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Transient expression of laminin immunoreactivity in the developing rat hippocampus

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Summary

We have isolated and characterized a polyclonal antibody that recognizes the extracellular matrix glycoprotein laminin. In the developing rat hippocampus, in addition to staining the basal laminae of blood vessels and the glia limitans, this antibody detects a punctate deposit of non-basal lamina laminin that appears transiently, disappearing on E18. The punctate laminin is distributed throughout the hippocampus from the border of the ependyma and the neuropil to the pial surface, but is particularly concentrated in the presumptive molecular layer (marginal zone). Electron microscopy showed that the punctate laminin was in the extracellular space on the surfaces of cell bodies and, more commonly, their processes. In double-labelling experiments in which neurites were labelled with tau or MAP2 antibodies, there were occasional cases where neurites came into direct contact with punctate laminin. These observations suggest a role for laminin in hippocampal development and possibly in neurite outgrowth.

Introduction

Laminin is the major non-collagenous glycoprotein of the basal lamina (for review, see Von der Mark & Kühl, 1985). It is a large, cruciform-shaped, multifunctional molecule with a molecular weight of over a million. It has binding sites for cell surface receptors and for other extracellular matrix (ECM) proteins. In basal laminae it probably functions as a cross-linking protein, anchoring cells that contact a basal lamina, such as epithelial cells, muscle cells and Schwann cells, to components of the ECM, such as collagen and heparan sulphate proteoglycan (Timpl *et al.*, 1983).

In addition to its function in the basal lamina, laminin has been proposed to play a role in supporting neurite outgrowth in the developing CNS (reviewed by Lander, 1987). The evidence to support this idea comes from a variety of circumstantial observations. For instance, in cell culture, laminin has been shown to be the most effective of all substrates so far tested in supporting neurite outgrowth and elongation of neurons from both the CNS and PNS (Baron-van Evercooren *et al.*, 1982; Manthorpe *et al.*, 1983; Rogers *et al.*, 1983; Edgar *et al.*, 1984; Faivre-Bauman *et al.*, 1984; Liesi *et al.*, 1984a,b; Smallheiser *et al.*, 1984; Calof & Reichardt, 1985). It is transiently expressed in early developing fibre tracts in the CNS in a form that is particulate and not associated with basal laminae (Cohen *et al.*, 1987; Letourneau *et al.*, 1988 but see Sosale *et al.*, 1988) and permanently present in regions of the CNS in which axons are added continuously throughout adult life, such as the rat olfactory system (Liesi, 1985b) and the goldfish optic nerve (Hopkins *et al.*, 1985). Furthermore, growth cones possess membrane receptors that bind laminin (Horwitz *et al.*, 1985; Kleinman *et al.*, 1988; Tomaselli *et al.*, 1988).

Although these observations are highly suggestive of a role for laminin in neurite outgrowth, they do not amount to proof. More conclusive evidence might come from observing the effects of antibodies to laminin introduced into appropriate regions of the nervous system during development. In addition, it would also be useful to know what cell processes contact laminin in the neuropil and in particular if these include neuronal growth cones.

In the present paper we document the transient appearance of particulate, non-basal lamina laminin in the developing rat hippocampus using a monospecific antibody to laminin. We show that this form of laminin is present on the surface of cell processes and that neuronal processes occasionally contact this laminin.

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Materials and methods

Laminin antibody production

Two female New Zealand White rabbits received injections of purified laminin (Engelbreth Holm-Swarm (EHS) derived laminin, 0.5 mg, emulsified in Freund's complete adjuvant) by intramuscular and interscapular subdermal routes on four separate occasions at monthly intervals. The last injection was with incomplete adjuvant. Seven days after the last injection, the animal was anaesthetized and bled to death via a carotid cannula. Pre-immune serum was obtained from the marginal ear vein.

Immunoglobulin fractions were prepared by ammonium sulphate precipitation (Mayer & Walker, 1980). Laminin antibodies were purified by affinity chromatography on laminin coupled to Sepharose-4B according to the manufacturers' instructions. Antibodies were characterized initially by ELISA and immunoblotting.

ELISA

The wells of Dynatec Immulon II ELISA plates were incubated with laminin or fibronectin diluted in coating buffer (sodium carbonate/bicarbonate, pH 9.6) overnight at 4° C. No attempt was made to determine the amount of laminin or fibronectin bound to the microplate well following incubation and therefore the amounts of laminin are nominal. A comparative analysis is, however, valid. Following washing in Tris-buffered saline (TBS), wells were incubated in blocking buffer (3% bovine serum albumin, 0.2% polyvinyl pyrollidone in TBS) for 1 h at room temperature, followed by primary antibody diluted in blocking buffer for 2 h at room temperature. After washing in TBS, wells were incubated with goat anti-rabbit Ig conjugated to horseradish peroxidase (HRP) diluted to 1:250 in blocking buffer for 2 h at room temperature. Following a final wash in TBS, wells were developed with O-phenylenediamine (1 mg ml^{-1} in phosphate buffer, pH 6.3). The reaction was stopped after a standard time (10 min) with citric acid (0.5 M) and the optical density read at 492 nm using a Flow plate reader.

Measurements were carried out in duplicate and controls included omitting the target from the coating buffer or the primary antibody or the secondary antibody.

Gel electrophoresis and immunoblotting

Samples of EHS laminin were electrophoresed in sodium dodecyl sulphate–polyacrylamide slab gels (7.5% acrylamide) using buffers described by Laemmli (1970). Gels were blotted on to nitrocellulose paper by transverse electrophoresis (0.8 mA cm^{-2}) for 1 h in a graphite-electrode blotter using a three-phase buffer system (Kyhse-Andersen, 1984). Blots were calibrated using molecular weight marker kits.

The blots were quenched by incubation in blocking buffer containing 5% normal horse serum in TBS for 1 h at room temperature and then further incubated overnight in primary antibody (anti-laminin) at 1:1000 in blocking buffer at 4° C. They were then thoroughly washed in TBS and incubated in goat anti-rabbit Ig HRP conjugated (1:200) in blocking buffer for 2 h. Finally, blots were washed in TBS and developed in 4-chloronaphthol.

Immunoblots were photographed on Kodalith ortho type 3 using a yellow filter.

Immunohistochemistry

Pregnant Wistar rats were deeply anaesthetized with pentobarbitone and embryos dissected free from the uterus. Embryos (E14 to E21, where E0 is day of vaginal plug) were fixed by immersion in 2.5% formaldehyde with or without 0.1% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.4, for 1–2 h at room temperature. Following fixation, embryos were stored in PBS containing sodium azide at 4° C. At least two litters were used for each age studied, and from each litter at least three brains were examined for immunohistochemistry and at least two brains for immunoelectron microscopy.

Embryos were embedded in albumin/gelatin polymerized with glutaraldehyde and sections, 50 μm thick, were cut on a Vibroslice (Campden Instruments). Sections were incubated free-floating in blocking buffer (5% normal horse serum, 5% normal goat serum, 0.2% L-lysine, 0.2% Triton X-100 in PBS) for 1 h at room temperature. Sections were then incubated in primary antibody (anti-laminin, anti-tau or anti-MAP2 or, in double-labelling experiments, anti-laminin with anti-tau or anti-MAP2; 1:400 to 1:5000) for 1 h at room temperature and then for 24–72 h at 4° C. They were washed thoroughly in PBS and then incubated in the appropriate bridging antibody (goat anti-rabbit or goat anti-mouse Ig, 1:40 in blocking buffer) for 2 h, washed thoroughly in PBS and finally incubated in rabbit or mouse peroxidase anti-peroxidase (PAP; 1:100 in blocking buffer) for 2 h at room temperature before finally washing in PBS. In some cases a biotinylated secondary antibody was used (goat anti-rabbit Ig, 1:200 in blocking buffer for 2 h), followed by streptavidin-peroxidase (2.5 μg ml^{-1} blocking buffer for 2 h). Peroxidase was developed with diaminobenzidine (0.5 mg ml^{-1}) in PBS containing 0.01% H_2O_2.

Following staining, sections were washed in PBS, some after brief osmication (1% OsO_4 in 0.1 M Na cacodylate) or counterstaining in cresyl violet, air-dried on to gelatin-coated glass slides, dehydrated in alcohol and mounted in DPX. Sections were photographed on Pan F film using a green filter.

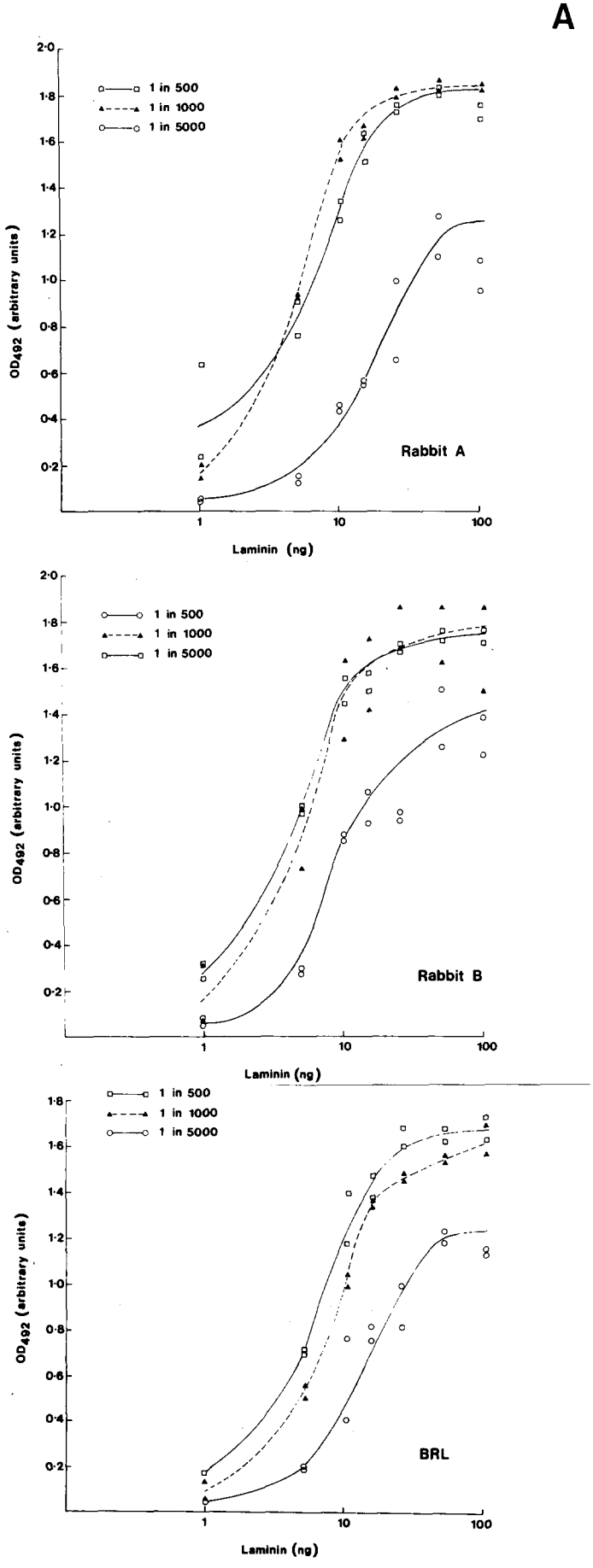
Immunoelectron microscopy

Some sections were further processed for electron microscopy, in which case they were fixed in 3% glutaraldehyde for 10 min after peroxidase development, osmicated as above for 30 min, block stained in saturated aqueous uranyl acetate, dehydrated in ethanol and embedded in Epon. When sections were processed for electron microscopy, Triton X-100 was usually omitted from the blocking buffer. Serial thin sections were cut on a diamond knife, mounted on to Formvar-coated, one-hole grids or hexagonal uncoated grids and viewed, unstained, in a Jeol 100 CX electron microscope at 80 kV.

Controls

Four types of control were performed.

1. Omitting the primary antibody from the blocking buffer.
2. Substituting pre-immune serum for the primary antibody.
3. Incubating the primary antibody with antigen (laminin). Laminin (4 μg) was incubated with 1 ml of affinity-purified anti-laminin, diluted 1:100 in blocking buffer, for 2 h at room



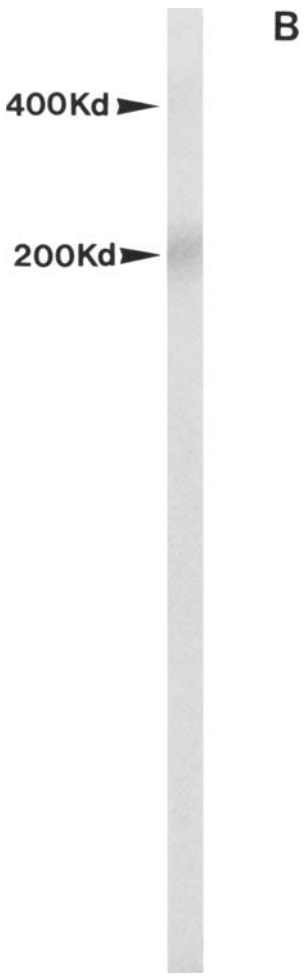


Fig. 1. (A) ELISA analysis of the binding of rabbit A, rabbit B and BRL anti-laminin sera to EHS laminin. Optical density readings have been corrected for 'background', defined as the optical density measured when the primary antibody is omitted. Values are the means of duplicate measurements. (B) Immunoblot of EHS laminin probed with anti-LaB showing that the antibody recognizes both heavy and light chains.

temperature followed by centrifugation at 17 000 g_{max} for 15 min. The supernatant was substituted for primary antibody. 4. Substituting the unbound proteins from the laminin affinity column for primary antibody.

Protein assay

Proteins were assayed by the method of Bradford (1976) using bovine serum albumin as standard.

Materials

The L-lysine (free base), normal horse serum, normal goat serum, rabbit and mouse PAP, goat anti-rabbit Ig, diaminobenzidine, 4-chloronaphthol, polyvinyl pyrollidone and *O*-phenylenediamine were all purchased from Sigma. Triton X-100, scintillation grade, was from BDH. Molecular weight markers, SDS and nitrocellulose were from BioRad. Laminin and anti-laminin antibodies were from Bethesda Research

Laboratories and porcine plasma fibronectin from Bioprocessing. Affinity-purified rabbit polyclonal antibody to purified bovine brain tau was the kind gift of Dr D. N. Drechsel (Drubin *et al.*, 1986), rabbit polyclonal antibodies to MAP2 were kindly provided by Drs P. Bayley and P. Sheterline (Sheterline, 1980) and a mouse monoclonal anti-MAP2 by Dr A. Matus (Huber & Matus, 1984).

Results

Characterization of laminin antibodies

Of the two rabbits (A and B) immunized with mouse laminin derived from the EHS sarcoma (Timpl *et al.*, 1979), rabbit B produced serum with the highest titre as determined by ELISA (Fig. 1A). For instance, at a dilution of 1 in 5000, rabbit B antiserum gave an OD_{492} reading of 0.83 (arbitrary units) against 10 ng of laminin, whereas the corresponding values for rabbit A and the BRL antiserum were 0.43 and 0.60 respectively. Rabbit B serum was therefore selected for affinity chromatography and further study. Both of our rabbit antisera were more sensitive in detecting laminin than a commercially available antibody (BRL, Fig. 1A). No detectable cross-reactivity between fibronectin and any of these antibodies was observed in ELISA.

Affinity-purified anti-laminin from rabbit B (anti-LaB) was further characterized by immunoblotting and shown to recognize both heavy (400 kD) and light (200–220 kD) chains of reduced EHS laminin (Fig. 1B).

Distribution of laminin immunoreactivity in the hippocampus: light microscopy

As anticipated from the known distribution of laminin in basal laminae, anti-LaB strongly stained the pia membranes and the basal lamina tubes of blood vessels at all ages studied (E14 to E21; Fig. 2). The blood vessel basal lamina staining was continuous, and anti-LaB staining sprouts extending from capillary walls were occasionally observed (cf Eriksdotter-Nilsson *et al.*, 1986). Anti-LaB also stained the basal lamina tubes of skeletal muscle and peripheral nerves (S. Kemplay, unpublished observations). We conclude from the biochemical and immunocytochemical data that anti-LaB is specific for laminin.

At E14, the earliest age studied, anti-LaB produced a distinctive, punctate staining pattern throughout the entire thickness and length of the telencephalic vesicle wall (not shown; see also Liesi, 1985a), in addition to the basal lamina staining. By E15, a dramatic loss of punctate laminin staining is seen such that the only region in the forebrain showing appreciable non-basal lamina is the hippocampus and the adjacent cortical mantle (Fig. 2A and 3). The hippocampal laminin staining persisted until E18. By E19 all punctate laminin immunoreactivity had disappeared from the hippocampus.

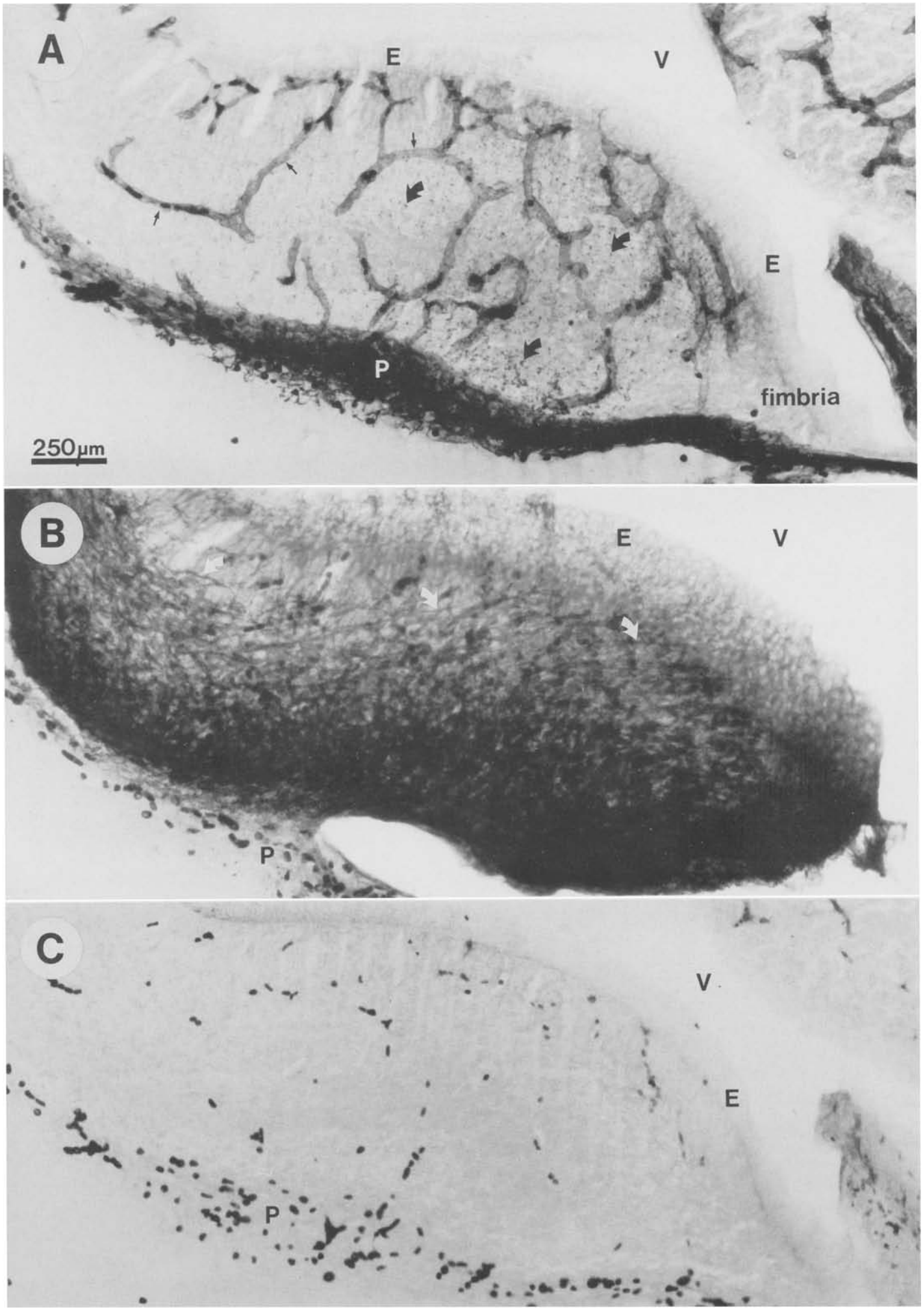
Serial section analysis revealed a complex, three-dimensional distribution of punctate laminin staining in the developing hippocampus (Fig. 3). In more dorsal regions the laminin staining spanned the entire thickness of the wall from the junction between the ependyma and the neuropil to the pial surface (Fig. 3B and C), whereas in ventral regions the staining originated from a localized region at the pial surface and passed laterally through a zone between the ventricular and marginal zones, extending beyond the hippocampus into the cortical mantle (Fig. 3F and G). The highest density of staining was in the future marginal zone immediately beneath the pia. There was no staining in the ependyma (Fig. 2A).

Rabbit A anti-serum and the BRL anti-serum produced similar staining patterns to anti-LaB in the developing hippocampus, although background staining was unacceptably high (not shown). All controls were negative (Fig. 2C).

We looked for a correlation between the distribution of the punctate laminin staining in the developing hippocampus and neurite outgrowth by staining alternate, consecutive sections with anti-LaB and with antibodies to the neuron-specific, microtubule-associated proteins MAP2 and tau which specifically stain neurites in developing brain (Riederer & Matus, 1985; Brion *et al.*, 1988).

Both MAP2 and tau antibodies strongly stained fibres entering the hippocampus from the adjacent cortical mantle – a region from which the adult hippocampus receives a major afferent input (Fig. 2B). Furthermore, there was considerable overlap between this staining pattern and that for laminin (cf. Fig. 2A and B). However, with this approach it is not possible to determine whether neurites come into contact with laminin. To address this question we used immuno-electron microscopy.

Fig. 2. Adjacent, vibroslice sections of E17 rat hippocampus cut in the horizontal plane and stained with anti-LaB (A) and tau (B) antibodies. (C) Control, in which anti-LaB was pre-absorbed with laminin. Anti-LaB, in addition to staining the pial membranes (P) and basal lamina tubes of capillaries (small arrows) in the hippocampus, also produces a punctate staining pattern (curved arrows in A) in the neuropil from the pial surface to approximately the junction between the ependyma (E) and the neuropil. The tau antibodies in (B) stain neural elements in the hippocampus and in particular neural processes (curved white arrows) entering from the entorhinal cortex. Note that in control sections (C) the erythrocytes stain because of endogenous peroxidase. V, Ventricle.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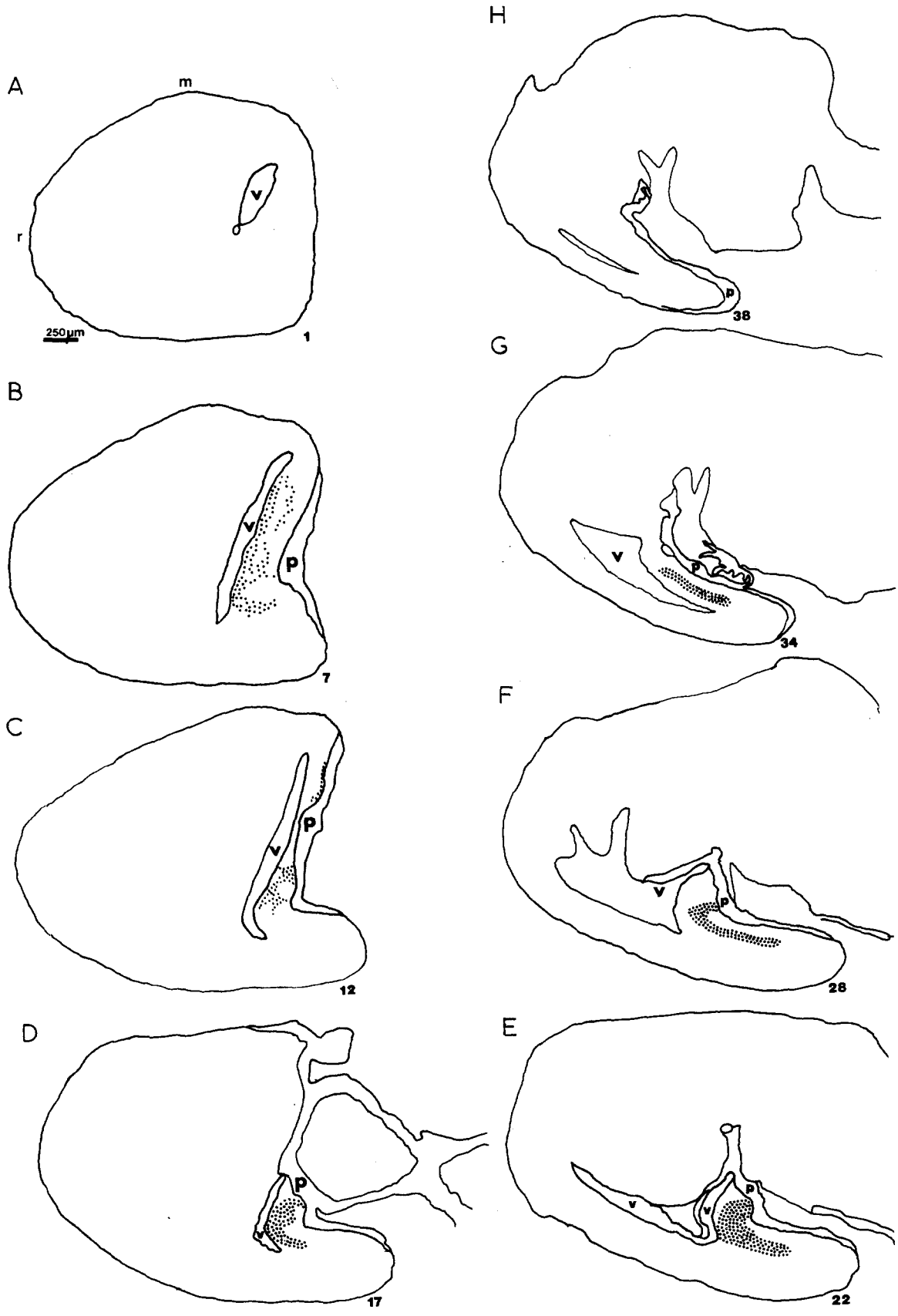


Fig. 3. Camera lucida drawings of vibroslice sections of E16 rat brain cut in the horizontal plane and stained with anti-LaB. The left half of the brain only is shown. The distribution of punctate laminin staining is indicated by dots; P shows pia sectioned obliquely; V, ventricle. Numbers in the bottom right-hand corner indicate the section number, counting from dorsal to ventral. Orientation is indicated in (A); r, rostral; m, medial.

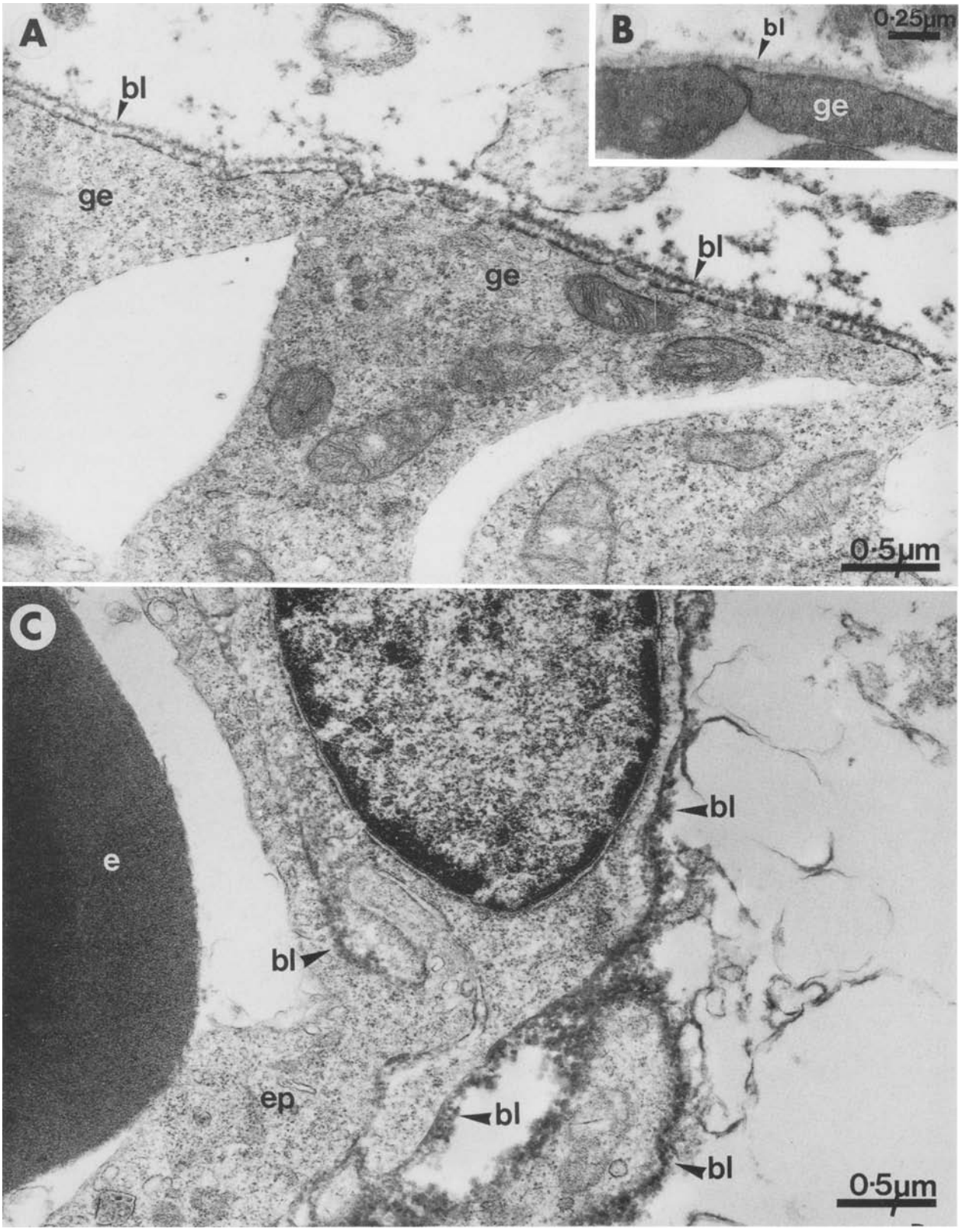


Fig. 4. Electron micrographs from E17 hippocampus stained with anti-LaB. In (A) the pial surface is shown with the glial endfeet (ge) forming the glial limitans. Anti-LaB has stained the basal lamina (bl). Inset (B) shows a similar region in a control section incubated with pre-absorbed anti-LaB. The apparent patchy staining of the plasma membrane of the glial endfeet was seen in all types of control (see Methods). In (C) staining of blood vessel basal lamina (bl) in the neuropil is shown. E, Erythrocyte; ep, endothelial cell process.

Distribution of laminin immunoreactivity in the hippocampus: electron microscopy

As expected from the light microscopy results, laminin immunoreactivity was found in three locations in the hippocampus under the electron microscope: in the basal laminae of blood vessels and in the glial limitans and between cell processes in the neuropil (Figs 4 and 5). At all these locations the staining was entirely extracellular, there being no evidence of intracellular staining even in those sections that were exposed to detergent in the incubation buffers (Triton X-100, see Methods), despite the fact that under these circumstances the plasma membranes of cells were solubilized and could not, therefore, offer a barrier to antibody penetration into the cytoplasm (not shown). The ependyma did not stain with anti-laminin antibodies, confirming the light microscopy observations.

At the glial limitans, anti-LaB stained the basal lamina of the glial endfeet in its entirety (Fig. 4A). Two regions of the basal lamina were stained strongly, corresponding to the lamina densa and a narrow zone at the external surface of the glial endfeet plasma membrane (see also Halfter & Fua, 1987). Coated vesicles that were contiguous with the basal lamina-facing plasma membrane of glial endfeet were also stained. Occasionally, discrete patches of laminin staining were also seen on the surface of glial endfeet facing the neuropil.

The basal laminae of blood vessels were also completely stained by anti-LaB (Fig. 4C). The basal lamina staining was present on endothelial cells and around the cell body and processes of cells associated with blood vessels. The blood vessel basal lamina was only partially apposed by glial cell processes so that regions of the basal lamina were directly exposed to the neuropil. This was also the case with the basal lamina surrounding the cells associated with blood vessels.

In the neuropil, laminin immunoreactivity was found in discrete patches on the surfaces of cell processes (Fig. 5). These punctate deposits were about 0.5 μm in diameter and were not associated with the basal lamina of blood vessels, as demonstrated by serial section analysis and by the fact that they were not within a basal lamina. Non-basal lamina patches of laminin immunoreactivity were found on cell bodies and, more frequently, on cell processes. In many cases, several morphologically distinct types of cell process came into contact with laminin patches (Fig. 5). In one type the process had an irregular outline and contained intermediate filaments, glycogen particles and free ribosomes. These features may identify these processes as glial, but in developing tissue they are not diagnostic. However, in favourable instances, some of these processes could be traced in consecutive sections to glial endfeet at the pial surface. The other type of process that came into contact with laminin particles were more regular in shape, usually cylindrical, and their cytoplasm was lighter and contained microtubules rather than intermediate filaments. In many cases in individual sections, the particulate laminin was completely surrounded by cell processes, which therefore effectively partitioned off the laminin from the neuropil.

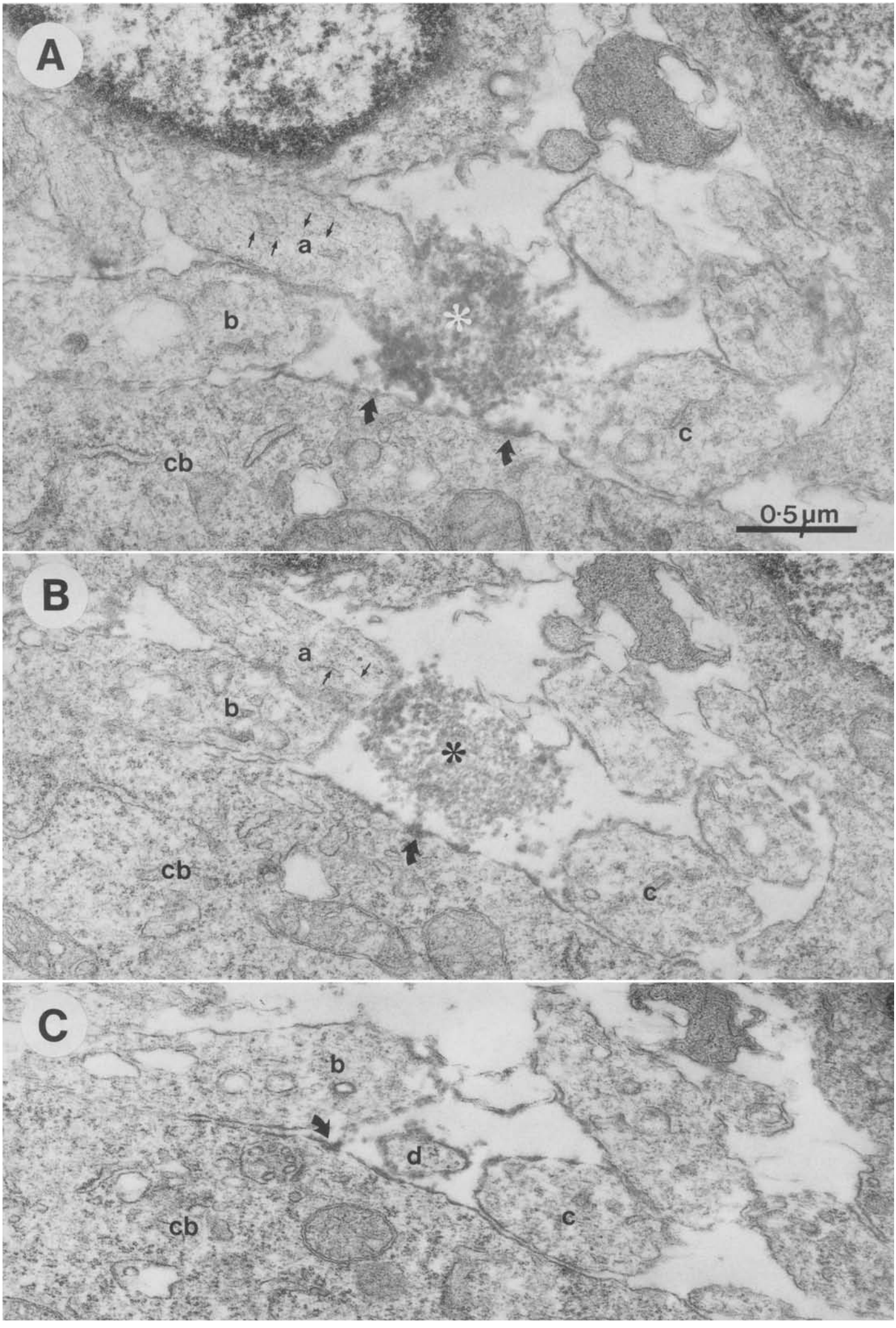
MAP2 and tau staining

We found that in fixed embryonic hippocampus it was not necessary to permeabilize plasma membranes with Triton X-100 to obtain staining with antibodies against intracellular antigens (MAP2 and tau). However, without Triton X-100 a proportion of neuronal processes do not stain with MAP2 or tau. We have not measured this proportion, though it seems to be small. The MAP2 and tau antibodies stained intracellular structures in the embryonic hippocampus exclusively. Both antibodies stained neuronal cell bodies and neuronal processes. There was no evidence of glial cell staining. In double-labelling experiments it was found that, in some cases, processes contacting punctate laminin deposits and having a neuronal morphology were stained with MAP2 and tau antibodies (Fig. 6), confirming that they were axonal or dendritic processes. Contacts between tau- or MAP2-positive processes and laminin particles were most commonly seen in the marginal zone.

Discussion

Previously, two main observations have supported the notion that laminin plays some part in neurite outgrowth and guidance (reviewed in Lander, 1987). The first observation is that laminin, as a tissue culture substrate, is extremely potent in promoting and sustaining neurite outgrowth *in vitro* (Baron-van Evercooren *et al.*, 1982; Manthorpe *et al.*, 1983; Rogers *et al.*, 1983; Edgar *et al.*, 1984; Liesi *et al.*, 1984a; Smallheiser *et al.*, 1984; Calof & Reichardt, 1985). Furthermore, some neuronal growth cones possess surface membrane receptors that bind laminin (Horwitz *et al.*, 1985;

Fig. 5. Electron micrographs of serial sections of the hippocampus from an E17 rat embryo stained with anti-LaB. The series shows a punctate laminin deposit (asterisks) stained with anti-LaB and contacted by a variety of cell processes (a–d), at least one of which contains intermediate filaments (arrows in A and B). A cell body (cb) also contacts the laminin and its surface is extensively stained by the antibody (curved arrows). One section has been omitted between A and B and three sections between B and C.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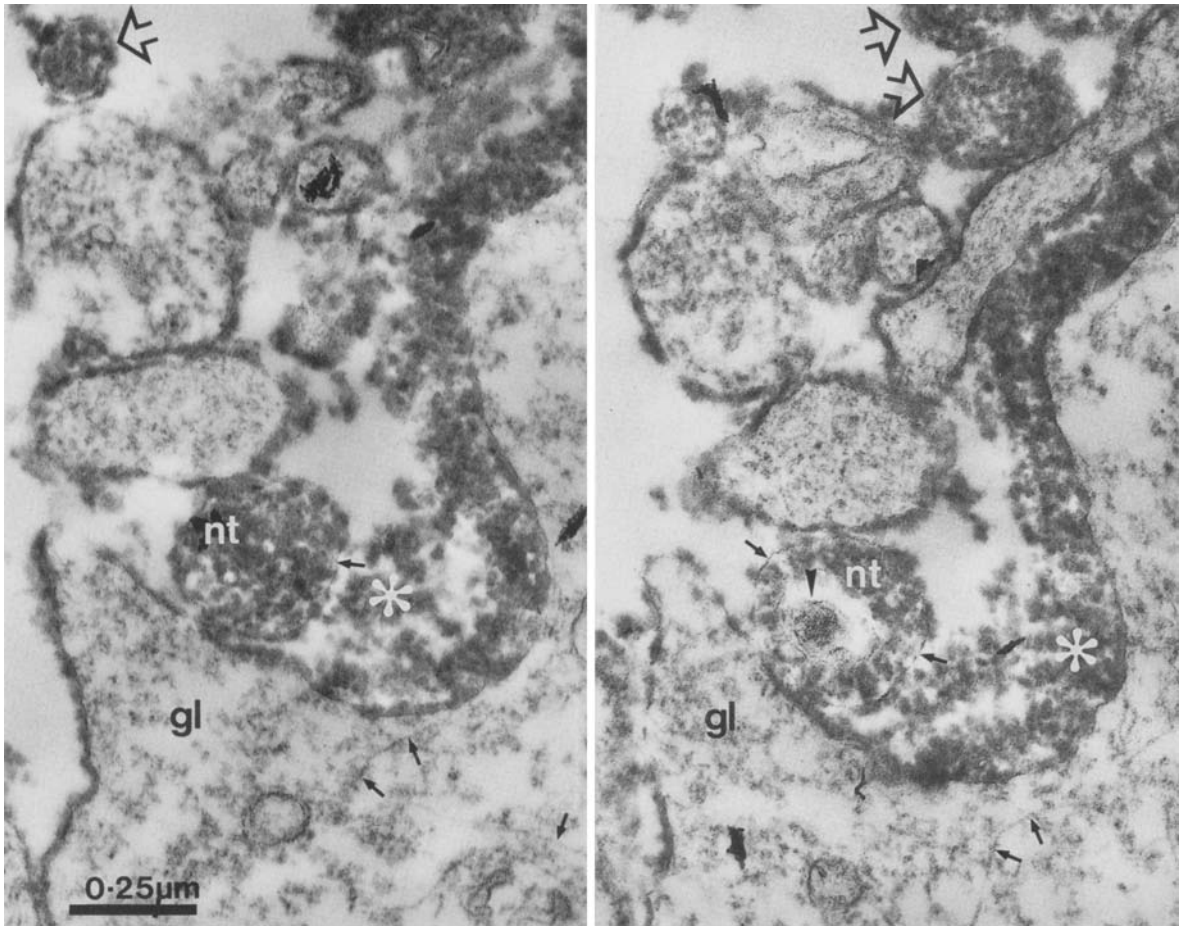

Fig. 6. Electron micrographs of serial sections of the rat hippocampus from an E17 rat embryo double-labelled with anti-LaB and anti-tau antibodies. A laminin deposit (asterisks) on the surface of a tau-negative cell process (gl: possibly a glial cell, note the intermediate filaments shown by arrows) is contacted by a tau-positive neurite (nt) identified by intracellular peroxidase reaction product. The plasma membrane of the neurite is visible (arrows) and also an organelle (arrowhead in B; possibly a multi-vesicular body) within the neurite. Other tau-positive neurites are indicated by open arrows. One section between these two has been omitted.

Cohen *et al.*, 1986; Kleinman *et al.*, 1988; Tomaselli *et al.*, 1988). The second observation is that laminin is transiently expressed in the developing CNS during the formation of some early tracts (Cohen *et al.*, 1987; Letourneau *et al.*, 1988), but not all (Sosale *et al.*, 1988). These latter observations, however, were made at the light microscopic level, and it was not possible, therefore, to determine whether neurites or their growth cones actually came into contact with the punctate laminin.

In this paper we show that laminin, in a punctate, non-basal lamina form, is transiently expressed in the developing rat hippocampus. Immunoelectron microscopy further showed that the punctate laminin is entirely extracellular and associated with cell bodies or their processes. In many cases the punctate laminin was completely surrounded by processes in individual ultrathin sections. Some processes had the appropriate morphology for neurites; they were cylindrical and contained bundles of microtubules, whereas others were irregularly shaped and contained intermediate filaments and ribosomes/glycogen particles. These may have been glial cell processes; however, this identification cannot be made solely on ultrastructural criteria and will have to await more definitive evidence, e.g. immunocytochemical. Some of these processes were MAP2- and tau-positive, antigens known to be specific for neuronal processes in developing rat brain (Riederer & Matus, 1985; Brion *et al.*, 1988). The transient expression of punctate laminin in the hippocampus and the observed contact between neurites and punctate laminin is consistent with a role for laminin in guiding neurite outgrowth.

Neurogenesis of the pyramidal cells in the mouse hippocampus begins on about E10 and continues until about E18, with peak rates of formation around E14

(Angevine, 1965). The granule cells of the dentate gyrus, however, continue to be born postnatally. A similar time-course probably also occurs in the rat, a related altricial rodent. It is during this time period that we observe a punctate staining for laminin in the rat hippocampus. It is conceivable, therefore, that the role of punctate laminin is related to pyramidal cell migration from the neuroepithelium (ependymal layer), where these cells are born, to the stratum pyramidale layer of the hippocampus, or related to granule cell migration either from the ependymal layer or from their secondary zone of proliferation in the hilar region, to the dentate gyrus (Nowakowski & Rakic, 1981). Neuronal migration probably occurs along the surface of radial glial fibres in the hippocampus (for review, see Rakic, 1985) and, in culture, cerebellar granule cells can migrate on laminin (Selak *et al.*, 1985). In the hippocampus there is a change in the arrangement of radial glial fibres during development, from an arrangement where fibres span the cortical mantle orthogonal to the ependymal layer, to one where fibres are arranged tangentially (Rickmann *et al.*, 1987) to form a prominent bundle beneath the pial surface that radiates into the dentate anlage. It has been suggested that this change underlies the migration of granule cells from their zones of proliferation to form the dentate gyrus (Hausmann *et al.*, 1987; Rickmann *et al.*, 1987) and indeed there is a striking similarity between the pattern of tangential radial glial fibres and the non-basal lamina staining observed here. However, in the electron microscope we only occasionally saw punctate laminin between the surfaces of cell bodies – most of the punctate laminin was contacted by cell processes. Nevertheless, some of these processes may be those of migrating neurons.

MAP2- and tau-positive neurites contacting laminin particles were most commonly found in the marginal zone of the hippocampus subjacent to the pial surface. This is the region of the hippocampus where the punctate laminin staining is densest. It is also a region where neuronal cell processes are abundant and form a plexus that will eventually become the relatively cell-free molecular layer. A punctate staining pattern for laminin has also been seen in the marginal zone of the mouse myelencephalon (Letourneau *et al.*, 1988), where it was suggested to play a role in the formation of the ventral longitudinal pathway.

In sections prepared for immunofluorescence microscopy with laminin antibodies it is often difficult to see the punctate staining in the marginal zone because of the intense fluorescence in the pial membranes (Liesi, 1985a). For this reason, and because of a requirement for immunoelectron microscopy, we chose to use the PAP method, which is at least as sensitive as immunofluorescence. The laminin particles were, on average, about 0.5 μm in diameter. This dimension is likely to be exaggerated by the signal amplification of the PAP method used here, and we will have to await results of immunogold labelling studies, currently in progress in our laboratory, to determine the true size of these particles.

Our observation that one of the cellular elements contacting laminin particles may be glial is consistent with the suggestion that glial cells (astrocytes) synthesize laminin (Liesi *et al.*, 1983; Hopkins *et al.*, 1985; Selak *et al.*, 1985). In the PNS, where, unlike the CNS, laminin is present in the adult, Schwann cells produce the laminin (Cornbrooks *et al.*, 1983). The eventual fate of secreted punctate laminin, particularly during E18 in the hippocampus, is not known. Perhaps glial cells are involved in the degradation of laminin as well as its production. It will be interesting to probe the developing hippocampus for laminin using *in situ* hybridization to test directly which cells are producing the molecule.

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References

ANGEVINE, J. B. (1965) Time of neuron origin in the hippocampal region. An autoradiographic study in the mouse. *Experimental Neurology* Suppl. **2**, 1–70.

BARON-VAN EVERCOOREN, A., KLEINMAN, H. K., OHNO, S., MARGANOS, P., SCHWARTZ, J. P. & DUBOIS-DALCQ, M. (1982) Nerve growth factor, laminin, and fibronectin promote neurite growth in human fetal sensory ganglion cultures. *Journal of Neuroscience Research* **8**, 179–93.

BRADFORD, M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–54.

BRION, J. P., GUILEMINOT, J., COUCHIE, D., FLAMENT-DURAND, J. & NUNEZ, J. (1988) Both adult and juvenile tau microtubule-associated proteins are axon specific in the developing and adult rat cerebellum. *Neuroscience* **25**, 139–46.

CALOF, A. L. & REICHARDT, L. F. (1985) Response of purified chick motoneurons to myotube conditioned medium: laminin is essential for the substratum binding, neurite-

outgrowth promoting activity. *Neuroscience Letters* **59**, 183–9.

COHEN, J., BURNE, J. F., McKINLAY, C. & WINTER, J. (1987) The role of laminin and the laminin/fibronectin receptor complex in the outgrowth of retinal ganglion cell axons. *Developmental Biology* **122**, 407–18.

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

CORNBROOKS, C., CAREY, D., McDONALD, J., TIMPL, R. BUNGE, R. (1983) *In vivo* and *in vitro* observations on laminin produced by Schwann cells. *Proceedings of the National Academy of Sciences USA* **80**, 3850–4.

DRUBIN, D., KOBAYASHI, S. & KIRSCHNER, M. (1986) Association of tau protein with microtubules in living cells. *Annuals of the New York Academy of Science* **466**, 257–68.

EDGAR, D., TIMPL, R. & THOENEN, H. (1984) The heparin binding domain of laminin is responsible for its effect on neurite outgrowth and neuronal survival. *EMBO Journal* **3**, 1463–88.

ERIKSDOTTER-NILSSON, M., BJÖRKLUND, H. & OLSON, L. (1986) Laminin immunohistochemistry: a simple method to visualize and quantitate vascular structures in the mammalian brain. *Journal of Neuroscience Methods* **17**, 275–86.

FAIVRE-BAUMAN, A., PUYMIRAT, J., LOUDES, J., BARRET, A. & TIXIER-VIDAL, A. (1984) Laminin promotes attachment and neurite elongation of fetal hypothalamic neurons grown in serum-free medium. *Neuroscience Letters* **44**, 83–9.

HALFTER, W. & FUA, C. S. (1987) Immunohistochemical localization of laminin, neural cell adhesion molecule, collagen type IV and T-61 antigen in the embryonic retina of the Japanese quail by an *in vivo* injection of antibodies. *Cell and Tissue Research* **249**, 487–96.

HAUSMANN, B., HARTMANN, D. & SIEVERS, J. (1987) Secondary neuropithelial stem cells of the cerebellum and the dentate gyrus are attached to the basal lamina during their migration and proliferation. In *Mesenchymal–Epithelial Interactions in Neural Development* (edited by WOLFF, J. R., SIEVERS, J. & BERRY, M.), pp. 279–91. NATO ASI Series, Vol. H5. Springer-Verlag.

HOPKINS, J. M., FORD-HOLEVINSKI, T. S., McCOY, J. P. & AGRANOFF, B. W. (1985) Laminin and optic nerve regneration in the goldfish. *Journal of Neuroscience* **5**, 3030–8.

HORWITZ, A., DUGGAN, K., GREGGS, R., DEKER, C. & BUCK, C. (1985) The cell substrate attachment (CSAT) antigen has properties of a receptor for laminin and fibronectin. *Journal of Cell Biology* **101**, 2134–44.

HUBER, G. & MATUS, A. (1984) Differences in the cellular distributions of two microtubule-associated proteins, MAP1 and MAP2, in rat brain. *Journal of Neuroscience* **4**, 151–60.

KLEINMAN, H. K., OGLE, R. C., CANNON, F. B., LITTLE, C. D., SWEENEY, T. M. & LUCKENBILL-EDDS, L. (1988) Laminin receptors for neurite formation. *Proceedings of the National Academy of Sciences USA* **85**, 1282–6.

KYHSE-ANDERSEN, J. (1984) Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *Journal of Biochemical and Biophysical Methods* **10**, 203–9.

LAEMMLI, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (London)* **227**, 680–5.

LANDER, A. D. (1987) Molecules that make axons grow. *Molecular Neurobiology* **1**, 213–45.

LETOURNEAU, P. C., MADSEN, A. M., PALM, S. L. & FURCHT, L. T. (1988) Immunoreactivity for laminin in the developing ventral longitudinal pathway of the brain. *Developmental Biology* **125**, 135–44.

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

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

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 astrocytes of adult brain by injury. *EMBO 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.

MAYER, R. J. & WALKER, J. H. (1980) *Immunochemical Methods in the Biological Sciences: Enzymes and Proteins*. Academic Press.

NOWAKOWSKI, R. S. & RAKIC, P. (1981) The site of origin and route and rate of migration of neurons to the hippocampal region of the rhesus monkey. *Journal of Comparative Neurology* **196**, 129–54.

RAKIC, P. (1985) Mechanisms of neuronal migration in developing cerebellar cortex. In *Molecular Bases of Neural Development* (edited by EINAR GALL, W. & COWAN, W. M.), pp. 139–60. New York: John Wiley & Sons.

RICKMANN, M., AMARAL, D. G. & COWAN, W. M. (1987) Organization of radial glial cells during the development of the rat dentate gyrus. *Journal of Comparative Neurology* **264**, 449–79.

RIEDERER, B. & MATUS, A. (1985) Differential expression of distinct microtubule-associated proteins during brain development. *Proceedings of the National Academy of Sciences USA* **82**, 6006–9.

ROGERS, S. L., LETOURNEAU, P. C., PALM, S. L., McCARTHY, J. B. & 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.

SELAK, I., FOIDART, J. M. & MOONEN, G. (1985) Laminin promotes cerebellar granule cells migration *in vitro* and is synthesized by cultured astrocytes. *Developmental Neuroscience* **7**, 278–85.

SHETERLINE, P. (1980) Immunological characterisation of the microtubule-associated protein MAP2. *FEBS Letters* **111**, 167–70.

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

SOSALE, A., ROBSON, J. A. & STELZNER, D. J. (1988) Laminin distribution during corticospinal tract development and after spinal cord injury. *Experimental Neurology* **102**, 14–22.

TIMPL, R., ENGEL, J. & MARTIN, G. R. (1983) Laminin – a multifunctional protein of basement membranes. *Trends in Biochemical Sciences* **8**, 207–9.

TIMPL, R., ROHDE, H., GEHRON ROBEY, P., RENARD, S. I., FOIDART, J. M. & MARTIN, G. R. (1979) Laminin – a glycoprotein from basement membranes. *Journal of Biological Chemistry* **254**, 9933–7.

TOMASELLI, K. J., NEUGEBAUER, K. M., BIXBY, J. L., LILIEN, J. & REICHARDT, L. F. (1988) N-Cadherin and integrins: Two receptor systems that mediate neuronal process outgrowth on astrocyte surfaces. *Neuron* **1**, 33–43.

VON DER MARK, K. & KÜHL, U. (1985) Laminin and its receptor. *Biochimica et Biophysica Acta* **823**, 147–60.