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Fine observation on nerves colonizing the regenerating tail of the lizard *Podarcis sicula*

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Summary. During the regeneration of lizard tail, nerves sprouting from ganglia and the spinal cord invade the blastema as far as the apical epidermis. Electron microscopical observations reveal axons storing dense granules (dg) and dense core vesicles (dcv) which are concentrated in nerve terminals or in axoplasmatic regions. In the regenerating spinal cord (SC) these terminals resemble aminergic-peptidergic endings and grow as far as the distal portion of the SC, which is made up of irregularly arranged ependymal cells. Some axons storing dcv contact blastematic cells and other nerve terminals show a plasma membrane incomplete or broken. Whether this latter aspect is due to fixation artifacts or physiological rupture is unknown. Nerves containing dcv and a few dg also originate from spinal ganglia innervating the regenerating tail. The accumulation of material into these endings is probably slow and a possible trophic influence on the regeneration of lizard tail is discussed.

Key words: Lizard, Tail, Regenerating nerves, Neurosecretion

Introduction

During regeneration of a vertebrate appendage the nervous tissue produces a trophic action on the regenerating tissues (Goss, 1969; Thorton, 1970). This phenomenon is particularly well known in amphibians where either sensory and motor neurons are involved in the production of the trophic factor (Singer, 1978).

Regarding regeneration in the lizard tail, it has been argued that the trophic influence is derived from the ependymal tube of the regenerating spinal cord (SC) meanwhile little importance has been given to the regenerating nerves of the tail (Cox, 1969; Simpson, 1970). In spite of these latter authors there are indications for an active participation of peripheral nerves to promote tail and limb regeneration in lizards (Singer, 1961; Zika and Singer, 1965; Whimster, 1978; Evans and Bellairs, 1983; Alibardi et al., 1988).

The discovery of some neurons and their axons inside the regenerating SC in lizard (Alibardi and Sala, 1986, 1988; Alibardi and Meyer-Rochow, 1988), threw new insight on the nervous influence.

Neurotrophic material and neuropeptides are thought to be stored in specific secretory organeles, namely dense core vesicles (dcv) or granules with broad dimensions (80-200 nm or more), (Arsdall and Lentz, 1968; Thureson-Klein et al., 1986).

On the basis of these ideas it would be interesting to observe in great detail what type of organeles are present in the nerves colonizing the regenerating tail of lizard.

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Materials and methods

Autotomy was induced in 40 adult lizards (*Podarcis sicula)* kept in cages at summer conditions (27-35° C during sunlight). After the formation of regenerating blastema (8-12 days) a sampling of regenerating tails began, from the 15th to the 45th days (scaled tail).

Some samples were fixed in a solution of Bouins or 10% formaldehyde (2-7 days) and processed for paraffin-wax embedding. Other pieces (1-2 mm long) derived from 20 animals were removed from the central axis of the regenerated tail in order to sample the new SC. Other small pieces were derived from the regenerating blastema.

A solution of 2.5% glutaraldehyde in Ringer or in cacodylate buffer (0.1 M, pH 7.2) served as fixative (5-8 hours at 0-4°C).

After rinsing with the buffers, the pieces underwent a 2 hours post-fixation process in 1% OsO_4, followed by alcohol dehydration, propylene oxide and Epon embedding.

Paraffine-wax embedded tissues were sectioned at a 7-12 μm thickness and stained by the hematoxilin-eosin and the Ag-Palmgreen techniques for nerve fibres (Mazzi, 1977).

Selected thick sections were collected from epon-embedded tissues by a LKB ultratome III and stained with 0.5% toluidine blue.

Thin sections collected from specific areas were mounted on copper grids, stained with uranyl-acetate and lead citrate and observed with a Philips EM-300 and Hitachi H-600 electron microscope.

Results

A) Optical Microscopy

The regenerating tail was supplied by nerves derived from the old SC as well as spinal ganglia (Figs. 1-4). Some nerves grew out of the apical ependymal ampulla and invaded the blastema reaching the apical epidermal cup (Figs. 5-7).

The apical ependyma was made up of irregularly arranged cells: they were not organized in a compact epithelium and the basal membrane was missing.

B) Electron Microscopy

1) Ependimal tube: as previously revealed by silver stain, the axons descending from the original SC were generally located close to the basal membrane as well as among ependymal cells (Fig. 8). These axons were of different diameter (average 2.5-0.4 μm) and a few extended as far as the apical ampulla.

Many of such electronclear amyelinic axons showed a few dense granules (dg) and dense core vesicles (dcv) of 70-90 nm width. However dcv were particularly concentrated in some axons or in particular regions along the axons (Figs. 9, 10).

Other dcv were present within the axons sprouted from the cerebro-spinal-fluid-contacting-neurons (CSFCNs) which are located among the ependymal cells of the regenerated SC (Fig. 11).

Axons with many dcv, resembling aminergic-peptidergic terminals were not rarely encountered by the apical ependymal tube (Figs. 12, 13). Some dcv terminals were close to the blastematic cells surrounding the ependymal tube (Fig. 14).

Around the regenerating SC some free axons or axons bundles were seen and not delimited by the basal membrane of the ependyma. These latter axons were generally amyelenic and they frequently stored dcv (Fig. 15), sometimes in large amounts (Fig. 16).

2) Nerves from spinal ganglia: the large neurons of the spinal ganglia contained bundles of neurofilaments among ER cisternae as well as many mitochondria (Fig. 17). In the cytoplasm some large granules (average diameter 0.2-0.8 μm) were visible: they showed medium-high electron density (Figs. 18, 19). The granules were commonly encountered but they were not very numerous in a ganglion neuron, rarely more than 8-10 per neuron section.

Dcv were occasionally observed in ganglion cells cytoplasm, while clear vesicles of 50-80 nm were commonly found near the Golgi region (Figs. 17-19).

Nerve bundles (myelenic and amyelinic) which were derived from ganglia, showed occasional dg or dcv while clear vesicles were quite common (Figs. 19-21).

These peripheral nerves, as seen in the central axons, stored groups of dcv only in some axoplasmic regions, perhaps representing accumulating terminals.

In the brief regenerative period here surveyed, myelinated axons did not enter into the blastema but extended into it as amyelinic fibers.

3) Axons innervating the blastema: amyelinic or naked axons (3-0.4 μm width) reached close to the cells of the regenerating blastema (Fig. 22). Some axons were ensheated by electron clear cells, probably Schwann elements (Figs. 23, 24). Electron dense Schwann cells were also present.

A few dg and dcv (60-90 nm width) were generally seen within the axoplasm, sometimes in particular terminal axons apposed to blastematic cells (Figs. 25-27).

Dcv terminals were also encountered by the apical epidermis of the blastema (Figs. 28, 29). It is uncertain if the alteration of mitochondria and plasma membrane of these latter terminals was due to a poor fixation or a physiological degeneration.

Discussion

This study shows that the innervation of lizard blastema is derived either from spinal ganglion cells as well as nerves surrounding the apical ependyma of the regenerating SC (Fig. 30).

Even if the specific origin of nerves from motor, sensory or autonomous areas (Terni, 1920, 1922; Zannone, 1953; Charvat and Kral, 1969) was not traced in this study, dcv were more frequent in amyelinic axons.

The axons of lizard blastema have analogous feature described in newt limb blastema (Salpeter, 1965; Hay, 1966; Lentz, 1967a) but the large dg were not as frequent in lizard as in the newt (Arsdall and Lentz, 1968; Thorton, 1970).

Some of the apical nerves were growing out of the pathway traced by ependymal channels (Simpson, 1983). In fact, the apical ependyma was irregularly arranged and the limiting basal membrane often resulted incomplete (Alibardi and Sala, 1989).

Trophic material could be discharged from the apical aminergic-peptidergic terminals (Dellmann, 1973; Vigh et al., 1981) derived from growing nerves or from CSFCNs within regenerating SC (Alibardi and Sala, 1988; Alibardi and Meyer-Rochow, 1988; Alibardi et al., 1988). As observed in this study some of these dcv terminals contact the blastematic cells so that materials could be descharged.

Previous studies (Simpson, 1968, 1983; Egar et al., 1970) also described dcv in regenerating axons of *Anolis*

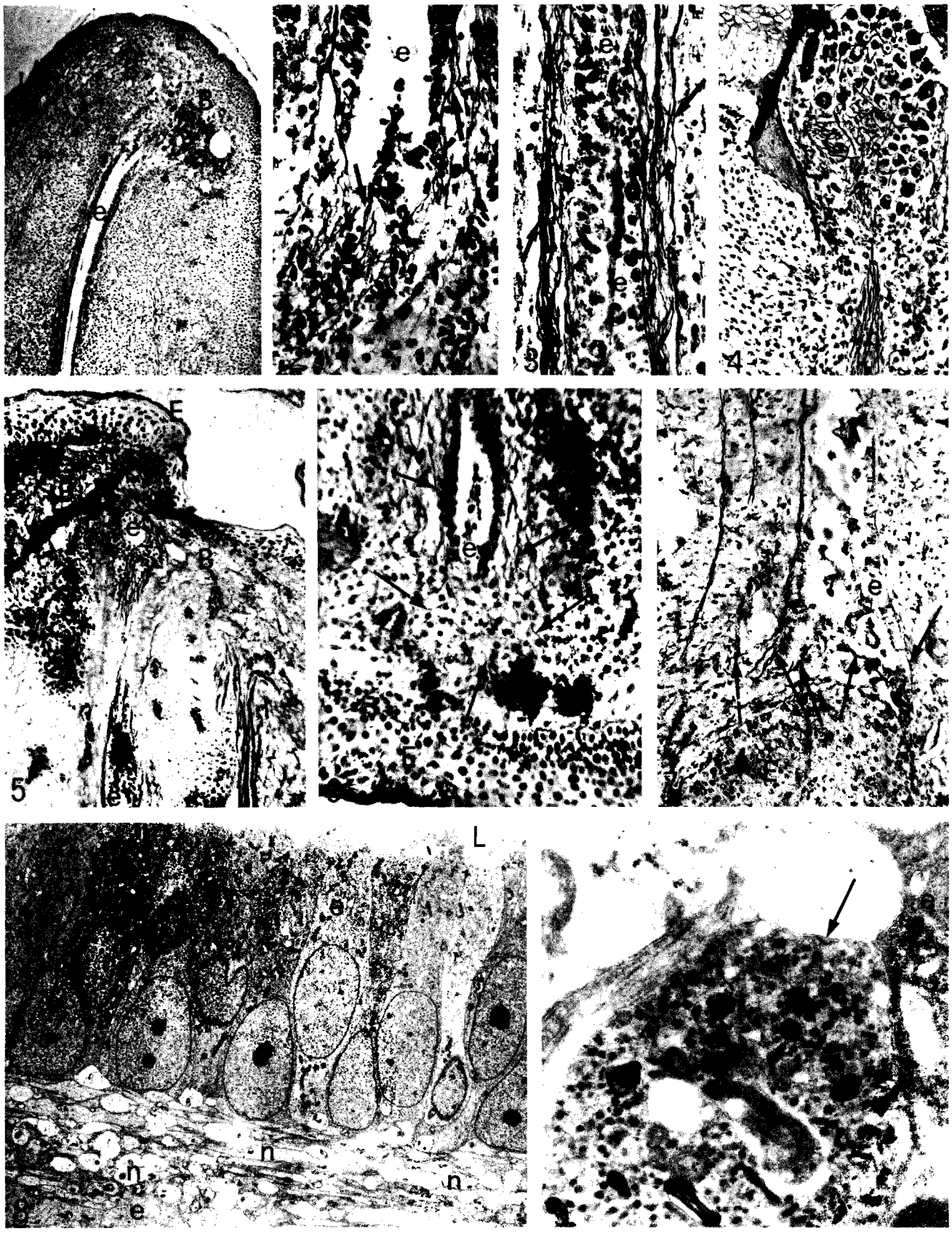

Fig. 1. General view of regeneration tail. E, apical epidermis; e, ependymal tube; B, apical blastema. Palmgreen. × 40
Fig. 2. Distal ependymal tube showing nerves (arrows) among ependymal cells (e). Palmgreen. × 190
Fig. 3. Intermediate portion of regenerating ependyma. Fascicles of nerves (arrows) are located peripherally in the ependymal tube (e). Palmgreen. × 190
Fig. 4. Spinal ganglion (G) innervating the blastema. n, spinal nerve sprouted from the ganglion toward the regenerating blastema. Palmgreen. × 96
Fig. 5. Regenerating apical blastema (B): note a large nerve (n) and the ependyma tube (e) reaching near the apical epidermis (E). Palmgreen × 100
Fig. 6. Apical ependyma (e) from which some nerves are sprouting toward blastematic cells (arrows). E, apical epidermis. Palmgreen. × 165
Fig. 7. Some nerve fibres (arrows) from ependyma (e) reach areas underlying the apical epidermis (E). Palmgreen × 165
Fig. 8. Electron microscopic longitudinal section of regenerating ependymal tube. The thin electronclear nerves (n) are located mostly at the basal side of ependymal cells (e). L, lumen of ependymal tube. × 2,250
Fig. 9. Distal ependyma showing an axon in cross section which is filled with dcv. The arrow points out a dense pit, perhaps representing a discharging vesicle. × 19,100

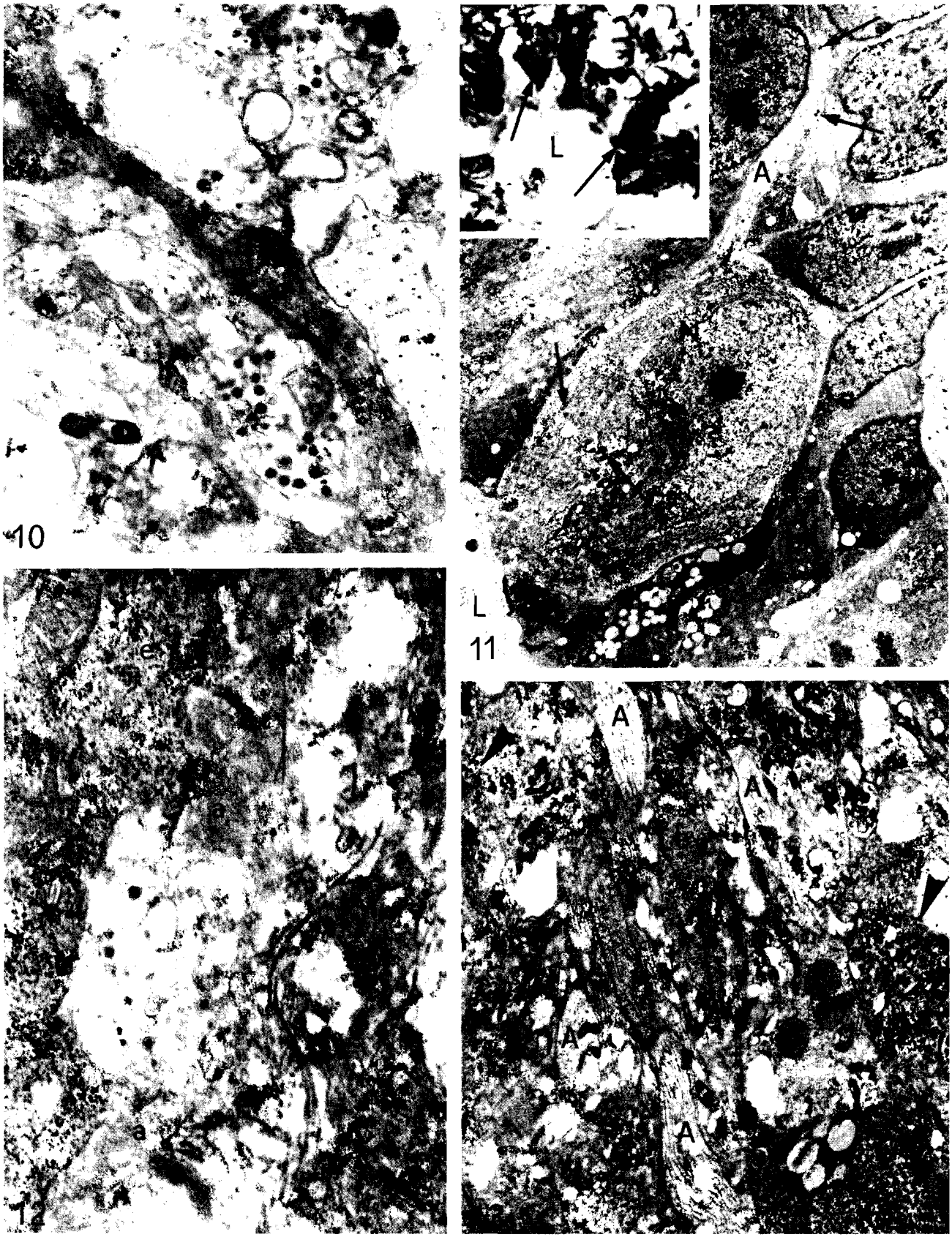

Fig. 10. dcv stored in axons of the intermediate ependymal tube (see corresponding area in Fig. 3). e, ependymal process. × 21,600

Fig. 11. Panoramical view of CSFCN (N) among ependymal cells (e). Some dg (arrows) are stored in the apical cytoplasm as well as in the axon (A). L, lumen of the central canal, × 5,550. Inset: two CSFCNs (arrows) as revealed by silver stain according to Palmgreen. × 720

Fig. 12. Apical ependyma: irregular boundary between ependymal and surrounding blastematic mesenchima. Some dcv are observed out the basal portion of ependymal cells (e), maybe stored in a damaged nerve ending. Instead of the basal membrane, an amorphous substance is present (a) with sparse collagen fibrils. × 26,000

Fig. 13. Apical ependyma: tangential section featuring axons (A) among ependyma (e). Some of them are filled up with dcv (arrowheads). × 6,250

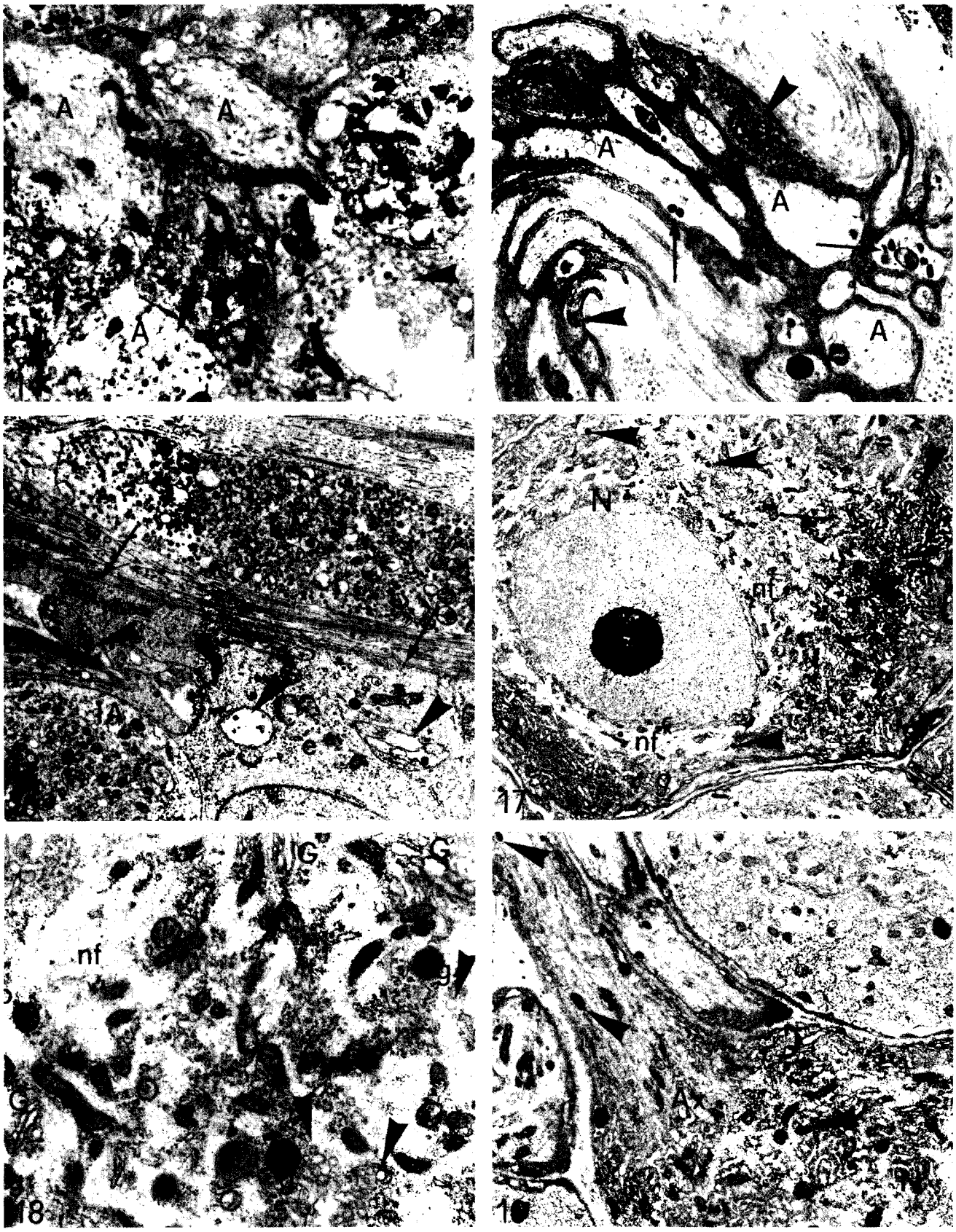

Fig. 14. Aspect of some axons (A) by the apical ependymal tube at the boundary with blastematic cells. Dcv are particularly numerous in some areas (arrowheads). × 10,300
Fig. 15. Intermediate ependymal tube: section of extraependymal amyelinic axons (A). Some of them show dcv (arrowheads) or dg (arrows). C, cytoplasm of ensheating Schwann cell. × 13,300
Fig. 16. Ependymal tube in a non-apical area: note extraependymal (EA) and intraependymal (IA) dcv containing axons. e, ependymal cells; arrowheads point out thin axons between ependymal cells; arrows show the external basal membrane. × 17,300
Fig. 17. Spinal ganglion cell (N, see general aspect in Fig. 4) innervating a regenerating blastema. The cytoplasm is full of neurofilaments (nf) and mitochondria. Arrowheads point to dg. × 3,300
Fig. 18. Magnification of a ganglion cell cytoplasm. Among neurofilament heaps (nf) some dg (g) and many smaller clear vesicles (arrowheads) are visible. G, Golgi areas. × 15,800
Fig . 19. Close-up on an axon hillock of a ganglion neuron (N). Among clear neurofilaments some dg are present (arrowheads). × 6,450

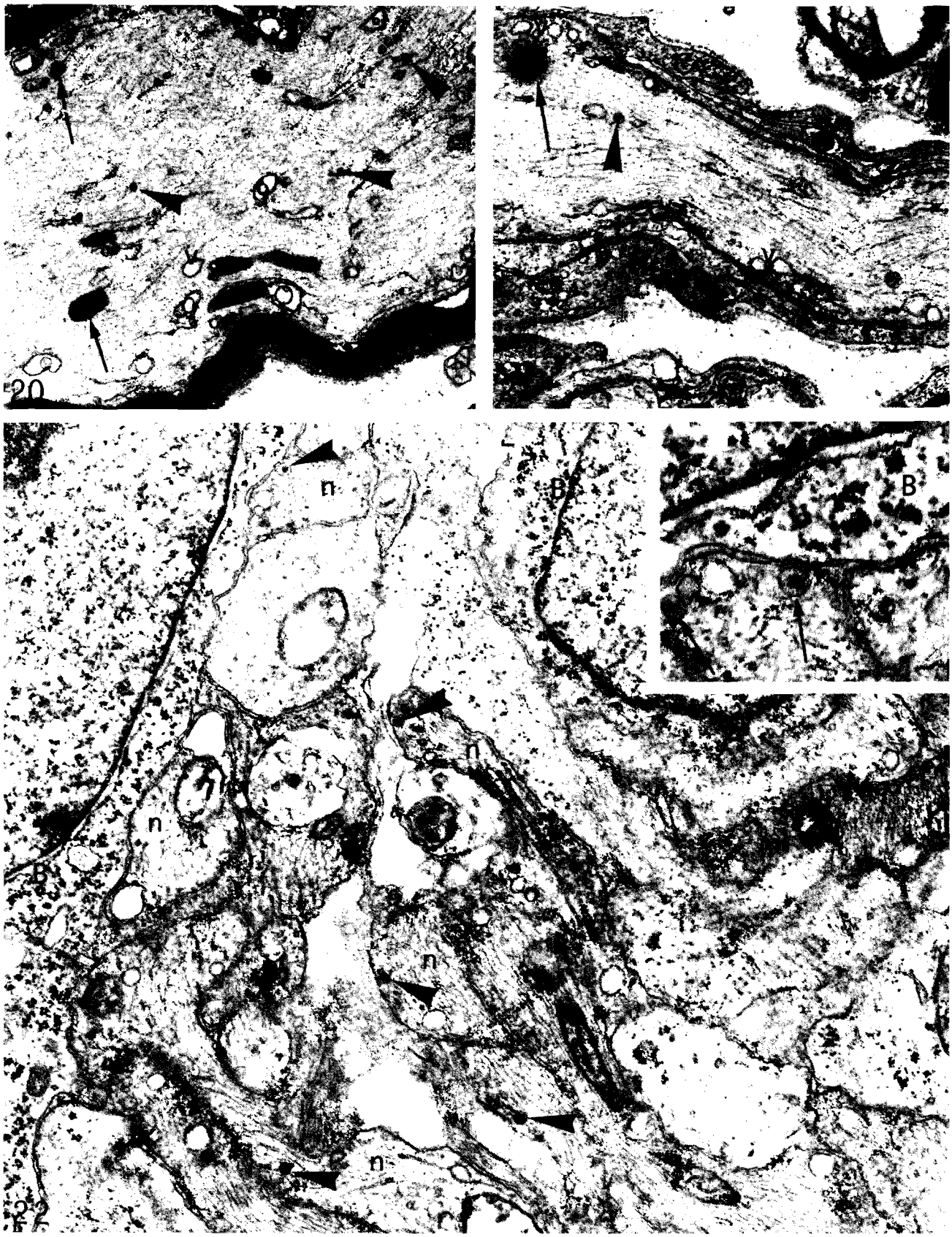

Fig. 20. Myelinated fibers within a nerve sprouted from a spinal ganglion (see n in Fig. 4). Note dg (arrows), dcv (arrowheads) and clear vesicles (v). × 14,000

Fig. 21. Amyelinic fibers within a nerve derived from a spinal ganglion. Observe dg (arrow), dcv (arrowhead) and clear vesicles (v) within the axoplasm. C, Schwann cell cytoplasm. × 17,700

Fig. 22. Nerve terminals or preterminals (n) on blastematic cells (B) which are located laterally with respect to the apical ependyma. Some dcv (arrowheads) and various clear vesicles are seen in the axoplasm. × 24,800. Inset: two dcv (arrows) close to the plasma membrane facing a blastematic cell (B). × 42,700

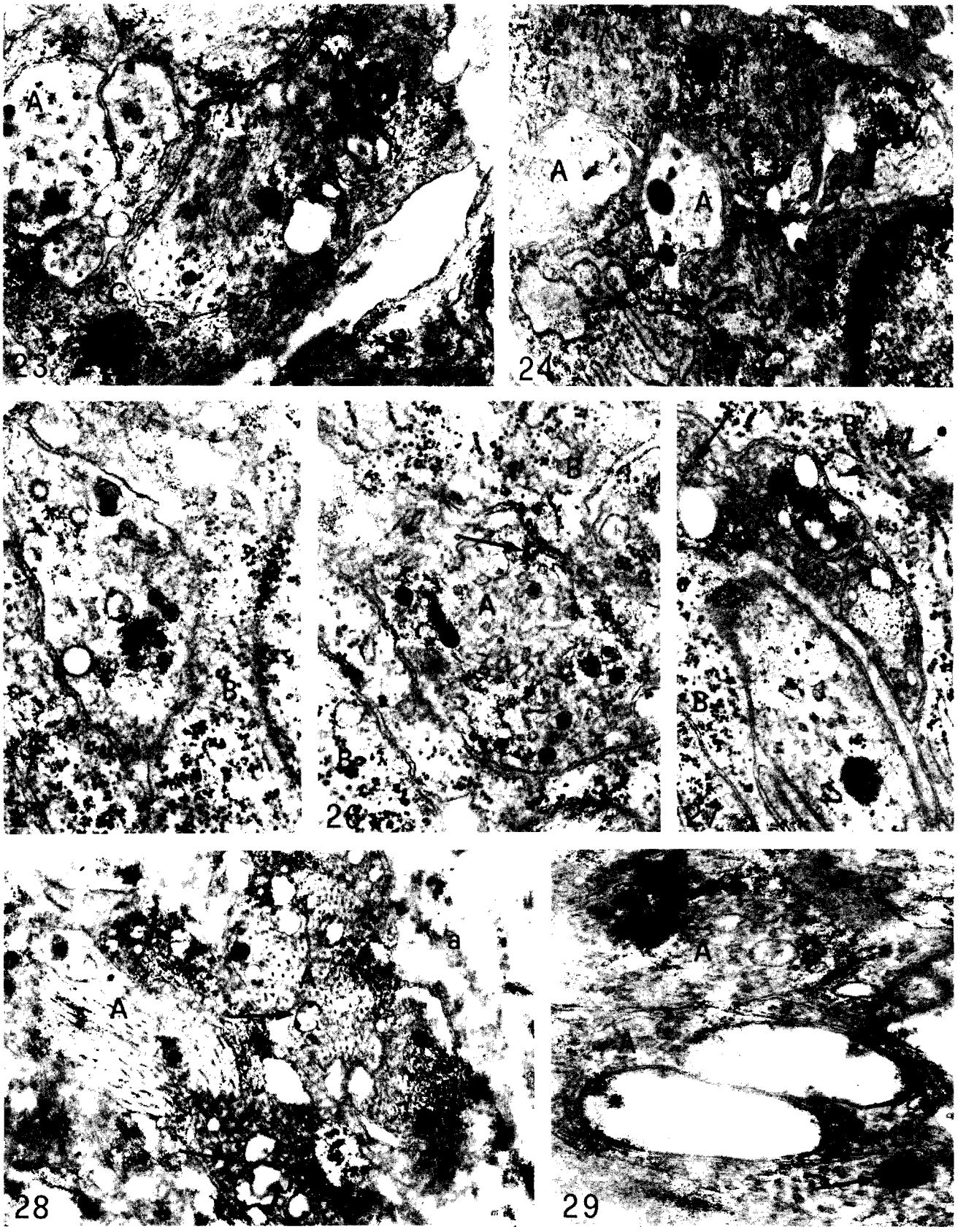

Fig. 23. Cross section of axons storing dcv (A) in an amyelinic nerve inside the blastema. C, Schwann cell cytoplasm. × 25,800
Fig. 24. Cross section of thin axon passing through a blastematic (or Schwann) cell (C). Some axons store dg (arrows) or dcv (arrowhead). × 16,500
Fig. 25. Axon with dcv apposed to blastematic cells (B). × 35,000
Fig. 26. dcv and clear vesicles in a terminal or preterminal axon (A) contacting a blastematic cell (B). Arrowhead on a coated pit facing a blastematic cell, perhaps descharging some material. × 37,200
Fig. 27. Terminal axon on a blastematic cell (B). It appears filled mainly with clear vesicles. The arrow points out the only dcv present in this area. × 26,400
Fig. 28. dg and dcv within axons (A) located at the tip of the blastema under the apical epidermis. a, amorphous matrix in the extracellular space. Arrows point out altered mitochondria. × 13,000
Fig. 29. Magnification of a very apical axon (A) close to the apical epidermal cup. Cluster of dcv and dg (arrow) are stored in the axoplasm. × 35,700

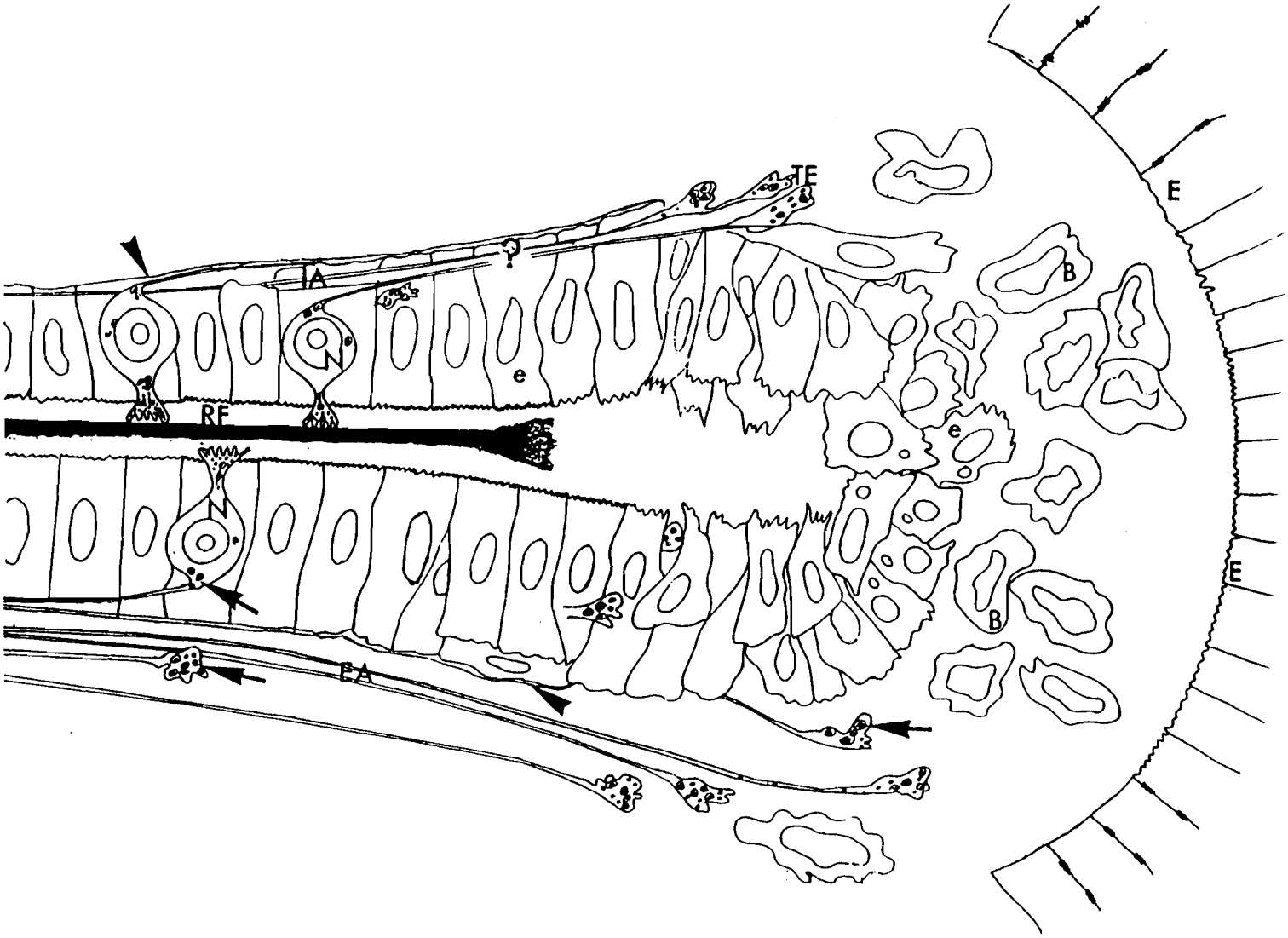

Fig. 30. Schematic drawing featuring the fine structure of the apical regenerationg SC in lizard as resulted from this study. E, apical epidermis; B, blastematic cells; EA, periependymal axons; IA, intraependymal axons; TE, terminal endings of nerves; e, ependyma; RF, Reissner's fiber in the lumen; N, CSFCNs; Arrows point out dcv; Arrowheads point out the basal limiting membrane around ependyma; ?, indicates uncertain cell continuity between CSFCNs and apical nerve endings.

but no mention was made about aminergic-peptidergic terminals located in the very apical ependyma. This latter was not completely sealed and cerebro spinal fluid could permeate out through blastematic cells. If a trophic material is present in the cerebro spinal fluid, a stimulating action on blastematic cells could be produced.

Other trophic material may derive from the release or degeneration of dcv terminals into the ependymal channels.

Also extraependymal nerves can contribute to the liberation of trophic substances, namely from the dcv they store.

Other aminergic-peptidergic terminals were derived from ganglion cells (dcv diameter 70-90 nm) in particular from light neurons previously described in lizard by Pannesse (1963a,b). With respect to CSFCNs the ganglion neurons and the axons derived from them store a few dcv and these aspects were also observed in *Triturus* ganglia during limb regeneration (Lentz, 1967b). This fact suggests for these latter cells a low but continuous production of dcv and dg which progressively accumulate distally into the nerve endings.

Dcv or dg may also accumulate in particular axons regions which function as releasing sites (Dellmann, 1973; Thureson-Klein et al., 1986) through blastematic cells.

Some axons storing mostly clear vesicles probably advance toward myoblasts present among the growing tail blastema in order to establish the future motor end plates (Hughes and New, 1959). The progressive formation of specialized end plates between myoblast and axons storing clear vesicles of cholinergic type, has been well documented during the limb regeneration of *Triturus* (Lentz, 1969).

According to Singer (1978) for amphibians and Zika and Singer (1965) for lizards, the regenerative response to nerves depends on the total number of axons (or

axoplasm) present on the surface of amputation and not on their cholinergic or adrenergic nature (Thorton, 1970; Egar and Singer, 1971; Taban et al., 1977).

In lizards the presence of aminergic-peptidergic terminals derived from spinal nerves or ganglion cells could explain the trophic stimulation of tail and limb outgrowths by augmentation of nerve supply (Singer, 1961; Whimster, 1978; Alibardi et al., 1988).

This fine observation emphasized the role of nerve cells and their axons more than ependyma as a source of the hypotized trophic factor stimulating the regeneration of the tail in lizard (Simpson, 1970; Turner and Singer, 1973). Ependymal cells are probably less specialized than neural cells in sintetizing specific substances. Besides in lizard no particular secretory activity was noticed from the apical regenerating ependyma through the surrounding blastematic cells meanwhile it was intense through the ependymal lumen (Turner and Singer, 1973; Alibardi and Sala, 1989).

The isolament in culture of the regenerating SC of lizard (Simpson and Cox, 1967) is presently under analysis in order to clarify the different secretory activity of the regenerating ependymal and CSFCNs.

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