Intrahypothalamically Transected Neurosecretory Axons do not Regenerate in the Absence of Glial Cells

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SUMMARY

Fifteen days after transection of the hypothalamo-neurohypophysial tract at the lateral retrochiasmatic hypothalamic area, neurosecretory axons had vigorously regenerated into transplants of explanted hypophysial neural lobe, to a lesser extent into sciatic nerve transplants, and least into optic nerve transplants. Regenerating axons were always closely associated with the specific glial cells of these grafts. When these glial cells were killed by cryotreatment prior to transplantation, neurosecretory axons did not regenerate into the abundant extracellular matrix of the transplants, including persisting basal lamina tubes in neural lobe and sciatic nerve grafts. The presence of viable glial cells is a prerequisite for neurosecretory axon regeneration.

KEY WORDS

neurosecretory neurons, transection, neurophysin immunohistochemistry, electron microscopy, glial cells, regeneration

INTRODUCTION

Contrasting with the robust regeneration of neurosecretory axons that occurs following transection of the hypothalamo-neurohypophysial tract at the median eminence /7,19,31,32,37/ is the virtual lack of a regenerative response when the same tract is transected intrahypothalamically /6,27,34/; the latter neurosecretory axons do, however, regenerate to a limited degree into perivascular connective tissue spaces in the vicinity of the transection site, or into suitable microenvironments provided by tissues transplanted into contact with the proximal stumps of the severed axons. Syngeneic grafts of hypophysial neural lobe /12/, sciatic nerve /10/, optic nerve /14/, and hypophysial neural lobe explants /4/ are invaded to varying degrees by regenerating neurosecretory axons, which form perivascular plexuses around the microvessels of the grafts. When the ventral hypothalamic surface is damaged during the transection procedure, vigorous regeneration occurs into the leptomeninges /13/.

Neurosecretory axon regeneration always occurs in close association with glial cells, i.e., pituicytes in neural lobe grafts, neurolemmocytes in sciatic nerve grafts, astrocytes in optic nerve grafts, and neurolemmocyte-like cells in the meninges. Glial cells thus seem to play an essential role in the regeneration process. In order to verify that role, we have killed glial cells by cryotreatment of the above tissue types prior to transplantation, to determine whether their absence from the grafts precludes neurosecretory axon regeneration.

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MATERIAL AND METHODS

Adult male Holtzman rats (250-275 g) were anesthetized with ketamine-pentobarbital (60 mg ketamine/kg/i.m.; 20 mg pentabarbital/kg/i.p.). The hypothalamo-neurohypophysial tract was transected bilaterally with a 1.5 mm wide wire loop knife at the lateral retrochiasmatic area /30/, using stereotactic procedures (in relation to bregma: AP = 1.2 mm; LM = 0.8-1.0 mm; DV = 8.5 mm). Lesions were then assigned to a control group in which no transplant was placed, or to one of six groups based on the source and treatment of transplanted tissue. The six types of transplants placed with a 19-gauge spinal needle into the transected hypothalamo-neurohypophysial tract in the lateral retrochiasmatic area were: (1) hypophysial neural lobe explants (after 30 days *in vitro*) /9/ (n=5); (2) cryotreated hypophysial neural lobe explants (n=9); small pieces (0.25 mm^3) of (3) sciatic nerve (n=3); (4) cryotreated sciatic nerve (n=8); (5) optic nerve (n=9); (6) cryotreated optic nerve (n=6). The cryotreatment consisted of three freeze-thaw cycles prior to transplantation.

Fifteen or 20 days after transplantation, pentobarbital-anesthetized rats were fixed by aortic perfusion with a phosphate-buffered (0.2 M, pH 7.2) 2% glutaraldehyde-3% paraformaldehyde solution. Brains were removed and placed in the same fixative for an additional 6 to 12 hours. Alternate 50 μm thick, vibratome-cut horizontal sections of the hypothalamus were then processed for immunohistochemistry and electron microscopy. The immunohistochemical procedure, which identifies both vasopressin-associated and oxytocin-associated neurophysin, was as previously published /13/. For electron microscopy, sections were postfixed in 2% OsO_4 and 0.75% potassium ferricyanide, stained for 3 h in 2% uranyl acetate and embedded in eponaraldaite. This sections were stained with lead citrate.

RESULTS

Detailed accounts of the immunohistochemical and fine structural characteristics of non-cryotreated neural lobe explants, sciatic nerve and optic nerve have been published previously /4,8,10,14/; therefore only a concise description of these tissues is included here to permit comparison with the cryotreated transplants.

Transplants of neural lobe explants

In non-cryotreated neural lobe explants, neurosecretory axons were frequently more concentrated peripherally, at the interface between graft and host, and were abundant throughout the grafted tissue (Fig. 1a). Usually a punctate immunostaining prevailed, indicative of neurovascular contact regions (Fig. 1b) and in some areas the reaction was weak (Fig. 1a). In cryotreated neural lobe explants no neurophysin immunoreactivity was detected (Fig. 2a).

The fine structural characteristics of non-cryotreated neural lobe explants were similar to those of intact neural lobes /12/, except for a greater abundance of connective tissue in some areas. Axons filled with densely packed neurosecretory granulated vesicles were fully or partially ensheathed by pituicyte processes, and they terminated at perivascular basal laminae or their evaginations into the surrounding neuropil (Figs. 1b, c). Both fenestrated and continuous capillaries were abundant.

Cryotreated transplants of explanted neural lobe lacked pituicytes, and neurosecretory axons were also absent, except peripherally (see below) (Fig. 2b). Scattered fibrocytes, many basal lamina remnants (Fig. 2c), macrophages alone or in groups, and scarce fenestrated and continuous capillaries lay among densely packed and abundant collagen fibrils with little amorphous ground substance (Fig. 2b).

Transplants of sciatic nerve

Non-cryotreated sciatic nerve transplants contained numerous neurosecretory axons (Fig. 3a), as evidenced by neurophysin immunoreactivity. Cryotreated transplants, in contrast, did not support neurosecretory axon regeneration (Fig. 4a).

At the fine structural level, non-cryotreated transplants were characterized by bundles of neurosecretory axons invested by basal lamina-ensheathed Schwann cells. Endoneurial collagen

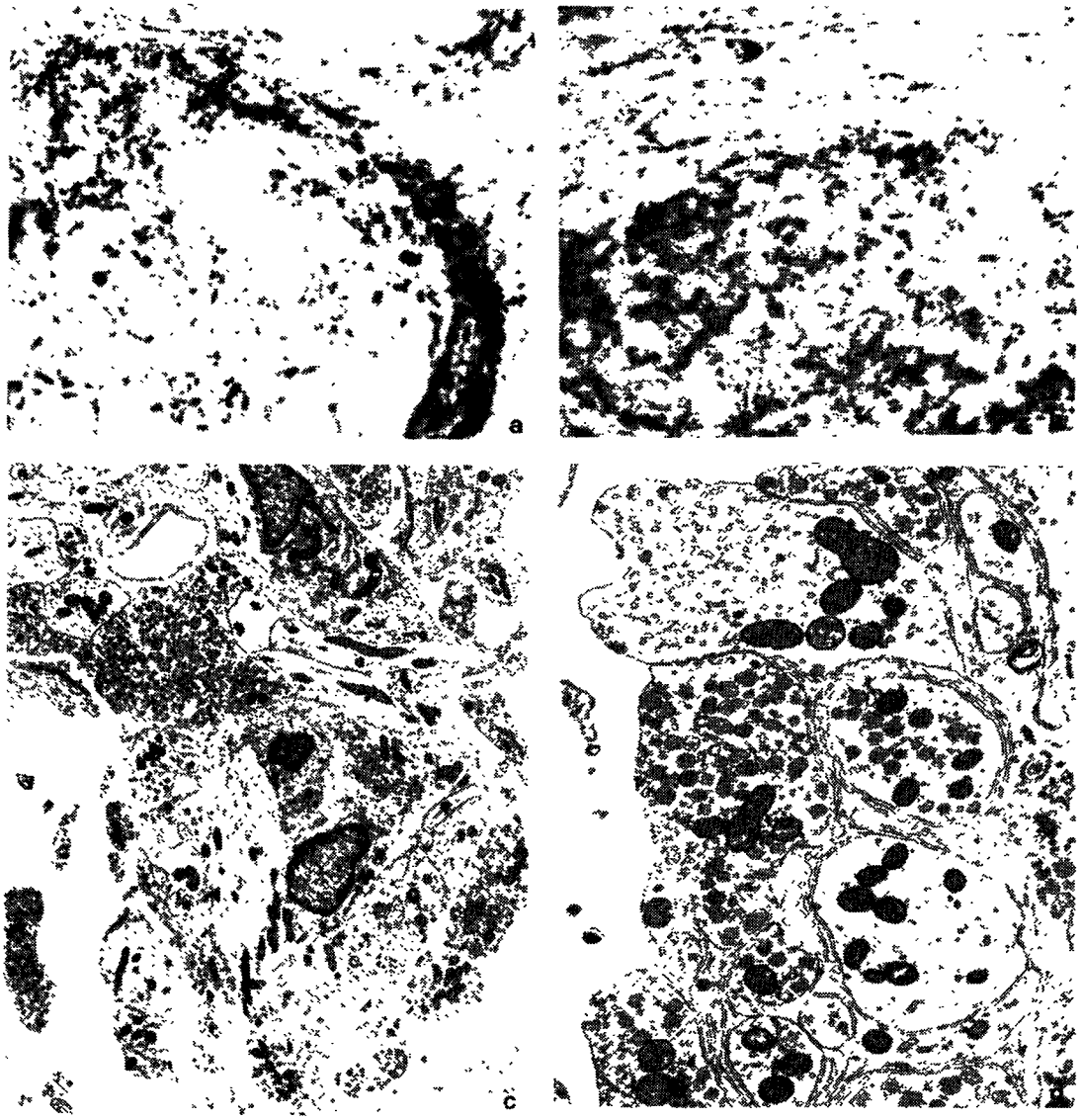

Fig. 1: Transplants of non-cryotreated neural lobe explants. **a**: A dense layer of neurosecretory axons surrounds this transplant and numerous axons are present in the transplant in which areas of more intense immunoreactivity indicate perivascular terminals. x 460. **b**: This transplant lacks the surrounding layer of neurosecretory axons. Neurovascular contact regions are more extensive than in **a**. The punctate immunoreactive material represents axon terminals and groups of axon terminals, while the empty appearing regions are capillaries. x 460. **c**: Profiles of neurosecretory axons and axon terminals have the same relationship with pituicytes as in the intact neural lobe. x 7600. **d**: Axons and axon terminals abutting the perivascular basal lamina are partially or entirely invested by pituicyte lamellopodia. x 19200.

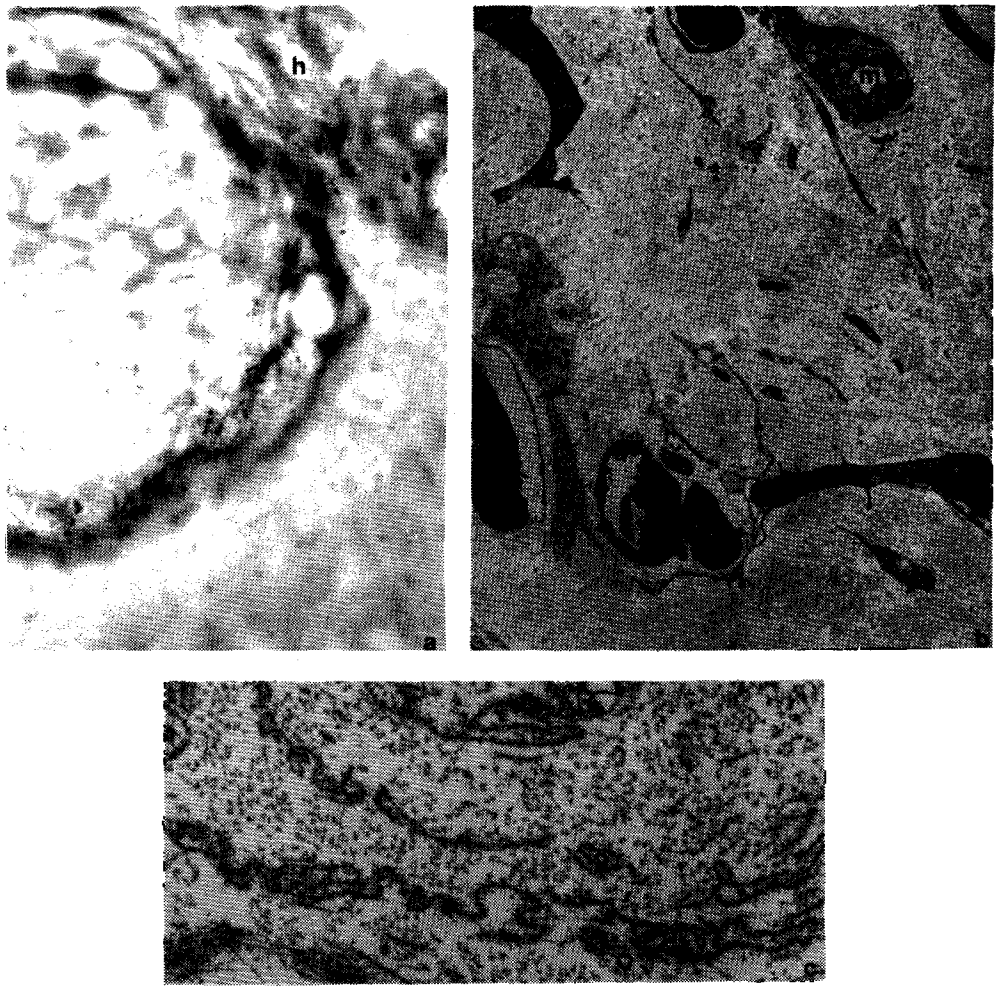

Fig. 2: Transplants of cryotreated neural lobe explants. **a**: Neurosecretory axons are present only in the host (h) and at the interface between host and graft. Axons seemingly penetrating the transplant belong to the interface layer. x 460. **b**: Within the predominant extracellular matrix, notice continuous capillaries and a fenestrated capillary (arrowhead), fibrocytes and part of a macrophage (m). x 3500. **c**: Basal lamina remnants in the extracellular matrix. x 21000.

fibrils and fibrocytes surrounded the bundles (Fig. 3b).

In cryotreated transplants, Schwann cells were no longer present, but their collapsed empty basal lamina tubes remained, and were surrounded by abundant extracellular connective tissue matrix (Figs 3c, 4b). Most capillaries were continuous, but a few fenestrated capillaries were observed (Fig. 4b). Fibrocytes and a few macrophages were scattered throughout the transplants (Fig. 4b). Neurosecretory axons were absent, except peripherally (see below).

Transplants of optic nerve

Non-cryotreated transplants contained regenerating immunoreactive neurosecretory axons that had a distinct affinity for the microvasculature, around which they formed plexuses of varying density (Fig. 5a). Cryotreated transplants failed to promote neurosecretory axon regeneration (Fig. 6a), except peripherally (Fig. 6b) (see below).

With the electron microscope, single neurosecretory axons were observed between densely packed astrocytes in non-cryotreated

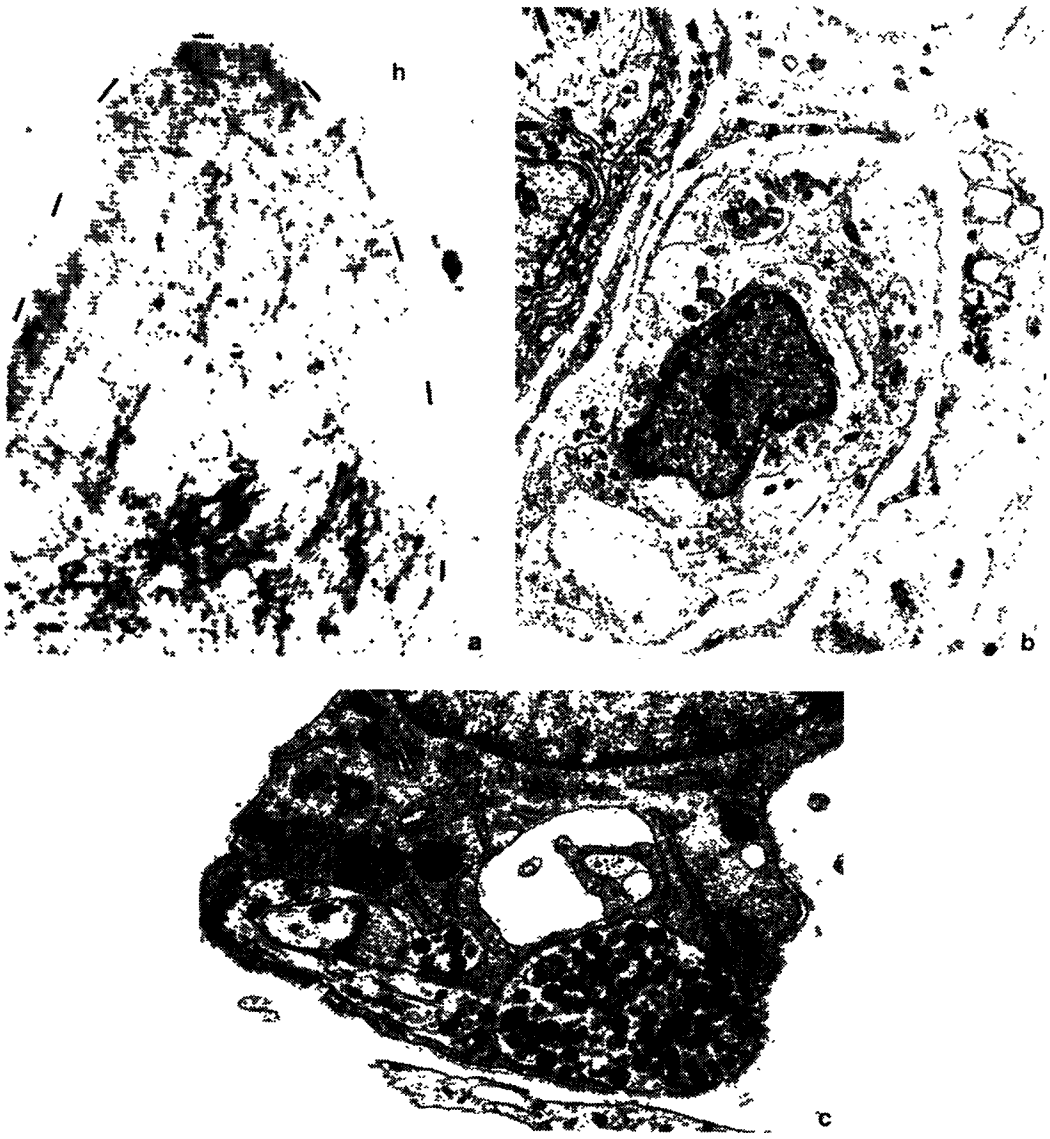

Fig. 3: Transplants of non-cryotreated sciatic nerve. **a**: Many neurosecretory axons have penetrated the transplant (t) that is clearly delineated (interrupted line) from the host (h). Neurosecretory axons are denser in a piece of transplant (bottom of picture) that is connected to the host at a different level. x 460. **b**: Bundles of neurosecretory axons containing neurosecretory granulated vesicles (asterisks) and/or microvesicles indicative of hormone release, are related to Schwann cells in a manner similar to that observed in intact peripheral nerves. x 7600. **c**: The typical relationship between neurosecretory axons and a basal lamina-enclosed Schwann cell is shown in this micrograph. x 20000.

transplants. Bundles of neurosecretory axons associated with basal lamina-enclosed astrocyte processes were especially numerous in perivascular connective tissue spaces (Fig. 5b).

In cryotreated transplanted, collagen fibrils were considerably less abundant than in the other graft types. Macrophages were the predominant cell type; astrocytes and oligodendrocytes were no longer identifiable (Fig. 6b). Grafts contained both continuous capillaries and a few fenestrated capillaries (Fig. 6b). Neurosecretory axons were only observed in the graft periphery (Figs. 6b, c).

Fig. 4: Transplants of cryotreated sciatic nerve. **a**: Neurosecretory axons are absent from this transplant but present in the hypothalamus proximal to it (p). Distally (d) degenerating neurosecretory axons have formed immunoreactive "retraction balls". x 460. **b**: Fibrocytes and fenestrated capillaries are surrounded by copious extracellular matrix. x 4200. **c**: Collapsed basal lamina tubes remain after the disappearance of the Schwann cells. x 8400.

All transplants

Regenerating neurosecretory axons that did not form terminals occasionally penetrated the periphery of all types of transplants. Invariably, these axons were accompanied by basal lamina-surrounded astrocytic processes from the host (Figs. 6c, 7a, b). Neurosecretory axons were never observed without investment of host glia.

DISCUSSION

In previous investigations we have shown that intrahypothalamically transected neurosecretory axons regenerate into transplants of intact neural lobe, neural lobe explants, sciatic nerve and optic nerve /4,10,12,14/. Perivascular connective tissue spaces of hypothalamic blood vessels likewise provide a conducive microenvironment for regenerating neurosecretory axons /1,11/, and the meninges support neurosecretory axon regeneration over long distances /13/. In all these diverse microenvironments, regenerating neurosecretory axons are almost always associated with glial cells; only exceptionally are naked neurosecretory axons observed. These observations suggest that a close

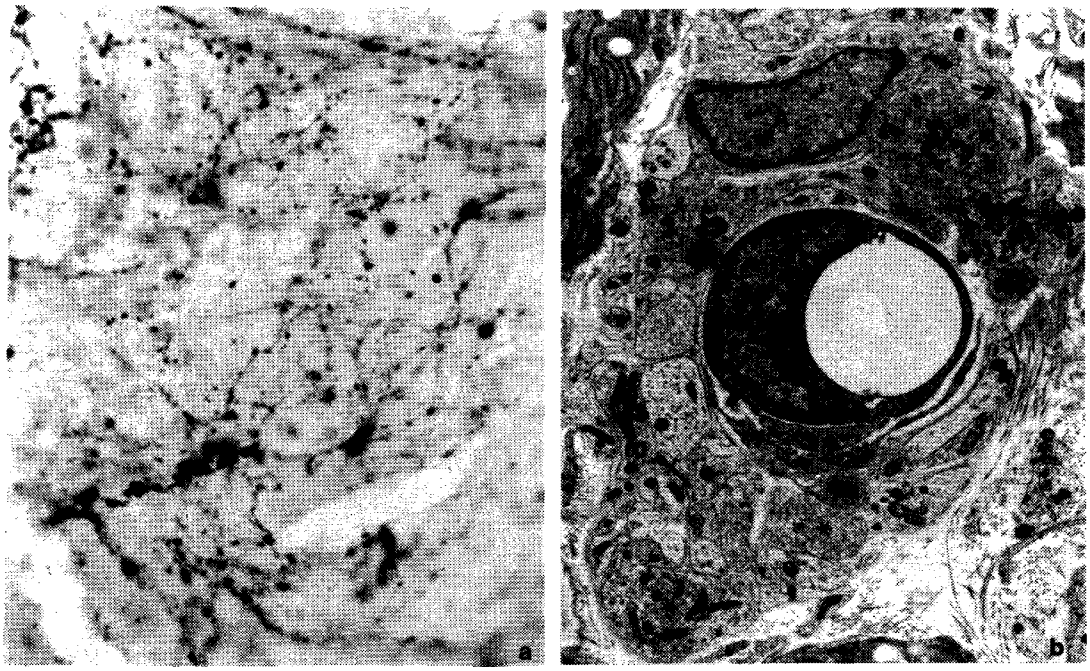

Fig. 5: Transplants of non-cryotreated optic nerve. **a:** Rather vigorous regeneration has taken place into this transplant. The more densely staining areas in the bottom half of the picture represent neurovascular contact regions. x 460. **b:** Perivascular bundles of regenerated neurosecretory axons containing neurosecretory granulated vesicles (asterisks) and/or microvesicles are invested by an astrocyte (A) and astrocyte processes. x 7600.

association between glia and transected axons is a prerequisite for regeneration. The data reported here strongly support the notion that the presence of viable glial cells is indispensable for neurosecretory axon regeneration.

Cryotreatment effectively kills neurolemmocytes in peripheral nerves /17,18,29,36/, pituicytes in neural lobe explants (4) and astrocytes and oligodendrocytes in optic nerves (present investigation). Fifteen days after transplantation, cryotreated neural lobe and sciatic nerve grafts are primarily composed of fibrocytes (whether indigenous to the graft or of host origin is unknown), abundant collagen and amorphous extracellular matrix, and some macrophages. These cells predominate in cryotreated optic nerve transplants, and connective tissue components are distinctly less abundant than in the other two types of transplants, which is likely due to the relative scarcity of these elements in the intact optic nerve, especially when the organ is divested of its meningeal covering, which was the case in our transplants. In neural lobe and sciatic nerve grafts fragments of basal lamina and collapsed basal lamina tubes are also present, but neither of these elements supports neurosecretory axon regeneration.

Basal lamina components have been suggested as substrates for axonal regeneration (for review see /17/), and basal lamina tubes have been shown to support regeneration in the peripheral nervous system /15,17,18,20/. Empty basal lamina tubes are commonly found in both predegenerated sciatic nerve grafts and neural lobe grafts, but neurosecretory axons have not been observed to regenerate into these tubes without accompanying neurolemmocytes or pituicytes /8,12/. Therefore, basal lamina alone does not support regeneration of neurosecretory axons, even in tissue that has not been cryotreated. The absence of glial cells in cryotreated grafts, therefore, is clearly the reason that regeneration does not occur. This conclusion also holds true for non-neurosecretory CNS axons /18,36/.

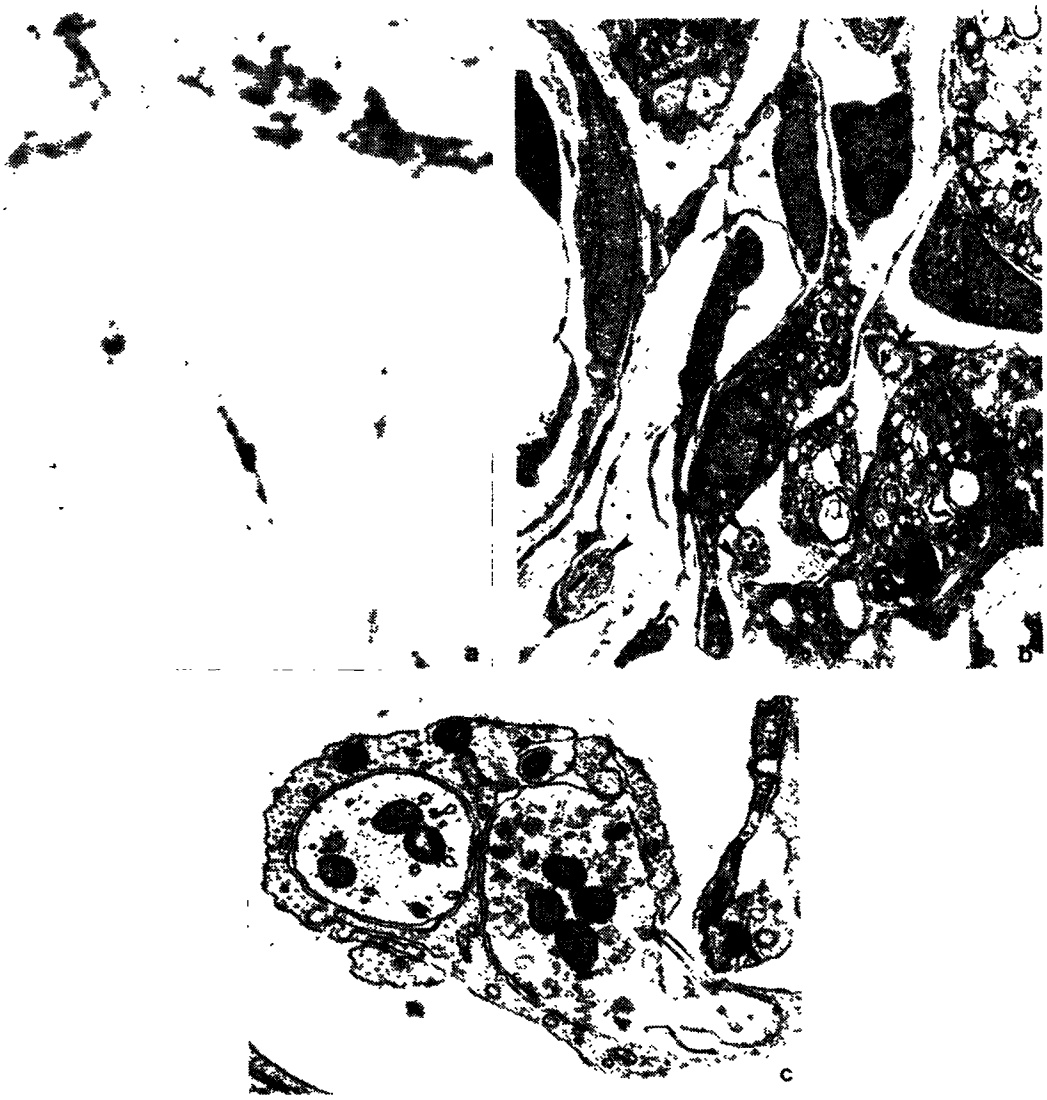

Fig. 6: Transplants of cryotreated optic nerve. **a**: A single neurosecretory axon has penetrated this transplant. x 460. **b**: Fibrocytes and macrophages are surrounded by extracellular matrix in the vicinity of a fenestrated capillary. Four neurosecretory axons invested by astrocyte processes are present (arrowheads; micrograph taken in the graft periphery). x 5250. **c**: Higher magnification of the double arrow-labeled bundle in Fig. 5b to illustrate the relationship between astrocyte processes and neurosecretory axons. x 30000.

Blood-borne factors have been implicated in the initiation of regenerative events /21/, and in fact regeneration of neurosecretory axons appears to occur only where a blood-brain barrier is absent /13; unpublished observations/. Blood-borne factors are available at the transection site immediately after placement of the graft due to extravasation at the site, and remain available throughout the observation period within grafts, since fenestrated capillaries are present in all grafts. However, it seems clear that in the absence of glial cells in cryotreated grafts, the availability of serum derived factors is insufficient to initiate neurosecretory axon regeneration.

It is logical to conclude at this point, that glial cells are an essential element in the regenerative process. What role then can glial cells be presumed to play in that process? Glial cells could mediate

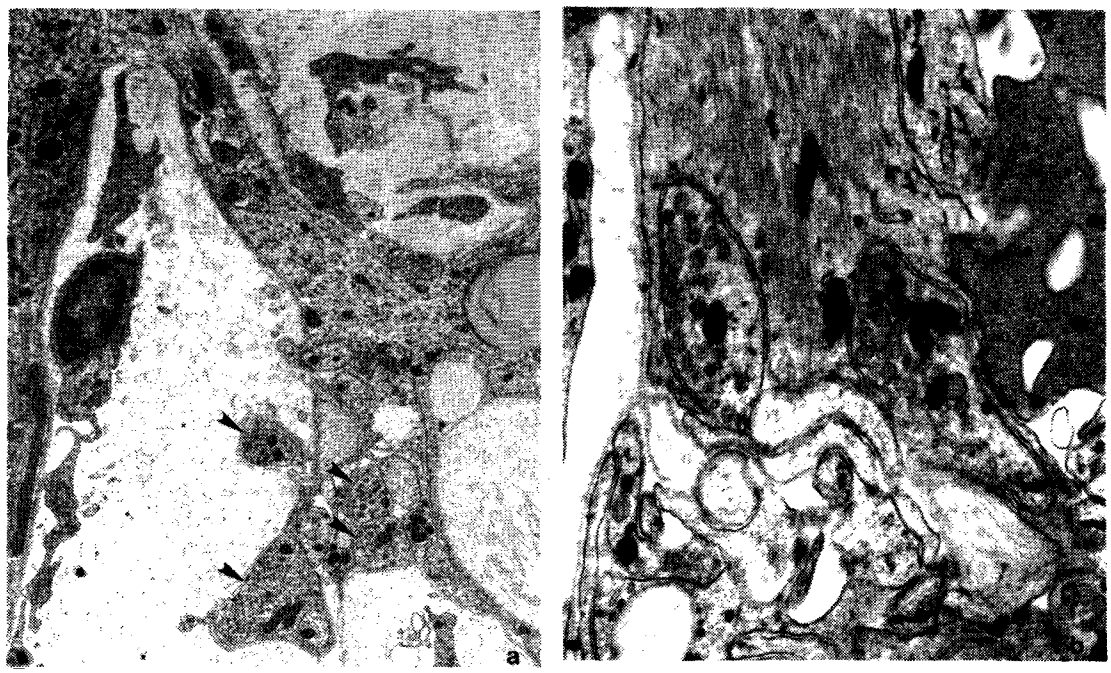

Fig. 7: **a**: Neurosecretory axons have penetrated a short distance into the periphery of this cryotreated neural lobe transplant along astrocyte processes from the adjacent hypothalamus. x 7200. **b**: Neurosecretory axons are closely invested by a basal lamina-enclosed astrocyte process in the periphery of a cryotreated sciatic nerve transplant. x 21000.

regeneration through direct interaction with the axon, or through release of trophic substances, or both. The close juxtaposition of all 3 types of glial cells with the regenerating neurosecretory axons suggests that axon extension takes place along the glial cell surface; therefore, adhesion molecules are probably involved in the process /33/. Candidate molecules are L1, which has been implicated in PNS axonal regeneration /2,5,26,28/, and NCAM, which has recently been shown to occur in the hypothalamo-neurohypophysial system /38/. The observations that neurosecretory axons regenerate into cryotreated grafts only when accompanied by basal lamina-ensheathed astrocyte processes, and that they do not grow into the grafts beyond these glial processes, strongly support the notion that the glial cell surface provides a guidance substrate. On the other hand, the fine structural characteristics of transplanted pituicytes are typical of highly secretory cells, and our recent *in vitro* studies have provided evidence for the existence of a pituicyte-derived trophic factor for developing neurosecretory neurons (unpublished observations), which could conceivably be operative in adult neurosecretory neurons as well. Such a factor could initiate and sustain regeneration of transected neurosecretory axons. NGF /16,23,40/, astrocyte-derived factors /22,25/ and bFGF /3,24,35,39/ have been shown to be secreted by neurolemmocytes and astrocytes, and could conceivably initiate and promote neurosecretory axon regeneration.

Our experiments clearly support the concept that glial cells are indispensable for neurosecretory axon regeneration, and that serum-derived factors with basal lamina and other extracellular matrix elements alone, provide an insufficiently supportive microenvironment.

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