Structure of laminin substrate modulates cellular signaling for neuritogenesis

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Summary

Laminin, a major component of basement membranes, can self-assemble in vitro into a typical mesh-like structure, according to a mass-action-driven process. Previously, we showed that pH acidification dramatically increased the efficiency of laminin self-assembly, practically abolishing the necessity for a minimal protein concentration. Here we have characterized the morphologies of laminin matrices produced in either neutral or acidic conditions and compared their capacities to induce neuritogenesis of rat embryonic cortical neurons. Although laminin matrices formed in neutral buffer presented aggregates of heterogeneous morphology, the acidic matrix consisted of a homogeneous hexagonal sheet-like structure. The latter was comparable to the matrix assembled in vivo at the inner limiting membrane of the retina in newborn rats, shown here, and to matrices secreted by cultivated cells, shown elsewhere. The average neurite length of cortical neurons plated on acidic matrices was 244.9 μm, whereas on neutral matrices this value dropped to 104.1 μm. Increased neuritogenesis on the acidic matrix seemed to be associated with a higher degree of neuronal differentiation, since cell proliferation was immediately arrested upon plating, whereas on neutral matrices, the cell number increased six-fold within 24 hours. Investigation of the mechanisms mediating neurite outgrowth on each condition revealed that the extensive neuritogenesis observed on the acidic matrix involved activation of protein kinase A, whereas moderate neuritogenesis on neutral laminin was mediated by activation of protein kinase C and/or myosin light-chain kinase. Explants of cerebral cortex from P2 rats did not grow on the neutral laminin substrate but presented extensive cell migration and neurite outgrowth on the acidic laminin matrix. We propose that laminin can self-assemble independently of cell contact and that the assembling mode differentially modulates neuritogenesis and neuroplasticity.

Key words: Laminin, Neuritogenesis, Protein kinase A

Introduction

Laminin is a large cross-shaped glycoprotein ubiquitously found in basement membranes, as well as in the non-basement membrane extracellular matrix of the developing central nervous system (Timpl, 1989; Beck et al., 1990; Tryggvason, 1993; Luckenbill-Edds, 1997; Colognato and Yurchenco, 2000). The molecular network directly in contact with the cellular surface consists of polymerized laminin, which can self-assemble even in the absence of other basement membrane components (Yurchenco et al., 1992). Besides its structural role, laminin associated to basement membranes modulates several cellular functions such as cell adhesion, proliferation, migration and tumor metastasis (Kleinman et al., 1993; Aumailley and Smyth, 1998). In particular, the morphogenesis of the nervous system is strongly modulated by laminin, whose expression is both spatially and temporally regulated during development (Liesi, 1985; Liesi and Silver, 1988; McLoon et al., 1988; Reichardt and Tomaselli, 1991; Nurcombe, 1992). In this context, laminin has been shown to influence neurite outgrowth (Manthorpe et al., 1983; Adler et al., 1985; Edgar et al., 1988; Chamak and Prochiantz, 1989), axonal guidance (Cohen et al., 1987; McLoon et al., 1988; Hammarback et al., 1988), differentiation (Cohen et al., 1986) and cell proliferation (Drago et al., 1991; Frade et al., 1996).

In vitro studies of the effects of laminin on cell function have been achieved by using artificial matrices, prepared by adsorbing the protein on the surfaces of cell-culture plastic or glassware. It is usually assumed that adsorbed laminin will form a molecular network retaining the overall properties of laminin matrices in vivo. This assumption, however, is not necessarily correct. First, laminin polymerization does not correspond to random protein aggregation, but, instead, it is a well defined assembly process, involving interactions of specific domains of the protein (Schittny and Yurchenco, 1990; Yurchenco and Cheng, 1993). Second, there is evidence that laminin matrices assembled on either cultured astrocytes or Schwann cells can vary their morphologies, which has been considered of biological relevance (Garcia-Abreu et al., 1995a; Garcia-Abreu et al., 1995b; Farwell and Dubord-Tomasetti, 1999; Tsiper and Yurchenco, 2002). In addition, analysis of laminin distribution in developing rat brain revealed the occurrence of polymers of distinct morphologies also in vivo (Zhou, 1990).

Laminin had previously been shown to self-assemble in

solution at a minimal protein concentration of approximately 60 nM (Yurchenco et al., 1985). More recently, we showed that solution polymerization could alternatively be triggered at low laminin concentrations by acidification of bulk pH (Freire and Coelho-Sampaio, 2000). We hypothesized that acidic polymerization may occur in vivo in regions of the plasma membrane where circumstantially the negatively charged groups provided by glycoproteins, glycolipids and proteoglycans reduce the local pH (Wettreich et al., 1999; Freire and Coelho-Sampaio, 2000). In this work, we investigated whether manipulation of solution pH generates morphologically distinct polymers from purified laminin and whether such polymers would induce distinct phenotypes on overlaying cells. We showed that laminin polymerized at neutral or acidic condition self-assembled into structurally distinct matrices and that such matrices favored either neuritogenesis or cell division through activation of distinct signaling pathways.

Materials and Methods

Materials

Natural mouse laminin isolated from Engelbreth-Holm-Swarm tumor, trypsin and BSA were purchased from GIBCO BRL (Gaithersburg, MD). Triton X-100, DMEM-F12, N-propyl gallate, DAPI and BrdU were from Sigma Chemical Co. Staurosporine and H-89 were obtained from Calbiochem (La Jolla, CA). Repel silane was from Amersham Pharmacia Biotech (Uppsala, Sweden) and the iodination kit Iodo Beads from Pierce (Rockford, IL). Primary antibodies were rabbit anti-Tau (Sigma; 1:200 dilution) (Figs 2, 4 and 9), rabbit anti-β tubulin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:50 dilution) (Figs 8 and 9), mouse anti-β tubulin (Sigma: 1:500 dilution) (Figs 3 and 6), rat anti-BrdU (Accurate Chemical & Scientific Corp., Westbury, NY; 1:500 dilution) (Fig. 7) and rabbit anti-laminin (Sigma; 1:30 dilution) (Figs 1 and 6). Secondary antibodies were conjugated with FITC (goat anti-mouse, Accurate Chemical & Scientific Corp.; 1:200 dilution) (Figs 3 and 6), biotin (goat anti-rat, Vector Laboratories, Burlingame, CA; 1:400 dilution) (Fig. 7) or with Cy-3 (goat anti-rabbit, Sigma; 1:50 dilution) (Figs 1, 2, 4, 6, 8 and 9). Texas red-streptavidin-conjugate (Fig. 7) was from Vector Laboratories. All other reagents were of analytical grade.

Light scattering and fluorescence measurements

Light scattering and intrinsic fluorescence were both measured on an ISSPC-PCI spectrofluorometer (ISS Inc., Champaign, IL) at 35°C. In the first case the wavelength of the incident light was fixed at 400 nm and scattered light was collected at 90° between 350 and 450 nm. In fluorescence measurements excitation was at 295 nm and emission between 300 and 400 nm. Laminin self-polymerization was initiated by diluting the protein from a stock solution to a final concentration of 50 μg/ml in either 20 mM sodium acetate, pH 4 or 20 mM Tris-HCl, pH 7 (final volume of 600 μl). Laminin stock solutions (1 mg/ml) were kept at 4°C until they were diluted into assay media previously warmed to 35°C in the sample compartment of the instrument. Under these conditions, i.e. at 4°C, aggregates are not formed in the stock solutions until the exact time when dilution in pre-warmed buffer takes place (Yurchenco et al., 1985).

After addition of laminin, samples were gently mixed and the measurements were taken within approximately 1 minute. Data were corrected by subtracting appropriate blanks containing buffer only. Prior to use, cuvettes were pre-treated with Repel silane overnight to avoid adherence of laminin to the quartz surface.

Preparation of laminin matrices

Laminin was diluted to a final concentration of 50 μg/ml in either 20 mM sodium acetate, pH 4 or 20 mM Tris-HCl, pH 7, both containing 1 mM $CaCl_2$. Aliquots of 200 μl were immediately placed on 5.5 mm diameter glass coverslips and incubated at 37°C for 12 hours. Coverslips were then washed three times with PBS, pH 7 and used either directly for immunocytochemical analysis or as a substrate for cell plating. The percentage of laminin adsorbed on coverslips was calculated by measuring the radioactivity remaining in solution after 12 hours of incubation of the iodinated protein with glass coverslips. Laminin labeling was carried out using the Iodo beads iodination reagent as described by the manufacturer.

Retinal whole-mounts

Whole mounted retinas were prepared as previously described (Linden and Perry, 1982). Briefly, newborn Lister hooded rat pups were instantaneously killed by decapitation with a single cut of sharp scissors, and their retinae were dissected out of the eyeballs with fine forceps and whole-mounted onto gelatinized glass slides with the vitreal side up. A few radial cuts were made with a sharp scalpel blade to flatten the retina onto the slides. Care was taken to avoid touching the vitreal surface of the retinae to prevent disruption of the extracellular matrix. Following fixation with 4% paraformaldehyde in phosphate buffer pH 7.2, the retinae were processed for laminin immunostaining as described below but omitting the permeabilization step.

Neuron primary cultures

Primary cultures of neurons were prepared from cerebral cortex of Wistar rats (UFRJ, Rio de Janeiro, Brazil) at embryonic day 14, as previously described (Gomes et al., 1999; Fróes et al., 1999). Briefly, single cell suspensions were obtained by dissociating cells of cerebral cortex in DMEM/F12 medium supplemented with glucose (33 mM), glutamine (2 mM) and sodium bicarbonate (3 mM). 100,000 cells were plated on each laminin-coated coverslip previously placed on a 24-well plate. Neuron cultures were kept in DMEM/F12 medium without serum or supplements for up to 24 hours at 37°C in humidified, 5% CO and 95% air atmosphere. In some experiments, instead of dissociated cells, cortex tissue was cut into small pieces with sharpened forceps and placed on culture wells as explants. Explants of postnatal day 2 rat pups were also prepared.

Immunocytochemistry

For immunocytochemistry, cultured cells were fixed with 4% paraformaldehyde for 20 minutes, washed three times with PBS and permeabilized with 0.2% triton X-100 for 5 minutes at room temperature. After permeabilization, cells were washed again twice with PBS. Immunocytochemistry was performed as previously described (Garcia-Abreu, 1995a; Gomes et al., 1999). Cells were blocked with 5% BSA in PBS for 1 hour and subsequently incubated with the specific primary antibodies diluted in blocking solution, overnight, at room temperature. Cells were then washed three times with blocking solution and incubated with secondary antibodies for 2 hours, at room temperature. In the case of biotin-conjugated antibodies, development of secondary antibodies was performed by incubating the cells with the Texas-Red-streptavidin conjugate according to the specifications of the manufacturer. Nuclei were labeled with DAPI. Negative controls were performed by omitting primary antibodies. In all cases no reactivity was observed when the primary antibody was absent. Cell preparations were mounted directly on N-propyl gallate. The coverslips were visualized using a Zeiss Axioplan microscope.

Trypan blue viability assay

Cell viability was assayed at 24 hours on neuronal cultures by

replacing culture medium with 0.4% trypan blue solution in PBS for 1 minute. At least 5 fields of attached cells were counted per well.

Bromodeoxyuridil incorporation and detection

Neuronal cultures were incubated for 24 hours in the presence of 1 μg/ml of BrdU. In order to prevent BrdU interfering with cell adhesion, cortical cells were allowed to settle for 2 hours before addition of BrdU. After 24 hours of incubation, cells were fixed with 4% paraformaldehyde for 20 minutes. Cultures were then washed twice with distilled water and incubated in 2 N HCl at 50°C for 15 minutes. Subsequently, neuronal cultures were washed twice with 0.1 M borate buffer for 10 minutes at room temperature. After washing with PBS, cells were incubated with anti-BrdU antibody as described above and visualized using a Zeiss Axioplan microscope. The percentage of proliferating cells was quantified by counting the percentage of labeled cells in at least 10 different fields per coverslip.

Morphometry and statistical analyses

Neurons stained for either Tau or β tubulin were photographed in a Zeiss Axioplan microscope. Photos were scanned and the number of neurites and the total neurite length were analyzed using the Sigma Scan Pro Software (Jandel Scientific). Two forms of quantitative analyses are presented. In the first one, 100 neurons in six or seven fields chosen randomly were considered independently of their size. In the second one, only the 30 longest neurites were averaged. Each experiment was repeated at least three times producing very similar results. Statistical analyses were performed using the Microsoft Excel version 7.0. Error bars in histograms represent s.e.m.

Results

Characterization of laminin matrices assembled at neutral or acid pH

Self-assembly of laminin was studied either in solution, using Repel-silane-treated glass cuvettes (Fig. 1, graphic) or on the surface of non-treated glass coverslips (Fig. 1A-D). In solution, thawing/dilution of a concentrated stock of laminin directly into acidic buffer led to immediate formation of high molecular weight aggregates, whereas dilution in neutral buffer did not induce aggregation (Fig. 1, inset in graph). Acid-triggered laminin aggregation was probably not caused by protein unfolding since the spectrum of intrinsic fluorescence observed at acidic condition was similar to the spectrum at neutral pH. The lack of spectral shift and the fact that the average emission was centered at approximately 330 nm both indicate preservation of the native structure (Lakowicz, 1983) (Fig. 1, graph). When laminin polymerization occurred with contact with the surface of non-treated glass, progressive adsorption was observed. Using iodine-labeled laminin, it was determined

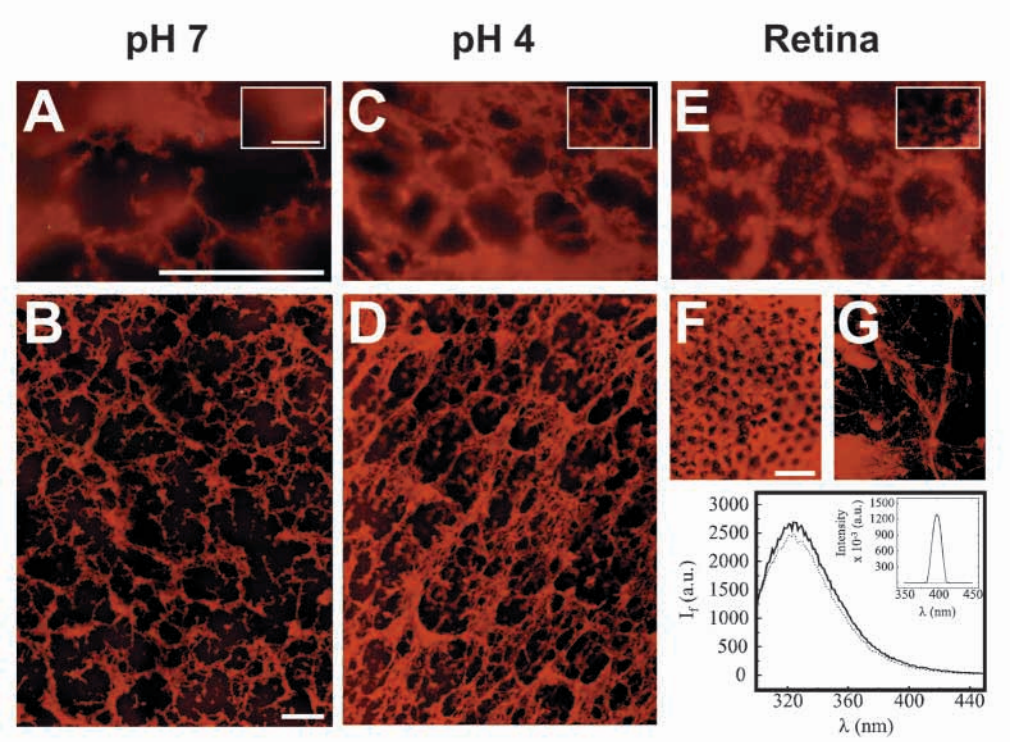

Fig. 1. Self-assembly of laminin in neutral or acidic buffer. Frozen aliquots of laminin were dissolved in either Tris-HCl, pH 7 or in sodium acetate, pH 4, both containing 2 mM $CaCl_2$. Dilutions were made either in a pre-silanized cuvette (graphic) or in drops of buffer previously placed onto non-silanized coverslips (pictures). The graphic shows fluorescence spectra obtained for laminin in neutral (continuous line) and acidic buffer (dotted line) using excitation at 275 nm. The inset shows light-scattering intensity, using incidental light at 400 nm, for neutral (lower trace) and acidic condition (upper trace). Panels A to D show immunocytochemical analyses of laminin matrices formed on glass coverslips at pHs 7 (A,B) or 4 (C,D). Note that the apparent difference in the amount of bound protein in panels A and C is due to the impossibility of homogeneously focusing laminin aggregates throughout the neutral matrix. Insets show details of the corresponding main panels. Panels E to G show immunohistochemistry for laminin on whole-mount preparations of the retina of newborn rats (P0). A honeycomb pattern is typically seen on the periphery of the whole mount (E and F), whereas unorganized deposits characterize the center of the retina (G). The scale bars in panel A, main, and B correspond to 50 μm and apply to panels A, C and E or to panels B and D, respectively. The bar in the inset of panel A represents 10 μm and applies to all insets. In panel F, the bar corresponds to 100 μm in panels F and G.

that in 12 hours, 60% of the total radioactivity bound to the coverslip, regardless of the buffer pH.

In order to compare the morphologies of the polymers formed at each pH, laminin deposited onto coverslips at either pH 7 or 4 were immunostained with anti-laminin antibodies. Matrices formed at either pH presented clearly distinct morphologies, as seen by comparing panels A and B with panels C and D of Fig. 1. At pH 7, the laminin matrix consisted mainly of large aggregates, protruding from the surface of the coverslip, and could not be homogeneously focused on a single optical plane (Fig. 1A,B; see also Fig. 6A). At pH 4, laminin produced a flat network, where regular polygons could be distinguished (Fig. 1C,D). Laminin organization in polygonal flat arrays had previously been observed on the surfaces of cultured myotubes, Schwann cells and on embryoid bodies (Colognato et al., 1999; Lohikangas et al., 2001; Tsiper and Yurchenco, 2002), which suggests that the artificial matrix obtained at low pH is structurally similar to some cell-assembled laminin polymers.

To investigate whether the polygonal structure of the artificial laminin matrix produced at low pH is also found in vivo, we carried out an immunohistochemical analysis of the surface of whole-mounted retinae. In most tissues laminin can be assessed only in sections and therefore cannot be visualized as a naturally deposited layer. The inner (vitreal) surface of the developing retina expresses laminin (e.g. Linden et al., 1999), promotes neuritogenesis (Halfter et al., 1987) and can be directly accessed in the whole-mount preparation. As seen in Fig. 1, panels E and F, laminin at the periphery of the immature retina is also organized in a polygonal pattern, visualized in a single optical plane. Interestingly, laminin deposited at the center of the whole mount was not similarly organized and presented protruding laminin aggregates, as seen on the artificial laminin matrix produced at neutral pH. Because retinal maturation progresses from the center to the periphery (Rapaport and Stone, 1984), it is conceivable that the organized laminin matrix occurring at the periphery of the retina of newborn rats is involved in early events of axonal extension from ganglion cells.

Because previous studies have implicated calcium ions in the modulation of laminin polymerization (Schittny and Yurchenco, 1990; Lallier and Bronner-Fraser, 1991), we compared the morphologies of acidic matrices obtained in the presence of 2 mM $CaCl_2$ (Fig. 1) with matrices obtained in the presence of contaminating calcium only and in the presence of 2 mM EDTA. In the absence of added calcium (contaminating Ca^{2+}) matrices presented an intermediate morphology, that is, flat, organized, but disrupted polymers alternated with rare large aggregates (data not shown). In the complete absence of calcium ions (added EDTA), matrices were not formed (data not shown).

Neurite outgrowth on different laminin matrices

Artificial laminin matrices were compared with respect to their abilities to promote neurite outgrowth of embryonic neurons. Dissociated cells from the cerebral cortex of E14 rat embryos were added to coverslips previously coated with either neutral or acidic laminin. After 24 hours, immunocytochemistry for the neuron marker Tau revealed a clear difference between cells plated on the two surfaces (Fig. 2A,B). On the neutral matrix, we observed poor neuritogenesis and a clear tendency for cells to clump together. Measurement of only the 30 longest neurites (which is the usual procedure detailed in the literature) showed that the average neurite length was of 104.1±6.0 and 244.9±14.9 μm for neurons plated onto neutral and acidic matrices, respectively (Fig. 2C). When 100 neurites from randomly chosen cells were averaged, the respective values were 46.7±4.6 and 141.9±9.4 μm (Fig. 2D-E). The discrepancy between values obtained using each approach was mainly due

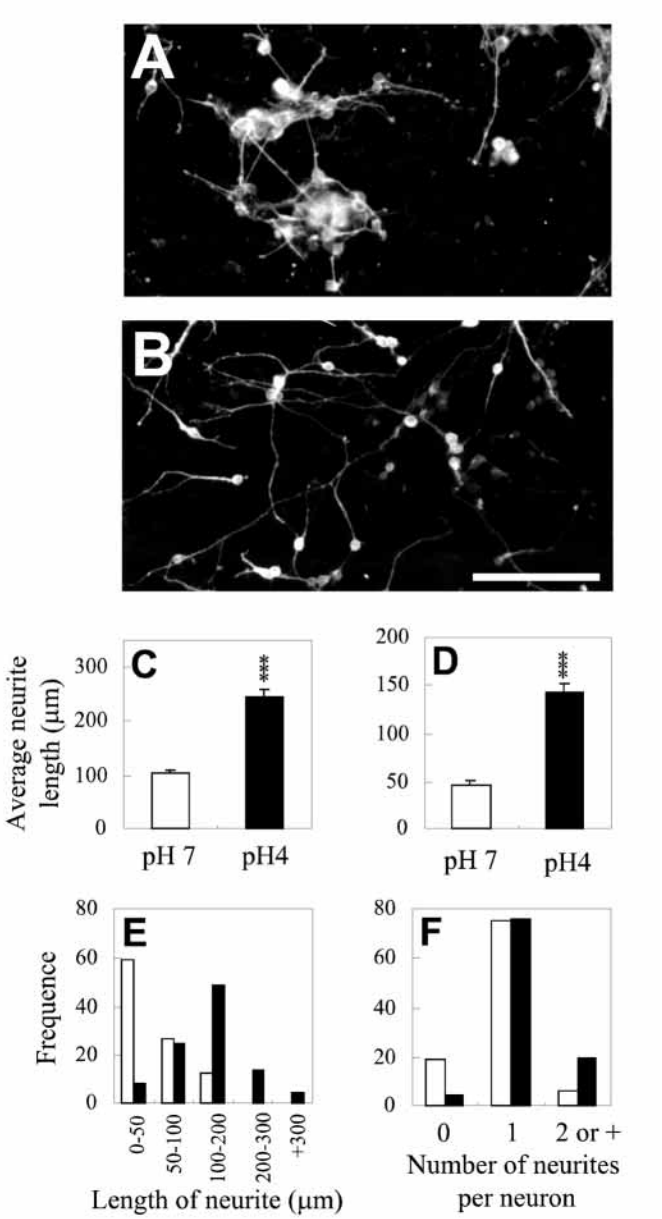

Fig. 2. Neurite outgrowth on neutral or acidic laminin matrices. Dissociated cortical cells were incubated for 24 hours over laminin matrices obtained at neutral (A) or acid pH (B). Immunocytochemistry for neuronal cells was carried out using a polyclonal anti-Tau reagent as the primary antibody and Cy3-labeled anti-rabbit as the secondary antibody. Panels C-F show quantitative analyses of data obtained in panels A (pH 7, open bars) and B (pH 4, closed bars). Analyses were carried out considering only the 30 longest neurites (C) or 100 neurons randomly chosen (D-F). Three asterisks correspond to $P<0.0001$. Bar, 100 μm.

to the fact that there were many more cells without neurites on the neutral than on the acidic matrix (Fig. 2F). Laminin matrices obtained at intermediate pH values between 7 and 4 induced neuritogenesis with increasing efficiencies, for example, matrix assembled at pH 6 promoted significantly higher neurite outgrowth than matrix obtained at pH 7 (data not shown). In another control we observed that neurites did not develop on substrates coated with laminin in the presence of EDTA. Finally, we evaluated the behavior of cortical cells plated directly onto plastic, glass and on poly-ornithine-coated glass using DMEM/F12 in the absence of serum or other supplements. In such conditions, cells attached very poorly to the substrate, and the few attached cells were not viable within 24 hours (data not shown). It is noteworthy that cortical cells cultured in Neurobasal medium plus B-27 supplement (Invitrogen) attach and develop neurites after 48 hours on either untreated or on poly-ornithine or laminin-coated substrates (data not shown).

Further, we compared neuritogenesis induced by the two laminin matrices using brain explants. In this case, overall neurite outgrowth was again more pronounced at pH 4 than at pH 7 (Fig. 3). As also seen in Fig. 2, neuronal cell bodies clumped on the neutral (Fig. 3B) but not on the acidic matrix. The few isolated cells seen on the neutral matrix had short neurites (Fig. 3A, inset). It is interesting to note that both laminin matrices were capable of promoting cell migration out of the explant, although migration on the acidic matrix seemed slightly more effective.

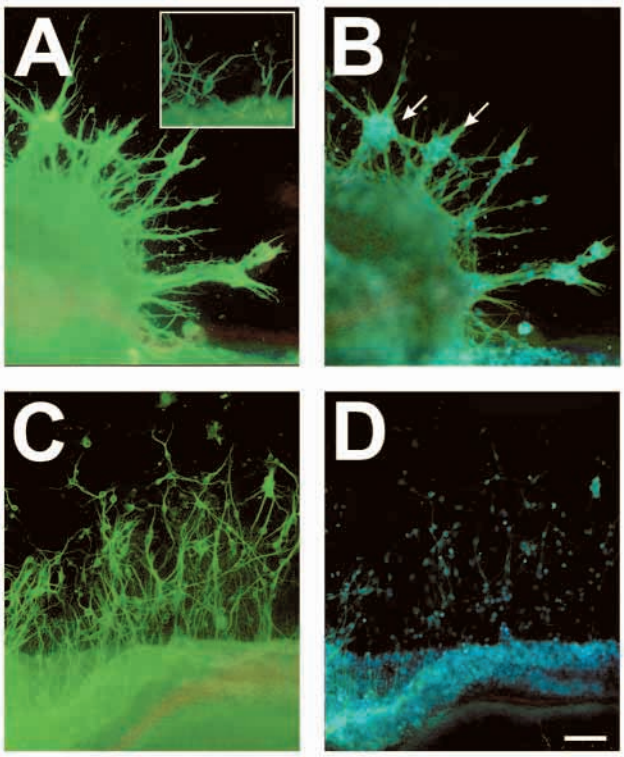

Fig. 3. Neurite outgrowth from explants plated on neutral or acidic laminin matrices. Brain explants were incubated for 24 hours on laminin matrices obtained at neutral (A,B) or acidic pH (C,D). Explants were developed for the neuronal marker β tubulin III (A,C) and for total cells, using DAPI (B,D). Arrows point to typical aggregates of cell bodies occurring on the neutral matrix. The inset in A shows short neurites emerging from a rare region of the explant where aggregation of cell bodies did not occur. Bar, 100 μm.

Closer inspection of isolated neurons revealed that neurites appearing in the acidic matrix after 6 hours of culture already have multiple fine filopodia along their axes, whereas neurites formed on the neutral matrix remained unbranched even after 24 hours. Furthermore, growth cones in the acidic matrix exhibited lamellipodia and filopodia extending for large areas, whereas growth cones from neurons on the neutral matrix were reduced, usually without filopodia (Fig. 4). The increase in the lamellar and filopodial form of neurons on the acidic matrix could be due to superior adhesive properties of this matrix. To test this hypothesis we measured cell attachment to each matrix at different times either using ^{35}S-labeled neurons or by directly counting cells remaining in the culture supernatant (Lallier and Bronner-Fraser, 1991). Twenty minutes after plating, cell attachment to either neutral or acidic matrix was about 60%. Two hours later, attachment increased to 80% in both cases. These results indicate that differential effects of laminin matrices on cell morphology were not dictated by selectivity of cell attachment.

Neuritogenesis was additionally investigated at increasing incubation times ranging from 2 to 24 hours (Fig. 5). The kinetics of neurite outgrowth on each substrate were significantly different: on the acidic matrix neurites grew linearly with time up to 24 hours, on laminin assembled at neutral pH neurite extension tended to stabilize at an average length of approximately 100 μm at around 16 hours.

Cell growth on distinct matrices

Comparison of panels A and B on Fig. 2 shows that, besides a lower neuritogenic potential, the neutral-assembled laminin matrix induced clumping of cell bodies. The appearance of

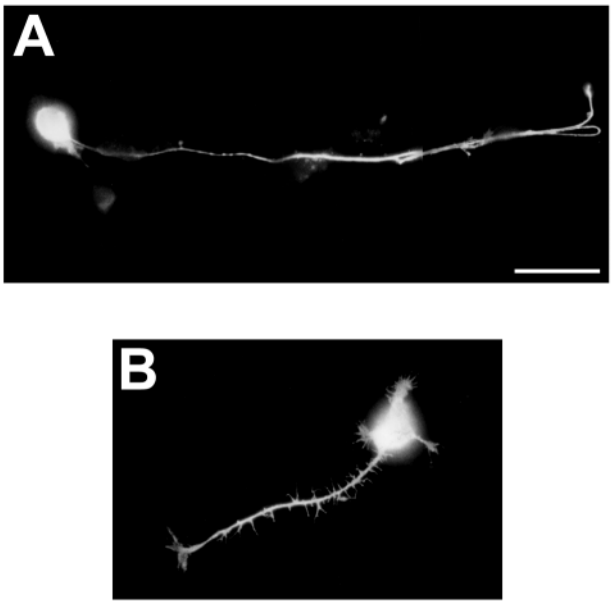

Fig. 4. Comparison between individual neurons on neutral and acidic matrices. A shows an isolated neuron after 24 hours on a laminin matrix obtained at neutral pH. B shows a neuron after 6 hours on a laminin matrix obtained at acid pH. Cells were immunostained for Tau. Bar, 25 μm.

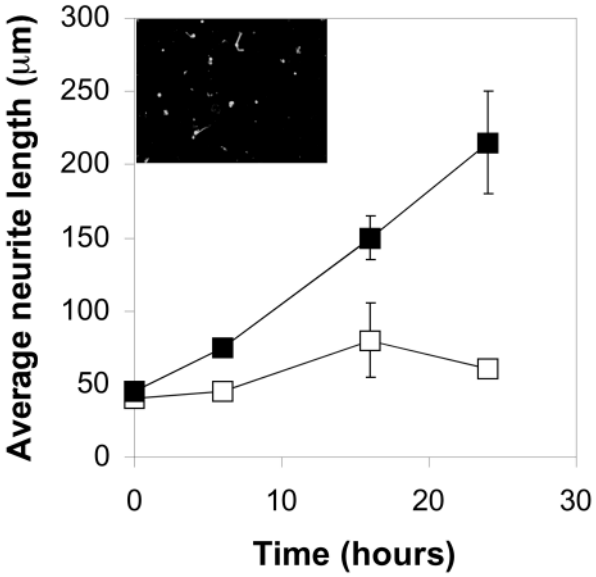

Fig. 5. Kinetics of neurite outgrowth on neutral and acidic laminin matrices. Cells were plated onto neutral (open symbols) or acidic laminin matrices (closed symbols) and fixed after 2, 6, 16 or 24 hours. After immunostaining for Tau, the average sizes of the 30 longest neurites were averaged and plotted against the time of incubation. Inset shows the absence of cell clumps within 2 hours of plating on the neutral matrix.

clumped cells could be attributed to several effects. First, it could be due to cell death. To examine this possibility, we estimated cell viability using trypan blue in both conditions and found that after 24 hours more than 90% of the cells were viable in both cases (data not shown). As second possibility is that cell bodies may preferentially attach to the large laminin aggregates present on neutral matrices. This could be the case because clumps appearing 24 hours after plating cells over the neutral matrix indeed colocalize with large laminin aggregates (Fig. 6). Nevertheless, after 2 hours, a time frame sufficient to guarantee maximal cell attachment (see above), clumps were still not present, ruling out this possibility (Fig. 5, inset). We thus hypothesized that clumps could be due to proliferating cells. To test this hypothesis we treated cells with the thymidine analogue, BrdU, which labels cells in S phase. Visual inspection revealed that the majority of cells in contact with the neutral matrix showed overlapping labeling for both the nuclear marker DAPI and for BrdU, whereas only a fraction of cell nuclei stained with DAPI was co-stained for BrdU on the acidic matrix (Fig. 7). Quantitative analysis showed that the fractions of proliferative cells in culture after 24 hours were 60 and 30% for cultures on neutral and acidic matrices, respectively (Fig. 7G). The observation that the neutral matrix supported a larger fraction of proliferating cells was confirmed by directly counting cell number at increasing times. On the neutral matrix, cells doubled after 2 hours and then increased three-fold in the following 22 hours, leading to a total increase of six-fold in 24 hours. In the same time interval cells plated on the acidic matrix did not increase in number. Taken as a whole these results indicate that, although the two laminin matrices promote cell adhesion with similar efficiency, the acidic matrix favors neuritogenesis while the neutral matrix favors cell proliferation.

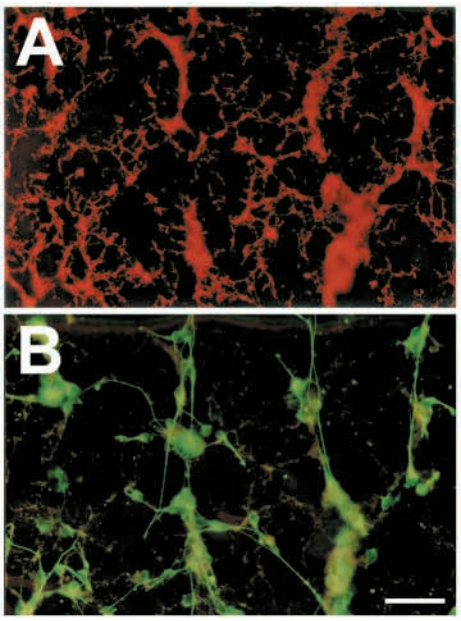

Fig. 6. Double staining for laminin and neuronal cells. Cortical cells were plated over laminin polymerized in neutral buffer. After 24 hours immunocytochemical analyses for laminin (rabbit polyclonal) and for the neuronal marker, β tubulin (mouse monoclonal), were performed. A shows the laminin matrix and B neuronal cell bodies accumulating on regions corresponding to large laminin aggregates. The scale bar corresponds to 50 μm.

Differential effects of kinase inhibitors upon neurite outgrowth on distinct laminin matrices

To investigate whether neuritogenesis on the two laminin matrices involves similar signal transduction pathways, we tested the effects of selective inhibitors of protein kinases upon neuritogenesis of embryonic cortical cells plated on each matrix. Staurosporine, a wide spectrum inhibitor of protein kinases, with particular effects upon protein kinase C (PKC) and myosin light chain kinase (MLCK), reduced the average neurite length by 40% when added to cultures on the neutral laminin matrix (Fig. 8). On the other hand, neuritogenesis on the acidic matrix was not significantly affected by this inhibitor. Conversely, H-89, which inhibits protein kinase A, did not affect neuritogenesis on the neutral matrix but decreased the average neurite length from a mean value of 244.9 to 126.2 μm when tested in cultures plated on the acidic matrix. These results are in agreement both with previous studies using artificial matrices assembled in neutral buffer, which reported that modulation of neuritogenesis by laminin involved activation of protein kinase C (Bixby, 1989; Ary-Pires and Linden, 2000), as well as with evidence for the involvement of MLCK in neurite growth (Jian et al., 1996; Ramaker et al., 2001). Notwithstanding, our data raise the possibility of alternative activation of protein kinase A in the transduction of neuritogenic signals. Based on the emerging concept that increased intracellular levels of cyclic AMP can prompt neurons to neuroplasticity events (Bailey et al., 1996; Cai et al., 2001), we investigated whether contact with the acidic laminin matrix would promote neuritogenesis in cells that are otherwise non-responsive to laminin. In this investigation, we used cortical explants of newborn rats, which

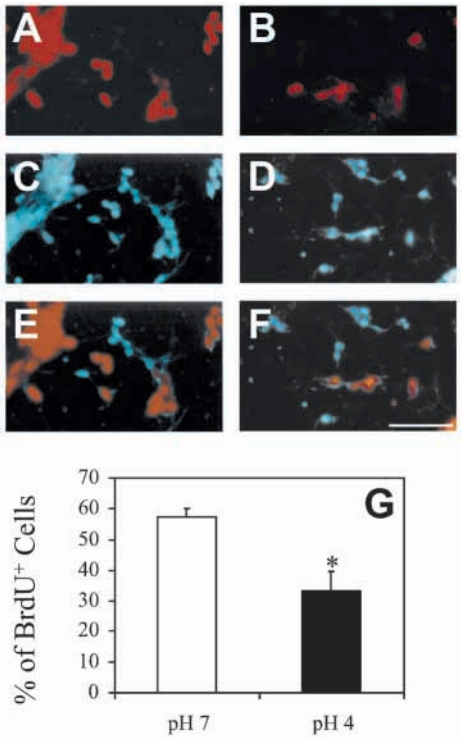

Fig. 7. BrdU incorporation by cells plated on neutral or acidic laminin matrices. Neurons were plated on neutral (A, C and E) or acidic laminin matrices (B, D and F). After 2 hours BrdU was added and kept in culture for the following 22 hours, after which cells were fixed, permeabilized and analyzed for the incorporation of BrdU. A and B show cells that incorporated BrdU, C and D show the same fields analyzed for the nuclear marker, DAPI and E and F show the BrdU and DAPI images overlaid using the software Photoshop. Bar, 100 μm. G represents the quantitative analysis of data in panels A to F. One asterisk indicates $P<0.05$.

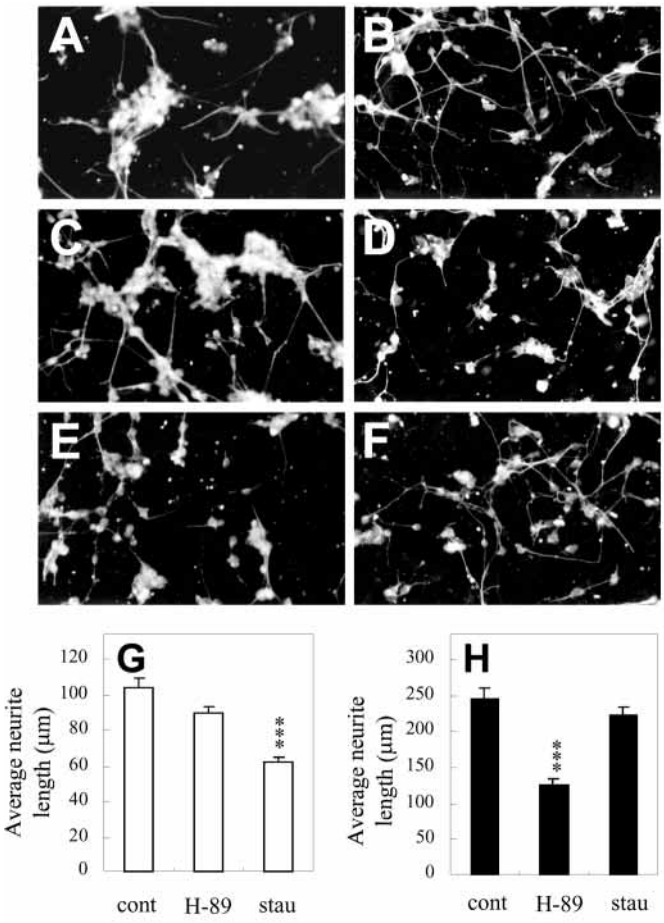

Fig. 8. Effect of protein kinase inhibitors on neuritogenesis. Cortical cells were plated over laminin matrices polymerized under either neutral (A, C, E and G) or acidic conditions (B, D, F and H) and incubated for 24 hours in the absence of inhibitors (A and B) or in the presence of 0.1 μM H-89 (C and D) or 0.15 nM staurosporin (E and F). The scale bar corresponds to 100 μm. The lowest panels show average neurite lengths (quantified as the 30 longest neurites) in each case. Three asterisks correspond to $P<0.001$.

do not respond to neutral laminin, when plated in total absence of serum or growth factors (see below).

Migration and neurite outgrowth of postnatal neurons

Explants of cerebral cortex of postnatal day 2 rats were plated either on the neutral or on the acidic laminin matrix and examined after 24 hours. As seen in Fig. 9, explants did attach to the neutral matrix but cells were virtually unable to migrate or develop neurites. On the other hand, cells from explants plated on the acidic matrix migrated outwards and developed long neurites. Since virtually all migrating cells were double-labeled for the nuclear marker DAPI and for the neuronal cytoskeletal protein Tau, it is likely that they are committed to neuronal differentiation

Discussion

In this work we show that laminin can self polymerize in different ways, giving rise to supramolecular networks of distinct patterns. Previous studies have hypothesized that laminin in solution alternatively self-assemble into either two or three-dimensional polymers depending on protein concentration or on the availability of calcium ions (Schittny and Yurchenco, 1990; Lallier and Bronner-Fraser, 1991). Thereafter, Garcia-Abreu and co-workers showed that the laminin matrices deposited by astrocytes in culture could be organized in two different patterns, described by the authors as punctate and fibrillar (Garcia-Abreu et al., 1995a; Garcia-Abreu et al., 1995b). Recently, Tsiper and Yurchenco reported that exogenous laminin assembled into reticular or fibrillar deposits on the surface of Schwann cells (Tsiper and Yurchenco, 2002). In addition, laminin has been shown to be organized at the extracellular matrix of developing brain as aggregates of distinct morphologies, namely small and large puncta, sheath and somal (Zhou, 1990). In most cases, differences in the supramolecular organization of laminin were either attributed to interactions with other macromolecules, such as proteoglycans or cellular receptors or due to specific properties of the various laminin isoforms. On the other hand, the distinct polymers shown here were obtained using a laminin preparation not chemically treated or proteolytically digested, in the absence of other added extracellular matrix components and, therefore, should result from interaction between laminin molecules at different conformations.

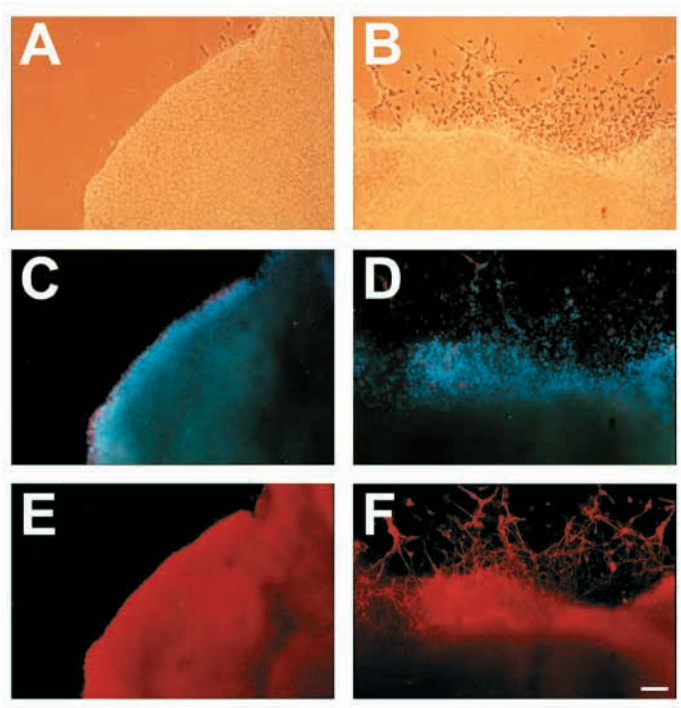

Fig. 9. Neurite outgrowth from explants of newborn rat brains. Brain explants were obtained from the cerebral cortex of postnatal day 2 rats and plated over laminin matrices obtained at neutral (A, C and E) or acidic pH (B, D and F). Explants were developed after 24 hours for DAPI (C,D) and for the neuronal marker Tau (E,F). A and B show corresponding phase-bright images. Bar, 100 μm.

The morphologically distinct laminin matrices described here presented different functional properties. Laminin polymers obtained at neutral pH had already been shown to promote neuritogenesis of neurons from both the peripheral and central nervous system (Rogers et al., 1983, Hammarback et al., 1988; Chamak and Prochiantz, 1989). Here we confirmed that neurite outgrowth is promoted by the neutral laminin matrix, but we observed that neritogenesis was further increased on the acidic matrix. A correlation between the morphology of a cell-associated laminin matrix and its neuritogenic potential was described in cultivated astrocytes, isolated from different regions of rat brain (Garcia-Abreu et al., 1995a). Interestingly, a similar structure/function correlation occurred in brain cells isolated at different developmental stages (E.F. and T.C.-S., unpublished). These observations support the notion that, in the nervous system, glial cells can use differential laminin assembly modes to control their biological properties in vivo. Our finding that laminin is organized in vivo in either permissive or non-permissive morphologies, respectively, at the periphery and center of newborn retina is consistent with this view.

Comparison of the distributions of cell bodies on the two matrices revealed that the neutral matrix led to cell clumping. Such effect had previously been observed in primary cultures of rat embryonic neurons either plated on laminin (Ivins et al., 1998) or in co-cultures with astroglial cells (Garcia-Abreu et al., 1995a), and in both cases cell clumping correlated with poor neuritogenesis. Here we showed that the clumps of cell bodies observed after 24 hours of culture resulted from an increase in cell proliferation. It can be speculated that the distinct functional properties of the two matrices, that is, their capacities for favoring either neuritogenesis or cell division, are related to differential exposure of laminin domains in the polymers. In this regard, it has previously been proposed that distinct laminin fragments, namely E8 and P1 control neurite outgrowth and proliferation, respectively, in cultured retinal neuroepithelial cells (Frade et al., 1996).

A consistent body of evidence has defined a crucial role for the isoform 1 of laminin (LN-1) as a positive cue during development of the nervous system. Besides inducing neuronal migration and differentiation in vitro, LN-1 has been implicated in neuritogenesis in vivo (reviewed by Luckenbill-Edds, 1997; Colognato and Yurchenco, 2000). For instance, LN-1 has been shown to localize along the routes of migrating neuroblasts and growing fiber tracts in the embryonic brain (Liesi, 1985; Letourneau et al., 1988; Liesi and Silver, 1988; Hunter et al., 1992). Furthermore, mice lacking the laminin-specific α6 integrin chain present abnormal laminar organization in the developing cerebral cortex and retina (Georges-Labouesse et al., 1998). Late in development, neurons lose their ability to respond to LN-1, which contributes to the impairment of regeneration in the adult central nervous system. Lack of response to LN-1 has been ascribed to either downregulation of integrin receptors or a decrease in their activation state (Cohen et al., 1986; Cohen et al., 1987; Hall et al., 1987; de Curtis et al., 1991; de Curtis and Reichardt, 1993; Ivins et al., 2000). Nevertheless, adult neurons can still respond to the isolated long arm (fragment E8) of LN-1 or to 'activated' LN-1, that is, LN-1 blocked with antibodies directed to the short arms (Calof et al., 1994; Ivins et al., 1998). In this study we report that acidic polymerization can reverse the lack of response to full-length LN-1 in explants of newborn rat cortex. This suggests that, upon acidic polymerization, laminin can hide its short arms and predominantly expose the distal region of the long arm to recognition by cellular receptors. This hypothesis is compatible with our experimental data (Fig. 1), showing that the acidic matrix presents a sheet-like arrangement, which indicates prevalence of the interactions occurring in a single spatial plane (between short arms). By contrast, the neutral laminin matrix assembles into 3D aggregates, indicative of higher contribution of interactions involving domains located at different planes (between short and long arms). Several laminin receptors have been implicated in neuritogenesis, namely integrins α1β1, α6β1 and α3β1, dystroglycan, the amyloid precursor protein (reviewed in Luckenbill-Edds, 1997; Powell and Kleinman, 1997) and the cellular prion protein (Graner et al., 2000). Except for α1β1, all these receptors bind to laminin sequences located at the distal region of the long arm, which is probably available for cell recognition in both neutral and acid matrices. Conversely, the N-terminal globular domain of laminin α1 recognized by the α1β1 integrin (Colognato-Pyke et al., 1995) is located in a short arm and expected to be committed to laminin-laminin interactions in the acidic polymer. It is thus conceivable that the proliferative response of cortical cells observed here on neutral laminin involves recognition by α1β1 integrins.

Laminin-induced neuritogenesis involves activation of PKC (Bixby, 1989; Ary-Pires and Linden, 2000). Here we have shown that staurosporin, which inhibits both PKC and MLCK

activation, selectively inhibits neurite extension on the neutral matrix. This result is not surprising since activation of these two kinases can be connected to the Ras/MAP kinase signaling cascade, which mediates integrin signal transduction (Hood and Cheresh, 2002). In addition, ligation of dystroglycan has also been connected to the same signaling pathway, precisely through activation of the adaptor protein Grb-2 (Yang et al., 1995). Interestingly, neuritogenesis on acidic laminin was not affected by staurosporin but, instead, by the specific inhibitor of PKA, H-89. Increased levels of endogenous cAMP are associated with enhancement of neuritogenesis induced by neurotrophic factor (e.g. Meyer-Franke et al., 1995), whereas decreased levels have recently been identified as the cause for the developmentally regulated loss of neuronal response to myelin (Cai et al., 2001). Despite the identification of a connection between PKA and neuritogenic potential downstream from cAMP, no correlation between activation of PKA and occupancy of laminin receptors has been established so far. One possibility is that cell attachment to the acidic, flat, matrix would permit better cell spreading and, consequently, transduction of mechanochemical signals through simultaneous engagement of integrin receptors. Such a mechanism has recently been described in endothelial cells attached to a fibronectin substrate (Meyer et al., 2000).

The question of how acidification controls laminin self-assembly in vivo remains to be answered. Despite the morphological similarities between artificial matrices assembled at acidic pH and the natural matrix in the retinal inner limiting membrane, it cannot be concluded that acidification plays a role in vivo. In vitro acidification could simply mimic a putative physiological event, such as a conformational change, thereby privileging interactions between laminin short arms (necessary for organization into a sheet-like matrix). Alternatively, it is possible that acidification of bulk pH actually simulates an in vivo situation, because the accumulation of negative charges at the outer surface of cell membranes leads to a net decrease in local pH. If this is the case, then laminin binding to cell receptors [in particular to integrins containing the β1 subunit, reported to localize to lipid rafts along with gangliosides (Claas et al., 2001)], would trigger acid-induced conformational changes and the corresponding self-assembly process. In this regard, we have observed that laminin matrices formed over films of gangliosides in neutral buffer presented the same morphological and functional properties as the acidic matrix described here (E.F., unpublished).

In conclusion, the alternative modes of assembly of laminin matrices lead to differential growth of neurites, with the acidic matrix, which shows a morphology similar to that found in vivo on a permissive surface for axonal growth, favoring neuritogenesis over cell proliferation. These results, together with recent findings demonstrating the pivotal role of laminin for in vivo regeneration in the central nervous system (Grimpe at al., 2002), point to the possibility of using acidic laminin matrices to stimulate axonal regeneration.

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