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REORGANIZATION OF THE GABAergic SYSTEM FOLLOWING BRAIN EXTIRPATION IN THE EARTHWORM (*EISENIA FETIDA,* ANNELIDA, OLIGOCHAETA)*

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The reorganization of the GABAergic system was studied by means of immunohistochemistry after the symmetrical and asymmetrical (unilateral) extirpation of the brain of the annelid *Eisenia fetida.* GABA-immunoreactive neurons were first observed in the wound tissue on the 3rd postoperative day. Thereafter the number of labelled cells gradually increased, and by postoperative days 76–80 all GABA-immunoreactive cells (approx. 140 neurons) could be found in their final positions in the symmetrically regenerated brain. After asymmetrical brain extirpation, nearly all cells (70–75) could be detected in the regenerating hemisphere by postoperative days 50–56. In the early stages of the asymmetrical regeneration of the brain, more GABAergic cells were concentrated dorsally and laterally in the preganglion than during the symmetrical type of regeneration.

In both types of regeneration, the immunoreactive neurons in the regenerated brain originated in part from undifferentiated neuroblasts situated in different parts of the body, and in part from dividing neurons localized mainly in the pharyngeal nerve plexus.

Both exogenous GABA and picrotoxin, applied during the early stages (days 10–12) of brain regeneration, inhibited the development of the wound tissue and the migration of the neuroblasts and the enteric neurons. At the same time, exogenous GABA application accelerated the proliferation of the pharyngeal neurons. No effect on the process of regeneration could be demonstrated when exogenous GABA and picrotoxin were given together.

Keywords: Brain regeneration – GABA immunocytochemistry – GABA and picrotoxin treatments – earthworm – oligochaetes – *Eisenia fetida*

INTRODUCTION

A characteristic feature of some lower invertebrates (e.g. cnidarians, flatworms and annelid oligochaetes) is their regenerative capability [14, 19, 22, 34]. This process includes not only asexual multiplication, but the regeneration of different organs and tissues. It is generally accepted that the regenerative capacity has gradually decreased during evolution [4, 17].

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The regeneration of the nervous elements in lower invertebrates includes not only the regeneration of axons [18, 26], but also the renewal of neural perikarya in the central nervous system (CNS) itself [7, 10, 15, 21, 26, 38]. The origin of the new neurons is a fundamental question. These neurons might derive from stem cells or neuroblasts or from the proliferation of differentiated neurons [9, 16, 27]. Only a few data are available on the renewal of earthworm neurons following injury or during the regeneration of a ganglion [10, 21, 43].

The process of regeneration is influenced by both external (e.g. temperature [35]) and internal factors (e.g. the type of the neuron, the development of the animal and wound hormone) [2, 16, 21, 28, 37, 38, 39]. The activity of neurosecretory cells has been shown to increase during regeneration [25]. Neurosecretory materials such as neuropeptides and neurohormones play regulatory roles in the reorganization of the CNS [25]. Neuropeptides have also been shown to activate the neuroblasts [2], and to have a stimulatory or inhibitory effect on cell proliferation [5, 11].

Gamma-aminobutyric acid (GABA) has been found to be among the first neuroactive substances which appears during the embryonic development of mammals, and to have an effect on the growth of the neurites [24, 31, 42]. GABA has also been proven to stimulate neuroblast proliferation [1]. On the other hand, there are no data on the possible role of GABA in the regeneration of the CNS of invertebrates. The presence of GABA in the CNS of *Lumbricus terrestris* has been demonstrated biochemically [12]. In the course of an earlier study, we mapped the distribution of the population of GABA-immunoreactive (IR) neurons in the CNS of the earthworm *Lumbricus terrestris* [40], demonstrating that GABA-IR neurons are present in all parts of the CNS. The total number of GABA-IR neurons in the brain is lower than in other parts (ventral ganglia) of the CNS. In the present study, we have analysed the reorganization of the GABAergic system during brain regeneration, and describe how and when the population of GABA-IR neurons reappears in the regenerating brain of the earthworm *Eisenia fetida,* following either total or unilateral brain extirpation. We have also investigated how exogenously applied GABA and picrotoxin (PTX) influence the process of regeneration and the reorganization of the GABAergic system.

MATERIALS AND METHODS

Adult specimens of *Eisenia fetida* (Annelida, Oligochaeta) were used for this study. Following anaesthesia in carbonated water, the whole or a hemisphere of the cerebral ganglion (CG, brain) was dissected. After operation, the animals were kept in a climate box at standard temperature (10 °C) and 60% humidity. The anterior part of the body (segments 1–10) was fixed on postoperative days 3, 5, 10, 15, 20, 34, 56, 76 and 80. Altogether 15–20 animals with both types of brain extirpation were used on each postoperative day.

For GABA immunostaining, the anterior body segments of the animals were fixed in a mixture of 15 ml saturated picric acid, 5 ml 25% glutaraldehyde and 0.2 ml

glacial acetic acid [6] for 5 h at 4 °C. Following fixation, the body segments were washed for 3×20 min in phosphate-buffered saline (PBS) containing 1% Triton X-100 (pH 7.4). The fixed materials were dehydrated in a graded ethanol series and embedded in Paraplast. Sections 8 μm in thickness were cut serially and mounted on slides coated with chrome alum gelatine.

Immunocytochemistry

After deparaffination and rehydration, the sections were treated as follows: i) 3×20 min washes in PBS-Triton X-100; ii) preincubation for 1 h in 10% normal goat serum diluted in PBS containing 0.25% bovine serum albumin, 0.25% Triton X-100 and 0.01% Na-azide; iii) incubation for 16 h with a rabbit, polyclonal anti-GABA antiserum (Sigma) diluted 1 : 5000; iv) incubation for 1 h with biotinylated goat anti-rabbit IgG (Sigma) diluted 1 : 20; and v) incubation for 1 h with ExtrAvidin complex (Sigma) diluted 1 : 20.

All antisera were diluted in PBS containing 0.25% Triton X-100 and 0.25% bovine serum albumin. Incubations with all antisera were performed at room temperature and each incubation was followed by 3×20 min washes in PBS-Triton X-100.

To develop the peroxidase reaction, sections were incubated for 10 min in 0.05 M TRIS-HCl buffer containing 0.05% 3,3'-diaminobenzidine and 0.01% H_2O_2. Following the diaminobenzidine reaction, sections were dehydrated in graded ethanol, cleared in xylene and coverslipped in Canada balsam. Immunoreactivity was never detected when the same procedure was applied but the primary antibody was omitted. The specificity of the antiserum was previously tested by Telkes et al. [40].

Cell counting

Serial sections of both intact and regenerated brain were stained with toluidine blue solution [20]. Only nerve cells containing a well-visible nucleus were counted.

Pharmacological treatments

Experiments on the effects of exogenous GABA were carried out according to the following protocol. Two μl of 100 μM GABA (Sigma) diluted in Ringer's solution [32] was injected twice into body segment 4 at the site of the removed CG. The first injection followed immediately after the operation, while the second one was given on postoperative day 3.

Another group of earthworms was given 2 μl 10 μM $GABA_A$ receptor antagonist picrotoxin (PTX) (Sigma) diluted in Ringer's solution, similarly as described for GABA treatment.

To a third group of earthworms the same quantities of exogenous GABA and PTX were given together.

Following the second injection of each treatment, on postoperative day 6, the anterior body part of the animals was fixed and GABA immunostaining was carried out on the paraffin sections. Six series of each treatment were performed.

RESULTS

Regeneration of GABA-IR elements in the brain

After the removal of the entire brain or one hemisphere, the stages of regeneration are similar. First, at the site of the operation, both layers of the body wall (epidermis and muscle layers) appear. This process lasts for 1–2 days.

From postoperative day 2, primary loose wound tissue develops at the site of the dissected brain or brain hemisphere. At the same time, GABAergic elements appear in it (Figs 1A and 3A). In the case of symmetrical regeneration, the wound tissue containing GABA-IR elements begins to separate into two symmetrical halves on postoperative day 3 (Fig. 1A). In the course of both types of regeneration, labelled fibres can be observed growing towards the wound tissue, in addition to GABA-IR cell bodies. The majority of these fibres originate from the neurons located in the subesophageal ganglion (SOG).

In the case of symmetrical brain regeneration, the wound tissue is gradually isolated by a capsule from postoperative days 25–27 (Fig. 1B); this remains open on the ventral side until the end of regeneration (postoperative day 80). In asymmetrical regeneration, this capsule develops already from postoperative day 16 (Fig. 3A). Following the appearance of the capsule, new GABA-IR neurons of the preganglion seem to enter either from the SOG via the circumpharyngeal connectives, or from the pharyngeal plexus (Figs 4A, C).

On postoperative day 56 of symmetrical brain regeneration, 40 dorsomedial and 16 dorsolateral GABA-IR cells can already be found in their final positions (Fig. 1C), and the lateral GABA-IR cell groups consisting of 24 cells have also begun to be formed (Figs 2A, B).

Unilateral brain regeneration is finished by postoperative day 56. On day 56 after unilateral brain extirpation, all GABA-IR cell groups with the final cell number can

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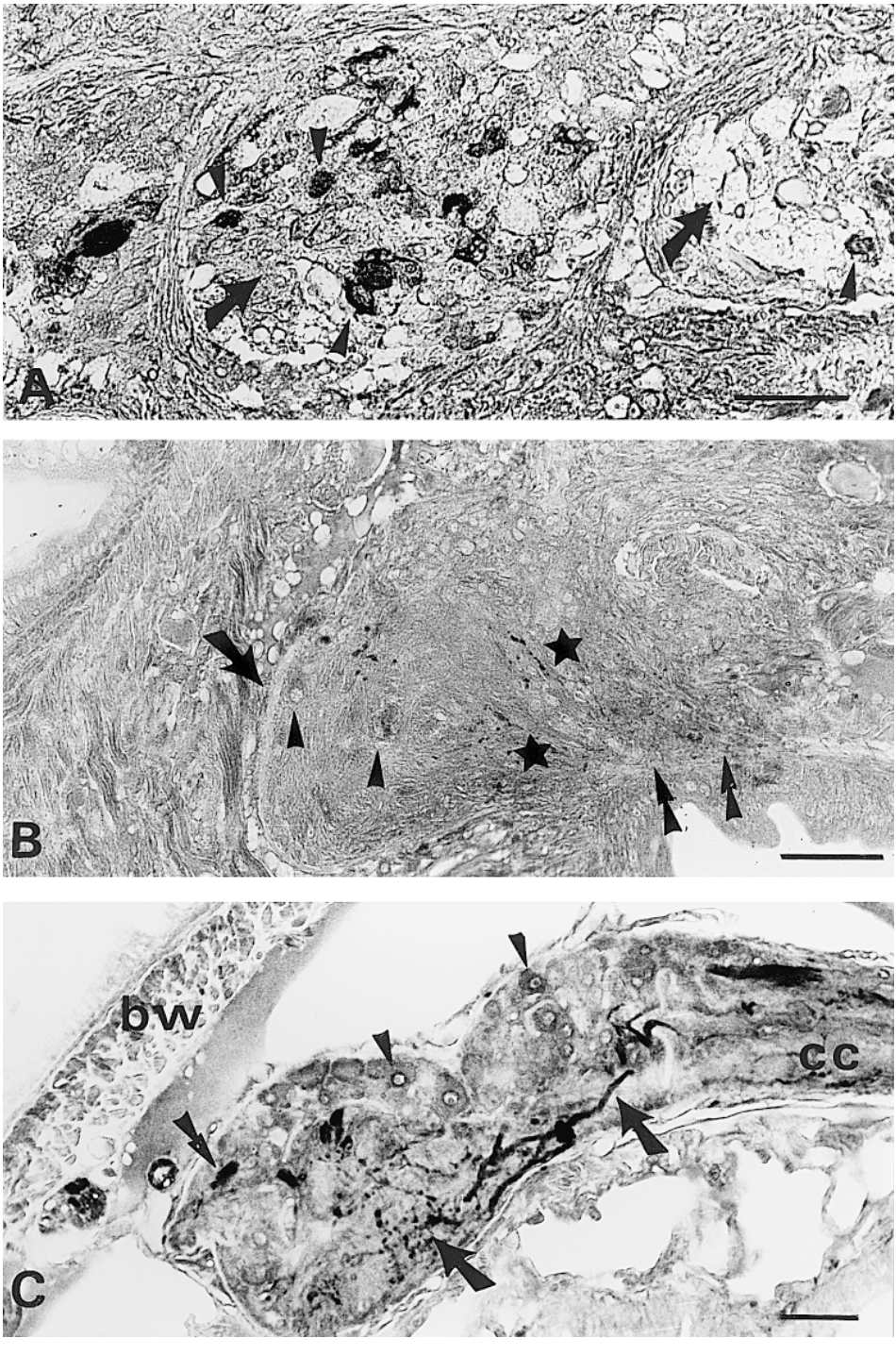
Fig. 1. Regeneration of the GABA-IR system of the brain in *Eisenia fetida* after total (symmetrical) brain extirpation. **A:** On postoperative day 3, several GABA-IR neurons (arrowheads) are present in the wound tissue (arrows), already separated into two parts. bw: body wall. Scale bar: 70 µm. **B:** A capsule (arrow) is formed dorsally and laterally around the preganglion between postoperative days 25 and 27. The preganglion remains open on its ventral side (double arrowheads). Arrowheads: GABA-IR neurons within the preganglion; asterisks: GABA-IR fibres. Scale bar: 70 µm. **C:** On postoperative day 56, GABA-IR neurons are located in the dorsomedial (arrowheads) and dorsolateral (double arrowhead) cell groups. Arrows: GABA-IR fibres; bw: body wall; cc: circumpharyngeal connective. Scale bar: 70 µm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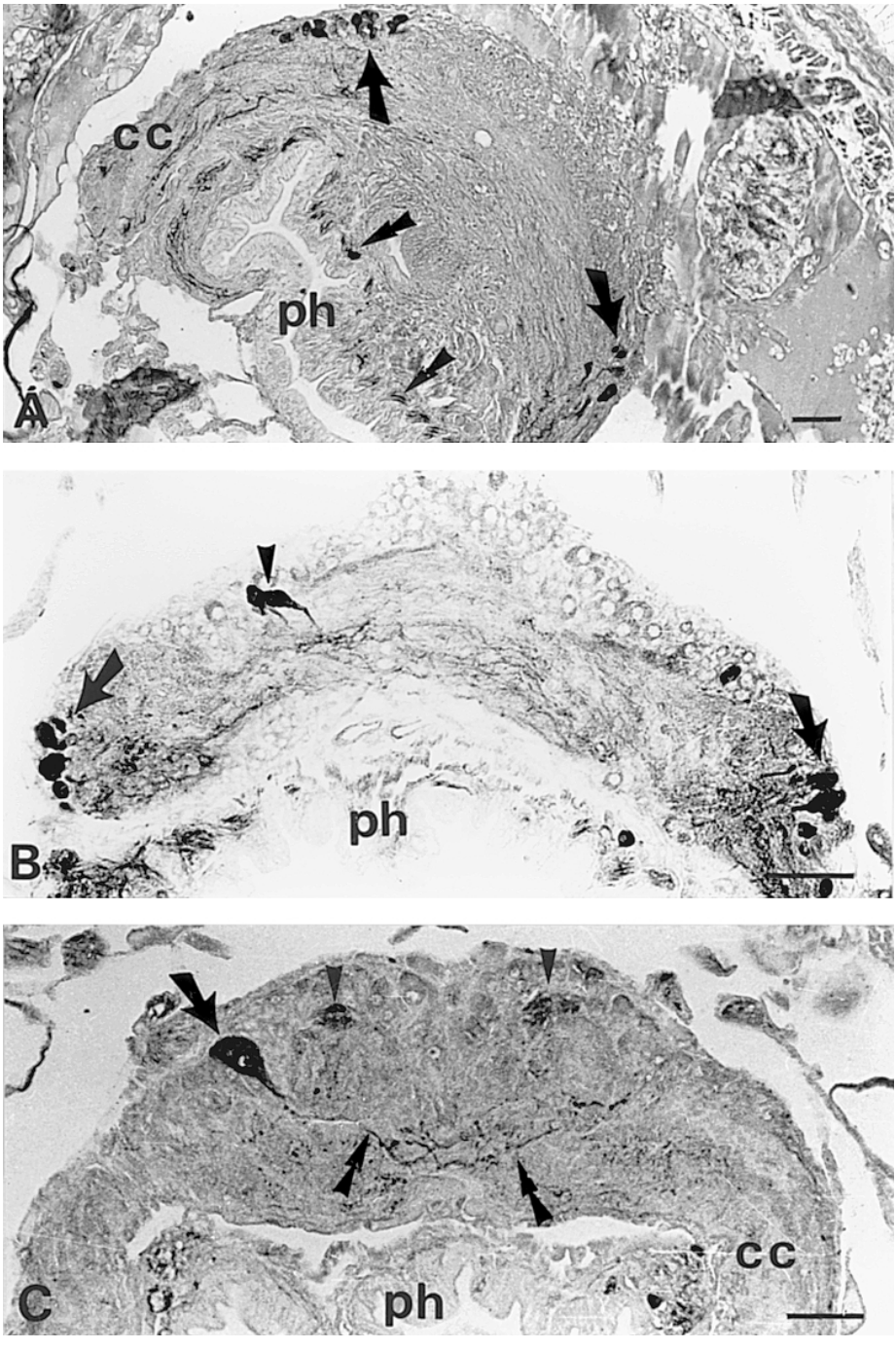

Fig. 2

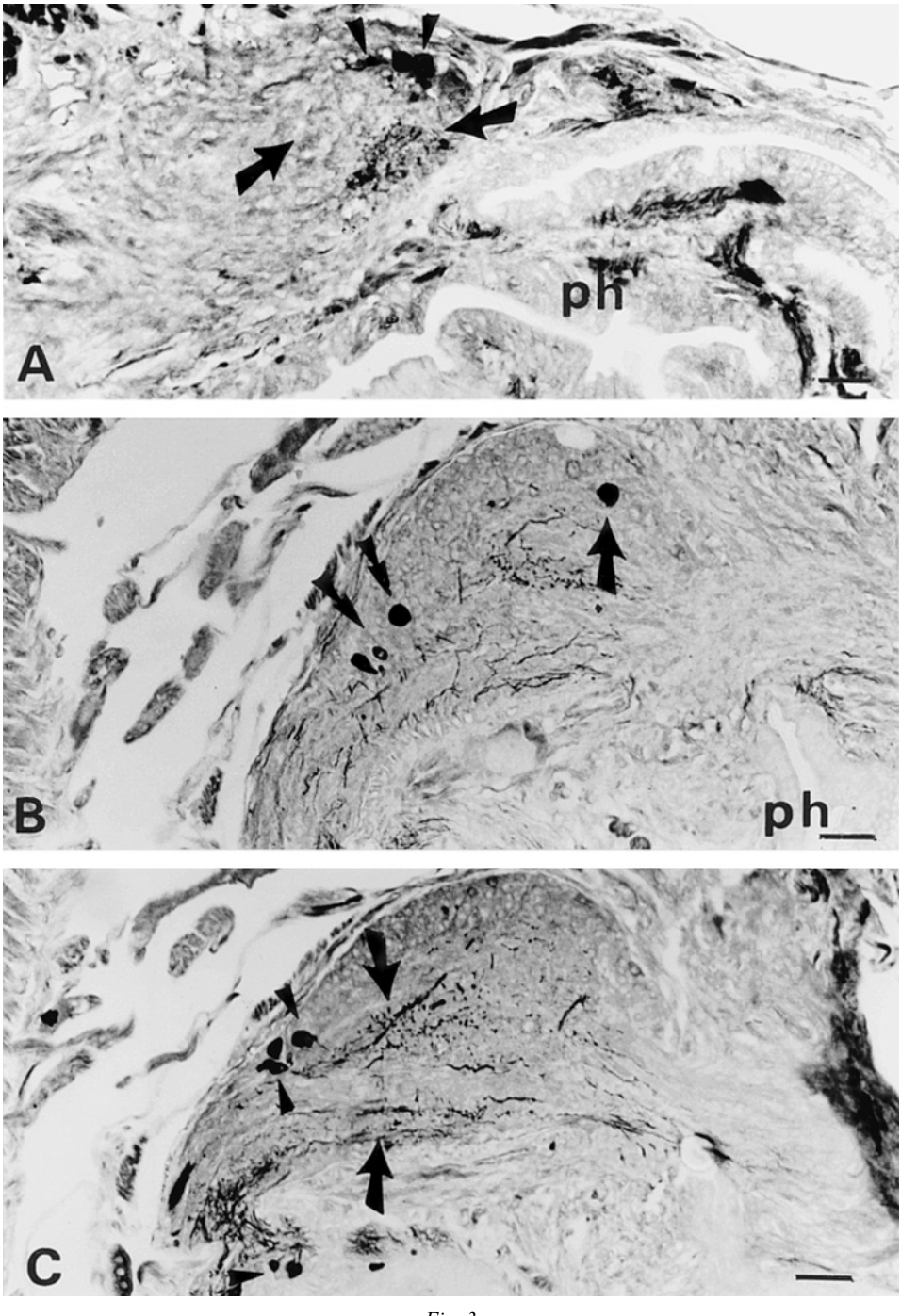

Fig. 3

Fig. 2. Regeneration of the GABA-IR system following total brain extirpation in *Eisenia fetida.* **A:** The lateral GABA-IR neurons (arrows) begin to form from postoperative days 56–80. Double arrowheads: pharyngeal plexus; cc: circumpharyngeal connectives; ph: pharynx. Scale bar: 70 μm. **B:** On postoperative day 56 the lateral GABA-IR cell groups (arrows) are formed. Arrowhead: dorsolateral GABA-IR neurons; ph: pharynx. Scale bar: 70 μm. **C:** A few dorsomedial (arrowheads) and dorsolateral (arrow) GABA-IR neurons can be found in the final positions by the end of regeneration. Double arrowheads: IR fibres in the central commissure; cc: circumpharyngeal connective; ph: pharynx. Scale bar: 70 μm

Fig. 3. Reorganization of the GABAergic system after unilateral (asymmetrical) brain extirpation. **A:** On postoperative day 3, wound tissue (arrows) with a few GABA-IR neurons (arrowheads) appears. ph: pharynx. Scale bar: 70 μm. **B:** By postoperative day 56, dorsomedial (arrow) and dorsolateral (double arrowhead) GABA-IR cells can be observed. ph: pharynx. Scale bar: 70 μm. **C:** By the end of gangliogenesis (postoperative day 56), commissures (arrows) containing numerous GABA-IR fibres of the brain can be observed. Arrowheads: GABA-IR neurons. Scale bar: 70 μm

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be observed in the newly formed brain hemisphere (Figs 3A, B and 6): 20 GABA-IR neurons in dorsomedial, 9 in dorsolateral, 13 in lateral, and 12 in ventrolateral positions; at the origin of the circumpharyngeal connectives, 19 cells are seen (Fig. 3B).

In the case of symmetrical brain regeneration, the total number and the distribution of GABA-IR neurons on postoperative day 80 are similar to those observed in the intact brain (Figs 2C, 6 and 7). In the intact brain of *Eisenia fetida*, a posterolateral GABA-IR group can also be observed. At this stage of regeneration, however, this cell group cannot be distinguished from the group located at the origin of the circumpharyngeal connectives.

During regeneration, the number of IR fibres in the preganglion increases continuously. These fibres are partly located in the commissures (Figs 1C, 2C and 3C). First, the ventral commissure-containing GABA-IR fibres appear, followed by the dorsal and central commissures (Fig. 3C).

After extirpation of the entire brain or one of the hemispheres, the number of GABA-IR nervous elements can also be observed in the intact ventral ganglia of the neighbouring body segments. These GABA-IR fibres display a more intense staining, primarily in the circumpharyngeal connectives connecting the brain to the SOG. In the connectives, GABA-IR cells can be seen from postoperative days 2–3, and their number thereafter gradually increases. These neurons accumulate at the cut edge of the circumpharyngeal connectives (Fig. 4A), and enter the preganglion together with the labelled fibres. In the intact *Eisenia fetida*, neurons can not be

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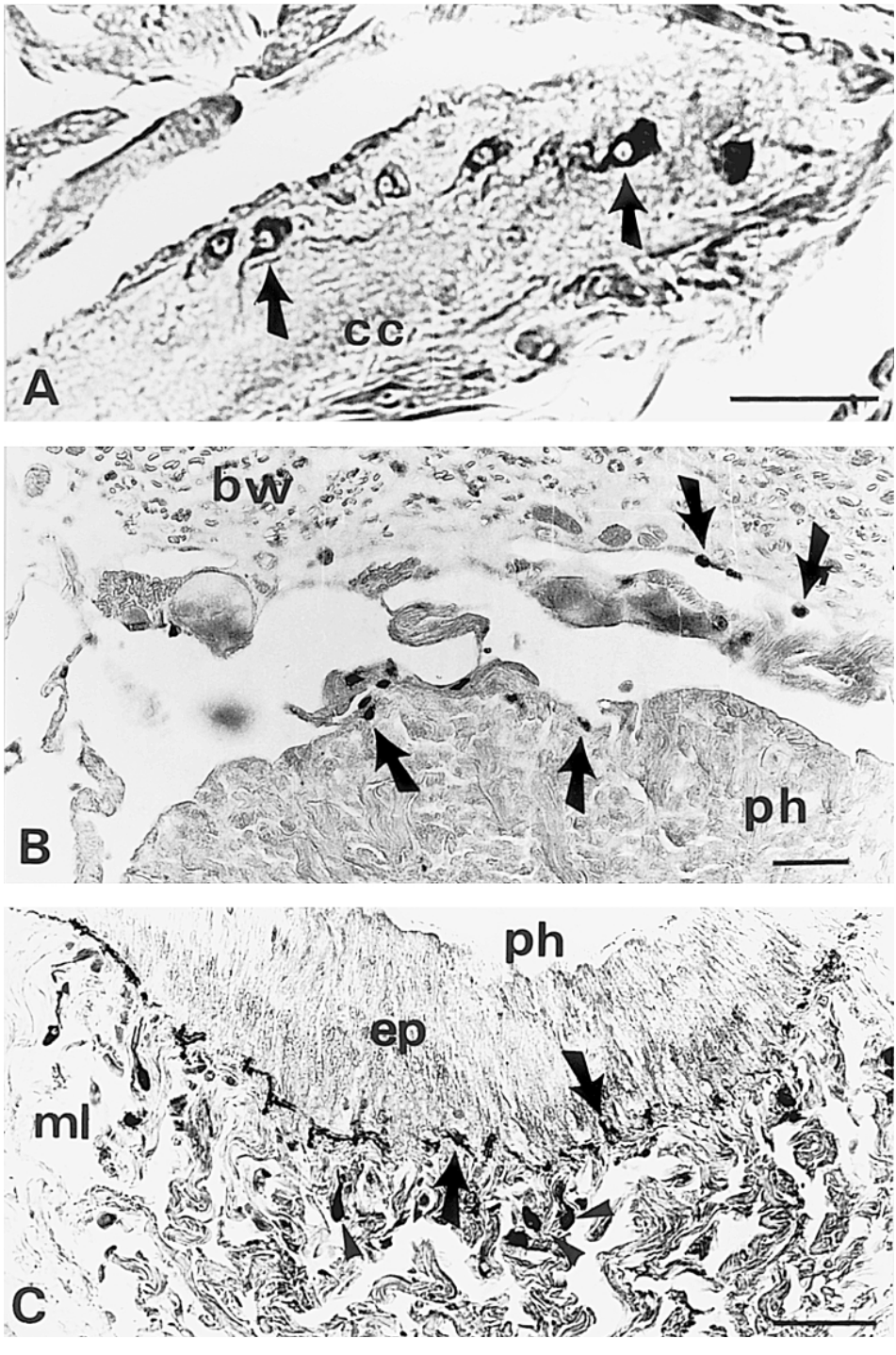
Fig. 4. Origin of the GABA-IR neurons in the regenerating brain of *Eisenia fetida.* **A:** GABA-IR neurons (arrows) accumulating at the cut edge of the circumpharyngeal connective (cc) from postoperative day 3. Scale bar: 70 μm. **B:** GABA-IR cells (arrows) in the pleura from postoperative day 3. bw: body wall; ph: pharynx. Scale bar: 70 μm. **C:** GABA-IR neurons (arrowheads) appear in the deeper muscle layer of the pharynx (ml) from postoperative day 3. Arrows: enteric plexus; ep: epithelial layer; ph: pharynx. Scale bar: 70 μm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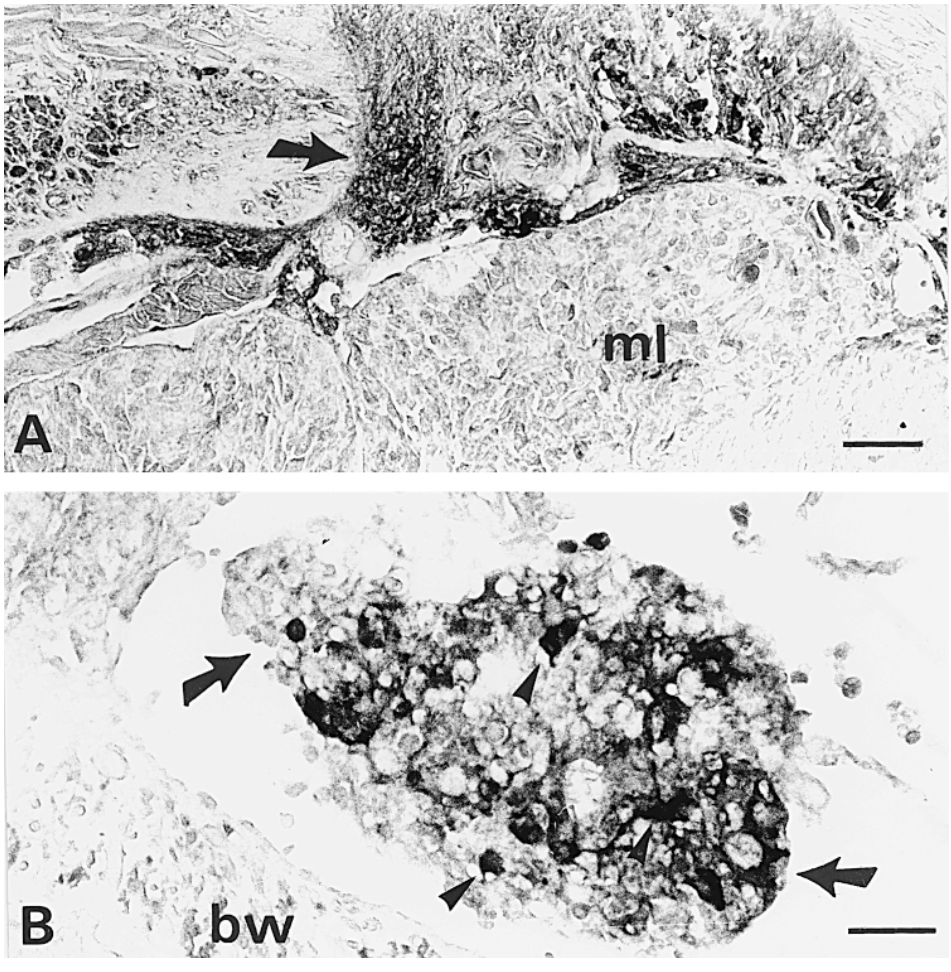

Fig. 5. **A:** Reorganization of the body wall (arrow) on postoperative day 12 after the application of exogenous GABA. ml: muscle layer of the pharynx. Scale bar: 70 µm. **B:** The wound tissue (arrows) contains GABA-IR cells (arrowheads) on postoperative day 21 following the application of exogenous GABA. bw: body wall. Scale bar: 70 µm

together with the labelled fibres. In the intact *Eisenia fetida*, neurons can not be found in the circumpharyngeal connectives.

From postoperative day 3, GABA-IR cells can be observed in the outer and inner membranes of the pleura (Fig. 4B), and also within the muscle layer of the dorsal side of the pharynx (Fig. 4C). Many, possibly dividing GABA-IR neurons with double nucleus can be found in this layer. We presume that these neurons may enter the preganglion through the uncapsulated ventral side.

Effect of exogenous GABA and PTX

After exogenous GABA application, the main process of brain regeneration was found to be similar to that in the regenerating brain of untreated earthworms. However, there are differences as regards the durations of the different phases, as follows: i) the reorganization of the body wall is finished only by postoperative day 12 (Fig. 5A); ii) the organization of the wound tissue starts by about postoperative day 12; iii) GABA-IR cells can be seen first from postoperative day 21 (Fig. 5B).

At the cut edges of the circumpharyngeal connectives and in the pharyngeal plexus, GABA-IR cells accumulate and enter the wound tissue from postoperative day 24. From postoperative days 30–31, the capsule around the preganglion begins to form. By postoperative day 50, the dorsomedial, dorsolateral and lateral GABA-IR neurons take up their final positions in the newly formed brain. By postoperative days 70–76, the cerebral ganglion is almost completely reorganized and the GABA-IR neurons are distributed similarly as seen in the regenerated brain of untreated, operated worms (Fig. 7). Hence, the total time required for brain regeneration is slightly shorter in the GABA-treated animals than in the untreated operated worms.

When PTX is given after brain extirpation, regeneration begins only on postoperative days 6–7 and the first GABA-IR neurons appear later, from postoperative day 24. There are no further differences in the process as compared to the regeneration of the untreated worms.

When PTX and GABA are given together, the process and time course of regeneration do not differ from those observed in the untreated operated animals.

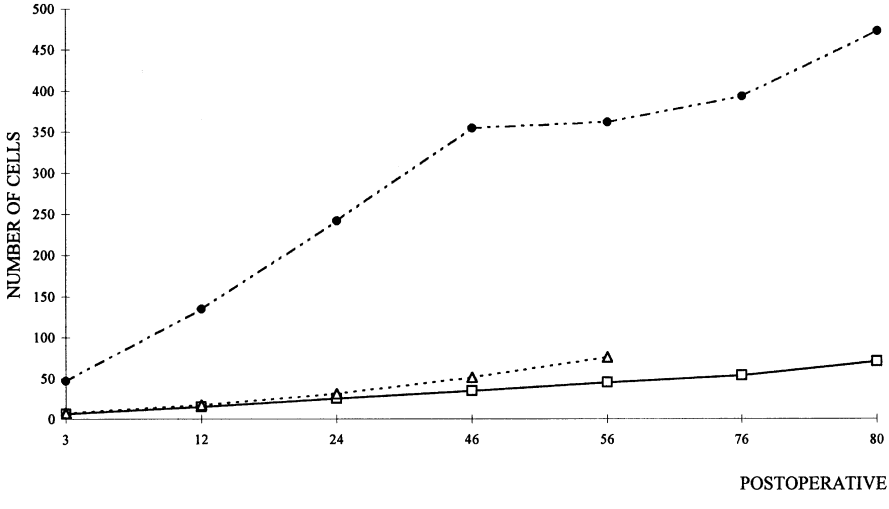

Fig. 6. Changes in number of neurons during the regeneration of *Eisenia* brain. Dotted line with solid dots: changes in the total cell number (after Nissl – staining). The changes in the number of GABA-IR neurons at the symmetrical (black line with squares) and asymmetrical (dotted line with triangles) brain regeneration. Note: the data represent one hemisphere

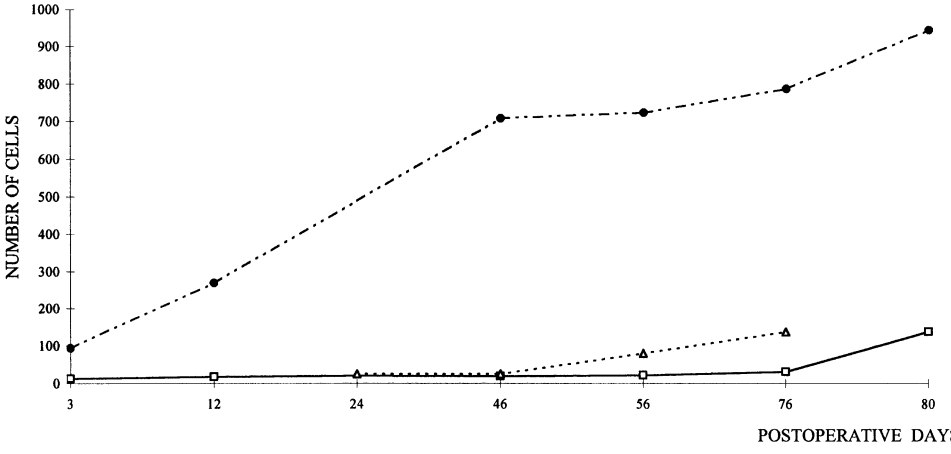

Fig. 7. Changes in number of neurons in the regenerating *Eisenia* brain. Dotted line with solid dots: regenerating brain of the untreated worm (after Nissl – staining); black line with squares: regenerating brain with GABA-IR neurons; dotted line with triangles: regenerating brain with GABA-IR neurons after exogenous GABA application

DISCUSSION

The present study is the first that describes and compares the reorganization of the GABAergic system during symmetrical and asymmetrical regeneration of the brain in an invertebrate, the earthworm *Eisenia fetida*.

The main features of the regeneration of the *Eisenia* brain are similar in symmetrical and asymmetrical regeneration, although asymmetrical regeneration is completed about 25 days earlier, than symmetrical regeneration.

The brain regeneration events can be separated into two phases. At the beginning of regeneration, reorganization of the body wall at the site of the operation, and the formation of wound tissue do not form part of real gangliogenesis [11]. This begins on postoperative day 3, when the first GABA-IR elements (both cell bodies and fibres) appear in the wound tissue. The formation of the preganglion starts when a capsule appears around the wound tissue which already contains GABA-IR elements. The organization of the GABA-IR neurons into groups (localized in dorsomedial, dorsolateral, lateral and ventromedial positions and at the origin of the circumpharyngeal connectives) begins from postoperative day 26 and is finished by postoperative day 80 in the animals with total brain extirpation.

In the case of unilateral brain regeneration, the appearance and arrangement of the GABA-IR neurons in the hemiganglion are similar to those seen in total brain regeneration, but the process is finished by postoperative day 56. In both regeneration types, by the end of the process the number of GABA-IR neurons is similar to that observed in the intact *Eisenia*.

In invertebrates, neural regeneration involves both axon regeneration and the replacement of neurons. Many studies have dealt with the regeneration of the nervous elements in annelids (e.g. *Lumbricus terrestris*: [21, 26, 27] and leech: [8, 18, 33, 41] and have shown that axon regeneration is faster than the generation of new neurons. This process is similar in *Eisenia* during brain regeneration.

The processes of symmetrical and asymmetrical regeneration of the GABAergic system are similar to those observed in the reorganization of the monoaminergic system [11]. Comparison of the reorganization of the monoaminergic and GABAergic systems indicates that: i) the first serotoninergic and GABAergic cells appear at the same time during regeneration (postoperative day 3), ii) both monoaminergic and GABAergic neurons occur in the main cell groups of the newly formed brain, but their numbers differ and iii) there is a difference between the origins of the IR elements of the two signal systems.

One question regarding the process of regeneration in annelids is the origin of the nerve cells in the newly formed ganglion. On the basis of our earlier and present investigations, we suggest that the new nerve cells originate at least in part from the neuroblasts situated in the pleura and around and within the intact ganglia [3, 10, 11]. It has been suggested that the neuroblasts become proliferating following brain extirpation and the new cells migrate to the preganglion.

Another possibility for the replacement of the lost neurons is the mitosis of the neurons. From the beginning of brain regeneration (postoperative days 2–3), dividing GABA-IR neurons can be observed in the enteric plexus of the pharynx of *Eisenia*, similarly as found in the course of reorganization of the tyrosine hydroxylase-IR neurons during brain regeneration [11].

There are data proving that monoamines (serotonin, noradrenaline and dopamine) stimulate regeneration and mitosis in planarians [2, 28]. Studies on the regeneration of planarians have revealed that neuropeptides exert an even greater effect. However, there are no data on GABA in this respect, whereas GABA has been shown to play a role in the early developmental events in the mammalian brain [1, 23, 29]. The fact that GABA-IR nervous elements appear in the early stage of *Eisenia* brain regeneration suggests that GABA may have a role not only in the development of the CNS, but in the general process of regeneration too.

Our data from the pharmacological experiments lend support to the influence of GABA on brain regeneration in the annelid *Eisenia fetida.* It appears that reorganization of the brain following exogenous GABA application begins later than in operated animals not treated with GABA. After the application of exogenous GABA, the formation of wound tissue is inhibited, the new neurons can not enter the future preganglion, and the process of regeneration begins later (from postoperative day 24). Later, when the effect of GABA on the non-neural elements has ceased, the migration of the new nerve cells can start again, resulting in a shortening of the duration of brain regeneration.

At the same time, GABA has an excitatory effect on the regeneration of the axons. At the beginning of gangliogenesis, numerous GABA-IR fibres can be seen in the wound tissue. A large majority of these fibres belong to the neurons located in the

SOG/or ventral ganglia which are cut during brain extirpation. *In vivo* experiments in mammals have proved that GABA promotes neurite outgrowth [36]. We suggest that exogenously applied GABA exerts a similar effect to that seen in *in vivo* experiments.

An excitatory effect of GABA could be observed not only on the outgrowth of neurites, but also on the neuronal proliferation, especially in the pharyngeal plexus. When exogenous GABA is injected into animals after brain extirpation, the migration and proliferation of the cells of the future wound tissue are inhibited, but the proliferation of the neurons of the pharyngeal plexus is undisturbed. The new neurons can not enter the wound tissue and accumulate on the outer surface of the muscle layer of the pharynx.

The next level of the GABA effect can be observed in the differentiation of the neuroblasts. During the early stages of regeneration (postoperative days 2–24), the neuroblasts of the different parts of the body (the pleura, around and within the intact ganglia) begin to divide. The proliferation of the neuroblasts situated within the nearly intact ganglion is reflected in the higher cell number during the first phase of regeneration. Later, the newly formed cells migrate to the scar tissue forming the new ganglion, and this migration results in a reduction in the number of cells in the intact ganglion during the further stages of regeneration [3, 11]. These observations are confirmed by other authors in reports on annelids and planarians [9, 16, 27, 43].

The effect of GABA on the regenerating CNS can effectively be blocked by the non-competitive $GABA_A$ receptor antagonist PTX in *Eisenia*. Similar experiments during development of the cortex of the brain in mammals, have proved that the $GABA_A$ receptor regulates the proliferative capacity of the cortical cells [24, 29, 30, 42]. The same effect has been observed when other neuroactive compounds are applied (a number of growth factors, neurotrophins [13]). On the basis of these findings, interactions between GABA and other neuroactive substances in regulation of the cell number changes have been suggested [1].

All these effects of GABA may be due either to different concentrations at different sites within the regenerating nervous system, or to different receptors on different target cells.

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