia of the central nervous system in earthworm (GÜNTHER, 1971 a, b).

The subesophageal ganglion and the ganglia of the ventral cord contain numerous serotoninergic cells. There is a gradual decrease in the number of serotonin cell bodies relative to the position of ganglia, i.e. the more posterior the ganglon is the less serotoninergic cells are found. There are two distinct cell groups in a "typical" ganglion (Table I, Figs. 1, 2). The perikarya of these cells can be characterized by their shape and location. One cell group is situated in ventro-medial position bilaterally (Table I, Figs 1, 2). They are large cells which send their axons into the neuropil, both uni- and bilaterally. Occasionally these processes can be traced into the origin of the segmental nerves. Another cell group is located more laterally, at the level of 2nd and 3rd segmental nerves (Table I, Fig. 3). These neurons are smaller, and their processes are mostly distributed unilaterally and referred as intermediate cells. It is very important to emphasis that serotoninergic fibers of a "typical ganglion" occupy the entire neuropil offering an easily detectable framework in the ganglion. This makes serotonin immunostaining a very useful tool studying the regeneration process.

In the cerebral ganglion, the serotonin immunopositive cells are situated in two groups (Table I, Fig. 4, Table II, Fig. 1). One group is found in the dorsal part of the ganglion and the perikarya look like those which are considered neurosecretory cells according to the classical description. Another serotoninergic cell group is located more laterally (Table II, Fig. 2), close to the junction between the ganglion and the circumpharyngeal commissure. Additional serotonin positive cells are in the stomatogastric ganglia. The connectives contain large number of serotoninergic fibers. Ultrastructurally the serotoninergic neurons are large, unipolar and pyriform in

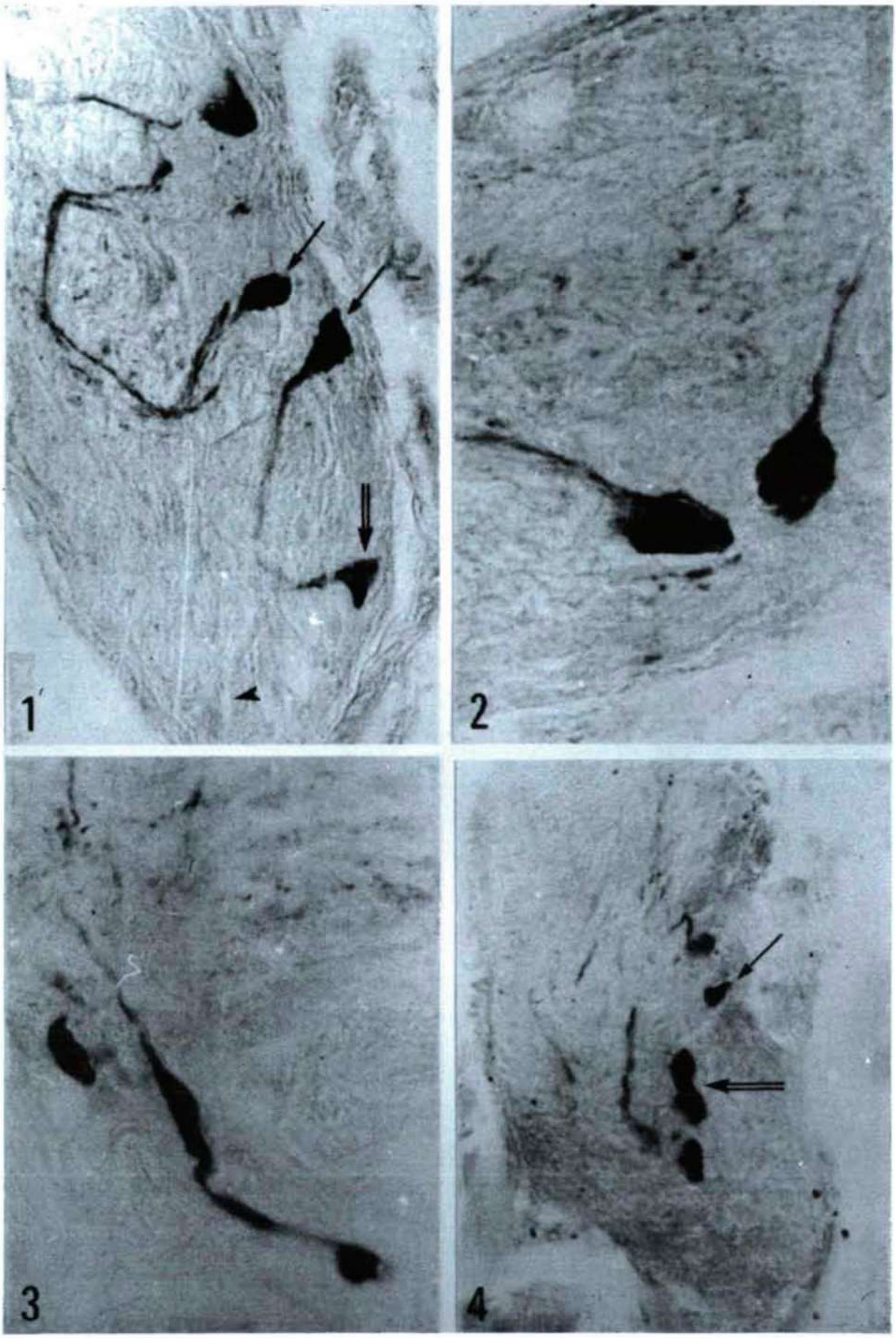
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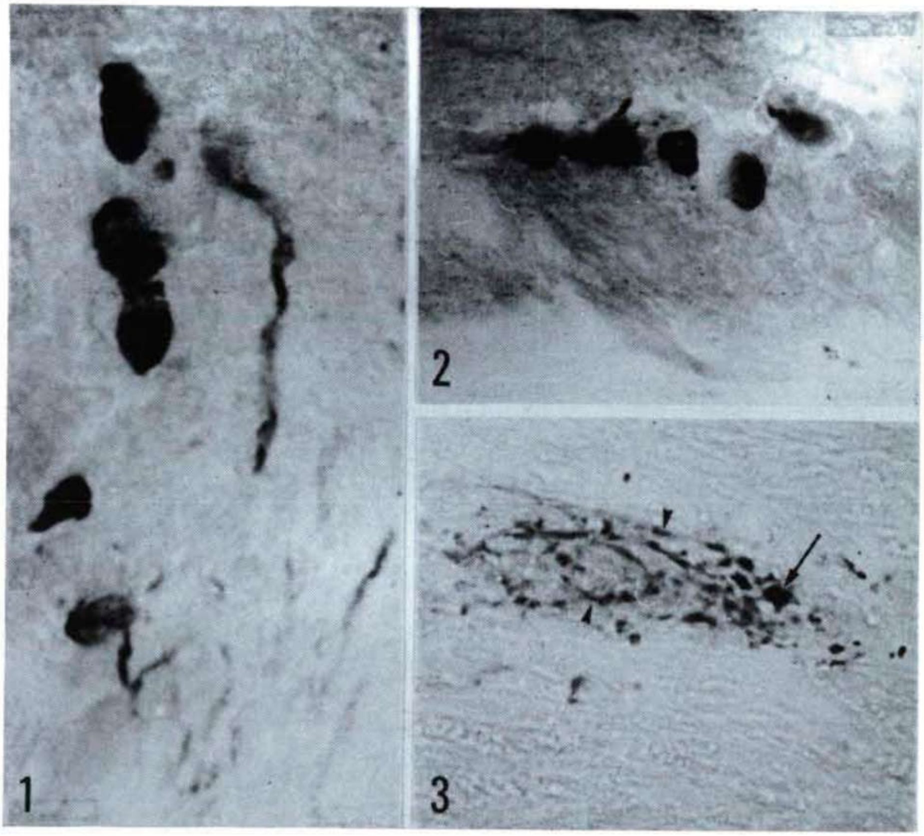
Table II. Fig. 1. Dorsally located serotoninerg perikarya of the cerebral ganglion. 900x

Fig. 2. Lateral serotoninergic cells of the cerebral ganglion. 450x

Fig. 3. Regenerated cerebral ganglion with serotoninergic cells (arrows) and fibers (arrowheads). 450x

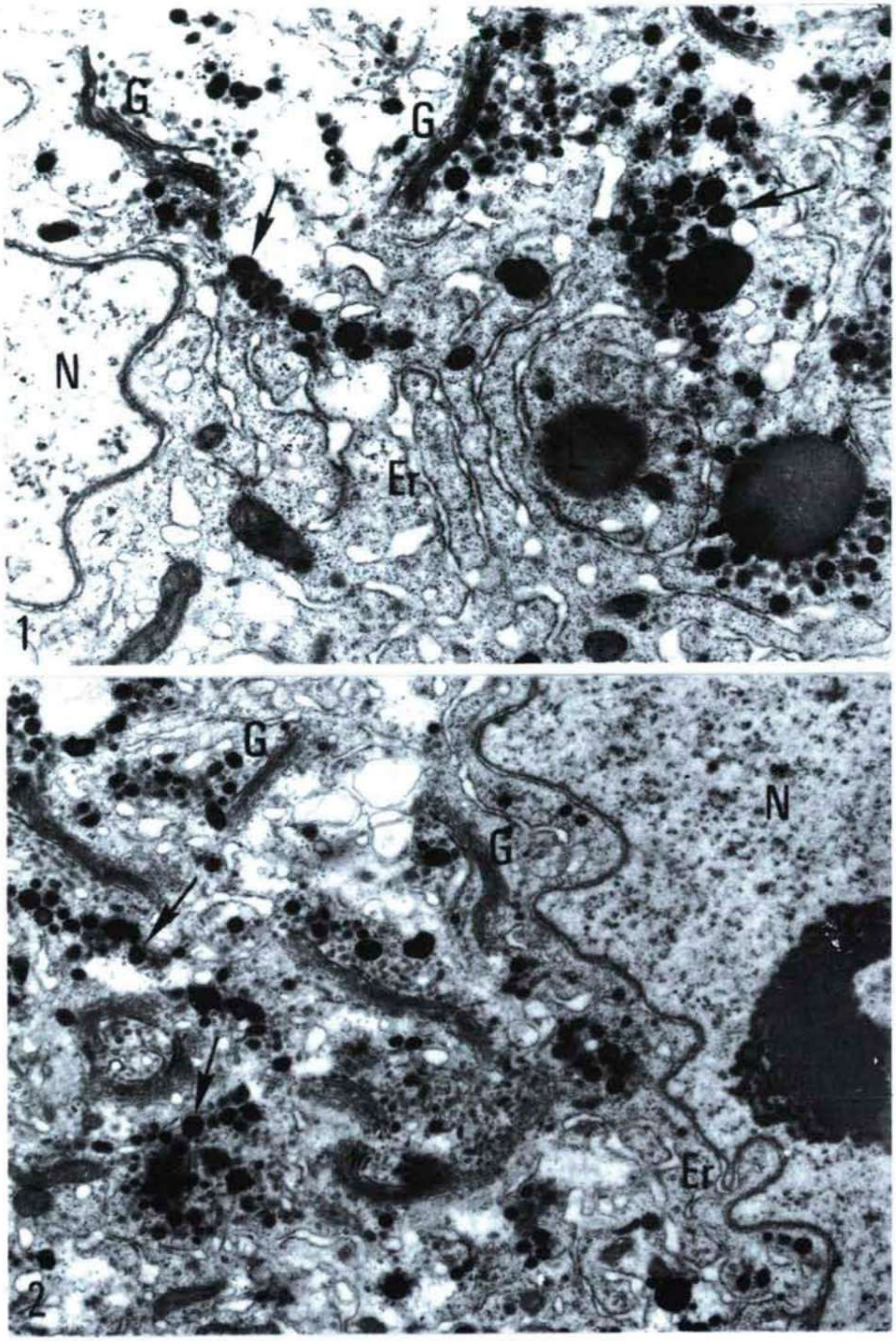


⇦ Table I. Fig. 1. Transverse section of a segmental ganglion. Note the large medioventral (arrows) and lateral (double arrow) serotoninergic cells, as well as nonreactive lateral cells (arrowheads) and serotoninergic fibers in the neuropil. 300x

Fig. 2. Medioventral serotoninergic cells with ipsilaterally projecting axons. 600x

Fig. 3. Serotoninergic cell close to the origin of 2nd and 3rd segmental nerves. 850x

Fig. 4. Horizontal section of the subesophageal ganglion showing medial (arrow) and lateral (double arrow) serotoninergic perikarya, as well as serotoninergic fibers. 300x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their shape. They have a large clear nucleus often with an irregular outline. The nucleus lies eccentrically, apposite to the axon hillock. The granular endoplasmic reticulum is mostly found in the perinuclear zone (Table III, Fig. 1). The mithocondria are scattered throughout the cytoplasm randomly. Golgi-complex and many granules and vesicles are grouped in the periphery of the cell near the axon hillock. The granules are spherical. In addition, there are large round lipid globules, either as single units or as aggregates. A characteristic feature of the intermediate cells is that their contain both dense vesicles, as well as dense granules (Table III, Fig. 2). Serotonin immunopositive cells of the cerebral ganglion are similar ultrastructurally (Table IV, Figs. 1, 2).

Earlier fluorescence microscopy studies have shown serotonin and primary catecholamines in polychaetes (ANCTIL et al., 1990; BIANCHI et al., 1988; CLARK, 1966). These substances have also been demonstrated in the leech both histochemically and with microspectrofluorometry (EHINGER et al., 1968, 1971; RUDE et al., 1969). Our earlier (LENGVÁRI et al., 1992) and present results confirm these previous findings.

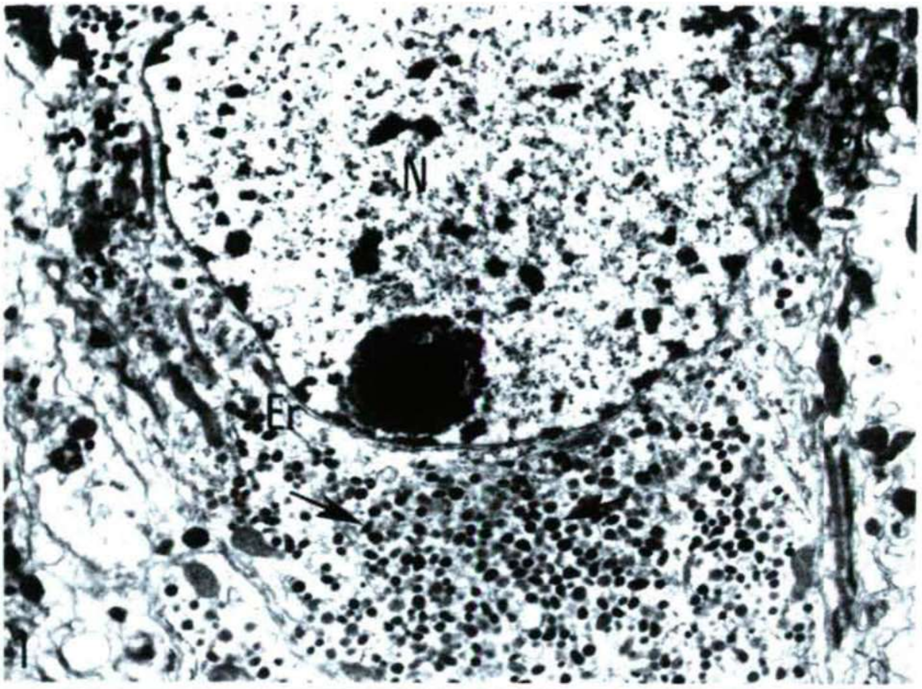
The newly formed cerebral ganglion is closer to the dorsal wall of the pharynx than to the epidermis. When the regeneration tissue disappears, the new cerebral ganglion is separated from the epidermis while it still remains in contact with the wall of the pharynx. These observations indicate that the pharyngeal epithelium is more important concerning the regeneration of the cerebral ganglion than the epidermis.

During the regeneration process of the subesophageal ganglion serotonin immunopositive fibers appear in the "scar tissue" first, followed by the emergence of serotonin positive cells (Table II, Fig. 3). The ganglion has no connective tissue capsule and the fibers run parallel in dorsoventral direction in this early phase of regeneration.

After the 10th day, the capsule develops and the general appearence of serotoninergic elements resembles to the normal ganglion. Although no quantitative analysis has been made yet, it is obvious that there are less serotoninergic elements in the regenerated ganglion than in the intact one. The cerebral ganglion emerges as two bundles originating from the anterior pole of the regenerated subesophageal ganglion, forming the connectives first. Around the 15th day they unify and give rise to a tissue mass which slowly increases, and forms the cerebral ganglion. Both in the regenerating connectives and the brain serotonin immunopositive fibers appear first, and around the 20th day, serotonin immunopositive cells are present. Their number and distribution, however differ from the normal ganglion.

⇦ Table III. Fig. 1. Medioventral serotoninergic cell of a segmental ganglion. Er: endoplasmic reticulum; G: Golgi-complex, L: lipid droplet; N: nucleus. Arrows show dense-core vesicles. 18500x

Fig. 2. Laterally located serotoninergic cell of a segmental ganglion. Er: endoplasmic reticulum; G: Golgi-complex; N: nucleus. 22500x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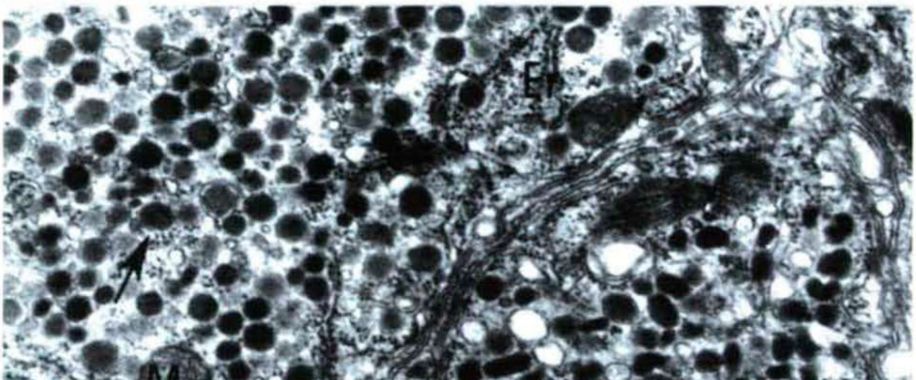
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Quantitative estimates of monoamine content of the nervous system in earthworm have been performed utilizing less accurate methods (EULER, 1949; MYHRBERG, 1967; ÖSTLUND, 1954; RUDE, 1969). It is concluded that there is more serotonin in the central nervous system of annelids and mollusca than in that of arthropods (EVANS, 1980; GARDNER and CASHIN, 1975; WELSH and MOOREHEAD, 1960).

The main purpose of the present work is to detect the changes of serotonin content during the regeneration of different parts of the nervous system in earthworm. According to our preliminary study dynamic changes occur during the regeneration process. The first period lasts for three days after removing the cerebral ganglion and it is characterized by a reduction of serotonin content in intact ganglia, although the degree of reduction varies. The explanation for this lower serotonin content can either be a reduced production or an increased utilization.

After the third day regeneration the serotonin level gradually increases until the 17th postoperative day, when it is 2.5-6 fold higher than in control specimens. By day 20 (the longest postoperative period studied so far), the serotonin level tends to decrease again.

BIANCHI et al. (1988) and DE VRIES-SCHOUMACKER (1977) presumed that the monoaminergic cells in the nervous system of the earthworm play an endocrine role. Our present finding strongly support that there is an interrelationship between the serotonin content and the regeneration process of the nervous system in earthwoms, although the exact nature of this still remains obscure.

Summary

Numerous serotoninergic neurons and fibers are present in the subesophageal as well as segmental ganglia of the earthworm, *Lumbricus terrestris*. These cells were demonstrated by immunohistochemical and electron microscopy methods. During the regeneration of the brain the new serotoninergic element (fibers and cells) and changes in the serotonin contents of the regenerating nervous tissue were studied. The new serotoninergic elements appeared similar to the normal by the 17th postoperative day. To this time the level of serotonin content gradually increases, later it decreases.

Our result support the hypothesis that serotonin affects the regeneration processes of the nervous system in the earthworm.

⇦ Table IV. Fig. 1. Dorsal serotoninergic cell of the cerebral ganglion. Er. endoplasmic reticulum; N: nucleus. Arrows show dense-core vesicles. 12000x

Fig. 2. Lateral serotoninergic cell of the dorsal ganglion. Er: endoplasmic reticulum; M: mitochondrium; N. nucleus. Arrows show dense-core vesicles. 24000x

Acknowledgements

The authors wish to thank Dr. T. GÖRCS for providing the serotonin antiserum, and Mrs. EDIT KISS for her skillful technical assistance.

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