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MOLECULES THAT BECOME REDISTRIBUTED DURING REGENERATION OF THE LEECH CENTRAL NERVOUS SYSTEM

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Summary

A search has been made for molecules other than laminin that change their distribution during axonal regrowth after injury. Two monoclonal antibodies, generated against a protein extract of leech central nervous system (CNS), stain distinct regions of leech CNS and, following lesions, show changes in distribution with time.

1. Monoclonal antibody NP17 stained two bands of M_r 80×10^3 and 60×10^3 on Western blots of protein extracted from whole CNS. On cryosections of leech CNS, staining was predominantly within the neuropile.

2. A second antibody, mAb CT16, stained a collagen-associated molecule of the extracellular matrix. It labelled the outer capsule, which surrounds the neuronal cell packets, and the inner capsule, which envelops the neuropile. In the connectives, CT16 immunoreactivity was restricted to the connective capsule and was not associated with nerve fibres or glia.

3. When the connectives were crushed, immunocytochemical studies revealed changes in distribution of both mAb NP17 and mAb CT16. After 3 days, a time when fibres begin to sprout and form connections, an increase in NP17 immunoreactivity appeared at the site of the lesion. Staining levels remained elevated for several weeks. In contrast, CT16 immunoreactivity did not change for several days after damage. After 10 days, fibre-like staining appeared at the site of the crush; for several weeks it continued to spread throughout the connective.

4. These results show that regeneration of the leech CNS involves the redistribution of at least two molecules. Using monoclonal antibodies, these two molecules, which are situated in distinct regions of the CNS, have been visualized at different stages of the repair process. It has been shown that they alter their distribution at distinct times during regeneration.

Introduction

A key question in regeneration of the nervous system concerns the identity of factors required for growth and synapse formation to occur. To what extent does the target, by virtue of secreting diffusible factors, play a role and to what extent do environmental factors encountered by the growing axons direct regeneration? It is now clear that cell-

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adhesion molecules as well as extracellular matrix molecules can appear at the site of a lesion coincident with regrowth and synapse formation (Martini and Schachner, 1988; Gatchalian *et al.* 1989; Paschke *et al.* 1992).

One such molecule, which has been studied extensively in vertebrates as well as in the leech, is laminin. It is a glycoprotein of the extracellular matrix and is an effective substratum for a wide range of neurones in culture (Manthorpe *et al.* 1983). The domains responsible for its effects on neurite outgrowth and neuronal survival have been identified (Edgar *et al.* 1984). In the central nervous system (CNS) of the adult leech, laminin has a distribution characteristic of a basement-membrane protein. In the nerve connectives, which contain the axons running from one ganglion to the next, laminin is restricted to the capsule tissue surrounding the nerve fibres. Thus, neurones are not in contact with laminin in the CNS of the adult leech. However, when connectives are severed, resulting in massive axonal damage, glial cell injury and the development of a neuropile (Fernández and Fernandez, 1974), it has been shown that leech laminin becomes closely associated with areas of neurite growth at the site of lesion (Masuda-Nakagawa *et al.* 1990, 1993).

An interesting question concerns the involvement of proteins other than laminin in axonal regrowth. Initiation of growth, axon elongation, termination of growth and synapse formation might be directed by a variety of molecules. Accordingly, a major goal of this study was to look for the presence of molecules other than laminin at the site of lesion during axonal regeneration. Two monoclonal antibodies, raised against proteins of the leech CNS, have been identified that recognize antigens appearing at different times during regeneration.

Materials and methods

Experimental animals

Leeches, *Hirudo medicinalis* (L.), were purchased from Ricarimpex (France) or from Biopharm (England) and maintained at 16 °C in artificial pond water.

Preparation of a high-pH extract

Ganglionic chains were dissected and put into 10 mmol l^{-1} Tris–HCl buffer, pH 7.4, containing 2 % Triton X-100 (E. Merck GmbH, Darmstadt, Germany) and protease inhibitors (2 mmol l^{-1} phenylmethylsulphonylfluoride, 5 mmol l^{-1} *N*-ethylmaleamide, 1 μg ml^{-1} antipain, 0.5 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin, 5 μg ml^{-1} aprotinin; Boehringer Mannheim). The ganglia were homogenized in a 2 ml Dounce homogenizer using a mechanical rotor and rotated at 4 °C overnight. The following day, the insoluble material was centrifuged at 15 000 ***g*** for 3 min and washed three times with 50 mmol l^{-1} Tris–HCl. The pellet was extracted in 20 mmol l^{-1} Caps [3-(cyclohexyl-amino)-1-propanesulphonic acid], pH 11, containing 10 mmol l^{-1} EDTA, 150 mmol l^{-1} NaCl and protease inhibitors, for 18–24 h at 4 °C. 100 μl of Caps buffer was used per chain of ganglia. The extract was dialysed against Tris-buffered saline (TBS) (50 mmol l^{-1} Tris–HCl, pH 7.4, 150 mmol l^{-1} NaCl) before further use.

For fractionation on a glycerol gradient, 5 ml of extract corresponding to 50 ganglia chains was concentrated to a final volume of 0.75 ml using Sephadex G100. The extract

was applied to a 14 ml 15 %–40 % continuous glycerol gradient in 0.2 mol l^{-1} NH_4HCO_3, pH 8.0. The proteins were separated by ultracentrifugation at 302 500 ***g*** for 18 h at 4 °C in a Kontron TST 41.14 rotor and a Kontron ultracentrifuge. 0.5 ml fractions were collected from the bottom of the tube.

Binding of antigen to Concanavalin A

To 1 ml of dialysed Caps extract, $CaCl_2$, $MgCl_2$ and $MnCl_2$ were added to a final concentration of 1 mmol l^{-1}. This was added to 500 μl of Concanavalin A–sepharose (Pharmacia) equilibrated in the same buffer and incubated at room temperature (24 °C) for 1 h. Extensive washing preceded elution with 0.5 ml of 500 mmol l^{-1} methylglucopyranoside.

Production of monoclonal antibodies

Balb/c mice were purchased from Madörin AG Kleintierfarm, Füllinsdorf, Switzerland. 140 μl of fraction 14 of the glycerol gradient was suspended in the same volume of complete Freund's adjuvant (Gibco Laboratories, Grand Island, NY) and injected intraperitoneally into a mouse. Four weeks later, the mouse was boosted by intraperitoneal injection of 150 μl of fraction 14 in incomplete Freund's adjuvant. The mouse was killed 8 days later and the spleen removed. Spleen lymphocytes were fused with 5×10^3 myeloma cells (known as 'PAI') and the hybridoma cells were grown as previously described (Chiquet *et al.* 1988). Instead of using macrophage feeder cells, interleukin-6 (Institute of Immunology, Basel, Switzerland) was added for initial growth. Culture supernatants were screened by an ELISA on microtitre wells (no. 3911; Falcon Labware, Oxnard, CA) coated with Caps extract diluted 100-fold. Seventy hybridoma lines were selected for further screening on cryosections of leech CNS and by Western blotting. Selected hybridomas were cloned by serial dilution and, after rescreening, the clones (one confluent dish per mouse) were injected into pristane-primed (Aldrich Chemical Co., Milwaukee, WI) Balb/c mice to generate ascites tumours. Mice were killed approximately 10 days later and ascites fluid was collected. Immunoglobulins were precipitated with 50 % saturated NH_4SO_4 and dissolved in the original volume of buffer for subsequent coupling to CNBr-activated Sepharose. The identity of the immunoglobulin type was determined with an Ouchterlony test, using antibodies purchased from Nordic Immunology, Tilburg, Netherlands. Both mAb CT16 and mAb NP17 were identified as IgMs.

Crush operations

The crush operations were kindly performed by John G. Nicholls. Leeches were anaesthetized in 8 % ethanol for 20 min. Using forceps, the connectives were crushed on both sides of one ganglion per leech and allowed to regenerate for 1–34 days (Masuda-Nakagawa *et al.* 1990). The ganglia and adjacent crushed connectives were dissected out of the leech, frozen in Tissue Tek, sectioned and prepared for immunocytochemistry.

Immunocytochemical studies on cryosections

Ganglia and connectives of the leech CNS or leech body wall were embedded in Tissue Tek (Miles Laboratories, Inc., Lenaxa, KS) and frozen on dry ice. Sections 10 μm thick

were cut using a cryostat (Slee Mainz type HS) and mounted onto gelatin-coated slides. Sections were preincubated with phosphate-buffered saline (PBS) containing 1 % bovine serum albumin (BSA) and then incubated for 3 h with mAb CT16 or mAb NP17 diluted 200-fold in PBS/1 % BSA. After washing the sections with PBS, FITC-labelled secondary antibody (Cappel) was added at a dilution of 1:100 in PBS/1 % BSA for 1 h. When sections were double-labelled, Hoechst 33258 dye was subsequently added for 10 min at a concentration of 5 μg ml^{-1} PBS before washing with PBS and mounting in PBS containing 50 % glycerol and 0.5 % *N*-propylgalate. Samples were viewed using an Olympus microscope equipped with 40×1.3 NA and 10×0.4 NA objectives.

To examine whether mAb CT16 binds to collagen or to a collagen-associated protein, its staining pattern was analysed on cryosections treated with collagenase. Slides were preheated to 100 ˚C for 2 min, before adding bacterial collagenase (Clostridiopeptidase type VII, Sigma, Giessenhofen, Germany) at 40 units ml^{-1} (units defined by Sigma). The tissue was treated with the enzyme in the presence of 5 mmol l^{-1} $CaCl_2$ and then washed several times in PBS. mAb CT16 or control mAb CS39, a monoclonal antibody against an intermediate filament protein, was added and immunostaining of the slides was performed as described above.

Gel electrophoresis and Western blotting analyses

SDS–PAGE was performed according to Laemmli (1970) with reagents from the Bio-Rad Laboratories (Richmond, CA). Samples of protein extracted from whole CNS and fractionated on a glycerol gradient were run with or without reduction by dithiothreitol (Sigma Chemical Co., St Louis, MO) on 3 %–15 % gradient acrylamide gels. Gels were stained with 0.2 % Coomassie Brilliant Blue. Relative molecular mass standards for the gels were obtained from Sigma Chemical Co. Rainbow markers were used for Western blots and purchased from Amersham International. Western blots were performed as described by Towbin *et al.* (1979). Proteins were transferred and the nitrocellulose (BA-85; Schleicher and Schuell, Inc., Dassel, Germany) was preincubated with PBS containing 5 % milk powder (SanoLait, Coop, Switzerland) and 0.05 % Tween-20 and then incubated for 3 h at room temperature (24 ˚C) in mAb CT16 or mAb NP17 (1:200). After washing the filter in PBS, 5 % milk powder and 0.05 % Tween-20, it was incubated with the secondary antibody goat anti-mouse IgG (1:100; Cappel Laboratories, Cochranville, PA) for 1 h at room temperature (24 ˚C). This was followed by a 1 h incubation in mouse peroxidase anti-peroxidase (PAP) (1:3000; Jackson Immunoresearch Laboratories, Avondale, PA) before the blots were developed with 4-chloro-1-naphthol (E. Merck GmbH) as described by Imhof *et al.* (1983).

Results

Proteins extracted from leech CNS were separated on a 15 %–40 % glycerol gradient by ultracentrifugation and a fraction, number 14, running with a sedimentation coefficient of approximately 10S (10^{-12} s) was selected for production of monoclonal antibodies. Two other proteins of the leech CNS, laminin and tenascin, were not present in this fraction, since they occur in fractions with lower sedimentation coefficients (Masuda-

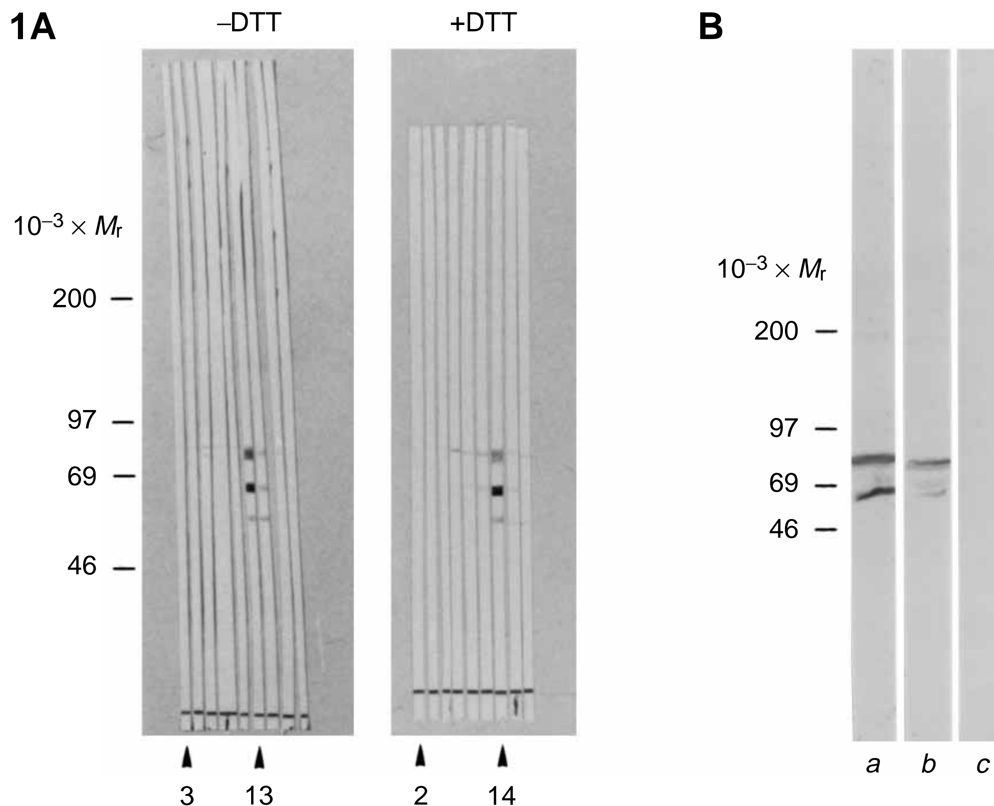

Fig. 1. (A) Analysis of the NP17 antigen on a Western blot. Leech CNS proteins were separated on a 15 %–40 % glycerol gradient and the fractions were run on a 3 %–15 % gradient SDS–polyacrylamide gel. Alternating fractions beginning with fractions 3 and 2 were applied to the gel in the presence or absence of reducing agent and transferred to nitrocellulose. Under both conditions mAb NP17 recognized two main bands at approximately 80×10^3 and 60×10^3 M_r. The bands were specific to fractions 13 and 14. (B) NP17 antigen does not bind to Concanavalin A. 1 ml of Caps extract from the leech CNS (*a*) was incubated with Concanavalin A–sepharose and the non-binding proteins were removed (*b*). The sepharose was washed before eluting the Concanavalin-A-binding proteins (*c*) with 0.5 ml of 0.5 mol l^{-1} methylglucopyranoside. 80 μl was applied to each lane. DTT, dithiothreitol.

Nakagawa and Nicholls, 1991; Masuda-Nakagawa and Wiedemann, 1992). After fusion, the hybridomas were screened for differences in staining patterns on cryosections of intact and regenerating nerve fibres of leech CNS. Two antibodies with distinctive properties, which selectively stained neuropile (mAb NP17) or connective tissue (mAb CT16), were selected.

Biochemistry of the NP17 antigen

To assess the properties of the NP17 antigen, a CNS extract was prepared for Western blotting. As seen in Fig. 1, mAb NP17 recognized two main bands with relative molecular masses of approximately 80×10^3 and 60×10^3. Running the antigen in the

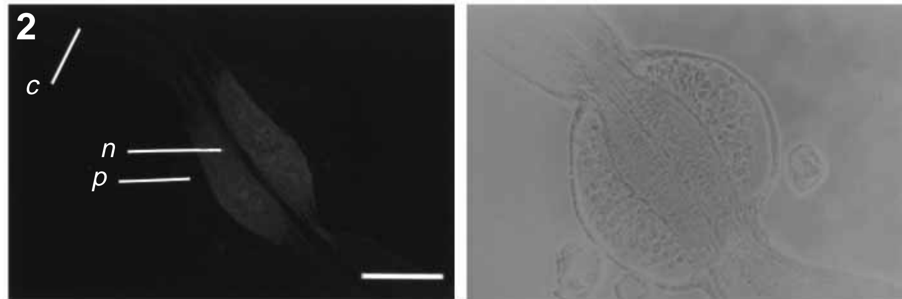

Fig. 2. Distribution of NP17 immunoreactivity within the leech CNS. Cryosections of ganglia were stained with mAb NP17 followed by FITC-labelled secondary antibody. Label was found within the neuropile (*n*). Weak staining was present in some neuronal perikarya within the packets (*p*) and in the fibre tracts within the connectives (*c*). No staining was seen when secondary antibody was used alone. A phase contrast picture is shown on the right. Scale bar, 200 μm.

presence of reducing agent (dithiothreitol) did not result in a change in its electrophoretic mobility, indicating that the antigen was not linked by disulphide bonds. The antibody was tested on the various fractions of CNS extract separated by ultracentrifugation. As shown in Fig. 1A, the bands appeared in fractions 13 and 14, confirming the specificity of this antigen to the fraction used for immunization.

To test whether the NP17 antigen was glycosylated with mannosyl or glycosyl oligosaccharides, CNS extract was applied to a Concanavalin A–sepharose column, and the bound proteins were eluted with $0.5\,\mathrm{mol\,l^{-1}}$ methylglucopyranoside. The CNS extract, the flow-through proteins and the proteins eluted with $0.5\,\mathrm{mol\,l^{-1}}$ methylglucopyranoside were separated on a gel and transferred to nitrocellulose. Fig. 1B shows that the 80×10^3 and $60\times10^3\,M_r$ bands are seen only in lanes *a* and *b*, and did not bind to Concanavalin A. These results indicate that mAb NP17 antigen may not be glycosylated and may thus represent an intracellular protein.

Staining pattern of leech CNS with mAb NP17

mAb NP17 recognized an antigen situated predominantly in the neuropile, the structure where synaptic connections are made. Slight staining was also observed in neuronal perikarya and within the fibre tracts of the connectives (Figs 2 and 3).

Fig. 3. NP17 immunoreactivity of regenerating CNS connectives (see Discussion). Cryosections of regenerating connectives were double-stained with the Hoechst stain 33258 (left-hand side) and with mAb NP17 followed by FITC-conjugated secondary antibody (right-hand side). Arrows point to the crush sites. 2 days after lesion of the connectives (A), NP17 immunoreactivity had a spot-like appearance at the border of the crush. 4 days after the crush (B) there was increased staining at the site of damage, which is indicated by the presence of Hoechst-33258-stained microglial cells (see explanation in the text). At 16 days (C), increased label was coincident with the increase in microglial cells in the connectives. A high magnification of a 5-day crush site (D) is depicted at the bottom to show the distribution of immunoreactivity. Scale bars, 2, 4 and 16 days, 200 μm; 5 days, 20 μm.

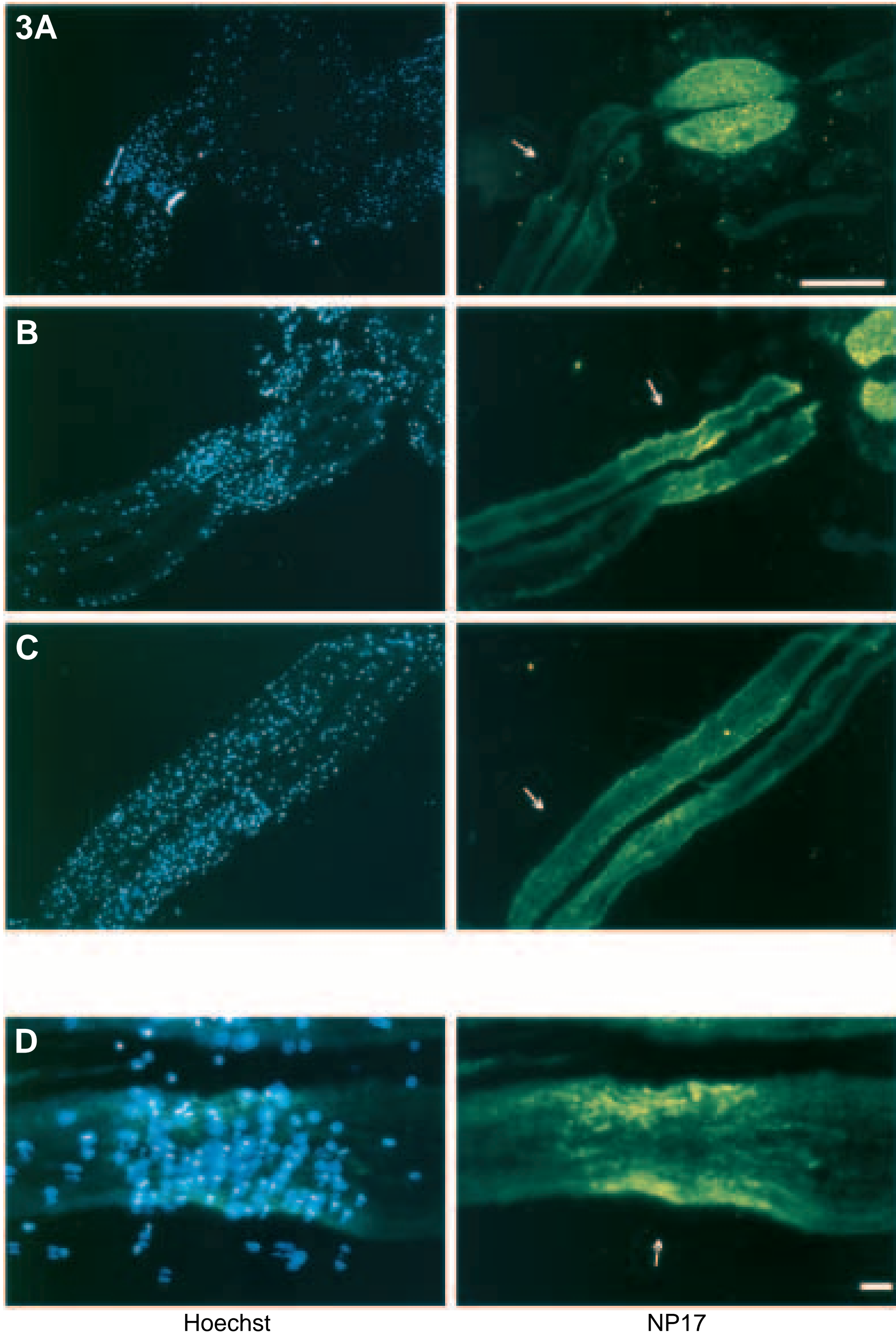

Fig. 3

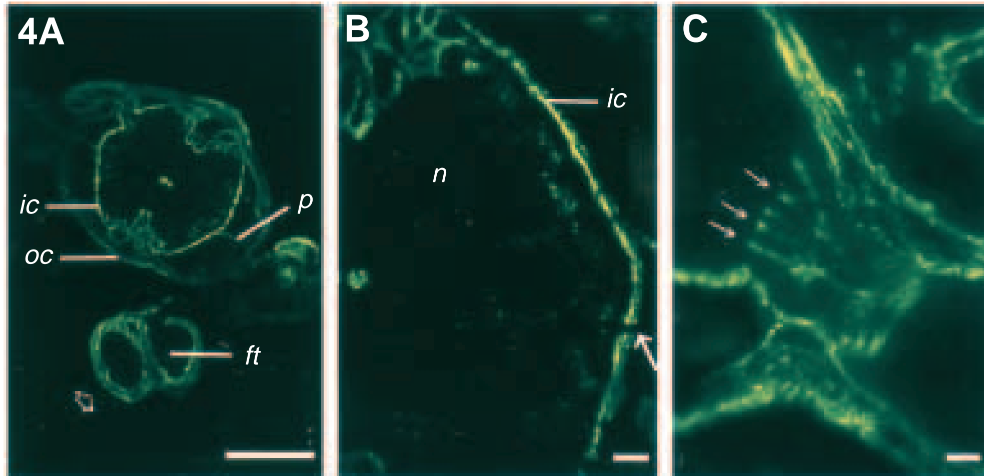

Fig. 4. Distribution of CT16 immunoreactivity within the leech CNS revealed by staining cryosections of segmental ganglia with mAb CT16 followed by FITC-labelled secondary antibody. (A) A section through a ganglion. Staining was observed within the connective tissue of the inner (*ic*) and outer capsule (*oc*) and of the partitions that separate neuronal cell packets (*p*). A cross section of a connective nerve is also present (arrowhead). The connective capsule sheath was labelled, whereas the fibre tracts (*ft*) were not. Scale bar, 200 μm. (B) High-magnification photograph of the neuropile (*n*) and the inner capsule (*ic*). Openings within the inner capsule are apparent (arrow). Scale bar, 20 μm. (C) A high-magnification photograph of the ganglion–connective boundary. The connective tissue partitions that separate fibres into distinct tracts are stained (arrows). Scale bar, 20 μm.

To determine whether the NP17 antigen was localized intracellularly or extracellularly, neurones were isolated from the CNS and plated on coverslips coated with Concanavalin A. The cells were then stained with mAb NP17 in the presence and in the absence of 1 % Triton X-100. There was a slight increase in staining after permeabilization with 1 % Triton X-100 (not shown) with immunoreactivity visible in neuronal cell bodies as well as in the neurites, suggesting that, under these conditions, the NP17 antigen has an intracellular location.

mAb NP17 immunoreactivity increases during regeneration

When regenerating fibres were analysed for NP17 immunoreactivity in crushed connectives, punctate staining was first seen at the border of the lesion after 2 days (Fig. 3). At 4 days, when regrowing fibres had crossed the lesion and when synapses had begun to be formed, increased staining was detected at the crush site. To indicate the site of lesion, sections were simultaneously stained with the Hoechst dye 33258, which labels the microglial cells that accumulate there (Morgese *et al.* 1983). These remain at the crush site for several weeks, but in time also spread along the connectives. In a 16-day regenerating connective, spread of immunoreactivity was observed through a wide area containing large numbers of microglial cells. A few days later, however, the staining in crushed connectives became indistinguishable from that of control connectives (not shown). Thus, the NP17 antigen, which is localized predominantly in the neuropile of the intact CNS, was localized within the connectives at the crush site.

Staining pattern of leech CNS with mAb CT16

Fig. 4 shows the staining pattern of mAb CT16 in a ganglion of the leech CNS. Immunoreactivity was present in connective tissue of the outer and inner neural capsules, as well as in the partitions that subdivide each ganglion into packets (Fig. 4A). At higher magnification, some label was also seen within the neuropile, especially in areas close to the inner capsule (Fig. 4B). This staining probably represents extracellular material that can be found surrounding the neural arborizations (Gray and Guillery, 1963; Coggeshall and Fawcett, 1964). Some openings were seen within the inner capsule (arrow Fig. 4B). These may represent the areas through which the apical processes of the neuronal perikarya enter the neuropile (Fernández, 1978). In Fig. 4C, finger-like structures are visible at the ganglion–connective junction zone. These are the connective tissue partitions, derived from the adjacent inner capsule of the packets, that funnel axons into distinct fibre pathways as they leave the ganglion.

mAb CT16 distribution was examined in other tissues of the leech. The body wall of leech is largely made up of three layers of muscles running circumferentially, obliquely and longitudinally. Staining this area revealed extracellular CT16 immunoreactivity surrounding single muscle fibres (not shown).

To determine whether the antigen might have an intracellular location in neurones, cells were stained with mAb CT16 in the presence and in the absence of 1 % Triton X-100. No staining was apparent in either permeabilized or non-permeabilized cells (not shown). Staining of a section through the outer capsule of a leech ganglion showed a mesh-like pattern, reminiscent of a collagen or collagen-associated antigen (Fig. 5A).

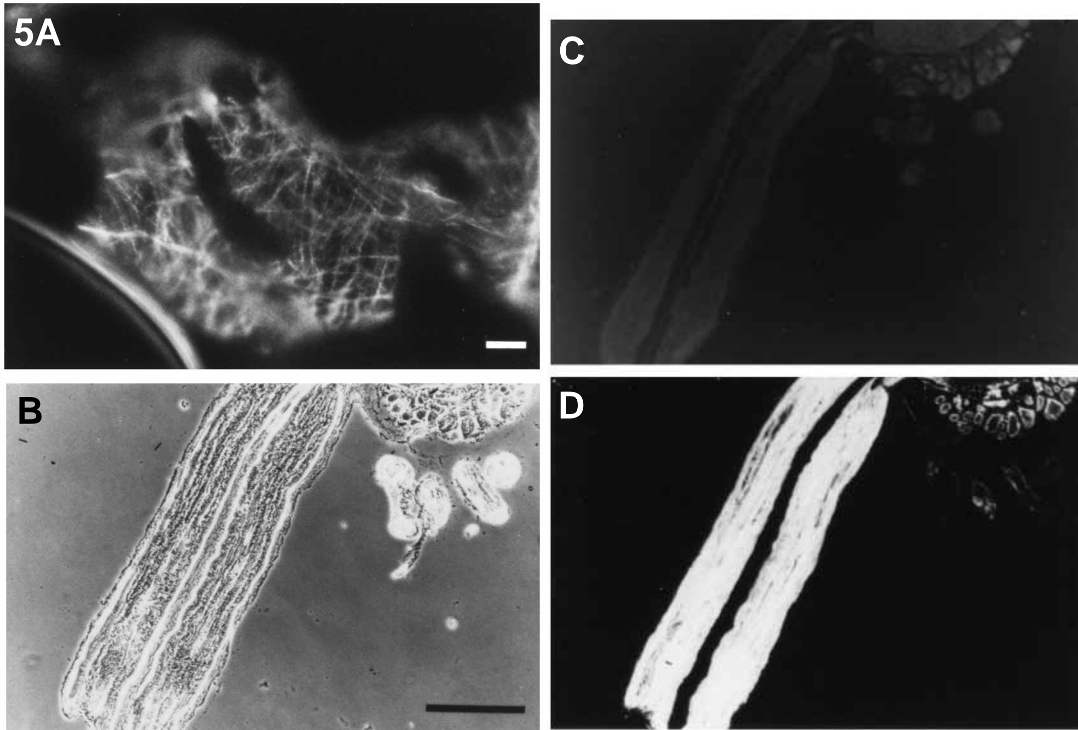

Fig. 5. mAb CT16 recognizes a collagen-associated molecule. (A) CT16 immunoreactivity of a section through the outer capsule of a leech ganglion. Scale bar, 20 μm. (B) Cryosections of connective nerve were treated with collagenase as described in the text and subsequently stained with mAb CT16 (C) or with a control mAb CS39 (D), followed by FITC-labelled secondary antibody. Characteristic CT16 immunoreactivity on the outer capsule of the nerve connective disappeared after collagenase treatment, whereas cytoskeletal CS39 immunoreactivity within the fibre tracts did not. B, C, D, scale bar, 200 μm.

CT16 immunoreactivity disappeared in cryosections digested with collagenase (Fig. 5C), whereas a control mAb recognizing a cytoskeletal antigen of the leech CNS (T. E. Lüthi, D. L. Brodbeck and P. Jenö, in preparation) retained its staining pattern after similar treatment (Fig. 5D). These results suggest that mAb CT16 recognizes a collagen or collagen-associated protein of the extracellular matrix.

mAb CT16 immunoreactivity is altered during regeneration

Since molecules of the extracellular matrix have been implicated in neuronal outgrowth and regeneration, crush experiments were performed to examine the staining pattern of mAb CT16 during regeneration of the leech CNS. Fig. 6 shows connectives stained with mAb CT16 1 day and 9 days after injury. No changes in CT16 immunoreactivity were observed compared with control connectives. Label remained confined to the connective tissue capsule surrounding the fibres. The crush site could be unambiguously recognized by the presence of microglial cells. After 10 days, punctate

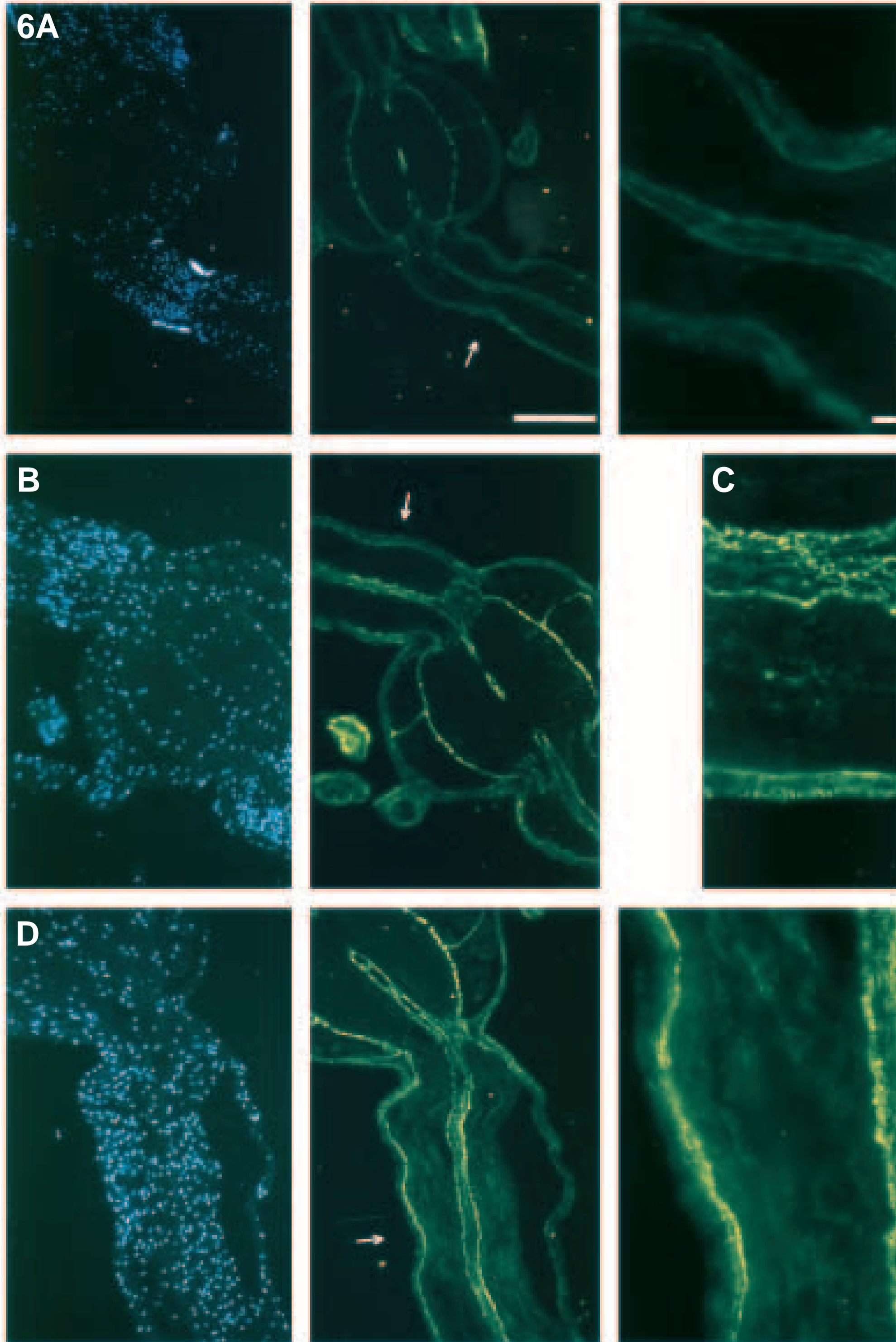

Fig. 6

Fig. 6. CT16 immunoreactivity on regenerating connectives of the CNS. Cryosections of regenerating connectives were double-stained with the Hoechst stain 33258 (blue stain) and with mAb CT16 followed by FITC-coupled secondary antibody (green stain). At 1 day after lesion (A), CT16 immunoreactivity was restricted to the connective capsule sheath. At 9 days after crush (B), the situation had not changed. Punctate staining appeared at the site of lesion 10 days after damage (C). At later stages, the staining pattern became more fibre-like, as shown in a 16-day preparation (D). The crush sites are indicated by the Hoechst stain 33258 and by arrows as explained in the text. Scale bars, centre, 200 μm; right-hand side, 20 μm.

staining was first detected at the site of crush on sections incubated with mAb CT16. Thereafter, fibre-like immunoreactivity spread throughout the connective, as shown in a section of a connective 16 days after injury. Interestingly, in two instances the staining was observed to extend away from the crush site towards the nearest ganglion (Fig. 7). Smith *et al.* (1991) have proposed that non-neuronal elements of the nervous system provide the critical microenvironment for neuronal repair in insects. They observed migration of glial cells from the ganglion to the lesion site. Thirty-four days after the crush, axon tracts within the connectives were still stained with a fibre-like pattern. No changes in the staining pattern were observed within the ganglia.

Discussion

Regeneration of the leech central nervous system involves a characteristic sequence of events. Microglial cells move to the site of injury immediately after damage and remain in contact with regrowing axons as the glial cells of the connective eventually degenerate (McGlade-McCulloh *et al.* 1989). Severed axons begin to sprout fine processes 2 days

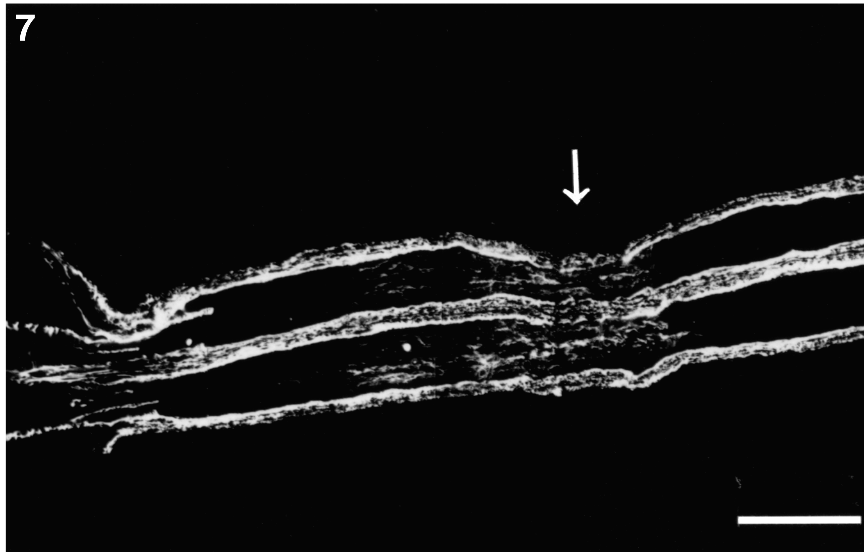

Fig. 7. CT16 immunoreactivity of a connective crushed 26 days previously. The staining seen within the axon tracts at the crush site extended beyond the immediate area of damage towards the closest adjacent ganglion. The crush site is marked with an arrow. Scale bar, 0.2 mm.

after injury, a time when the neurite-promoting molecule laminin has been shown to appear in regions where nerve outgrowth occurs (Wallace *et al.* 1977; Masuda-Nakagawa *et al.* 1990). At 4 days, fibres of S-cells meet across the lesion and form synapses on their severed segments (Carbonetto and Muller, 1977). Restoration of connections is achieved by 1–2 months after injury.

Little is known about the molecular events accompanying regeneration, and the aim of the present experiments was to analyse the molecular processes involved in nerve fibre regrowth. It seems likely that damage to the CNS induces local signals between cells, initiating a series of events that lead to functional recovery. The study presented here demonstrates that the appearance of molecules other than laminin is part of the response to nerve injury.

One molecule, the NP17 antigen, appears in the connectives at the site of lesion at increased levels after approximately 3 days and the immunoreactivity remains elevated for several weeks. In the intact animal, the NP17 antigen is restricted to the neuropile and only a very little immunoreactivity is observed within the fibre tracts of the connectives. The association of the NP17 antigen with the site of synaptic junctions and the appearance of increased immunoreactivity within the fibre tracts after 4 days is interesting. Fernández and Fernandez (1974) have shown that crushing connectives results in the induction of a neuropile at the site of injury. Whether this antigen plays a role in synapse stability or function remains to be determined. Two lines of evidence point to an intracellular location of the NP17 antigen. First, permeabilization of leech neurones with Triton X-100 resulted in increased staining, indicating that neurones may synthesize this molecule. Second, Concanavalin A did not bind to the antigen. This suggests that, as one would expect for an intracellular molecule, it may not be glycosylated. It will be of interest to examine the localization of this antigen at a subcellular level.

Another molecule, the CT16 antigen, is, like laminin, located in the extracellular matrix of the leech CNS. However, there are differences in the staining pattern shown by this antibody and by mAbs 203 and 206, which bind to leech laminin (Chiquet *et al.* 1988). The CT16 antigen is not restricted to the basement membrane; instead, it labels connective tissue in a more general way and staining is not found around the nerve cell bodies. The mesh-like staining of the outer connective capsule and the lack of staining on cryosections treated with collagenase suggest an association of this antigen with collagen. It cannot, however, be ruled out that this antibody is recognizing collagen itself. It is distinct from the laminin antigen in its temporal distribution during regeneration. Whereas laminin staining appears at the crush site after 2 days, when nerve fibres initiate regrowth, CT16 immunoreactivity remains unaltered for 10 days after lesion. Only then does staining occur at the sites of fibre regrowth. The reason for this late expression has yet to be determined. The cells that are activated to produce the newly appearing molecules have not been identified. Molecules of the extracellular matrix have been implicated in neuronal outgrowth and regeneration (Sanes, 1989). However, because of the nature of the immunoglobulin, it has not been possible to purify sufficient amounts of antigen to test for neurite-promoting properties.

A number of molecules have been implicated in nerve regeneration in other systems.

Changes in the expression of tenascin during nerve repair in adult mouse sciatic nerve and in the chicken neuromuscular junction have been demonstrated (Daniloff *et al.* 1989; Martini *et al.* 1990). The application of antibodies directed against tenascin to regenerating frog neuromuscular junctions resulted in delayed or inhibited reinnervation (Mège *et al.* 1992). Levels of N-CAM and Ng-CAM increase in the neurones and Schwann cells surrounding the lesion site following damage to mouse sciatic nerve (Martini and Schachner, 1988). Another candidate cell adhesion molecule, neurolin, has been shown to be re-expressed in the fish retinal tectal system after optic nerve transection (Paschke *et al.* 1992) and significant levels of ciliary neurotrophic factor (CNTF) protein have been detected at sites distal to nerve lesions (Sendtner *et al.* 1992).

The NP17 and CT16 antigens described in this study add possible candidates to the list of molecules that appear in a specific spatio-temporal distribution during regeneration. Analysis of the distinct steps involved in regeneration and the characterisation of the various molecules involved may allow a more detailed understanding of this phenomenon.

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