Article

Tenascin is accumulated along developing peripheral nerves and allows neurite outgrowth in vitro

WEHRLE-HALLER, Bernhard, CHIQUET, Matthias

Abstract

The extracellular matrix protein, tenascin, appears in a restricted pattern during organ morphogenesis. Here we studied the expression of tenascin along developing peripheral nerves in chick embryos and tested its activity as a substrate for cultured neurons. Motor axons grow out through the tenascin-rich, anterior part of the sclerotome. Shortly after, tenascin surrounds axon fascicles of ventral roots. At the limb levels, outgrowing axons accumulate in the tenascin-containing girdle region forming a plexus. In the limb, tenascin first appears in bracket-like structures that surround the precartilage cell condensations of the femur and humerus, respectively. These regions coincide with the channels along which axons first grow in from the girdle plexus to form the limb nerves. Later, the major tenascin staining is associated with the cartilage and tendon primordia, and not with the limb nerves. We used tenascin as a substrate for cultured neural explants and single cells in order to test for its function in neurite outgrowth. Dissociated embryonic neurons of various types attached to mixed polylysine/tenascin substrates [...]

Reference

Tenascin is accumulated along developing peripheral nerves and allows neurite outgrowth *in vitro*

BERNHARD WEHRLE and MATTHIAS CHIQUET

Department of Biophysical Chemistry, Biocenter, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

Summary

The extracellular matrix protein, tenascin, appears in a restricted pattern during organ morphogenesis. Here we studied the expression of tenascin along developing peripheral nerves in chick embryos and tested its activity as a substrate for cultured neurons. Motor axons grow out through the tenascin-rich, anterior part of the sclerotome. Shortly after, tenascin surrounds axon fascicles of ventral roots. At the limb levels, outgrowing axons accumulate in the tenascin-containing girdle region forming a plexus. In the limb, tenascin first appears in bracket-like structures that surround the precartilage cell condensations of the femur and humerus, respectively. These regions coincide with the channels along which axons first grow in from the girdle plexus to form the limb nerves. Later, the major tenascin staining is associated with the cartilage and tendon primordia, and not with the limb nerves. We used tenascin as a substrate for cultured neural explants and single cells in order to test for its function in neurite outgrowth. Dissociated embryonic neurons of various types attached to mixed polylysine/tenascin substrates and sprouted rapidly after a lag of several hours. Outgrowth was inhibited and neurites were detached by anti-tenascin antibodies. On substrates coated with tenascin alone, neurite outgrowth was achieved from 3 day spinal cord explants. Whereas growth cones were well spread and rapidly moving, the neurites were poorly attached, straight and rarely branched. We speculate that *in vivo* tenascin allows axonal outgrowth, but inhibits branching and supports fasciculation of newly formed axons.

Abbreviations: ECM, extracellular matrix; TBS, Tris-buffered saline; SDS, sodium dodecyl sulfate; mAb, monoclonal antibody; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; TRITC, tetramethyl rhodamine isothiocyanate; IgG, immunoglobulin gamma; FCS, fetal calf serum; DMEM, Dulbecco's minimal essential medium; TU, tubulin; TN, tenascin; FN, fibronectin; pL, polylysine; LN, laminin.

Key words: tenascin, neurite outgrowth, peripheral nerve, chick embryo.

Introduction

Tenascin (also called cytotactin) is a large, oligomeric extracellular matrix protein with a six-armed 'hexabrachion' structure (for reviews see Erickson and Bourdon, 1989; Chiquet, 1989; Chiquet-Ehrismann, 1990). Unlike fibronectin with which it shares sequence homologies (Spring *et al.* 1989), tenascin is not as potent and universal cell adhesion molecule *in vitro* (Lotz *et al.* 1989). Cells that do attach to tenascin do not respond by spreading (Halfter *et al.* 1989; Lotz *et al.* 1989). In fact, excess tenascin interferes with fibronectin-mediated spreading of cultured fibroblasts (Chiquet-Ehrismann *et al.* 1988) and inhibits the migration of mesodermal cells during amphibian gastrulation (Riou *et al.* 1990). Seemingly, tenascin can exert diverse effects on various cell types and can modulate the activities of other ECM molecules (Chiquet, 1989).

A main interest in tenascin stems from its highly restricted and regulated pattern of expression during morphogenesis (Chiquet and Fambrough, 1984a; Chiquet-Ehrismann *et al.* 1986; Crossin *et al.* 1986). Especially fascinating is its association with the developing nervous system. Tenascin is expressed first by the chorda-mesoderm and then by the neuroepithelium in response to neural induction (Riou *et al.* 1988) and is accumulated by glial and mesenchymal cells in defined areas of the developing central (Chuong *et al.* 1987; Steindler *et al.* 1989; Crossin *et al.* 1989) and peripheral (Crossin *et al.* 1986; Rieger *et al.* 1986) nervous system. It is reexpressed during nerve regeneration (Sanes *et al.* 1986; Daniloff *et al.* 1989). In the embryo, tenascin accumulates along neural crest pathways in the head (Bronner-Fraser *et al.* 1988) and in the trunk in the anterior halves of the sclerotomes (Tan *et al.* 1987; Mackie *et al.* 1988), although this happens presumably after the major wave of crest cell migration (Stern *et al.* 1989). The anterior halves of the sclerotomes are also the areas where a bit later the first motor axons grow out from the spinal cord establishing the ventral roots;

they are joined later by sensory fibers from the dorsal root ganglia to form the peripheral nerves (Rickmann *et al.* 1985).

In the work described here, we studied the expression of tenascin along peripheral nerves in normal development. We were especially interested in the correlation between the tenascin pattern and the forming neuronal plexus and nerves at the limb level. This was because the precartilage areas of the girdle and upper limb regions are known to influence the nerve pattern decisively (Tosney and Landmesser, 1984), and because tenascin is specifically expressed in and around precartilage elements (Chiquet and Fambrough, 1984a). Molecules like tenascin might be relevant to the morphogenesis of nerves either as promotors or as inhibitors of axonal growth (Edgar *et al.* 1984; Caroni and Schwab, 1988). In order to probe for a possible function of tenascin in neurite outgrowth, we therefore performed cell culture experiments with neural explants and dissociated neurons using tenascin, fibronectin and laminin as culture substrates.

Materials and methods

Extracellular matrix proteins

Tenascin was isolated from medium conditioned by 11 day chick embryo fibroblasts by affinity chromatography to mAb M1-Sepharose as described previously (Chiquet and Fambrough, 1984b), with a few modifications. To wash the bound tenascin on the antibody affinity column, we used a buffer containing 1 M NaCl, 0.05 % Triton X-100, 20 mM Tris–HCl pH 7.4. The low concentrations of SDS used in the original washing buffer were omitted because of toxic effects of the preparations. After washing the column extensively with 150 mM NaCl, 20 mM Tris–HCl pH 7.4 (TBS), tenascin was eluted with 150 mM NaCl, 100 mM triethylamine pH 11.0, neutralized with 1 M Tris–HCl pH 6.8, dialyzed against TBS and frozen. On SDS–polyacrylamide gels, the preparations revealed after reduction the three subunit isoforms (230, 200 and $190 \times 10^3 M_r$) (Fig. 1a); under nonreducing conditions, subunit hexamers and trimers were the major components (cf. Spring *et al.* 1989). Chick embryo fibroblast fibronectin stemmed from the same conditioned medium as did tenascin. It was eluted with 4 M urea from a gelatin–Sepharose column used to preabsorb the medium before the subsequent isolation of tenascin (Chiquet and Fambrough, 1984b). The fibronectin preparations showed a major band of $450 \times 10^3 M_r$ before reduction (not shown) and of $230 \times 10^3 M_r$ after reduction (Fig. 1b) on SDS–polyacrylamide gels (Chiquet and Fambrough, 1984a). We checked by immunoblotting (Chiquet *et al.* 1988) with specific mAbs (see below) that the tenascin preparations were not contaminated with fibronectin and *vice versa* (Fig. 1c–f). Laminin–nidogen complex isolated from mouse Engelbreth–Holm–Swarm tumor (Paulsson *et al.* 1987) was a gift of Dr Mats Paulsson (Sandoz AG, Basel).

Antibodies

The following monoclonal antibodies (mAbs) were used: M1 (Chiquet and Fambrough, 1984a) and Tn68 (Chiquet-Ehrismann *et al.* 1988) against chick tenascin; M6 against chick fibronectin (Pearson *et al.* 1988); and ID-5 against the C-terminal peptide· of de-tyrosinated mouse alpha-tubulin (Wehland and Weber, 1987; gift of Dr Juergen Wehland, Braunschweig). All four mAbs are mouse IgGs. A culture
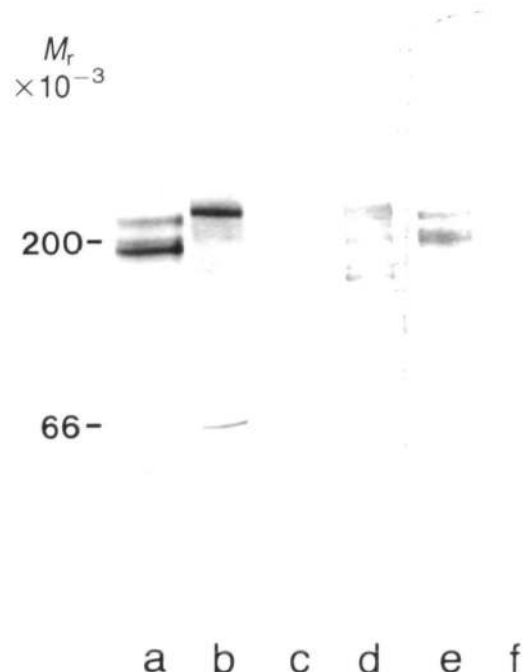
Fig. 1. Analysis by SDS–gel electrophoresis and immunoblotting of the chick tenascin and fibronectin preparations used as culture substrates. Coomassie-stained 3–15 % acrylamide gradient gel shows 10 μg each of tenascin (a) and fibronectin (b) run under reducing conditions. The contamination at $M_r=66\,000$ in the fibronectin preparation is BSA. Parallel samples of tenascin (c, e) or fibronectin (d, f) were blotted to nitrocellulose and probed with mAb M6 against chick fibronectin (c, d) or mAb Tn68 against chick tenascin (e, f), respectively.

supernatant of ID-5 was used; the other mAbs were partially purified from ascites fluid by ammonium sulfate precipitation and dialysis against TBS (Chiquet and Fambrough, 1984a). The characterization of specific antisera against chick fibroblast tenascin and human plasma fibronectin has been published (Chiquet and Fambrough, 1984a). An antiserum against mouse laminin nidogen complex was obtained from Dr Mats Paulsson ˌ(Sandoz AG, Basel). Both the anti-fibronectin and the anti-laminin antisera crossreact with, and are specific for, the corresponding chick proteins; the anti-tenascin antiserum reacts with neither fibronectin nor laminin on immunoblots (not shown). No qualitative differences in the immunofluorescence staining patterns were seen with the monoclonal *versus* the polyclonal antibodies against the same ECM protein (cf. Chiquet and Fambrough, 1984a). Igs were precipitated from antisera with ammonium sulfate, redissolved in the original volume, and dialyzed against TBS (Chiquet and Fambrough, 1984a).

Immunocytochemistry

Fertilized chick White Leghorn eggs (Gallipor, Wil, Switzerland) were incubated in a humified chamber at 37°C. Embryos were removed from eggs, staged according to Hamburger and Hamilton (1951), fixed for 6 h in 4 % paraformaldehyde in Dulbecco's phosphate-buffered saline (PBS), and immersed overnight in 25 % sucrose in PBS (Tucker *et al.* 1988). They were embedded in Tissue Tek (OCT compound; Miles), frozen on dry ice, and 15–20 μm sections were cut on a Slee cryomicrotome at −18°C. In some

instances, embryos were embedded and cut without fixation. Air-dried sections were blocked with 5 mg ml^{-1} BSA (Serva) in PBS. Antibodies were diluted in the same solution either 1:200 (anti-tenascin antiserum), 1:50 (anti-laminin and anti-fibronectin antisera), 1:100 (mAb M1 and M6 ascites), or 1:10 (ID-5 supernatant). Sections were double-labeled with different combinations of primary antibodies for 45 min at room temperature, washed with PBS/BSA, and incubated for another 45 min with second antibodies (FITC- or TRITC-labeled goat anti-mouse and goat anti-rabbit IgG, respectively; Cappel) which were diluted 1:100 in PBS/BSA. Slides were mounted in glycerol containing 10 mg ml^{-1} *n*-propyl gallate (Sigma) and 20 mM sodium phosphate, pH 7.4. They were viewed on a Olympus microscope equipped with epifluorescence optics and photographed on Ilford HP5 film.

Tissue and cell cultures

To prepare culture substrates, 12 mm circular glass coverslips were washed with 70 % ethanol, wiped with cotton gauze, air-dried, and coated with 1 mg ml^{-1} poly-L-lysine (M_r 15 000; Sigma) in 0.1 M sodium borate, pH 8.0, for 15 min. They were then washed three times with distilled water and once with PBS. A 50 μl drop of tenascin, laminin or fibronectin, respectively, diluted to 40 μg ml^{-1} in PBS was placed on each coverslip. Where indicated, cleaned coverslips not precoated with polylysine were directly incubated with ECM protein solutions. After 1–2 h at room temperature in a closed dish, coverslips were washed twice with PBS and placed into the wells of a 24-well culture dish containing DMEM (Gibco).

Sympathetic and sensory neurons were isolated and cultured essentially as described by Edgar *et al.* (1984). Sympathetic chains and dorsal root ganglia were dissected from 11 day chick embryos and placed into 0.25 % trypsin/EDTA in Puck's saline (Gibco) for 30 min at 37°C. Digestion was stopped by adding an equal volume of DMEM (Gibco) containing either 10 % FCS (Gibco) or 5 mg ml^{-1} heat-denatured (5 min, 70°C) BSA (Serva). A single cell suspension was obtained by trituration with a Pasteur pipet. Cells were centrifuged at 150 g for 5 min and resuspended in culture medium consisting of DMEM and Ham's F-12 (Gibco) mixed 1:1, to which 30 ng ml^{-1} 7S-NGF (Sigma) and either 0.3 % FCS or 0.5 mg ml^{-1} heat-treated BSA were added. Routinely, one sixth of the sympathetic neurons obtained from one embryo (about 50 000), or sensory neurons isolated from one dorsal root ganglion (about 20 000) were plated per 15 mm well in one ml of medium.

Motor neurons were isolated from 3 day embryonic spinal cord according to a procedure adapted from Henderson *et al.* (1984). The dorsal part of the embryo at the brachial region was dissected by removing the dorsal aorta and the flank mesenchyme. The tissue was incubated for 30 min at 37°C in 2 mg ml^{-1} collagenase/dispase (Boehringer Mannheim) dissolved in L-15 medium (Gibco). After washing with 5 mg ml^{-1} heat-treated BSA in L-15 medium, the neural tubes were carefully cleaned from remaining sclerotomal tissue with fine forceps. Single cell suspensions were obtained from spinal cords by trypsinization for 5 min as described above. Spinal cord neurons were resuspended and cultured in Ham's F-12 medium containing 0.5 mg ml^{-1} heat-treated BSA. Neural tissue explant cultures were prepared as described above, except that pieces of cleaned neural tube were not trypsinized, but instead plated directly onto coverslips coated with the different ECM proteins. Plain Ham's F-12 medium without supplements was used in this case.

Kinetics of neurite outgrowth

For measuring the rate of neurite outgrowth on different substrates, at least four separate culture dishes were prepared per substrate, and either sympathetic or spinal cord neurons were plated as described above. Two or more randomly chosen fields per culture dish (corresponding to 1 mm^2 each) were photographed with the inverted microscope at different time intervals. Cell number and neurite length per field were measured from the projected negatives using a graphics tablet, at a final magnification of 250×. Data are expressed as the total neurite length per field, divided by the number of sprouting neurons per field. For each time point and substrate, five or more measured values were averaged, and the standard deviations are indicated in Fig. 7 by error bars.

Results

Distribution of tenascin along developing peripheral nerves

To monitor the outgrowth of peripheral axons in developing chick embryos by immunofluorescence, we used the monoclonal antibody (mAb) ID-5 which recognizes the *C*-terminus of de-tyrosinated alpha-tubulin in stable microtubules (Wehland and Weber, 1987). At the stages investigated, this antibody was found to stain exclusively skeletal muscle fibers in the myotomes and, more brightly, the cell bodies and processes of differentiated neurons; background staining in other tissues was extremely low (Figs 2–5). In cell cultures, neuronal processes were labeled up to the base of the growth cones, whereas non-neuronal cells appeared negative (not shown; cf. Wehland and Weber, 1987). Distribution of tenascin, fibronectin and laminin along axonal pathways was detected by double immunofluorescence with the respective antisera and ID-5 antibody, or in consecutive sections with monoclonal anti-ECM antibodies (see Materials and methods).

The time course of axonal outgrowth in developing peripheral nerves was investigated in cross sections at the level of the hind limb buds. Here, the first ID-5 positive nerve fibers were detected growing out from the ventral roots of the spinal cord at embryonic day 3.5 (stage 19–20; Hamburger and Hamilton, 1951) (Fig. 2A). At this stage, tenascin had already accumulated in the ventral part of the sclerotome adjacent to the aortas, and from there extended dorsolaterally into the anterior half of each sclerotome (Fig. 2C; Tan *et al.* 1987; Mackie *et al.* 1988). Thus, a half-circle-like structure marked by anti-tenascin was formed in every sclerotome that surrounded the (still tenascin-negative) presumptive precartilage areas immediately adjacent to the notochord (Fig. 2C). The motor axons took a pathway that coincided with this tenascin-rich fibrillar matrix (Fig. 2D, E), growing first laterally before turning ventrally somewhat later (at stage 22) (Fig. 3; Rickmann *et al.* 1985). In contrast to tenascin, fibronectin staining was more homogeneous throughout the sclerotome (Fig. 2B).

At embryonic day 4 (stage 22), the proximal part of each ventral root at the hind limb level consisted of several axon fascicles which joined with sensory fibers arriving from the dorsal root ganglia (Figs 3A; 4A and B). Tenascin was strongly concentrated along and
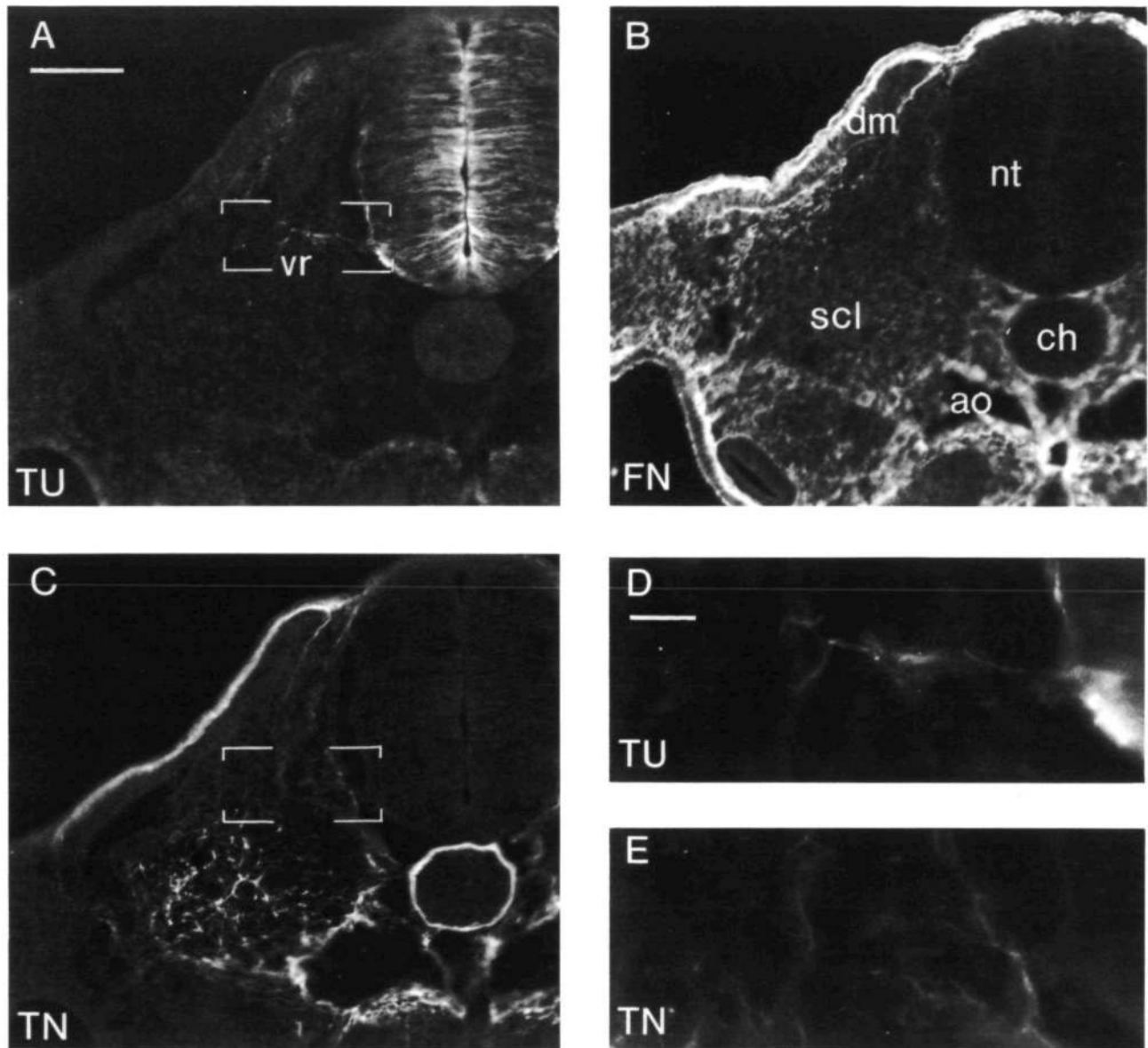
Fig. 2. Distribution of tenascin in cross sections at the hind limb level of a chick embryo, at the time when the first motor axons penetrate into the sclerotome (day 3.5; stage 19–20). Staining with anti-tubulin mAb ID-5 (A) reveals the distribution of axons in the spinal cord and the developing ventral root. For comparison, a consecutive section was labeled with anti-fibronectin antiserum (B). In C, the same section as shown in A was double-labeled with anti-tenascin antiserum. High power views in D and E correspond to the regions indicated in A and C, respectively. nt, Neural tube; vr, ventral root; ch, notochord; dm, dermamyotome; scl, sclerotome; ao, aorta. Bars, 100 μm in A–C and 25 μm in D, E.

within ventral roots (Figs 3B; 4C), but was almost absent in the presumptive precartilage between roots at this stage (Figs 3F; 4C). In oblique parasagittal sections which cut the roots longitudinally, accumulation of tenascin on the surface of axon fascicles was especially obvious (Fig. 4E). In contrast to their accumulation of tenascin, the ventral roots stained poorly for fibronectin in comparison to the surrounding mesenchyme (Figs 3C; 4D). Also, we could not observe with our antibodies that laminin was specifically enriched in the more distal parts of the ventral roots (cf. Rogers *et al.* 1986). At this stage, laminin was found in the basement membranes of the spinal cord and dermamyotomes and

in fine spots in the sclerotome. In addition, it started being expressed proximally along axon fascicles of the ventral roots, presumably in association with glial cells (Figs 3D, 4F; Rogers *et al.* 1986).

Distally, the front of outgrowing axons accumulated at day 5 (stage 25–26) in a plexus in the presumptive pelvic region (Fig. 5A, A'). Again, this region was tenascin-positive in comparison to the surrounding mesenchyme, and the distal neurites seemed to be embedded in a tenascin-containing, fibrillar ECM (Fig. 5B, B'). In the more proximal pelvic region, however, tenascin was now excluded from axon fascicles and instead marked their boundaries (Fig. 5A',
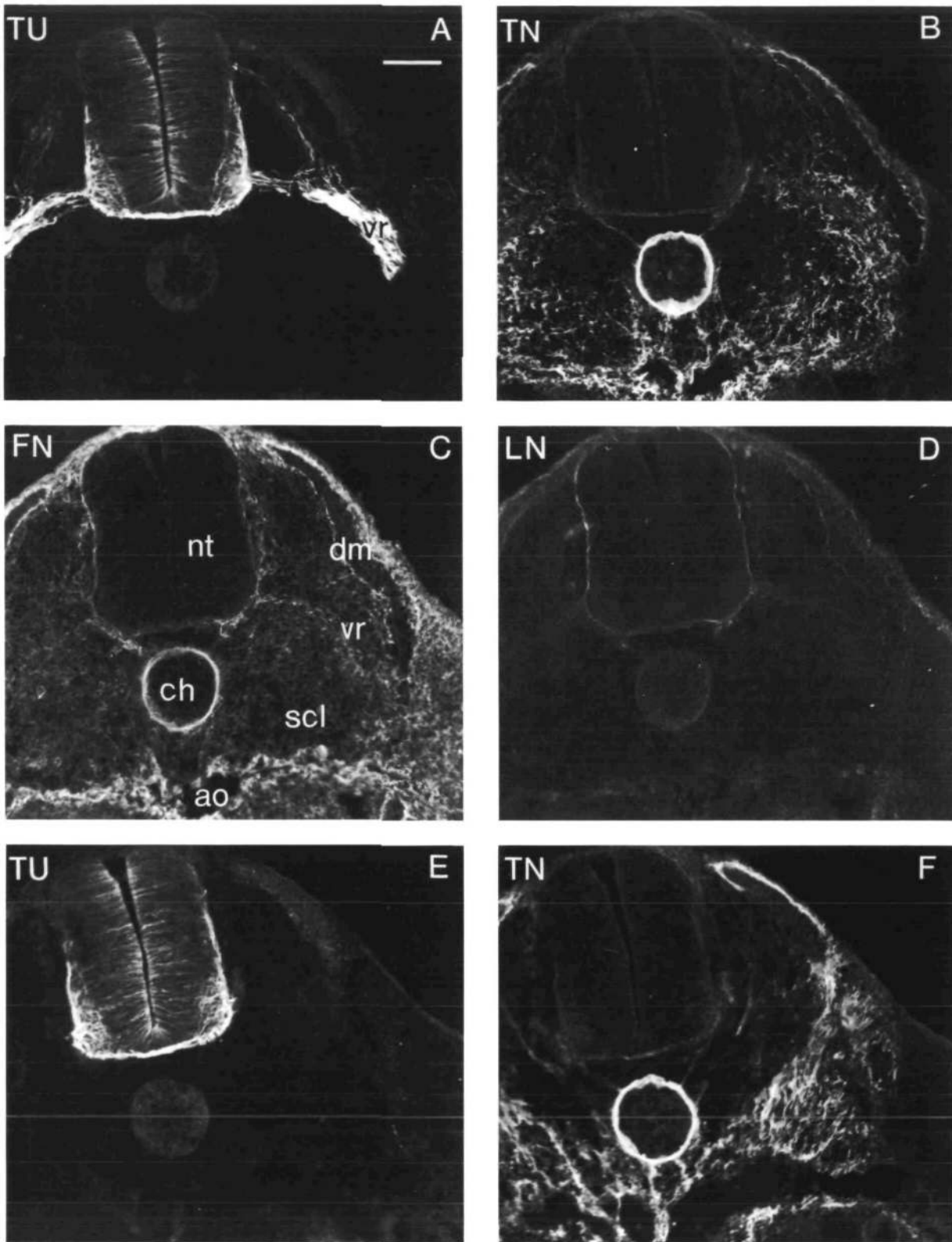
Fig. 3. Cross sections through a 4 day (stage 22) chick embryo at the level of the hind limb. Section A is through the anterior part of a somite and is labeled with anti-tubulin mAb ID-5 to show the ventral roots which have turned ventrally within the sclerotome; (B) same section double-labeled with anti-tenascin antiserum. A consecutive section stained with anti-fibronectin mAb M6 is shown in C and double-labeled with anti-laminin antiserum in D. Section E through the posterior *part of a somite, i.e. between two pairs of ventral roots, is stained with anti-tubulin; double-labeling of the same section* with anti-tenascin antiserum (F) shows heavy staining in the lateral sclerotome but not adjacent to the neural tube (compare to B). nt, Neural tube; vr, ventral root; ch, notochord; dm, dermamyotome; scl, sclerotome; ao, aorta. Bar, 100 μm.
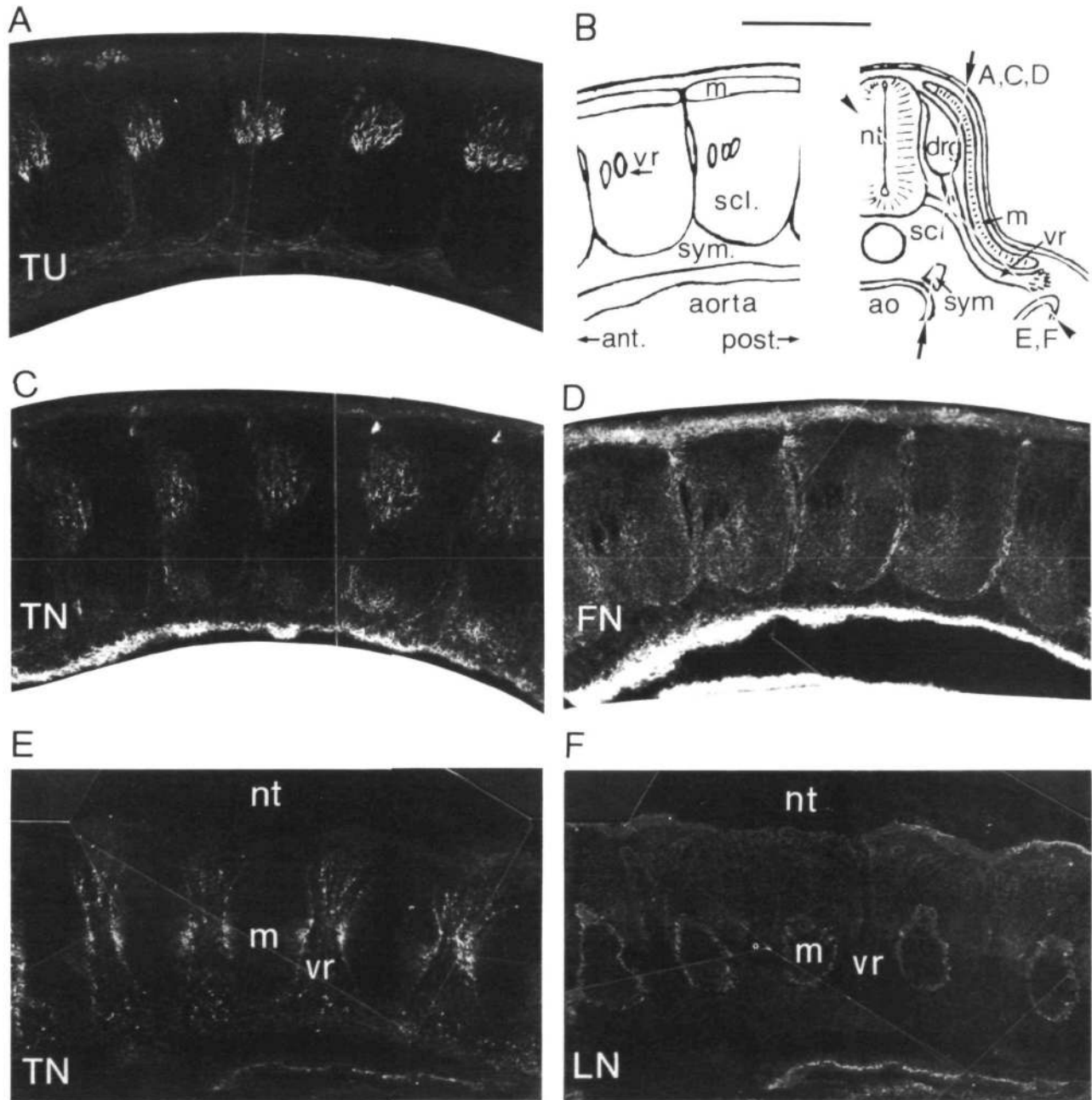
Fig. 4. Sagittal sections through the trunk region of a 4 day/stage 22 embryo, showing the accumulation of tenascin along peripheral nerves. (A, C, D) Consecutive sagittal sections which cross the ventral roots, stained with anti-tubulin mAb ID-5 (A), anti-tenascin mAb M1 (C), and anti-fibronectin mAb M6 (D). (E, F) Oblique parasagittal section in the plane of the ventral roots, double-labeled with anti-tenascin mAb M1 (E) and with anti-laminin antiserum (F). The scheme in B at left indicates the positions of different structures in the sagittal sections (A, C, D). The schematic cross section in B at right shows the planes of sections (A, C, D) (arrows) and (E, F) (arrowheads), respectively. m, Myotome; vr, ventral root; scl, sclerotome; sym, sympathetic ganglion chain; drg, dorsal root ganglia; ao, aorta; nt, neural tube; ant, anterior; post, posterior. Bar, 400 μm.

B′). The ventral roots now started to be encased completely in a precartilage matrix very rich in tenascin (Fig. 5B, B′). From proximal to the most distal levels, staining with anti-fibronectin was found to be reduced or even absent in the areas containing ID5-positive axons (Fig. 5C, C′).

At the same stage (25–26), the first tenascin-positive

labeling was also detected in the limb buds. Whereas fibronectin was found to accumulate in the precartilage cell condensations of the femur head (Fig. 5C″), anti-tenascin antibodies marked ribbon-like structures that surrounded the femur primordia (Fig. 5B, B″). Tangential sections through the limbs clearly demonstrated how the tenascin-containing areas
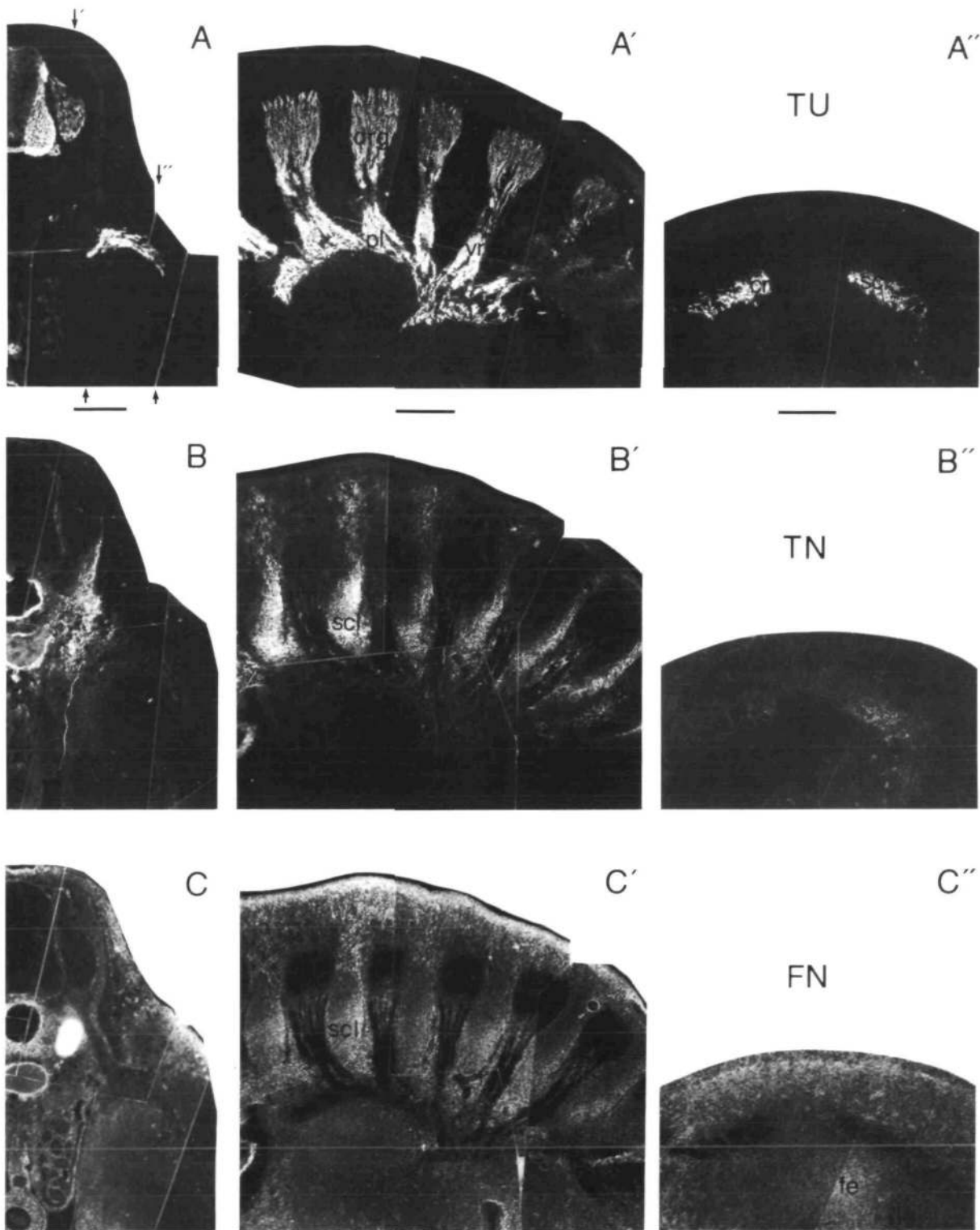
Fig. 5. Cross (A–C) and sagittal (A'–C', A"–C") sections through the hind limb region of a 5 day/stage 25–26 embryo, stained for axons with anti-tubulin mAb ID-5 (A, A', A"), for tenascin with anti-tenascin antiserum (B) or with mAb M1 (B', B"), and for fibronectin with mAb M6 (C) or with anti-fibronectin antiserum (C', C"). The image pairs (B', C'), and (B", C") each are photographed from one double-labeled section. The arrows in A mark the cutting planes of the sections (A') and (A"). Sagittal sections (A'–C') are at a proximal level, cutting the dorsal root ganglia, the ventral roots and the neural plexus longitudinally. Sections (A"–C") are also sagittal, but a more distal level within the limb bud, crossing the crural and the sciatic nerve primordia as well as the precartilage cell condensation of the femur. Note the colocalization of the plexus and limb nerves with tenascin-rich mesenchyme. drg, Dorsal root ganglia; vr, ventral roots; scl, sclerotome; pl, neural plexus; cr, crural nerve; sc, sciatic nerve; fe, femur primordium. Bars, 300 μm.

were regionally specified on the surface of the femur anlage: not the whole perimeter of the precartilage was labeled by anti-tenascin at this developmental stage (Fig. 5B″). Interestingly, these tenascin-positive 'channels' again coincided with the pathways that axons took while growing into the limb buds to establish the primordia of the crural and sciatic nerve (Fig. 5A″). We found a good correlation of ID5-positive neuronal processes with anti-tenascin staining of ECM within the 5 day limb buds (Fig. 5A, B); fibronectin again appeared less concentrated in these regions compared to the surrounding limb tissue (Fig. 5C).

At embryonic day 5.5 to 6 (stage 27–29) and later, the pattern of tenascin within the upper limbs changed, and little correlation with the nerves could be observed. Instead, perichondrium and tendon primordia began to be heavily labeled by anti-tenascin (not shown; cf. Chiquet and Fambrough, 1984a).

These immunohistochemical studies revealed that, at all stages investigated, the distal parts of developing peripheral nerves extend into a fibrillar ECM which is characterized by its pronounced accumulation of tenascin as compared to the surrounding mesenchyme. In contrast, no such positive correlation is found with the distribution of fibronectin or laminin. The pattern of tenascin along developing peripheral nerves is however, rapidly changing. At any time, tenascin is excluded from and surrounds axon fascicles in more proximal (i.e. developmentally more advanced) regions of the nerves; later, it is restricted mainly to the perineurium (cf. Chiquet and Fambrough, 1984a; Crossin et al. 1986).

Neurite outgrowth by dissociated neurons on polylysine/tenascin substrates

What is the fuction of tenascin that is accumulated in close association with the developing peripheral nervous system? To address this question, we tested how dissociated embryonic neurons respond, in terms of cell attachment and neurite outgrowth, to tenascin substrates *in vitro*. Since neurite outgrowth by embryonic sensory and sympathetic neurons on fibronectin and on laminin substrates is well studied (Humphries *et al.* 1988; Edgar *et al.* 1984), and since tenascin is expressed to some extent in developing sympathetic and dorsal root ganglia (Figs 3–5; Crossin *et al.* 1986), we started by using these neurons for reference. Most experiments reported here were done with isolated sympathetic neurons from 11 day embryos; results obtained with 8–11 day dorsal root ganglion neurons were essentially the same. Culture conditions were adjusted such that neuronal attachment and survival were optimal, but neurite outgrowth minimal, on a polylysine-coated control coverslip (Edgar *et al.* 1984).

Sympathetic and sensory neurons dissociated with trypsin did not attach well to glass or plastic substrates coated with tenascin alone (not shown). Good attachment was however observed when the culture substrate was pretreated with polylysine before adsorbing tenascin (Fig. 6A). In this case, similar numbers of cells were found to attach and survive at 24 h in culture

$(231\pm61$ per mm$^2)$ as on polylysine alone $(207\pm46$ per mm^2 (Fig. 6D). For comparison, these numbers were 246 ± 55 per mm^2 on polylysine/laminin and 191 ± 28 per mm^2 on polylysine/fibronectin, respectively. After 20 h in culture, a network of neurites had formed on polylysine/tenascin (Fig. 6A), which was less dense than on polylysine/laminin (Fig. 6B) but still considerable compared to the pure polylysine control (Fig. 6D). On polylysine/fibronectin, fewer neurites had formed, but more contaminating non-neuronal cells were found than on tenascin (Fig. 6C).

We quantified the rate of neurite outgrowth on the different substrates by measuring neurite length per sprouting neuron at different time intervals. In accordance with the results of Edgar *et al.* (1984), the sympathetic neurons started sprouting on polylysine/laminin at a fast rate immediately after plating (Fig. 7A). In contrast, neurite outgrowth was observed on polylysine/tenascin substrates only after a lag phase of several hours. Once neurites began to form, their rate of progression compared favorably to that observed on polylysine/laminin and was higher than on fibronectin-coated or pure polylysine (Fig. 7A). As a consequence of the lag phase, total neurite length per sprouting neuron on polylysine/tenascin was close to half of that observed on polylysine/laminin after 20 h although at earlier time points the difference was much larger (Fig. 7A). It should also be noted that the number of sprouting neurons as a percentage of the total cell population was higher on laminin than on tenascin and on the other substrates. At 22 h when the maximum was reached on all substrates, these values amounted to $86\pm5\%$ on laminin/polylysine, $45\pm8\%$ on tenascin/polylysine, $43\pm10\%$ on fibronectin/polylysine, and $35\pm7\%$ on polylysine alone, respectively.

To exclude the possibility that neurite outgrowth on polylysine/tenascin was due to some unrelated mechanism, we looked at the effects of anti-tenascin antibodies. Fig. 8A, B demonstrates that when anti-tenascin antiserum was added to the culture medium at the time of plating, neurite outgrowth by dorsal root ganglion neurons was completely inhibited on polylysine/tenascin. The same result was obtained with sympathetic neurons (not shown). One might have expected to still observe the small intrinsic activity of polylysine when neurons are plated on polylysine/tenascin in the presence of anti-tenascin. We assume that the additional antibody coat sterically blocks residual polylysine activity.

Anti-tenascin antibodies were also able to detach neurites after they had formed on polylysine/tenascin substrates. When anti-tenascin antiserum was added to sympathetic neurons after 24 h in culture, neurites retracted on tenascin- but not on laminin-coated polylysine substrates (Fig. 8D, E); the reverse result was obtained with anti-laminin antiserum (Fig. 8C, F). These results showed that the substrate-bound tenascin itself was involved in neurite outgrowth under these conditions.

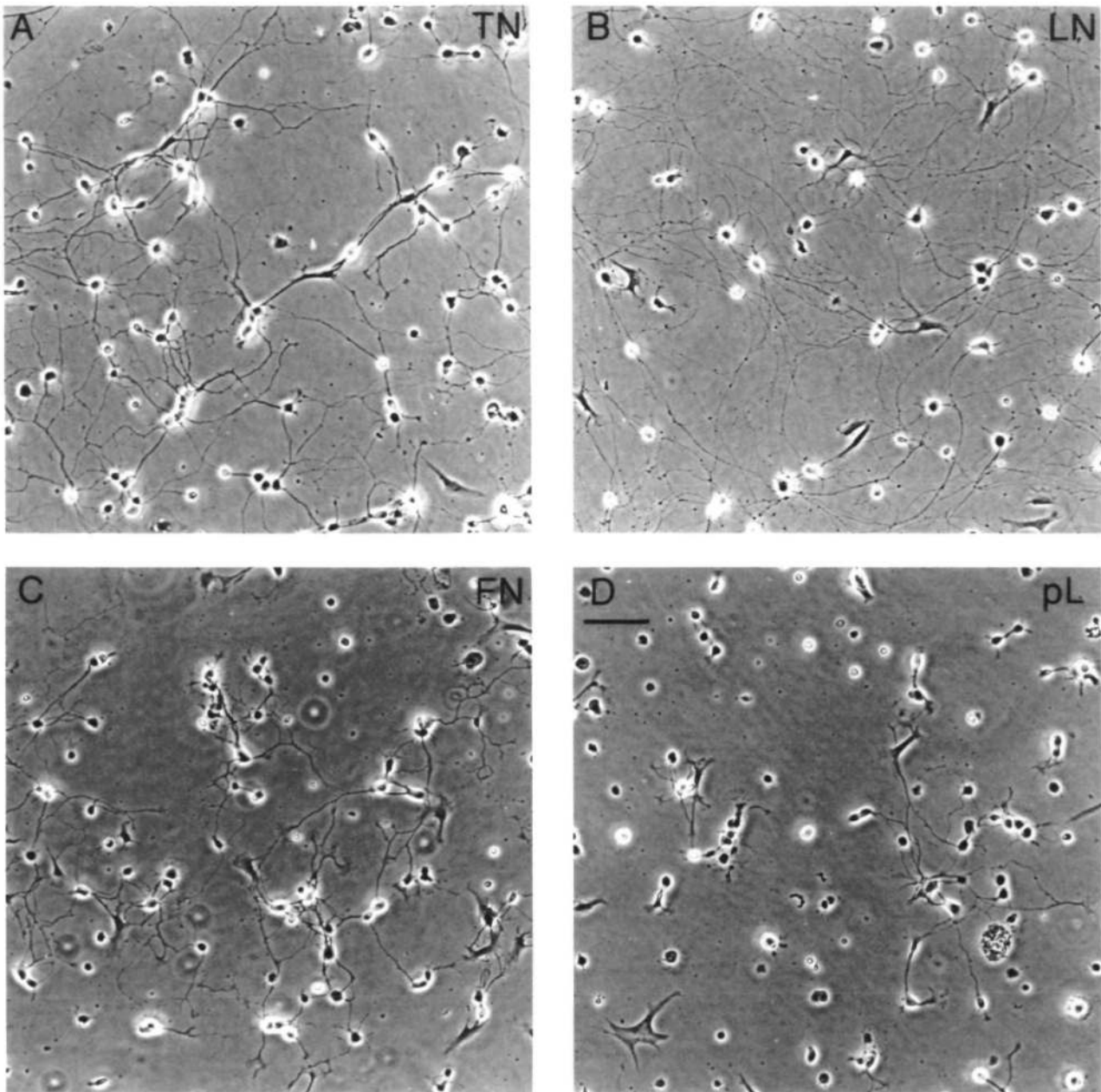Since tenascin is associated with developing ventral

Fig. 6. Sympathetic neurons from 11 day embryos cultured on glass coverslips which were coated first with polylysine, then with tenascin (A), laminin (B), or fibronectin (C), or with polylysine alone (D). Photographs were taken at 20 h. Bar, 100 μm.

roots and motor nerves *in vivo* (Figs 2–5), we also asked whether cultured embryonic motor neurons grew neurites on tenascin substrates. As a source for dissociated cells, 3 day (stage 18) embryonic spinal cords were chosen because at this time the motor neurons constitute the majority of differentiated neurons there, and because their axons just start to leave the cord through the ventral roots *in vivo* (Holliday, 1980; Fig. 2). As shown in Fig. 9A, dissociated 3 day spinal cord cultures contained many neurons with long processes on a polylysine/tenascin substrate after 18 h in culture. For this type of neurons, the growth pattern (Fig. 9A, B) and growth rates (Fig. 7B) on tenascin-

versus laminin-coated polylysine looked remarkably similar. Polylysine/fibronectin-coated substrates promoted neurite outgrowth as well, but at a lower rate (Fig. 7B), and in addition many spread non-neuronal cells were observed in these cultures (Fig. 9C). In contrast, poor outgrowth was obtained on polylysine alone in the serum- and growth-factor-free medium which was used (Fig. 7B, 9D).

Since spinal cord cultures contain many non-neuronal and undifferentiated neuronal cells, the percentage of cells that formed neurites was expectedly much lower than in sympathetic neuron cultures (cf. Figs 6 and 9), but very similar on all substrates (between 11 % and
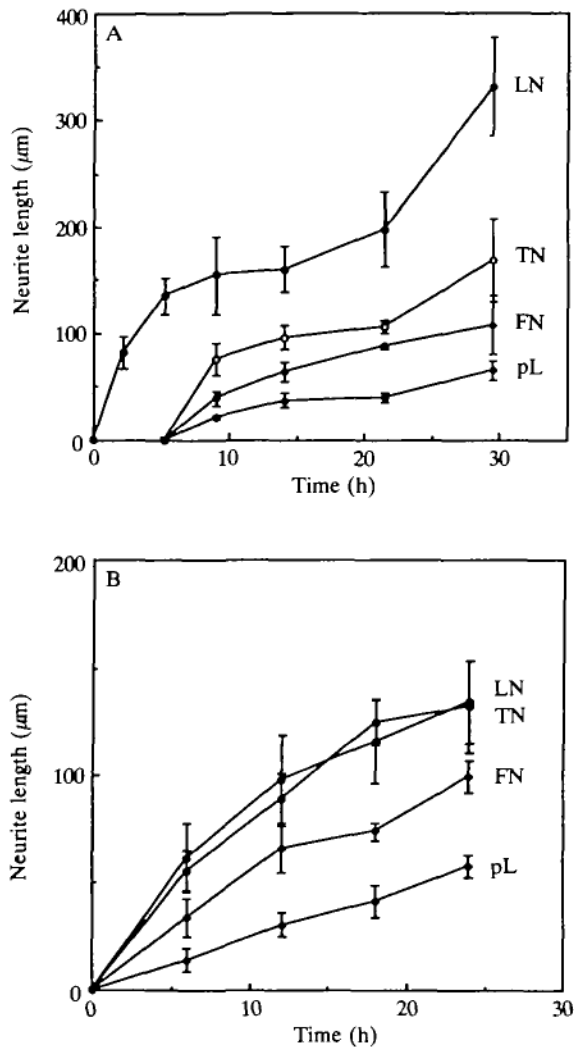
Fig. 7. Kinetics of neurite outgrowth by 11 day sympathetic neurons (A) or 3 day spinal cord neurons (B) which were plated on glass coverslips coated with polylysine alone (◇), or with polylysine followed by fibronectin (◆), tenascin (○), or laminin (●). For experimental procedures and evaluation of data, see Materials and methods.

14 %). In the case of spinal cord neurons, similar results were obtained when the polylysine precoating was omitted. That is, attachment of neurons and subsequent sprouting could be observed on plain tenascin substrates (not shown).

*Neurite outgrowth by neuronal explants on plain tenascin substrates*

We asked whether neurite outgrowth by motor neurons could be obtained from undissociated explants of spinal cord on tenascin substrates. Under these conditions, enzymatic treatment to disperse the neurons is avoided, and attachment of the neuronal cell bodies to the culture substrate is not a prerequisite for the establishment of growth cones and for sprouting. Fig. 10 shows spinal cord explants from 3 day embryos plated for 25 h on either polylysine-coated or untreated glass to which tenascin, laminin, or fibronectin was adsorbed. Surpris-

ingly, many but short neurites were produced on all three ECM substrates when polylysine was used for precoating (Fig. 10A, C, E), and the growth pattern was similar to that of the polylysine control (Fig. 10G). Better and more discriminative results were obtained when glass was directly coated with ECM proteins. Neurites with well-spread growth cones at their tips were found on plain tenascin (Fig. 10B), laminin (Fig. 10D) and fibronectin (Fig. 10F), but few on uncoated glass (Fig. 10H). Maximal neurite length at a given time was similar on all three ECM proteins, but their morphology differed (Fig. 10B, D, F). In contrast to fibronectin, tenascin caused the neurite shafts to be very straight, mostly unbranched and poorly attached. Observations of single neurites with time showed that the growth cones advanced very rapidly on plain tenascin, but that the neurites were unstable and tended to collapse (not shown). Branching and bending of processes were frequent on fibronectin compared to tenascin; neurites on laminin showed an intermediate morphology (Fig. 10).

From these findings, we conclude that a plain tenascin substrate allows the adhesion and movement of growth cones at the tips of advancing neurites, but mediates only poor attachment of the neurites themselves. Presumably as a consequence of weak attachment, branching of processes on tenascin alone is infrequent.

Discussion

The molecular mechanism by which outgrowing peripheral nerves are guided into the developing limb buds of vertebrate embryos are still largely unknown (Holliday, 1980). At the time when the first motor axons leave the spinal chord, they rely on local cues rather than on signals from target limb muscles which only start to develop (Lewis *et al.* 1983; Tosney and Landmesser, 1984). Transplantation experiments have shown that motor neuron pools acquire the specificity to innervate certain limb muscles *before* their axons leave the spinal chord (Lance-Jones and Landmesser, 1980). Nevertheless, motor axons first grow in bundles along common pathways whose spatial and temporal patterns are specified by the somite and limb mesenchyme (Lewis *et al.* 1983), and they will accept an ectopic pathway if offered one experimentally (Lance-Jones and Landmesser, 1981). Since adhesive ECM glycoproteins are thought to be involved in specifying migratory pathways in the embryo (Boucaut *et al.* 1984) and are known to promote neurite outgrowth *in vitro* (Edgar *et al.* 1984; Humphries *et al.* 1988), we looked at the distribution of fibronectin, laminin and tenascin along developing peripheral nerves. Of the three proteins, tenascin was found to be enriched in a fibrillar ECM in pathways of peripheral axons at their distal ends. This pattern of expression is transient in the sense that tenascin-positive matrix is rapidly excluded from and surrounds axon fascicles. Later, the developing nerves are often (but not always) in contact with or encased by a
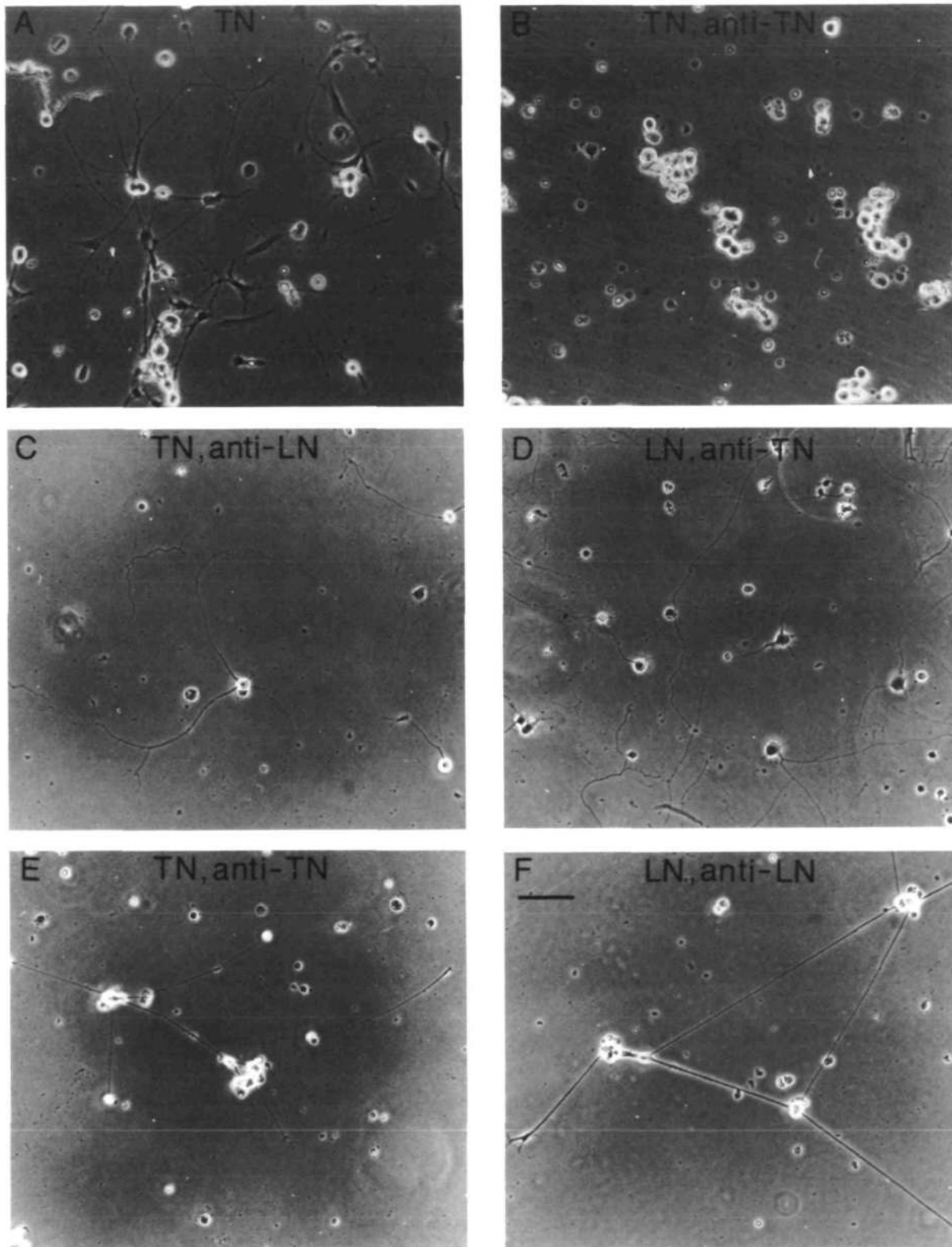
Fig. 8. Effect of anti-tenascin antiserum on neurite outgrowth on substrates coated with polylysine and then either with tenascin or laminin. A control culture of dorsal root ganglion neurons from 11 days embryos on a polylysine/tenascin substrate is shown in A. To a parallel culture, anti-tenascin antiserum (diluted 1:50) was added at the time of plating. Neurons in A, B were photographed at 20 h. In C–F, 11 day sympathetic neurons were allowed to grow neurites for 24 h on polylysine/tenascin or polylysine/laminin before the addition of the respective antisera (diluted 1:100), and cultures were photographed 24 h later. (C) Tenascin substrate, anti-laminin added; (D) laminin substrate, anti-tenascin added; (E) tenascin substrate, anti-tenascin added; (F) laminin substrate, anti-laminin added. Bar, 100 μm.
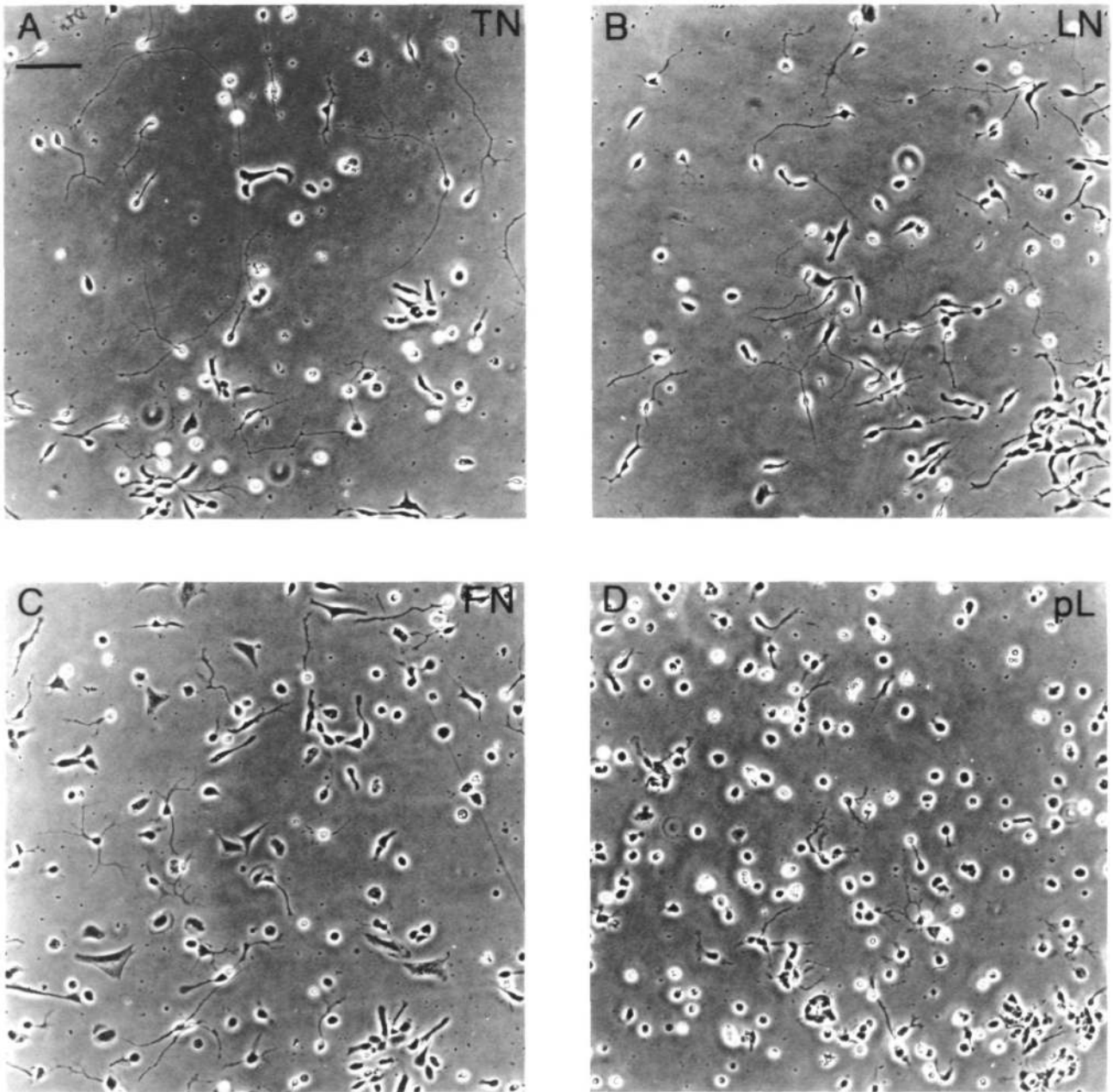
Fig. 9. Spinal cord neurons from 3 day embryos grown on glass coverslips which were coated with polylysine followed by tenascin (A), laminin (B), or fibronectin (C), or with polylysine alone. Photographs taken at 20 h. Bar, 100 µm.

tenascin-rich matrix such as perichondrium and precartilage, but are themselves devoid of tenascin (Chiquet and Fambrough, 1984a; Crossin *et al.* 1986). In adult peripheral nerves, tenascin is found in the perineurium (Chiquet and Fambrough, 1984a) and at the nodes of Ranvier (Rieger *et al.* 1986).

The association of tenascin-rich ECM with the tips of growing peripheral nerves of course raises the question about the biosynthetic origin of this material. Is tenascin produced in response to the ingrowing axons, or is its expression pattern specified independently by mesenchymal cells? Only in the latter case, tenascin could be involved in defining axonal pathways. A similar argument has been raised concerning the accumulation

of tenascin along neural crest pathways in the anterior half of the sclerotomes (Tan *et al.* 1987; Mackie *et al.* 1988), which seems to occur as a consequence of crest cell migration (Stern *et al.* 1989). Stern *et al.* (1989) therefore claimed that neither the segmented pattern of neural crest cells, nor that of motor axons can be caused by the differential distribution of tenascin in the sclerotome. Their argument was based on the observation that ablation of the neural crest changed the tenascin pattern in the sclerotome (Stern *et al.* 1989), yet the motor nerve pattern appeared normal in an earlier set of experiments where the same operation had been made (Rickmann *et al.* 1985). However, the actual distribution of tenascin along subsequently developing
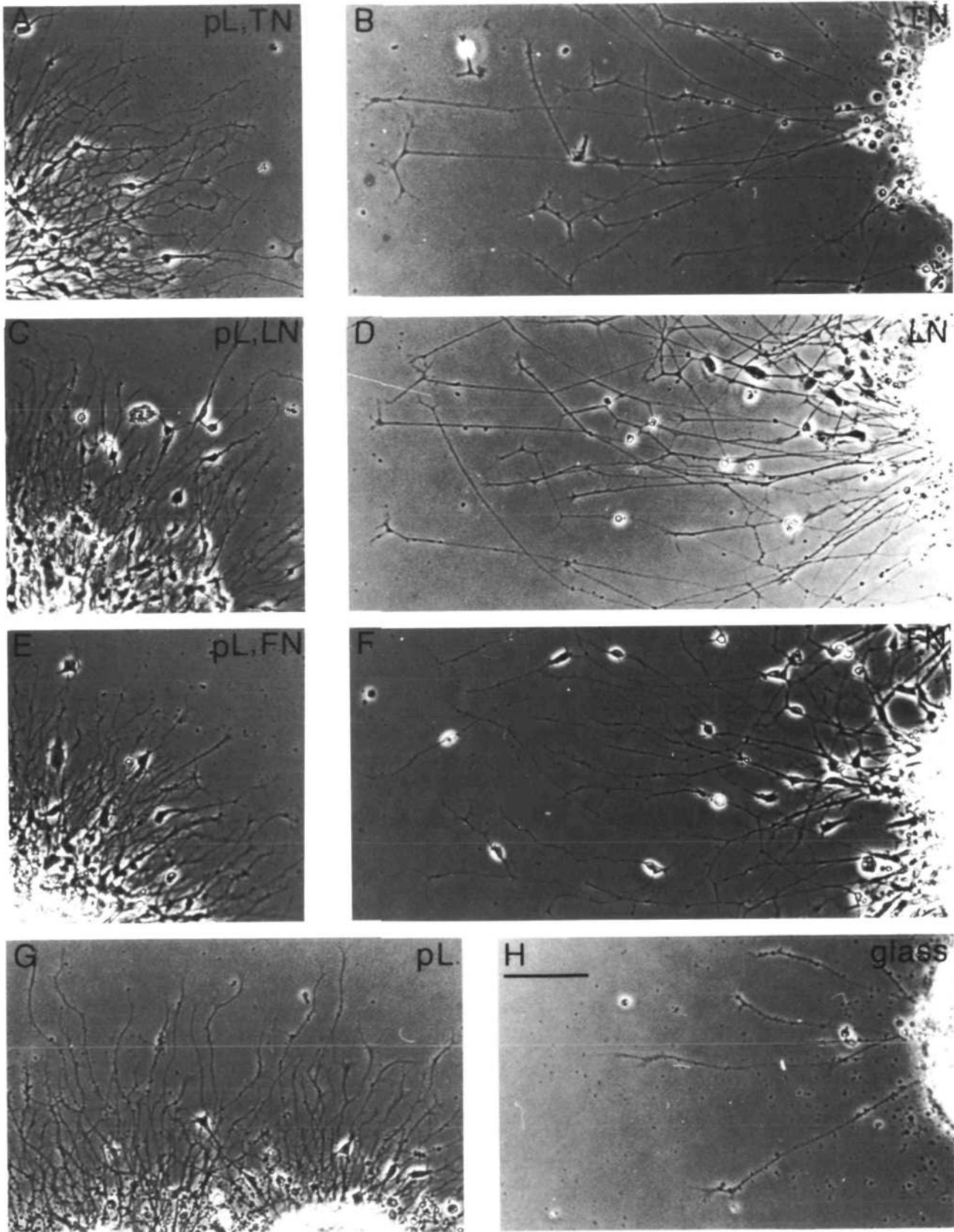
Fig. 10. *Spinal cord explants from 3 day embryos cultured for 25 h on polylysine-treated (left row; A, C, E, G) or untreated* (right row; B, D, F, H) glass coverslips that were coated in addition with tenascin (A, B), laminin (C, D) or fibronectin (E, F). Polylysine and pure glass control substrates are shown in G and H, respectively. Bar, 100 μm.

ventral roots has not been looked at by these authors, either in normal or in operated embryos (Stern *et al.* 1989).

With the current study, we did not try to solve the difficult problem of whether the localized tenascin expression is one of the causes or a mere consequence of the peripheral nerve pattern. Both mesenchymal and glial cells are able to produce tenascin (Chiquet *et al.* 1984b; Grumet *et al.* 1985), and glial cells are known to follow the outgrowing peripheral axons very closely (Noakes *et al.* 1988). It is thus possible that much of the tenascin found between axon fascicles is of glial, not mesenchymal origin. However, we have preliminary evidence indicating that the early tenascin pattern in the limb is produced at least in part by mesenchymal cells independently of the ingrowing nerves. Very young (stages 18–20) limb buds were transplanted into the coelomic cavity of host embryos where they developed without being invaded by nerves. Although tenascin expression was somewhat delayed in the grafts and weaker compared to controls, its ribbon-like pattern around the precartilage areas of the grafts looked quite normal (B. Wehrle, in preparation).

A more immediate problem concerns the function of the tenascin, which is associated with developing nerves, irrespective of its cellular origin. Since tenascin surrounds axon fascicles in more proximal regions of the nerves, does it prevent axons leaving their tracts? Inhibitors of axonal growth are thought to be an important principle of neural development (Caroni and Schwab, 1988), and a similar function has been suggested for the J1/tenascin which transiently marks the barrel field boundaries in the developing somatosensory cortex (Steindler *et al.* 1989; Crossin *et al.* 1989). Excess exogenous tenascin interferes with mesenchymal cell spreading and migration mediated by fibronectin (Chiquet-Ehrismann *et al.* 1988; Riou *et al.* 1990), and a similar mechanism could have been expected to act on growing axons. Alternatively, does tenascin at the tips of growing nerves promote growth cone movements and axon extension? Although these two mechanisms seem to be mutually exclusive, our cell and tissue culture experiments indicate that both might apply. Clearly, plain tenascin is not a potent substrate for the attachment of neuronal cell bodies and neurites. However, the migratory organelles of neurons, the growth cones, *are* able to attach and locomote on tenascin alone. In fact, a similar behavior has been observed for neural crest cells on tenascin substrates: whereas the cell bodies as a whole were not able to attach and spread efficiently, they did extend microspikes and filopodia onto the tenascin substrate, resulting in rapid (yet undirected) cell locomotion (Halfter *et al.* 1989). Thus, we speculate that the response of cell surfaces to tenascin might be regionally specified. For example, migratory organelles but not the rest of the cell body might express tenascin receptors, or different types of receptors might be found in different areas of the cell surface.

In addition, different neuron types might express ECM receptors in a stage-specific manner (Hall *et al.*

1987) and thus exhibit various responses to tenascin, laminin and fibronectin in their environment. We found here that sensory and sympathetic neurons from 11 day old embryos do not attach to plain tenascin, in contrast to 3 day spinal cord neurons. We do not know yet whether and how these two cell types differ in their temporal expression of tenascin receptors which, in this case, have not been identified. So far, a tenascin-specific integrin has been reported from glioma cells (Bourdon and Ruoslahti, 1989).

Our results clearly demonstrate that a tenascin substrate is permissive, not inhibitory, for growth cone movements, and that growing axons are able to invade tenascin-containing ECM. Thus, tenascin cannot simply inhibit axons leaving their fiber tracts (Caroni and Schwab, 1988). However, since the neurite shafts attach poorly to tenascin, their fasciculation *via* cell surface adhesion molecules might be stimulated and branching might be inhibited. It is characteristic for developing motor nerves that they grow out in bundles from the very beginning and only diverge and branch when contacting their target muscles. A functional interplay between different cell attachment molecules (N-CAM and L1) is involved in the control of the final branching of motor axons on developing muscle fibers (Landmesser *et al.* 1988). In addition, ECM proteins like laminin, fibronectin and tenascin might shift the balance between nerve fasciculation and branching in opposite ways, by modulating the activity of cell attachment molecules.

References

BOUCAUT, J. C., DARRIBERE, T., POOLE, T. J., AOYAMA, H. AND YAMADA, K. M. (1984). Biologically active synthetic peptides as probes of embryonic development: A competitive inhibitor of fibronectin function inhibits gastrulation in amphibians embryos and neural crest cell migration in avian embryos. *J. Cell Biol.* **99**, 1822–1830.

BOURDON, M. A. AND RUOSLAHTI, E. (1989). Tenascin mediates cell attachment through an RGD-dependent receptor. *J. Cell Biol.* **108**, 1149–1155.

BRONNER-FRASER, M (1988). Distribution and function of tenascin during cranial neural crest development in the chick. *J. Neurosci. Res.* **21**, 135–147.

CARONI, P. AND SCHWAB, M. E. (1988). Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. *Neuron* **1**, 85–96.

CHIQUET, M. (1989). Tenascin/J1/cytotactin: The potential function of hexabrachion proteins in neural development. *Devl Neurosci.* **11**, 266–275.

CHIQUET, M. AND FAMBROUGH, D. M. (1984a). Chick myotendinous antigen. I. A monoclonal antibody as a marker for tendon and muscle morphogenesis. *J. Cell Biol.* **98**, 1926–1936.

CHIQUET, M. AND FAMBROUGH, D. M. (1984b). Chick myotendinous antigen. II. A novel extracellular glycoprotein complex consisting of large disulfide-linked subunits. *J. Cell Biol.* **98**, 1937–1946.

CHIQUET, M., MASUDA-NAKAGAWA, L. AND BECK, K. (1988). Attachment to an endogenous laminin-like protein initiates sprouting by leech neurons. *J. Cell Biol.* 107, 1189–1198.

CHIQUET-EHRISMANN, R. (1990). What distinguishes tenascin from fibronectin? *FASEB J.* (in press).

CHIQUET-EHRISMANN, R., KALLA, P., PEARSON, C. A., BECK, K. AND CHIQUET, M. (1988). Tenascin interferes with fibronectin action. *Cell* 53, 383–390.

CHIQUET-EHRISMANN, R., MACKIE, E. J., PEARSON, C. A. AND SAKAKURA, T. (1986). Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell* 47, 131–139.

CHUONG, C. M., CROSSIN, K. L. AND EDELMAN, G. M. (1987). Sequential expression and differential function of multiple adhesion molecules during the formation of cerebellar cortical layers. *J. Cell Biol.* 104, 331–342.

CROSSIN, K. L., HOFFMAN, S., GRUMET, M., THIERY, J.-P. AND EDELMAN, G. M. (1986). Site-restricted expression of cytotactin during development of the chicken embryo. *J. Cell Biol.* 102, 1917–1930.

CROSSIN, K. L., HOFFMAN, S., TAN, S.-S. AND EDELMAN, G. M. (1989). Cytotactin and its proteoglycan ligand mark structural and functional boundaries in somatosensory cortex in the early postnatal mouse. *J. Cell Biol.* 136, 381–392.

DANILOFF, J. K., CROSSIN, K. L., PINCON-RAYMOND, M., MURAWSKI, M., RIEGER, F. AND EDELMAN, G. M. (1989). Expression of cytotactin in the normal and regenerating neuromuscular system. *J. Cell Biol.* 108, 625–635.

EDGAR, D., TIMPL, R. AND THOENEN, H. (1984). The heparin binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. *EMBO J.* 3, 1463–1468.

ERICKSON, H. P. AND BOURDON, M. A. (1989). Tenascin: an extracellular matrix protein prominent in specialized embryonic tissues and tumors. *Annu. Rev. Cell Biol.* 5, 71–92.

GRUMET, M., HOFFMAN, S., CROSSIN, K. L. AND EDELMAN, G. M. (1985). Cytotactin, an extracellular matrix protein of neural and non-neuronal tissues that mediates glia-neuron interaction. *Proc. natn. Acad. Sci. U.S.A.* 82, 8075–8079.

HALFTER, W., CHIQUET-EHRISMANN, R. AND TUCKER, R. P. (1989). The effect of tenascin and embryonic basal lamina on the behavior and morphology of neural crest cells *in vitro*. *Devl Biol.* 132, 14–25.

HALL, D. E., NEUGEBAUER, K. M. AND REICHARDT, L. F. (1987). Embryonic neural cell response to extracellular matrix proteins: developmental changes and effects of the cell substratum attachment antibody (CSAT). *J. Cell Biol.* 104, 623–634.

HAMBURGER, V. AND HAMILTON, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* 88, 29–92.

HENDERSON, C. E., HUCHET, M. AND CHANGEUX, J.-P. (1984). Neurite-promoting activities for embryonic spinal neurons and their developmental changes in the chick. *Devl Biol.* 104, 336–347.

HOLLIDAY, M. (1980). Motoneuron histogenesis and the development of limb innervation. *Current Topics in devl Biol.* 15, 181–215.

HUMPHRIES, M. J., AKIYAMA, S. K., KOMORIYA, A., OLDEN, K. AND YAMADA, K. M. (1988). Neurite extension of chicken peripheral nervous system neurons on fibronectin: relative importance of specific adhesion sites in the central cell binding domain and the alternatively spliced type III connecting segment. *J. Cell Biol.* 106, 1289–1297.

LANCE-JONES, C. AND LANDMESSER, L. (1980). Motoneuron projection patterns in the chick hind limb following early partial spinal cord reversals. *J. Physiol. Lond.* 302, 581–602.

LANCE-JONES, C. AND LANDMESSER, L. (1981). Pathway selection by embryonic chick motoneurons in an experimentally altered environment. *Proc. R. Soc. Lond.* B 214, 19–52.

LANDMESSER, L., DAHM, L., SCHULTZ, K. AND RUTISHAUSER, U. (1988). Distinct roles for adhesion molecules during innervation of embryonic chick muscle. *Devl Biol.* 130, 645–670.

LEWIS, J., AL-GHAITH, L., SWANSON, G. AND KHAN, A. (1983). The control of axon outgrowth in the developing chick wing. In *Limb*

Development and Regeneration (ed. J. F. Fallon and A. I. Caplan) Part A, pp. 195–205. New York: Alan Liss.

LOTZ, M. M., BURDSAL, C. A., ERICKSON, H. P. AND McCLAY, D. R. (1989). Cell adhesion to fibronectin and tenascin: quantitative measurements of initial binding and subsequent strengthening response. *J. Cell Biol.* 109, 1795–1805.

MACKIE, E. J., TUCKER, R. P., HALFTER, W., CHIQUET-EHRISMANN, R. AND EPPERLEIN, H. H. (1988). The distribution of tenascin coincides with pathways of neural crest cell migration. *Development* 102, 237–250.

NOAKES, P. G., BENNETT, M. R. AND STRATFORD, J. (1988). Migration of Schwann cells and axons into developing chick forelimb muscles following removal of either the neural tube or the neural crest. *J. comp. Neurol.* 277, 214–233.

PAULSSON, M., AUMAILLEY, M., DEUTZMANN, R., TIMPL, R., BECK, K. AND ENGEL, J. (1987). Laminin-nidogen complex: extraction with chelating agents and structural characterization. *Eur. J. Biochem.* 166, 11–19.

PEARSON, C. A., PEARSON, D., SHIBAHARA, S., HOFSTEENGE, J. AND CHIQUET-EHRISMANN, R. (1988). Tenascin: cDNA cloning and induction by TGF-beta. *EMBO J.* 7, 2977–2981.

RICKMANN, M., FAWCETT, J. W. AND KEYNES, R. J. (1985). The migration of neural crest cells and the growth of motor axons through the rostral half of the chick somite. *J. Embryol. exp. Morph.* 90, 437–455.

RIEGER, F., DANILOFF, J. K., PINCON-RAYMOND, M., CROSSIN, K. L., GRUMET, M. AND EDELMAN, G. M. (1986). Neural cell adhesion molecules and cytotactin are colocalized at the node of Ranvier. *J. Cell Biol.* 103, 379–391.

RIOU, J.-F., SHI, D.-L., CHIQUET, M. AND BOUCAUT, J.-C. (1988). Expression of tenascin in response to neural induction in amphibian embryos. *Development* 104, 511–524.

RIOU, J.-F., SHI, D.-L., CHIQUET, M. AND BOUCAUT, J.-C. (1990). Exogenous tenascin inhibits mesodermal cell migration during amphibian gastrulation. *Devl Biol.* 137, 305–317.

ROGERS, S. L., EDSON, K. J., LETOURNEAU, P. C. AND McLOON, S. C. (1986). Distribution of laminin in the developing peripheral nervous system of the chick. *Devl Biol.* 113, 429–435.

SANES, J. R., SCHACHNER, M. AND COVAULT, J. (1986). Expression of several adhesive macromolecules, N-CAM, L1, J1, NILE, uvomorulin, laminin, fibronectin, and a heparan sulfate proteoglycan in embryonic, adult, and denervated adult skeletal muscle. *J. Cell Biol.* 102, 420–431.

SPRING, J., BECK, K. AND CHIQUET-EHRISMANN, R. (1989). Two contrary functions of tenascin: dissection of the active sites by recombinant tenascin fragments. *Cell* 59, 325–334.

STEINDLER, D. A., COOPER, N. G. F., FAISSNER, A. AND SCHACHNER, M. (1989). Boundaries defined by adhesion molecules during development of the cerebral cortex: The J1/tenascin glycoprotein in the mouse somatosensory cortical barrel field. *Devl Biol.* 131, 243–260.

STERN, C. D., NORRIS, W. E., BRONNER-FRASER, M., CARLSON, G. J., FAISSNER, A., KEYNES, R. J. AND SCHACHNER, M. (1989). J1/tenascin-related molecules are not responsible for the segmented pattern of neural crest cells or motor axons in the chick embryo. *Development* 107, 309–319.

TAN, S.-S., CROSSIN, K. L., HOFFMAN, S. AND EDELMAN, G. M. (1987). Asymmetric expression in somites of cytotactin and its proteoglycan ligand is correlated with neural crest cell distribution. *Proc. natn. Acad. Sci. U.S.A.* 84, 7977–7981.

TOSNEY, K. W. AND LANDMESSER, L. T. (1984). Pattern and specificity of axonal outgrowth following varying degrees of chick limb bud ablation. *J. Neurosci.* 4, 2518–2527.

TUCKER, R. P., BINDER, L. I. AND MATUS, A. I. (1988). Neuronal microtubule-associated proteins in the embryonic avian spinal cord. *J. comp. Neurol.* 271, 44–55.

WEHLAND, J. AND WEBER, K. (1987). Turnover of the carboxy-terminal tyrosine of alpha-tubulin and means of reaching elevated levels of detyrosination in living cells. *J. Cell Sci.* 88, 185–203.