Journal of Neurocytology **21**, 79–93 (1992)

Changes in nerve growth factor receptor-like immunoreactivity in the spinal cord after ventral funiculus lesion in adult cats

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Received 10 December 1990; revised 30 April and 7 August 1991; accepted 19 August 1991

Summary

Spinal motoneurons have a capability to regenerate CNS-type axons after intramedullary lesions in the adult cat. Regrowing axons have been traced through CNS-type scar tissue in the ventral funiculus of the spinal cord and into adjacent ventral root fascicles. This scar tissue, which appears to support and sustain regenerating axons, has been shown to have a persistent defect in the blood–brain barrier. It has been suggested that the blood–brain barrier may play a vital role in CNS regeneration by regulating the access of blood-borne trophic factors to the lesion area. In the present study, the binding of antibodies to the human nerve growth factor receptor in the cat spinal cord was examined with immunohistochemical methods 2 days to 8 weeks after a ventral funiculus lesion. The results show that, while no neurons in the ventral horn of the control material contained nerve growth factor receptor-like immunoreactivity as revealed by fluorescence microscopy, affected motoneurons expressed nerve growth factor receptor after ventral funiculus lesion. Nerve growth factor receptor-like immunoreactivity associated to both capillaries and interstitium was present in the scar tissue. Electron microscopic examination of sections labelled with the immunogold-silver method showed that perivascular nerve growth factor receptor-like immunoreactivity was located exclusively to non-pericytic perivascular cells. These cells were abundant in the expanded capillary perivascular spaces adjacent to the traumatic lesion. Similar cells, with or without relation to blood vessels, were observed in the scar tissue and in the pia mater. In a separate set of specimens it was observed that a ventral funiculus lesion combined with ventral root avulsion, which removes denervated PNS tissue, resulted in an expression of nerve growth factor receptor-like immunoreactivity which was similar to the one observed after ventral funiculus lesion only. The results of the present study show that affected motoneurons and cells in the scar tissue express nerve growth factor receptor after ventral funiculus lesion which implies that neurotrophic factors related to nerve growth factor may be of importance for the regenerative response.

Introduction

The formation of scar tissue after a traumatic lesion in the CNS is a complex process in which both glial and various mesodermal cells participate. Haematogenous cells and fibroblasts derived from the meninges contribute in this reaction (Berry *et al.*, 1983). The relation between this cicatrix and failure of CNS regeneration is enigmatic. The possibility that the scar could represent a mechanical barrier to growing axons has been widely discussed (see Kiernan, 1979). Recent data show that CNS myelin and oligodendrocyte membranes contain two minor proteins with strong inhibitory effects on growing neurites (Caroni & Schwab, 1988; Schwab, 1990). In a scar tissue deprived of most its oligodendrocytes (David *et al.*, 1984; Franson, 1985), though, the significance of inhibitory oligodendrocyte-associated proteins has yet to be settled. Glial hyaluronate-binding protein (GHAP), a glycoprotein produced by white matter astrocytes, has been suggested to represent a non-permissive factor for regenerating axons (Bignami *et al.*, 1988). Glial hyaluronate-binding protein is not, however, expressed in astrocytes following penetrating traumatic injury (Mansour *et al.*, 1990). Some data indicate that activated astrocytes, by secreting nerve growth factor (NGF), could facilitate posttraumatic sprouting (Gage *et al.*, 1988). Other studies have emphasized the presence or absence of certain components in the extracellular matrix, such as the basal lamina associated glycoprotein laminin (Manthorpe *et al.*, 1983; Berry *et al.*, 1988; David, 1988; Liesi & Silver, 1988). A lesion-induced defect in the blood-brain barrier (BBB) (Kiernan, 1985) may provide a route for circulating

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substances and mononuclear phagocytes, which release endothelial and fibroblast mitogens as well as activators of collagen synthesis (Berry *et al.*, 1983). The BBB function is normally restored in the lesion area within about 4 weeks (Kiernan, 1979, 1985). This roughly coincides with cessation of the axonal sprouting elicited by the lesion (Ramon y Cajal, 1928; Kiernan, 1979). Theoretically, blood-borne trophic substances (see Stephani *et al.*, 1987) could reach injured axons through a persistent defect in the BBB (Kiernan & Contestabile, 1980). Vascular changes at the site of lesion may in this way contribute to the regenerative response.

In previous studies, we have demonstrated that spinal motoneurons, after lesions in the ventral funiculus or ventral root replantation, are able to regenerate axons through CNS scar tissue into denervated ventral roots (Risling *et al.*, 1983; Lindå *et al.*, 1985; Cullheim *et al.*, 1989). These lesions have been shown to be associated with a durable defect in the BBB (Risling *et al.*, 1989, 1990; Sjögren *et al.*, 1991). Against this background it is of obvious interest to examine the distribution of various growth factors and their receptors in this particular type of CNS scar tissue, which lacks BBB function and appears to sustain the growth of axonal sprouts. In the present study we performed an immunohistochemical analysis of the binding of antibodies to the NGF receptor (NGFr) in injured spinal cord tissue after a ventral funiculus lesion.

Materials and methods

Twelve young adult cats were anaesthetized using Rompun Vet (2 mg kg^{-1} s.c.; Bayer, Leverkusen, FRG) and Mebumal Vet (30 mg kg^{-1} i.p; NordVacc, Stockholm). The lumbosacral spinal cord was exposed and a longitudinal incision was made in the left ventral funiculus of the spinal cord segment L7 (Fig. 1), as described in detail previously (Risling *et al.*, 1983). In nine cats, the ventral funiculus incision was combined with additional lesions (cf Fig. 4), in order to determine the possible influence of denervated PNS tissue distal to the lesion. In these animals, the ventral funiculus lesion involved both the L6 and L7 segments after avulsion of the ventral roots L7 and S1 at the surface of the spinal cord.

After a post-operative survival time of 2 days to 8 weeks the operated cats and four age-matched controls were anaesthetized (as described above) and perfused through the descending aorta with Tyrode's solution followed by ice-cold 4% formaldehyde with 0.2% picric acid in phosphate buffer. The picric acid was replaced by 0.5% glutaraldehyde in one operated cat (8 weeks survival) and one of the control cats. These two animals were used for electron microscopic evaluation. After perfusion, the lumbosacral spinal cord was gently dissected out from the operated animals. Specimens from the brain stem, at the level of the area postrema, the optic nerve, olfactory bulb, the cerebral cortex and the lumbo-sacral cord were trimmed out from the control animals. All specimens were immersed in the fixative for 90 min and rinsed overnight in phosphate buffer.

Specimens from animals perfused with formaldehyde and picric acid were cut in a cryostat. Transverse sections 14 μm thick were collected on gelatine-coated glass slides. Speci-

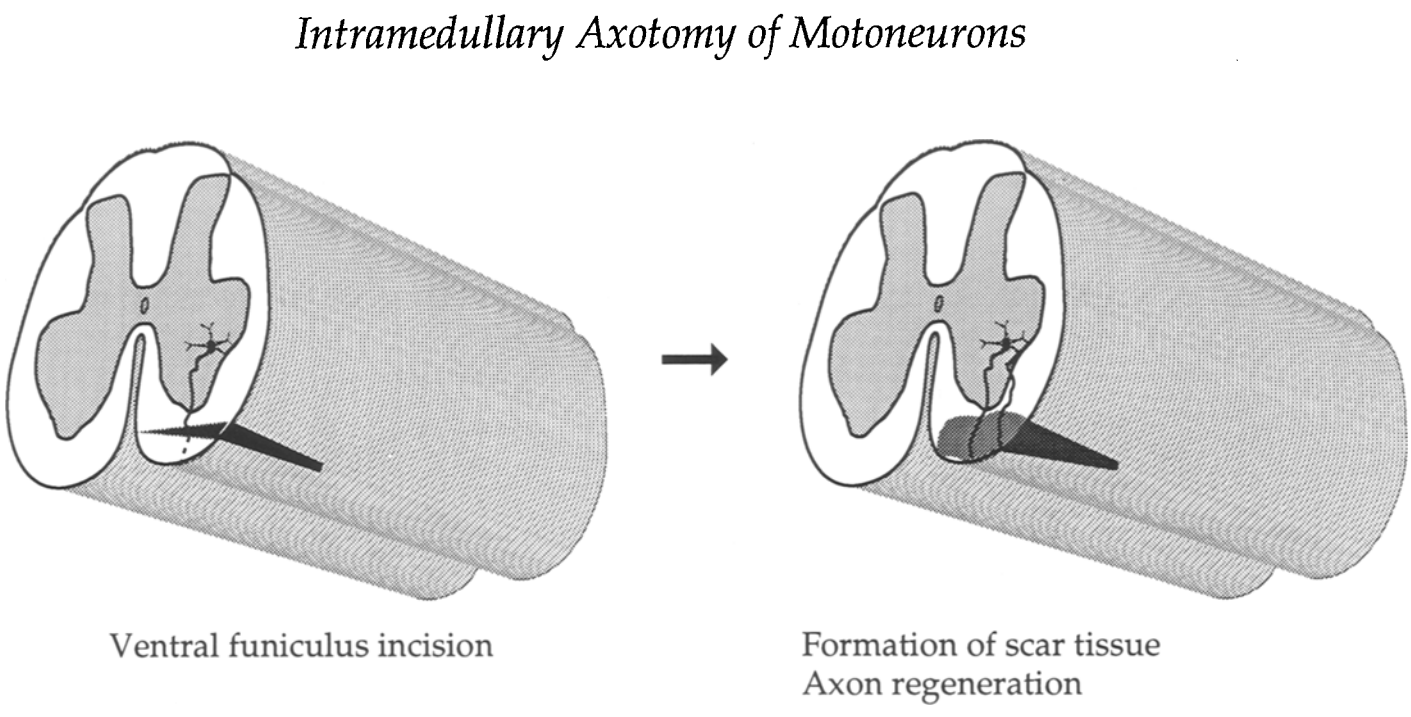


Fig. 1. Schematic drawings illustrating the location of the lesion in the ventral funiculus.

mens subjected to ventral funiculus lesion, ventral funiculus lesion combined with ventral root avulsion, ventral root avulsion only and controls were cut in one block and laid on a single glass slide for identical immunohistochemical treatment. The sections were incubated with antibodies to human NGFr (dilution 1:100) for 24 h at 4° C in a humid atmosphere. This antibody (IgG1-kappa) was produced from a mouse hybridoma 200–3–G6–4 (see Ross *et al.*, 1984). The cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The binding characteristics of this antibody to the normal feline spinal cord has recently been described (Fried *et al.*, 1990). For controls some sections were incubated with a monoclonal antibody S2C6 directed against the human B-lymphocyte and carcinoma-associated antigen CDw40 (IgG1), which is known to have an extensive structural homology with the NGFr (Stamenkovic *et al.*, 1989). The antibodies were purified on protein A-sepharose (Pharmacia, Uppsala, Sweden) from culture supernatants and were used at a final concentration of 5 $\mu g\ ml^{-1}$. In order to determine the amount of gliosis in the scar tissue, some sections were incubated with a polyclonal antibody to glial fibrillary acidic protein (GFAP) raised in rabbits (Dakopatts, Denmark; 1:100) or with a mixture of GFAP and NGFr antibodies. After incubation with primary antibodies, the sections were rinsed in phosphate buffered saline (PBS) and incubated with rhodamine-conjugated goat-anti-mouse antibodies (dilution 1:100; Boehringer Mannheim, Indianapolis, USA) or with fluorescein-isothiocyanate (FITC)-conjugated swine anti-rabbit antibodies (dilution 1:10; Dakopatts, Denmark), for 30 min at 22° C. All antibodies were diluted in 0.01 M PBS containing 0.3% Triton X-100. Whole-mount stretch preparations of the spinal pia mater in control animals were incubated with NGFr antibodies as described above. Finally, the sections and pia mater preparations were mounted, coverslipped and examined in a Nikon Microphot-FX microscope equipped for epifluorescence.

Slices (50 μm) of specimens from animals perfused with the mixture of formaldehyde and glutaraldehyde were obtained using an Oxford Vibratome®. Some of the sections were transferred through a series of graded ethanols and then rehydrated in order to improve the penetration of the antibodies. The sections were treated with sodium-borohydride (see Priestley, 1984; Varndell & Polak, 1986), preincubated in 5% BSA and incubated with the NGFr antibody (diluted 1:200 in 0.01 M PBS containing 0.5% IGSS gelatin; Jansen Biotech, Belgium) for 24 h at 4° C in a humid atmosphere. The sections were rinsed in PBS and incubated with 1 or 5 nm gold-particle-conjugated goat-anti-mouse antibodies (Auroprobe One and Auroprobe LM, Jansen Biotech, Belgium; diluted 1:50 in 0.01 M PBS containing 0.5% IGS gelatin) for 60 min at 22° C. The specimens were rinsed in PBS and osmicated overnight according to a modification of the Marchi-method (Hildebrand & Aldskogius, 1976). The osmicated sections were rinsed in PBS and the binding of gold particles was visualized using a silver enhancement reaction (Intense M, Jansen Biotech, Belgium; Basbaum, 1989; Fried & Risling, 1991). After light-microscopic examination and documentation, the sections were dehydrated in acetone and embedded in Vestopal W® (Carlstedt, 1977). Whole mount preparations of the pia mater were treated in the same way. Thin silver-grey transverse sections from selected areas were collected on 460 hex grids (Polaron, UK) using glass knives and an LKB Ultrotome III. Finally these sections were contrasted with uranyl acetate and lead citrate and examined in a Philips CM12 electron microscope.

Results

Nerve growth factor receptor-like immunoreactivity (NGFr-LI), as observed by light microscopy, was present in neurons, nerve fibres and blood vessels at various locations in the CNS. The distribution of NGFr-LI as revealed by examination with immunofluorescence technique was very similar to that observed in sections incubated with gold-conjugated secondary antibodies and enhanced with silver. Best results with the gold/silver method were obtained when incubation was preceded by a subtotal dehydration and visualized using 1 nm gold-conjugated secondary antibodies. Neuronal labelling in normal animals corresponded to results obtained in previous immunohistochemical studies (Allen *et al.*, 1989; Yan & Johnson, 1989; Fried *et al.*, 1990; Pioro & Cuello, 1990) and will not, with exception of the motor nucleus of the spinal cord, be further commented on. Neurons in the ventral horn contained no NGFr-LI as revealed by fluorescence microscopy. However, in vibratome sections incubated with gold-conjugated secondary antibodies, a weak neuronal labelling in the ventral horn sometimes was perceived after silver enhancement. Blood vessels in all examined parts of the intact CNS presented NGFr-LI (Fig. 2). The labelling appeared to be restricted to large blood vessels surrounded by perivascular spaces. Nerve growth factor receptor immunoreactive blood vessels prevailed in the superficial parts of the cerebral cortex and spinal cord. The immunoreactivity was very dense in the area postrema (Fig. 2c–e), the superficial part of the olfactory bulb (Fig. 2f) and in the most distal portion of the optic nerve, i.e. the optic nerve head (ONH) region (Hildebrand *et al.*, 1985) (Fig. 2g). Stretch preparations of the spinal pia mater displayed a network of thin NGFr immunoreactive structures (Fig. 2h).

Fluorescence microscopic examination of cryostat sections from operated animals showed that scar tissue resulting from the ventral funiculus incision contained a large number of thin elongate structures bearing NGFr-LI (Fig. 3a and b). The scar tissue labelling, which was continuous with the pia mater, could be observed four days after the operation but seemed to reach a higher density after three weeks survival time. An increased number of blood vessels displaying NGFr-LI could be observed in the scar tissue and the adjacent white and grey matter. No ventral funiculus scar tissue displaying NGFr-LI could be detected after ventral root avulsion only. However, the pia mater surrounding the avulsion site was

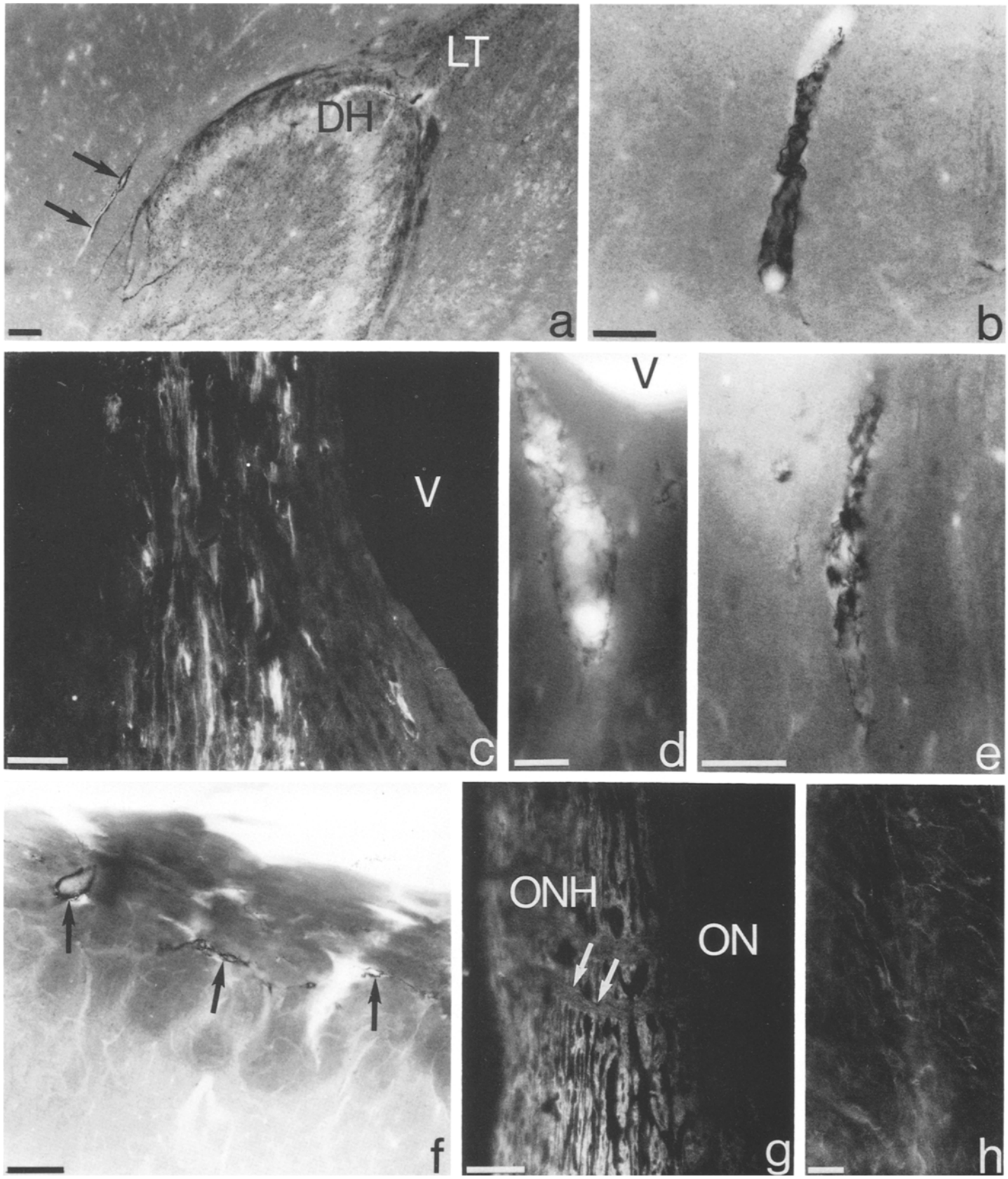


Fig. 2. Light micrographs showing 50 μm vibratome slices (a,b,d–f), 14 μm cryostat sections (c,g) and a whole mount preparation of the pia mater (h) from normal adult cats, after incubation with NGFr antibodies. Nerve growth factor receptor-like immunoreactivity was visualized using gold-conjugated secondary antibodies which after silver enhancement provided a dark reaction product (a,b,d–f) or with rhodamine-conjugated sheep-anti-mouse antibodies (c,g,h). Vascular NGFr-LI in the cerebral cortex and the spinal cord (a and b) was restricted to vessels of the arteriolar size range. Note the labelling of nerve fibres in the superficial layers of the dorsal horn (DH) and the tract of Lissauer (LT). A dense labelling was observed in the area postrema (c–e) (V = 4th ventricle), where NGFr-LI appeared to be associated with vessels of all sizes. Numerous vessels displaying NGFr-LI were observed in the superficial layers of the olfactory bulb (f). An intense NGFr-LI was present in the optic nerve head region (ONH) but not in the optic nerve proper (ON in Fig. g). A network of thin NGFr labelled structures, lacking obvious vascular relations, was observed in the pia mater (h). Scale bars = 50 μm.

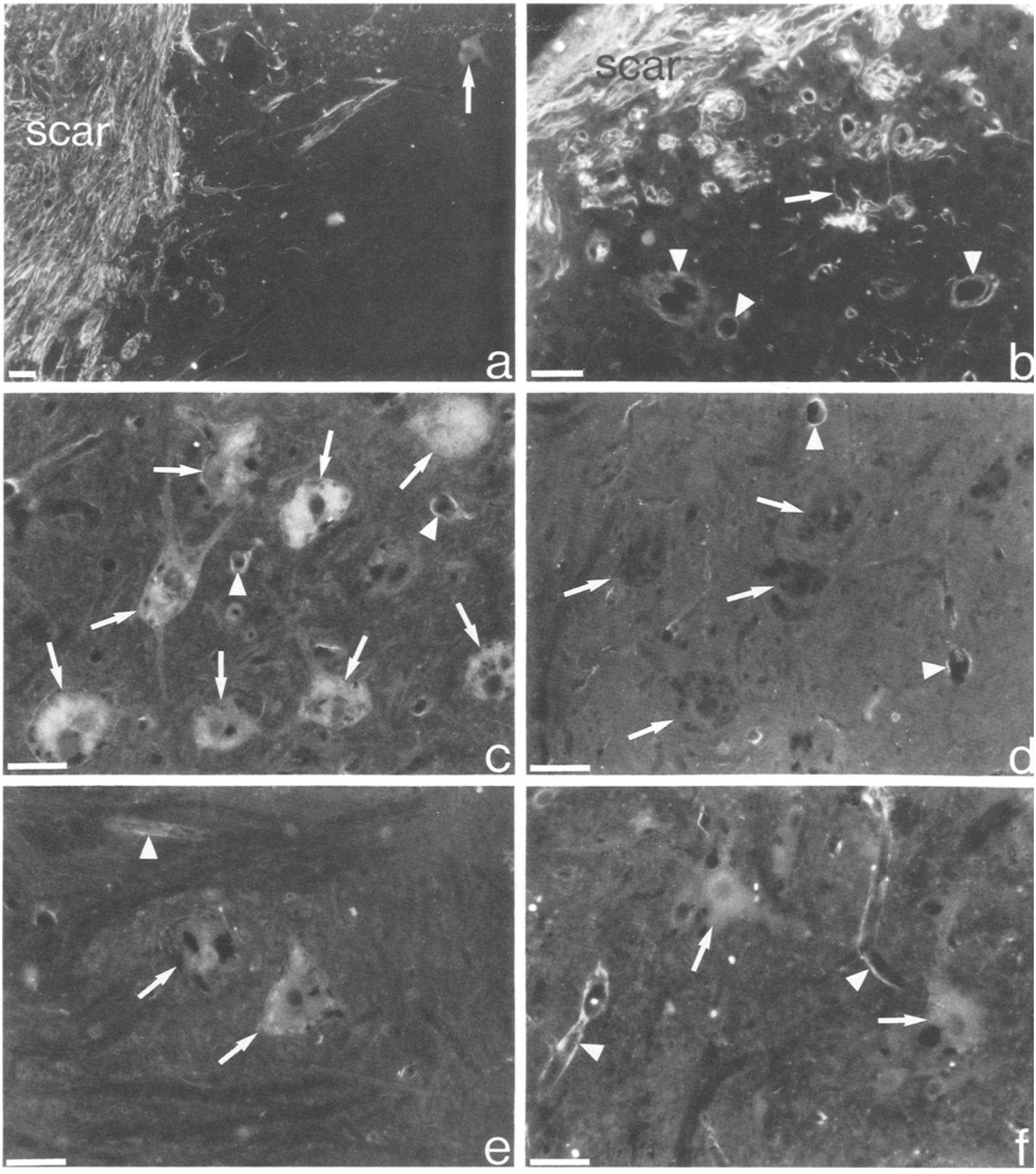


Fig. 3. Immunofluorescence micrographs showing transverse cryostat sections from the lumbosacral spinal cord in operated animals. A dense NGFr-LI was observed in the scar tissue three weeks after ventral funiculus lesion (a). Note the labelled neuron adjacent to the lesion area (arrow). Binding of NGFr antibodies to the scar tissue was still evident at eight weeks after the lesion (b). Large and small diameter blood vessels which appeared to have expanded perivascular spaces (arrowheads) and thin elongate processes, in the vicinity of the scar tissue (arrow in b), were labelled. Examination of the motor nucleus three weeks after ventral funiculus lesion combined with root avulsion showed that most neurons within the alpha-motoneuron size range possessed NGFr-LI on the experimental side (c) but not on the control side (d). Avulsion lesion only resulted in a less evident expression of NGFr-LI. Two neurons which displayed a weak NGFr-LI, three weeks after ventral root avulsion, are shown in (e). A few neurons were still found to be labelled with NGFr antibodies eight weeks after ventral funiculus lesion (f). Large neurons within the motor nucleus are represented by arrows in (c–f) and NGFr positive blood vessels are indicated by arrowheads. Scale bars = 50 μm.

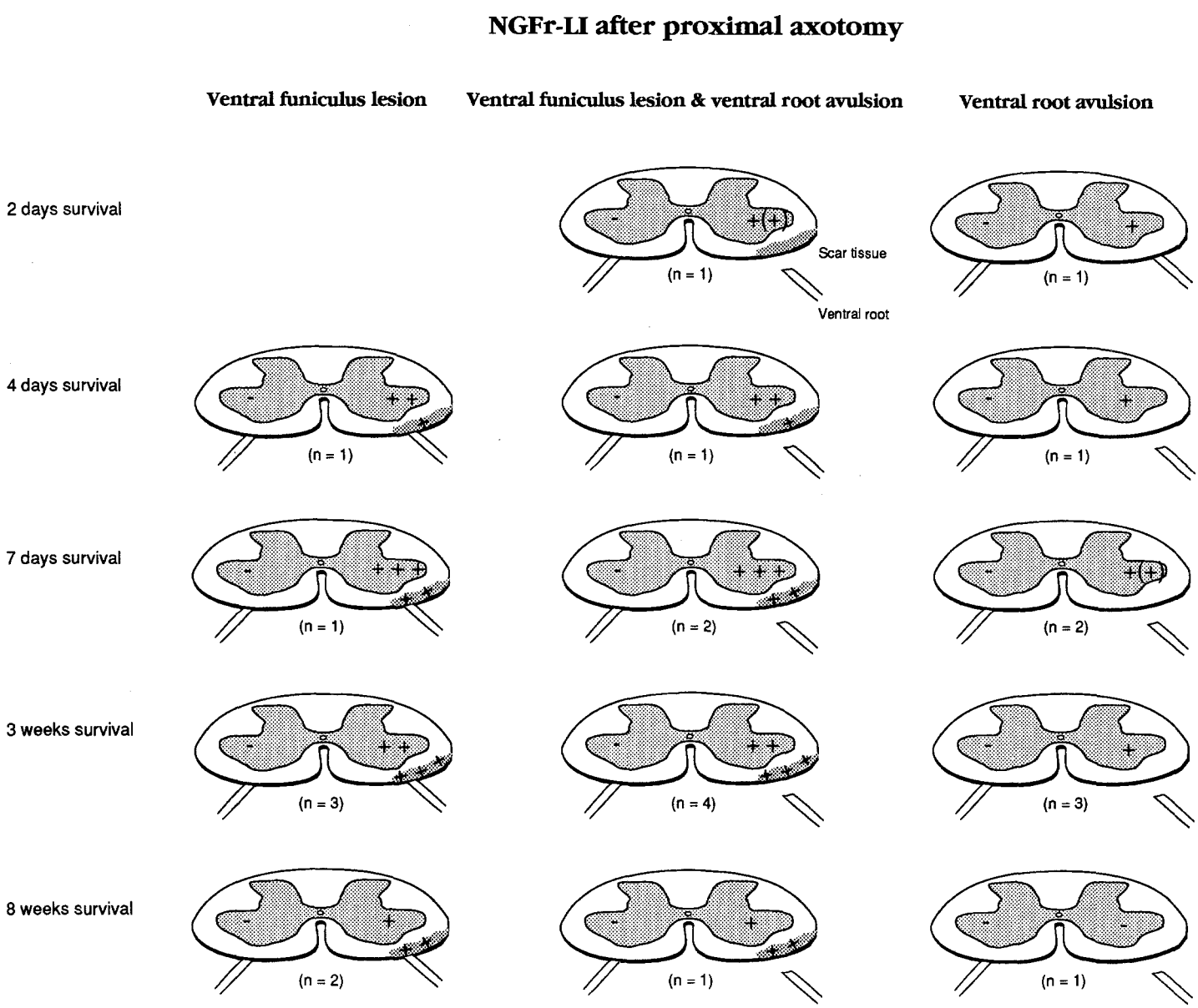


Fig. 4. A schematic representation of the semiquantitative data obtained in the present study. NGFr-like immunoreactivity (NGFr-LI) in motoneurons has been indicated as + (0–33% of the affected motoneurons showed NGFr-LI), ++ (34–66%), +++ (67–100%) or − (absent). NGFr-LI in the scar tissue has been expressed as + (weak), ++ (moderate) or +++ (strong). Note that in nine of the 11 animals that were used for this part of the study, the ventral funiculus lesion was combined with root avulsions. Theoretically, each of these cases could have generated three different types of specimens. The total number of possible observations would then be 29. However, due to incomplete lesions, some of the specimens were excluded from this semi-quantitative figure. For further explanation see text.

usually hypertrophic and contained an increased amount of NGFr-LI.

NGFr-LI in ventral horn neurons was only assessed in the spinal cord segments L6, L7 and S1. No labelled neurons could be detected in the ventral horn in the control animals. A small number of labelled neurons were observed in the ventral horn on the experimental side four days after ventral funiculus lesion. Virtually all large neurons in lamina IX on the experimental side showed NGFr-LI at seven days after ventral funiculus lesion. This labelling was grossly maintained at three weeks after the lesion (Fig. 3c). Eight weeks after ventral funiculus lesion NGFr-LI was present in only some few scattered neurons (Fig. 3f). A comparison between specimens subjected to ventral funiculus lesion alone and a combination of ventral funiculus lesion and ventral root avulsion indicated that neither the intensity of the labelling nor the number of NGFr-immunoreactive neurons was affected by the presence of the denervated ventral root. Ventral root avulsion alone, however, resulted in a less prominent neuronal labelling compared to ventral funiculus lesion (Fig. 3e). No NGFr-immunoreactive neurons were observed on the control side in operated animals (Fig.

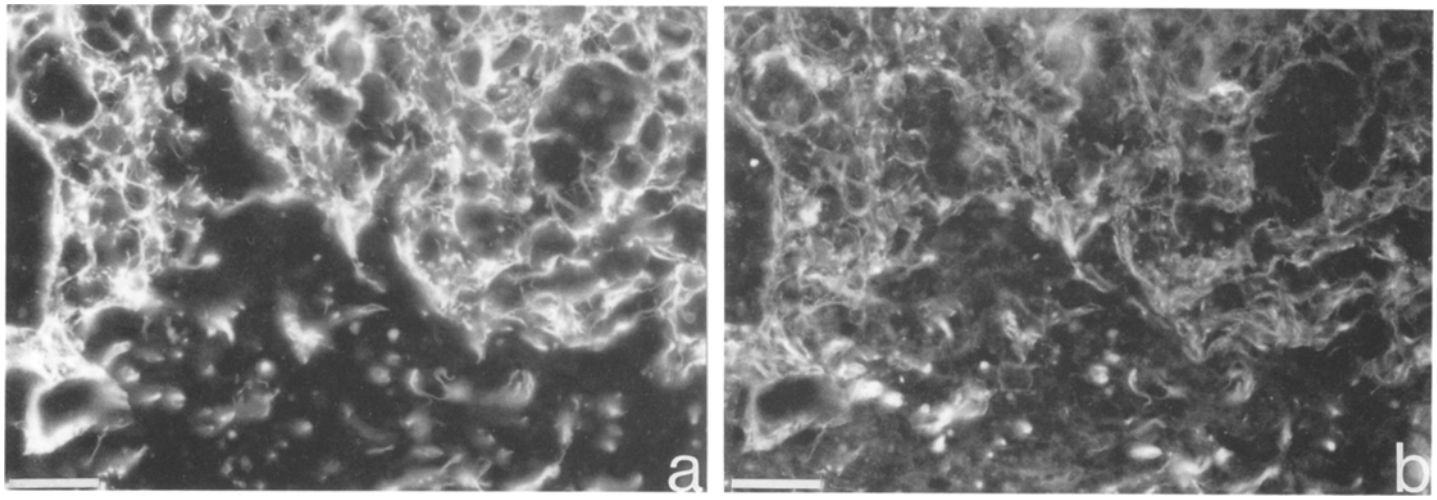


Fig. 5. Immunofluorescence micrographs showing a 14 μm transverse section through the scar tissue three weeks after ventral funiculus lesion. Double incubation with GFAP ((a), FITC fluorescence) and NGFr ((b), rhodamine fluorescence) antibodies. Note that the immunostaining obtained with the two antibodies have a similar distribution in the scar tissue. Scale bars = 50 μm.

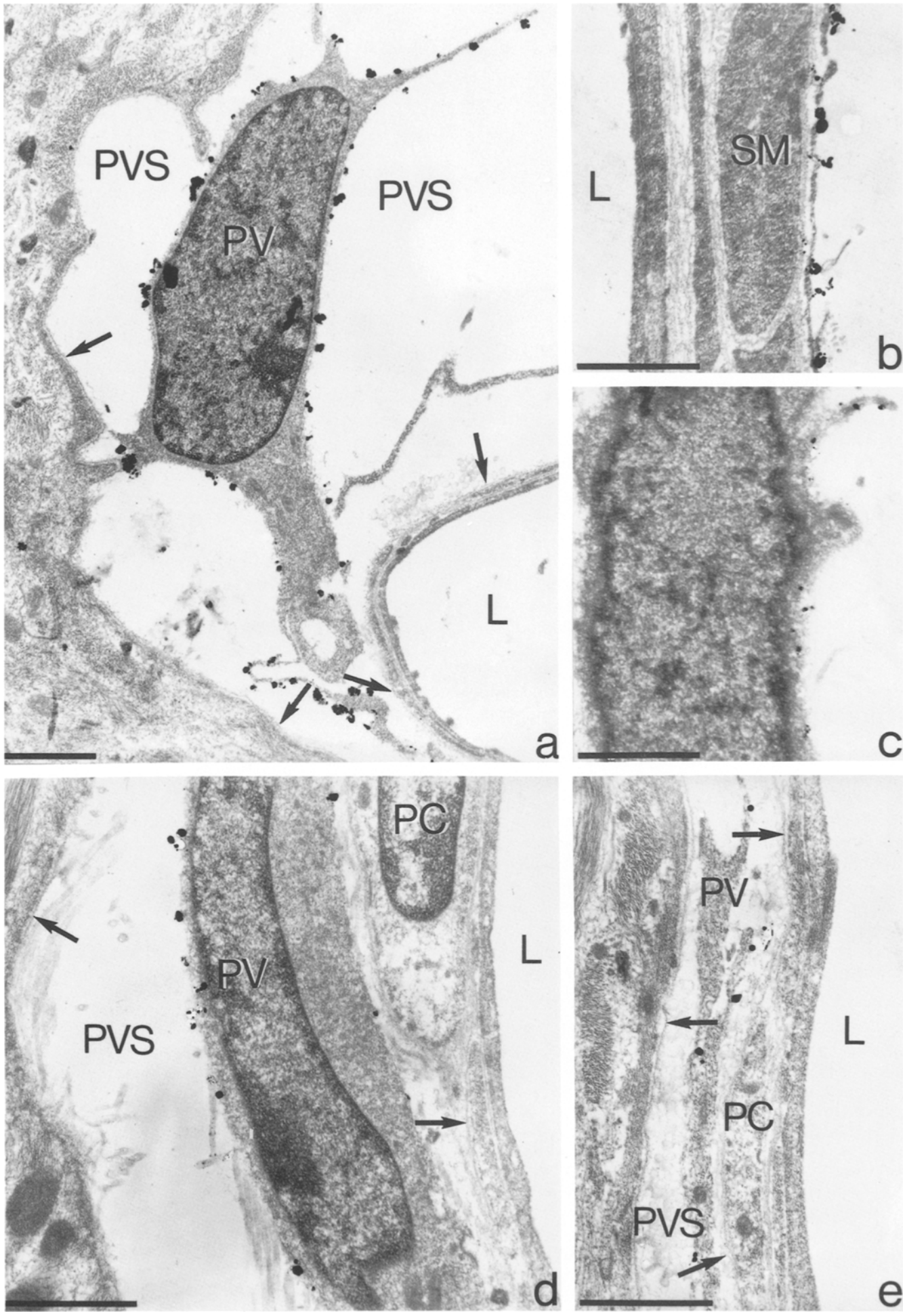
3d). These semiquantitative data have been compiled in Fig. 4. Examination of sections incubated with GFAP antibodies revealed that the intensity of GFAP immunoreactivity in the scar increased markedly during the first postoperative weeks. Double-labelling experiments showed that GFAP- and NGFr immunoreactivity often had a similar distribution within the trabecular superficial cicatrix (Fig. 5a and b). Sections incubated with the secondary antibody only or with control primary antibodies presented no specific binding to neurons or blood vessels, in either operated or control animals.

Selected vibratome sections were re-embedded and subjected to thin sectioning. Electron microscopic examination showed that the 1 nm gold particles after silver enhancement provided an electron dense reaction product, within the 5–120 nm size range. Vascular NGFr-LI, as observed in the electron microscope, was located to the surface of elongate cells, present between the endothelial and parenchymal basal laminae (Fig. 6). These cells lacked a basal lamina and were found to have a darker cytoplasm than pericytes, which are enclosed by the endothelial basal membrane. Large numbers of NGFr-immunoreactive non-pericytic cells were observed in the wide capillary perivascular spaces of the area postrema in normal animals (Fig. 6a). NGFr immunoreactive cells were present in perivascular spaces surrounding arterioles in the cerebral cortex (Fig. 6b) and spinal cord. Gold-labelled cells with a similar morphology, with or without vascular relation, were also observed in the pia mater (Fig. 6c).

For a comprehensive electron microscopic description of the scar tissue and associated changes in the vascular morphology after ventral funiculus lesion, the reader is referred to previous reports (Risling *et al.*, 1983, 1989). A prominent feature of this lesion is that the capillaries in the ventral horn adjacent to the scar tissue are surrounded by expanded perivascular spaces with a jagged outline. In such regions, perivascular cells of non-pericytic appearance and elongate cell processes labelled with gold-silver particles were abundant (Fig. 6d and e). A large number of gold-labelled cells and processes, lacking a vascular relation, were present in the scar tissue (Fig. 7a–c). These cells strongly resembled non-perivascular cells.

Fig. 6. Electron micrographs showing sections incubated with NGFr antibodies and 1 nm gold-conjugated secondary antibodies. The gold labelling was visualized with silver enhancement. Vascular NGFr-LI was found to be located to the surface of non-pericytic perivascular cells. These cells were observed in perivascular spaces surrounding capillaries in regions known to lack blood-brain barrier function, such as the area postrema (a). In the cerebral cortex, vascular labelling was restricted to a thin cellular layer in perivascular spaces associated with larger calibre vessels (b). Examination of the pia mater revealed NGFr-LI on the surface on a cell type which resembled the non-pericytic perivascular cells (c). Capillaries with expanded perivascular spaces were observed adjacent to the ventral funiculus scar tissue. Abundant non-pericytic cells displaying NGFr-LI were present in these perivascular spaces. Arrows indicate basal laminae. (L = lumen of blood vessel; PVS = perivascular space; SM = smooth muscle cell; PC = pericyte). Scale bars = 1 μm.

Fig. 7. Electron micrographs showing NGFr-LI in the ventral funiculus scar tissue. The gold-silver complexes were observed on the surface of cellular elements resembling non-pericytic perivascular cells but not associated with axons (Ax), astrocytic processes (A) or collagen fibres (C). Scale bars = 1 μm.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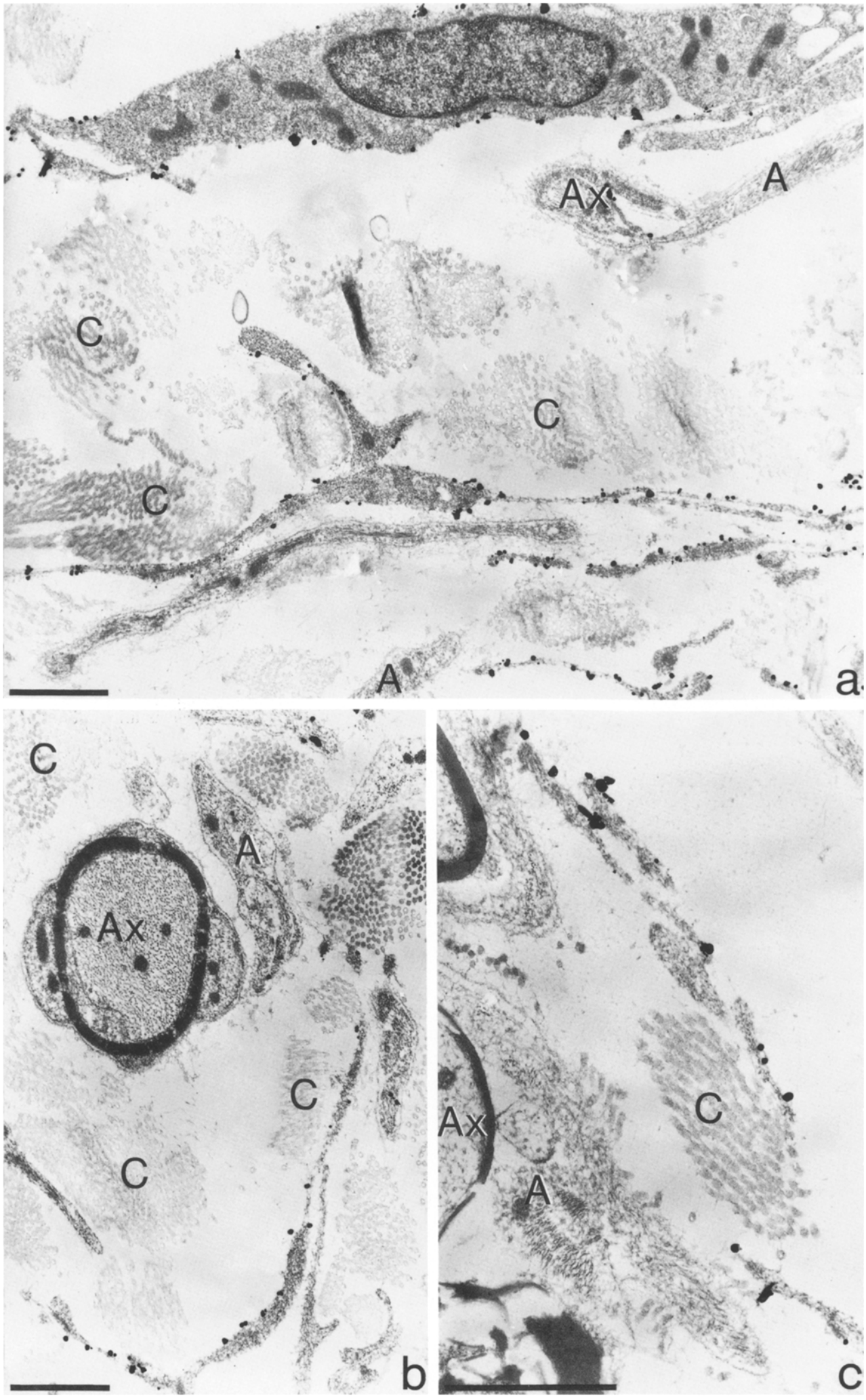
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Myelinated axons, glial processes, basal laminae or collagen fibres in the scar tissue were unlabelled with gold particles. Some occasional profiles, resembling unmyelinated axons, presented a weak immunolabelling. A few unlabelled Schwann cells, related to axons, were observed in the superficial layers of the scar.

Discussion

The target-derived neurotrophic factor NGF has a well-known ability to prevent retrograde cell death and to promote axon regeneration in primary sensory neurons and central cholinergic neurons (See Lindsay, 1988; Brown *et al.*, 1991; Hagg *et al.*, 1991). It seems still to be uncertain precisely how NGF exerts these effects, which represent important determinants for the outcome after lesions in the nervous system. On the cellular level, though, NGF has been shown to regulate levels of microtubule-associated protein and neuropeptides (e.g. Aletta *et al.*, 1988; Lindsay *et al.*, 1989). Nerve growth factor seems to initiate its effects on responsive cells by interacting with cell surface receptors, (i.e. NGFr). Two different classes of NGF binding sites have been described. The majority of the receptor sites display low affinity binding of NGF. *In vitro* experiments indicate that biological effects require interactions of NGF with a high affinity site (see Hempstead & Chao, 1989; Snider & Johnson, 1989; Marchetti & McManaman, 1990). Receptors in the process of endocytosis seem to represent high affinity receptors while low affinity receptors occur free on the surface. The two receptor species can be interconverted by agents that manipulate the receptor internalization cycle (Eveleth & Bradshaw, 1988). Recent data suggest that high affinity binding requires coexpression of at least two different receptor proteins. The so-called low affinity NGF receptor is a glycosylated transmembrane protein which generates only low affinity binding sites for NGF (Johnson *et al.*, 1986). Coexpression of another protein, the *trk* protooncogene product, is required for the formation of specific high affinity binding sites for NGF (Hempstead *et al.*, 1991). Monoclonal antibodies to the low affinity receptor protein may block the effects of NGF and cause neural degeneration *in vivo* but not *in vitro* (Johnson *et al.*, 1989). Such NGFr antibodies have been used to survey the distribution of NGFr-LI in the normal adult nervous system (e.g. Allen *et al.*, 1989; Yan & Johnson, 1989; Fried *et al.*, 1990; Pioro & Cuello, 1990; Fried & Risling, 1991).

High and low affinity NGFr are present at significant levels in motoneurons during prenatal development (Raivich *et al.*, 1985; Eckenstein, 1988; Yan & Johnson, 1988; Yan *et al.*, 1988; Marchetti & McManaman, 1990) but, with few exceptions, not in the normal adult stage. A group of motoneurons, probably corresponding to the nucleus of Onufrowicz, has been shown to maintain NGFr immunoreactivity also in adults (Koliatsos *et al.*, 1991b). It is noteworthy that this nucleus appears to be spared by degenerative diseases like amyotrophic lateral sclerosis (Mannen *et al.*, 1977). Recent studies have revealed a re-expression of NGFr in motoneurons after peripheral nerve lesions in adult rats. This re-expression of NGFr is more pronounced after nerve crush than after nerve resection and has been shown to cease at the time when regeneration is completed (Ernfors *et al.*, 1989; Wood *et al.*, 1990; Koliatsos *et al.*, 1991a; Saika *et al.*, 1991). Nerve growth factor is retrogradely transported by motoneurons from peripheral nerve terminals in the newborn rat (Yan *et al.*, 1988). Treatment of newborn rats with NGF does not, however, increase motoneuron size or synthesis of neurotransmittor enzymes and does not appear to prevent retrograde neuronal degeneration after axotomy (Yan *et al.*, 1988). Motor axon sprouting after lesions in the PNS does not seem to be dependent on NGF (e.g. Brown *et al.*, 1991). The possibility that NGFr in motoneurons mediates effects of neurotrophic factors other than NGF should therefore be considered. Brain-derived neurotrophic factor (BDNF) and hippocampal neurotrophic factor, i.e. neurotrophin-3, are structurally and functionally related to NGF (Ernfors *et al.*, 1990; Hohn *et al.*, 1990). Although these factors have unique biological activities, it has been shown that they bind to and interact with the low affinity NGFr (Ernfors *et al.*, 1990; Rodriguez-Tebar *et al.*, 1990). These data indicate that the low affinity NGFr protein is an important component in the specific receptors for a family of neurotrophic factors that may cooperate to support the development and maintenance of various types of neurons (see Ragsdale & Woodgett, 1991). The expression of NGFr in motoneurons may then signify a dependency of a neurotrophic factor that has yet to be identified (cf Thoenen, 1991).

The results of the present study reveal a re-expression of NGFr in adult cat spinal motoneurons after intramedullary axotomy. This particular type of spinal cord lesion has been shown to be supervened by an indubitable CNS axon regeneration (Risling *et al.*, 1983). This regenerative activity is maintained for several weeks and so was the NGFr-LI in the scar tissue. Re-expression of NGFr-LI in motoneurons was, however, shown to reach an early maximum and decreased markedly between three and eight weeks postoperatively, i.e. at a time when regeneration is not completed (cf. Risling *et al.*, 1983). Ventral funiculus lesion seemed more effective than ventral root avulsion in inducing re-expression of NGFr. No difference was observed between cases subjected to ventral funiculus lesion alone or to a combination of ventral funiculus lesion and ventral root avulsion. This implies that contact with a denervated PNS structure, i.e.

the ventral root, is of limited importance for NGFr-expression in motoneurons after ventral funiculus lesion. It has been suggested that re-expression of NGFr in motoneurons after peripheral nerve lesions should be the result of a loss of a target-derived message (Koliathos *et al.*, 1991a) or to be directly associated with periods of axonal growth and maturation (Wood *et al.*, 1990). One additional hypothesis should however be considered. It has been shown that NGF and BDNF upregulate expression of the low affinity NGFr in central or peripheral neurons (Cavacchioli *et al.*, 1989; Alderson *et al.*, 1990; Lindsay *et al.*, 1990). Increased levels of NGFr in motoneurons after ventral funiculus lesion may thus reflect an increased access of neurotrophic factors. Theoretically, such factors could enter the scar tissue from the circulatory system (Stephani *et al.*, 1987) through the defective BBB (Risling *et al.*, 1989). This would be consistent with the present finding that NGFr expression in motoneurons was more pronounced after intramedullary injury than after avulsion lesions.

Nerve growth factor receptor antibodies bind to a fraction of the blood vessels in the normal CNS (Allen *et al.*, 1989; Yan & Johnson, 1989; Fried *et al.*, 1990) and it has been suggested that the antigen might be located in perivascular nerve fibres (Allen *et al.*, 1989). The area postrema has previously been observed to stain intensely with NGFr antibodies (Yan & Johnson, 1989). A robust perivascular NGFr-LI was observed also in the distal portion of the optic nerve and the superficial layers of the olfactory bulb as well as in the ventral funiculus scar tissue. These represent territories which are known to lack BBB function (Balin *et al.*, 1986; Risling *et al.*, 1989; Sjögren *et al.*, 1991). Thus, the results of the present study show that an intense perivascular NGFr immunostaining parallels the absence of BBB function. Regions devoid of BBB are characterized by the presence of wide perivascular spaces containing numerous (non-pericytic) perivascular cells (cf. Hildebrand *et al.*, 1985; Risling *et al.*, 1989). This is in contrast to the general appearance of the extremely narrow perivascular spaces surrounding capillaries in the CNS where non-pericytic perivascular cells are lacking (Peters *et al.*, 1976). Our observations in the normal cat suggest that labelling of perivascular cells rather than perivascular nerve fibres should account for the vascular NGFr-LI. The identity and origin of the perivascular cell types in the CNS is controversial (Graeber *et al.*, 1989, 1990; Streit *et al.*, 1989). The absence of a basal lamina and location of the NGFr-positive cells, though, suggest that they should represent modified leptomeningeal cells rather than pericytes (Peters *et al.*, 1976). This view is supported by the finding of NGFr immunoreactive cells with a comparable morphology in the normal pia mater. In operated cats, NGFr-immunoreactive cells of the type described above constituted a substantial part of the cellular elements in the trabecular scar tissue. In this context it is of interest that NGFr-LI has previously been observed in other non-neuronal cell types, such as Schwann cells, both during development and after nerve lesions (see Johnson *et al.*, 1988). It has been suggested that NGF binds to fast-dissociating (low affinity) NGFr and thereby becomes concentrated on the surface of the Schwann cells. Regenerating axons are then guided along the Schwann cell surface by the binding of NGF to their own receptors (Johnson *et al.*, 1988). If scar tissue cells may play a similar role in promoting axonal regeneration by concentrating neurotrophic factors remains to be resolved.

The possibility that the labelling of scar tissue cells may not reflect synthesis of NGFr in the cicatrix should be considered. Truncated forms of the NGFr are excreted to plasma and other body fluids during development and after peripheral nerve lesions (DiStefano & Johnson, 1988). Such molecules have similar binding affinities to NGF as the intact low affinity NGFr protein (Zupan *et al.*, 1989) and have been suggested to play a regulatory role in nervous system development (DiStefano & Johnson, 1988). Immunohistochemical detection of NGFr-LI in the area postrema or the lesion area could then be due to the presence of the truncated form of the NGFr sequestered on perivascular cells rather than to a local production of the receptor in the cell. Biochemical studies indicate, however, that the major species of NGFr in area postrema is indeed the intact receptor (Yan & Johnson, 1989). A tentative local production of NGFr in the area postrema and the scar tissue could be induced by an influx of serum-NGF (Stephani *et al.*, 1987) across a deficient BBB (Risling *et al.*, 1989). It cannot be excluded, though, that mesodermal components of the scar tissue might represent a source of neurotrophic factors. It is of interest that Ramon y Cajal, as early as the beginning of the century, suggested that CNS axon regeneration can be 'powerfully stimulated by means of active or trophic substances liberated by the mesodermic scar and diffused in the spinal wounds and their edges' (Ramon y Cajal, 1928). Later on, it was observed that neoplastic mesodermal tissue may initiate fibre outgrowth from the spinal cord (Duncan & Bellegie, 1948). In fact, NGF was first isolated from some mouse sarcomas (Levi-Montalcini & Angeletti, 1968) and has also been demonstrated in human liposarcoma (Wadell *et al.*, 1972). A hypothetical local production of neurotrophic factors in the scar could be regulated by substances like interleukin-1 (Lindholm *et al.*, 1987; Spranger *et al.*, 1990), 12-O-tetradecanoyl phorbol 13-acetate (D'Mello & Heinrich, 1990) and glucocorticoid hormones (Lindholm *et al.*, 1990). The cytokine interleukin-1 has also been shown to stimulate astrogliosis and neovascularization (Giulian *et al.*, 1988). Such molecules could possibly reach the scar tissue via a

defect in the BBB such as the one demonstrated in the lesion model used here (Risling *et al.*, 1989).

Neurotrophic factors could possibly exert effects primarily on other neuronal systems or on the scar formation and thereby have some indirect effects on the regeneration of motor axons. For example, a recently demonstrated increase in enkephalin-like immunoreactivity in the ventral horn after ventral funiculus lesion (Lindå *et al.*, 1990) could conceivably be dependent on neurotrophic factors in the lesion area. Increased levels of enkephalin may in turn induce a decrease in effectiveness of the BBB in the lesion area (cf. Baba *et al.*, 1988). In this context, it should be noted that NGF appears to have a wider biological activity than supporting growth and survival of neurons. Thus, labelling with NGFr antibodies has been observed in various non-neuronal tissues including *e.g.* mononuclear blood cells belonging to the B cell lineage (Thomson *et al.*, 1988). The possibility that NGF may have an immunoregulatory activity, contributing to tissue inflammation or repair, as suggested by these findings, has indeed been discussed (Morgan *et al.*, 1989; Tsuda *et al.*, 1990).

The regenerative activity after a ventral funiculus lesion can be assumed to be regulated by several factors, which may interact. A strong inherent regenerative capacity of spinal motoneurons cannot be excluded. High levels of ciliary neurotrophic factor in the ventral root may prevent the retrograde degeneration of motoneurons (Sendtner *et al.*, 1990). An increased volume of basal lamina associated glycoproteins like laminin, due to an expansion of the perivascular spaces in the lesion area, could have implications for the regenerative activity (Manthorpe *et al.*, 1983; Liesi & Silver, 1988). The re-expression of NGFr in injured motoneurons suggests a possible role also for NGF or related neurotrophic factors. The effects of NGF may require additional molecules, which can be difficult to control in an experimental situation. For instance, *in vitro* experiments indicate that a growth associated protein (GAP-43) may potentiate the action of NGF on neurite initiation and regeneration (Yankner *et al.*, 1990). Thus, interactions with other neuronal systems, glial cells as well as an adaptation of the metabolic machinery and the vascular functions in the lesion area could be of vital importance for the observed regeneration of motor axons. Such complex changes can be difficult to imitate *in vitro* or *in vivo* by increasing the level of one neurotrophic factor only.

Acknowledgements

This study was supported by grants from the Swedish Medical Research Council (proj. no.s. 6815, 8654 & 8657), Karolinska Institutet, Stiftelsen Karl Jeppsens minne, Tore Nilssons fond för medicinsk forskning, Torsten and Ragnar Söderbergs Stiftelser, Folksam and Trygg Hansa. Antibodies were kindly provided by Dr S. Paulie (Dept. of Immunology, Stockholm University) and purified by Dr A. M. Sjögren (Dept. of Bacteriology, Karolinska Institutet, Stockholm).

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