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# Laminin and heparan sulphate proteoglycan in the lesioned adult mammalian central nervous system and their possible relationship to axonal sprouting

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**Summary**

Several *in vitro* studies indicate that the extracellular matrix (ECM) glycoprotein laminin can promote neurite outgrowth from CNS (central nervous system) neurons. Laminin has been detected immunohistochemically in astrocytes in the embryonic but not the uninjured adult mammalian CNS. In the injured adult CNS, it is found in some reactive astrocytes located near the site of CNS lesions. In the present study, we have attempted to examine the relationship between these laminin$^+$ astrocytes and the axonal sprouting that occurs after CNS injuries. This was studied in the intracranially transected adult rat optic nerve which consists of a cranial segment devoid of all retinal ganglion cell axons, and a retinal segment attached to the retina which contains some viable axons that undergo sprouting. Laminin$^+$ reactive astrocytes were found in the cranial segment, but not in the retinal segment. In addition, the cut ends of the retinal and cranial segments were capped by an intensely laminin$^+$, glial fibrillary acidic protein negative (GFAP) region. Axonal sprouts from the transected retinal ganglion cell axons, identified by anterogradely transported rhodamine isothiocynate (RITC), were confined to laminin$^-$, GFAP$^+$ regions of the retinal segment. These results suggest that injury-induced axonal sprouting in the adult mammalian CNS *in vivo* may be promoted by molecules other than laminin, that may be associated with astrocytes.

The presence of heparan sulphate proteoglycan HSP G was also examined in the transected optic nerve because the neurite outgrowth promoting factors found in conditioned media derived from several cell types *in vitro* have been shown to consist of a complex of laminin and heparan sulphate proteoglycan. No significant changes in heparan sulphate proteoglycan-like immunoreactivity was observed after transection. The presence of laminin and HSPG were also examined in the lesioned adult rat cerebral cortex.

## Introduction

Studies of peripheral nerve regeneration (Ranson, 1912; Ramon y Cajal, 1928; Thomas, 1974), and CNS regeneration in which segments of peripheral nerve were introduced into the CNS (Richardson *et al.*, 1980; David & Aguayo, 1981; Aguayo, 1985) clearly demonstrate that the peripheral nerve non-neuronal environment is capable of promoting long distance axonal regrowth from peripheral and central neurons *in vivo*. In denervated peripheral nerve segments, Schwann cells align themselves into cords that are surrounded by a basal lamina (reviewed by Thomas 1974). Even though the regenerating axons which grow along these cords of Schwann cells are in contact with both the basal lamina and the Schwann cell membrane (Nathaniel & Pease, 1963; Schwab & Thoenen, 1985; Hall, 1986), there is some evidence that axonal growth can occur in the absence of viable Schwann cells, provided the basal lamina is present (Ide *et al.*, 1983). Studies such as these have helped to focus attention on various extracellular matrix molecules present in basal lamina, and on their role in promoting axon growth (reviewed by Carbonetto, 1984). One of these molecules, the non-collagenous glycoprotein laminin can promote neurite outgrowth *in vitro* from a variety of central and peripheral neurons (Baron von Evercooren *et al.*, 1982; Manthorpe *et al.*, 1983; Rogers *et al.*, 1983; Liesi *et al.*, 1984a; Smalheiser *et al.*, 1984). In addition, laminin has been detected immunohistochemically in the embryonic but not the adult mammalian CNS (Liesi, 1985a,b; Carbonetto *et al.*, 1987). However, it is found in the adult CNS of lower vertebrates capable of CNS

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regeneration (Liesi, 1985b). In the adult rat CNS, it has been found in reactive astrocytes after kainic acid lesions of the striatum (Liesi *et al.*, 1984b), but not observed in the optic nerve after enucleation (Liesi, 1985b; Ford-Holevinski *et al.*, 1986; McLoon, 1986). This difference in the response of astrocytes in the adult mammalian CNS might be due either to differences in the types of CNS injuries or to differences in the ability of astrocytes in different CNS regions to respond to injury. We have, therefore, re-examined the presence of laminin in the adult rat optic nerve and cerebral cortex after mechanical lesions.

Another consequence of CNS injuries is the occurrence of regenerative sprouting from injured axons (Ramon y Cajal, 1928). In the present study, we have investigated the relationship between injury-induced axonal sprouting and the presence of laminin in the intracranially transected adult rat optic nerve. Such a lesion results in a cranial segment in which all the ganglion cell axons degenerate and a retinal segment which retains some viable axons that undergo axonal sprouting (Grafstein & Ingoglia, 1982; Richardson *et al.*, 1982).

Some recent *in vitro* studies suggest that the neurite outgrowth promoting factor found in conditioned media from a variety of cell types consists of a complex of laminin and heparan sulphate proteoglycan (HSPG), another ECM molecule (Lander *et al.*, 1983; 1985; Mathew & Patterson, 1983; Chiu *et al.*, 1986). Therefore, the presence of HSPG in the lesioned optic nerve and cerebral cortex was also examined.

We have found laminin$^+$ astrocytes in the cranial optic nerve segment and in the lesioned cerebral cortex, but not in the retinal segment. The significance of these findings to axonal sprouting and vascularization are discussed.

## Materials and methods

### *Optic nerve transections*

In 26 adult female Sprague–Dawley and Black-hooded rats, the intracranial portion of the right optic nerve was exposed under chloral hydrate (420 mg kg$^{-1}$ b.w.) or sodium pentobarbital (52 mg kg$^{-1}$ b.w.) anaesthesia. This region of the optic nerve was exposed by removing a portion of the right frontal lobe by suction and the optic nerve was transected with a pair of microscissors. The integrity of the retinal blood supply was monitored post-operatively. Only animals retaining a good retinal blood supply were used in these experiments. Intracranial optic nerve transections were done in order to yield an optic nerve segment attached to the retina in which some ganglion cell axons survive transection, and a cranial segment continuous with the optic chiasm, in which all the ganglion cell axons degenerate (Fig. 1; Grafstein & Ingoglia, 1982; Richardson *et al.*, 1982).

### *Immunohistochemistry*

At 5, 10, 14 and 21 days after optic nerve transection, the animals were deeply anaesthetized and killed by transcardiac perfusion with 0.4% *p*-benzoquinone (Anachemia) in 0.1 M phosphate buffer pH 7.4 (Liesi, 1985b). The lesioned frontal lobe, the entire retinal and cranial stumps of the transected right optic nerve and the intact left optic nerve were removed immediately, immersed in the same fixative for 1 h and rinsed in 0.1 M phosphate buffer overnight at 4° C. Cryostat sections (10 μm) of the lesioned frontal lobe and longitudinal sections of the various optic nerve segments were picked up on gelatin coated glass slides. Non-specific binding was blocked by incubating the tissue sections with 3% ovalbumin (Sigma) overnight at 4° C. This was followed by an overnight incubation at 4° C with either polyclonal rabbit anti-laminin antisera which were raised against laminin derived from mouse EHS sarcoma (BRL diluted 1:100; or obtained from Dr S. Carbonetto), or a polyclonal rabbit anti-HSPG antiserum (diluted 1:125, obtained from Dr J. Hassell). The binding of these antibodies to tissue sections was detected by a 1 h incubation at room temperature (RT) with a goat anti-rabbit immunoglobulin (Ig) conjugated to fluorescein (Hyclone, diluted 1:200). The sections were then double labelled with a monoclonal anti-GFAP antibody (Lab Systems, diluted 1:100) overnight at 4° C. The binding of this antibody was visualized by a 1 h incubation at RT with a goat anti-mouse Ig conjugated to rhodamine (Hyclone, diluted 1:100). Cell nuclei in the tissue sections were labelled by dipping the slides for 60 s in a 0.001% solution of the fluorescent dye Nuclear Yellow (Hoechst S769121). Between each of these steps, the sections were rinsed with several changes of 0.1 M phosphate buffer for at least 1 h. Slides were mounted in sodium carbonate buffered glycerol (pH 9.0) and viewed with a Leitz fluorescence microscope equipped with N2 (rhodamine) and L2 (fluorescein) filters. The latter does not permit any breakthrough of rhodamine fluorescence.

### *Controls*

Control sections were incubated with mouse myeloma ascites fluid or normal rabbit serum instead of the monoclonal antibody or the polyclonal primary antibodies respectively. In another set of controls, the specificity of the labelling with the anti-laminin antiserum was determined by pre-absorbing the antiserum with purified laminin. To do this, laminin purified from the EHS sarcoma (Kleinman, 1982) was further purified by HPLC using a Mono-Q column (Pharmacia). The pre-absorption was done after immobilizing the purified laminin to Affigel-10 (Bio Rad).

### *Intraocular rhodamine injections*

The distribution of growth cones at the distal tip of the retinal optic nerve stump was visualized by labelling the axons with an intraocular injection of RITC. As described previously, 6 μl of a 2.5% solution of RITC was injected into the right vitreal cavity (Thanos *et al.*, 1987) 3 days prior to killing at 5, 10 and 19 days after transection. The animals were perfused with 0.4% *p*-benzoquinone and 10 μm thick longitudinal cryostat sections of the retinal optic nerve segment were obtained. After photographing the rhodamine labelled axons and growth cones, the sections were

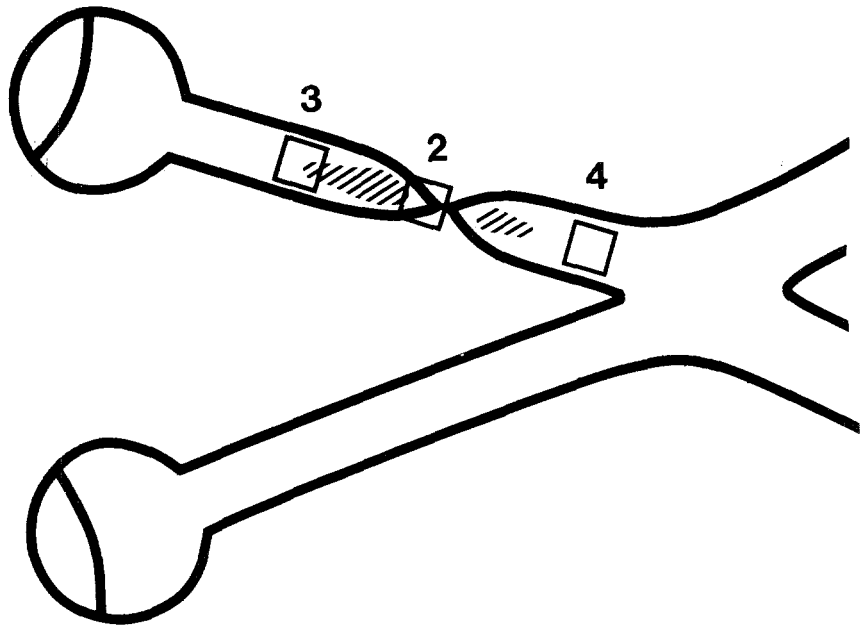


**Fig. 1.** Schematic representation of the intracranial optic nerve transection which results in a retinal segment attached to the eye and a cranial segment that is continuous with the chiasm. A few days after transection, the cut ends of the retinal and cranial segments become tapered. The hatched areas represent areas of central necrosis. The areas within the boxes labelled 2, 3 and 4 represent regions illustrated in Figs 2, 3 and 4 respectively.

labelled with anti-laminin, anti-HSPG or anti-GFAP antibodies, as described above.

*Analysis of vascular profiles*

Intracranial optic nerve transections were carried out as described above. Five days after nerve transection, animals were deeply anaesthetized with chloral hydrate and killed by transcardiac perfusion with 1.5% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The normal optic nerve, and the retinal and cranial segments of the transected optic nerve were dissected and placed in the same fixative for 2 h, post-fixed in 1% osmium tetroxide for 1 h, dehydrated and embedded in Epon. Cross-sections of 0.5 μm were stained with Toluidine Blue for light microscopy. Sections of the transected optic nerve segments were obtained from the region beyond the area of central necrosis, and from similar regions of the normal nerve. Camera lucida drawings of the vascular profiles were made using a Leitz Ortholux II light microscope. Measurements of the total circumferential length of the blood vessels within cross-sections of the optic nerve were made from the camera lucida drawings using a Zeiss image analysis system (IBAS I).

## Results

IMMUNOHISTOCHEMISTRY

An indirect immunofluorescence assay was used to detect changes in laminin and HSPG-like immunoreactivity in the retinal and cranial stumps of intracranially transected adult rat optic nerves. These changes were examined at 5, 10, 14 and 21 days after transection. Longitudinal sections were double labelled with anti-laminin/anti-GFAP or anti-HSPG/anti-GFAP antibodies. In addition, a similar analysis was carried out on the lesioned frontal cortex. Suction lesions of the frontal cortex were made in order to expose the intracranial portion of the optic nerve for transection. Changes in these three regions are described separately. No distinction will be made between Sprague–Dawley and Black-hooded rats, since no differences were observed between these two strains.

*Retinal optic nerve segment*

At 5, 10 and 14 days after transection, the tip of the retinal optic nerve segment was capped by an intensely laminin$^+$, HSPG$^+$ region (Fig. 2B,E). This region, which was GFAP$^-$ (Fig. 2A,D), decreased in size at later survival times. Nuclear yellow labelling indicated that this laminin$^+$, HSPG$^+$, GFAP$^-$ area was cellular (Fig. 2C,F), and appeared continuous with the meninges surrounding the remainder of the optic nerve.

Five days after transection, there were small focal patches of degeneration within the distal portion of the retinal segment. At later survival times, the size of these patches enlarged to form an area of central necrosis that extended up to the cut end of the nerve segment. The region of central degeneration was laminin$^-$ and HSPG$^-$ (Fig. 3B,E). This area was also cellular, but devoid of axons and astrocytic cell

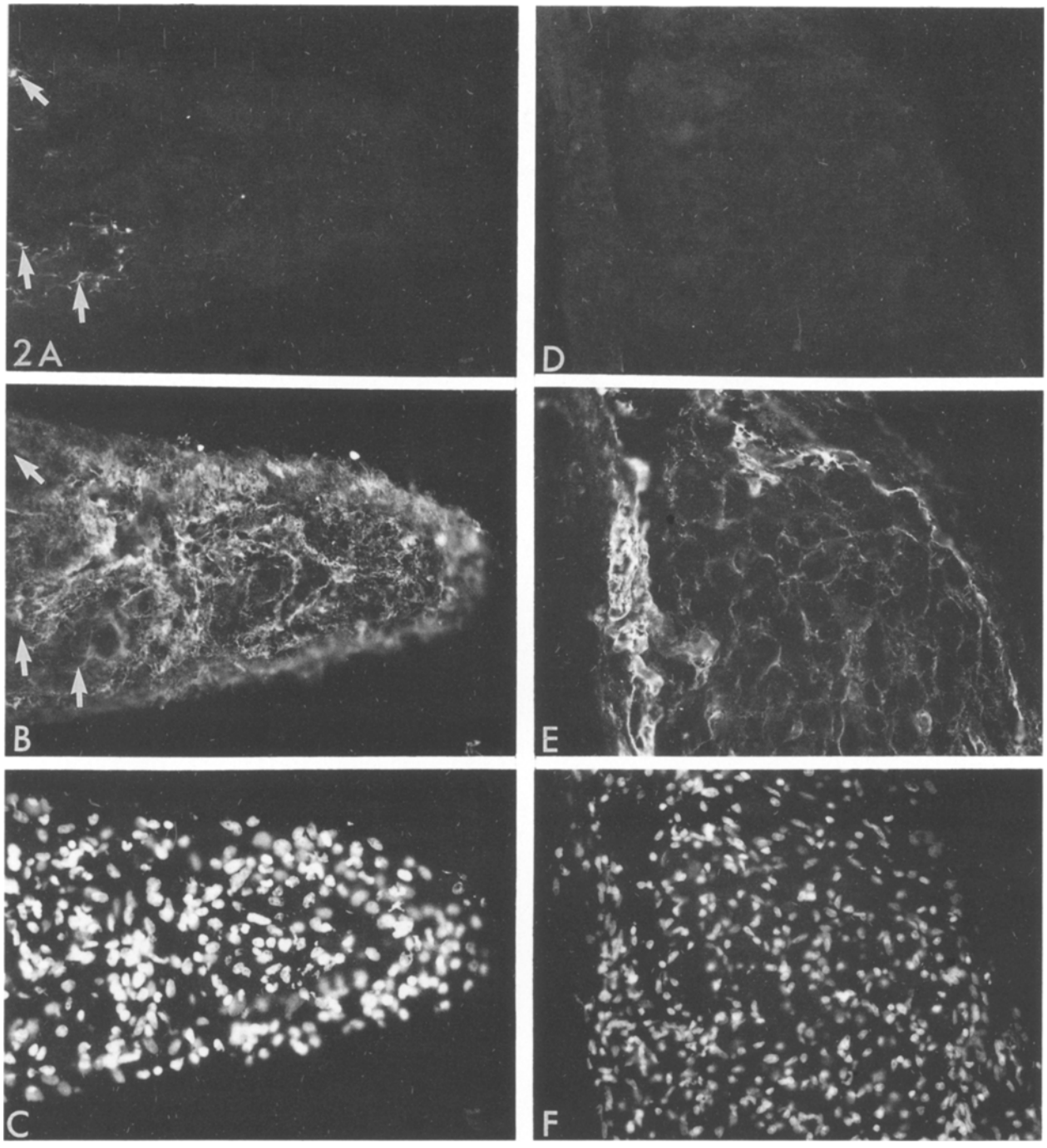


**Fig. 2.** Double indirect immunofluorescence labelling of the cut end of the retinal optic nerve segment 5 days (A,B,C) and 10 days (D,E,F) after transection. The tip of this segment is capped by a $GFAP^-$ (A and D), $laminin^+$ (B), $HSPG^+$ (E) region which is cellular, as indicated by Nuclear Yellow labelling (C and F). The $GFAP^+$ areas near the tip at 5 days after transection (A, arrows) are $laminin^-$ (B, arrows). × 156.

bodies, but contained $GFAP^+$ processes around blood vessels, as evidenced by the absence of rhodamine labelled axons (see below), lack of $GFAP^+$ cell bodies (Fig. 3A,D) and numerous NY labelled nuclei (Fig. 3C,F).

The $GFAP^+$ regions surrounding the area of central necrosis (Fig. 3A) including regions near the tip (Fig. 2A) were $laminin^-$ (Figs 2B and 3B). $Laminin^+$ or $HSPG^+$ astrocytic cell bodies were not found in the retinal segment at any of the survival times.

$GFAP^+$ astrocytic processes surrounded blood vessels. In addition, the blood vessels were also $laminin^+$ and $HSPG^+$. This was particularly evident in the area of central degeneration (Fig. 3B,E).

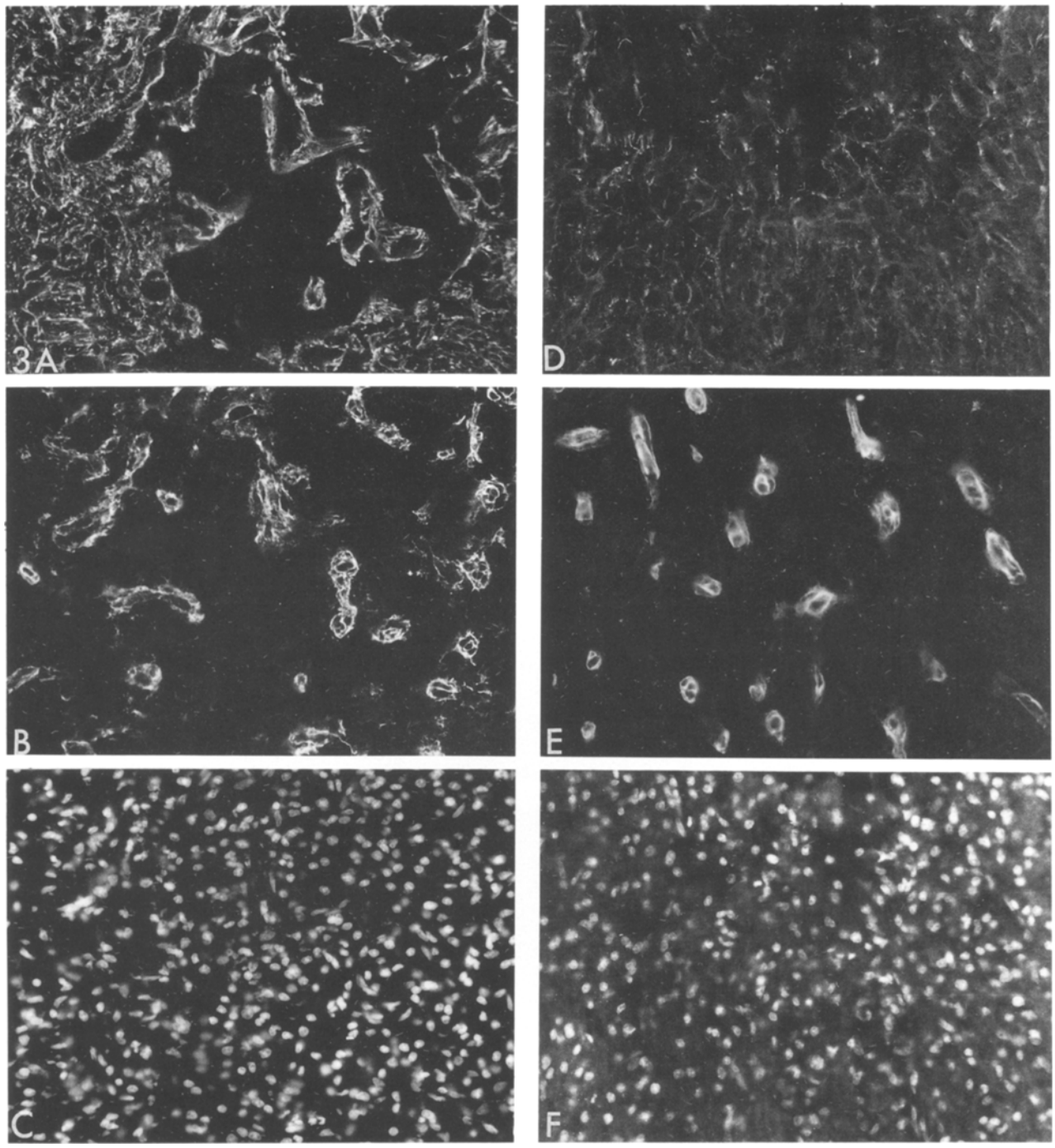


**Fig. 3.** Double indirect immunofluorescence labelling of the area of central degeneration in the distal portion of the retinal optic nerve segment 10 days (A,B,C) and 5 days (D,E,F) after intracranial nerve transection. The area of degeneration lacks $GFAP^+$ labelling, except for the blood vessels (A and D). Anti-laminin (B) and anti-HSPG (E) also label only the blood vessels. The Nuclear Yellow labelling (C and F) clearly shows that the area of central degeneration is highly cellular. × 156.

Despite the apparent coincidence of the two labels, the $laminin^+$ and $HSPG^+$ labelling is most likely to be confined to the basal lamina surrounding blood vessels, since the $laminin^+$ and $HSPG^+$ outline of the blood vessels is much smaller than the anti-GFAP labelling (Fig. 3A,B,D,E).

*Cranial optic nerve segment*

As in the retinal segment, the cranial segment also showed: (i) An intensely $laminin^+$, $HSPG^+$, $GFAP^-$ area which capped the tip of the segment. (ii) A cellular area of central degeneration which was

devoid of astrocytic cell bodies but contained GFAP$^+$ processes which surrounded blood vessels. These vascular profiles were laminin$^+$, HSPG$^+$, and were more numerous than that present in the retinal segment. (iii) The presence of intensely GFAP$^+$ astrocytes.

The most important difference between the retinal and cranial segments was the presence of laminin$^+$ astrocytes in the latter (Fig. 4A–F). These laminin$^+$/GFAP$^+$ double labelled cells were found maximally at 5 days after transection and were virtually absent by 14 days. The laminin$^+$ astrocytes were located approximately 500 μm from the apex of the area of central necrosis, and the end of the cranial segment, which was a distance of about 2 mm. These laminin$^+$ reactive astrocytes were located within the central region of the nerve and were often seen adjacent to blood vessels. The astrocytes that form the glia limitans at the periphery of the nerve were laminin$^-$ HSPG$^+$ astrocytic cell bodies were not seen at any survival times.

*Brain*

The lesion-cavity was lined by an intensely laminin$^+$, HSPG$^+$, GFAP$^-$, cellular zone (Fig. 5A–F) which was between 300 μm and 600 μm in thickness. Laminin$^+$ astrocytes were found near the lesion at all survival times, but were most numerous 5 days after transection. The majority of these laminin$^+$/GFAP$^+$ double labelled astrocytes were found between 450 and 1800 μm from the boundary between the GFAP$^+$ and GFAP$^-$ regions at the lesion site (Fig. 6). These double labelled astrocytes were frequently located adjacent to blood vessels or extended GFAP$^+$,

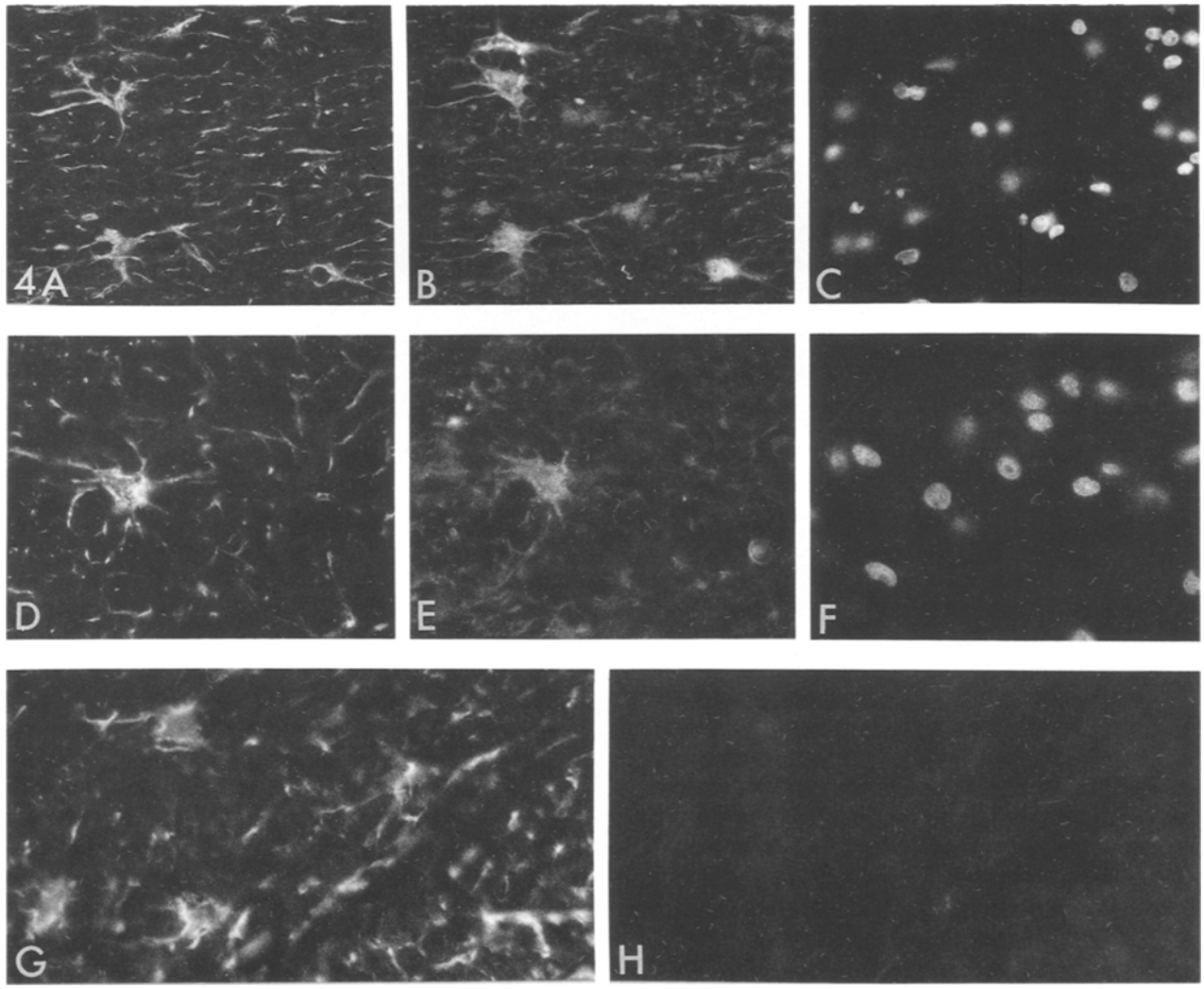


**Fig. 4.** Astrocytes in the cranial optic nerve segment 5 days after transection were double labelled with anti-GFAP (A and D) and anti-laminin antibodies (B and E). Note the presence of Nuclear Yellow labelled cell nuclei which corresponds to the double labelled astrocytes. The cell illustrated in D, E and F is located adjacent to a capillary. Control sections (G & H) incubated with anti-GFAP (G) and pre-absorbed anti-laminin antiserum (H). A,B,C × 296; D,E,F × 473; G,H × 244.

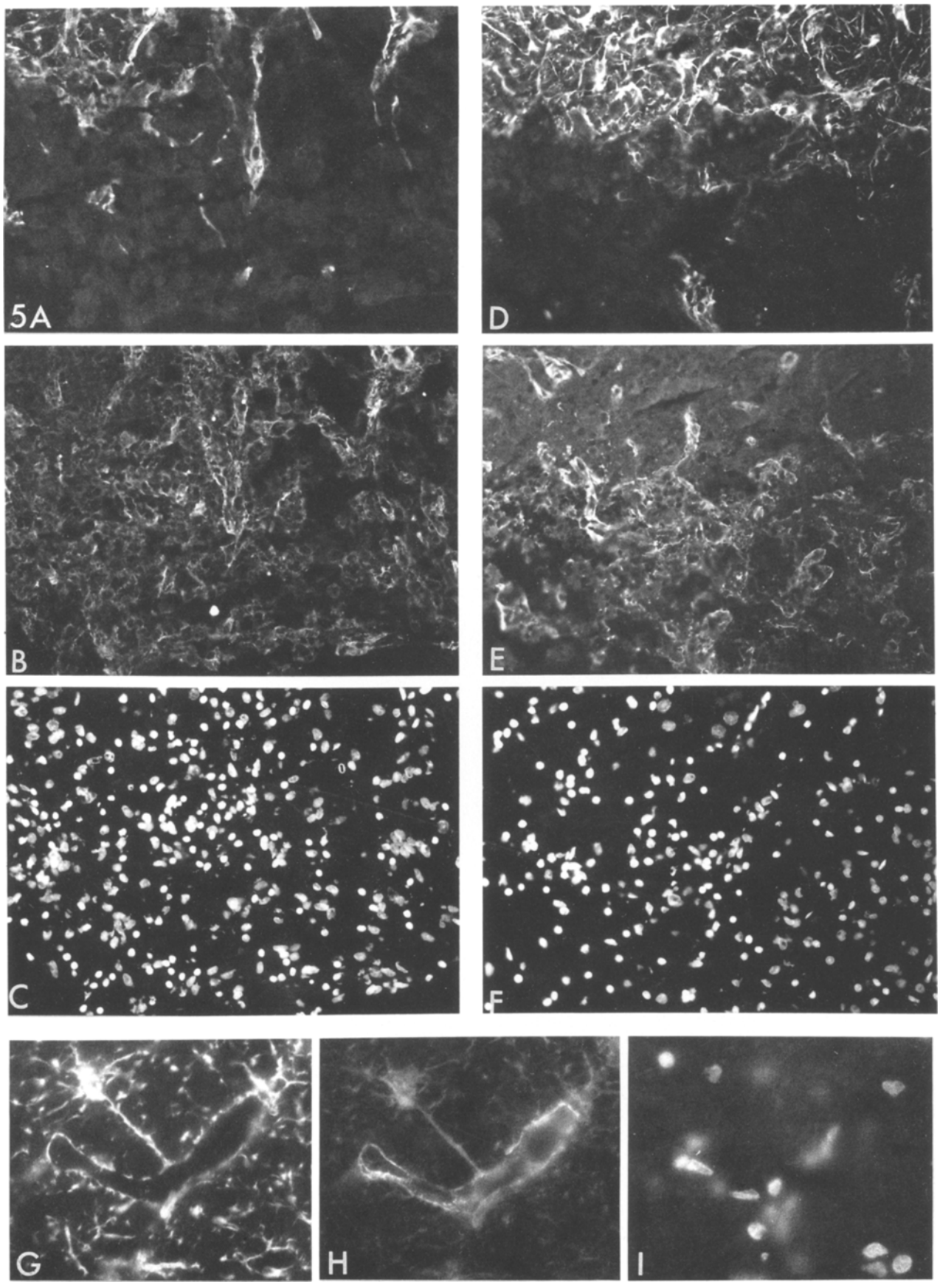


**Fig. 5.** Double indirect immunofluorescence labelling of the wall of the cortical lesion, labelled with anti-GFAP (A) and anti-laminin (B); anti-GFAP (D) and anti-HSPG (E). Note the laminin$^+$, HSPG$^+$, GFAP$^-$ region which lines the lesion cavity which is towards the bottom of the micrographs. This region is cellular as indicated by Nuclear Yellow labelling (C, F). $\times 156$. A GFAP$^+$ (G), laminin$^+$ (H) astrocyte in the cortex close to the site of lesion. This laminin$^+$ astrocyte extends a process to a blood vessel. Nuclear Yellow labelling (I). $\times 200$.

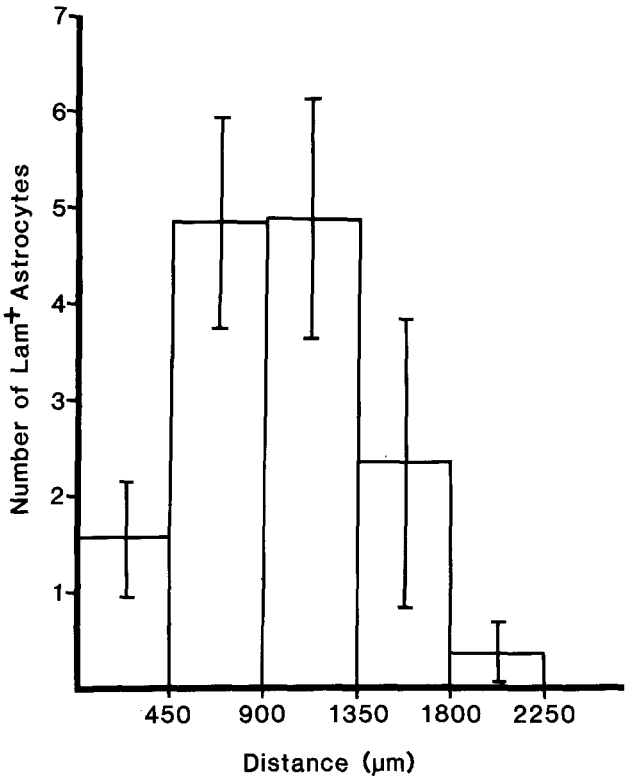


**Fig. 6.** Histogram showing the distribution of laminin$^+$ reactive astrocytes in the lesioned adult rat cerebral cortex as a function of distance from the lesion cavity. Counts were made from an area 290 μm × 2700 μm. The GFAP$^-$, laminin$^+$, HSPG$^+$ region lining the lesion cavity was not included in the measurements. The values represent Means ± S.D. of ten counts from randomly chosen sections from 2 animals.

laminin$^+$ processes which abut vascular profiles (Fig. 5G,H,I). Not all astrocytes in this region were laminin$^+$. Astrocytes were never found to be HSPG$^+$.

*Controls*

No labelling was obtained in optic nerve sections incubated with preabsorbed anti-laminin antiserum (Fig. 4G, H). Since the pre-absorption was done using purified laminin, it is felt that the labelling observed in the experimental groups is specific for laminin. In addition, no labelling was observed in control sections incubated with myeloma ascites fluid and normal rabbit serum instead of the monoclonal and polyclonal primary antibodies, respectively.

ANTEROGRADE AXONAL LABELLING

Axons in the retinal segment were anterogradely labelled with rhodamine, which was injected intraocularly 3 days prior to killing at 5, 10 and 19 days post-transection. In rats injected with rhodamine on the second day after the lesion and killed 3 days later i.e. 5 days after transection, the region of central degeneration was generally devoid of rhodamine labelling. Intact and degenerating axons were found in the remainder of the nerve, including the narrow strip of GFAP$^+$ tissue located around the area of central degeneration. At 10 days post lesion, many rhodamine labelled axons were also present (Fig. 7A). The distal tip of some of these axons had structures that resembled growth cones (Fig. 7B–D). The front of these RITC labelled putative growth cones advanced between 10 and 19 days after transection. These putative axonal sprouts, were always confined to GFAP$^+$ regions of the nerve including the narrow strip of GFAP$^+$ tissue surrounding the area of central necrosis. Growth cone-like structures were never found within the highly cellular GFAP$^-$ region of central degeneration (Fig. 7A,D). Nor did they extend into the intensely laminin$^+$, HSPG$^+$, GFAP$^-$ region that capped the tip of the retinal segment. Occasionally, a labelled axon followed an aberrant course at right angles to its normal trajectory and terminated in a growth cone-like structure at the border of the area of central degeneration (Fig. 7D).

*Changes in vascular profiles*

There was a significant increase in the number of blood vessels in cross-sections of the retinal (58%) and cranial (112%) optic nerve segments obtained after nerve transections, as compared to the normal optic nerve. However, there were even greater changes in the size of the blood vessels after nerve transection. As can be seen in Table 1, the total circumferential length of the vascular profiles in cross-sections of the retinal segment is 4.5 fold greater than that in the normal optic nerve, whereas that in the cranial segment is 6.5 fold above normal. Since blood vessels in the CNS are surrounded by a basal lamina (Peters *et al.*, 1976), changes in the circumferential length of the vascular profiles in the retinal and cranial segments provides an estimate of the changes in the relative amounts of basal lamina

**Table 1.** Table showing the total circumferential length of all blood vessels in Epon-embedded cross-sections of the normal optic nerve and the retinal and cranial segments of the transected optic nerve. The values represent Mean ± S.D. of measurements made from four cross-sections obtained from two animals.

| | *Normal nerve* | *Retinal segment* | *Cranial segment* |
|---|---|---|---|
| Total circumferential length of blood vessels in cross-sections of the nerve | 621 ± 40 | 2805 ± 89 | 4105 ± 210 |

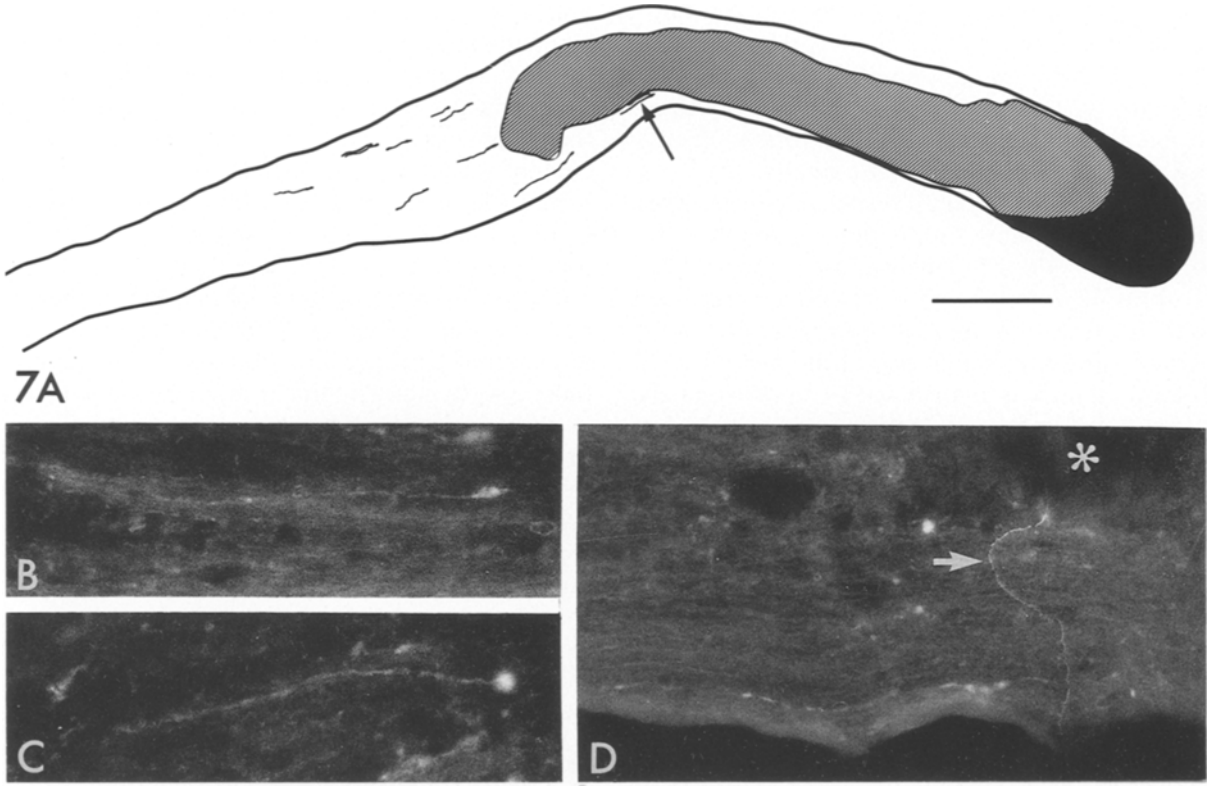


**Fig. 7.** Anterograde labelling of axons in the retinal optic nerve segment by RITC 10 days after intracranial nerve transection. Camera lucida drawing of RITC labelled axonal profiles in a longitudinal section of the nerve (A). The shaded area represents the region of central degeneration. The dark area at the tip represents the laminin$^+$, GFAP$^-$ region which caps the cut end of the nerve. Labelled axons are located only in the GFAP$^+$ (clear region) of the nerve. Axon indicated by the arrow is illustrated in B. Note the different terminal morphologies of the axons (B and C). A labelled axon running at right angles to its normal course within the nerve is illustrated in D. This axon terminates in a growth cone-like structure at the border of the region of central degeneration (*). Calibration bar in A = 250 μm; B,C × 400; D × 340.

after nerve transection, assuming that basal lamina of 'stable' and 'reacting' blood vessels are quantitatively and qualitatively similar.

## Discussion

We have used an indirect immunofluorescence assay to detect laminin and HSPG, two ECM molecules, in the lesioned adult rat CNS. Two regions of the CNS were studied, (i) the optic nerve, after intracranial nerve transection, and (ii) the cerebral cortex, after suction lesions. We have also examined the relationship between laminin and injury-induced axonal sprouting in the transected adult rat optic nerve. The two main findings in this study are: (i) laminin$^+$ astrocytes are present in the cranial optic nerve segment and in the lesioned cerebral cortex, but not in the retinal optic nerve segment, and (ii) axonal sprouts that appear in the retinal segment are confined to laminin$^-$, GFAP$^+$ regions of the nerve.

In the lesioned optic nerve and cerebral cortex, HSPG-like immunoreactivity was localized to blood vessels and to a GFAP$^-$ cellular zone at the site of lesion. The latter is also laminin$^+$, and is believed to be comprised of leptomeningeal cells (Kruger *et al.*, 1986). Thus the distribution of HSPG labelling in the lesioned CNS is similar to that in the normal adult CNS (Laurie *et al.*, 1983; Carbonetto *et al.*, 1987).

### *Laminin$^+$ astrocytes in the lesioned CNS*

Axons in the cranial segment of the transected optic nerve undergo Wallerian degeneration because they are severed from their cell bodies which are located in the retina. Astrocytes in this segment show intense GFAP-like immunoreactivity. Some of these reactive astrocytes in the cranial segment express laminin, being most numerous 5 days after nerve transection. The timing of the appearance of laminin-like immunoreactivity in reactive astrocytes in the optic

nerve is consistent with the appearance of laminin$^+$ reactive astrocytes in the lesioned cerebral cortex. Liesi *et al.*, (1984b) have also described a similar timing in the appearance of laminin$^+$ astrocytes in the corpus striatum after kainic acid lesions. Thus, the type of lesion, chemical or traumatic, does not appear to influence the overall response of astrocytes located near the lesion, to express laminin. The laminin$^+$/GFAP$^+$ double labelled astrocytes in the cranial optic nerve segment were located between about 500 μm from the apex of the region of central degeneration and the cranial end of the nerve (up to the chiasm), which is a distance of approximately 2 mm. It is not known if laminin$^+$ astrocytes are present beyond this 2 mm distance. In the lesioned cerebral cortex, laminin$^+$ astrocytes were located for a distance of up to 2.25 mm from the wall of the lesion.

These findings in the intracranially transected adult rat optic nerve differ from those of other investigators who have failed to observe laminin$^+$ reactive astrocytes in the degenerating adult rat optic nerve after enucleation (Liesi, 1985b; Ford-Holevinski *et al.*, 1986; McLoon, 1986). There are several possible explanations for these differences, such as differences in fixatives, antibodies, or sites of lesion. In order to resolve this, we re-examined the anti-laminin labelling after enucleation using our antibodies and fixation protocol. The results we obtained (data not shown) were consistent with the previously reported findings (Liesi, 1985b; Ford-Holevinski *et al.*, 1986; McLoon, 1986) that laminin$^+$ astrocytes were not present in the degenerating rat optic nerve after enucleation. One issue that should be considered further in assessing the reliability of the laminin$^+$ labelling observed after intracranial optic nerve transections is the specificity of the anti-laminin antisera. We have used two different antisera (BRL; and that obtained from S. Carbonetto) which were generated against laminin purified from mouse EHS sarcoma using the protocol described by Kleinman *et al.*, (1982). It is possible that both these antisera are directed against laminin as well as some other components of the ECM which co-purify with laminin, or that the antisera cross-react with some other antigens found in reactive astrocytes. However, these antisera were found to be highly specific for laminin and do not bind to fibronectin or collagen. Furthermore, labelling was abolished by pre-absorbing the anti-laminin antiserum with HPLC purified laminin. In addition, we can also exclude the possibility that this is a technical artifact since these antisera do not label astrocytes in the retinal segment which were processed along with the cranial segments in an identical fashion.

For these reasons we feel that the presence of laminin$^+$ astrocytes in the intracranially transected optic nerve and their absence in enucleated animals, might be due to differences in the site of the lesion along the optic nerve. Although we do not know why intraorbital and intracranial lesions should produce such differing results, differences along the length of the optic nerve, such as differences in the glial content (Skoff *et al.*, 1986) may contribute to such results. It is known, for example, that the cross-sectional area occupied by astrocytes is 34% in the orbit and 8% near the chiasm (Skoff *et al.*, 1986).

The presence of laminin$^+$ astrocytes in the cranial but not the retinal optic nerve segment was an unexpected finding. One possible explanation for these results could be that it might reflect an increase in the number or size of the blood vessels i.e. an increase in the amount of basal lamina in the cranial segment as compared to the retinal segment. Astrocytes *in vitro* are capable of producing certain components of the basal lamina (Liesi *et al.*, 1983; Liesi, 1986; Ard & Bunge, 1986). If astrocytes *in vivo* contribute to the formation of the basal lamina surrounding blood vessels (Lawrence *et al.*, 1984), then an increase in the number and/or size of blood vessels might result in an increased demand on the astrocytes to produce basal lamina components, such as laminin. The frequent association of laminin$^+$ astrocytes with blood vessels, supports such a role. We have also found that compared to the normal optic nerve, there is a 6.5 fold increase in the total circumferential length of the blood vessels in the cranial optic nerve segment, but only a 4.5 fold increase in the retinal segment. It is possible that the 4.5 fold increase in the retinal segment is not sufficient to induce an immunohistochemically detectable increase in laminin, whereas the 6.5 fold increase in the cranial segment may do so. Therefore, differences in the extent of revascularization, together with other differences along the nerve, such as the content of glial cells (Skoff, 1986) may contribute to the appearance of laminin$^+$ astrocytes in the cranial optic nerve segment.

The laminin$^+$ astrocytes in the cranial optic nerve segment are located within the central region of the nerve, not at the periphery. Two biochemically different types of astrocytes called type 1 and type 2 have been described in the rat optic nerve (Raff *et al.*, 1983a,b; Miller & Raff, 1984). Type 1 astrocytes are located along the periphery of the nerve where they form the glia limitans, while type 2 astrocytes occupy the central region of the nerve (Miller & Raff, 1984). In the present study, the location of laminin$^+$ astrocytes in the central portion of the cranial optic nerve segment suggests that they might be type 2 astrocytes. Therefore, type 2 astrocytes appear to express laminin soon after lesioning (5 days). At longer survival times, these type 2 astrocytes will die (Miller *et al.*, 1986; David *et al.*, 1984), possibly for the lack of some axonal influence.

*Laminin and axonal sprouting* in vivo

In rodents, at least one third of the retinal ganglion cells and their axons are present two weeks after intracranial optic nerve lesions (Richardson *et al.*, 1982; Grafstein & Ingoglia, 1982). Axonal sprouting in the injured optic nerve has been observed previously by silver staining (Ramon y Cajal, 1928; Cankovic, 1968; Muchnick & Hibbard, 1980; Grafstein & Ingoglia, 1982) and by electron microscopy (Richardson *et al.*, 1982). The retinal origin of these axonal sprouts has been demonstrated by EM-autoradiography using anterogradely transported $^3$H-amino acids (Richardson *et al.*, 1982). In the present study, we have labelled retinal axons anterogradely with intraocular injections of RITC which would be expected to label sprouting as well as degenerating axons. Many of these rhodamine labelled axons have terminal structures that resemble the RITC labelled growth cones of the developing chick retinal ganglion cell axons (Thanos & Bonhoeffer, 1983, 1984). Therefore, on the basis of their morphology we have identified some of these labelled structures as being putative axon sprouts. Further evidence that these are likely to be axonal sprouts with growth cones rather than retraction bulbs at the end of degenerating axons, is the finding that (i) some of these axons with growth cone-like structures deviate sharply from their normal trajectory within the optic nerve, and (ii) that the front of RITC labelled putative growth cones advanced between 10 and 19 days after lesion. An interesting observation in the present study was that all the RITC labelled axons, including the putative axonal sprouts were located within the GFAP$^+$, laminin$^-$ regions of the nerve. Some of these axons could be seen to terminate at the border between GFAP$^+$ and GFAP$^-$ regions. The latter include the highly cellular GFAP$^-$ area of central degeneration and the intensely laminin$^+$/GFAP$^-$ region which caps the tip of the retinal optic nerve segment. These results indicate that axonal sprouts arising from injured CNS axons are confined to regions of the nerve containing astrocytes, even though these regions are laminin$^-$. These results are in agreement with several *in vitro* studies which show that neurite outgrowth from CNS neurons is promoted preferentially by astrocytes rather than by non-glial cells (Noble *et al.*, 1984; Fallon, 1985; David, 1988) even though the non-glial cells have greater amounts of laminin on their surface than do astrocytes (David, 1988).

Neurite outgrowth on astrocytes *in vitro* has recently been shown to be mediated largely via a laminin-independent mechanism (Ard & Bunge, 1986; Cohen *et al.*, 1986; David & Crossfield-Kunze, 1986; Tomaselli *et al.*, 1986; David, 1988). Our present results suggest that axonal sprouting *in vivo* from the tips of injured CNS axons may also be promoted by molecules other than laminin that may be associated with astrocytes located near the site of a lesion. Such permissive glial influences at the site of lesion may work in concert with intrinsic neuronal mechanisms (Grafstein & McQuarrie, 1978) to initiate and promote axonal sprouting. The reasons for the failure of axonal sprouts to regenerate over long distances in the injured adult mammalian CNS are not yet known, but may include differences between the properties of astrocytes located near the site of a lesion and those located further away from the lesion. Astrocytes located near a lesion may be able to provide a suitable substrate for axonal sprouting, whereas those located away from the lesion may be unable to provide such a substrate for continued axonal growth.

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## References

AGUAYO, A. J. (1985) Axonal regeneration from injured neurons in the adult mammalian central nervous system. In *Synaptic Plasticity*, (Edited by C. W. COTMAN), Guilford Press: New York.

ARD, M. D. & BUNGE, R. P. (1986) Tissue culture observations on the interactions of astrocytes, extracellular matrix, and neurites. *Neuroscience Abstracts* **12**, 395.

BARON VAN EVERCOOREN, A., KLEINMAN, H. K., OHNO, S., MARANGOS, P., SCHWARTZ, J. P. & DUBOIS-DALQ, M. E. (1982) Nerve growth factor, laminin and fibronectin promote neurite growth in human fetal sensory ganglion cultures. *Journal of Neuroscience Research* **8**, 179–94.

CANKOVIC, J. C. (1968) Contribution to the study of regenerative–degenerative qualities of the fasciculi optica in mammals under experimental conditions. *Acta Anatomica* **70**, 117–123.

CARBONETTO, S. (1984) The extracellular matrix of the nervous system. *Trends in Neurosciences* **7**, 382–7.

CARBONETTO, S., EVANS, D. & COCHARD, P. (1987)

Nerve fibre growth in culture on tissue substrata from central and peripheral nervous systems. *Journal of Neuroscience* **7**, 610–20.

CHIU, A. Y., MATTHEW, W. D. & PATTERSON, P. H. (1986) A monoclonal antibody that blocks the activity of a neurite regeneration-promoting factor: Studies on the binding site and its localization *in vivo*. *Journal of Cell Biology* **103**, 1383–98.

COHEN, J., BURNE, J. F., WINTER, J. & BARTLETT, P. (1986) Retinal ganglion cells lose response to laminin with maturation. *Nature (Lond)* **322**, 465–7.

DAVID, S. (1988) Neurite outgrowth from mammalian CNS neurons on astrocytes in vitro may not be mediated primarily by laminin. *Journal of Neurocytology* **17**, 131–44.

DAVID, S. & AGUAYO, A. J. (1981) Axonal elongation into peripheral nervous system "bridges" after central nervous system injury. *Science* 214, 931–3.

DAVID, S. & CROSSFIELD-KUNZE, T. (1986) Immunohistochemical detection of laminin and heparan sulphate proteoglycan in cultures of CNS non-neuronal cells. *Neuroscience Abstracts* **12**, 514.

DAVID, S., MILLER, R. H., PATEL, R. & RAFF, M. C. (1984) Effects of neonatal transection on glial cell development in the rat optic nerve: evidence that the oligodendrocyte-type 2 astrocyte cell lineage depends on axons for its survival. *Journal of Neurocytology* **13**, 961–74.

FALLON, J. R. (1985) Preferential outgrowth of central nervous system neurites on astrocytes and Schwann cells as compared with nonglial cells *in vitro*. *Journal of Cell Biology* **100**, 198–207.

FORD-HOLEVINSKI, T. S., HOPKINS, J. M., MCCOY, J. P. & AGRANOFF, B. W. (1986) Laminin supports neurite outgrowth from explants of axotomized adult rat retinal neurons. *Developmental Brain Research* **28**, 121–6.

GRAFSTEIN, B. & INGOGLIA, N. A. (1982) Intracranial transection of the optic nerve in adult mice: Preliminary observations. *Experimental Neurology* **76**, 318–30.

GRAFSTEIN, B. & MCQUARRIE, I. G. (1978) Role of the nerve cell body in axonal regeneration. In *Neuronal Plasticity* (edited by COTMAN, C. W.) pp. 155–95. Raven Press: New York.

HALL, S. M. (1986) Regeneration in cellular and acellular autografts in the peripheral nervous system. *Neuropathology and Applied Neurobiology* **12**, 27–46.

IDE, C., TOHYAMA, K., YOKOTA, R., NITATORI, T., & ONODERA, S. (1983) Schwann cell basal lamina and nerve regeneration. *Brain Research* **288**, 61–75.

KLEINMAN, H. K. MCGARVEY, M. I., LIOTTA, L. A., ROBEY, P. G., TRYGGVASON, K. & MARTIN, G. R. (1982) Isolation and characterization of type IV procollagen, laminin and heparan sulfate proteoglycan from the EHS sarcoma. *Biochemistry* **21**, 6188–93.

KRUGER, S., SIEVERS, J., HANSEN, C., SADLER, M. & BERRY, M. (1986) Three morphologically distinct types of interface develop between adult host and fetal brain transplants: Implications for scar formation in the adult central nervous system. *Journal of Comparative Neurology* **249**, 103–16.

LANDER, A. D., FUJII, D. K. & REICHARDT, L. F. (1985) Laminin is associated with the 'neurite outgrowth-promoting factors' found in conditioned media. *Proceedings of the National Academy of Science, USA* **82**, 2183–7.

LANDER, A. D., TOMASELLI, K., CALOF, A. L. & REICHARDT, L. F. (1983) Studies on extracellular matrix components that promote neurite outgrowth. *Cold Spring Harbor Symposia on Quantitative Biology* **48**, 611–24.

LAURIE, G. W., LEBLOND, C. P. & MARTIN, G. R. (1983) Light microscope immunolocalization of type IV collagen, laminin, heparan sulphate proteoglycan and fibronectin in the basement membranes of a variety of rat organs. *American Journal of Anatomy* **167**, 71–82.

LAWRENCE, J. M., HUANG, S. K. & RAISMAN, G. (1984) Vascular and astrocytic reactions during establishment of hippocampal transplants in adult host brain. *Neuroscience* **12**, 745–60.

LIESI, P. (1985a) Do neurons in the vertebrate CNS migrate on laminin? *European Molecular Biology Organization Journal* **4**, 1163–70.

LIESI, P. (1985b) Laminin-immunoreactive glia distinguish regenerative adult CNS systems from non-regenerative ones. *European Molecular Biology Organization Journal* **4**, 2505–11.

LIESI, P. (1986) The neurite-outgrowth promoting domain of laminin is in the alpha-helical region of the B2-chain. *Neuroscience Abstracts* **12**, 395.

LIESI, P., DAHL, D. & VAHERI, A. (1983) Laminin is produced by early rat astrocytes in primary culture. *Journal of Cell Biology* **96**, 920–4.

LIESI, P., DAHL, D. & VAHERI, A. (1984a) Neurons cultured from developing rat brain attach and spread preferentially to laminin. *Journal of Neuroscience Research* **11**, 241–51.

LIESI, P., KAAKKOLA, S., DAHL, D. & VAHERI, A. (1984b) Laminin is induced in adult brain astrocytes by stereotaxic lesioning. *European Molecular Biology Organization Journal* **3**, 683–6.

MANTHORPE, M., ENGVALL, E., RUOSLAHTI, E., LONGO, F. M., DAVIS, G. E. & VARON, S. (1983) Laminin promotes neuritic regeneration from cultured peripheral and central neurons. *Journal of Cell Biology* **97**, 1882–90.

MATHEW, W. D. & PATTERSON, P. H. (1983) The production of a monoclonal antibody that blocks the action of a neurite outgrowth-promoting factor. *Cold Spring Harbor Symposia on Quantitative Biology* **48**, 625–31.

MCLOON, S. C. (1986) Response of astrocytes in the visual system to Wallerian degeneration: An immunohistochemical analysis of laminin and glial fibrillary acidic protein (GFAP). *Experimental Neurology* **91**, 613–21.

MILLER, R. H., ABNEY, E. R., DAVID, S., FFRENCH-CONSTANT, C., LINDSAY, R., PATEL, R., STONE, J. & RAFF, M. C. (1986) Is reactive gliosis a property of a distinct subpopulation of astrocytes? *Journal of Neuroscience* **6**, 22–9.

MILLER, R. H., DAVID, S., PATEL, R., ABNEY, E. R. & RAFF, M. C. (1985) A quantitative immunohistochemical study of macroglial cell development in the rat optic nerve: in vivo evidence for two distinct astrocyte lineages. *Developmental Biology* **111**, 35–41.

MILLER, R. H. & RAFF, M. C. (1984) Fibrous and protoplasmic astrocytes are biochemically and developmentally distinct. *Journal of Neuroscience* **4**, 585–92.

MUCHNICK, N. & HIBBARD, E. (1980) Avian retinal ganglion cells resistant to degeneration after optic nerve lesion. *Experimental Neurology* **68**, 205–16.

NATHANIEL, E. J. H. & PEASE, D. C. (1963) Regenerative changes in rat dorsal roots following Wallerian degeneration. *Journal of Ultrastructure Research* **9**, 533–49.

NOBLE, M., FOK-SEANG, J. & COHEN, J. (1984) Glia are a unique substrate for the in vitro growth of central nervous system neurons. *Journal of Neuroscience* **4**, 1892–903.

PETERS, A., PALAY, S. & WEBSTER, H. deF. (1976) *The Fine Structure of the Nervous System: Neurons and Supporting Cells*. W.B. Saunders, Philadelphia.

RAFF, M. C., ABNEY, E. R., COHEN, J., LINDSAY, R. & NOBLE, M. (1983a) Two types of astrocytes in cultures of developing rat white matter: differences in morphology, surface gangliosides and growth characteristics. *Journal of Neuroscience* **3**, 1289–300.

RAFF, M. C., MILLER, R. H. & NOBLE, M. (1983b) A glial progenitor cell that develops *in vitro* into an astrocyte or an oligodendrocyte depending on the culture medium. *Nature* **303**, 390–6.

RAMON Y CAJAL, S. (1928) *Degeneration and Regeneration of the Nervous System*. (Translated by MAY, R. M.) Oxford University Press, London.

RANSON, S. W. (1912) Degeneration and regeneration of nerve fibers. *Journal of Comparative Neurology* **22**, 487–545.

RICHARDSON, P. M., ISSA, V. M. K. & SHEMIE, S. (1982) Regeneration and retrograde degeneration of axons in the rat optic nerve. *Journal of Neurocytology* **11**, 949–66.

RICHARDSON, P. M., MCGUINNESS, U. M. & AGUAYO, A. J. (1980) Axons from CNS neurones regenerate into PNS grafts. *Nature* **284**, 264–5.

ROGERS, S. L., LETOURNEAU, P. C., PALM, S. L., MCCARTHY, J. & FURCHT, L. T. (1983) Neurite extension by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. *Developmental Biology* **98**, 212–20.

SCHWAB, M. & THOENEN, H. (1985) Dissociated neurons regenerate into sciatic but not optic nerve explants in culture irrespective of neurotrophic factors. *Journal of Neuroscience* **5**, 2415–23.

SKOFF, R. P., KNAPP, P. E. & BARTLETT, W. P. (1986) Astrocyte diversity in the optic nerve: a cytoarchitectural study. In: *Astrocytes* **Vol. 1**. (edited by FEDEROFF, S. & VERNADAKIS, A.) pp. 269–71, Academic Press, New York.

SMALHEISER, N. R., CRAIN, S. M. & REID, L. M. (1984) Laminin as a substrate for retinal axons *in vitro*. *Developmental Brain Research* **12**, 136–40.

THANOS, S. & BONHOEFFER, F. (1983) Investigations on the development and topographic order of retinotectal axons. Anterograde and retrograde staining of axons and perikarya with rhodamine *in vivo*. *Journal of Comparative Neurology* **219**, 420–30.

THANOS, S. & BONHOEFFER, F. (1984) Development of the transient ipsilateral retinotectal projection in the chick embryo: a numerical fluorescence-microscopic analysis. *Journal of Comparative Neurology* **224**, 407–14.

THANOS, S., VIDAL-SANZ, M. & AGUAYO, A. J. (1987) The use of rhodamine–B–isothiocyanate (RITC) as an anterograde and retrograde tracer in the adult visual system. *Brain Research* **406**, 317–21.

THOMAS, P. K. (1974) Nerve injury. In: *Essays on the Nervous System*. (Edited by BELLAIRS, R. & GRAY, E. G.), pp. 44–70, Oxford University Press, London.

TOMASELLI, K. J., REICHARDT, L. F. & BIXBY, J. L. (1986) Distinct molecular interactions mediate neuronal process outgrowth on non-neuronal cell surfaces and extracellular matrices. *Journal of Cell Biology* **103**, 2659–72.

WEINBERG, H. J. & SPENCER, P. S. (1978) The fate of Schwann cells isolated from axonal contact. *Journal of Neurocytology* **7**, 555–69.