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Dual Action of a Carbohydrate Epitope on Afferent and Efferent Axons in Cortical Development

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During development of the mammalian cerebral cortex, ingrowing afferents from the thalamus take a path that is different from that of axons leaving the cortical plate. Thalamic axons arrive at the cortex at the time before their target cells of layer 4 are generated in the ventricular zone, but they invade the cortex only shortly before these cells have migrated to their final position in the cortex. Growth-promoting molecules are upregulated in the developing cortical plate during this period. To identify such molecules, we have generated monoclonal antibodies against membrane preparations from rat postnatal cortex. In Western blots, one antibody (mAb 10) recognized a carbohydrate epitope of a glycoprotein with an apparent molecular weight extending from 180 to 370 kDa. Immunohistochemical staining revealed that the staining pattern of mAb 10 at embryonic stages delineates the pathway of thalamocortical axons, with only very faint labeling of the corticofugal pathway. *In vitro* assays in combination with time-lapse imaging indicated that mAb 10 has opposite effects on the growth of thalamic and cortical axons. The growth speed and axonal elongation of thalamic fibers on postnatal cortical membranes preincubated with mAb 10 was reduced compared with untreated cortical membranes. In contrast, cortical axons grew faster and stopped their growth less frequently after addition of mAb 10 to a cortical membrane substrate. Taken together, these results suggest that a carbohydrate moiety of a membrane-associated glycoprotein plays a role in the segregation of afferent and efferent cortical axons in the white matter. Moreover, the epitope recognized by mAb 10 might also contribute to regulation of the timing of the thalamocortical innervation at later developmental stages.

Key words: cortical development; thalamocortical connections; segregation of afferent and efferent cortical projections; axonal growth rate; extracellular matrix; carbohydrate epitope; monoclonal antibodies; time-lapse imaging

During development, the guidance of axons to their targets is controlled by molecules in the environment of the growth cone. These are either diffusible factors or constituents of the cell surface and the extracellular matrix (Bixby and Harris, 1991; Goodman and Shatz, 1993; Lander, 1993). Receptors on the axonal growth cone mediate the recognition of guidance molecules, leading to changes in the cytoskeleton of the growth cone and influencing the orientation of axons (Lin et al., 1994). The response of the neuron to a given substrate is also thought to be dependent on the spatial context in which it is presented. Whether the molecule is distributed as a uniform substrate, a sharp boundary, or a gradient can determine the behavior of the neurite (Walter et al., 1987b; Baier and Bonhoeffer, 1992; Lochter and Schachner, 1993). Axonal subpopulations may respond to a changing environment by expressing different sets of receptors and adhesion molecules (Dodd et al., 1988; De Curtis et al., 1991).

In developing neocortex, thalamic afferents follow a pathway that is distinct from the adjacent pathway taken by axons leaving the cortex (De Carlos and O'Leary, 1992; Bicknese et al., 1994). Afferent and efferent fibers also differ in their ability to respond to growth-promoting molecules in the cortex. Although rat cortical axons grow on a membrane preparation from either embryonic or postnatal cortex, thalamic axons tend to avoid an embryonic cortical explant and show poor outgrowth on embryonic membranes (Götz et al., 1992). This behavior reflects the situation *in vivo*. In several mammalian species, it has been demonstrated that fibers from the thalamus arrive before the generation of their cortical target neurons in layer 4 (Lund and Mustari, 1977; Rakic, 1977; Shatz and Luskin, 1986; Catalano et al., 1991; Ghosh and Shatz, 1992; Götz et al., 1992; Kageyama and Robertson, 1993; Miller at al., 1993). Thalamic axons are confined first to the subplate zone beneath the developing cortical layers and enter the cortical gray matter only after layer 4 cells have migrated to their final position. This time delay between arrival of thalamic fibers and formation of their target layer is short in rodents but can cause a waiting period of up to 2 months in the developing human cortex (Kostovic and Rakic, 1990).

The mechanisms that regulate the timing of afferent cortical innervation are not understood completely. *In vitro* experiments indicated that membrane-associated molecules promoting the growth of thalamic fibers are upregulated in the cortex in parallel with its innervation by thalamic axons (Götz et al., 1992; Bolz et al., 1993; Hübener et al., 1995; Tuttle et al., 1995). As a first attempt to characterize such molecules, we generated monoclonal antibodies (mAb) against postnatal cortical membranes. We describe here three antibodies binding to antigens whose expression

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patterns correlate with cortical invasion by thalamic fibers. One antibody (mAb 10) proved to be especially interesting, because its staining revealed a molecular heterogeneity in the intermediate zone. The biochemical analysis indicated that mAb 10 recognizes a carbohydrate epitope of a matrix-bound glycoprotein. *In vitro* assays showed that this antibody interferes with the outgrowth of thalamic and cortical axons in an opposing fashion, i.e., it inhibits growth of thalamic axons on a cortical membrane substrate, but at the same time enhances growth of cortical axons. The spatiotemporal distribution and the dual mechanism on axonal elongation of this epitope suggests that it might be involved in regulating the cortical invasion of thalamic axons as well as in segregating afferent and efferent projections in the developing white matter.

A preliminary report of some of these findings has been presented in abstract form (Henke-Fahle et al., 1994).

MATERIALS AND METHODS

Generation of mAbs. Six-week-old female Balb/c mice were immunized with membrane preparations from postnatal day 6 (P6) rat cortex (Lewis strain) suspended in RAS (Ribi's-Adjuvans-System; Pan Systems). Mice were injected intraperitoneally at biweekly intervals 4 d after the last boost spleen cells were fused with NS-1 hybridoma cells, according to established methods (Fazekas de St. Groth and Scheidegger, 1980), and distributed into 96-multiwell dishes containing a feeder layer of peritoneal macrophages. Hybridoma supernatants were screened on fixed frozen sections of embryonic day 16 (E16), E19, and P6 cortex from Lewis rats (day of sperm detection = E1). Cell lines of interest were subcloned several times by limiting dilution.

Immunohistochemistry. Brains were removed, immediately frozen on dry ice, and cut on a cryostat at a thickness of 10 μm. Frontal sections from presumptive sensorimotor cortex were fixed for 10 min in ice-cold methanol and washed several times in PBS. Incubation with tissue culture supernatants was carried out overnight at 4°C. Unbound antibody was washed off with PBS, and the sections were then incubated with rhodamin-coupled rabbit-anti-mouse IgG + IgM (1:250; Jackson ImmunoResearch Labs, West Grove, PA). Alternatively, for immunostaining with mAb 10, sections were incubated with biotinylated rabbit-anti-mouse IgM (1:200; Vector Labs, Burlingame, CA), briefly washed with PBS, and then incubated with Cy3-conjugated streptavidin (1:100; Amersham, Buckinghamshire, UK). With this more sensitive detection method, the tissue culture supernatant was diluted 1:20, which considerably decreased the background staining without significantly reducing the specific antibody staining. To label cell nuclei, sections were counterstained with bisbenzimide solution (1 μg/ml in PBS, 5 min at room temperature; Sigma, St. Louis, MO).

Membrane preparation. Membranes were prepared according to Götz et al. (1992). Blocks of cortex were dissected in Gey's balanced salt solution (GBSS) supplemented with glucose (6.5 mg/ml), and the pia was removed. Slices (thickness, 200–300 μm) were cut with a McIlwain tissue chopper and homogenized in homogenization buffer consisting of 10 mM Tris-HCl, 1.5 mM $CaCl_2$, 1 mM spermidine, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 5 μg/ml pepstatin, and 15 μg/ml 2,3-dehydro-2-desoxy-*N*-acetylneuraminic acid (all from Sigma), pH 7.4. The homogenate was centrifuged for 10 min at 25,000 rpm in a sucrose step gradient (upper phase 150 μl of 5% sucrose, lower phase 350 μl of 50% sucrose) in a Beckman TLS 55 rotor. The interband containing the membrane fraction was washed twice with PBS without Ca^{2+} and Mg^{2+} at 14,000 rpm in an Eppendorf Biofuge, and after resuspension the membrane concentration was determined by its optical density measured with a photospectrometer at 220 nm. The actual protein content was determined according to the method of Bradford (1976). In these cell membrane preparations, molecules from the extracellular matrix co-purify.

Quantitative growth assay. Coverslips (12 × 24 mm) were boiled for 10 min in absolute ethanol, air-dried, and then sterilized for 24 hr at 150°C. Pairs of coverslips were coated with 2 μg of laminin (Sigma) in 100 μl of GBSS as a "sandwich" for 60 min at 37°C under sterile conditions. The coverslips were then separated, washed with PBS, and air-dried. They were then coated again as a sandwich with 100 μl of membrane suspension (optical density 0.1 or 0.2) at 37°C for 1–2 hr. After separation, the coverslips were placed in Petriperm dishes (Bachofer, Reutingen, Germany) with 0.75 ml of culture medium, with or without antibodies. The medium consisted of 50% Eagle's basal medium, 25% HBSS, and 25% horse serum; in addition, 0.1 mM glutamine, 6.5 mg/ml glucose, 4 mg/ml methylcellulose, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin were added. Thalamic or cortical explants were then pipetted onto the coverslips. Explants from E16 rats were prepared from slices of the whole dorsal thalamus or the cortical hemispheres by cutting them into 200 × 200 × 200 μm pieces with a McIlwain tissue chopper. After ~30 min, most of the explants had adhered to the coated coverslips, and another 1.25 ml of medium was added. Cultures were kept at 37°C under 5% CO_2 in air atmosphere.

Antibody-containing tissue culture media were concentrated with either a Filtron-Ultrasette or Filtron-Centricon membranes. Antibodies were added to the culture medium at 5–7 μg/ml. In another series of experiments, membranes were preincubated with the concentrated antibody solution diluted 1:10 for 2–4 hr at 4°C (resulting concentration 7 μg/ml), and the membranes were then used in the test assay. After 2–4 d *in vitro*, the explants were fixed with 4% paraformaldehyde. The number of distal ends of the outgrowing axons was counted using an inverted microscope with phase-contrast optics. To confirm the neuronal origin of the fibers, several explants were stained with antibodies directed against neuronal markers SMI31 (Sternberger and Meyer, Inc.) and MAP5 (Sigma) and glial markers vimentin (Sigma) and GFAP (Bioscience, Bethlehem, PA). Statistical differences between antibody-treated cultures and controls were determined with the unpaired *t* test.

We also measured the length of cortical and thalamic axons in the presence and absence of mAb 10. For this, several prints from video images of an explant were taken at a final magnification of 120× to cover the full length of all axons extending from this explant. On these video prints, concentric circles with increasing diameter were drawn; the smallest, innermost circle was fitted by eye to the rim of each explant. The spacing of the circles corresponded to 100 μm. We then counted the number of distal ends of the axons in each annulus, and in each case we checked under the microscope with a 20× objective to determine that the axons possessed a well-defined growth cone and exhibited no signs of degeneration. In this way we obtained the total axonal length in multiples of 100 μm. To illustrate the data, a graphic representation introduced by Chang et al. (1987), which plots the percentage of axons longer than a given length versus axonal length, was used. As a characteristic for the effect of antibody treatment, the "L_{50} value," defined as the length exceeded by 50% of all axons, was determined from these plots.

Time-lapse video microscopy. For time-lapse imaging, a Petriperm dish containing a coverslip with cortical or thalamic explants was transferred to a closed chamber on the stage of an inverted microscope (Zeiss Axiovert) equipped with phase-contrast optics. Temperature (35°C) and CO_2 concentration (5%) were kept constant. Video images were taken every 60 sec with a sensitive CCD camera (Imac). To minimize photo damage, a computer-controlled shutter closed the light path after the images had been captured with an image analysis system (Hamamatsu, Bridgewater, NJ). The images were contrast-enhanced and stored for later analysis on video tape. As reported previously, cultures that had been filmed continuously for 3 d revealed no differences in growth-rate compared with cultures that were kept in the incubator during this time (Hübener et al., 1995).

The video recordings were analyzed with a Panasonic video editing controller. At every fifth frame (corresponding to 5 min intervals), the positions of the center of the growth cones were marked on a transparent overlay over the video monitor to reconstruct the trajectories of the axons. Only axons that were imaged continuously for at least 90 min and did not contact other axons during the recording period were analyzed. From these trajectories we determined the mean and maximal growth speed as well as the number and duration of the pauses during axon elongation. We then also calculated the net growth speed, i.e., the average speed during the growth phase of the axons.

Immunoblotting. Cortex from E19 and P6–P7 animals was homogenized in 10 mM Tris-HCl, and 1.5 mM $CaCl_2$, pH 7.0, containing protease inhibitors (50 μg/ml phenylmethylsulfonylfluoride, 25 μg/ml aprotinin, 25 μg/ml leupeptin, and 5 μg/ml pepstatin). The homogenate was centrifuged for 30 min in a Sorvall centrifuge at 20,000 × *g*. The sediment was either solubilized directly in sample buffer (0.1 M phosphate buffer, 1% SDS, 10% glycerol, 0.1 M dithiothreitol; 5 min at 100°C) or extracted with 6 M urea in PBS on ice, cleared by centrifugation for 50 min at 105,000 × *g*; the supernatant was dialyzed against two changes of 10 mM phosphate buffer and 0.1 M NaCl, pH 7.0. Protein (70 μg each) was digested with 40 mU of the enzymes chondroitinase ABC, chondroitinase AC, heparitinase, and keratinase (all from Sigma; protease-free chondroitinase ABC also from ICN and from Boehringer Mannheim, Mannheim, Germany)

overnight at 37°C in the presence of protease inhibitors as above and 5 mM EDTA. Incubation with PNGaseF (BioLabs) was according to the instructions of the manufacturer. Digested samples and controls kept at 37°C were applied to 7% and 10% SDS-PAGE gels and run under reducing conditions in Laemmli buffer. Transfer onto nitrocellulose filters (BA85; Schleicher & Schüll, Keene, NH) was carried out for 4 hr at 20 V in Laemmli buffer containing 0.0001% SDS. Filters were blocked with 5% nonfat dry milk in PBS (1 hr, RT) and incubated overnight at 4°C with hybridoma supernatants diluted 1:1 with washing buffer (PBS with 0.1% Tween 20). Bound antibody was visualized by reaction with peroxidase-labeled goat-anti-mouse IgG + IgM (Jackson ImmunoResearch) for 1 hr at RT and subsequent development with chloronaphthol/H_20_2. Dot blots were performed by filtrating 100 μl of a protein solution (1 mg/ml protein concentration) onto a nitrocellulose membrane by means of a dot-blot apparatus (Millipore).

RESULTS

mAbs were generated using a membrane preparation from P6 rat cortex that supports thalamic fiber outgrowth *in vitro*. Hybridoma supernatants were tested on cryostat sections from E16, E19, and P6 cortex to detect antibodies specifically binding to components that are regulated developmentally. Because we attempted to identify candidates of membrane-associated proteins that play a role during the development of thalamocortical projections, we screened for mAbs whose spatiotemporal staining pattern corresponded closely to the thalamocortical invasion. Of >7000 hybridoma cell lines tested, four cell lines fulfilled this criterion, and three of these cell lines (mAbs 10, 111, and 942) were stably established and their antibodies were analyzed in more detail.

Developmental expression of antigens 10, 111, and 942

Axons arising from neurons in the thalamus pass through the internal capsule and then enter the lateral wall of the telencephalon at approximately E16. Once within the neocortex, the pathway of thalamocortical axons is centered on the subplate and the upper part of the intermediate zone. The fibers reach the dorsomedial aspect of the telencephalon by E17. At this stage, faint staining with all three antibodies can be detected in the subplate region (data not shown). By E19, the antibodies strongly stain the subplate and intermediate zone. The cortical plate and the ventricular zone display little but discernible label, whereas the fiber-rich marginal zone is clearly stained. At P6, prominent antigen is visible in all cortical layers (Fig. 1). The location of all three antigens within the tissue seems to be indicative of extracellular matrix components. The cell membranes, if positive, do not stand out as particularly more stained than the surrounding matrix; however, the exact location can be determined only at the electron microscopic level.

There are, however, distinct differences in the staining patterns between the three different antibodies. The most notable differences are between mAb 10 and the other antibodies, mAbs 111 and 942. At E19, labeling with mAb 10 was inhomogeneous in the intermediate zone, with very little expression in the lower part adjoining the ventricular zone. In contrast, mAbs 111 and 942 strongly stained throughout the intermediate zone (Fig. 1). Moreover, there was a clear mediolateral gradient in the distribution of the mAb 10 epitope at E19. In the medial regions, labeling was restricted to the subplate zone, and in more lateral regions mAb 10 staining spread into the upper part of the intermediate zone (Fig. 2). Finally, with mAb 10, there was also faint staining within the subventricular zone.

Functional assays

Because of the close correlation between cortical invasion by thalamic fibers and the staining pattern of all three antibodies, we tested their ability to interfere with the growth of thalamic as well as cortical axons. Various *in vitro* test situations were used to analyze their influence on axonal growth.

In the first experimental paradigm, a membrane preparation from early postnatal cortex was offered as substrate, and outgrowth of fibers from E16 thalamic and cortical explants was determined in the presence of antibodies and compared with the values obtained without antibody addition. Cortical membranes from postnatal days 2–7 were used, because thalamic explants had shown dense fiber extension in a quantitative growth assay using membranes from these developmental stages (Götz et al., 1992). Figure 3 illustrates axonal outgrowth from thalamic and cortical explants on P4 cortical membranes after 4 d *in vitro* under control conditions (Fig. 3*A,C*) and after addition of 6 μg/ml mAb 10 to the culture medium (Fig. 3*B,D*). mAb 10 exerted a differential effect on the growth of thalamic and cortical axons: it diminished the number of fibers extending from thalamic explants and enhanced fiber outgrowth from cortical explants. On average, the presence of mAb 10 reduced the outgrowth from thalamic explants to <50% of the control value ($p < 0.001$, $n = 357$ explants; Fig. 4*A*) and enhanced cortical outgrowth to 139% ($p < 0.01$, $n = 351$ explants; Fig. 4*C*). In the same test situation but using the EHS tumor-derived laminin as a substrate, there was no significant influence of mAb 10 on thalamic and cortical fiber extension (Fig. 4*B,D*). The mAb 942 did not exert a significant effect on either fiber subpopulation, independent of whether it was tested on membranes (Fig. 4*A,C*) or laminin (Fig. 4*B,D*). Because this antibody belongs to the same immunoglobulin class as mAb 10 (IgM), we take this result as evidence that the addition of an antibody solution as such does not interfere with axonal growth. Fiber extension in the presence of 7 μg/ml mAb 111 (IgG) was reduced for thalamic explants on cortical membranes ($p < 0.01$; Fig. 4*A*), but remained unchanged with cortical axons (Fig. 4*C*) and when laminin was used as substrate (Fig. 4*B,D*). Taken together, these experiments suggest that those epitopes recognized by mAbs 10 and 111 render cortical membranes more permissive for thalamic growth. In addition, the mAb 10 epitope is also involved in creating a less favorable environment for the growth of cortical axons.

To rule out the possibility that the antibodies exert their function by binding directly to growth cones, we repeated some experiments with pretreated membrane preparations. The membranes were first incubated with the respective antibody (7 μg/ml), excessive antibody was washed off, and they were then offered as substrate without the addition of more antibody to the culture medium. Under these conditions, results obtained with mAbs 111 and 10 (Fig. 5) were comparable to those described above. In fact, the effects were even more pronounced, as shown by a 72% reduction in growth for thalamic axons after pretreatment with mAb 10 and an almost threefold (278%) enhancement for cortical axons. This indicates that the blocking of a growth-promoting epitope present in the membrane preparation—rather than a general impairment of growth cones—causes reduced outgrowth of thalamic fibers.

In the experiments described above, we analyzed axonal outgrowth by counting the number of distal ends of fibers extending from thalamic and cortical explants after 2–4 d *in vitro*. Because we found more cortical fiber endings in the presence of mAb 10 than under control conditions, one possible explanation of this result could be that mAb 10 induces branch formation and/or defasciculation of cortical axons but has no effect on axonal outgrowth per se. We therefore also measured the length of

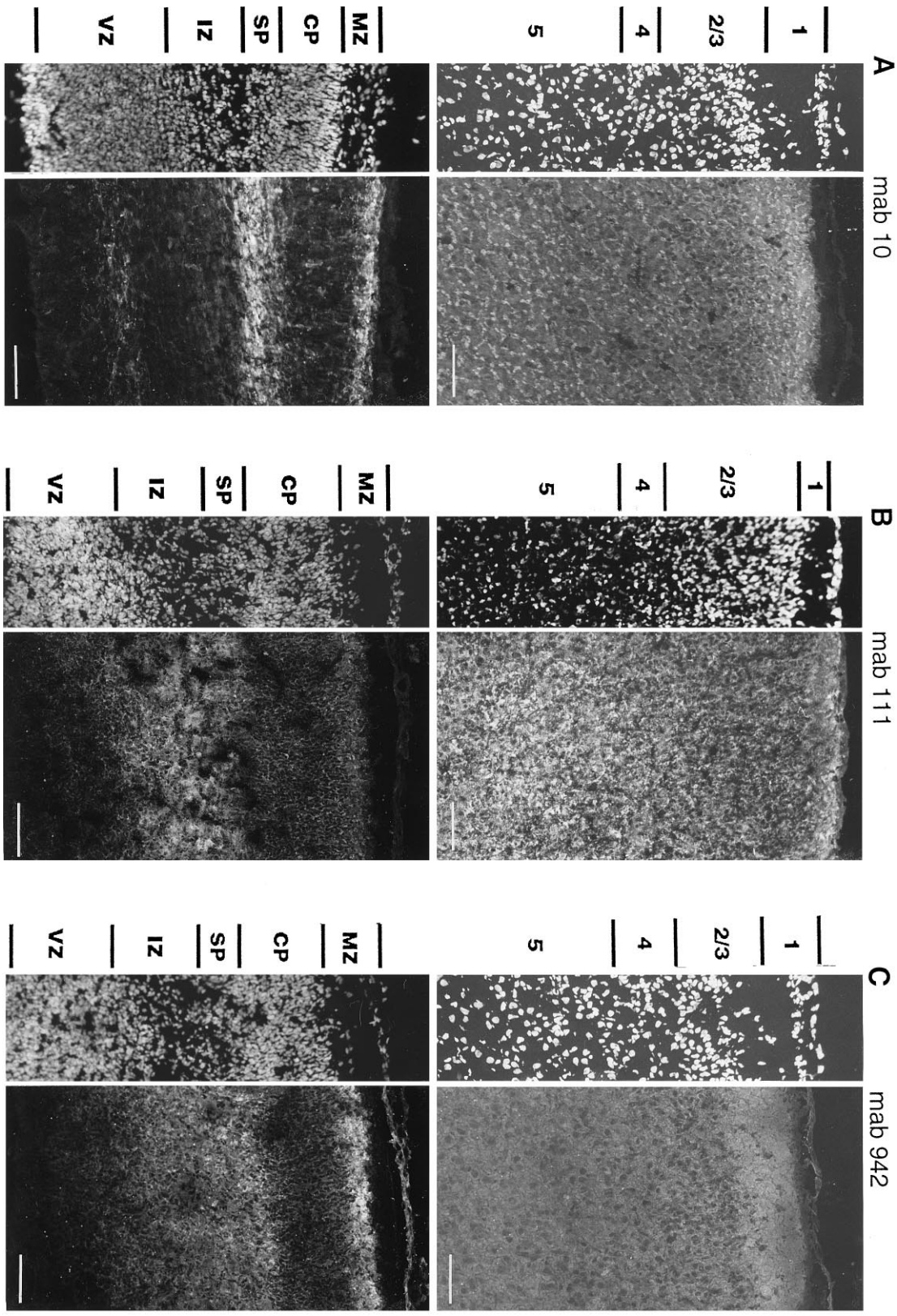

Figure 1. Immunofluorescence labeling with three different mAbs directed against rat postnatal cortical membrane preparations in the developing rat cortex. *A*, *mab 10*; *B*, *mab 11*; *C*, *mab 942*. *Top*, Frontal sections from P6 cortex; *bottom*, frontal sections from E19 cortex. To the *left* of each fluorescent image is a micrograph of the left half of the same section counterstained with bisbenzimide to illustrate the cortical layering. *MZ*, Marginal zone; *CP*, cortical plate; *SP*, subplate zone; *IZ*; intermediate zone; *VZ*, ventricular zone. Scale bars, 100 μm.

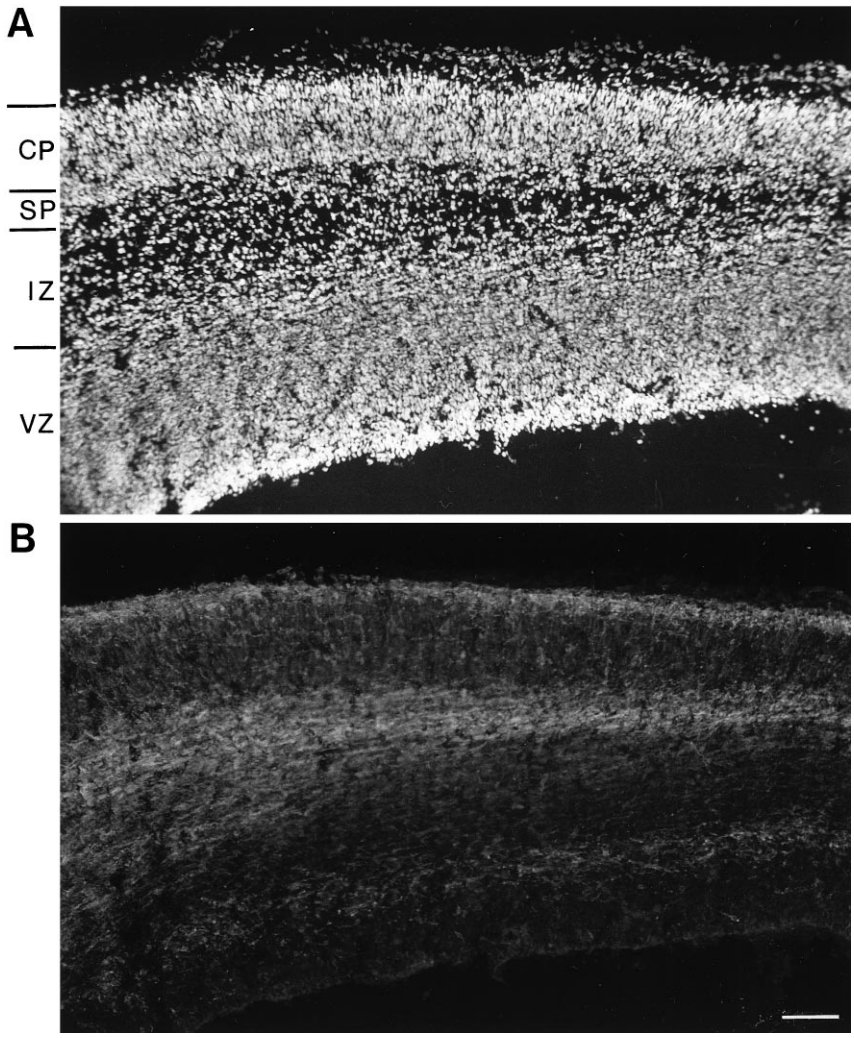

Figure 2. mAb 10 labels the path of thalamocortical afferents and does not stain the corticofugal pathway. Double-labeling of a frontal section from E19 cortex; lateral is to the *left*, medial to the *right*. *A*, Bisbenzimide staining to reveal the cortical layering; *B*, immunohistochemical localization of mAb 10 antigen. In the medial cortex, staining with mAb 10 is restricted to the *SP*. In the lateral cortex, mAb 10 labeling spreads into the upper part of the *IZ*, but the deep part of the IZ, the path of efferent cortical fibers, is not labeled. Same abbreviations as in Figure 1. Scale bar, 100 μm.

cortical and thalamic axons extending on postnatal cortical membranes in the presence and absence of mAb 10, and we used time-lapse video microscopy to study the influence of this antibody on the growth dynamic of axons. Figure 6 illustrates the distribution of the length of cortical and thalamic axons growing on cortical membranes after 2 d *in vitro*. The distribution for cortical axons on membranes preincubated with mAb 10, in comparison with the distribution on native membranes, was shifted to the right, i.e., toward larger axonal length (Fig. 5*B*). The L_{50} value, the length exceeded by 50% of all axons (see Material and Methods), increased from 214.3 μm on native membranes to 310.7 μm on membranes treated with mAb 10 ($p < 0.0001$; $n = 655$ axons). The opposite effect was observed with thalamic axons; here the curve was shifted to the left, i.e., toward shorter axonal length, after incubation of the membranes with mAb 10 (Fig. 5*A*). For thalamic axons, the L_{50} value decreased from 464.3 μm on native membranes to 314.3 μm on membranes preincubated with mAb 10 ($p < 0.0001$; $n = 308$ axons).

These results indicate that the epitope recognized by mAb 10 influences the growth rate of cortical and thalamic axons in an opposing manner. To examine more closely the bifunctional action of this epitope, we used time-lapse imaging to study the growth behavior of cortical and thalamic axons growing on postnatal cortical membrane substrates with and without preincubation with mAb 10. As reported previously for cortical fibers growing *in vivo* (Halloran and Kalil, 1994) and thalamic fibers growing *in vitro* (Hübener et al., 1995), axons did not grow at a constant speed but rather frequently changed their growth-rate, and sometimes the fibers even stopped growing and then started to grow again. Such stops either were accompanied by growth cone collapse and fiber retraction or the axons stopped elongating without growth cone collapse. In these cases, the growth cones continued to extend and retract their filopodia, but there was no net forward movement of the growth cones. Cortical axons extending on native postnatal cortical membranes halted their growth 1.3 times per hour, and the average pause duration was 14.2 min (Table 1). The mean growth rate was 33.1 μm/hr; the net speed, the rate of extension during the growth phase, was 46.7 μm/hr. On average, cortical axons reached a maximal growth rate of 101.3 μm/hr (Table 1). There was no statistical significant difference between cortical and thalamic axons in any of the five parameters analyzed (Table 1).

After preincubation of cortical membranes with mAb 10, the mean growth rate, as well as the net and maximal growth speed of cortical axons, was increased significantly compared with untreated membranes. The addition of mAb 10 to the membranes had no effect on the number of pauses, but the pause duration decreased by 33% (Table 1). Figure 7 depicts representative reconstructions from time-lapse recordings of trajectories of representative cortical axons growing on a membrane substrate in the

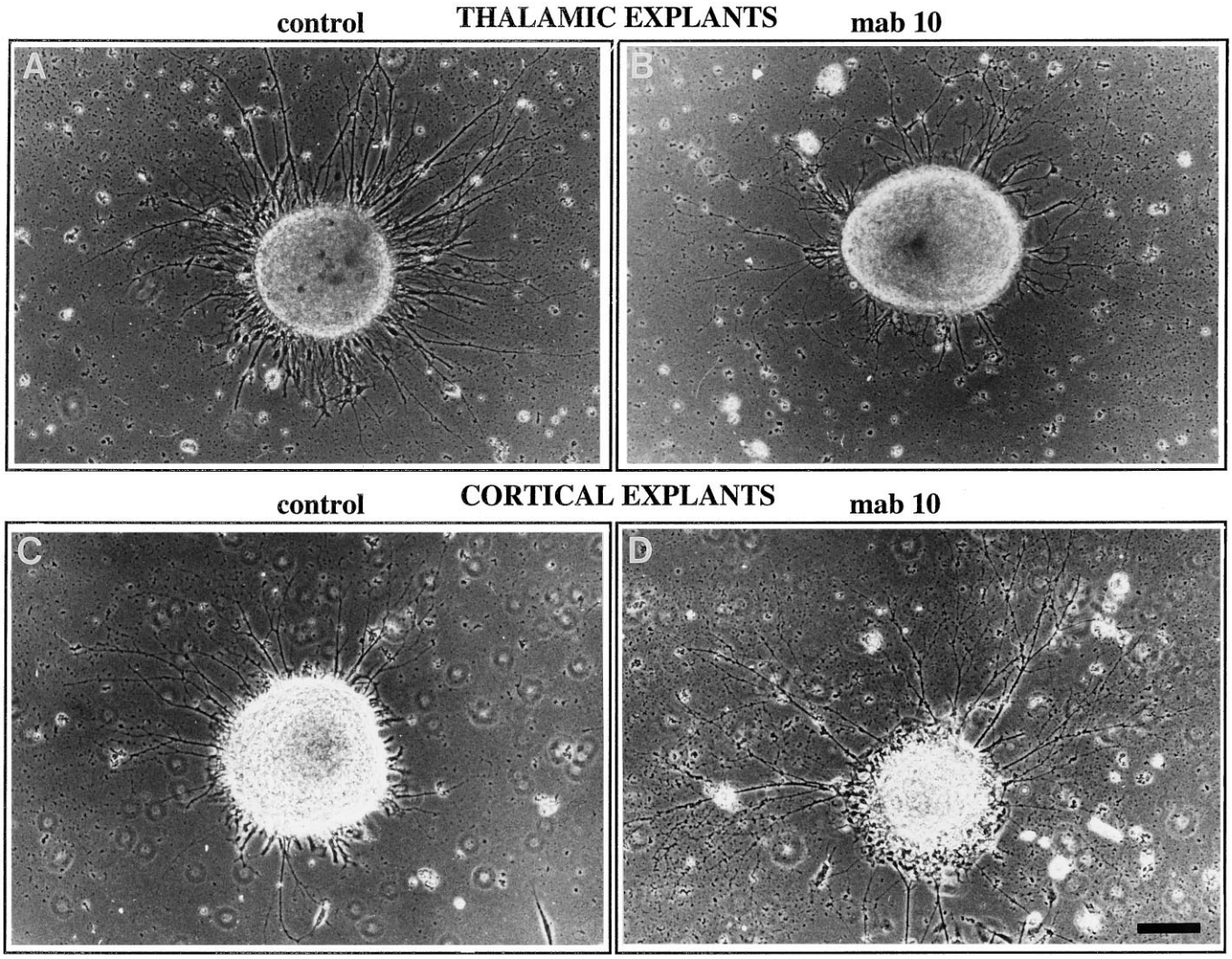

Figure 3. Effect of mAb 10 on axonal outgrowth. Phase-contrast micrographs of E16 (*A, B*) thalamic and (*C, D*) cortical explants on postnatal cortical membranes after 4 d *in vitro*. *A, C*, Outgrowth under control conditions and (*B, D*) after addition of mAb 10 (*mab 10*). mAb 10 reduces the extension of thalamic axons, whereas outgrowth of cortical axons is enhanced. Scale bar (shown in *D*): 100 μm.

presence and absence of mAb 10. For thalamic axons, each of the three parameters related to growth speed decreased significantly by >30% after incubation of the cortical membranes with mAb 10; however, mAb 10 had no influence on the number and duration of the growth pauses of thalamic axons (Table 1).

Biochemical characterization of the antigens recognized by mAbs 10, 111, and 942

As an initial attempt to analyze the antigens that the antibodies (described above) recognize in cortex, immunoblotting was performed with material from embryonic and postnatal rat cortex. We first determined the solubilization properties of the antigens by extracting the tissue under various conditions (20 mM Tris-HCl, 2 M NaCl, and 6 M urea or 0.1% Chaps) and monitoring the extraction in dot blots. Among these, only 6 M urea proved to be effective, suggesting that the molecules might be constituents of the extracellular matrix. Urea extracts were then applied to 10% SDS-PAGE gels and run under reducing conditions. After electrophoretic transfer, no signal was found with mAb 942, whereas mAbs 10 and 111 both stained a broad band of similar molecular weight. Because some ECM molecules such as proteoglycans have very high molecular weights that hinder them from entering the gel matrix, enzyme digestions were performed after dialysis of urea-extracted material and then analyzed after separation in 7% and 10% SDS-PAGE and subsequent transfer onto nitrocellulose. Results for mAb 10 are shown in Figure 8. A broad smear extending from 180 to 370 kDa is visible after separating proteins in a 7% gel (Fig. 8*A*), unaltered by digestion with chondroitinase ABC, chondroitinase AC, keratinase, and heparitinase, respectively. This result proves that the antigen is not a proteoglycan. Molecular weights extending over broad ranges are also found for glycoproteins; therefore, the experiment was repeated and included a digestion with the glycosidase PNGase F (peptide: *N*-glycosidase F; Fig. 8*B*). Under these conditions, the signal is abolished completely, indicating that the epitope recognized by mAb 10 is a carbohydrate epitope.

To verify that this carbohydrate epitope is associated exclusively with the high molecular weight glycoprotein present in the urea extract, cortical tissue was homogenized, and the resulting sediment after centrifugation was solubilized in the sample buffer. After separation in 10% SDS-PAGE and electrophoretic transfer,

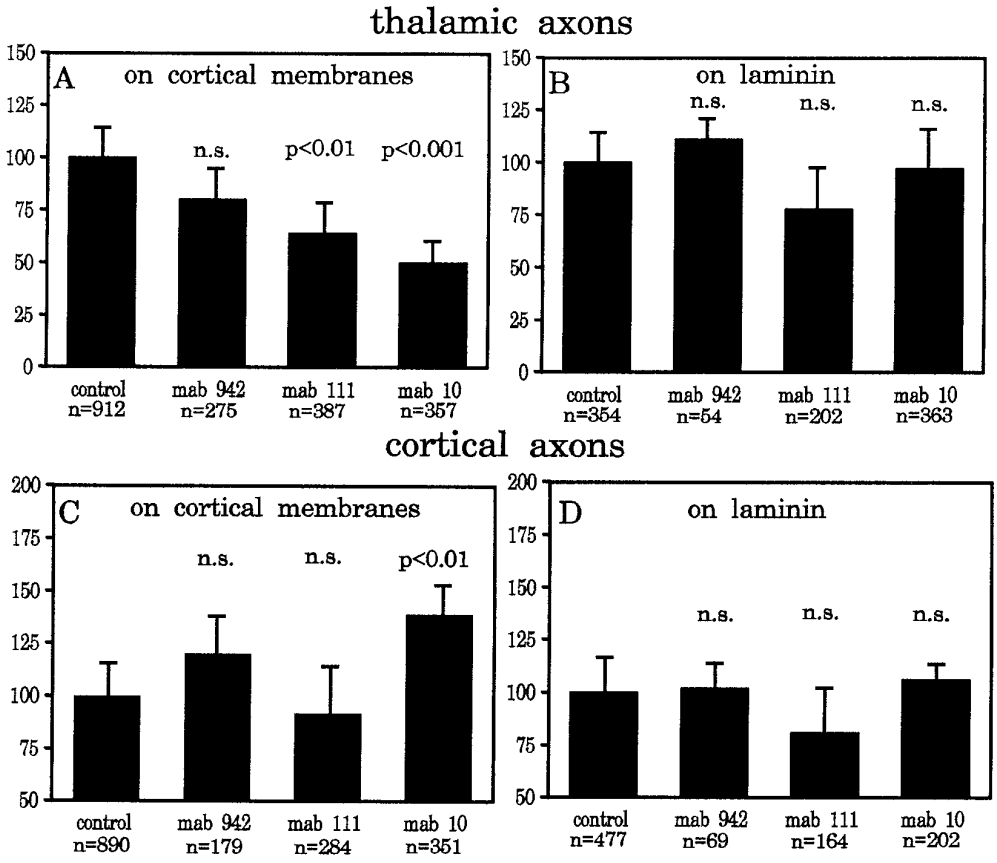

Figure 4. Histograms of axonal outgrowth from E16 thalamic and cortical explants after the addition of mAbs to the medium. The vertical axes depict the average number of outgrowing fibers per explant relative to the outgrowth of explants in the absence of the antibodies (*control*). *A*, Outgrowth of thalamic axons on cortical membranes is reduced significantly in the presence of mAbs 111 and 10, whereas mAb 942 exerts no significant effect (*n.s.*). *B*, Outgrowth of thalamic fibers is not influenced by these antibodies on a laminin substrate. *C*, Cortical axons growing on cortical membranes respond with enhanced outgrowth to the presence of mAb 10, indicating that the antibody blocks an epitope inhibitory for these fibers. *D*, Outgrowth of cortical axons on laminin is not altered significantly by any of the antibodies. Error bars indicate the SEM; n = number of explants.

incubation with mAb 10 revealed a band of the same molecular weight as in the urea extracts (Fig. 8*B*, *lane 7*). Because no reactivity was found in either the soluble supernatant or with detergent extracts, we conclude that the 180–370 kDa glycoprotein is the only cortical protein associated with the mAb 10 epitope.

For mAb 942, incubation with chondroitinase ABC generates a core protein of 68 kDa; all other treatments (enzymes as above) do not result in changes as compared with the control (data not shown). Faint staining is visible at the application site of the stacking gel, which becomes a clear signal when a second filter is applied during the electrophoretic transfer. Thus the 942 antigen seems to be a high molecular weight chondroitin sulfate proteoglycan with a core protein of apparent molecular weight 68 kDa. The molecular weight of the undigested proteoglycan is not yet known.

DISCUSSION

Glycosylated molecules previously have been suggested to be involved in regulating cortical invasion by thalamic axons (Götz et al., 1992; Bolz et al., 1993; Tuttle et al., 1995). To identify such molecules, we raised mAbs against membrane preparations from postnatal cortex and tested their ability to interfere with axonal growth from thalamic and cortical explants on a membrane substrate. One antibody (mAb 10) recognizing a carbohydrate epitope of a membrane-associated glycoprotein had opposite ef-

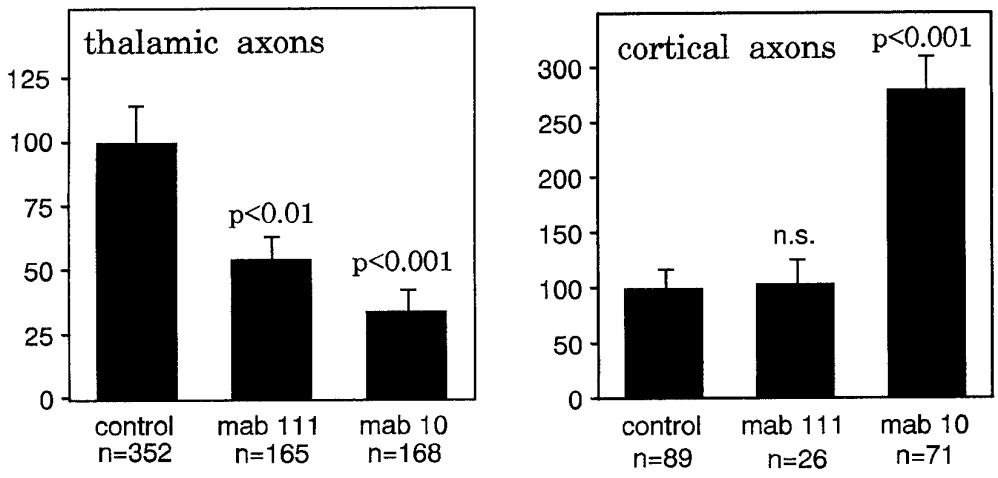

Figure 5. Histograms of axonal outgrowth from explants prepared at E16 on cortical membranes preincubated with mAbs as indicated. The vertical axes depict the average number of fibers per explant relative to control explants; the SEM and statistical significance are indicated at the top of each column; n = number of explants.

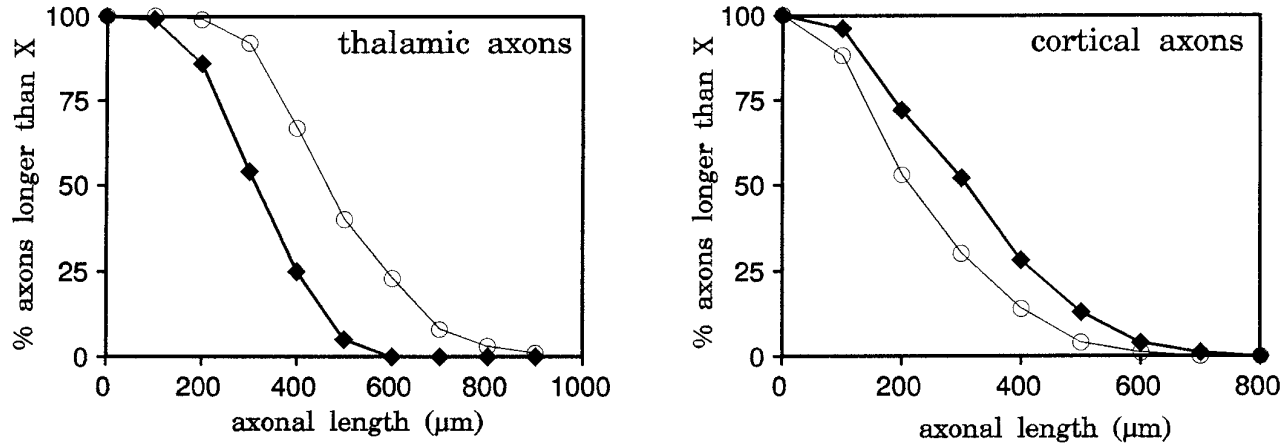

Figure 6. Opposite effects of mAb 10 on the growth rate of thalamic and cortical axons. The plots depict the distribution of axonal length versus the percentage of axons longer than a given length *X* of thalamic (*left*) and cortical (*right*) fibers after 2 d *in vitro*. *Thick lines, diamonds*, Axons growing on cortical membranes preincubated with mAb 10; *thin lines, circles*, axons extending on native membranes.

fects on afferent and efferent fibers: it reduced the growth rate of thalamic axons, whereas it enhanced the growth speed of cortical axons. In addition, cortical axons stopped their growth for shorter periods of time on cortical membranes preincubated with mAb 10 compared with untreated membranes. As discussed below, this dual action of mAb 10 might explain the segregation of afferent and efferent cortical pathways. Moreover, the spatiotemporal distribution of the carbohydrate epitope defined by mAb 10, together with its action on growing thalamic axons, suggests that it might play an important role in regulating the timing of thalamocortical innervation.

Molecular heterogeneity of axonal pathways in developing cortex

The three antibodies that were characterized in the present study were selected because the developmental expression pattern of their respective antigens correlated with the time course of cortical innervation by thalamic axons. Immunohistological investigations using antibodies directed against other constituents of the extracellular matrix also revealed an expression of the respective antigens regulated spatially and temporally in developing cortex (Stewart and Perlman, 1987; Chun and Shatz, 1988; Sheppard et al., 1991; Bignami et al., 1992; Hunter et al., 1992; Bicknese et al., 1994; Oohira et al., 1994). Among these, laminin and fibronectin, which have been described as supporting the extension of neurites especially from the peripheral nervous system but also from various central neurons (Sanes, 1985), are present at early stages of cortical development. Expression is highest in the subplate (Chun and Shatz, 1988; Hunter et al., 1992), where the path of thalamic fibers is centered during the phase of intracortical growth toward their target area. The level of expression, however, declines with ongoing development, and no substantial amounts of either laminin or fibronectin are detectable in the cortical plate and the cortical layers generated at later stages (Stewart and Perlman, 1987; Chun and Shatz, 1988; Sheppard et al., 1991). Although this distribution might suggest an involvement of these molecules in early ingrowth of thalamic afferents, the lack of expression in the ultimate cortical layers also supports the interpretation that they most likely do not contribute to the later-occurring cortical invasion by thalamic axons.

A closer spatial and temporal correlation with cortical innervation could be demonstrated for the expression pattern of chondroitin sulfate proteoglycans (Bicknese et al., 1994; Miller et al., 1995). The growth of thalamocortical afferents into the cortical plate coincides with a progressive increase of CSPGs from deep to superficial cortical plate. Other molecules that increase in the cortical plate during thalamocortical afferent invasion include the ECM glycoprotein tenascin (cytotactin) and the membrane-associated cell adhesion molecules L1 and TAG-1 (Fushiki and Schachner, 1986; Chung et al., 1991; Sheppard et al., 1991; Wolfer et al., 1994; Tuttle et al., 1995). Although the immunohistological and cytochemical studies reveal a complex molecular heterogeneity of developing cortex and suggest a role of these molecules in promoting cortical invasion by thalamic axons, this has yet to be demonstrated directly in most cases. Furthermore, our results and those of others (Faissner and Kruse, 1990; Wehrle and Chiquet, 1990; Colamarino and Tessier-Lavigne, 1995) indicate that axonal subpopulations respond differentially to individual molecules or molecular domains, and the expression patterns as such do not necessarily allow prediction of the influence that these molecules exert on different axons.

Table 1. Effect of mAb 10 on the growth of cortical and thalamic axons

	Mean growth rate (μm/hr)	Maximum growth rate (μm/hr)	Net growth rate (μm/hr)	Number of pauses per hour	Pause duration (min)
Cortex: control	33.1 ± 3.7	101.3 ± 8.7	46.7 ± 3.5	1.3 ± 0.3	14.2 ± 2.7
Cortex: mAb 10	59.3 ± 4.5***	152.6 ± 8.9***	74.6 ± 4.9***	1.4 ± 0.1 n.s.	9.5 ± 1.7*
Thalamus: control	37.7 ± 2.6	114.2 ± 9.4	49.2 ± 2.3	0.8 ± 0.1	18.2 ± 2.7
Thalamus: mAb 10	23.8 ± 3.1**	76.5 ± 6.8***	30.7 ± 3.0***	0.8 ± 0.2 n.s.	17.1 ± 3.6 n.s.

Cortical and thalamic axons extended on postnatal cortical membranes in the presence and absence (control) of mAb 10. Data are mean ± SEM; differences between control and mAb 10 incubated membranes. n.s., Not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

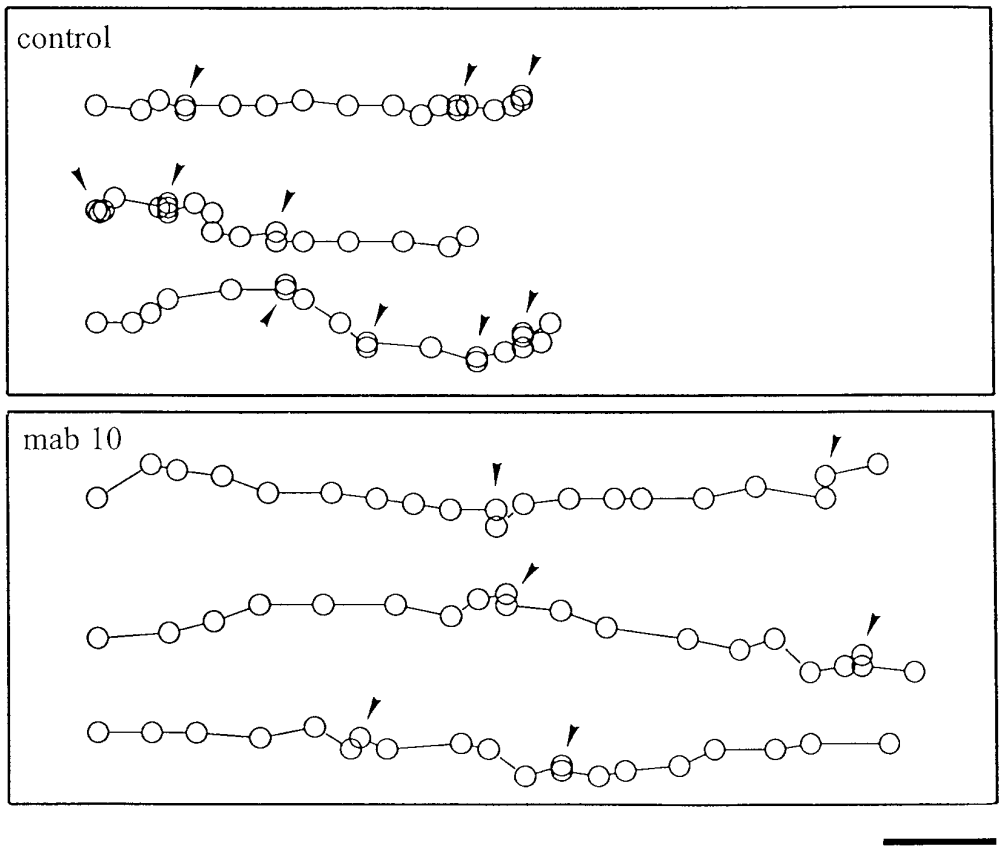

Figure 7. Reconstructions of axonal trajectories from time-lapse imaging, each recorded for 100 min, of cortical fibers growing on native cortical membranes (*top*) and on membranes preincubated with mAb 10 (*bottom*). The positions of the growth cones are plotted every 5 min; the site of the *circles* corresponds approximately to the size of a growth cone. *Arrows* point to growth pauses. Incubation of cortical membranes with mAb 10, compared with untreated membranes, increased the growth speed and decreased the pause duration of cortical axons. Scale bar, 30 μm.

Involvement of carbohydrate epitopes in pathfinding and targeting of axons

Our experiments performed with mAb 10 demonstrate that a glycoprotein-associated carbohydrate epitope can perform a dual function in axonal growth. Although this epitope influences extension of thalamic axons in a positive fashion, it decreases the growth speed of cortical axons. Carbohydrates on the neural surface or in the matrix are being associated with many developmental events involving the regulation of cell adhesion or recognition. Highly acidic carbohydrates seem to contribute to axonal branching and guidance (Landmesser et al., 1990; Wang and Denburg, 1992; Grumet et al., 1993). In some cases, their differential distribution underlies selective reinnervation during regeneration (Martini et al., 1992). Neutral carbohydrates have been implicated in neuronal migration and axonal growth (Lehmann et al., 1990) as well as pathfinding and targeting (Dodd and Jessell, 1986; Schwarting et al., 1992; Song and Zipser, 1995). Although the biochemical analysis of the carbohydrates involved in influencing the growth of thalamic and cortical axons as described in the present study does not allow assignment of the activity to a single identified carbohydrate, the cleavage characteristics of the enzyme used (PNGase F) suggest that the epitope specifically recognized by mAb 10 belongs to the group of N-linked carbohydrates of either the complex or high mannose type. In addition, these results also propose that other carbohydrate structures are adding to the preferred growth of thalamic fibers on postnatal membranes. Previous investigations showed that incubation with peanut agglutinin reduces the growth-supporting properties of the membranes (Götz et al., 1992). Because this lectin binds to a core structure [Gal-(β1–3)-GalNAc] present in O-linked carbohydrates that are common to many membrane glycoproteins, it seems unlikely that mAb 10 and peanut agglutinin recognize the same epitope. Thus, several different protein domains as well as carbohydrate epitopes might contribute to the observed phenomenon, which is also evidenced by the fact that neither of the reagents completely abolishes outgrowth of thalamic axons.

Do growth-promoting molecules define thalamocortical pathways?

Initial intracortical growth of thalamic fibers is confined to the subplate and the upper intermediate zone. Short collateral branches extend upward toward the cortical plate and reach into the lower cortical plate in more mature areas of the cortical wall (De Carlos and O'Leary, 1992; Bicknese et al., 1994); however, axons grow into the cortex only when the definite layers are formed. So far, the mechanisms that restrict the path of thalamic axons have remained elusive. In various parts of the nervous system, inhibitory or repulsive factors that contribute to axonal pathfinding have been described (Kapfhammer and Raper, 1987; Walter et al., 1987a; Davies et al., 1990; Moorman and Hume, 1990; Pini, 1993). Many molecules that inhibit axonal growth cause a collapse of the growth cone and thereby restrict axonal elongation to specific pathways (Cox et al., 1990; Luo et al., 1993). Although such mechanisms might also operate in the immature cortex, to date most investigations have not provided evidence for inhibitory factors guiding thalamic fibers into cortex (Hübener et al., 1995; Tuttle et al., 1995). Evidence presented so far indicated that growth-promoting molecules define the pathway for thalamocortical afferents, although it must be noted that the data were obtained *in vitro* and molecules might be lost or masked by the preparation procedure. Our current results obtained with mAbs 10 and 111 support the previous findings. Both antibodies interact

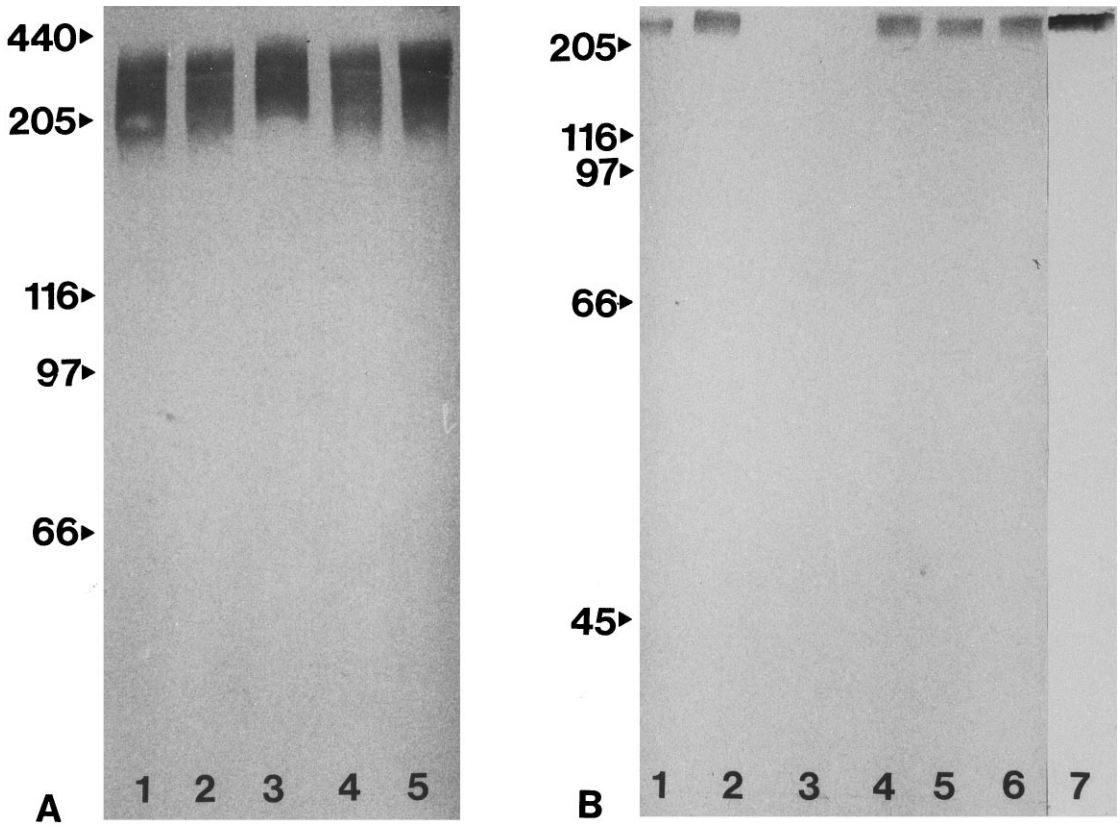

Figure 8. Biochemical characterization of the mAb 10 antigen. Western blots of 6 M urea extracts obtained from P6 cortex, dialyzed, and incubated with enzymes as indicated. *A*, Separation of proteins by a 7% SDS-PAGE gel under reducing conditions. Samples were digested with (1) keratinase, (2) heparitinase, (3) chondroitinase AC, or (4) chondroitinase ABC, or (5) incubated without enzyme. *B*, Separation of proteins by a 10% SDS-PAGE gel under reducing conditions after incubation with (1) keratinase, (2) heparitinase, (3) *N*-glycosidase F, (4) chondroitinase ABC, or (5) chondroitinase AC, (6) without enzyme, and (7) cortical tissue solubilized directly in sample buffer. mAb 10 binds to a carbohydrate epitope of a 180–370 kDa glycoprotein. Molecular weight of marker proteins in kilodaltons.

with growth-supporting components present in the membrane preparation without directly influencing the growth cone, as evidenced by experiments using the EHS tumor-derived laminin as substrate or by preincubating membranes with antibodies before attachment to coverslips. The enrichment of these components in the subplate and later in the developing intermediate zone, possibly together with already described or as yet unidentified molecules, might explain the growth of axons along this rather narrow sheet of cells.

Regulation of cortical invasion: a molecular basis for waiting periods

A time delay between the arrival of thalamic fibers in the cortex and invasion into the developing cortical layers has been described in several species. Afferent fibers reach the cortex before most cortical neurons have been born or layers have been formed. It has been suggested that the lack of sufficiently high levels of growth-promoting molecules in the undifferentiated cortical plate might hinder the afferents from invading a tissue that still lacks the target neurons (Götz et al., 1992; Bolz et al., 1993; Tuttle et al., 1995). Our observations on expression of several epitopes recognized by mAbs 10, 111, and 942 support the notion that such molecules are missing in the embryonic cortex. They do not necessarily need to act by providing a growth-permissive function themselves, but instead might contribute to create a favorable environment by binding and subsequently presenting growth factors to cells and growth cones, as has been demonstrated for various proteoglycans (Andres et al., 1989; Yayon et al., 1991). By acting in concert, these molecules could provide the molecular basis for cortical invasion.

A dual mechanism for segregation of cortical inputs and outputs

Thalamic afferents follow a pathway that is different from the path taken by efferent fibers. The trajectories of the two axonal populations are separated within the white matter (Woodward et al., 1990), and this segregation is already apparent at early developmental stages (De Carlos and O'Leary, 1992; Miller et al., 1993). Among the mechanisms that underlie the segregation of afferent and efferent axons, fiber–fiber interactions and specific interactions with growth-permitting or inhibiting molecules might play an important role (Blakemore and Molnar, 1991; De Carlos and O'Leary, 1992; Miller et al., 1995). A selective fasciculation of cortical axons with other cortical axons has been described *in vitro* (Bagnard et al., 1995; Bolz et al., 1995). In addition, cortical fibers tend to avoid thalamic fibers. Although these findings could explain the segregation into different fiber tracts, they do not explain the preference for a certain localization within the developing white matter. Our results obtained with mAb 10 might contribute to an understanding of how these positions are selected. The first axons arising from subplate neurons leave the cortex before thalamic fibers have arrived. The later descending cortical plate neurons probably use axons from the subplate as pioneer fibers (McConnell et al., 1989, 1994); however, they take

an even deeper position in the intermediate zone than those axons and grow below but possibly along them (McConnell et al., 1989; Ghosh and Shatz, 1992; Bicknese et al., 1994), thus indicating that they either avoid an unfavorable molecular environment in the upper intermediate zone or are attracted by molecules expressed just above the ventricular zone. The differential distribution of the mAb 10 epitope in the intermediate zone, together with its function in decreasing outgrowth of cortical axons—as evidenced by the increased growth rate after masking this epitope by antibody incubation—makes it a good candidate for a molecule that is avoided by cortical axons, thus causing them to grow in the deep intermediate zone where only small amounts of the epitope are present. At the same time, the same molecule might attract afferent fibers into areas expressing a higher level of this epitope. Whether this behavior depends on different sets of receptors expressed by efferent and afferent cortical fibers or on the same receptors connected to different signaling pathways remains unclear. A combination of fiber–fiber interactions and the unequal distribution of substrate-bound molecules interpreted in opposing ways by different fiber populations might thus underlie the observed segregation and localization of axons in the white matter.

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