

Modulation of Growth Cone Morphology by Substrate-Bound Adhesion Molecules

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The growth cone, a terminal structure on developing and regenerating axons, is specialized for motility and guidance functions. In vivo the growth cone responds to environmental cues to guide the axon to its appropriate target. These cues are thought to be responsible for position-specific morphological changes in the growth cone, but the molecules that control growth cone behavior are poorly characterized. We used scanning electron microscopy to analyze the morphology of retinal ganglion cell growth cones in vitro on different adhesion molecules that axons normally encounter in vivo. L1/8D9, N-cadherin, and laminin each induced distinctive morphological characteristics in growth cones. Growth cones elaborated lamellipodial structures in response to the cell adhesion molecules L1/8D9 and N-cadherin, whereas laminin supported filopodial growth cones with small veils. On L1/8D9, the growth cones were larger and produced more filopodia. Filopodial associations between adjacent growth cones and neurites were frequent on L1/8D9 but were uncommon on laminin or N-cadherin. These results demonstrate that different adhesion molecules have profoundly different effects on growth cone morphology. This is consistent with previous reports suggesting that changes in growth cone morphology in vivo occur in response to changes in substrate composition.

Key words: N-cadherin, L1, laminin, neurite outgrowth, neuronal guidance, filopodia

INTRODUCTION

One of the most fascinating types of cell motility is the migration of an axon over very long distances to its target. A key to understanding both neuronal guidance and the specificity of neuronal connections lies in the growth cone. This terminal appendage of developing and regenerating axons has structures specialized for motility and guidance functions. Growth cones extend and retract both lamellipodia, which advance the cell margin, and filopodia, which are thought to direct and augment axon elongation. Studies of growth cones in vivo have revealed that their morphologies can change, apparently in response to local environmental cues. This is most apparent at points where axon bundles divide or fuse. Examples of these “decision regions” are the optic chiasm and the lumbar plexus. In decision regions where the need for accurate direction finding is the most acute, growth cone structure frequently becomes most elaborate [Tosney and Landmesser, 1985; Caudy and Bentley, 1986; Bovolenta and Mason, 1987]. The guidance cues

to which growth cones normally respond are poorly defined at present. Likely candidates for such environmental signals include chemoattractant gradients, growth inhibitory molecules, and adhesive pathways defined by neural or glial adhesion molecules (see [Strittmatter and Fishman, 1991] for review). Growth cone guidance has been manipulated in vitro on surfaces patterned with different substrate molecules [Letourneau, 1975; Gundersen, 1987; Vielmetter et al., 1990; Lemmon et al., in press]. Various substrate-bound molecules strongly influence neuritic outgrowth characteristics [Roufa et al., 1983; Gundersen, 1987; Gundersen, 1988; Lemmon et al., 1989], but relatively little is known about the effect of these materials on growth cone structure. There-

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fore, we have investigated the influence of different adhesion molecules on growth cone morphology.

The substrates tested in this study were chosen for their likely relevance *in vivo*. L1/8D9 is a CAM found predominantly on long projection axons [Lemmon and McLoon, 1986]. Immunohistochemical studies indicate that L1-like molecules are more highly concentrated on axons than on cell bodies [Rathjen and Rutishauser, 1984; Fischer et al., 1985; Stallcup and Beasley, 1985; Lemmon and McLoon, 1986; Rathjen et al., 1987]. N-cadherin is a calcium-dependent CAM found on glia and on neuronal soma, dendrites, and axons [Hatta et al., 1987]. Both purified L1 and N-cadherin support axon growth *in vitro* [Lagenaur and Lemmon, 1987; Bixby and Zhang, 1990]. Laminin is a glycoprotein component of basal laminae, and it is expressed transiently at non-basal laminae sites in the developing optic pathway in the chick [Cohen et al., 1987] and rat [McLoon et al., 1988]. Laminin also promotes the extension of CNS neurites in culture [Rogers et al., 1983; Drazba and Lemmon, 1990]. Poly-L-lysine (PLL) was also tested because it is such a widely used substrate in neuronal cell culture.

We analyzed the morphology of retinal ganglion cell growth cones on L1/8D9, N-cadherin, laminin, and PLL to evaluate structural differences that might account for the widely divergent neurite outgrowth behavior that has been reported on these substrates. We found that growth cones elaborate lamellipodial structures in response to the CAMs L1/8D9 and N-cadherin, whereas laminin supported filopodial growth cones with small veils. Growth cones on PLL were the smallest and they had small lamellipodia. On L1/8D9, the growth cones were larger and more filopodia were extended than on other substrates. Filopodial associations between adjacent growth cones and neurites were frequent on L1/8D9, but they were uncommon on laminin or N-cadherin. These results demonstrate that growth cones exhibit fundamentally different responses to different substrates. This is consistent with the evolving idea that growth cones are specialized to sense extracellular signals via transduction systems that modulate the intracellular mechanical systems for determining axonal growth [Strittmatter and Fishman, 1991].

MATERIALS AND METHODS

Substrate Preparation

The cell adhesion molecules L1/8D9 and N-cadherin were purified by immunoaffinity chromatography from embryonic chicken brain with monoclonal antibodies 8D9 (L1/8D9) or NCD-2.1 (N-cadherin) conjugated to columns of Affi-Gel 10 (Bio-Rad) as described previously [Lemmon and McLoon, 1986; Bixby and Zhang, 1990]. Solvent-resistant plastic coverslips (Electron Mi-

croscopy Sciences) were sterilized by ultraviolet irradiation and placed in tissue culture dishes. Dishes with coverslips or empty dishes were coated with nitrocellulose (NC; Schleicher & Schuell, type BA85) by the method of Lagenaur and Lemmon [1987] or with poly-L-lysine (PLL; Sigma) as described previously [Drazba and Lemmon, 1990] to facilitate binding of the substrate molecules to the culture surface. Laminin (Upstate Biotechnologies) was attached to PLL-coated dishes by incubation of the coverslips in 10 μ g laminin per milliliter of calcium- and magnesium-free saline at 37°C for 18–24 hours. Laminin, N-cadherin, and L1/8D9 were bound to NC-coated dishes by incubation at 22°C for 10 min with 3 μ l of substrate solution per cm^2 . The dishes were then incubated with fetal bovine serum (FBS) for 10 min to block the remaining binding capacity. Small stainless-steel bars were placed at the edges of the coverslips to keep them flush with the bottom of the dishes. The FBS was removed by aspiration and replaced with Dulbecco's modified Eagle's medium /10% FBS/5% chick embryo extract/penicillin (10,000 units per ml)/streptomycin (10,000 μ g per ml)/fungizone. The dishes were incubated for approximately 1 h at 37°C in an atmosphere of 5% CO_2 /95% air while retinal explants were prepared.

Preparation of Retinal Explant Strips

Distinct growth cone morphologies are exhibited by different types of neurons [Kapfhammer and Raper, 1987]. Thus, to analyze the effects of different substrates on growth cone morphology, we examined the growth cones exhibited by a single class of cells, i.e., retinal ganglion cells [Halfter et al., 1983]. Retinal explant cultures provided another advantage to us in the axonal nature of the neurites. The extremely long, unbranched neurites produced by ganglion cells are morphologically similar to axons, and they express axonal markers such as phosphorylated neurofilament protein, TuJ1 [Watanabe et al., 1991], and L1 [Lemmon and McLoon, 1986]. This maintenance of cell polarity avoided possible ambiguities that might be introduced by differences between axonal and dendritic growth cones. Fertilized chicken eggs (white leghorn) were incubated in a humidified, forced-air incubator at 39°C. Embryonic day 7 (corresponding to Hamburger and Hamilton stages 28–29 [Hamburger and Hamilton, 1951]) embryos were used to prepare retinal explant cultures by a procedure described previously [Halfter et al., 1983; Drazba and Lemmon, 1990]. Briefly neural retinae dissected from E7 chick eyes were flattened, photoreceptor side down, onto NC membrane filters made adhesive by treatment with concanavalin A. The retinae and attached filters were cut perpendicular to the optic fissure into 0.35-mm wide strips. The retina-membrane strips were inverted onto substrates prepared in 35-mm dishes, the ganglion cell

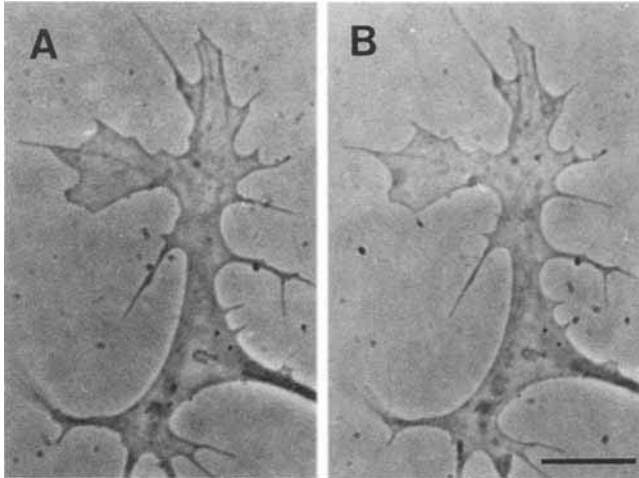


Fig. 1. Effect of chemical fixation on growth cone morphology. Phase-contrast light microscopy of explants plated on laminin/PLL and incubated for 2 days at 37°C. The growth cone was photographed in the living state (A), washed with cold medium, and fixed by the protocol described for SEM for 30 min. B: The same growth cone after fixation. The fixation protocol induced only minor changes in morphological parameters of most growth cones. Bar = 10 μ m.

layer facing the substrate. A small modification was introduced to ease the manipulations involved in processing the explants. Explants for electron microscopy were placed on solvent-resistant plastic coverslips in dishes. The cultures of retinal explant strips were incubated at 37°C in 5% CO₂/95% air for 48 h to allow neurite extension and then were fixed for scanning electron microscopy.

Fixation and Processing for SEM

Explants with long neurites were rinsed briefly in fresh medium cooled to 4°C. The culture medium was replaced immediately with cold 4% glutaraldehyde/4% sucrose in 0.1 M sodium cacodylate buffer, pH 7.4; the explants were fixed for 2 h at 4°C. The fixed specimens were washed for 1 h at 24°C with 4% sucrose in 0.1 M sodium cacodylate, pH 7.4, then incubated for 30 min in 1% osmium tetroxide in 0.1 M cacodylate buffer. A conductive coating was chemically deposited onto the specimen surface by a procedure [Postek et al., 1980] that consisted of 15-min incubations in 1% tannic acid buffered with 0.1 M sodium cacodylate, pH 7.4, followed by 1% osmium tetroxide in 0.1 M sodium cacodylate (2 cycles). The specimens were dehydrated in an ethanol series, transferred from 100% ethanol to Freon 113, and dried in a critical point dryer. The dried coverslips were mounted onto aluminum stubs, sputter-coated with gold, and viewed with a scanning electron microscope.

Statistical Evaluation

For comparison of growth cone characteristics on the various substrates, images of 10 solitary growth cones on the longer neurites in each preparation were recorded at 2500 \times from untilted specimens. Photographs were analyzed with a Bioquant image analysis system, and statistical analysis of the data was performed using the StatView II data analysis program. The Scheffe F test was employed to determine whether substrates produced significantly different growth cone characteristics.

RESULTS

Outgrowth Morphology on Different Substrates

Within a few hours of plating, neurites emerged from ganglion cells in the retinal explants and ventured onto the various substrates: L1/8D9, N-cadherin, laminin/NC, laminin/PLL, or PLL. Subsequent neurite extension during the 2-day incubation generated a characteristic outgrowth pattern on each test substrate [Lemmon et al., in press]. Neurites and growth cones on L1/8D9 formed a dense but defasciculated monolayer that typically was 0.7–0.8 mm wide. N-cadherin supported a profusion of neurites that filled a large outgrowth zone (1.8–2.4 mm wide) around the explant. In contrast, on laminin/NC-coated dishes, the processes formed fascicles of 5–10 tightly bundled neurites. Individual neurites often extended beyond the fascicle. The fascicles and individual neurites reached maximal lengths of 0.8–1.2 mm. Laminin/PLL supported radial neuritic outgrowth with many fine fascicles. Neurites typically were longer (1.2–2.0 mm) and less fasciculated when laminin was presented in this way. PLL was less effective than the other substrates in promoting neurite outgrowth. Few solitary neurites ventured far onto this substrate. The neurites that extended onto PLL formed a compact interwoven mesh 0.2 mm wide. Longer (0.4 mm), extended fascicles were formed occasionally.

Growth Cone Morphology

We used scanning electron microscopy to evaluate growth cone structure and the organization of interneuritic associations. Cultivation of the explants on plastic coverslips for SEM did not affect neuritic outgrowth morphology, except that the outgrowth length on PLL was somewhat reduced. The effect of fixation on growth cone morphology was tested by examining live growth cones with a 100 \times objective, fixing the cultures using the same method as that used for SEM, and then reexamining the same growth cones. The explant was cultured on a glass coverslip in a specially constructed

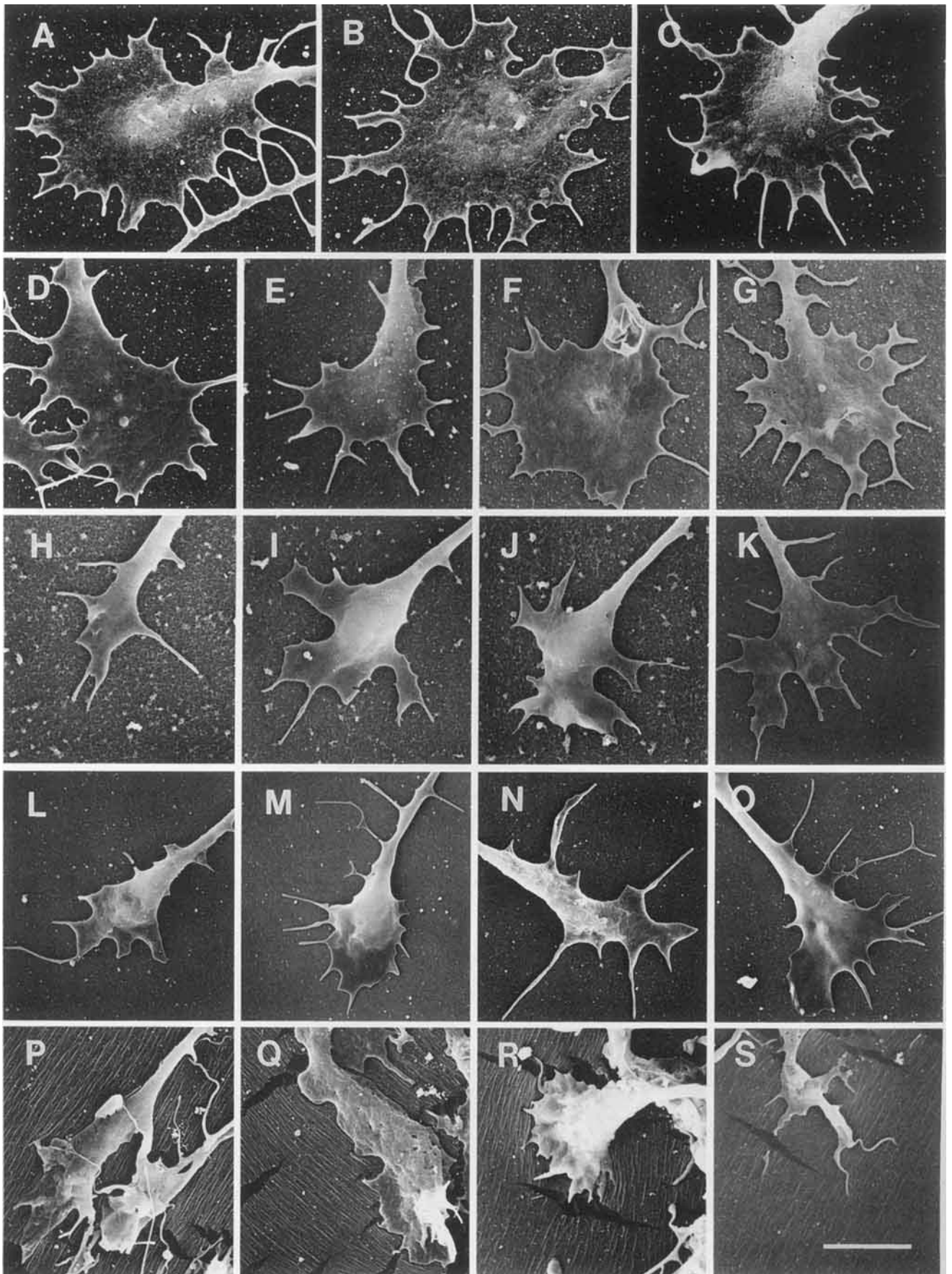


Fig. 2.

TABLE I. Effect of Substrate on Growth Cone Characteristics

Substrate	Shape	Size ^a	Filopodia per growth cone ^b	Filopodia per perimeter ^c	Filopodium length ^d
L1/8D9	Lamellipodia dominated	395 ± 60 ^c	13.9 ± 1.2 ^g	1.28 ± 0.07	2.9 ± 0.11
N-cadherin	Lamellipodia dominated	228 ± 16 ^f	12.8 ± 1.3 ^g	1.35 ± 0.1	2.9 ± 0.13
Laminin/NC	Filopodial with veils	127 ± 10	8.0 ± 0.7	1.12 ± 0.11	4.3 ± 0.26 ^h
Laminin/PLL	Filopodial with veils	106 ± 7	5.1 ± 0.5	.84 ± 0.12	3.1 ± 0.22
PLL	Variable	90 ± 14	5.8 ± 0.7	1.09 ± 0.14	2.6 ± 0.25

^aMean area (μm^2) ± S.E.M.

^bMean number of filopodia per growth cone ± S.E.M.

^cMean number of filopodia per 10 microns of growth cone perimeter ± S.E.M.

^dMean filopodial length (μm) ± S.E.M. Filopodium length was measured from the tip to the basal constriction (0.8 μm in diameter). Only microspikes longer than 0.8 μm were counted as filopodia.

^eL1/8D9 had a significantly larger area than other substrates at the .01 level.

^fN-cadherin had a significantly larger area than laminin at the .01 level.

^gSignificantly had more filopodia than laminin and PLL at the .01 level.

^hLaminin/NC had significantly longer filopodia than other substrates at the .01 level.

chamber dish to permit high-resolution light microscopy. No significant effects on the growth cones were observed on process retraction, swelling, or shrinking (Fig. 1). In the following analysis of growth cones, we use the terminology of Sheetz et al. [Sheetz et al., 1990]. The term *body* identifies a central region of the growth cone that has little membrane curvature. Lamellipodia and filopodia are included in a "periphery" of highly curved membranes that generally lie within 0.5 μm from the edge.

We observed that growth cones displayed a variety of shapes on each substrate (Fig. 2). Despite this variability, each substrate produced growth cones with morphological characteristics that fell within distinct limits. Growth cone shapes on each substrate exhibited both lamellipodial and filopodial elements. Purely lamellipodial growth cones were not observed under these conditions. Occasional blunt-ended neurites were observed on each substrate that probably represented quiescent or collapsed growth cones [Muller et al., 1990], and they were not included in our analysis. We analyzed enlarged SEM images to quantitate structural parameters of growth cones cultivated on each substrate (Table I).

On L1/8D9, lamellipodial features of growth cone structure predominated. The distal portion of the neurite was flattened and proximal portions of many filopodia were oval in cross section. The lamellipodia spread out in planar sheets that extended 5–10 μm from the growth

cone body. They were punctuated at regular intervals by filopodia and smaller microspikes. Growth cones that maintained contact with adjacent neurites often assumed elongated shapes with lamellipodial processes that extended 50–70 μm along the neurite terminus. Solitary growth cones were usually fan shaped. These growth cones were significantly larger than the growth cones on other substrates. The number of filopodia per growth cone on L1/8D9 was significantly greater than on laminin/NC, laminin/PLL, or PLL. Growth cone shapes on N-cadherin were predominantly lamellipodial with radiating filopodia; thus they resembled those on L1/8D9. N-cadherin growth cones also had as many filopodia as those on L1/8D9, but the former growth cones were significantly smaller and their neurites were flattened to a lesser extent. Growth cones on laminin/NC were similar to those on laminin/PLL in most respects, despite the differences in material used to bind the molecule to the coverslip. These growth cone shapes were fundamentally filopodial, with relatively small lamellipodial processes, which formed webs between the filopodia. These smaller lamellipodia usually were thicker than those on L1/8D9 or N-cadherin. The filopodia and neurites on laminin were rounded in cross section rather than flattened. The growth cone body was typically varicose (Fig. 3). Laminin supported growth cones that were smaller and exhibited fewer filopodia per growth cone. The filopodia were longer on laminin/NC than on laminin/PLL. Growth cone morphology on PLL was variable because the neuritic processes usually terminated in bizarre forms, often with curved filopodia. Lamellipodial formation was minimal on this substrate. There were significantly more filopodia on growth cones on L1/8D9 and N-cadherin compared to laminin and PLL. However, if the increased size of the growth cones on L1/8D9 and N-cadherin is taken into account by dividing the number of filopodia

Fig. 2. Influence of various substrates on growth cone morphology. Scanning electron micrographs. Growth cones on (A–C) L1/8D9 and on (D–G) N-cadherin elaborate characteristically large lamellipodia. Growth cones on (H–K) laminin/NC and on (L–O) laminin/PLL are dominated by filopodium formation with small lamellipodial veils. Filopodia of growth cones on (P–S) PLL are often curved into S shapes. There is very little lamellipodium formation on this substrate. Bar = 10 μm .

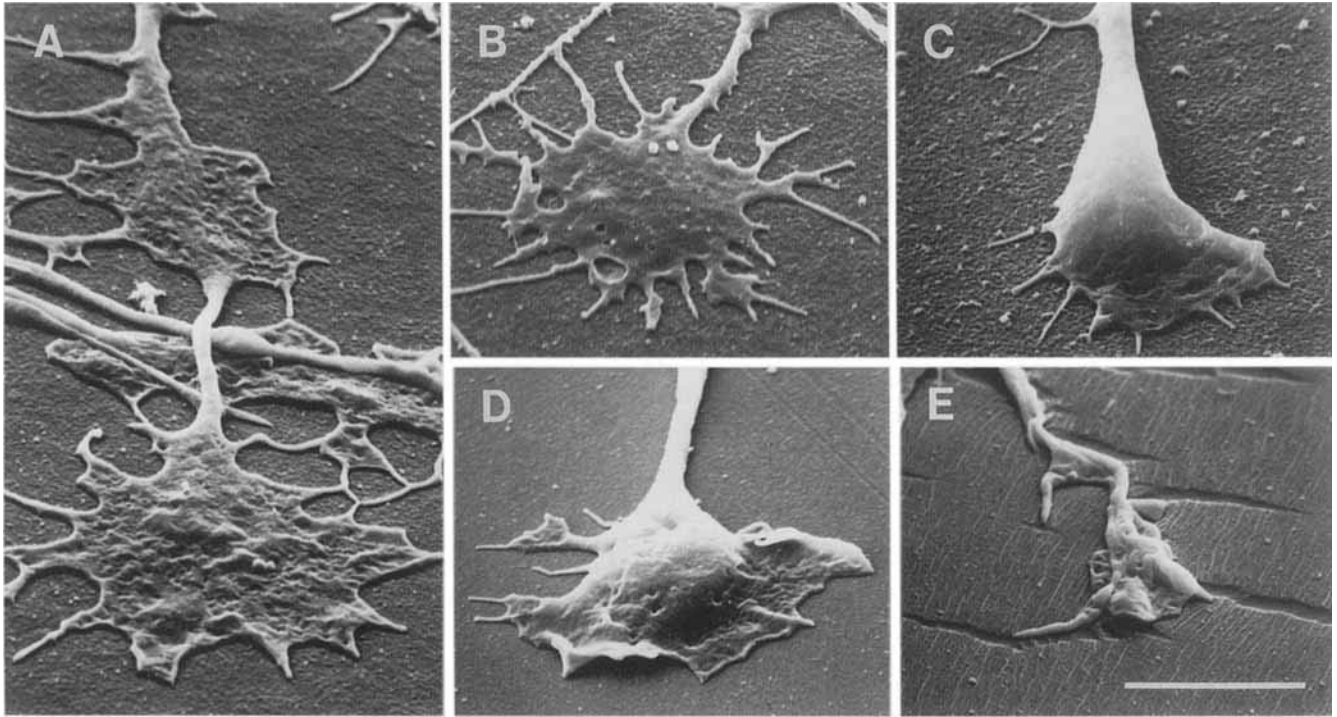


Fig. 3. Growth cone varicosities on various substrates. The growth cone base on (A) L1/8D9 or (B) N-cadherin is usually flattened into a fan shape. On (C) laminin/NC and on (D) laminin/PLL, the growth cone base is enlarged to form a varicosity at the neurite terminus.

Growth cones on (E) PLL are often varicose. Note also the *en passant* lamellipodial expansion of the neurite in A that is characteristic of subterminal segments of neurites on L1/8D9. Specimen tilt = 50°. Bar = 10 μ m.

by the perimeter, then the differences between substrates decreases dramatically (Table I).

Interneuritic Associations

The frequency of physical contacts between growth cones was a function of the substrate type. On laminin and N-cadherin, there was no obvious difference in growth cone morphology between regions of low growth-cone density and high growth-cone density. However, on L1 there was a significant change in growth-cone morphology in regions containing many growth cones (Fig. 4). The growth cones became elongated (many extended 50–70 μ m), and they maintained numerous contacts with adjacent growth cones and neurites. Most often, only the distal tips of filopodia touched, although filopodial-lamellipodial and lamellipodial-lamellipodial contacts were frequently observed. At low magnifications the processes frequently appeared to fuse, but closer examination usually revealed membrane discontinuities, suggesting that filopodial tips did not fuse. However, using the technique of SEM we were unable to determine whether membrane fusion occurred or to resolve the possible presence of gap junctions. Adjacent growth cones on N-cadherin made filopodial contacts, but these attachments were much less frequent than

the associations between L1/8D9 growth cones. Apparently individual growth cones were sometimes found upon closer inspection to be pairs of overlapping growth cones. This condition was most often seen on N-cadherin; paired growth cones were much less common on laminin/NC and laminin/PLL, and were never observed on L1/8D9. On PLL, frequent contacts were made between growth cones and neurites within a mesh. Lamellipodia were infrequent on top of underlying processes.

DISCUSSION

Our results show that substrate type clearly influences growth cone morphology and can alter the frequency of intercellular contact. Laminin supported predominantly filopodial growth cones with small veils, whereas L1/8D9 and N-cadherin induced lamellipodial expansion. Growth cones on L1/8D9 were the largest in area, and they had more filopodia as well. Filopodial physical associations are frequent on L1/8D9 but uncommon on laminin. We think that these responses reflect a morphological repertoire available to the growth cone *in vivo*. These data extend our earlier observations indicating that L1/8D9, N-cadherin, and laminin have very different effects on neurite outgrowth, growth-cone trans-

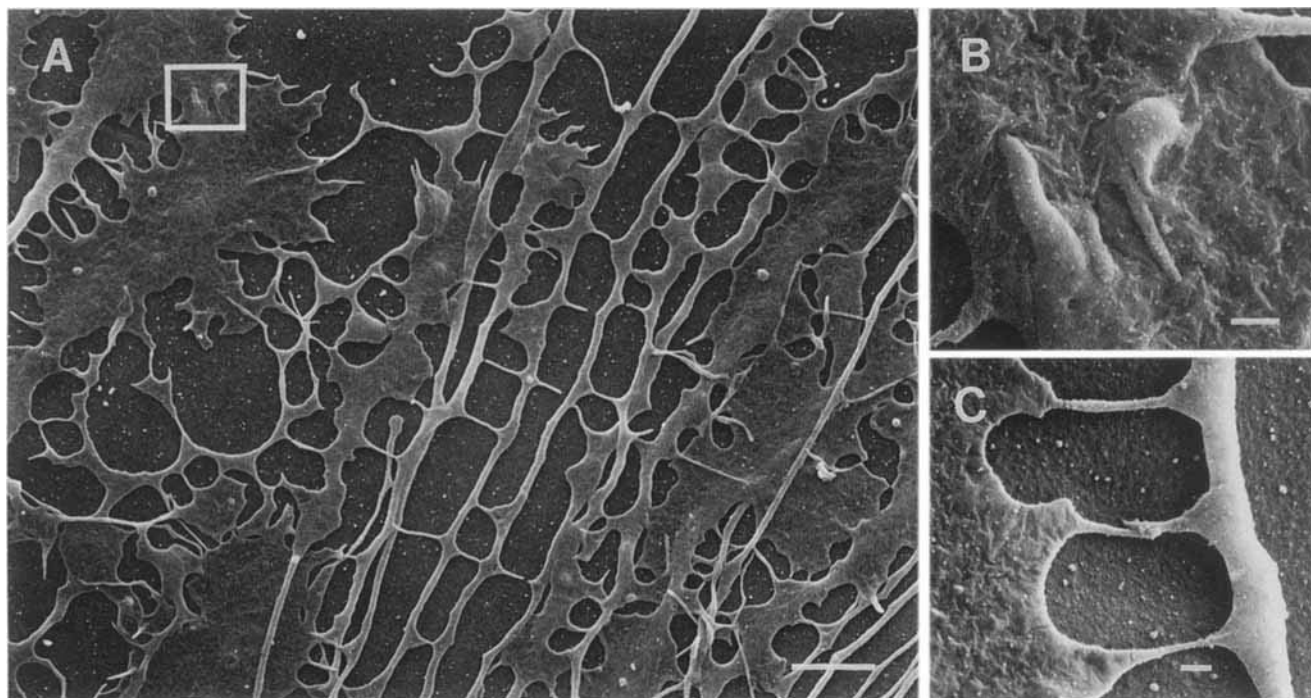


Fig. 4. Interneuritic associations. Growth cones on L1/8D9 establish many physical contacts with adjacent neurites and growth cones. Membrane discontinuities at contact sites (for example, the site boxed in **A** and enlarged in **B**) indicate incomplete anastomosis but cannot

eliminate the possibility of membrane fusion or gap junctions. The contacts usually involve attachment by filopodial tips (**C**). Bars = 10 μm in (**A**), 1 μm in (**B,C**).

location rate, growth cone adhesion, and neurite fasciculation [Lemmon et al., submitted].

Significantly more filopodia were extended by growth cones on L1/8D9 or N-cadherin than on laminin/NC, laminin/PLL, or PLL. However, this may simply be a function of filopodia production being dependent in part on the size of the growth cone, because we found fewer differences when we compared the number of filopodia per growth cone perimeter. Filopodia are thought to augment neurite extension, but their precise contribution to neurite advance has been disputed [Marsh and Letourneau, 1984; Bray and Chapman, 1985]. Other hypotheses, that filopodia mediate cell-to-cell interactions and that they are involved in steering the growth cone to its appropriate target, are better supported by *in vivo* observations [Bastiani and Goodman, 1984; Bentley and Toroian-Raymond, 1986; Bovolenta and Mason, 1987]. In our studies we found that contacts between growth cones usually involved filopodial tips as if they were specialized to mediate cell-to-cell contact. Transcellular labelling with carbocyanin dyes has been shown to occur between optic axons and neuroepithelial cells in the mouse chiasm, apparently at sites of close contact between cell membranes [Godement et al., 1987]. These contacts might involve intimate interactions, similar to those observed in electron microscopic analyses of

growth cones of the rodent optic nerve [Bovolenta and Mason, 1987] and of the grasshopper embryo [Bastiani and Goodman, 1984]. In these contacts, one growth cone inserts a filopodium into the invaginated surface of a nearby growth cone or guidepost cell. The intimate associations we observed between growth cones on L1/8D9 could mean that this CAM potentiates such associations *in vivo*, although the mechanism that mediates this effect is unclear.

Growth cones exhibit a wide spectrum of sizes and shapes *in vivo*, and some of these morphological differences are related to the type of neuron that extends the growth cone [Kapfhammer and Raper, 1987]. The morphological complexity of a growth cone is also strongly influenced both by its local environment and the developmental stage of the organism. For example, in the developing mouse visual system, growth cone morphology changes dramatically as retinal fibers travel from the optic nerve into the optic chiasm [Bovolenta and Mason, 1987]. Growth cones with relatively simple morphologies in the optic nerve are highly elongated, with convex and concave lamellipodial expansions that usually lack filopodia. In contrast, growth cones found in the chiasm, a "decision region," have strikingly different complex forms, with extensive filopodia emerging from an expanded body. This increase in morphological complexity

in the decision region is thought to reflect an active exploration of the environment of the optic chiasm [Bovolenta and Mason, 1987; Godement et al., 1990]. After growth cones pass the chiasm, they become more slender and again lack filopodia until they reach the target area. There, filopodia arise and point toward the target. Another example in which guidance cues appear to influence growth cone size and lamellipodial morphology is found in the lumbrosacral region of the chick embryo [Tosney and Landmesser, 1985]. Motor neuron growth cones at positions operationally defined as decision regions are larger, more lamellipodial, and have more complex trajectories than growth cones in other pathway regions. Growth-cone morphological complexity is also found to increase at segmental boundaries in the grasshopper embryo [Caudy and Bentley, 1986]. Pioneer growth cones consistently have more filopodia and circumferential lamellae along segment boundaries than in distal regions of limb segments. O'Connor et al. [1990] studied filopodial exploration by advancing growth cones in situ and observed that an individual filopodial contact with a guidepost neuron can steer axon growth in the direction of this high-affinity substrate. These examples suggest that growth-cone morphological complexity is an indicator of differences in extrinsic guidance cues. The presence of an adhesion gradient is often cited as a possible guidance cue, but this is not necessarily the case. Neurites grow in an undirected fashion on a laminin gradient [McKenna and Raper, 1988], and neurites can grow back and forth between substrates with dramatically different adhesivities [Lemmon et al., in press]. One might predict that growth cones would be larger on more adhesive substrates, but this is not what we observed [Lemmon et al., in press]. Growth cones were smallest on PLL, yet this was the second most adherent substrate among this group. Growth cones on N-cadherin were significantly larger than those on laminin, yet these two substrates were equally adhesive. Therefore, we think that substrate modulation of growth cone behavior is a complex process, with CAMs and SAMs exerting their effect via interactions with the cytoskeleton and other cytoplasmic components, and not due simply to the strength of growth cone binding to adjacent cells and matrix.

L1/8D9 and N-cadherin produced elaborate growth cone shapes in vitro that possessed more filopodia than growth cones on other substrates. It is tempting to consider the possibility of their influence in the increased intricacy of growth cone morphology at the optic chiasm. The slower growing, complex growth cones with more filopodia that are induced by L1/8D9 and N-cadherin would be optimal for sampling the environment of a choice point for guidance cues. These two CAMs are not restricted to the chiasm and they are present throughout

the optic path. Therefore, there may be a hierarchy of adhesion molecules, so that growth cone morphology is influenced by the more dominant molecule present. Thus, it may be informative to test combinations of adhesion molecules to determine how growth cones respond to multiple influences. It also will be important to map the precise distributions of different adhesion molecules along particular pathways to determine if there are local concentrations that might account for growth cone behavior.

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