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Maturation-Dependent Upregulation of Growth-Promoting Molecules in Developing Cortical Plate Controls Thalamic and Cortical Neurite Growth

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We have tested the hypothesis that maturation-dependent changes in the cortical plate affect the spatiotemporal growth patterns of developing thalamocortical and corticocortical axonal projections. Given a choice between alternating lanes of embryonic (E18–19) and neonatal (P0–1) rat cortical plate membranes, embryonic (E18–19) thalamic and cortical neurites prefer to extend on neonatal membranes. Thalamic and cortical explants do extend neurites on uniform carpets of E19 cortical plate membranes, but the outgrowth is consistently greater on uniform carpets of P1 cortical plate membranes. These experiments demonstrate a maturation-dependent enhancement in the ability of cortical plate to support neurite growth from thalamic and cortical explants. In contrast, retinal and cerebellar neurites, which do not grow into cortex *in vivo,* generally grew poorly on these membranes, suggesting a degree of specificity to the neurite growth response. Immunohistochemical analysis of developing cortex suggests that several extracellular matrix (ECM) and cell adhesion molecules are upregulated in cortical plate. However, immunocharacterization of membrane carpets for these same ECM and cell adhesion molecules suggests that the growth preferences of thalamic and cortical neurites *in vitro* are predominantly influenced by membrane-anchored, rather than ECM, molecules. Western analysis of E19 and P1 cortical plate membranes supports this conclusion, and indicates that the membrane-anchored cell adhesion molecules L1 and N-CAM are more abundant in the P1 cortical plate membrane preparation. Experiments in which cortical plate membranes were treated to remove molecules sensitive to phosphatidylinositol (PI)-specific phospholipase C demonstrate that neurite growth promoters present in E19 cortical plate membranes are predominantly PI linked, whereas those present in P1 membranes are predominantly non–PI linked. These findings indicate that the neurite growth preferences are mediated, at least in part, by an upregulation of neurite growth-promoting molecules in developing cortical plate that are not PI linked. Taken together, these findings suggest that a maturation-dependent upregulation of neurite growth-promoting molecules on cortical plate cells controls the invasion of the cortical plate by thalamocortical and corticocortical axons.

[Key words: axon guidance, thalamocortical development, cortical development, cell adhesion molecules, extracellular matrix, cortical plate, L1, N-CAM]

The early development of the afferent and efferent axonal projections in the mammalian cortex is characterized by stereotypic, spatiotemporal patterns of axon growth in relation to the cortical plate, from which layers 2–6 will later differentiate, and the subplate (for review see O'Leary and Koester, 1993). The first afferent axons to enter the cortex are from the thalamus (De Carlos and O'Leary, 1992; Miller et al., 1993; De Carlos et al., 1994); this projection forms the major afferent input to cortex. After passing through the internal capsule into the cortex, thalamocortical afferents do not immediately grow into the cortical plate, but instead take an intracortical path that is centered on the underlying and more mature subplate layer (Lund and Mustari, 1977; Miller et al., 1993; Bicknese et al., 1994; De Carlos et al., 1994). When thalamocortical afferents reach their appropriate cortical area, layer 4 neurons, their main target cells, are still being generated (Lund and Mustari, 1977; Kageyama and Robertson, 1993). However, layer 6 neurons, a secondary target for thalamocortical afferents, have been generated and most have already migrated into the cortical plate (Lund and Mustari, 1977). After a brief waiting period in rodents (Lund and Mustari, 1977; Blakemore and Molnar, 1990; Catalano et al., 1991; Kageyama and Robertson, 1993) or a lengthy one in cats and primates (Rakic, 1977, 1988; Shatz and Luskin, 1986; Ghosh et al., 1992), thalamocortical axons extend collaterals into the cortical plate. These afferent axons progressively invade more superficial parts of the cortical plate, paralleling the deep-to-superficial gradient in the maturation of cortical plate neurons (Lund and Mustari, 1977), and the majority of axons remain deep to the dense, or less mature, cortical plate (Catalano et al., 1991; Kageyama and Robertson, 1993; Miller et al., 1993; Schlaggar and O'Leary, 1994).

Like the thalamocortical afferents, cortical axons seem not to prefer immature cortical plate as a growth substrate. For instance, axons extended by neurons in the marginal zone (future layer 1), which directly overlies the cortical plate, are restricted to the marginal zone (Marin-Padilla and Marin-Padilla, 1982; De Carlos and O'Leary, 1992; Miller et al., 1993). Axons extended by neurons in the cortical plate take a direct radial path out of the cortical plate and adopt a tangential course in the intermediate zone deep to the subplate (Koester and O'Leary, 1993; Miller et al., 1993; Bicknese et al., 1994). Only later do

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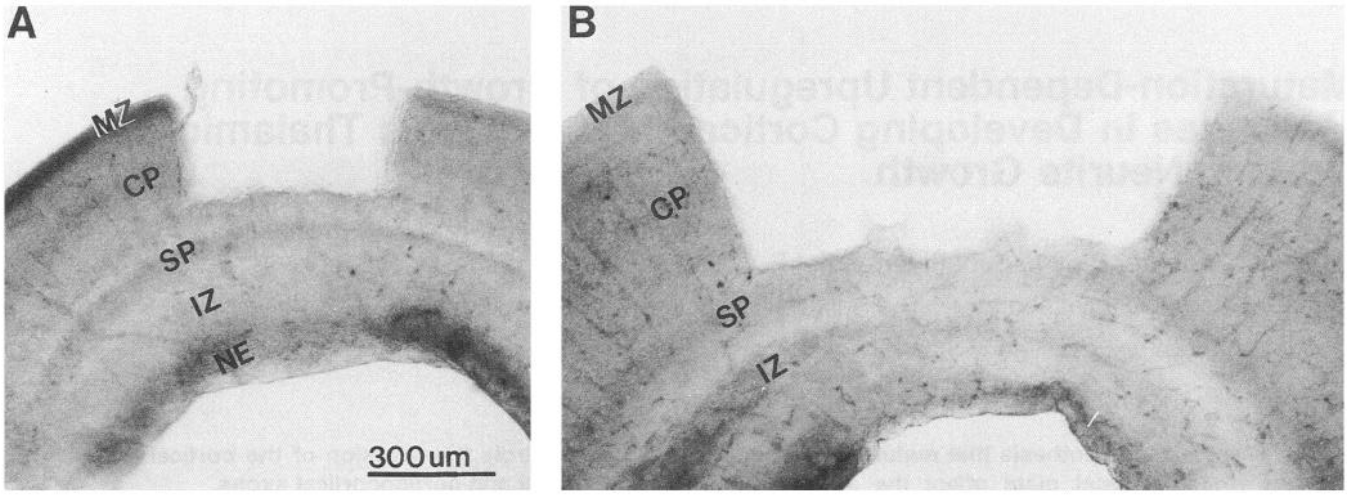


Figure 1. Cortical plate dissection from embryonic and neonatal rats. Bright-field photographs of coronal sections of E19 (*A*) and P1 (*B*) occipital neocortex in which a piece of cortical plate (*CP*) has been dissected away. The dissected cortical plate includes the overlying marginal zone (*MZ*), but excludes the underlying subplate (*SP*), intermediate zone (*IZ*), and neuroepithelium (*NE*).

the cortical plate axons branch along their proximal portions within the cortical plate (Katz, 1991; Callaway and Katz, 1992; Katz and Callaway, 1992). The invasion of cortical plate by callosal axons from contralateral cortex also parallels the maturation of cortical plate (Norris and Kalil, 1992). Thus, the elaboration of cortical axons within the cortical plate follows a spatial and temporal time course like that of the thalamocortical afferents.

These highly stereotyped patterns of axonal growth suggest that the cortical plate is initially a poor substrate for axon extension, but as it matures in an inside-out pattern, growth permissivity develops in parallel. In support of this hypothesis are immunohistochemical findings of changes in the distributions of the ECM molecules cytotactin (also called tenascin) and chondroitin sulfate proteoglycan (CSPG), and the membrane-anchored cell adhesion molecules L1 and N-CAM (Chung et al., 1991; Sheppard et al., 1991; Miller et al., 1992; Bicknese et al., 1994; Oohira et al., 1994) that parallel the development of thalamocortical and corticocortical projections (Lund and Mustari, 1977; Coogan and Burkhalter, 1988; Catalano et al., 1991; Kageyama and Robertson, 1993). Each of these ECM and cell adhesion molecules has been shown to affect CNS neurite growth *in vitro* (Lagenauer and Lemmon, 1987; Doherty et al., 1990; Iijima et al., 1991; Lochter et al., 1991; Snow and Letourneau, 1992; Friedlander et al., 1994). In tissue culture studies, Bolz and colleagues (Götz et al., 1992) have shown that E16 thalamic explants placed on a uniform carpet of embryonic day (E) 16 or postnatal day (P) 6 rat cortical membranes extend a greater number of neurite fascicles on the P6 membranes; on the other hand, E16 cortical explants did not display any differential growth response. Since these membrane preparations were derived from the entire cortical wall, it was not possible to localize the maturational changes to the cortical plate. On the other hand, Emerling and Lander (1994) have shown that thalamic cells placed on vibratome sections of brain exhibit an age-dependent increase in adhesion to cortical plate.

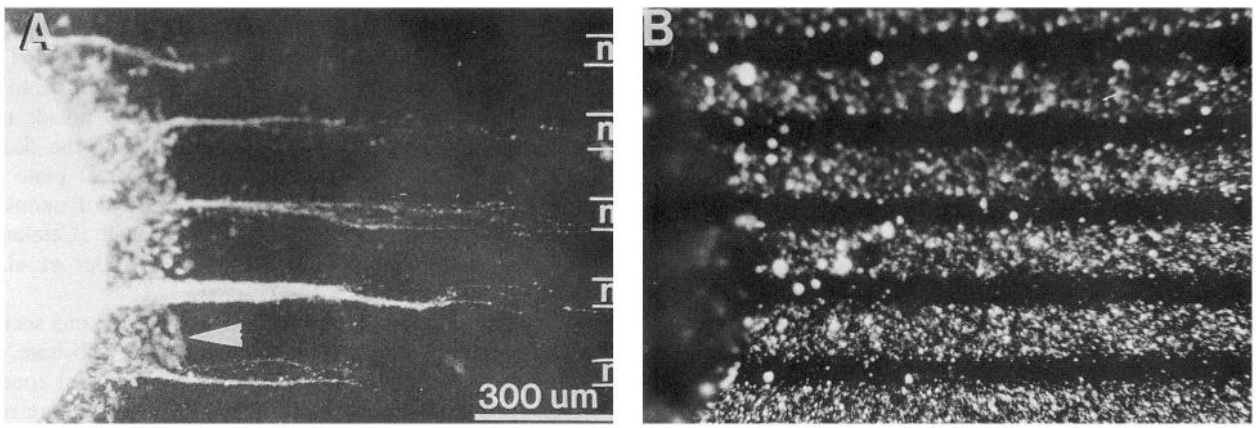


Figure 2. Thalamic neurites prefer neonatal over embryonic cortical plate membranes as a substrate for growth. *A*, Neurites from an explant of E18 LG display a strong preference for the lanes of neonatal (*n*) cortical plate membranes, which were the second set of lanes laid down. The neurites, visualized with a vital dye and FITC fluorescence illumination, extend well beyond a narrow halo of cells (*arrowhead*) that emigrated a short distance from the explant. *B*, UV illumination of the field in *A* reveals the lanes of embryonic cortical plate membranes which were marked by adding fluorescent beads to the membrane preparation. The alternating lanes of neonatal (*n*) and embryonic cortical plate membranes are indicated at the *right margin* in this and all subsequent figures.

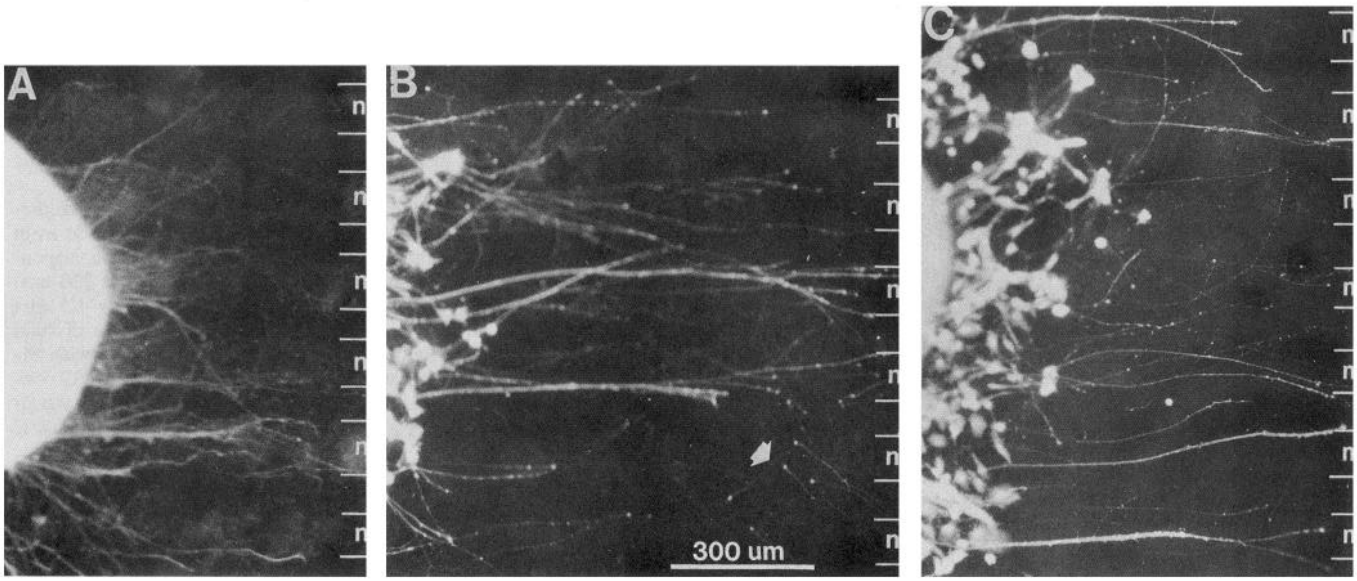


Figure 3. Thalamic and cortical neurites exhibit varying degrees of preference for neonatal cortical plate membranes. In each example illustrated, E18 or 19 LG explants were placed on carpets in which the lanes of neonatal cortical plate membranes were laid down first. Neurite preferences were categorized by the criteria of Walter at al. (1987b) as strong (*A* or Fig. 2*A*), intermediate (*B*), or weak (*C*). In *B,* the neurites in the *lower right corner* (*arrow*) are from a second explant.

To test the hypothesis that the cortical plate undergoes maturation-dependent changes that influence thalamic and cortical axon growth, we used the *in vitro* membrane stripe assay (Walter et al., 1987b) to create a substrate of alternating lanes of embryonic and neonatal cortical plate membranes prepared from rat cortical plate dissected free from the underlying subplate, intermediate zone, and neuroepithelium. The ages chosen for the cortical plate dissection were E18 or E19 and P0 or P1. At E19, thalamocortical afferents from the LG are just beginning to extend into the deepest part of the occipital cortical plate; by P0, the invasion of the cortical plate is well underway (Kageyama and Robertson, 1993). The growth preferences of rat thalamic and cortical neurites on these striped membrane carpets were examined, as was the growth behavior of neurites that do not normally project into cortex. Phosphatidylinositol-specific phospholipase C (PI-PLC) experiments were done to assess the contribution of PI-linked molecules in mediating the preferences observed *in vitro.* In addition, developmental changes in cortical plate ECM and membrane-anchored cell adhesion molecules that might affect thalamocortical and corticocortical axon growth were characterized immunohistochemically and by Western transfers.

A preliminary report of some of these findings has been presented (Tuttle et al., 1993).

Materials and Methods

Animals. The fetuses and offspring of timed pregnant Sprague–Dawley rats (Harlan Sprague Dawley, Inc.) were used. The day of insemination is designated E0; pups are normally born early on E22. The first 24 hr after birth is designated P0.

Preparation of explants. To collect embryonic tissue, pregnant rats were anesthetized with nembutal (5 mg/100 gm) and embryos removed by Cesarean section. Embryos were placed in cold, oxygenated L15 medium (Gibco) supplemented with 0.6% glucose (L15-glucose). Brains were dissected out, and, after the meninges were removed, embedded in 3% low-gelling-temperature agarose (FMC BioProducts) in L15-glucose. Coronal sections were cut at 300 μm in cold, oxygenated L15-glucose with a vibratome. Using incident light, the LG could be easily identified and dissected from sections of E18 and E19 thalamus. The LG explants measured approximately 300 μm thick, 350 μm wide, and about 700 μm long, and included the ventral and dorsal LG; the edge of the lateral posterior nucleus was often included. Explants of ventral diencephalon (VD) were dissected from the same sections as the LG explants; the VD explants were from the lateral surface of the diencephalon, ventral to the LG, and included lateral hypothalamus and subthalamic nuclei which do not project to cortex, as well as zona incerta, which does. The E18 or E19 cortical explants were dissected from sections through the occipital (i.e., posterior) cortex; tissue deep to the subplate (i.e., intermediate zone and neuroepithelium) was usually dissected away. Retina were dissected whole from E17 or E18 rat embryos and then cut into eight radially symmetric pieces. Cerebellar explants were cut from 250 μm vibratome sections of P7 rat brain, and included both external and internal granular cell layer.

After dissection, the explants were placed in suspension culture in a 5.5% CO_2, humidified incubator. The culture medium was DMEM/F12 supplemented with 2 mM glutamine, 0.6% glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, 5% heat-inactivated rat serum, and 10% heat-inactivated fetal bovine serum. Thalamic, retinal, and cerebellar explants were prepared 1 d before explanting onto the membrane stripes. During this 24 hr period, explants were exposed to 10 μM cytosine arabinoside to control proliferation of non-neuronal cells. Cortical explants were prepared either the day before or on the day of explantation; in neither case were they exposed to cytosine arabinoside.

Preparation of membrane stripes. Cortical plate was dissected from 200–300 μm sections of E18, E19, E20, P0, and P1 rat brain prepared as described above for explant preparation. Dissected "cortical plate" included the thin, overlying marginal zone; however, the underlying subplate, intermediate zone, and neuroepithelium were dissected away (Fig. 1). For the stripe assay, cortical plate was dissected only from the caudal half of the neocortex to avoid potential complications due to the rostrocaudal developmental gradient in the neocortex (Bayer and Altman, 1991).

Membranes were prepared according to the protocol originally described by Walter et al. (1987b) with some modifications. All solutions were sterile, 4°C, pH 7.4, and supplemented with protease inhibitors as previously described (Simon and O'Leary, 1992). Tissue was homogenized in hypotonic buffer containing 0.005% DNAase (Worthington) by three or four passes through a 27 gauge needle. The membrane fraction was separated by spinning the homogenate on a step gradient (5% and 50% sucrose in homogenization buffer with protease inhibitors in the 5% solution). The membrane fraction was washed once in a phosphate-buffered saline solution (PBS). The concentration of each membrane suspension was adjusted to an optical density of 0.2 at 220

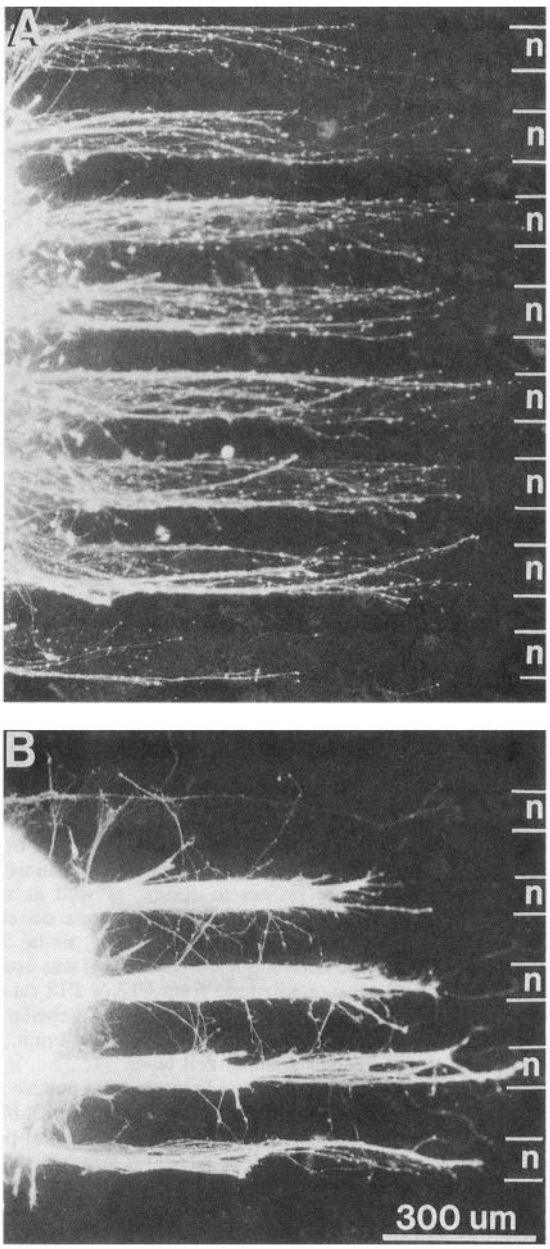


Figure 4. Cortical neurites prefer neonatal cortical plate membranes as a growth substrate. Neurites from E18/E19 cortical explants display strong preferences for neonatal membranes (*n*) laid down as either the first (*A*) or second (*B*) set of lanes.

nm using a Beckman spectrophotometer. At 0.2 nm optical density, the protein content of the P1 and E19 cortical plate membrane suspensions were determined to be roughly 120 and 60 μg/ml, respectively, using a bicinchoninic acid protein assay (Pierce).

Membrane stripes were prepared according to Walter et al. (1987b) with apparatus generously provided by F. Bonhoeffer. The membrane carpets were then placed in 2–3 ml of culture medium in a 35 mm petri dish and lowered to the bottom of the dish by placing stainless steel weights at the edges of the carpet. Explants were added to the dish and gently pushed onto the membrane carpet with forceps while the level of the culture medium was lowered; the surface tension of the medium generally prevented any subsequent movement of the explants and allowed them to attach. Cultures were maintained in a 5.5% CO_2, humidified incubator for 40–48 hours.

As described by Godement and Bonhoeffer (1989), the concentration of membranes, as judged by fluorescent bead density, tended to be lower in the second set of lanes laid down. Also consistent with previous studies, the second set of lanes laid down tended to be narrower than the first set (Godement and Bonhoeffer, 1989; Simon and O'Leary, 1992; von Boxberg et al., 1993). In some experiments a preference was observed for the second set of lanes independent of membrane type (Walter et al. 1987a,b; Vielmetter and Stuermer, 1989; Walter et al., 1990; Simon and O'Leary, 1992). To control for this second lane artifact, in each experiment the order in which the neonatal and embryonic cortical plate membranes were applied was reversed.

Phosphatidylinositol-specific phospholipase C. Membranes were prepared as described above, except that cortical plate was dissected from the entire rostrocaudal extent of the neocortex, and membrane suspensions were adjusted to an optical density of 0.3 (measured at 220 nm) in 10 mM Tris buffer (physiological pH at 37°C) containing 1.5 mM $CaCl_2$ and protease inhibitors. This membrane suspension was divided into three aliquots: two aliquots were incubated at 37°C, one with PI-PLC (1 U/ml; ICN Biochemicals) and one without enzyme (37°C control); the third aliquot was washed and resuspended in the same buffer solution but with a physiological pH at 4°C, and then left on ice (4°C control). After 1 hr, all three membrane suspensions were washed thoroughly in cold PBS containing protease inhibitors, and then resuspended in the same PBS solution. Uniform membrane carpets were made by pipetting roughly 200 μl of membrane suspension onto a filter placed over a uniform mesh and applying suction for up to 3 min.

Analysis of neurite growth. Neurites and cells were visualized with a fluorescent vital dye, 5 (and −6) carboxyfluorescein diacetate, succinimidyl ester (Molecular Probes), that labels all living cells and their processes (Tuttle and Matthew, 1991). This labeling technique allows one to determine whether neurites are directly on the membrane carpet or on cells that emigrated from the explants. A 6.15 mg/ml stock solution of dye was diluted 1:300 in PBS. Culture medium was removed from the dishes 40–48 hr after the explants were placed on the carpets, and 1–2 ml of the dye solution added for 2 min. To inhibit photobleaching, the dye solution was then replaced with a solution of 5 mM *p*-phenylenediamine (Kodak) in PBS. Neurite growth from the explant was examined and photographed with FITC optics on an epifluorescence microscope. Growth preferences for one or the other set of membrane stripes was assessed semiquantitatively using the criteria established by Walter et al. (1987a).

For PI-PLC experiments explants were placed on uniform carpets of either E19 or P1 cortical plate membranes. The amount of neurite growth from the explants was quantified from digitized images using NIH IMAGE software (version 1.49); for this, photomontages were digitized with a Microtek ScanMaker (Microtek International, Inc.). The total amount of fluorescence due to neurites was quantified to give an approximation of neurite mass. The distance from the edge of the explant to the tip of the longest neurite was measured at four positions around the explant, each separated by 90° of arc; for each explant, these four length measurements were averaged to give an estimate of neurite length. The neurite mass measurement was multiplied by the neurite length measurement to give an estimate of neurite growth on the membrane carpet. Incorporating both of these measurements distinguishes between an explant with a dense mat of neurites close to the explant, indicative of a tendency for neurites to grow on each other rather than on the membrane substrate (a result often observed on PI-PLC-treated E19 cortical plate membranes), and an explant with neurites of a comparable number and length that extend well away from the explant on the membrane substrate.

Immunolabeling. To prepare tissue sections, E19 and P1 rats were perfused with 4% paraformaldehyde in phosphate buffer (PB). Perfused brains were placed overnight in the same fix, rinsed, and cryoprotected in 30% sucrose in PB. Brains were frozen, and 12–14 μm coronal sections were cut on a cryostat and mounted on gelatin-coated slides. Uniform carpets of P1 cortical plate membranes were prepared using membranes at an optical density of 0.3 at 220 nm (Zhang et al., 1992). Carpets were immediately fixed overnight with 4% paraformaldehyde, and then washed thoroughly.

Both the sections and the carpets were incubated in PB containing 3–5% goat serum (PB-goat) for 20 min. The primary antibodies were all diluted in PB-goat and exposed to the sections and carpets for 2 hr at room temperature. The monoclonal antibody to chondroitin sulfate (CS; Avnur and Geiger, 1984; Sigma, CS-56) was diluted 1:200. Ascites against the brain CSPG neurocan (Margolis and Margolis, 1993) was diluted 1:100. Polyclonal antibodies to N-CAM (Chuong et al., 1982; Chuong and Edelman, 1984) and cytotactin (Crossin et al., 1989), generously provided by Drs. K. Crossin and G. Edelman, were diluted 1:50.

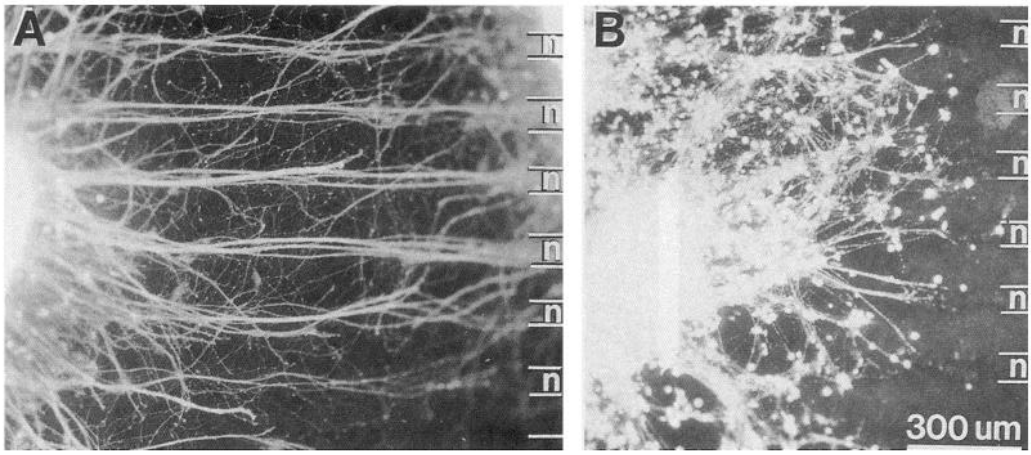


Figure 5. Neurites from different CNS regions vary in response to cortical plate membranes. *A,* Explants of E18/E19 ventral diencephalon (*VD*) exhibit a similar preference for neonatal cortical plate membranes as seen with LG and cortical neurites. A second VD explant is in the *upper right corner. B,* Neurites extending from P7 cerebellar explants tend to be coincident with cells that emigrate from these explants.

The polyclonal antibody to L1 (Chung et al., 1991; generously provided by Dr. C. Lagenaur) was diluted 1:1000. This incubation step was followed by three rinses in PB-goat. Secondary antibodies conjugated to FITC were all diluted 1:100 in PB containing 5% rat serum and allowed to sit at room temperature for 2 hr or more, followed by centrifugation. Sections and carpets were incubated with the secondary antibody for 1 hr, followed by three rinses in PB-goat. Secondary antibodies were either goat anti-rabbit Ig for the polyclonal antibodies, goat anti-mouse IgM for the monoclonal antibody to CS, or goat anti-mouse Ig for the ascites antibody to neurocan. Sections were photographed using fluorescence optics; for a given antibody, exposure times for the E19 and P1 tissue sections were the same. The sections were then counterstained with 0.5% thionin, dehydrated, cleared, coverslipped with DPX, and rephotographed with bright-field optics. Membrane carpets were photographed and printed using identical settings and conditions to reproduce relative levels of staining intensity.

Western transfer. Standard protocols were used for running SDS gels (Laemmli, 1970) and immunoblotting (Towbin et al., 1979). E19 and P1 membrane preparations were prepared as described under Preparation of membrane stripes and diluted to equivalent optical densities (0.15 at 220 nm). A volume of 10 μl of each membrane suspension was resolved on a 6 or 7.5% polyacrylamide gel under reducing conditions. Transfers were immunolabeled with the following antibodies: a polyclonal antibody made against immunoaffinity-purified N-CAM (generously provided by U. Rutishauser) used at 1:200; a polyclonal antibody to human cytotactin (Gibco) used at 1:500; and polyclonal antibodies to L1 and cytotactin (provided by the sources described above) used at 1:1000 and 1:200, respectively. Affinity-purified biotinylated anti-rabbit IgG secondary antibody (Vector Laboratories, Inc.) was used at a 1:500 dilution. Antibody binding was revealed by immunoperoxidase staining using the Vectastain Elite ABC Kit (Vector Laboratories, Inc.).

Results

Thalamic and cortical neurites prefer neonatal over embryonic cortical plate membranes

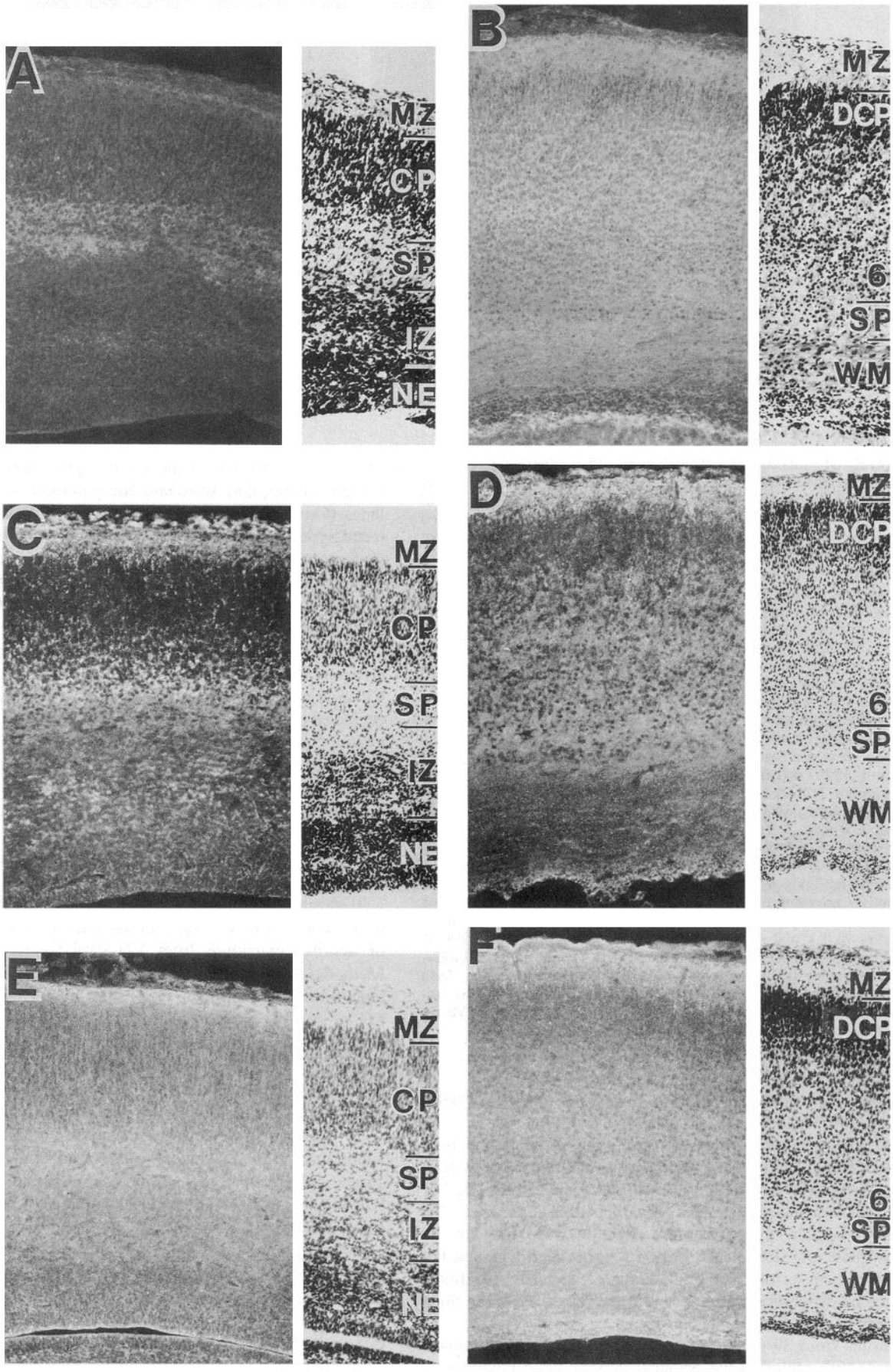
To test substrate preferences directly, explants of LG, cortex, and VD taken from E18 or E19 rats were presented with a choice between alternating narrow stripes of embryonic (E18 or E19) and neonatal (P0 or P1) cortical plate membranes. The majority of age comparisons were either between E18 and P0 cortical plate, or E19 and P1, with a few between E18 and P1. Neurite growth on the membrane carpets was analyzed after 2 d. Since no difference was detected in the results from the three age pairings, the data were pooled.

Neurites from 29 of 44 LG explants displayed a preference; of these, 86% preferred the neonatal cortical plate membranes. The preference for neonatal cortical plate membranes was observed whether they were laid down in the first or second set of lanes (Figs. 2, 3). As noted previously in studies of the retinotectal system (see Fig. 7 in Walter et al., 1987b), the degree of preference ranged from strong to weak (Fig. 3). These *in vitro* findings demonstrate that LG neurites respond to maturation-dependent changes in molecules associated with cortical plate membranes.

Neurites from embryonic cortical explants exhibited a preference similar to that of the LG explants (Fig. 4). Neurites from 47 of 51 cortical explants displayed a preference; of these, 63% preferred the neonatal membranes. As was the case for thalamic neurites, cortical neurites never displayed a preference for embryonic membranes when they were in the first set of lanes laid down; this suggests that the occasional preference for embryonic cortical plate membranes can be ascribed to the second lane artifact (see Materials and Methods). Therefore, cortical neurites also respond to maturation-dependent changes in molecules associated with cortical plate membranes.

To assess whether the thalamic growth preferences are unique to principal thalamic projection nuclei, we examined the growth of neurites extending from VD explants (see Materials and Methods). VD explants showed exuberant neurite growth on the membrane carpets which was often greater than that seen for LG or cortical explants (Fig. 5*A*). Neurites from all five VD explants tested showed a preference for neonatal cortical plate membranes. Thus, VD neurites grew robustly on cortical plate membranes and displayed a preference for the neonatal membranes similar to that observed for LG and cortical neurites.

To determine whether neurites might be able to detect more subtle maturational changes, we assessed growth preferences on striped carpets made with membrane preparations from E18 and E20 cortical plate. When given a choice between alternating lanes of E18 and E20 cortical plate membranes, neurites from 10 of 12 explants of LG, VD, or cortex exhibited a weak preference for one or the other set of lanes; of these, 80% preferred the E20 cortical plate membranes. These data support the contention that the maturation-dependent changes in cortical plate that underlie the neurite preferences are detectable in the bioassay by E20.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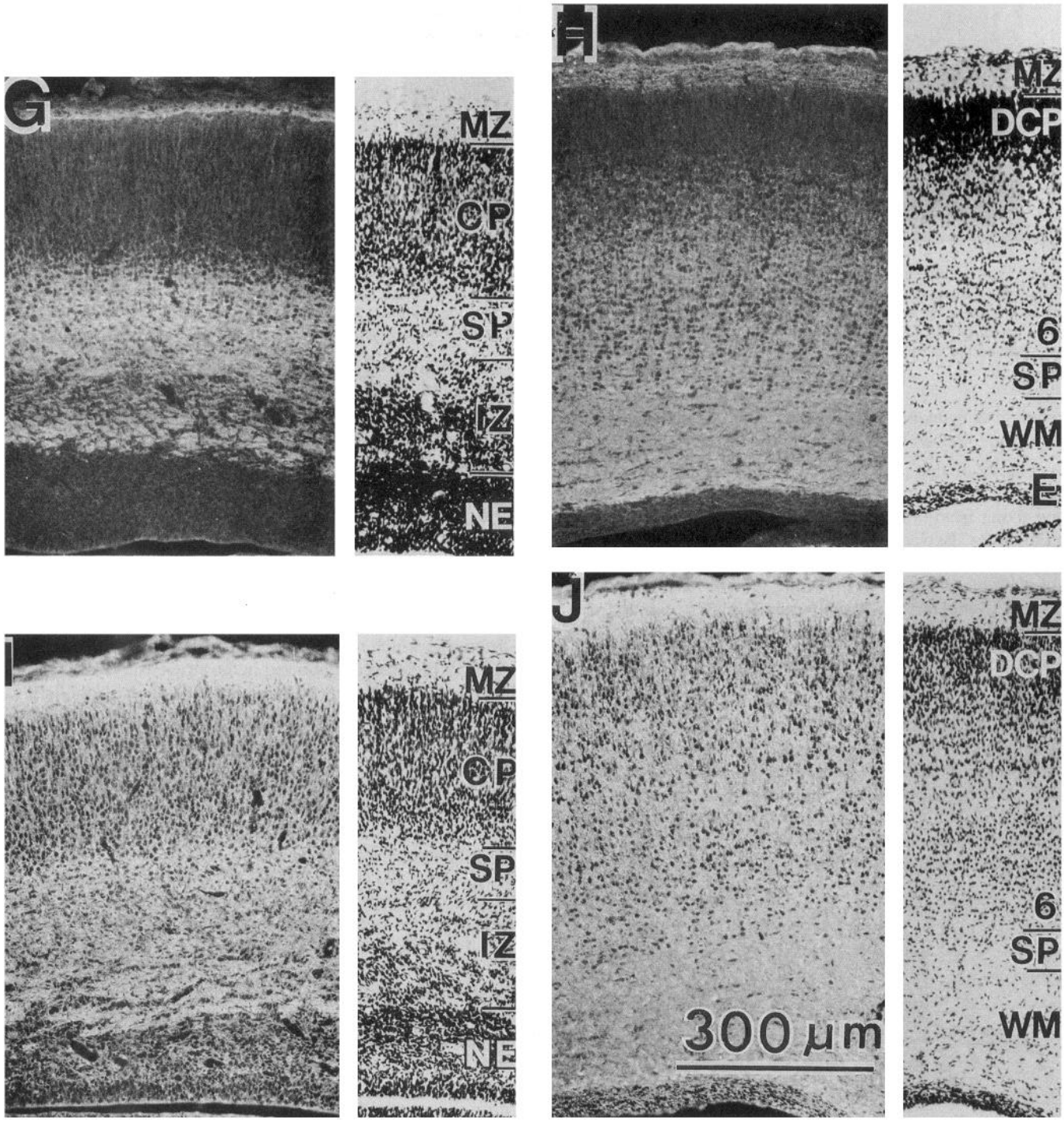


Figure 6. Immunolabeling patterns for ECM and cell adhesion molecules in cortex correlate spatially and temporally with the development of thalamocortical and intracortical projections. To the *right* of each fluorescent image is a photograph of the same section, Nissl stained to illustrate the cortical lamination. Ascites to the core protein of a brain CSPG ECM, neurocan (1F6; Margolis and Margolis, 1993), stain predominantly the subplate (*SP*) in E19 cortex (*A*); weak staining was apparent in the deep, maturing cortical plate (*CP*), the deep intermediate zone (*IZ*), and the marginal zone (*MZ*). At P1, neurocan staining is present throughout the cortical wall (*B*). A monoclonal antibody to CS (CS-56; Avnur and Geiger, 1984) stains the SP, MZ, and deep CP in E19 cortex, with patchy staining in the IZ (*C*). By P1, CS staining has greatly increased throughout the CP, but is weak in the dense cortical plate (*DCP*) and white matter (*WM; D*). A polyclonal antibody to the ECM, cytotactin (Crossin et al., 1989), intensely stains the SP and MZ, and moderately stains the IZ (*E*). By P1, the entire cortical wall, except the DCP, stains intensely for cytotactin (*F*). A polyclonal antiserum to the cell adhesion molecule, L1 (Chung et al., 1991), labels SP, MZ, and IZ at E19, as well as the deepest aspect of the CP (*G*). By P1, L1 staining is present throughout the entire cortical wall except the DCP and the ependyma (*E*); the staining intensity in the CP shows a graded deep-to-superficial pattern (*H*). A polyclonal antiserum to the cell adhesion molecule, N-CAM (Chuong et al., 1982; Chuong and Edelman, 1984), intensely stains the entire cortical wall at both E19 (*I*) and P1 (*J*), with the exception of the NE at E19 and the ependyma at P1.

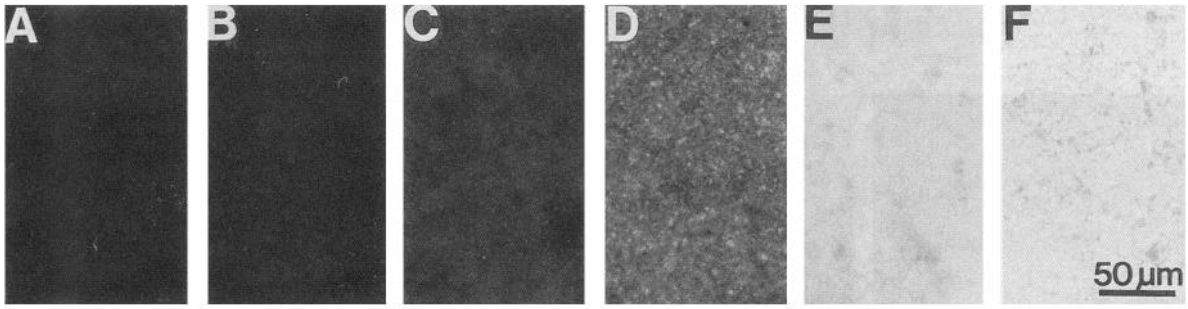


Figure 7. Cortical plate membrane carpets are deficient in ECM components and abundant in membrane-anchored molecules. Uniform carpets of P1 cortical plate membranes were stained with the antibodies described in Figure 6 and in the Materials and Methods. The negatives and prints were all exposed for the same length of time. Antibodies to neurocan (*B*) and CS (*C*) stained the carpets at levels that were barely distinguishable from the control (*A*; no primary antibody). A polyclonal antibody to cytotactin exhibited light staining (*D*). Antibodies to L1 (*E*) or N-CAM (*F*) intensely stained the carpets.

Embryonic and neonatal cortical plate membranes are not a good substrate for cerebellar or retinal neurite growth

The specificity of this preference of thalamic and cortical neurites for neonatal cortical plate membranes was studied by explanting tissue from other CNS regions which normally do not project to cortex. Explants of P7 cerebellum and E17–E18 retina were also placed on the striped carpets of embryonic and neonatal cortical plate membranes. Neurite growth from the retinal and cerebellar explants was usually much poorer than that observed from LG, VD, and cortical explants on the same membrane carpets, making it difficult to assess any potential preference for neonatal or embryonic cortical plate membranes. The cerebellar explants were typically surrounded by an expansive halo of cells which had emigrated from the explant (Fig. 5*B*). The neurites which did grow were usually found overlying these cells, apparently preferring them as a growth substrate over that provided by cortical plate membranes. Similarly, when embryonic cortical, thalamic, and retinal explants were placed on cryosections of neonatal CNS, only the cortical and thalamic neurites were able to extend on cortex; retinal neurites did, however, grow on thalamus (R. Tuttle, B. L. Schlaggar, J. E. Braisted, and D. D. M. O'Leary, unpublished observations). These data indicate either some specificity for the neurite growth promoters in the cortical plate membrane preparations (cf. Hankin and Lagenaur, 1994), or the presence of molecules in embryonic and neonatal cortical plate that are inhibitory for the growth of retinal and cerebellar neurites.

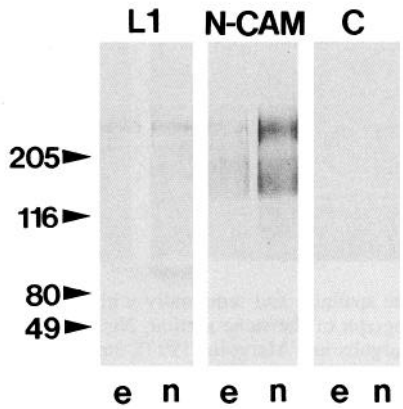


Figure 8. L1 and N-CAM are upregulated in cortical plate between E19 and P1. Western transfers of E19 and P1 cortical plate membranes stained with polyclonal antibodies to L1 and N-CAM and immunoperoxidase labeling. Ten microliters of membrane suspensions with equivalent optical densities were resolved on a 7.5% polyacrylamide gel under reducing conditions. The control lanes (*C*) were processed as the L1 and N-CAM lanes except that no primary antibody was used. Cytotactin was not detectable in these Western transfers (see Results). Molecular weight markers are indicated (205 kDa, myosin; 116 kDa, b-galactosidase; 80 kDa, bovine serum albumin; 49 kDa, ovalbumin).

ECM and membrane-anchored molecules are upregulated in developing cortical plate

To determine which molecules might be responsible for the neurite growth preferences observed in the stripe assay, E19 and P1 cortical cryosections were immunolabeled with antibodies to ECM and membrane-anchored cell adhesion molecules. The term "membrane anchored" is used in this paper to describe proteins which are either integral membrane proteins or are anchored in the lipid bilayer by covalent attachment to lipid. Antibodies to ECM components included ascites against the protein core of a brain CSPG, neurocan (1F6, Margolis and Margolis, 1993), a monoclonal antibody that recognizes chondroitin sulfate (CS-56, Avnur and Geiger, 1984), and a polyclonal antibody to cytotactin (Hoffman et al., 1988; Crossin et al., 1989). Polyclonal antibodies to the membrane-anchored cell adhesion molecules, L1 (Chung et al., 1991) and N-CAM (Chuong et al., 1982; Chuong and Edelman, 1984), were also used.

The immunohistochemical staining patterns for the three ECM components, neurocan, CS, and cytotactin, have similarities at both E19 and P1 (Fig. 6*A–F*; see legend for details). At E19, staining is concentrated in the subplate and marginal zone, and staining in the cortical plate is minimal. By P1, staining has progressed superficially from the subplate into the cortical plate and is robust in its deeper, more differentiated part. Similar to the staining patterns observed for the ECM components, L1 staining is minimal in the cortical plate at E19 and robust at P1 (Fig. 6*G,H*). In sharp contrast, a polyclonal antibody that recognizes the 120, 140, and 180 kDa isoforms of N-CAM stains both E19 and P1 cortical plate intensely, including the dense cortical plate (Fig. 6*I,J*).

Membrane preparations are ECM deficient and show a maturation-dependent increase in membrane-anchored molecules

The same antibodies were used to immunocharacterize the membrane preparations. For this, uniform carpets of P1 cortical plate membranes were chosen since, based on immunolabeling of cry-

osections, they should contain more of the ECM and membrane-anchored antigens than E19 membrane preparations. The neurocan and CS antibodies did not stain membrane carpets prepared using a membrane suspension with the same optical density (i.e., 0.2) used to prepare membrane carpets for the neurite growth assay (data not shown). Therefore, we immunolabeled carpets prepared with a membrane suspension of 0.3 optical density. However, even with these denser carpets, immunolabeling with the neurocan (Fig. 7*B*) and CS antibodies (Fig. 7*C*) resulted in staining only slightly brighter than the control carpets (Fig. 7*A*). This finding is not unexpected since CSPGs purified from developing CNS tissue are isolated from the soluble fraction (Oohira et al., 1988; Rauch et al., 1991; Grumet et al., 1993). The cytotactin antibody lightly stained the carpets (Fig. 7*D*). In sharp contrast, the L1 and the N-CAM antibodies stained the carpets intensely (Fig. 7*E,F*). These immunohistochemical data suggest that the membrane preparation method enriches for membrane-anchored molecules, such as cell adhesion molecules, but does not enrich for some ECM molecules, such as CSPG.

Western transfers were performed to determine whether L1 and N-CAM are upregulated on cortical plate membranes between E19 and P1 as suggested by the immunohistochemical data (Fig. 8). Membrane suspensions of E19 and P1 cortical plate were each adjusted to equivalent optical densities (0.15 at 220 nm), run on resolving gels, transferred, and probed with antibodies to L1, N-CAM, and cytotactin. A polyclonal antibody to L1 revealed three distinct bands with apparent molecular weights of 220, 180, and 85 kDa. L1 is often described as having an apparent molecular weight of 200 kDa in postnatal and adult mouse whole brain and cerebellum (Bock et al., 1985; Sadoul et al., 1988), but higher-molecular-weight forms have been described in PC12 cells and sensory neurons (Bock et al., 1985; Seilheimer and Schachner, 1988); the 180 and 85 kDa bands represent proteolytic products of the high-molecular-weight form (Sadoul et al., 1988). Each of these three bands is more prominent in the P1 lane, indicating that L1 is upregulated in cortical plate between E19 and P1. A polyclonal antibody to N-CAM detected diffuse high-molecular-weight bands characteristic of the highly polysialated, developmental form of N-CAM (Chuong and Edelman, 1984; Chung et al, 1991; Seki and Arai, 1991). These bands were also more prominent at P1, supporting previous suggestions that certain isoforms of N-CAM are upregulated in cortical plate (Chung et al., 1991). Cytotactin was not detectable in Westerns with the same volume and optical density of membrane suspension used to reveal L1 and N-CAM; this was the case whether 6 or 7.5% polyacrylamide resolving gels were used. Both polyclonal antibodies to cytotactin (see Materials and Methods) faintly labeled a high-molecular-weight doublet only when the maximal amount of membrane suspension (30 μl, or three times the volume used for L1 and N-CAM) was loaded and the transfer filters were overexposed (data not shown). This Western analysis supports our findings based on immunolabeling of P1 membrane carpets which suggest that the membrane suspensions are deficient in ECMs. Further, this analysis shows that the cortical plate membrane preparations contain more L1 and N-CAM at P1 than at E19, consistent with an upregulation of these membrane-anchored molecules in the cortical plate.

Non-PI-linked neurite growth promoters are upregulated in developing cortical plate

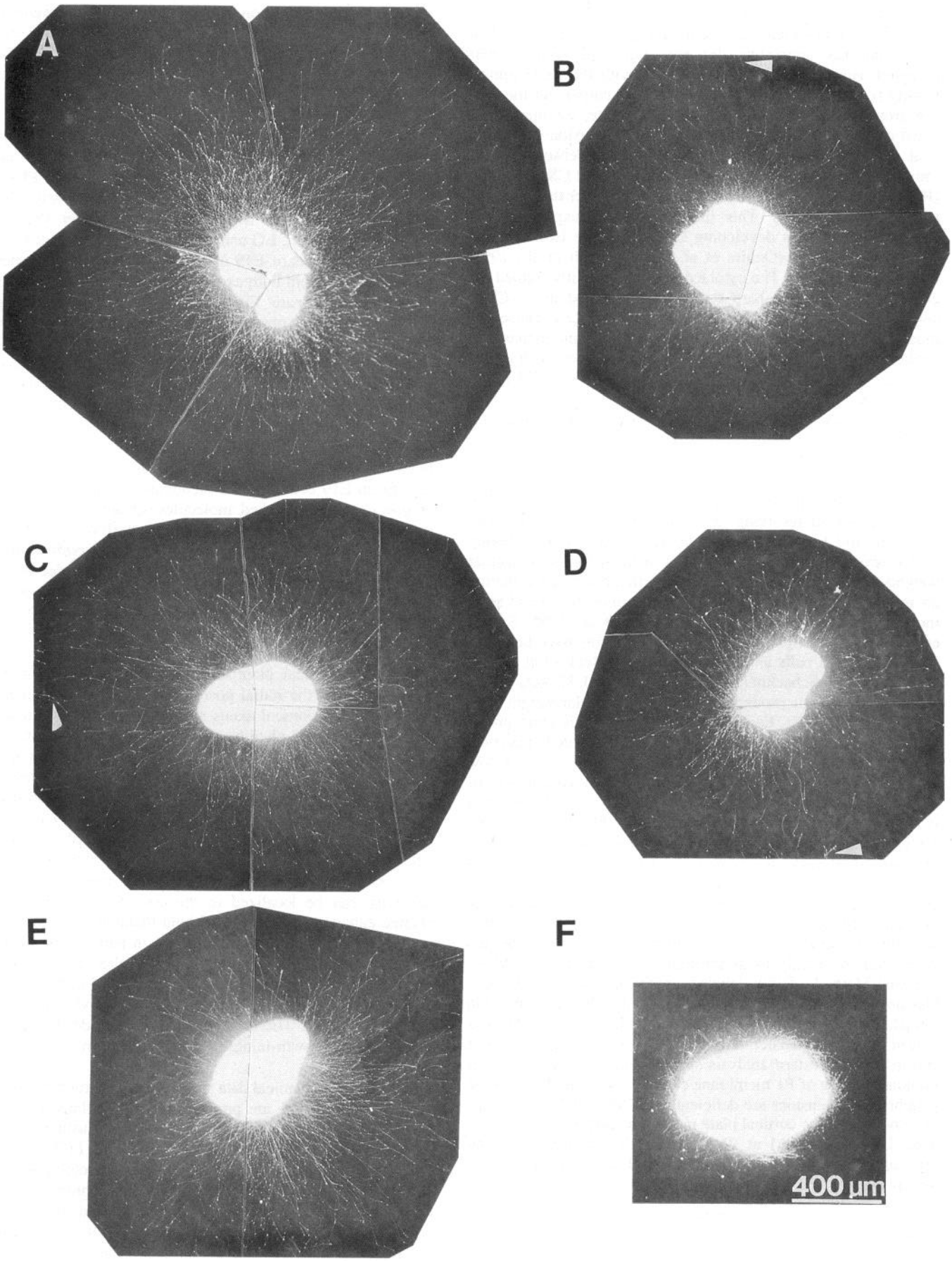
The experiments described above support the hypothesis that neurite growth-promoting molecules are upregulated in a maturation-dependent fashion in cortical plate. The immunological data implicate several cell surface and ECM molecules; only one of these, the 120 kDa isoform of N-CAM, is PI linked. It may, therefore, be possible to detect a maturation-dependent increase in non-PI-linked neurite growth-promoting molecules in the cortical plate membrane preparations.

To test this hypothesis, explants of LG or cortex were placed onto uniform carpets composed of either E19 or P1 cortical plate membrane preparations that had been treated with PI-PLC at 37°C. As controls, uniform carpets were made with identical membrane suspensions which were kept at either 4°C or 37°C without any PI-PLC. LG and cortical explants extended neurites on uniform carpets of E19 or P1 cortical plate membranes, but neurite growth from both explant types was much greater on the P1 carpets (compare Figs. 9*A,B*; 10*A,B*), corroborating the growth preferences observed on striped carpets. Neurite growth was good, although somewhat reduced, on P1 cortical plate membranes pretreated with PI-PLC (Figs. 9*E*, 10*C*), suggesting that PI-linked growth promoters are present in P1 cortical plate but that non-PI-linked growth promoters predominate. In contrast, LG and cortical explants exhibited greatly reduced or often no neurite growth on PI-PLC-treated E19 cortical plate membranes (Figs. 9*F*, 10*D*), suggesting that most of the neurite growth on E19 cortical plate membranes is due to the presence of one or more PI-linked molecules. Quantification of neurite growth on control and PI-PLC-treated uniform carpets is presented in Figure 11. These results indicate a prominent increase in non-PI-linked neurite growth-promoting molecules as the cortical plate matures; some of these molecules could, however, be PI linked but PI-PLC resistant (Wong and Low, 1994).

Discussion

Changes in cortical plate molecules that affect axon growth could influence the radial positioning of the intracortical pathway of thalamocortical axons and control the subsequent invasion of the cortical plate by thalamic and cortical axons. These spatiotemporal patterns of axonal growth could result from an initial deficiency of axon growth-promoting molecules in the immature cortical plate and their subsequent upregulation as the cortical plate matures. We addressed this issue by looking for maturation-dependent changes in the cortical plate that affect neurite growth and have made several novel findings. First, we demonstrate that a maturation-dependent change in growth permissivity can be localized to the cortical plate. Second, this change enhances the growth of both thalamic and cortical neurites *in vitro*. Third, it results, at least in part, from the upregulation of axon growth-promoting molecules. Fourth, types of membrane-anchored molecules that are not PI linked contribute to the increased neurite growth-promoting activity in the maturing cortical plate. These results do not exclude the possibility that neurite growth-inhibiting molecules are coincidentally downregulated.

Immunohistochemical data suggest that several molecules that affect neurite growth are progressively upregulated in developing cortical plate in a maturation-dependent fashion. Developmental changes in immunolabeling for the ECM molecules CSPG and cytotactin coincide with the spatiotemporal development of thalamocortical and corticocortical projections. Early on, the intracortical path of thalamocortical axons in the subplate is enriched for CSPGs (Bicknese et al., 1994; Oohira et al., 1994). Later, ingrowth of afferent axons into the cortical plate parallels a progressive deep-to-superficial immunolabeling pat-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tern for CSPGs. A brain CSPG, neurocan, has a similar distribution and undergoes the same spatiotemporal change (Miller et al., 1992; Oohira et al., 1994; present results). Some, if not all, of this CSPG is produced by cortical cells since it is apparent in the subplate, as well as in its forerunner, the preplate, before the arrival of afferent axons (Sheppard et al., 1991; Oohira et al., 1994). While CSPGs isolated from postnatal rat brain have been reported to promote the growth of embryonic rat cortical neurites (Iijima et al., 1991), neurocan has been reported to inhibit the growth of chick brain neurites (Friedlander et al., 1994). Therefore, CSPGs may play a role in influencing axon invasion of the cortical plate. Immunolabeling for cytotactin, which is produced by glia (Grumet et al., 1985; Bartsch et al., 1992), is initially most pronounced in subplate and intermediate zone but becomes apparent in cortical plate as it differentiates (Sheppard et al., 1991; present results). Although soluble cytotactin inhibits embryonic rat hippocampal neurite growth on a variety of ECM substrates, substrate-bound cytotactin promotes the growth of these neurites (Lochter et al., 1991). Immunological analysis of the membrane carpets as well as Western analysis of the membrane preparations suggest that cytotactin is present at low levels in our cortical plate membrane preparations, presumably in a substrate-bound, neurite growth-promoting form. However, since neurocan and cytotactin are deficient in the cortical plate membrane preparations, these cortical ECM components are unlikely to be mediating the thalamic and cortical neurite growth preferences that we observe *in vitro*.

The cell adhesion molecule L1 is expressed on the surfaces of maturing cortical plate neurons and their axons in a deep-to-superficial pattern coincident with the invasion of thalamocortical axons (Fushiki and Schachner, 1986; Chung et al., 1991; present study). Western data presented here supports the hypothesis that L1 is upregulated on maturing cortical plate membranes. *In vitro,* L1 promotes tectal and cerebellar neurite growth (Lagenaur and Lemmon, 1987) and neuron–neuron adhesion (Keilhauer et al., 1985) via homophilic interactions (Lemmon et al., 1989). Since L1 is also present on developing thalamocortical and cortical axons (Fushiki and Schachner, 1986), the upregulation of L1 in the maturing cortical plate could promote its invasion by these axons, as well as the elaboration of cortico-cortical connections, via a homophilic mechanism.

Like CSPGs, cytotactin, and L1, the cell adhesion molecule N-CAM undergoes a maturation-dependent increase in cortical plate. *In vitro* studies suggest that N-CAM can promote neurite growth from postnatal rat cerebellar neurons (Doherty et al., 1990) and postnatal mouse cerebellar or cortical neurons (Abosch and Lagenaur, 1993). The Western data presented here suggests that N-CAM is upregulated on maturing cortical plate membranes, although the high level of polysialic acid prevented the discrimination of the different isoforms of N-CAM. Immunolabeling with a monoclonal antibody specific for the 140 and 180 kDa transmembrane isoforms of N-CAM reveals a graded pattern of staining in the cortical plate similar to the L1 antibody staining pattern (Chung et al., 1991). On the other hand, immunolabeling with polyclonal antibodies that recognize all three isoforms of N-CAM, including the 120 kDa, PI-linked isoform, labels the entire extent of the cortical plate at both embryonic and neonatal stages (Fushiki and Schachner, 1986; Chung et al., 1991; present study). This suggests that the 120 kDa isoform is present in immature, and possibly also mature, cortical plate and that the 140 and/or 180 kDa isoforms are upregulated in maturing cortical plate. Consistent with this interpretation is our finding that PI-PLC treatment dramatically reduces thalamic and cortical neurite growth on E19 cortical plate membranes but not on P1 cortical plate membranes. Taken together, these data suggest that one or more of the three isoforms of N-CAM may be upregulated on cells in developing cortical plate; this upregulation may contribute to the increased neurite growth on P1 cortical plate membranes *in vitro,* as well as to axonal patterning *in vivo.*

Cortical and thalamic neurites can grow on embryonic cortical plate membranes *in vitro,* suggesting the existence of neurite growth promoters in embryonic cortical plate, or in marginal zone, which was included in the cortical plate dissection. Most of the neurite growth-promoting activity in E19 cortical plate membrane preparations is PI-PLC sensitive. Immunohistochemistry suggests that two PI-linked molecules, the 120 kDa isoform of N-CAM and TAG-1, are in embryonic cortical plate (Yamamoto et al., 1990; Chung et al., 1991; Wolfer et al., 1994). Since both of these molecules promote neurite growth (Furley et al., 1990; Doherty et al., 1991), the initial avoidance of the cortical plate by thalamic and cortical axons *in vivo* is not due to a lack of growth-promoting molecules in the immature cortical plate. It is possible that these molecules are present in immature cortical plate at concentrations which, *in vivo,* are subthreshold for thalamic and cortical axon growth (see, e.g., Doherty et al., 1991). In addition, subplate or intermediate zone may contain types or concentrations of axon growth promoters that make them preferred substrates during the tangential phase of thalamic and cortical axon growth, respectively (Bicknese et al., 1994). Consistent with these notions is the demonstration by Emerling and Lander (1994) that dissociated thalamic cells placed on vibratome sections of E15 mouse cortex adhere well to the subplate and superficial part of the intermediate zone, but poorly to the cortical plate. Finally, neurite growth inhibitors, which may not be enriched for or functional in the membrane preparations, could mask the function of neurite growth promoters in E19 cortical plate.

Studies by Bolz and colleagues (Götz et al., 1992) have also addressed the hypothesis that the cortical plate undergoes maturation-dependent changes in axon growth promotion. However, Bolz and colleagues prepared membranes from whole rat cortex, not just cortical plate as in our experiments; thus the changes that they observed cannot be attributed to changes in the cortical plate. Further, the activity associated with the full cortical wall that Bolz and colleagues examined enhanced thalamic but not

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Figure 9. Non-PI-linked molecules, upregulated in maturing cortical plate, promote thalamic neurite growth *in vitro.* E18 LG was explanted onto uniform carpets composed of P1 (*A, C, E*) or E19 (*B, D, F*) cortical plate membrane preparations that had been treated in one of three ways: left at 4°C for 1 hr (*A, B*), maintained at 37°C for 1 hr (*C, D*), or kept at 37°C for 1 hr in the presence of phosphatidylinositol-specific phospholipase C (PI-PLC; *E, F*). After PI-PLC treatment, P1 cortical plate membranes still promote neurite growth (*E*); identically treated E19 membranes, on the other hand, promote little if any LG neurite growth (*F*). It is also apparent that both the number and length of LG neurites is greater on the P1 compared to E19 membrane carpets (compare *A* and *B,* or *C* and *D*). All photographs are from a single experiment and represent the maximal amount of neurite growth for a given condition in that experiment. The tips of neurites from adjacent explants are visible at the perimeter of some of these montages (*arrowheads*).

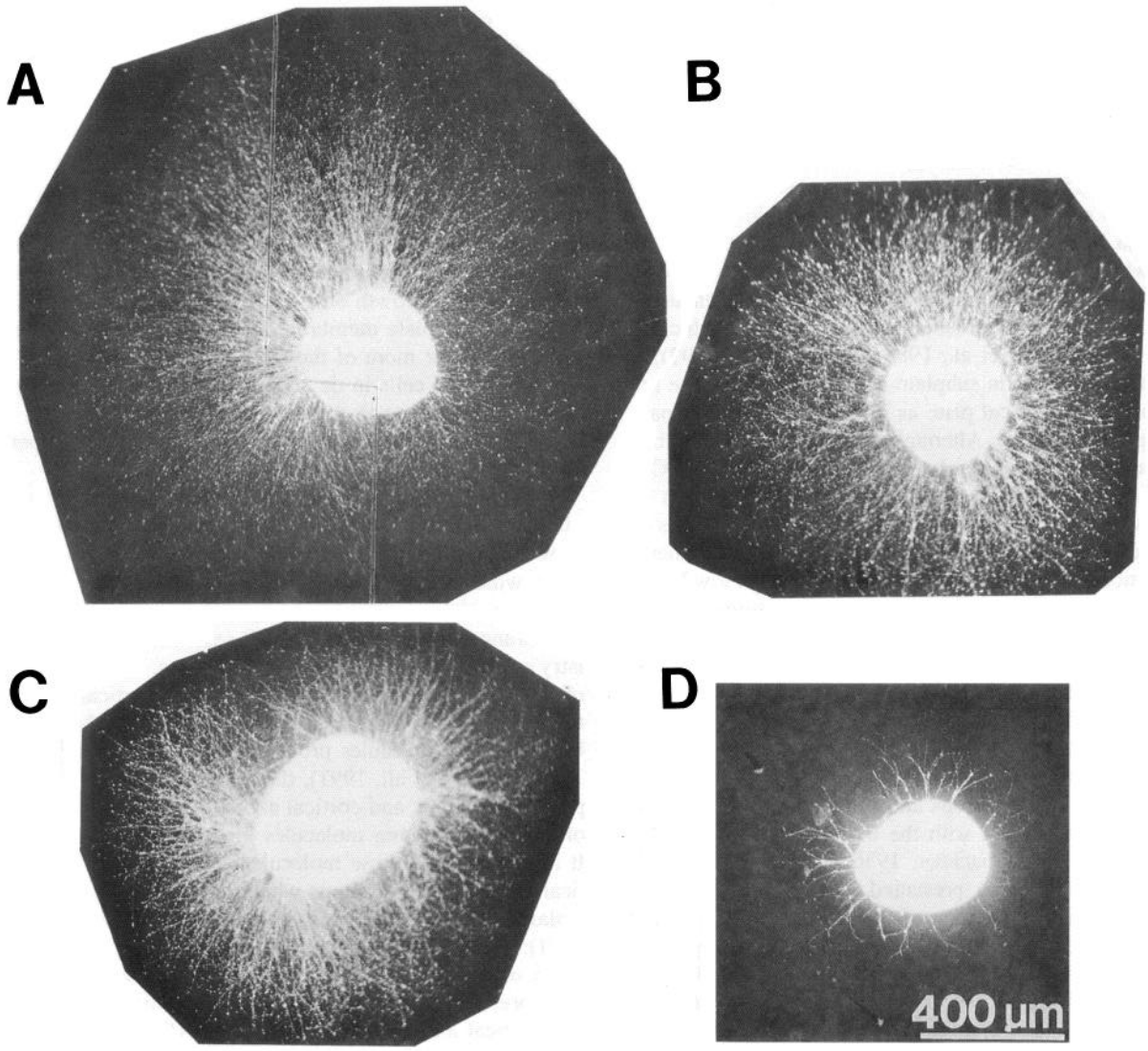

Figure 10. Non-PI-linked molecules, upregulated in maturing cortical plate, promote cortical neurite growth *in vitro*. Explants from E18 cortex were plated onto uniform carpets composed of P1 (*A, C*) or E19 (*B, D*) cortical plate membrane preparations that had been maintained at 37°C for 1 hr in the presence (*C, D*) or absence (*A, B*) of phosphatidylinositol-specific phospholipase C. After PI-PLC treatment, P1 cortical plate membranes still promote cortical neurite growth (*C*); identically treated E19 membranes, on the other hand, promote little if any neurite growth (*D*). It is also apparent that the length of cortical neurites is greater on the P1 than the E19 control carpets (compare *A* and *B*). All photographs are from a single experiment and represent the maximal amount of neurite growth for a given condition in that experiment.

cortical neurite growth, whereas the cortical plate activity that we observed enhanced the growth of both thalamic and cortical neurites. Bolz and colleagues found that E16 thalamic explants extended neurites on uniform carpets of either E16 or P6 cortical membranes, but the number of neurite fascicles was greater on P6 cortical membranes (Götz et al., 1992). On the other hand, E16 cortical explants had the same number of neurite fascicles on E16 and P6 cortical membranes (Götz et al., 1992), an observation inconsistent with our findings on both uniform and striped carpets. This inconsistency might be due not only to their use of whole cortex versus cortical plate for membrane preparations, but also to the wide (i.e., 12 d) developmental spread of the cortical ages compared. At E16, the cortical plate is exceedingly minor; therefore, cortical plate membranes would be significantly diluted by membranes from the neuroepithelium, intermediate zone, subplate, and marginal zone—all of which contain ECM and cell adhesion molecules at this stage which only later become apparent in the cortical plate (Fushiki and Schachner, 1986; Chung et al., 1991; Sheppard et al., 1991; present study), or, in the case of the ECM molecule fibronectin, are never apparent in the cortical plate (Stewart and Pearlman, 1987; Chun and Shatz, 1988; Sheppard et al., 1991). In addition, by P6 an enormous proportion of the cortical wall is composed of thalamic axonal membrane which could give rise to a cofasciculation artifact *in vitro*. In contrast, we have compared cortical plate membranes prepared from E18–E19, when afferents begin to invade the cortical plate, and P0–P1, during afferent invasion of the cortical plate; the molecules controlling the invasion of the cortical plate must be present over this 4 d developmental spread.

Although considerable data support the hypothesis that neurite growth promoters are upregulated in maturing cortical plate, we cannot exclude the possibility that inhibitors are coincidentally downregulated. The fact that explants were able to extend neu-

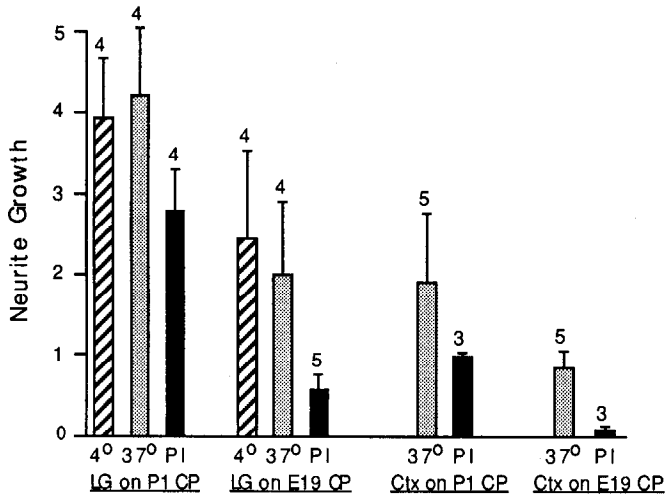

Figure 11. Quantitative analysis of one of three phosphatidylinositol-specific phospholipase C experiments illustrating that at least part of the maturation-dependent change in cortical plate membranes observed in the stripe assay is due to an increase in neurite growth-promoting molecules that are not PI linked. LG and cortical explants were placed onto uniform carpets made with P1 or E19 cortical plate membrane suspensions that had been treated in one of three ways: controls left at 4°C, controls left at 37°C, or membranes kept at 37°C in the presence of PI-PLC (PI). "Neurite growth" measurements were made using NIH IMAGE software (see Materials and Methods); the *numbers* are relative values representing the mean and standard error. *N* values appear above the standard error bars.

rites on carpets composed solely of E18 or E19 cortical plate membranes from occipital cortex might be interpreted as indicating that embryonic cortical plate is devoid of neurite growth inhibitors. However, it is possible that neurite growth inhibitors are excluded from the cortical plate membrane preparation, or are masked, possibly due to preferential enrichment of membrane-anchored neurite growth promoters or by habituation of the neurites to an inhibitor (Kapfhammer et al., 1986; Kapfhammer and Raper, 1987; Walter et al., 1987a). The PI-PLC experiments could also be argued to support the hypothesis that there is not a PI-linked neurite growth inhibitor in cortical plate since neurite growth on PI-PLC-treated cortical plate membranes was never enhanced. However, the poor neurite growth on PI-PLC-treated E19 cortical plate membranes could be due to PI-PLC treatment removing both neurite growth promoters and inhibitors. Alternatively, PI-PLC treatment may remove PI-linked neurite growth promoters in E19 cortical plate membranes leaving non-PI-linked neurite growth inhibitors intact.

In conclusion, we have shown that thalamic and cortical neurites respond to maturation-dependent changes in cortical plate membranes. Our findings suggest that an upregulation of non-PI-linked membrane-anchored cell adhesion molecules plays a role in mediating these *in vitro* growth behaviors. These molecules, perhaps working in concert with ECM molecules, may promote and regulate the timing and rate of axonal growth in the developing cortical plate, thereby contributing to the generation of the stereotypic, spatiotemporal patterns of developing thalamocortical and corticocortical projections.

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