Time-course of the regeneration of the earthworm cerebral ganglion with special reference to serotonergic elements

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SUMMARY

In the present study we give a detailed description of the time-course of regeneration following the removal of the cerebral ganglion in the earthworm, *Eisenia fetida*. Cerebral ganglia were removed by cutting the circumpharyngeal connectives and regeneration was examined at different time intervals by serotonin immunohistochemistry. The number of serotonergic fibers gradually increased between days 3-6, rebuilding the neuronal network of the cerebral ganglion. By day 10, the scar tissue was filled with an interwoven fiber network, which clearly outlined the central neuropil of the cerebral ganglion. The first immunoreactive nerve cells appeared on the 25th day following the removal of the cerebral ganglion. According to our observations, the cerebral ganglion regains its original structure 70 days after removal. It can be suggested based on our results that the cerebral ganglion mainly regenerates from the subesophageal ganglion through the circumpharyngeal connectives.

Key Words: Earthworm – Regeneration – Cerebral ganglion – Serotonin

INTRODUCTION

The annelid nervous system is a commonly used model in invertebrate neuroscience research. The central nervous system contains a relatively small number of neurons, and besides having a segmentally arranged ventral cord, in this phylum centralization first occurs during phylogenesis in the form of the appearance of a cerebral ganglion (Bullock and Horridge, 1965). Morphological and functional regeneration takes place not only if segments from the ventral cord are extirpated, but also after removal of the cerebral ganglion (Aros and Vigh, 1962; Bullock and Horridge, 1965). This remarkable regeneration capacity of the annelid nervous system has long been known and drew the attention of early neuroscientists (Iwanow, 1903; Krecker, 1923; Bailey, 1930). Non-specific methods for the detection of the neurosecretory elements were used to analyze the regeneration process of the cerebral ganglion (Aros and Vigh, 1962; Koritsanszky and Hartwig, 1974). No data, however, have been made available on the regeneration of the earthworm nervous system since specific immunohistochemical methods came into general use.

The capacity of the invertebrate nervous system to regenerate provides models for studying mechanisms of neural regeneration (Bullock, 1984; Roger et al., 1992; Scott et al., 1997). Regarding annelids during the past decade, the work has focused on the regeneration of the giant axon of the earthworm ventral cord, applying different methods and compounds designed to improve the fusion and growth of the lesioned or transplanted giant axon (Vining and Drewes, 1985; Yogev et al., 1991; Todorov et al., 1992;

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Krause et al., 1994; Lore et al., 1999). Another point of interest has been the study of regeneration in tissue cultures, the major focus being on another annelid class, the leech *Hirudo medicinalis* (Townsel and Thomas, 1987; Drapeau and Sanchez-Armass, 1988). One group of researchers has reported alterations of polyamine levels in posterior regeneration of the earthworm (Hamana et al., 1995). To our knowledge, very little or no attention has been paid recently to the exceptional regeneration capacity of the earthworm cerebral ganglion.

Serotonin is one of the major neurotransmitters in the nervous system of invertebrates, including annelids (Myhrberg, 1967; Hernádi et al., 1989; Gardner and Walker, 1982). The distribution of serotonin-containing nerve cells and fibers has been thoroughly investigated and described in detail in both the central and the peripheral nervous systems of earthworms using immunohistochemistry (Spörhase-Eichmann et al., 1987a, b; Fujii and Takeda, 1988; Csoknya et al., 1992; Lengvári et al., 1992; Reglődi et al., 1997). Serotonin has also been reported to play a role in regeneration processes in invertebrates (McCobb et al., 1988; Murrain et al., 1990; Villar and Schaeffer, 1993; Chiasson et al., 1994; Baker and Croll, 1996; Goldberg, 1998). According to our preliminary data, a correlation exists between the regeneration and the serotonin content of the nervous system (Csoknya et al., 1993).

In the present study we offer a detailed description of the time-course of the regeneration process following removal of the earthworm cerebral ganglion, using a highly specific serotonin immunohistochemical method. These results should serve as a basis for future investigation of basic neuroregeneration, using the earthworm as a model (Bullock, 1984).

Materials and Methods

Adult specimens of the earthworm, *Eisenia fetida* were purchased from a local supplier. The animals were kept in fresh moist soil until use. The earthworms were anesthetized in CO_2-containing water, and the cerebral ganglia were removed by cutting the circumpharyngeal connectives at their origins under operation microscope, through a small dorsal incision between the 2nd and 3rd segments.

After the operation, the animals were kept in fresh moist soil at room temperature for different periods of regeneration. Regeneration of the cerebral ganglion was examined daily during the first 10 days; every two days between days 10-20; every 5 days until day 45, and then every 10 days till the 70th postoperative day. Three animals were sacrificed at each time-point. The first 6 segments of the animals were removed and fixed in picric acid-formaldehyde fixative. For a detailed description of the immunostaining, we refer to our previous works using the same antiserum (Lubics et al., 1997; Reglődi et al., 1997). Briefly, serial 10 µm cryostate sections were stained with serotonin antiserum by the peroxidase-antiperoxidase method (double peroxidase-antiperoxidase method). The serotonin antiserum was a generous gift from Dr T. Görcs, and is a well defined serum that does not show cross-reactivity with any known antigen (Görcs et al., 1985). Although the distribution of the serotonergic elements is well known from previous descriptions (Spörhase-Eichmann et al., 1987a, b; Fujii and Takeda, 1988; Csoknya et al., 1992; Lengvári et al., 1992; Reglődi et al., 1997), the same segments from intact animals (n=5) were removed and processed parallel with the regenerating samples. Also, for general orientation, some sections were stained with haematoxilin-eosin.

Results

Mortality was highest during the first postoperative week: 12% of the operated animals died during this period. After 1 week of survival, mortality decreased to 3%. Examination of the HE-stained sections revealed that by the first day of regeneration following removal of the cerebral ganglion, the skin wound had already closed and the epidermal tissue was completely regenerated by day 3. The muscle of the body wall recovered only by day 7.

At the site of injury, the stump of the circumpharyngeal connectives was thickened and scar tissue filled the site of the removed ganglion. The scar tissue was in continuity with the healing epidermis and the underlying muscular wall on the dorsal side, and was strongly attached to the pharyngeal wall on the ventral surface. Serotonergic fibers grew out from the connectives towards the scar tissue, which were clearly visible from day 2 (Fig. 1). Between days 3-6, the number of serotonergic fibers gradually increased, outlining the rebuilding neuronal network of the cerebral ganglion (Fig. 2). From day 6, varicosities were also observed in the fiber network. The first group of fibers appeared in the ventral part of the regenerating cerebral ganglion, in continuity with the connectives. The first crossing fibers appear in the posterior part of the ganglion, close to the subesophageal ganglion. By day 8, these ventral fibers were observed to cross to the contralateral side in the ganglion (Figs. 3a, b, c). Fibers outlining the dorsal part appeared by day 9. By day 10, the scar tissue in the site of the cerebral ganglion was filled with an interwoven fiber network, which clearly outlined the central neuropil of the cerebral ganglion (Fig. 4).

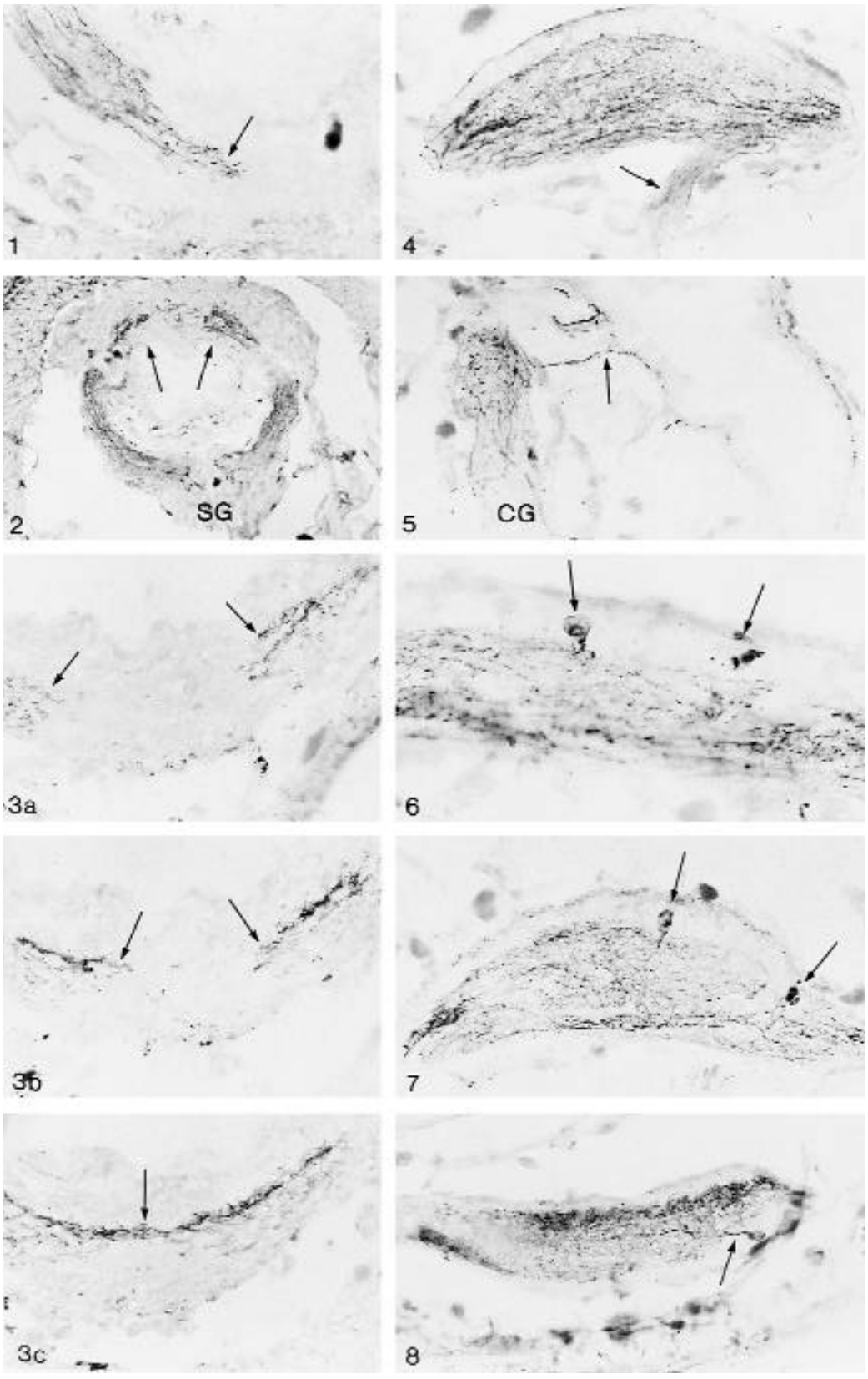

Figure 1.- 2nd day: serotonergic fibers grow out from the connectives into the scar tissue (*arrow*). x200.
Figure 2.- 5th day: rebuilding neuronal network (*arrows*) of the cerebral ganglion. (SG: subesophageal ganglion). x100.
Figure 3.- 8th day: regenerating fibers in the cranial (a) and middle (b) part of the cerebral ganglion (*arrows*). Crossing fibers appear first in the caudal part (c) (*arrow*). x200.
Figure 4.- 12th day: the cerebral ganglion is filled with interwoven fiber network. Fibers can be followed from the caudal part of the cerebral ganglion to the pharyngeal wall (*arrow*). x200.
Figure 5.- Serotonergic fibers from the dorsal surface of the pharynx (*arrow*) to the growing cerebral ganglion (CG). x200.
Figure 6.- 25th day: first immunoreactive cells appear near the origin of the connectives in the caudal cell group (*arrows*). x400.
Figure 7.- 40th day: serotonergic cells in the dorsal cell mantle of the regenerating cerebral ganglion (*arrows*). x200.
Figure 8.- 45th day: immunoreactive cell with its process entering the central neuropil (*arrow*). x200.

The pharyngeal wall also showed distinct changes as regards serotonergic fibers. These were strongly stained on the dorsal surface of the pharynx after removal of the cerebral ganglion, and the ventral surface showed no immunoreactivity. Immunoreactive fibers on the dorsal surface of the pharyngeal wall could be followed to the growing cerebral ganglion from day 5 (Figs. 4, 5). Some of these fibers were also connected to the stomatogastric ganglia or the connectives, while others coursed directly towards the scar tissue. Serotonergic fibers first appeared on the ventral side only at the level of the first segmental ganglion posterior to the subesophageal ganglion. Immunoreactive fibers in the prostomial nerves were observed from day 6.

The first immunoreactive nerve cells appeared relatively late: on the 25th day following removal of the cerebral ganglion. Immunopositive cells first appeared in the caudal cell group, near the origin of the connectives (Fig. 6), followed by cells in the dorsal cell mantle (Fig. 7). The number of serotonergic nerve cells gradually increased in both cell groups, and by day 70 the number of neurons reached the number found in intact ganglia. The caudal cell group contained neurons of 10-15 µm, whose processes could be followed to the connectives. The dorsomedial and dorsolateral cell groups contained neurons of 15-25 µm with processes entering the central neuropil (Fig. 8). The connective tissue capsule around the cerebral ganglion was completed by day 40, and hence the original shape of the ganglion was regained.

The regeneration of the cerebral ganglion was accompanied by changes observed in the subesophageal ganglion. During the first 10 days, the number of cells increased from 40 to 70 (Fig. 2). After day 10, 20-30 immunopositive cells were observed in the subesophageal ganglia. The original number (50) was regained at the end of the observation period.

DISCUSSION

In the present study, we report a detailed description of the regeneration of the cerebral ganglion of the earthworm, with special reference to the serotonergic elements. The remarkable ability of the earthworm cerebral ganglion to regenerate has long been known and drew the attention of neuroscientists at the beginning of the century (Iwanow, 1903; Krecker, 1923; Bailey, 1930). Decades later, two articles were published on the regeneration process (Aros and Vigh, 1962; Koritsanszky and Hartwig, 1974), but no recent data are available. In the past 10 years, investigators have focused on the regeneration of the giant axon of the ventral cord in annelids, and have used this as a model to study substances that promote this process (Gardner and Walker, 1982; Murrain et al., 1990; Yogev et al., 1991; Todorov et al., 1992). Examination of the regeneration of the earthworm cerebral ganglion should also serve as a model for further regeneration experiments (Bullock, 1984).

We found that morphological regeneration of the cerebral ganglion takes place rapidly after the removal of the ganglion. Forty days after the operation, the cerebral ganglion regained its original shape and structure, and the original cell number is reestablished by day 70. This is in accordance with the findings of Aros and Vigh (1962), who described the regeneration of the cerebral ganglion using classical histological stainings. Starting from only a few days after the removal of the cerebral ganglion, serotonergic fibers were gradually restored first in the ventral and then in the dorsal part of the regenerating ganglion. Most of the fibers grew out from the connectives, although a smaller portion of the fibers seemed to arise from the pharyngeal wall. During the first 2 weeks, the serotonergic nerves innervating the body and buccal wall (prostomial nerves) as well as the stomatogastric plexus were also reestablished.

In intact animals, the number of serotonergic nerve cells observed in the cerebral ganglion was 70-80, which is in accordance with the findings of Spörhase-Eichmann et al. (1987a,b). During regeneration, serotonergic perikarya appeared from day 25, and the original cell number was reached at the end of the examination period. In contrast, the number of serotonergic neurons in the subesophageal ganglion was lower than in intact animals throughout the entire observation period. In intact animals, the subesophageal ganglion contains 100-130 serotonergic nerve cells (Spörhase-Eichmann et al., 1987b). Our observations show that after the removal of the cerebral ganglion, the number of cells in the subesophageal ganglion is strongly reduced, followed by a slight increase during the first 10 days. The original cell number (after removal of the cerebral ganglion) is regained at the end of the observation period, but does not reach the number found in intact animals. In a preliminary study, we reported that the serotonin content is reduced during the first half of the regeneration in the intact ganglia as measured by HPLC (Csoknya et al., 1993). Similar observations have been made in snails (Chiasson et al., 1994; Baker and Croll, 1996), where serotonin is depleted in the buccal ganglia after lesion to the cerebrobuccal connectives. The authors claimed that this loss of serotonin may play a role in regeneration, as had been suggested in numerous reports documenting the inhibitory effect of serotonin on regenerating process in other species (McCobb et al., 1988; Murrain et al., 1990).

The origin of nerve cells forming the regenerating cerebral ganglion has long been a question of debate (Aros and Vigh, 1962; Bánvölgyi et al., 1994). It has been argued that regenerating nerve cells may arise from undifferentiated cells beneath the epithelium of the intact ventral ganglia; from mesodermal cells of the connective tissue sheaths, or from the epithelial lining of the pharyngeal wall (rev: Aros and Vigh, 1962; Bánvölgyi et al., 1994). According to the observations of Aros and Vigh (1962), the cerebral ganglion regenerates from the ventral nerve chain and the remaining stump of the circumpharyngeal connectives. The observed hypersecretion in the subesophageal ganglion suggests that the subesophageal ganglion temporarily takes over the secretory function of the cerebral ganglion and/or secretes substances necessary for the regeneration of the cerebral ganglion (Aros and vigh, 1962). Results from our previous work on the number of ganglionic cells in intact and regenerating cerebral ganglia are in accordance with these observations: during the first half of the regeneration process, the number of nerve cells shows a significant increase in the first intact ganglion (Bánvölgyi et al., 1994).

Our present findings support the above mentioned hypothesis on the origin of the regenerated nerves: from the first days of regeneration, an increasing number of serotonergic fibers grow from the circumpharyngeal connectives to the scar tissue, finally building up the neuronal network of the cerebral ganglion. According to these observations, it can be suggested that the serotonergic system of the cerebral ganglion mainly regenerates from the connectives. The changes found in the subesophageal (first intact) ganglion imply that the subesophageal ganglion plays a role in the regenerating process. Based on our findings, it is suggested that undifferentiated cells migrate from the subesophageal ganglion to the rebuilding cerebral ganglion, which explaines the changing cell number in the subesophageal ganglion and the first stained neurons near the connectives. Most probably these cells do not store serotonin in high quantity to be able to show the perikarya by immunostaining before day 25. This is in accordance with most previous observations (Aros and Vigh, 1962; Bánvölgyi et al., 1994). However, the ventral part of the growing cerebral ganglion is in close contact with the pharyngeal wall during the first 3 weeks of regeneration, and serotonergic fibers connect the pharyngeal wall to the ventral part of the cerebral neuropil as well as the stomatogastric system. Studies on the organization of the intact cerebral and stomatogastric system show that the ventral part of the cerebral ganglion partly innervates the pharyngeal wall through the stomatogastric system (Bullock and Horridge, 1965; Reglődi et al., 1997). Based on this organization pattern and our present observations, it cannot be excluded that a minor part of the *Eisenia* cerebral ganglion could regenerate from the pharyngeal wall, as has been suggested by some authors (Aros and Vigh, 1962). Our present results could serve as a basis for future investigation of basic neuroregeneration, and further studies are necessary to elucidate the origin of the regenerating cerebral ganglion.

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