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Extracellular matrix molecules in development and regeneration of the leech CNS

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SUMMARY

As neurons grow to their targets their processes elongate, branch and form specialized endings into which are inserted appropriate ion channels. Our aim has been to analyse the role of the extracellular matrix molecules laminin and tenascin in inducing growth and in determining the form and physiological properties of growing neurites. A preparation in which development and regeneration can be followed at the cellular and molecular level in the animal and in tissue culture is the central nervous system (CNS) of the leech. In leech extracellular matrix (ECM) both laminin and tenascin are present; the molecules are structurally similar but not identical to their vertebrate counterparts. Tenascin extracted from leech ECM shows a typical hexabrachial structure whereas laminin shows a typical cruciform structure in rotary shadowed preparations. Leech laminin purified by means of a monoclonal antibody is a molecule of about 1000 kDa, with a polypeptide composition of 340, 200, 180 and 160 kDa. Substrates that contain tenascin or laminin produce rapid and reliable outgrowth of neurites by identified cells. A remarkable finding is that the outgrowth pattern produced by an individual neuron depends in part on its identity, in part on the substrate upon which it is placed. For example, a Retzius cell grows in a quite different configuration and far more rapidly on laminin substrate than does another type of neuron containing the same transmitter (serotonin); and the pattern of outgrowth of the Retzius cell is different on laminin and on the plant lectin Con A (concanavalin A). Thus Con A induces the growth of processes that are shorter, thicker, more curved and contain fewer calcium channels than those grown on laminin. To determine whether laminin can also influence neurite outgrowth in the animal, immunocytological techniques have been used to follow its distribution in the extracellular matrix of normal, developing and regenerating leech CNS. In adult leeches neuronal processes in the CNS are not in contact with laminin which is confined to the surrounding extracellular matrix. In embryos however, laminin staining appears between ganglionic primordia along the pathways that neurons will follow. Similarly, after injury to the adult CNS, laminin accumulates at the very sites at which sprouting and regeneration begin. How the laminin becomes redistributed to appear in the region of injury has not yet been established. Together these findings suggest a key role for laminin and for other extracellular matrix molecules. One attractive speculation is that large molecules situated at a particular region of the CNS may give differential instructions to different types of neurons, causing branching in some, accelerated outgrowth in others and formation of specialized endings in still others. This represents an economical scheme by which relatively few molecules could exert diverse effects.

INTRODUCTION

After injury to a peripheral nerve regenerating axons grow to their targets along Schwann cells and extracellular matrix (ECM). Once arrived at their destination the axons branch, stop growing and form sensory endings in skin, or motor nerve terminals in muscle. While these changes are occurring at the periphery other processes of the same cells within the central nervous system remain unaffected. Similarly, during development one region of the arborization of a neuron may form synapses while another is retracting or forming sensory endings.

Numerous questions arise concerning the mechanisms by which molecules in the external environment of a growing or regenerating axon can determine its form and its physiological properties. Detailed information is now available about growth factors secreted by targets and about adhesion molecules on cell membranes that influence outgrowth, migration and fasciculation (see Berg 1984; Jessel 1988). In addition, it has been shown that large molecules with important growth-promoting properties are anchored in the extracellular matrix (Sanes 1989). For example, laminin can provide a substrate to which neurons adhere and grow in culture (Manthorpe *et al.* 1983; Hammarback *et al.* 1985; Smalheiser *et al.* 1984; Adler *et al.* 1985; Baron van Evercoon *et al.* 1982). The aim of the present experiments was to test the range of effects that laminin and another large molecule, tenascin, could produce on neuronal outgrowth in adult animals and embryos as well as in the dish.

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Suitable preparations are provided by the relatively simple CNS of the leech in which one can identify and culture various types of nerve cells with different, well-characterized morphological features and physiological characteristics. Before describing these experiments, we first review briefly the properties of laminin and tenascin extracted from vertebrate ECM.

(*a*) *Laminin and tenascin*

Laminin is a non-collagenous glycoprotein component of the basal lamina (Martin & Timpl 1987), a specialized extracellular matrix that surrounds muscle cells, fat cells, the peripheral nervous system and forms the basal layer of epithelial cells (Laurie *et al.* 1982). It also appears in medium conditioned by a variety of cells (Collins & Lee 1984; Davis *et al.* 1985; Lander *et al.* 1985*a*, *b*). Nerve cells isolated from central as well as peripheral nervous system adhere to and grow on laminin substrate in culture (Rogers *et al.* 1983; Manthorpe *et al.* 1983). The neurite promoting activity has been localized to a fragment at the end of the long arm (Edgar *et al.* 1984, 1988; Engvall *et al.* 1986) but the exact sequences have not yet been identified. It has been suggested that the sequence Ile-Lys-Val-Ala-Val may be important since peptides containing the sequence can promote process outgrowth from cerebellar neurons and PC12 cells (Tashiro *et al.* 1989). Detailed information about integrins (laminin receptors) and laminin binding proteins is discussed in Edgar (1989).

Evidence for the role of laminin *in vivo* has been provided by immunocytological correlation of laminin localization with growing axons in the peripheral nervous system. Thus laminin is present in the basal lamina of Schwann cells (Sanes 1982), Schwann cells in culture produce laminin (Cornbrooks *et al.* 1983), and regenerating rat sciatic nerve fibres can regrow through the scaffold of basal lamina that remains after Schwann cells have been killed (Ide *et al.* 1983). By immunoelectron microscopy laminin has been localized to the basement membrane and to the surface of axonal sprouts growing out from the regenerating sciatic nerve (Kuecherer-Ehret *et al.* 1990). Another example of laminin in regeneration of the peripheral nervous system occurs at the neuromuscular junction. Regenerating motor axons recognize their former synaptic sites on the basal lamina (Tello 1907; Sanes *et al.* 1978). At the denervated endplates there is present an isoform of laminin, s-laminin (Hunter *et al.* 1989*a*, *b*) which could provide the signal for cessation of growth and synapse formation. Moreover, an antibody which recognizes a laminin-heparan sulphate proteoglycan complex (INO, inhibitor of neurite outgrowth; Chiu *et al.* 1986) is known to inhibit neurite outgrowth on sections of peripheral nerve *in vitro* (Sandrock & Matthew 1987*a*) and regeneration in the iris *in vivo* (Sandrock & Matthew 1987*b*); the active component of the complex has been shown to be laminin. Less clear is the role of laminin in adult central nervous system where it is usually restricted to basement membrane surrounding blood vessels. Laminin is however produced by early rat astrocytes in culture (Liesi *et al.* 1983) and is present on astrocytes in injured and actively regenerating CNS regions; these include mammalian olfactory bulb, frog (Liesi 1985*b*; Carbonetto *et al.* 1987) and goldfish optic nerve (Hopkins *et al.* 1985) and rat brain after injury (Liesi *et al.* 1984).

In embryonic development the correlation is again good. Laminin has been found in the developing peripheral nervous system of the chick (Rogers *et al.* 1986) in the basement membrane surrounding the neural tube and as a meshwork in early ganglia and nerve roots. In the developing CNS laminin occurs in the ventral longitudinal pathway of the brain as punctate staining on the surfaces of neuroepithelial cells through which axons extend (Letourneau 1989) and in regions of neuronal migration (Liesi 1985*a*). In the optic nerve of the early developing rat, laminin is present in the ventral portion of the optic stalk where the first retinal axons grow (McLoon *et al.* 1988). And in Drosophila embryos CNS laminin has been localized to the longitudinal and commissural axon pathways as well as intersegmental and segmental roots (Fessler *et al.* 1987; Montell & Goodman 1989). Together these observations suggest a role for laminin in development and in regeneration. Many open questions remain about its precise localization, which has not usually been established by electronmicroscopy, and about the identity of the cells that produce it.

Another ECM molecule implicated in neuronal development and regeneration is tenascin, which is closely related to glioma-mesenchymal ECM antigen, hexabrachion, J1 and cytotactin (Chiquet-Ehrisman *et al.* 1986; Chiquet & Fambrough 1984; Kruse *et al.* 1985; Erickson & Inglesias 1984; Bourdon *et al.* 1985; Crossin *et al.* 1986). After rotary shadowing tenascin has a characteristic 6-armed structure under the electron microscope. Chick fibroblast tenascin has a composition of polypeptides of M_r 220, 200 and 190 kDa (Chiquet & Fambrough 1986; Chiquet-Ehrisman *et al.* 1986). It mediates neuron-glia interactions (Grumet *et al.* 1985) and is expressed at sites of neuronal migration during the development of the CNS, especially on glial pathways in the developing cerebellum and retina (Crossin *et al.* 1986). Anti-cytotactin antibodies inhibit granule cell migration so that cells accumulate on the molecular layer (Chuong *et al.* 1987). The antibodies label Schwann cells of developing peripheral nervous system, adult sciatic nerve (where it is found at nodes of Ranvier (Rieger *et al.* 1986), regenerating nerves and synaptic sites of denervated muscle (Gatchalian *et al.* 1989). In the neuromuscular system, on the proximal side of a nerve lesion cytotactin staining was detected around axons at the same time as regeneration began (Daniloff *et al.* 1989). Unlike laminin, tenascin is expressed in adult chick brain. Chiquet (1989) has reported that dorsal root ganglion neurons in culture attach to tenascin coated substrates; after a lag of a few hours growth cones start to form and after 18 h neurites appear. In the regenerating sciatic nerve J1/tenascin is expressed at the endoneurium (Martini *et al.* 1990). In summary, the function of tenascin in the nervous system has not been defined, but immunocytological staining suggests

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a role in cell–substrate interactions. Two other molecules of importance, not discussed in this paper are fibronectin and heparan-sulphate proteoglycan (see Akers *et al.* 1981; Carbonetto *et al.* 1983; Hantaz-Ambroise 1987).

(*b*) *Why the leech?*

The principal advantages of the leech for studies on regeneration and development accrue from the simplicity of its nervous system and the amount of detailed information about the individual nerve cells. Thus the CNS is made up of a chain of stereotyped ganglia linked by bundles of axons, the connectives (see figure 7). Each ganglion contains only about 400 nerve cells, many of which have known properties, functions, morphology, connections and transmitters (Nicholls 1987). This sets the stage for studies on development and regeneration (Bissen & Weisblat 1989; Weisblat & Shankland 1985; Nicholls 1987). The steps involved in the formation of ganglia in embryos, the cell lineages of neurons, their patterns of outgrowth and their relation to glia have been worked out in detail. In adult leeches the CNS shows remarkable powers of regeneration. Individual cells will grow back after injury to reform their original connections with the appropriate targets with a high degree of precision (Wallace *et al.* 1977; Macagno *et al.* 1985). Accurate regeneration of connections also occurs in preparations maintained in culture. Another unique feature is that since a single large glial cell envelopes all the axons in a connective it is possible to kill the glial cell surround in its entirety and show that regeneration proceeds as before (Elliott & Muller 1983).

In spite of its apparent simplicity the CNS of the leech is still too complex for direct studies of molecular mechanisms contributing to neurite outgrowth or synapse formation. To approach such problems we use single identified neurons of known function, with known electrical properties that have been isolated from the CNS. In suitable medium such cells survive for up to 4 weeks and maintain their membrane properties. For example, a Retzius cell in the ganglion produces impulses, which are different in shape from those in sensory or motor cells; it contains and secretes the transmitter serotonin; its appearance in electronmicrographs is recognizable by the configuration of light and dark vesicles and the texture of its cytoplasm. All these features persist in the cultured Retzius cell, as does its ability to grow and to form chemical or electrical synapses with certain specific target cells (Ready & Nicholls 1979; Stewart *et al.* 1989). Since those studies were initiated the use of single cell culture has been applied to other invertebrates and vertebrates (Lin & Levitan 1987; Schacher *et al.* 1985).

With identified cells in culture it becomes possible to make a step-by-step analysis of molecules that affect growth of leech neurons. A major advantage is that one can test for effects on a wide variety of different neurons with different properties. As the following sections show we have been able to analyse the role of endogenous and exogenous growth promoting molecules for the single cells in culture: there the composition of the substrate is of critical importance. And then with information available about candidate molecules in the CNS we have been able to assess their distribution and possible role in the whole animal as it forms or repairs its nervous system. In the following sections we review early work and present new findings on the role of ECM molecules in tissue culture and in animals.

METHODS

Techniques used in this paper have been, for the most part, described fully elsewhere (Masuda-Nakagawa *et al.* 1988; Chiquet *et al.* 1988; Chiquet & Acklin 1986). In brief, individual identified leech neurons, usually Retzius or AP cells, were isolated from the CNS by suction after mild enzyme treatment (collagenase dispase 2 mg ml^{-1}). AP cells have distinctive membrane properties but no known function. Cells were plated in Leibovitz 15 medium and 2% foetal calf serum with 30 mM glucose and garamycin (0.1 mg ml^{-1}) in microwells precoated with substrate. Assays of growth were made by measuring total neurite length at successive intervals up to 7 days.

(*a*) *Preparation of ECM extracts*

Ganglion chains were dissected out of the leech, extracted in 10 mM Tris-HCl pH 7.4 containing 2% Triton-x and protease inhibitors. The pellet was re-extracted with 10 mM EDTA, 10 mM Tris-HCl pH 7.4 and 150 mM NaCl for EDTA extract (Masuda-Nakagawa *et al.* 1988) or with 20 mM CAPS (3-[cyclohexyl-amino]-1-propanesulphonic acid) pH 11, 150 mM NaCl, 10 mM EDTA and protease inhibitors for CAPS extract.

(*b*) *Purification of laminin and tenascin*

Laminin was purified by passing EDTA extract over a column made with mAb 203 or 206 bound to Sepharose. Laminin bound to the column was eluted with 4 M urea in 10 mM Tris-HCl pH 7.4, 10 mM EDTA or 0.1 M-triethylamine, pH 11 (Chiquet *et al.* 1988). To purify tenascin, CAPS extract was applied on a 15–40% glycerol gradient in 0.2 M NH_4HCO_3 pH 8.0 in a 14 ml tube and run at 41000 rpm 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 top.

(*c*) *Electron microscopy*

ECM molecules: samples from mAb columns were dialysed in 0.2 M ammonium bicarbonate and fractions from the glycerol gradient were spread without dialysis onto mica sheets. Samples were dried *in vacuo* and rotary shadowed with platinum-carbon at 5–6 followed by carbon at 90. Replicas were examined with an electron microscope (Masuda-Nakagawa *et al.* 1988;

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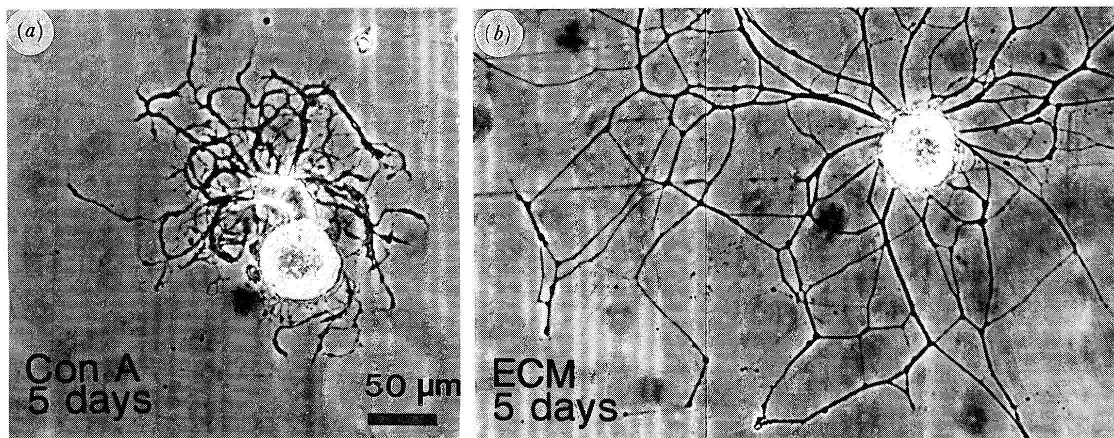

Figure 1. Neurite outgrowth from AP cells in culture after 5 days. Note the different patterns of outgrowth when neurons are plated on Con A (*a*) or ECM (*b*) substrates (after Grumbacher-Reinert 1989).

Chiquet *et al.* 1988). For sections, chains of ganglia were incubated in mAb 206 followed by horseradish peroxidase conjugated second antibody and diaminobenzidine. Tissue was fixed, embedded in Epon, sectioned and stained with lead (Masuda-Nakagawa *et al.* 1990).

(*d*) *Immunohistochemistry*

Frozen sections were fixed in 4% paraformaldehyde, shortly extracted with 0.5% Triton-X in PBS, incubated with mAb 206 or anti-serotonin rat mAb ascites followed by rhodamine or fluorescein conjugated second antibodies. Hoechst 33258 dye was used at 5 μg ml^{-1}. Sections were mounted in 90% glycerol in 20 mM phosphate buffer pH 7.4. Immunofluorescent staining was observed in an Olympus microscope (Masuda-Nakagawa *et al.* 1990).

(*e*) *SDS Gel electrophoresis and immunoblotting*

Extracts and samples were analysed by SDS–PAGE according to Laemmli 1970. Immunoblotting was done according to Towbin *et al.* 1979.

INFLUENCE OF SUBSTRATES ON NEURITE OUTGROWTH IN CULTURE

Leech neurons plated on a substrate of polylysine grow slowly and capriciously. Conditioned media, from ganglia or body wall, or lectins added to the culture have not produced more rapid or more extensive growth. By contrast Chiquet & Acklin (1986) showed that the same neuron plated on a substrate consisting of the plant lectin concanavalin A (Con A) grew within minutes or hours and continued to grow for days (figure 1). Foetal calf serum is not necessary for the initial outgrowth which takes place even in Ringer's fluid. Con A added to the medium did not promote growth of AP or Retzius neurons plated on BSA (BSA does not bind Con A). Growth on Con A was blocked by the addition of a hapten sugar specific for Con A, α-methylmannoside (Chiquet & Acklin 1986).

Of particular interest was that each type of cell grew with a characteristic recognizable fingerprint on Con A. One can immediately distinguish a Retzius cell from a sensory cell, an AP cell or a VL cell (see below) each of which branches quite differently. The VL cell is, like the Retzius cell, serotonergic. In culture it produced a bifurcated T pattern, highly similar to that seen in the ganglion arising from development (Acklin & Nicholls 1990). These results showed that substrate molecules can induce sprouting in culture and that intrinsic mechanisms contribute to the branching pattern. It was natural to wonder whether the CNS also contained molecules that would constitute substrates favourable for growth. (It seemed unlikely that plant lectins would be present in leeches, even in green ones.)

(*a*) *Outgrowth patterns in culture on extracts of extracellular matrix*

Crude extracts of the ECM surrounding the leech nervous system were even more effective than Con A in promoting outgrowth in culture (figures 1 and 2). Retzius or AP cells plated on ECM extract grew longer processes faster. The growth pattern was quite different from that on Con A, thin slender relatively unbranched processes (ECM) compared to thick curved branched processes (Con A). Again individual types of cells behaved differently. The VL cells that made a bifurcated T pattern on Con A also made a T on ECM substrate. But they began to grow far more slowly only after a delay of about 6 days (Chiquet & Acklin 1986; Acklin & Nicholls 1990).

If Con A produced one branching pattern and ECM extract another, how would a single neuron respond to contact with both? Grumbacher-Reinert (1989) made tests in which a single cell was placed exactly on the border separating Con A from ECM substrate, a difficult experiment. The result was clear: branches growing on each substrate took on the characteristic configuration for that substrate (figure 3). It was possible to observe single neurites or a single bundle of

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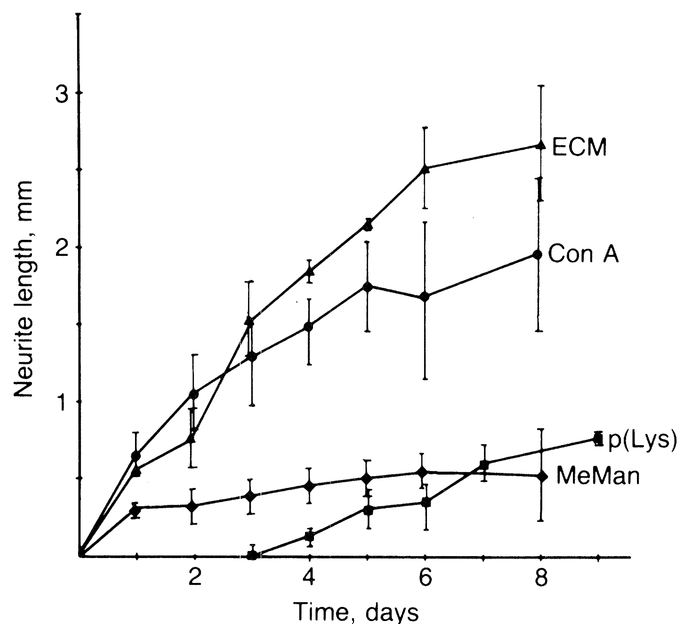

Figure 2. Timecourse of neurite outgrowth of single Retzius neurons cultured on the following substrates: ECM of ganglion capsules (▲); Con A (●); poly (L-lysine) (■); and Con A with 50 mM methyl α-D-mannoside added to the culture medium (◆). Each point represents the average neurite length per cell measured for at least 10 neurons; bars indicate the standard errors of the mean (from Chiquet & Acklin 1986).

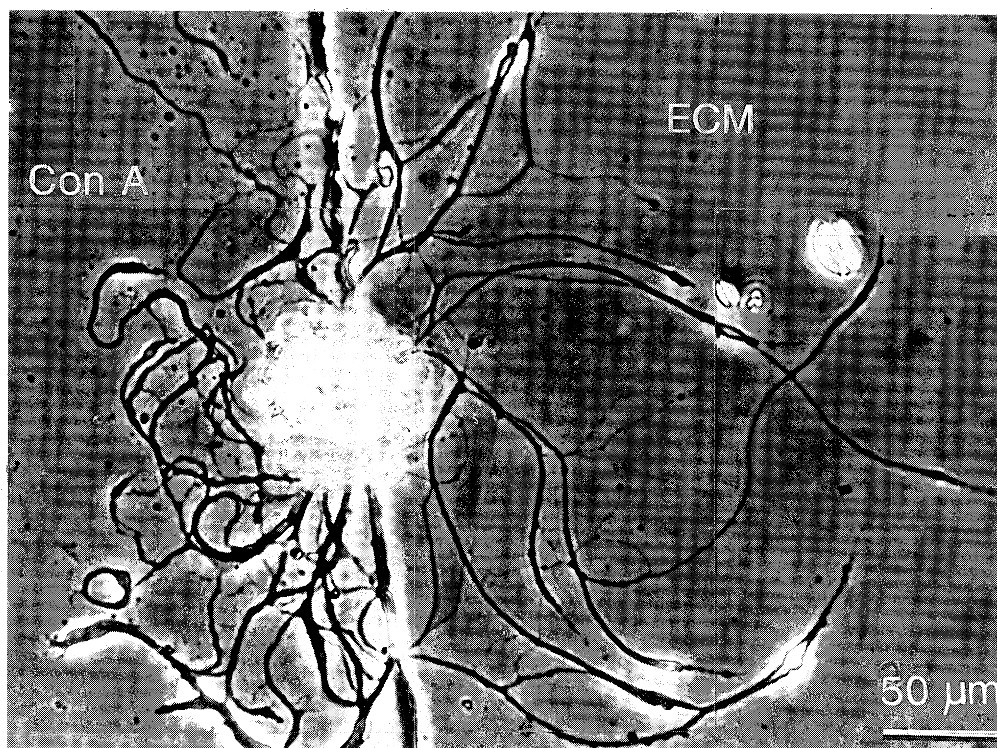

Figure 3. Neurite outgrowth of a single AP cell sprouting for 4 days in culture on a patterned substrate. On the left side of the border the plate was coated with Con A and on the right side with ECM. Note the different branching patterns made by the same cell on the two substrates (from Grumbacher-Reinert 1989).

neurites change from curved and branched to thin, straight and unbranched as soon as it crossed the border.

Together these experiments showed: (i) that extracts made from the ECM contained a growth promoting substance or substances; (ii) that *local* interactions between a neurite and the substrate it touched could determine its form. Another major effect of ECM extracts was on the calcium channels. The processes of neurons grown on Con A showed little calcium entry after stimulation. These measurements were made with optical recording techniques and the sensitive Ca^{2+}

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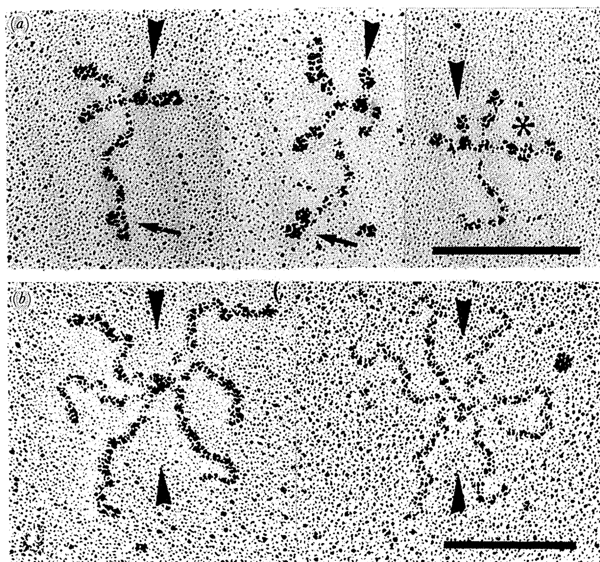

Figure 4. Electron micrographs of rotary shadowed leech molecules found in ECM extracts. (*a*) Laminin-like molecules show an asymmetric cross-shaped structure and globules resembling the appearance of mouse tumour laminin. Arrows mark the double-globular structure at the tip of the long arm. About 20% of the laminin-like molecules from EDTA extracts exhibit an additional extension at one of the short arms (arrowheads) resembling the mouse tumour laminin-nidogen complex. The asterisk marks the double globular structure in the elongated short arms found in approximately 40% of the molecules. (*b*) Tenascin-like molecules have a central globule that connects up to six arms. Arrowheads mark the symmetry axis: two groups of three arms are attached to opposite sides of the centre. Scale bars, 100 nm (after Masuda-Nakagawa *et al.* 1988).

indicator Arsenazo III, (Ross *et al.* 1987, 1988). The same neuron growing on ECM extract was very different: marked calcium entry was observable over the fine processes.

(*b*) *Proteins present in leech ECM*

Three major macromolecules were identified in extracts of ECM surrounding the leech nervous system. In rotary shadowed preparations observed by electron-microscopy these molecules resembled but were not identical to vertebrate laminin, tenascin (figure 4) and collagen IV. As a first step in purification laminin together with tenascin was separated from collagen by an ion exchange column and a sizing column. The laminin-tenascin mixed fraction produced profuse outgrowth as substrate but collagen did not (Masuda-Nakagawa *et al.* 1988). Up till now we have not been able to establish whether small amounts of fibronectin are present in ECM.

(*c*) *Purification of laminin*

Gel electrophoresis of active ECM fractions revealed two polypeptide components of 220 and 340 kDa at reduced conditions and two molecules of approximate molecular mass of 10^3 kDa co-migrating with mouse laminin under non-reducing conditions (Chiquet *et al.* 1988). Leech laminin (figure 4) has a cruciform shape with three short arms and one long arm.

The long arm, 94 nm, is longer than that of mouse laminin. In addition, the terminal globule of the long arm is subdivided into two domains. The short arms are 36 nm. In 40% of the molecules one of the short arms bore two globules in the centre and the length is 53 nm (figure 4, indicated by asterisks). In mild extractions with EDTA leech laminin had an extension resembling nidogen (arrowheads in figure 4) (Masuda-Nakagawa *et al.* 1988).

Two monoclonal antibodies (mAb 206 and mAb 203) selected from a hybridoma library generated against ECM extract reacted with the 340 and 220 kDa polypeptides respectively (figure 5 lane *e*, *f*). The laminin was purified by using an affinity column of mAb 203 and 206 bound to Sepharose. mAb 203 retained a component of one million relative molecular mass at non-reducing conditions that was resolved into polypeptides of 340 and 220 kDa. When the flow through material from the mAb 203 column was applied on a mAb 206 column two components of 10^3 kDa were retained and polypeptides of 340, 220 and 160–180 kDa were retained. Eluates from the mAb 203 and mAb 206 columns revealed only laminin molecules under the electron microscope of rotary shadowed preparations. When leech laminin purified by the mAb columns was coated on culture dishes, neurons grew extensive processes. Neurons grew processes on laminin eluted from the mAb 203 column as well as from mAb 206 column (figure 5). Very low residual activity was left after depleting the extract of leech laminin molecules (Chiquet *et al.* 1988).

(*d*) *Inhibition of growth by polyclonal antisera*

A polyclonal antiserum was generated against the SDS-denatured 220-kDa subunit of laminin. The antiserum blocked the growth of leech neurons on ECM extracts as well as on affinity-purified laminin. Thus the major neurite-promoting activity of ECM extract resides in a laminin-like molecule (Chiquet *et al.* 1988).

Although leech laminin is structurally similar to vertebrate laminin, embryonic rat dorsal root ganglion neurons did not grow on leech laminin and leech neurons did not grow on vertebrate laminin. Moreover antibodies to vertebrate ECM molecules did not cross react with leech components on immunoblots. In spite of similarities in the structure the molecules react differently with the neuronal surface (Chiquet *et al.* 1988).

(*e*) *Leech Tenascin*

Leech tenascin had a typical six-armed structure linked to a central globule (figure 4). Occasionally, five-armed molecules were also found. The average arm length was 114 nm. The axis of symmetry, where the three arms are connected to the central globule is

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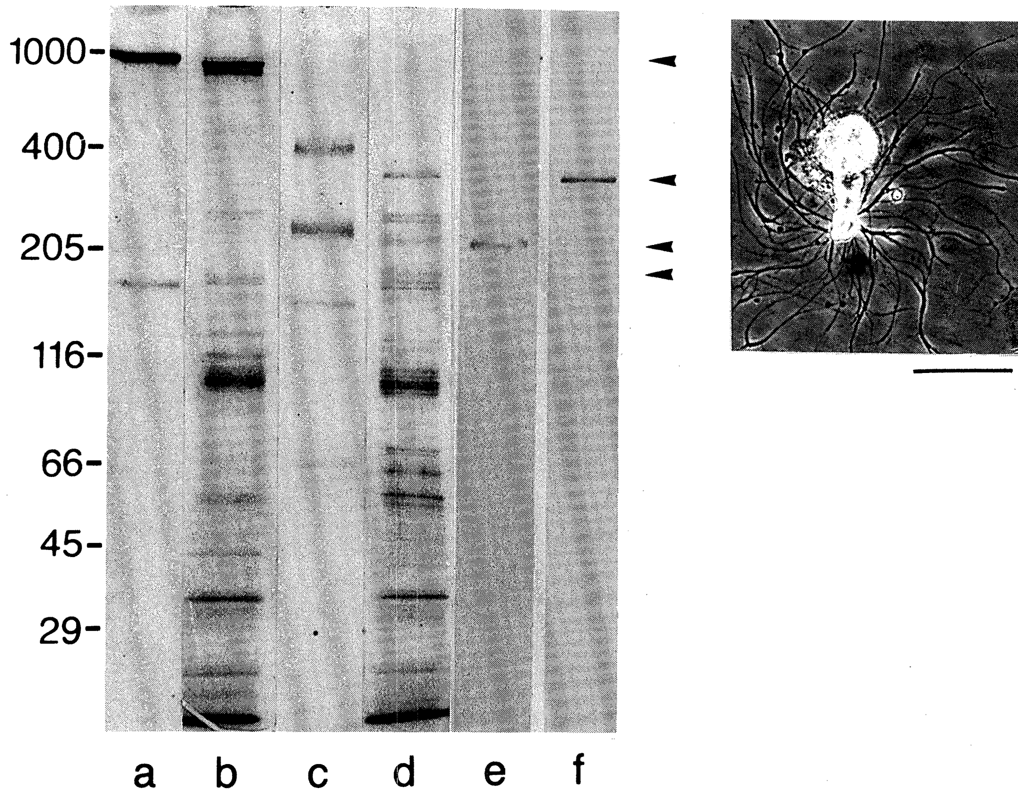

Figure 5. Analysis of neurite-promoting leech ECM extract on an SDS–polyacrylamide (3–15 % gradient) gel with and without reduction; comparison to mouse EHS laminin. Mouse laminin/nidogen complex (3 μg; lanes a and c) was run next to leech ganglion capsule EDTA extract (30 μl; lanes b and d) under nonreducing (lanes a and b) and reducing (lanes c and d) conditions, respectively. The two non-reduced bands of 10^3 kDa in EDTA extract (*b*) co-migrate with intact mouse laminin (a). (e, f) Immunoblotting of neurite-promoting leech ECM fractions with mAbs 203 (lane e) and 206 (lane f). Ganglion capsule EDTA extract was transferred to nitrocellulose to be developed with antibody 203 (lane e), 206 (lane f) as described in Methods. Arrowheads on the right side indicate the positions of the 10^3, 340, 220, 180–160 kDa bands, respectively from the top. Isolated Retzius neurons were plated on culture dishes coated with eluates from antibody 203 column. Estimated protein concentrations of the coating solutions were 10 μg ml^{-1}. Photographs were taken after 2 days. Scale bar, 100 μm (after Chiquet *et al.* 1988).

indicated by arrowheads. Tenascin co-purified with laminin in fractions from the Bio-gel 1.6 m column on which EDTA extracts had been applied (Masuda-Nakagawa 1988). To improve the yield of leech tenascin, extraction made with CAPS buffer pH 11 was run over a glycerol gradient ultra centrifugation according to the method of Erickson & Taylor (1987). Fractions were collected from the top and analysed by electron microscopy after rotary shadowing. Tenascin-like molecules were observed in fractions at around one-third from the bottom of the tube. These fractions were free of laminin but contained some proteoglycan-like material (Masuda-Nakagawa, unpublished data). When the same fractions were used as substrate and coated on culture dishes, AP and Retzius cells sprouted within hours (figure 6). The growth pattern was different from that on laminin and neurites were more curved and shorter (Lepre & Masuda-Nakagawa, unpublished data). We are now developing antisera and preparing a hybridoma library to isolate pure leech tenascin. On SDS–PAGE several bands at around 200 kDa are observed at reducing conditions in tenascin-like molecule-containing fractions; however at non-reducing conditions on 3–7.5 % gradient gel, no bands can be observed.

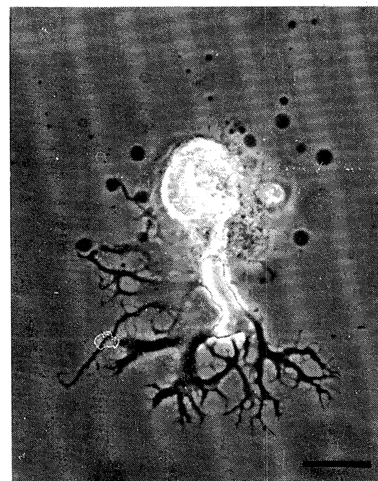

Figure 6. AP neuron plated on a dish coated with a tenascin containing fraction from a glycerol gradient of CAPS extract. Scale bar, 50 μm. (Lepre & Masuda-Nakagawa, unpublished data).

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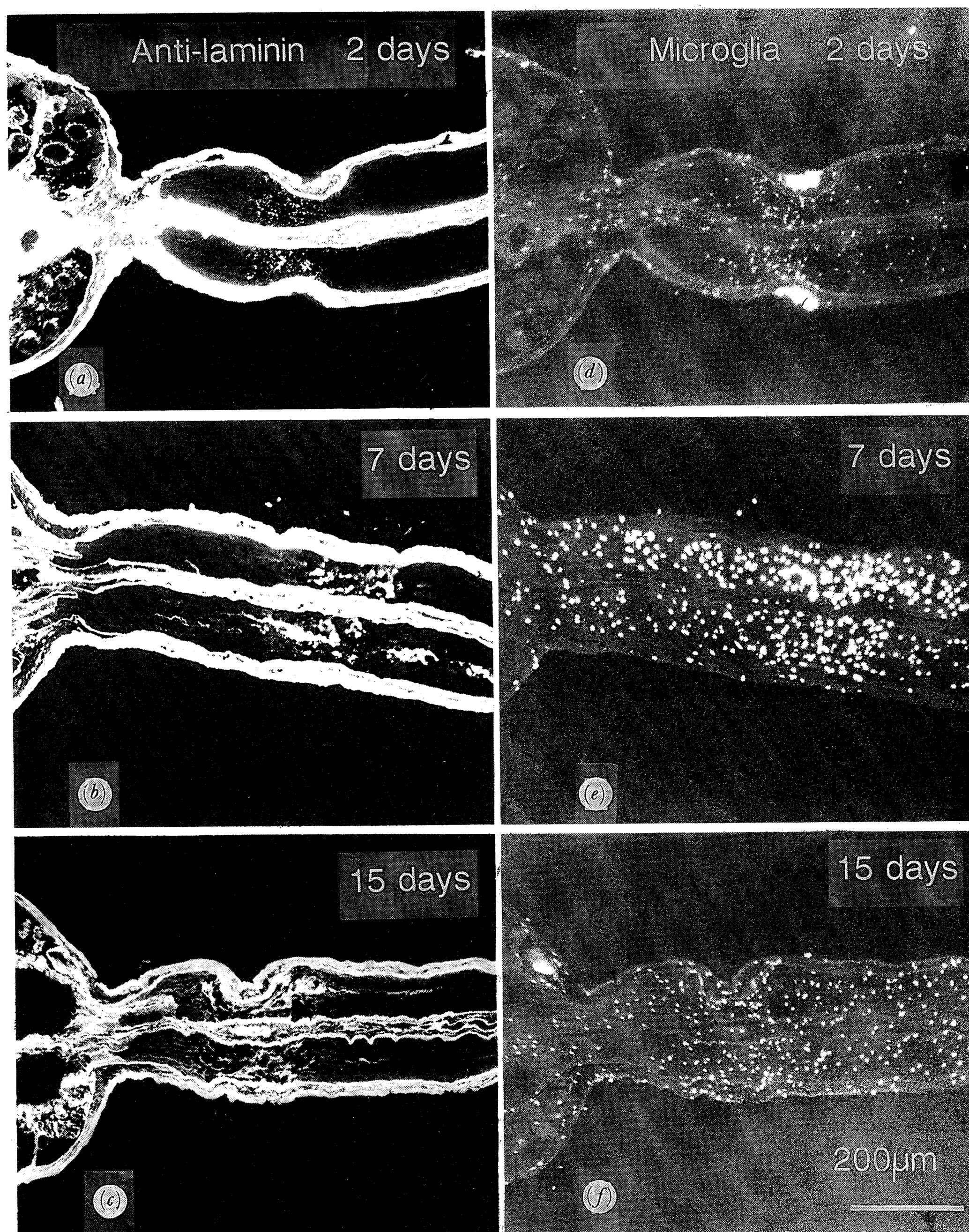

Figure 7. Longitudinal sections of leech CNS stained for laminin (L) and microglia (R). The connectives had been crushed 2 days (*a*, *d*), 7 days (*b*, *e*) and 15 days (*c*, *f*) previously. Part of the ganglion is seen on the left part of each photo. Laminin was labelled by mAb 206 and rhodamine conjugated anti-mouse IgG (*a*, *b*, *c*) and microglia nuclei were labelled with Hoechst stain (*d*, *e*, *f*). Two days after a crush laminin appeared within the connectives at the crush site (*a*) and microglial cells accumulated both outside and within the connectives (*d*). Seven days after a crush laminin aggregated into clumps and streaks at the site of injury (*b*) and microglial cells remained within the connectives at the crush site (*e*). Laminin stain was fibrillar 15 days after a crush (*c*) and microglia were more uniformly distributed along the connectives (*f*) (after Masuda-Nakagawa *et al.* 1990).

(*f*) *Distribution of laminin in normal and regenerating leech CNS*

Since laminin acts as a potent neurite-promoting molecule for leech neurons in culture, we have made experiments to assess its role in the intact animal, during regeneration after injury and during embryogenesis. In cryosections of adult leech CNS mAbs 203 and 206 reacted with basement membrane-like material. In ganglia, staining was localized on the surface of the capsule, on endothelial basement membrane and on the interface between the packet glia and the capsule. Some granular staining was present on the surface of the neurons but there was no staining inside the neuropile. In the connectives the staining correlated with basement membrane of the endothelial layer and the capsule matrix. Axons and axon bundles were not in contact with laminin reactive molecules (Chiquet *et al.* 1988).

Crushes were made in the connectives between pairs

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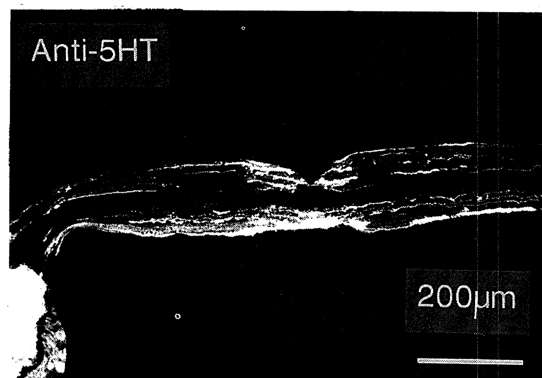

Figure 8. Profusely branched axons containing serotonin that have grown through the crush site at 2 days were stained with anti-5HT antibody and fluorescein-conjugated second antibody. In normal connectives the axons never branch.

of ganglia and the distribution of leech laminin was followed at various times after a lesion by staining cryosections with mAb 206 (Masuda-Nakagawa *et al.* 1990). In parallel, cryosections were also labelled with Hoechst 33258 dye to follow the appearance of microglial cells which are known to accumulate at the crush site (McGlade-McCulloh *et al.* 1989) and mark the lesion. Two days after a crush, punctate staining of laminin appeared within the connectives in the region of the crush. The staining of the basement membrane-like material of the connectives was indistinguishable from controls. As expected, microglial cells accumulated at the crush site. In addition to endogenous microglia a population of cells with small and brightly stained nuclei were seen on the surface of the damaged connectives (figure 7). In the same preparations regenerating serotonergic axons were stained with anti-5-HT antibody. The profuse outgrowth and branching occurring at the site of the lesion was similar to that observed in earlier experiments on regeneration (Nicholls 1987; Elliott & Muller 1983). Axons that were normally unbranched in the connective would start to grow numerous branched processes that regenerated across the lesion to reach the next ganglion (figure 8). One week after a lesion the laminin staining became fibrillar and more intense. Endogenous 'microglial cells' had disappeared and there was an apparent overall increase of microglia within the connectives. Two weeks after the lesion, the fibrillar staining appeared more pronounced and intense (figure 7). Even after two months laminin immunoreactivity did not disappear but the pattern of staining was different with a laminar distribution along the lesion. At no stage did obvious new laminin stain appear within ganglia. To investigate the source of the laminin appearing at new sites in connectives cryosections were made from nerve cords frozen immediately after they had been crushed. Laminin showed the normal distribution along basement membrane and connective tissue capsule. The inside of crushed connectives was free of laminin (Masuda-Nakagawa *et al.* 1990). Another set of experiments using protein synthesis inhibitors is now being conducted to clarify whether new laminin is produced by *de novo* synthesis.

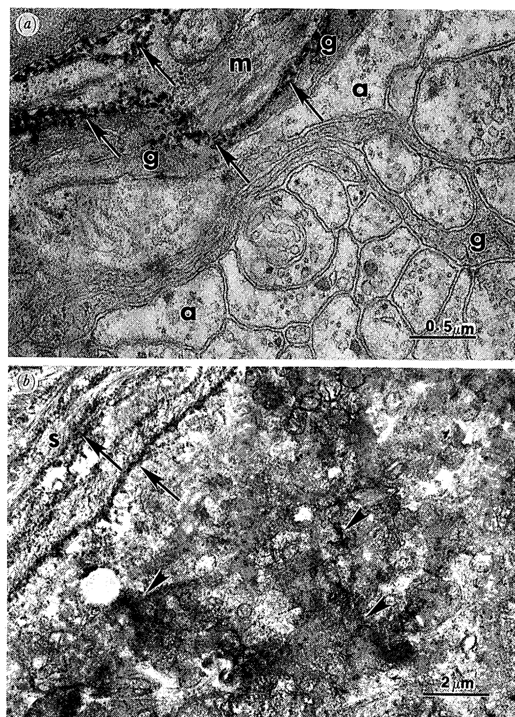

Figure 9. Electron micrographs showing laminin distribution in normal and regenerating connectives. (*a*) normal connective: leech laminin was situated outside the glial sheath over the surface of the CNS as shown in this cross-section. The living CNS had been stained with the aid of HRP-conjugated goat anti-mouse IgG secondary antibody after overnight incubation in mAb 206. The reaction product accumulated in the region occupied by basement membrane including basal lamina. Thus reaction product was localized between the connective sheath and the connective glial cell (g), and it surrounded the connective muscle (m). Axons (a) are not normally in contact with leech laminin. Control preparations (not shown) were incubated with another mAb (Lan3-2) which penetrated the connectives and (with the same secondary antibody) stained the surfaces of certain axons but not the perineurial sheath. This confirmed that the lack of staining by mAb 206 was not because antibody did not have access to axons. (*b*) low power electron micrograph of a connective crushed 4-days previously. The structure of the nerve cord was disrupted at the crush and microglial cells accumulated in the region. Immunoreactivity to mAb 206 persisted at the sheath (s) as indicated by arrows and was present within the region of the connectives previously occupied by axons and glia (arrowheads). (After Masuda-Nakagawa *et al.* 1990).

These results suggested: (i) that axons in the leech CNS are not normally in contact with laminin; (ii) that after injury laminin appears at the very sites through which axons will regenerate. Light microscopy would however fail to reveal a diffuse, non-punctate, low concentration of laminin within the connectives. Accordingly, Muller has made electronmicrographs of normal and crushed connectives stained with mAb 206 (Masuda-Nakagawa *et al.* 1990). The results obtained by immunocytochemistry at this level confirmed those described above. In normal preparations laminin staining was confined to ECM basal lamina with none observable around axons in the connectives. That this was not due to failure of penetration was shown by

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control antibody staining of axons. Four days after the connectives had been crushed laminin was stained at the site of injury but not elsewhere (figure 9). Moreover, growing axons came into direct contact with laminin.

(g) *Laminin in development of leech central nervous system*

The germinal plate of the leech embryo arises from divisions of five pairs of teloblasts, M, N, O, P, Q which give rise to bandlets of stem cells m, n, o, p, q (Bissen & Weisblat 1989). The larva consists of a membranous envelope that includes an outer flat epithelium and an inner discontinuous muscle layer that is associated with a nerve plexus. The germinal plate extends from the larval mouth, through which cocoon fluid is taken, to the teloblasts at the caudal tip. The right and left germinal bands coalesce on the future ventral midline. In the anterior sector ganglionic primordia and incipient somites are formed. There is a rostrocaudal gradient of development of the germinal plate so that the head part is more mature than the tail.

We have investigated the distribution of laminin in embryos at 8 days of development at 23 °C. At this stage 14–20 ganglionic primordia are formed with a characteristic ladder of development. In whole mount preparations as well as in cryosections laminin immunoreactivity was localized along the central nervous system of the embryo (figure 10). In fluorescent micrographs the developing ganglionic primordia appear black. Staining was especially intense in regions joining the paired ganglionic primordia from one segment to the other. This is the site at which the future connectives will develop. Some staining was also present in the neuropile. In the posterior part of the embryo the laminin appears as a strand delineating the midline (not shown here). Laminin also appeared outside the CNS associated with other organs, particularly around muscles and in paired structures which could be somites. The fine structural localization of laminin in early embryos is now under investigation (Masuda-Nakagawa & Muller, unpublished data).

DISCUSSION

Our findings suggest that laminin appears just where it is needed in the CNS if it is to promote growth. In normal adult leech CNS the axons have no contact with laminin, which is confined to basal lamina. At earlier stages while the embryo is forming its nervous system the pattern is different, with intense laminin staining between ganglionic primordia. Similarly after a crush to the adult CNS laminin becomes displaced, not immediately by mechanical translocation, but after a short delay to the pathway along which regenerating axons will sprout. A satisfactory aspect of these results is the good correlation between structure and function.

Less satisfactory is the information available about the intimate relation of laminin to accessory cells and growing axons. We do not yet know whether the laminin in embryonic structures destined to be connectives is located in basement membrane, in glial cells or in other types of cells. In regeneration (as in embryos) a major problem concerns the origin of the laminin. Among potential candidates are the axons themselves, the glial cells and the microglia. In regenerating adult CNS and in embryos the neurons seem an unlikely source: the laminin is there before the axons. Glial cells are also unlikely as Muller and his colleagues have shown that if the large glial cell in a connective has been killed undamaged axons start to sprout; moreover, laminin again appears in the region where neurons will grow (Elliott & Muller 1983; K. J. Muller & L. Masuda-Nakagawa, unpublished data). This leaves as prime candidates microglia which rapidly migrate to the site of growth after a lesion and after glial killing. Ongoing experiments, in which we block protein synthesis for prolonged periods, have not yet clarified whether new synthesis of laminin or displacement of pre-formed laminin are involved. And at the EM level we have no markers to label microglia and to see how closely they are associated with laminin in the animal or in culture.

What other extracellular matrix molecules promote axonal growth in the CNS of the leech? Tenascin is far less abundant than laminin and the small amounts we have been able to extract have limited the types of experiment that can be performed. Although it is clearly identifiable in electronmicrographs the amount of tenascin extracted is too low to be detected in gels. The tenascin enriched fractions that promote neurite outgrowth also contain proteoglycans. We cannot be certain of the role of tenascin itself, but what is clear is that the tenascin enriched substrate is virtually free of laminin; and it produces a growth pattern quite different from that on laminin. For fibronectin the situation is worse: only preliminary hints of a protein with similar characteristics in ECM have been obtained by T. Lüthi (unpublished data). Of considerable interest will be to see if there is an endogenous lectin comparable to Con A in its properties.

An attractive speculation emerges from these results obtained in embryos, adult leeches and cultured neurons. The large variety of cell types with their distinctive properties and morphologies are differentially affected by Con A, leech laminin and tenascin enriched substrates. The Retzius cell sprouts differently on each of these substrates; so does the AP cell, in patterns different from those of the Retzius cell. Not only their forms but the distributions of ion channels in their membranes are affected by the substrate. Yet another type of neuron, the VL grows only after a long delay on ECM laminin substrate. A large molecule such as laminin anchored in extracellular matrix could therefore give quite different instructions to different growing axons that reach a particular region of the CNS. In one type of cell branching and synapse formation could be promoted; in another, unbranched outgrowth to guide the axon to a more distant destination; in another, growth might be stopped, allowing for a change in direction. In this way a few molecules in combination could direct not merely growth but the formation of a patterned nervous system. With the opportunity offered by combined

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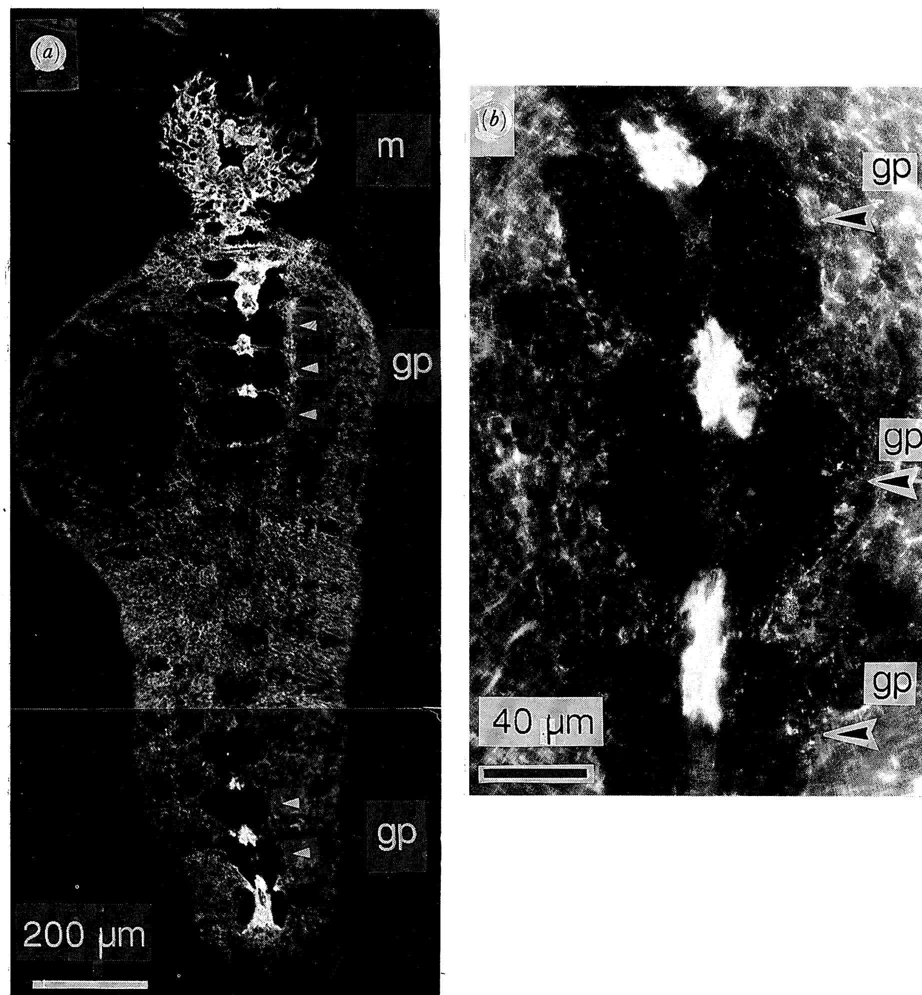

Figure 10. Longitudinal section of 8-day-old leech embryos stained with mAb 206 and rhodamine conjugated anti-mouse IgG. Low power (*a*) and high power (*b*). The ganglionic primordia (gp) are the sites on which future segmental ganglia will develop. With this stain they appear as black, unfused structures on either side of the midline. Note the intense staining at the sites of the future connectives along which axons will grow to connect ganglia. Top is anterior. Scale bar, 100 µm.

studies *in vitro* and *in vivo* on varieties of identified cells one can hope to test such ideas in greater detail.

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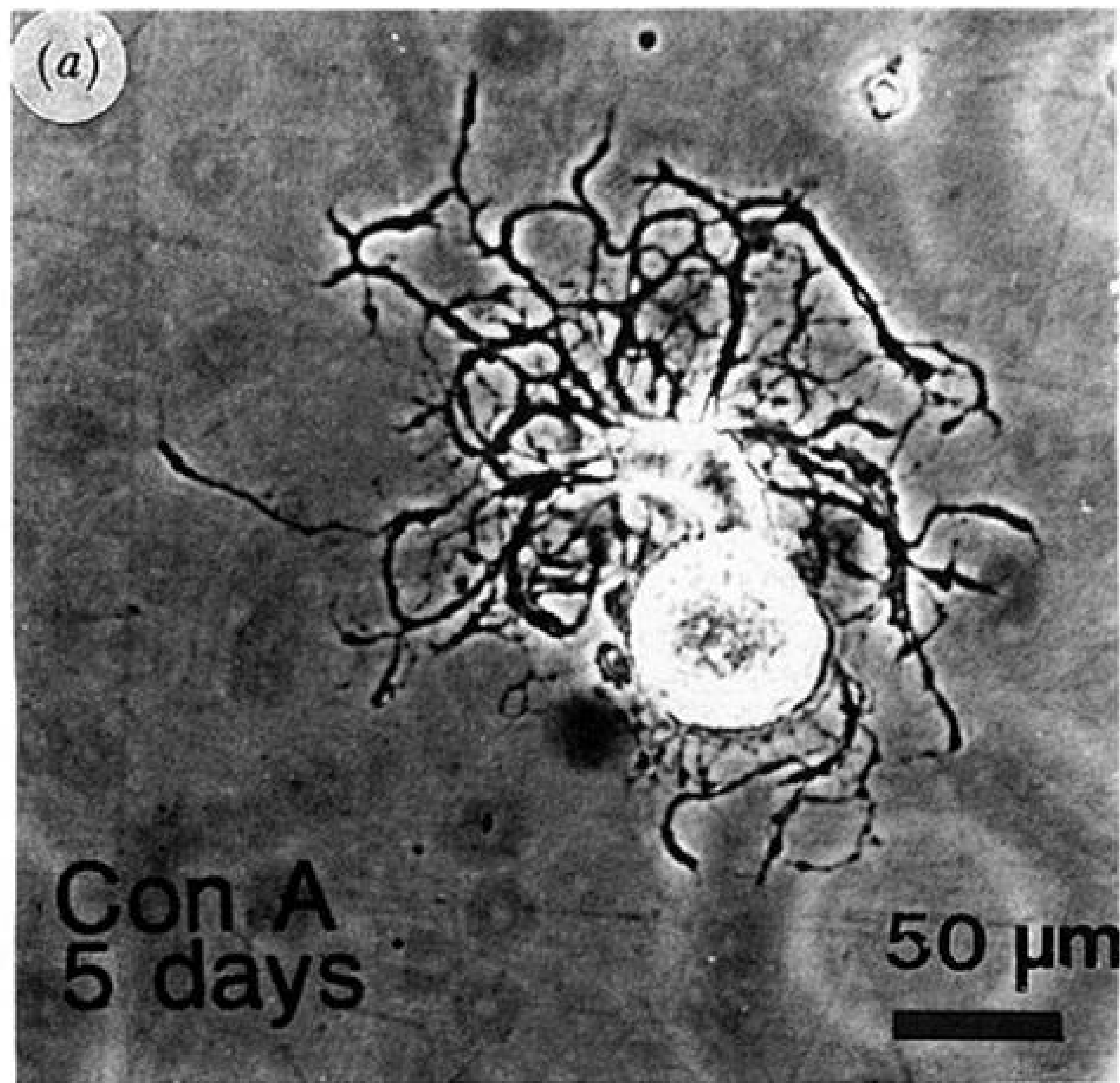

gure 1. Neurite outgrowth from AP cells in culture after 5 days. Note the different patterns of outgrowth when neurons are plated on Con A (*a*) or ECM (*b*) substrates (after Grumbacher-Reinert 1989).

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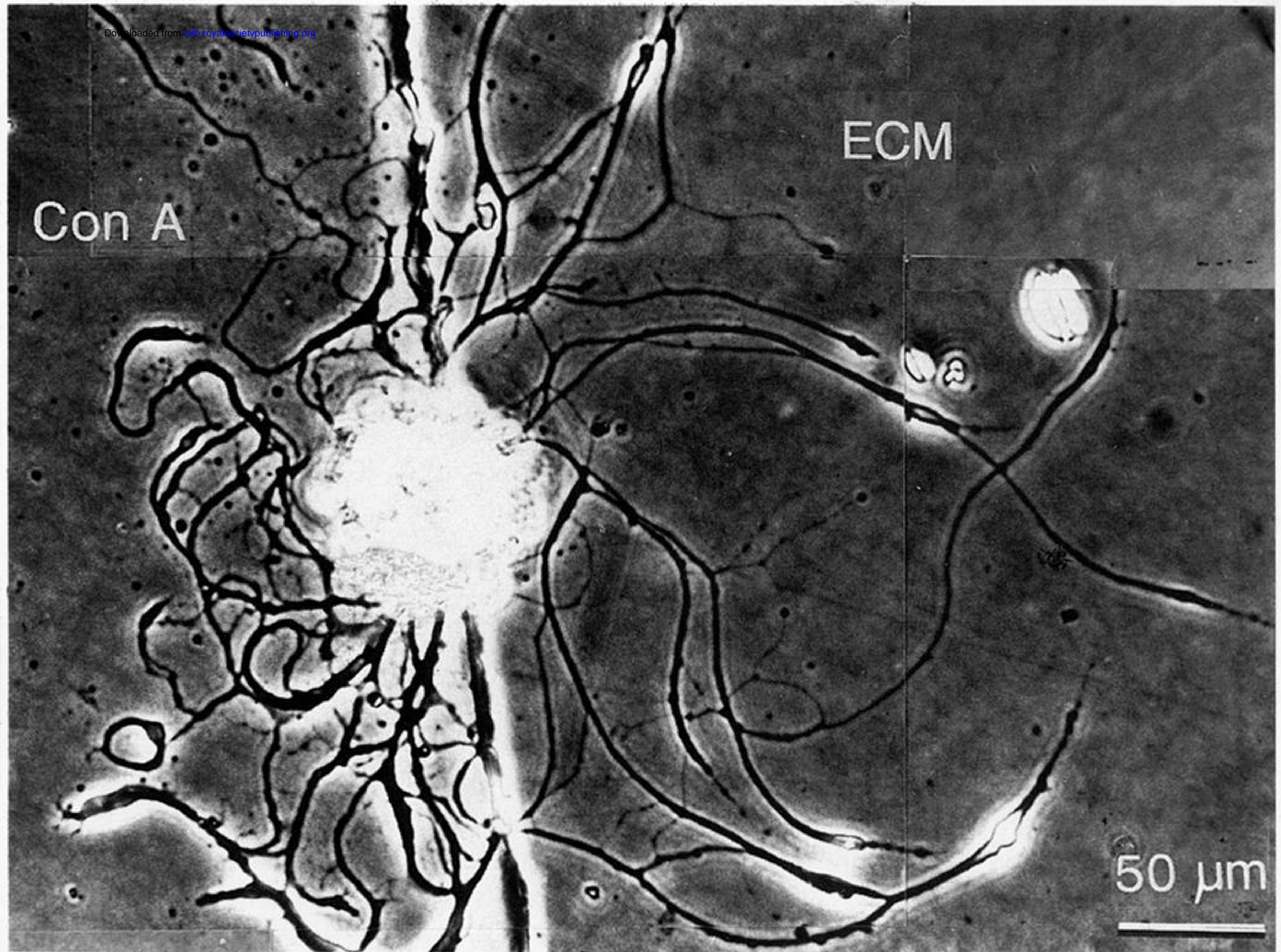
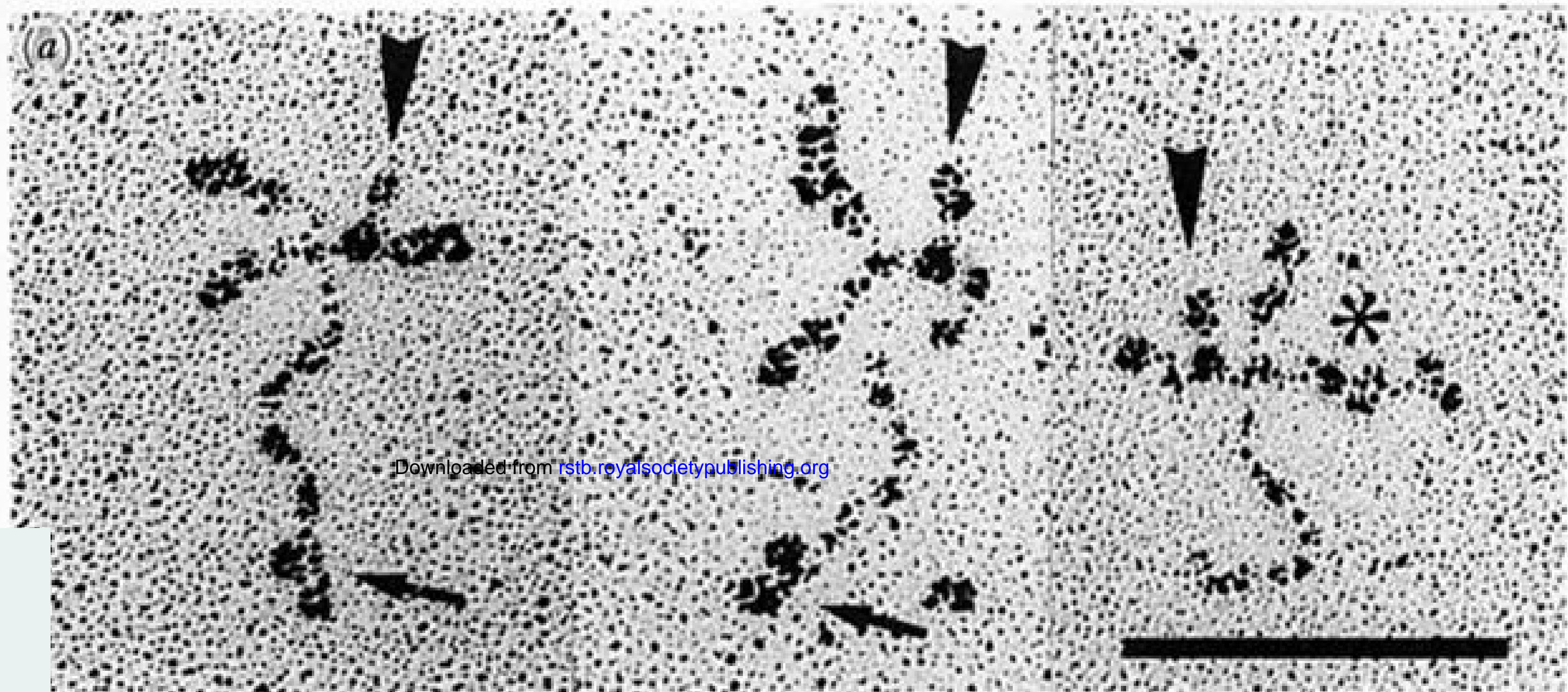


gure 3. Neurite outgrowth of a single AP cell sprouting for 4 days in culture on a patterned substrate. On the left le of the border the plate was coated with Con A and on the right side with ECM. Note the different branching tterns made by the same cell on the two substrates (from Grumbacher-Reinert 1989).



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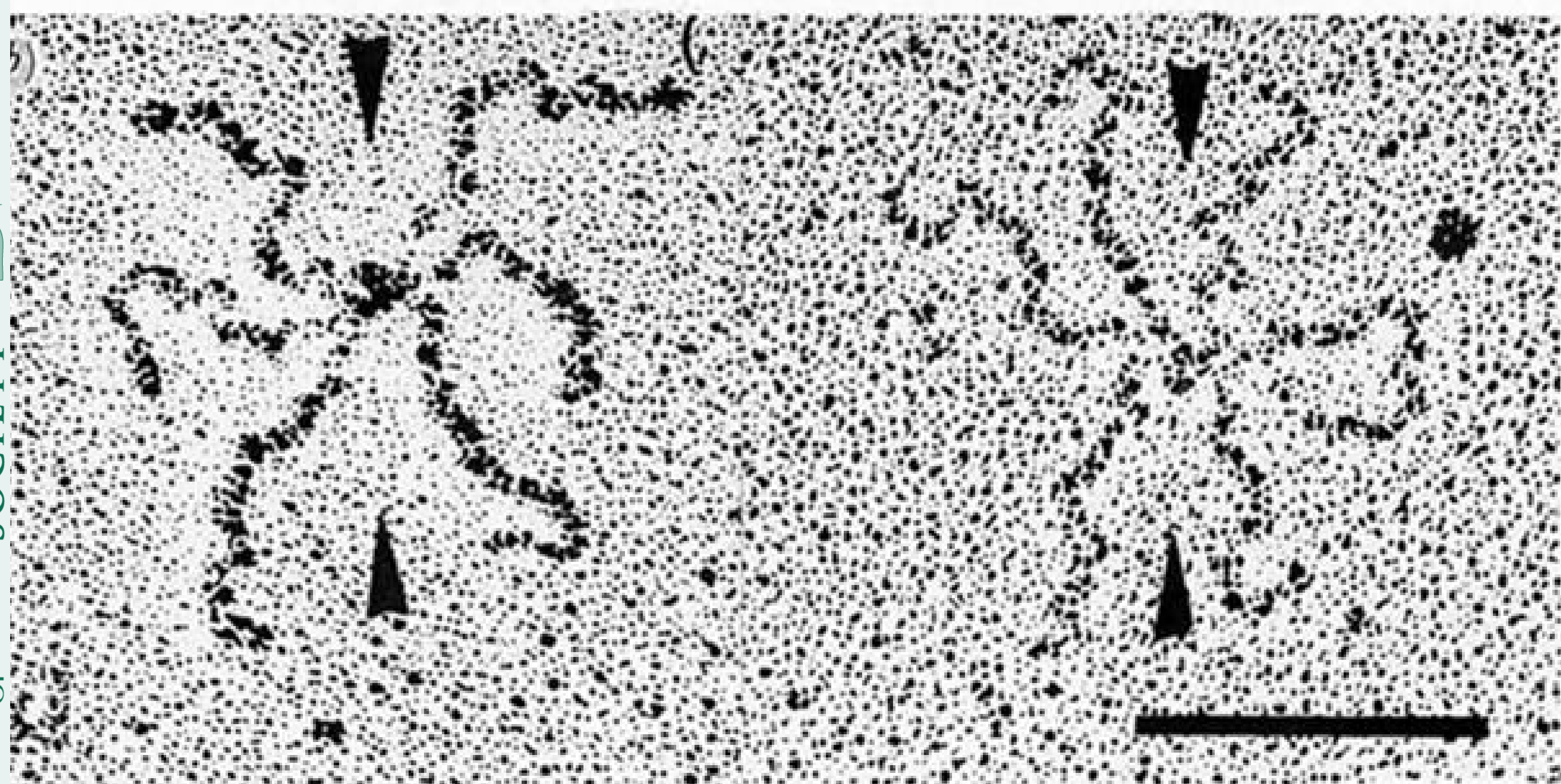

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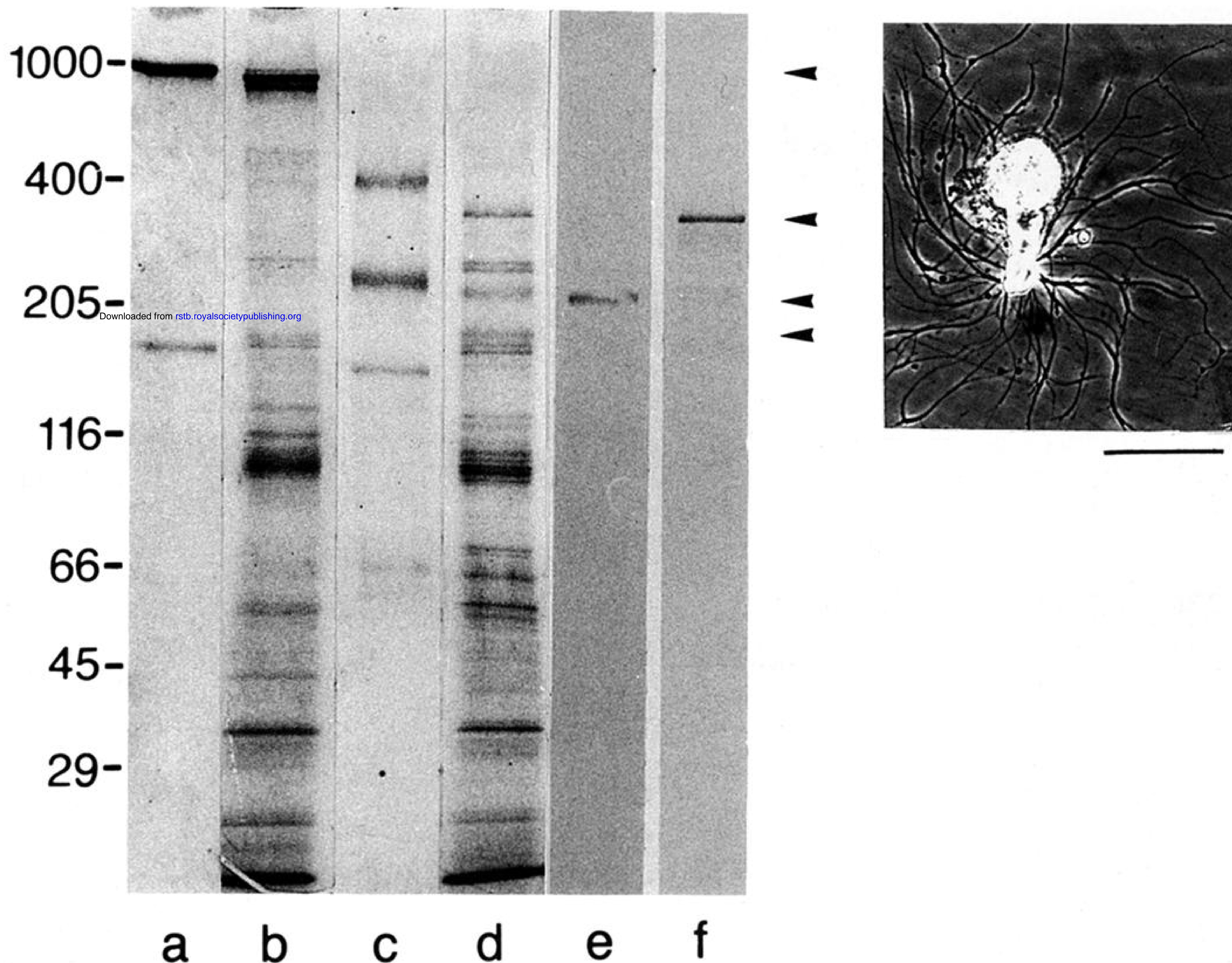
gure 4. Electron micrographs of rotary shadowed leech olecules found in ECM extracts. (*a*) Laminin-like moleles show an asymmetric cross-shaped structure and obules resembling the appearance of mouse tumour minin. Arrows mark the double-globular structure at the of the long arm. About 20% of the laminin-like molecules om EDTA extracts exhibit an additional extension at one of e short arms (arrowheads) resembling the mouse tumour minin-nidogen complex. The asterisk marks the double obular structure in the elongated short arms found in proximately 40% of the molecules. (*b*) Tenascin-like olecules have a central globule that connects up to six arms. rowheads mark the symmetry axis: two groups of three ms are attached to opposite sides of the centre. Scale bars, 0 nm (after Masuda-Nakagawa *et al.* 1988).



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gure 5. Analysis of neurite-promoting leech ECM extract on an SDS–polyacrylamide (3–15% gradient) gel with ıd without reduction; comparison to mouse EHS laminin. Mouse laminin/nidogen complex (3 μg; lanes a and c) as run next to leech ganglion capsule EDTA extract (30 μl; lanes b and d) under nonreducing (lanes a and b) and ducing (lanes c and d) conditions, respectively. The two non-reduced bands of 10^3 kDa in EDTA extract (*b*) -migrate with intact mouse laminin (a). (e, f) Immunoblotting of neurite-promoting leech ECM fractions with Abs 203 (lane e) and 206 (lane f). Ganglion capsule EDTA extract was transferred to nitrocellulose to be developed ith antibody 203 (lane e), 206 (lane f) as described in Methods. Arrowheads on the right side indicate the positions the 10^3, 340, 220, 180–160 kDa bands, respectively from the top. Isolated Retzius neurons were plated on culture shes coated with eluates from antibody 203 column. Estimated protein concentrations of the coating solutions were) μg ml^{-1}. Photographs were taken after 2 days. Scale bar, 100 μm (after Chiquet *et al.* 1988).

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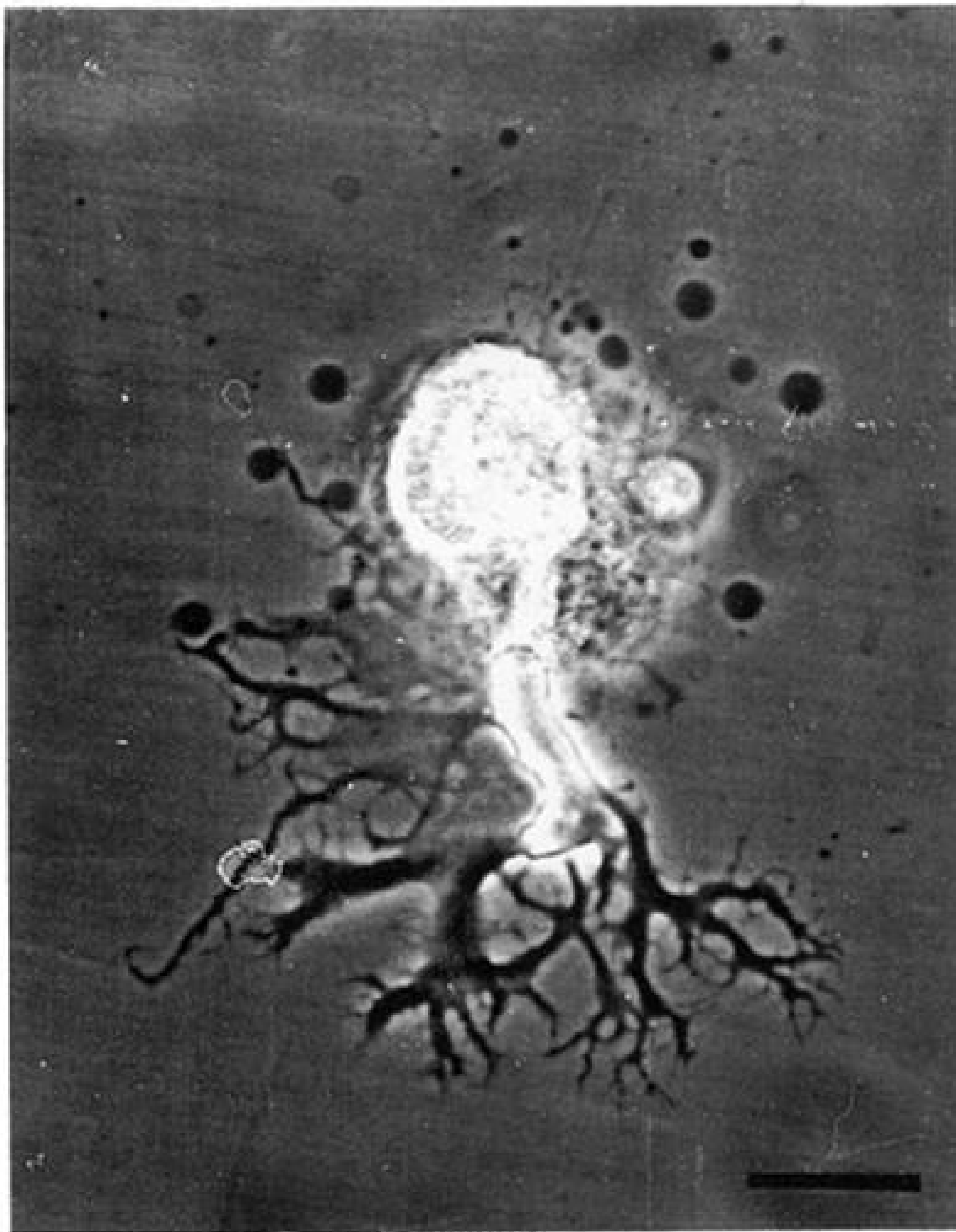
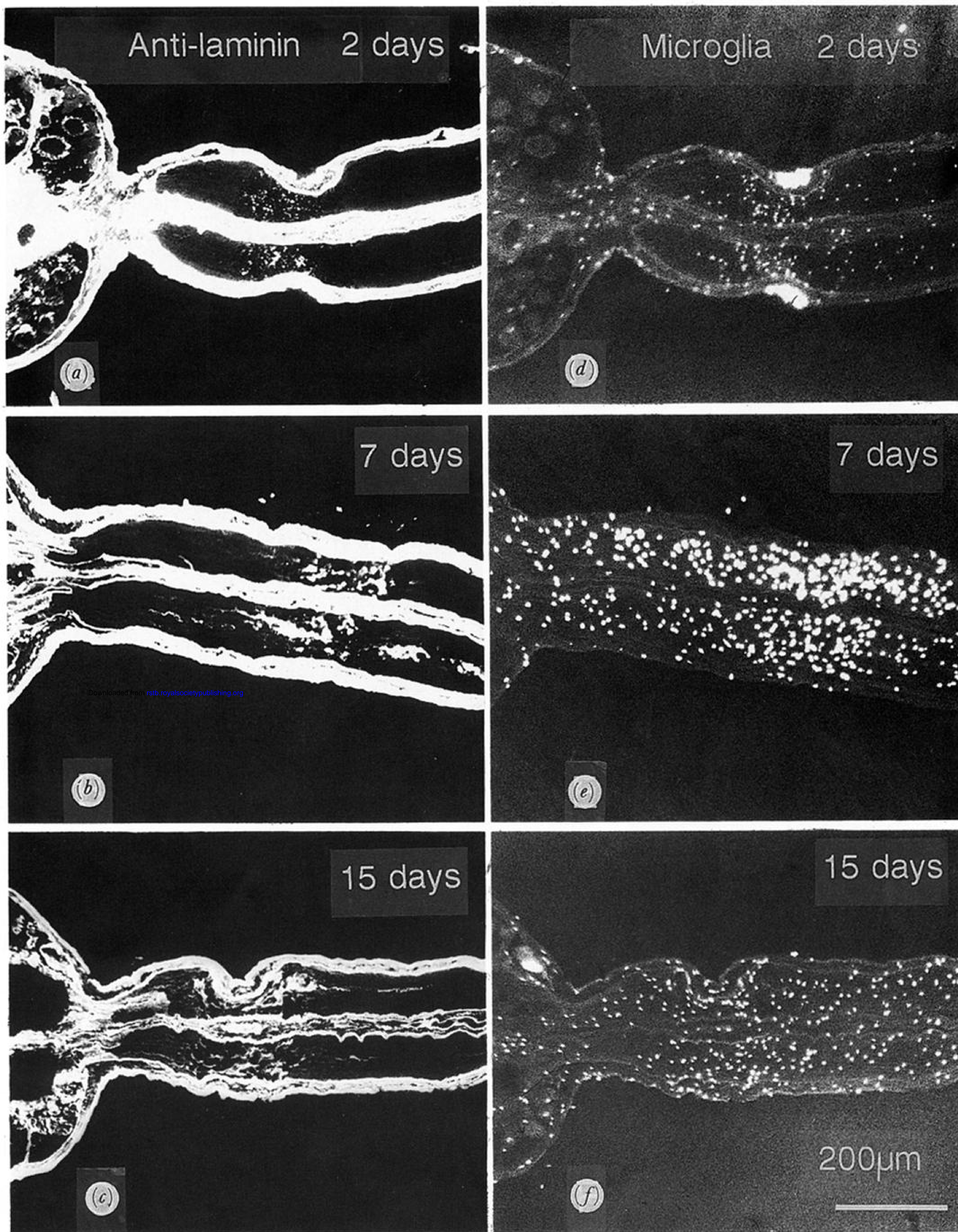


igure 6. AP neuron plated on a dish coated with a tenascin ontaining fraction from a glycerol gradient of CAPS extract. cale bar, 50 μm. (Lepre & Masuda-Nakagawa, unpublished ata).



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igure 7. Longitudinal sections of leech CNS stained for laminin (L) and microglia (R). The connectives had been rushed 2 days (*a*, *d*), 7 days (*b*, *e*) and 15 days (*c*, *f*) previously. Part of the ganglion is seen on the left part of ach photo. Laminin was labelled by mAb 206 and rhodamine conjugated anti-mouse IgG (*a*, *b*, *c*) and microglia uclei were labelled with Hoechst stain (*d*, *e*, *f*). Two days after a crush laminin appeared within the connectives the crush site (*a*) and microglial cells accumulated both outside and within the connectives (*d*). Seven days after crush laminin aggregated into clumps and streaks at the site of injury (*b*) and microglial cells remained within the onnectives at the crush site (*e*). Laminin stain was fibrillar 15 days after a crush (*c*) and microglia were more niformly distributed along the connectives (*f*) (after Masuda-Nakagawa *et al.* 1990).

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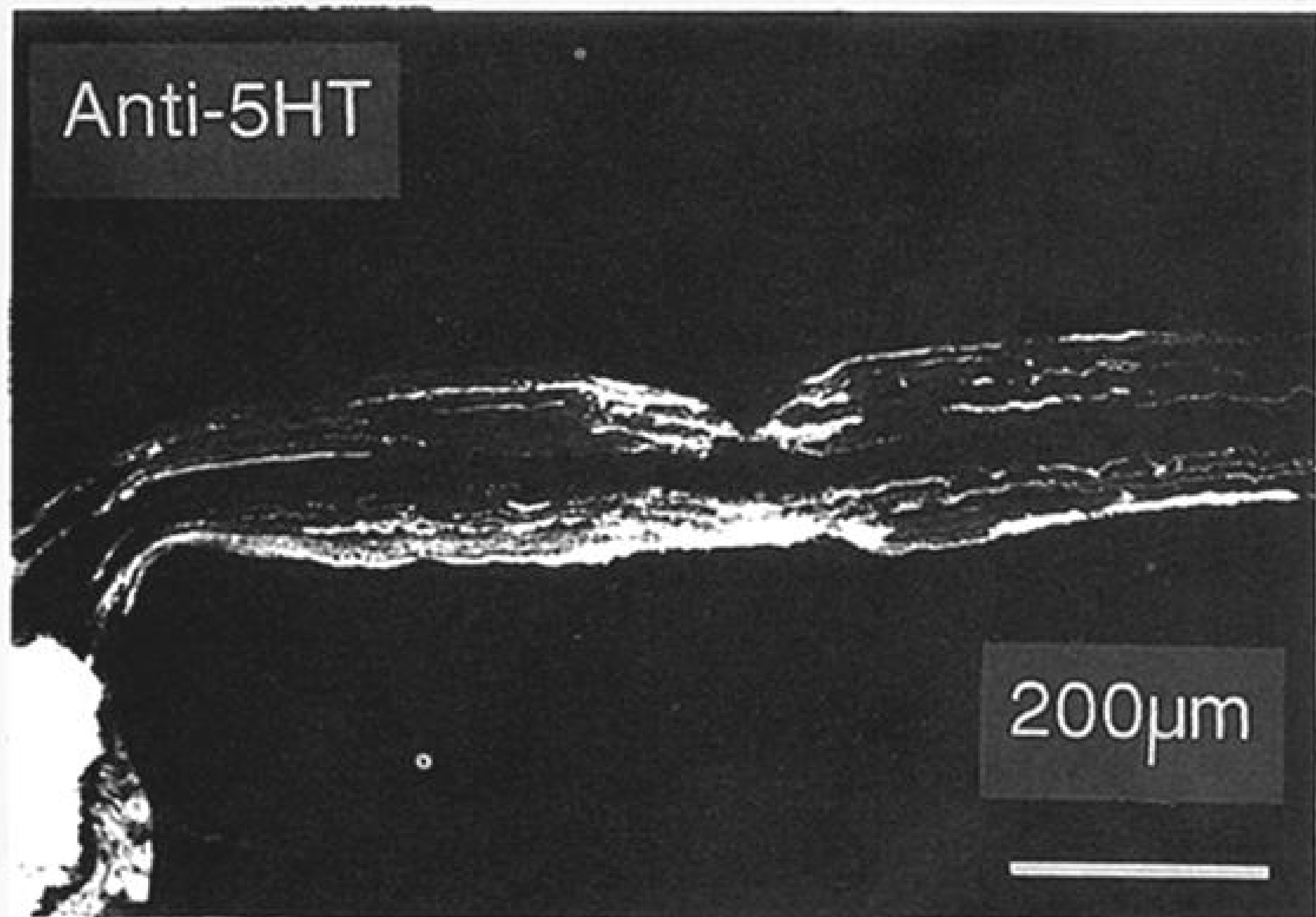

gure 8. Profusely branched axons containing serotonin that ve grown through the crush site at 2 days were stained with ti-5HT antibody and fluorescein-conjugated second anti-dy. In normal connectives the axons never branch.

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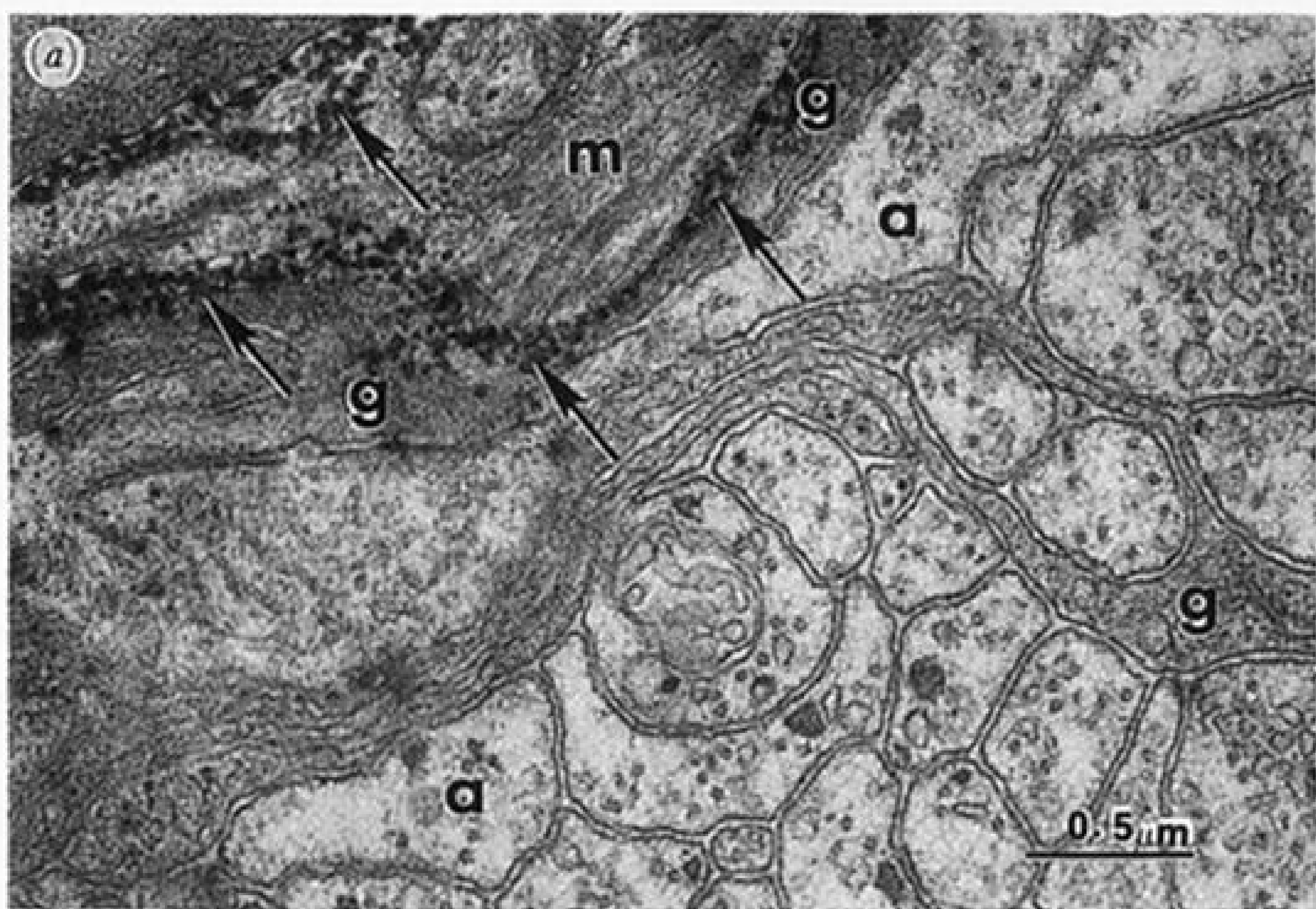

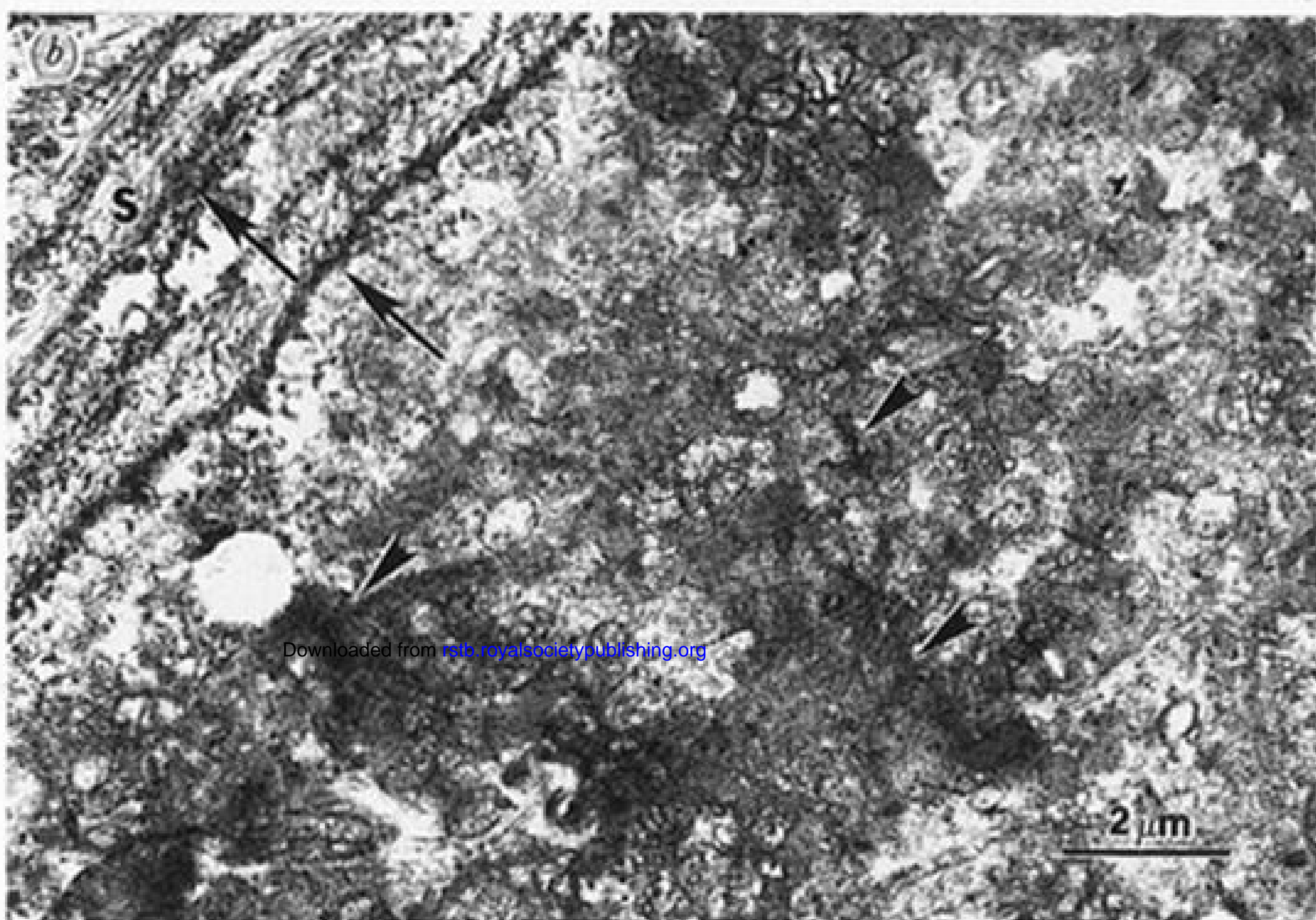

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gure 9. Electron micrographs showing laminin distribution normal and regenerating connectives. (*a*) normal con-:ctive: leech laminin was situated outside the glial sheath ver the surface of the CNS as shown in this cross-section. he living CNS had been stained with the aid of HRP-njugated goat anti-mouse IgG secondary antibody after vernight incubation in mAb 206. The reaction product :cumulated in the region occupied by basement membrane cluding basal lamina. Thus reaction product was localized :tween the connective sheath and the connective glial ll (g), and it surrounded the connective muscle (m). Axons) are not normally in contact with leech laminin. Control eparations (not shown) were incubated with another mAb ,an3-2) which penetrated the connectives and (with the me secondary antibody) stained the surfaces of certain :ons but not the perineurial sheath. This confirmed that the ck of staining by mAb 206 was not because antibody did ɔt have access to axons. (*b*) low power electron micro-aph of a connective crushed 4-days previously. The ructure of the nerve cord was disrupted at the crush and icroglial cells accumulated in the region. Immunoreactivity mAb 206 persisted at the sheath (s) as indicated by arrows id was present within the region of the connectives eviously occupied by axons and glia (arrowheads). (After 'asuda-Nakagawa *et al*. 1990).

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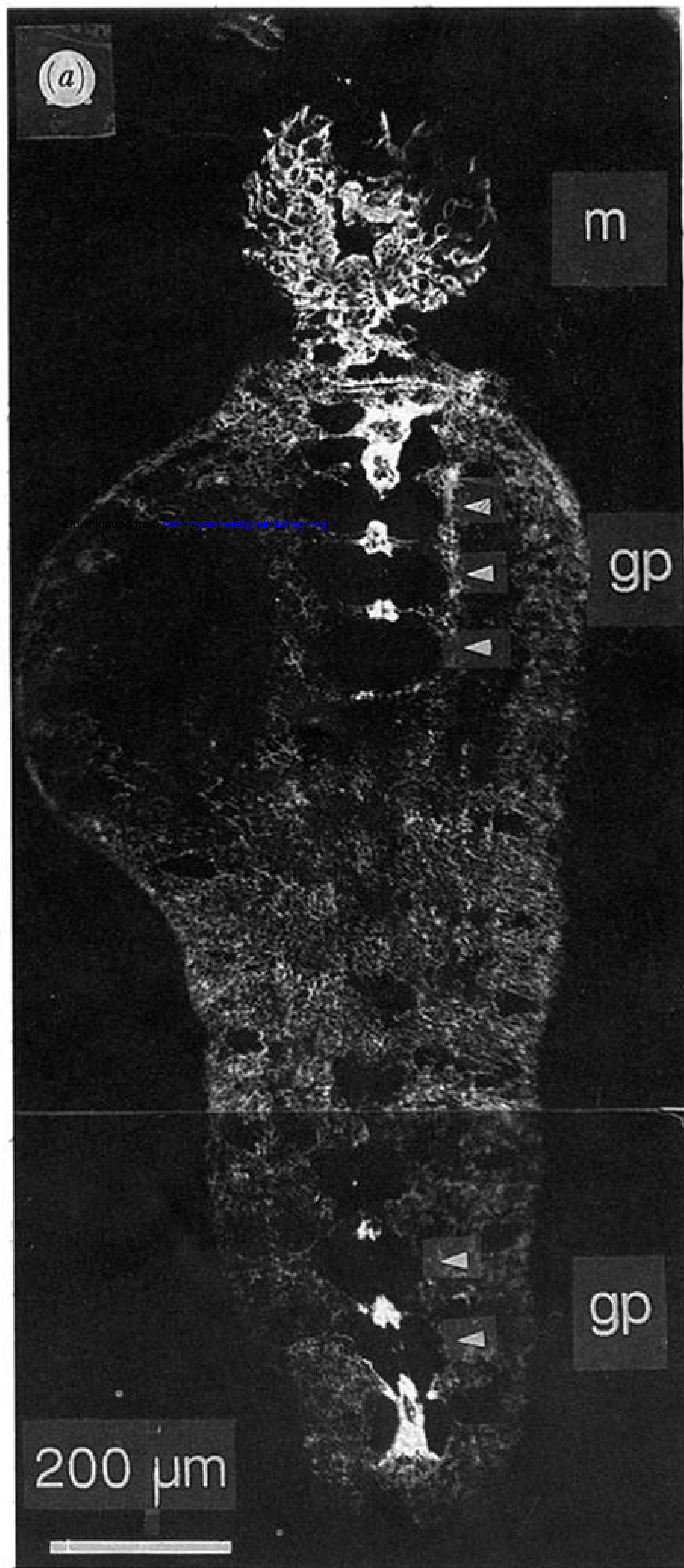

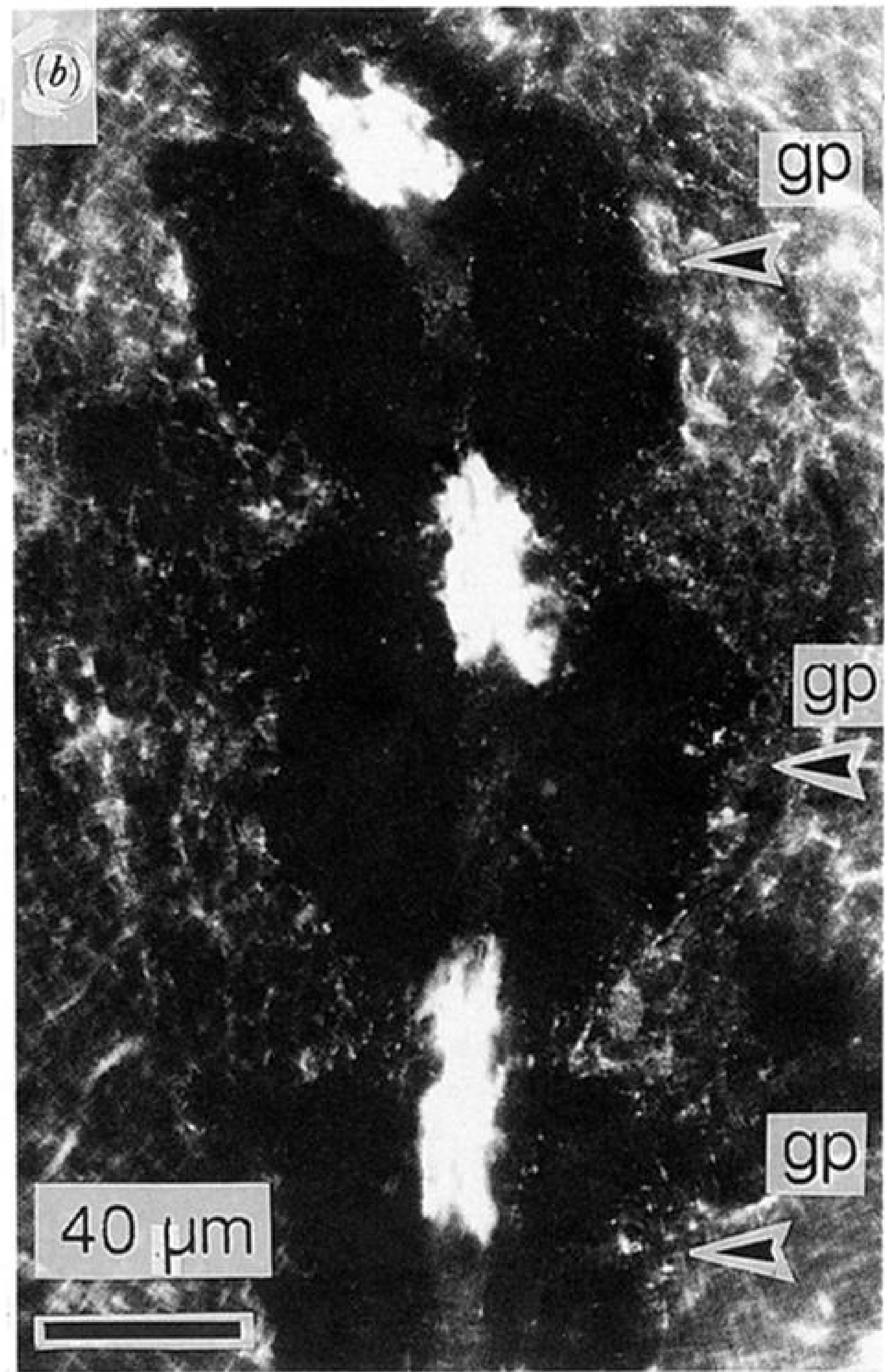

gure 10. Longitudinal section of 8-day-old leech embryos stained with mAb 206 and rhodamine conjugated anti-ouse IgG. Low power (*a*) and high power (*b*). The ganglionic primordia (gp) are the sites on which future segmental nglia will develop. With this stain they appear as black, unfused structures on either side of the midline. Note the tense staining at the sites of the future connectives along which axons will grow to connect ganglia. Top is anterior. ale bar, 100 µm.

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