Membrane-bound molecules in rat cerebral cortex regulate thalamic innervation

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Summary

During development of the thalamocortical projection, afferent fibers from the thalamus reach the cortex at a time when their target cells have just been generated but have not yet migrated to their final position. Thalamic axons begin to invade the cortex only shortly before their target layer 4 is formed. The mechanisms responsible for the innervation and termination of thalamic fibers in the cortex are not known. Here we show that the growth of thalamic axons in vitro is influenced by the age of cortical explants. Cortical explants of early embryonic stages were not invaded by thalamic explants, whereas thalamic fibers entered explants from postnatal cortices and terminated properly in their target layer 4 in vitro. Outgrowth assays on cortical cell membranes prepared at different developmental stages revealed that the growth of thalamic axons is selectively influenced by growth-promoting molecules that are up-regulated during development. Moreover, experiments with postnatal cortical membranes isolated from distinct layers revealed that the growth of thalamic axons is selectively reduced on membranes prepared from layer 4. These results provide evidence that membrane-bound molecules in the cortex are involved in both the regulation of thalamic innervation into the cortical layers and their termination in the correct target layer.

Key words: cerebral cortex, cortex development, axonal pathfinding, slice cultures, rat, thalamic innervation.

Introduction

The establishment of specific connections between neuronal populations and their distant target areas involves several steps. Growing axons have to find the correct pathways in order to reach the appropriate region of the brain and, once they arrive in their target area, they must then navigate and select the correct subsets of target cells. A striking feature in the development of the neocortex is that afferent fibers reach the cortex before most cortical neurons are born or cortical layers have been established (Levitt and Moore, 1979; Schlumpf et al., 1980; Lund and Mustari, 1977; Rakic, 1977; Shatz and Luskin, 1986; Uylings et al., 1990). In several mammalian species, it has been shown that fibers from the thalamus, which are the major afferent input to the cortex, arrive prior to the generation of their cortical target neurons in layer 4 (Lund and Mustari, 1977; Luskin and Shatz, 1986; Uylings et al., 1990). In several mammalian species, it has been shown that fibers from the thalamus, which are the major afferent input to the cortex, arrive prior to the generation of their cortical target neurons in layer 4 (Lund and Mustari, 1977; Luskin and Shatz, 1986; Uylings et al., 1990).

In the developing cortex, cells destined for one layer are generated at about the same time in the ventricular zone and then migrate towards the pia. Cortical layers are formed in an inside-out fashion, with cells in the deep cortical layers produced before the cells in the superficial layers (Berry and Eayrs, 1963; Berry and Rogers, 1965; Hicks and D’Amato, 1968; Rakic, 1972; Bayer et al., 1991). During phylogeny, the duration of cortical neuroblast migration increases and there is a corresponding increase in the time delay between the arrival of thalamic fibers and the formation of their target layer: thalamic fibers “pause” the longest, for about two months, in the developing human cortex (Bayer and Altman, 1991; Luskin and Shatz, 1985; Rakic, 1990, Kostovic and Rakic, 1990). The mechanisms that keep thalamic fibers out of the cortical plate during early development and that trigger the invasion of cortical layers at the appropriate time are not known.

Along with mechanisms that regulate the timing of afferent cortical innervation, information must also be available for thalamic fibers to allow them to recognize their correct target layer. It has been suggested that interactions with several cell types in the cortex are involved in the process of target cell selection. For example, interactions between growing thalamic axons and migrating layer 4 cells could occur in the subplate zone (Bayer and Altman, 1991). In addition, there is evidence that thalamic fibers make transient synaptic contacts with subplate cells (Shatz et al., 1988; Fríauf et al., 1990; Herrmann et al., 1991) and these contacts might be essential for the successful recognition of the later target cells in layer 4 (Shatz et al., 1988; Ghosh et al., 1990).

In order to get some insights into guidance cues that
might influence the growth of thalamic axons, we studied the interactions of thalamic fibers with cortical tissue using different in vitro approaches. The establishment of thalamocortical connections was examined in co-cultures of slices from rat thalamus and cortex prepared at different developmental stages. We found that the formation of thalamocortical connections in vitro depended crucially on the age of the cortical, but not on the age of the thalamic explants. Thalamic fibers of all ages tested did not invade cortical explants prepared early during development, around embryonic day 16 (E16). However, when cortical explants were prepared a few days later, thalamic fibers readily invaded the cortical explants and in cortical slices from postnatal cortex they terminated in their appropriate target layer in vitro. Using a quantitative growth assay, we found that membrane-bound molecules specifically promoting the growth of thalamic fibers were upregulated in the developing cortex, in parallel to its innervation by thalamic fibers in vivo and in cortical slice cultures. The invasion of thalamic fibers into cortical layers in vivo and in slice cultures closely correlated with the distribution of lectin-binding molecules suggesting that membrane-bound glycosylated proteins in the cortex are responsible for its innervation by thalamic fibers. In addition, the growth rate of thalamic fibers was specifically reduced on membranes from their isolated target layer. These results suggest that membrane-bound molecules in the cortex are involved in the innervation and termination of afferents from the thalamus.

Materials and methods

Preparation of slice cultures

Slice cultures were prepared using a roller culture technique (Gähwiler, 1981) as described in Caeser et al. (1989). Briefly, small blocks of cortical or diencephalic tissue were isolated in Gey’s balanced salt solution supplemented with glucose (6.5 mg/ml) and cut with a McIlwain tissue chopper to a thickness of 200-300 μm. The slices were stored for 30-45 minutes at 4°C in Gey’s balanced salt solution supplemented with glucose. The slices were then placed on a glass coverslip and embedded in 20 μl thrombin (0.2 mg/ml, 20 NIHU/ml Hoffmann-La Roche). Slice cultures were maintained in 0.75 ml balanced salt solution and 25% horse serum, containing 0.1 mM glutamine and 6.5 mg ml\(^{-1}\) glucose (all from Gibco) at 36°C under continuous rotation.

DiI-tracing

The lipophilic tracers Dil and DiA were used to study thalamocortical projections in situ and in co-cultures (Godemert et al., 1987). Embryos were obtained by Cesarian section from deeply anesthetized (400 mg/kg chloral hydrate), timed-pregnant rats (Lewis, day of sperm detection = E1). After decapitation of the embryos, brains were removed and stored in 4% paraformaldehyde in 0.1 M phosphate buffered (PB) and sections were cut on a vibratome at 100-200 μm. In thalamocortical co-cultures, small crystals of DiI were inserted into the thalamic explant after 4-23 days in vitro and then kept in PFA for several weeks. The trajectories of DiI-labeled fibers from the thalamus in vivo and in co-cultures were examined with a fluorescence microscope, photographed and drawn with the aid of a drawing apparatus.

BrdU-birthdating

In order to label cells at their birthday, timed pregnant rats were injected intraperitoneally with 250 mg bromodeoxyuridine (BrdU, Serva) per kg body weight. The brains were removed, immedi-ately frozen on dry ice and cut on a cryostat at a thickness of 10 μm. Sections were fixed for 10 minutes in ice-cold methanol and washed several times in PB. Cortical slice cultures were prepared from embryonic or postnatal animals of BrdU-injected timed-pregnant rats, and fixed after 2 or more days in vitro (DIV) with 4% paraformaldehyde in PB for 30 minutes. After several washings in the same buffer, fixed cultures and sections were incubated in 2N HCl for 30 minutes, followed by incubation in borate buffer, pH 8.5, twice for 15 minutes. BrdU-antiserum (Bioscience) was used at a 1:10 dilution in PB at 4°C overnight. After several washes, sections and cultures were incubated with a biotinylated secondary antibody solution (1:250) for 30 minutes at 4°C and further processed using avidin-biotin-peroxidase (Vectastain Kit), which was visualized by an enhanced DAB-reaction. Cultures and sections then were dehydrated in alcohol, cleared in xylene and embedded in Entellan. The location of BrdU-cells was drawn using a drawing apparatus on a microscope equipped with Nomarski optics.

Lectin-histochemistry

Cryostat sections were obtained as described above and were fixed for 10 minutes (slice cultures for 30 minutes) in 2% glutaraldehyde and 2% paraformaldehyde in 0.15 M sodium-cacodylate buffer, pH 7.3, followed by extensive washing in 1% (w/v) bovine serum albumin (BSA) in PBS with 1 mM CaCl\(_2\) and 1 mM MnCl\(_2\), pH 7.4, for several hours. Sections and cultures were then incubated overnight at 4°C in the same buffer containing either 50 μg/ml peanut lectin from Arachis hypogaea (Sigma), Jacalin from Artocarpus integrifolia (Medac), Concanavalin A from Canavalia ensiformis (Vektor), or the lectin from Erythrina cristagallis (Medac). All lectins were biotinylated and further processed using the avidin-biotin technique (Vectastain) and an enhanced DAB-reaction. Controls were incubated in the same lectin-solution containing the appropriate sugar concentration, e.g. 20 mM galactose and 50 μg/ml PNA.

Membrane preparation

Blocks of cortex or in some control experiments from the hindbrain, were dissected in Gey’s-balanced-salt-solution supplemented with glucose (6.5 mg/ml), and the pia was removed. Slices were cut with a McIlwain tissue chopper and pooled in the 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 (all from Serva) and 15 μg/ml 2,3-dehydro-2-desoxy-N-acetylneuraminic acid (Sigma), pH 7.4. For some experiments, layer 4 was isolated from each individual cortex slice by two cuts made parallel to the pial surface. Both isolated layer 4 slices, and the ventral part consisting of white matter, layer 6 and 5, were then collected separately in homogenization buffer. The homogenate was centrifuged for 10 minutes at 25,000 revs/minute 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 in PBS without Ca\(^{2+}\) and Mg\(^{2+}\) at 14000 revs/minute in an Eppen-
placed as near as possible to the first stripe. To identify and to determine the position of each well containing about 50 μl medium with 0.4% methylcellulose was added. Thalamic explants of nude mice (Harlan) were put into these drops across the coverslip. After 2-4 hours, membranes adhered to the coverslip, the capillary was removed and the coverslip washed with PBS. In experiments with two membrane stripes, the same procedure was repeated with the other membrane suspension and the second glass coverslip was placed as near as possible to the first stripe. To identify and to localize the cortical membranes, fluorescein- or rhodamine-labeled microspheres (Duke Scientific) were added in a 1:25,000 dilution to the membrane suspension. After the membrane stripes were located using an inverted Zeiss fluorescence microscope, the coverslips were placed in Petri dishes (Multimed) and medium containing 0.4% methylocellulose was added. Thalamic explants of about 200×200×200 μm were then pipetted in the regions of the membrane stripes. The axonal outgrowth was repeatedly checked and photographed with phase-contrast optics in an inverted Zeiss microscope.

**Results**

**Development of the thalamic projection in the cortex in vivo**

As a first step, we examined the establishment of the thalamocortical projection in vivo in relation to the location of their target cells from layer 4 and the developmental pattern of lectin-staining in the cortex (Fig. 1). The invasion of thalamic afferents in the cortex is depicted in Fig. 1B. As has been shown previously (Lund and Mustari, 1977; Catalano et al., 1991), the first thalamic afferents arrived at E17 in rat cortex and initially did not invade the cortical plate, but were restricted ventral to it in the subplate and intermediate zones (Fig. 1B). Around E20, individual thalamic fibers redirected their growth perpendicularly and started to enter the deep cortical layers (Fig. 1B). After several days, around P2-4, thalamic fibers then arrived at their target layer where they terminated their growth and formed terminal arbors. Thus, different phases of thalamic fiber growth can be distinguished during innervation of rat cortex which are also found during the development of the thalamocortical projection in other mammals (Shatz et al., 1988; Rakic, 1977; Kostovic and Rakic, 1990): initially, thalamic axons are restricted to the subplate zone; thereafter, they change their direction of growth, invade the cortical layers and finally arborize in layer 4. Since interactions of thalamic afferents with their target cells could influence the differential axonal growth, we studied the relation of thalamic fiber growth to the position of their target cells. Cortical target neurons destined for layer 4 were labeled with BrdU at their generation time, i.e. E17 in rat cortex (Miller, 1988). When the first fibers from the thalamus have reached the cortex, future layer 4 cells have just been generated in the ventricular zone (Fig. 1A,B). Then, BrdU-labeled cells destined for layer 4 migrated towards the pial surface for about a week and completed their migration several days after birth (Fig. 1A). At the time when thalamic axons redirected their growth and left the subplate zone, their target cells were already distributed throughout the whole cortex (Fig. 1A,B), indicating that thalamic fibers do not immediately follow their migrating future layer 4 cells.

Another possible influence on the growth of thalamic fibers in the cortex might be the existence of guidance molecules in the developing cortex. Since the guidance molecules for axonal growth so far identified are glycosylated (for review: Jessell, 1988; Stahl et al., 1990; Schwab, 1990; Furley et al., 1990) and various lectin-binding molecules have been described to influence axonal growth (Davies et al., 1990; Stahl et al., 1990), we examined the distribution of lectin-binding molecules during cortical development in vivo. We found a close correlation between the peanut agglutinin (PNA) staining and the growth of thalamic fibers throughout development in the cortex (Fig. 1B,C). In sections through the cortex at E17, staining with PNA was confined to a small band in the subplate/intermediate zone and in the marginal zone (Figs 1C and 4A; Raedler et al., 1981; Brückner et al., 1985). When thalamic fibers started to invade the cortical layers (E20), the PNA-positive band in the cortex had extended from the subplate zone into the deeper cortical layers (Figs 1C and 4B). During further development, PNA-staining spread into the superficial
layers until the whole cortex was uniformly stained with PNA in
the first postnatal week (Figs 1 and 4C). In sections from adult
cortices, PNA-staining was again very weak. A similar labeling
pattern was observed with the lectins Concanavalin A and Jacal,
whereas no staining in the cortex could be detected with the lec
tin from Erythrina cristagalli (data not shown). The close correla
tion between the growth of thalamic fibers and the distribution of PNA-
binding molecules was confirmed in double-labeling exper-
iments which revealed directly that DiI-labeled fibers from the
thalamus did not enter cortical layers with weak lectin-
staining. Instead thalamic axons were always confined to
those regions of the cortex that exhibited intense PNA-
staining and fibers were observed in the cortical plate only
after the PNA-staining had spread from the subplate zone
into the deep cortical layers.

Thalamic projections to co-cultured cortical slices of
different developmental stages
In order to examine the relationship between cortex matu-
ration and thalamic fiber growth in vitro, we co-cultured
thalamic explants with cortical slices prepared at different
times during development. When thalamic axons encoun-
tered cortical slices prepared at E16, in 88% of all cases
(Table 1; n=111) not a single thalamic fiber grew into the
E16 cortex explant (Figs 2D, 3), whereas axons from the
very same thalamic slice readily invaded co-cultured cor-
tical slices prepared at later developmental stages (Fig. 2A-
C). Axons extending from older thalamic explants (E20,
P0) also did not invade E16 cortical slices, but did inner-
vate explants prepared later during development (Table 1).

Table 1. Percentage of cortical cultures invaded by
thalamic axons

<table>
<thead>
<tr>
<th></th>
<th>E16</th>
<th>E19-21</th>
<th>P0-6</th>
<th>Ctx</th>
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<tr>
<td>Thal E16</td>
<td>12%</td>
<td></td>
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<td>90%</td>
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<tr>
<td>E19-20</td>
<td>26%</td>
<td>91%</td>
<td></td>
<td>88%</td>
</tr>
<tr>
<td>E21-P2</td>
<td>17%</td>
<td>100%</td>
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<td>87%</td>
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The direction from which growing axons approached the
cortical explants was not critical, since thalamic fibers grew
around an E16 cortical slice irrespective of whether they
approached the explant from the ventricular or the pial side.
Thalamic fibers exhibited a different growth in cortical
slices prepared at E19, where they grew randomly through-
out the explant and extended up to the pial side of the cor-
tical slice (Fig. 2B). Again, the growth of thalamic axons
in E19 cortical slice cultures was independent of the posi-
tion of the thalamic explant relative to the cortical slice
(Fig. 2B,C), and also independent of the age of the thala-
mic explants (Table 1). Previous birthdating studies have
shown that cells of layer 4 are scattered throughout the cor-
tical thickness at E19 and layer 4 is not formed in slice cul-
tures from E19 cortex during the time in vitro (Götz and
Bolz, 1992). In postnatal cortical explants, however, layer
4 is preserved as an organotypic layer (Götz and Bolz,
1992) and thalamic fibers invaded these explants, stopped
and arborized in their appropriate target layer in vitro (Fig.
2A; Yamamoto et al., 1989; Götz et al., 1990, Molnár and
Blakemore, 1991; Bolz et al., 1992). Thus, similar to the
growth of thalamic fibers during innervation of the cortex

Fig. 1. (A) Camera-lucida drawings of cells labeled with BrdU at embryonic day 17 (E17), (B) DiI-labeled fibers from the thalamus and
(C) staining with the peanut agglutinin (PNA) in frontal sections of the cortex of different developmental stages. The thick lines delineate
the pial surface of the cortical sections, and the thin lines the border between grey/white matter and cortical/subplate respectively. BrdU
was injected at E17 to label predominantly cells of layer 4 and the brains were processed at the developmental stages indicated in the
figure. Note that the ingrowth of thalamic fibers into the cortical layers (B) does not follow the migration of BrdU-labeled cells of layer 4
(A), but rather corresponds closely to the spread of PNA-staining during cortical development (C). Scale bar, 500 μm.
Fig. 2. (A-D) Camera-lucida drawings of DiI-labeled fibers from embryonic thalamic explants confronted with cortical slices at different developmental stages (A, P6; B,C, E19; D, E16). In A,B and D, cortical slices are oriented with the white matter and ventricular side, respectively, towards the thalamic explant, whereas in C the E19 cortical culture faces the thalamic slice with the pial surface. The dashed line in A indicates the border between the grey and white matter. (E) Camera lucida drawing of cells in an E16 cortical explant (pial side up) retrogradely labeled with DiI from a thalamic explant of the same age. For the sake of clarity, fibers emanating from the thalamic explant were not drawn. (F) Camera-lucida drawing of cells labeled with BrdU at E13 in a cortical slice culture (pial side up) prepared at E16. Cultures were fixed after 9 (A), 10 (B,C), 5 (D), 7 (E) and 5 (F) days in vitro respectively. Scale bars, (A,B) 500 µm, (C-F) 200 µm.

Fig. 3. (A) Fluorescence and (B) corresponding phase-contrast micrographs of a thalamocortical co-culture prepared at E16 after 4 days in vitro. The cortical slice is located in the centre of the micrograph, beneath the border of the thalamic explant. DiI-labeled thalamic fibers do not invade the embryonic cortical slice. Scale bar, 200 µm.
in vivo, differential growth of axons from thalamic explants was observed in cortical explants of different developmental stages in vitro.

**Lectin-staining in cortical slice cultures**

The close correlation between the developmental spread of PNA-staining and the invasion of thalamic fibers into the cortex in vivo prompted us to examine the distribution of PNA-binding molecules in cortical slice cultures. Fig. 4 illustrates the development of PNA-staining in cortical slice cultures (D-F) in comparison to the development in the cortex in vivo (A-C). In slice cultures prepared from E16 cortices, PNA-staining remained restricted to a small band in the subplate zone, similar to its distribution at the time of preparation (Fig. 4A,F). Most of the cortical thickness of E16 cortical slice cultures did not bind PNA. In contrast, intense staining with PNA was present throughout the cortical thickness of cultures prepared at E19 after few days in vitro (Fig. 4E). Since PNA-staining was not yet distributed throughout all cortical layers at the time of preparation (compare Fig. 4B-E), PNA-staining must have spread into the cortical plate in slice cultures prepared from E19 cortices. These results show that PNA-binding molecules cannot be located exclusively on thalamic axons or be secreted solely by afferent fibers, since thalamic axons are absent in isolated cortical slice cultures. Thus, at least a proportion of PNA-binding molecules must be located on cortical cells itself and spread in cortical slices in vitro. Moreover, the pattern of PNA-staining closely corresponds to the ingrowth of thalamic fibers in cortical slice cultures.
In cortical slice cultures prepared at E16, PNA-staining is restricted to a small band and these slices are not invaded by thalamic fibers, whereas in slice cultures from E19 cortices PNA-staining is present throughout the cortical layers and thalamic fibers enter these explants from all directions. However, few cortical explants prepared at E16 were invaded by thalamic fibers (Table 1). In these co-cultures (n=13), only axons from thalamic explants that approached the cortical slice parallel to the pial surface entered the cortical slice and these fibers extended exclusively in the region of the subplate zone. Thus, the only thalamic fibers that invaded E16 cortical explants grew along the PNA-positive subplate zone in vitro as is the case in the cortex in vivo.

The subplate zone in cortical slice cultures

During development of the cortex, the subplate zone is related to the establishment of the thalamocortical projections in two aspects: first, the growth of thalamic fibers is restricted to the subplate zone in early developing cortex (Fig. 1B) and, second, the first projection neurons that send their axons to the thalamus are cells of the subplate zone (McConnell et al., 1989). We therefore examined the subplate zone in embryonic slice cultures with BrdU-injections at E13/14, the time when subplate cells are generated in rat cortex (Bayer and Altman, 1990; Götz and Bolz, 1992). As depicted in Fig. 2F, cells labeled with BrdU at E13/14 formed a horizontal band in embryonic cortical slice cultures that was visible after several days in vitro. Since neurogenesis and migration ceases in this in vitro system, granular and supragranular layers are missing in E16-E20 cortical slice cultures (Götz and Bolz, 1992). Those layers, however, that already had been formed at the time of preparation, like e.g. the subplate zone, were equally well preserved in slice cultures from embryonic or postnatal cortices in this in vitro system (Götz and Bolz, 1992). Despite the presence of the subplate zone in E19 cortical slice cultures, thalamic fibers were not confined to this layer, but rather extended beyond it regardless whether they invaded the cortical slice from the ventricular or the pial side (Fig. 2B,C). In contrast, the few thalamic fibers that entered an E16 cortical explant in vitro all grew along the region of the subplate zone (n=13). As described above, the distribution of PNA-staining in E16 cortical slice cultures remains restricted to the subplate zone (Fig. 4F), whereas it spreads into the cortical plate in slice cultures from E19 cortex (Fig. 4D,E). Thus, the growth of thalamic fibers corresponds to the location of PNA-binding molecules, but not to the subplate zone by itself.

Similar to the situation in the cortex in vivo (McConnell et al., 1989), cells located in the subplate zone of slices from embryonic cortices were found to project to thalamic slices in vitro. In contrast to fibers from the thalamic explant that avoided the E16 cortical slice or extended only along the narrow subplate zone, axons from the cortical slice readily invaded E16 thalamic explants (Fig. 2E). About 65% of the corticothalamic projection neurons in E16 slice cultures (n=26) were located in the same region as cells of the subplate zone. Thus, despite the presence of corticothalamic projections from subplate cells, thalamic fibers only rarely invaded the E16 cortical slice. However, there were several examples where thalamic fibers invaded older cortical slices, but no corticofugal fibers were detected. Thus, it appears that efferent cortical projections are neither necessary nor sufficient for the invasion of cortical cultures by thalamic axons.

Thalamic fiber growth on cortical cell membranes from different developmental stages

The results obtained with the thalamocortical co-cultures suggested the presence of glycosylated molecules in isolated cortical slices that influence the growth of thalamic axons. Molecules that affect the growth of axonal processes can be either located in the extracellular matrix, or bound to cell membranes, or exist in a soluble form (Levi-Montalcini 1987; Stahl et al., 1990; Schwab, 1990; Furley et al., 1990; Davies et al., 1990; for review: Dodd and Jessell, 1988). Since diffusible molecules are stabilized in a graded way in our co-culture system (Bolz et al., 1990), we used cortical cell membranes to test whether soluble or membrane-bound molecules influence the growth of thalamic axons. Membranes prepared from cortices at different developmental stages were tested as substrata for thalamic axons in a quantitative growth assay (Vielmetter and Stürmer, 1989). Thalamic explants placed on E16 cortical membranes showed very little, if any, fiber outgrowth (Figs 5A, 6A), in contrast to explants placed on cortical membranes prepared at P7 that were surrounded by a dense fiber network after 2-4 days in vitro (Fig. 5B). An intermediate rate of thalamic fiber growth was observed on cortical membranes prepared at developmental stages E19 and 20 (Fig. 6A). On membrane substrata prepared from adult cortices, the rate of thalamic fiber growth was again reduced to a lower level (Fig. 6A). Thus, purified cortical membranes show growth-supporting properties for thalamic afferents (see below) that increase during development and peak in early postnatal development.

To test whether this differential effect on axonal growth was specific for thalamic fibers, we placed explants from the E16 cortex and hindbrain on cortical membranes prepared at E16 and P7. The number of cortical axons extending on cortical membranes far exceeded the axonal outgrowth from hindbrain explants (Fig. 6B). In contrast to thalamic axons, however, both of these axonal populations exhibited the same rate of outgrowth on embryonic and postnatal cortical membranes (Figs 5C,D; 6B). Since serotoninergic fibers from raphe nuclei in the hindbrain establish projections to the cortex in vivo (Uylings et al., 1990), explants from the hindbrain were also stained with an antibody directed against serotonin. Fibers immunoreactive for serotonin also showed no preference for one of the cortical substrata (on E16 cortex membranes; 6.8 axons (n=23); on P7 cortex membranes: 7.3 axons (n=32)). The large number of cortical axons extending on E16 cortical membranes (Fig. 5C) indicates that embryonic cortical membranes do not inhibit axonal outgrowth in general. Moreover, thalamic fibers do not avoid extending onto embryonic brain membranes in all cases, since more thalamic axons were found to extend on membranes prepared from E16 hindbrain than from E16 cortex (data not shown). Thus, there is a difference between embryonic and postnatal cortical
membranes which is specifically recognized by thalamic axons.

The differential outgrowth of thalamic fibers on E16 and P7 cortex can be explained in two ways: growth of thalamic axons may be either inhibited by molecules bound to membranes from E16 cortex or, conversely, membranes from E16 cortex may not contain growth-supporting molecules for thalamic axons. To address this issue, we mixed membranes prepared from E16 and postnatal cortex. If inhibitory molecules were associated with E16 cortical membranes, their addition should reduce the outgrowth of thalamic fibers on a growth-permissive substratum, as has been described previously for other inhibitory molecules (Davies et al., 1990). However, an equal number of axons extended on mixed cortical membranes as on P7 cortical membranes alone (Fig. 6C). Similarly, when the plastic dishes were coated with laminin, the addition of E16 cortical membranes did not reduce the rate of fiber outgrowth on postnatal cortical membranes, but the rate of fiber outgrowth on postnatal cortical membranes exceeded the number of thalamic axons on laminin alone (Fig. 6A). These data suggest that postnatal cortical membranes possess growth-promoting molecules for thalamic axons that are not present in the early embryonic cortex. To test this hypothesis further, cortical membranes of both ages were trypsinized for 45 minutes. The same number of thalamic axons extended on the trypsinized as on the control membranes prepared from E16 cortices (Fig. 6D). In contrast, the rate of thalamic fiber outgrowth was strongly reduced after trypsinization of P7 cortex membranes, almost to the low level found on E16 cortex membranes (Fig. 6D).

To examine the influence of PNA-binding molecules in cortical cell membranes on the outgrowth of thalamic fibers, we applied 50 µg/ml PNA in the quantitative growth assay. The rate of thalamic fiber growth on membrane substrata from postnatal cortices was diminished by more than 50%
Development of thalamocortical connections in the presence of PNA (Fig. 6E). In contrast, the addition of the lectin Jacalin did not influence the number of thalamic axons extending on postnatal cortical membranes (Fig. 6E). Thus, binding of the peanut agglutinin to glycosylated molecules present in membrane preparations from postnatal cortices seems to block the growth-promoting properties of this substratum for thalamic fiber growth.

The influence of different substrata on the direction of thalamic fiber growth

So far we have shown that the rate of thalamic fiber growth can be influenced by membrane-bound molecules in the cortex. However, changes in the direction of axonal elongation are also required, e.g. to confine the growth of thalamic axons to the subplate zone in early development (Fig. 1B,C). In order to examine which substrata might cause a redirection of axonal growth, thalamic axons were confronted with borders of different substrata. At the border between a pure laminin substratum and laminin with membranes from postnatal cortices in 95% of all thalamic explants (n=43) sharp turns of the growing axons were observed (Fig. 5E). Most of the fibers (97%; n=270) that encountered the border remained on the side where the postnatal cortical membranes were located and only 3% of thalamic axons entered the laminin substratum. In contrast, thalamic fibers that extended on pure laminin crossed the border to the region coated in addition with membranes from postnatal cortices in all cases examined (n=12). However, fibers showed almost no preference for membranes prepared from E16 cortex since, in only 2 out of 10 cultures examined, thalamic axons entered the laminin substratum. In contrast, thalamic fibers that extended on pure laminin crossed the border to the region coated in addition with membranes from postnatal cortices in all cases examined (n=12). However, fibers showed almost no preference for membranes prepared from E16 cortex since, in only 2 out of 10 cultures examined, thalamic axons respected the border between E16 cortical membranes and pure laminin. When axons from thalamic explants (n=15) approached the border between stripes containing E16 and P7 cortical membranes, 92% of all thalamic fibers (n=370) performed sharp turns and remained in regions where postnatal cortical mem-

Fig. 6. Histograms of axonal outgrowth from explants prepared at E16 on cortical membranes. Error bars indicate the standard error of the mean (s.e.m.). (A) The rate of thalamic growth increases on substrata from cortical membranes during development and decreases again on membranes from adult cortices. (B) Explants from E16 thalamus, cortex and hindbrain were placed on membranes from E16 or P7 cortices, respectively. Whereas the growth rate of thalamic fibers exhibits a pronounced difference between these substrata, axons from cortex and hindbrain do not recognize a difference between cortical membranes of different ages. (C) The rate of thalamic fiber growth on membrane substrata from both E16 and P7 cortices corresponds to their rate of outgrowth on postnatal cortical membranes alone. (D) Trypsinization of postnatal cortical membranes strongly reduces the outgrowth of thalamic fibers compared to their growth rate on control membranes, whereas trypsinization of embryonic cortical membranes does not exhibit any effect on the growth of axons from thalamic explants. (E) The growth rate of thalamic fibers on postnatal cortical membranes is strongly reduced after the addition of peanut agglutinin (PNA), but not after addition of Jacalin. (F) Many more thalamic fibers extend on membrane substrata from the deeper layers including the white matter of a postnatal cortex, compared to their growth rate on layer 4 membranes or a mixture of all cortical layers including the white matter.
branes were available (Fig. 5F). In control experiments, where two stripes of postnatal cortical membranes were applied adjacent to one another (n=15), no change in the number and direction of growing thalamic axons was observed at the border between the two postnatal membrane substrata. Thus, growing thalamic fibers show a strong preference for cortical membranes of postnatal origin and perform sharp turns to stay on this growth-supporting substratum while they avoid E16 cortex membranes.

**Thalamic fiber growth on membranes from isolated cortex layers**

These results revealed the presence of developmentally regulated membrane-bound molecules in the cortex that influence the growth of thalamic fibers. To elucidate a potential contribution of membrane-bound molecules to the layer-specific termination of thalamic fibers, we separated layer 4 of early postnatal cortex from the deeper layers, through which thalamic fibers grow in vivo. The same number of thalamic fibers extended on membranes from isolated layer 4 as on membranes derived from all cortical layers and the white matter (Fig. 6F). However, more than twice as many fibers extended from thalamic explants placed on membranes prepared only from the deeper cortical layers and the white matter (Fig. 6F). Thus, membrane-bound molecules that are differentially distributed in the cortical layers influence the growth of thalamic axons and specifically diminish the rate of thalamic fiber growth on membranes from layer 4, the target layer for thalamic afferents.

**Discussion**

There has been much discussion of how afferent fibers from thalamus make their appropriate connections with cortical targets (Wise and Jones, 1978; Lund and Mustari, 1977; Shatz et al., 1988; Ghosh et al., 1990). Along with the selection of their target cells, cortical afferents face the additional problem of a temporal mismatch between their early arrival and the late formation of their cortical target layer. Our in vitro results provide evidence that membrane-bound guidance molecules in the cortex are involved in both the regulation of thalamic innervation into the cortical layers and their termination in layer 4.

**Innervation of thalamic fibers into the cortex**

Thalamic fibers of all ages tested (E16-P1) recognize guidance cues in the cortex: whereas axons from thalamic explants terminated in layer 4 of postnatal cortical slices, they exhibited a random growth in cortical explants where layer 4 had not yet been established, and they avoided E16 cortical explants. Thus, the growth of thalamic fibers depends crucially on the age of the cortical tissue but not on the age of the thalamic explants. Preparations of cell membranes revealed that membrane-bound molecules in the cortex are responsible for the differential growth of thalamic axons and influence the growth of thalamic fibers in a developmentally regulated fashion: only few axons extend on cortical membranes isolated during early development and an increasingly higher rate of outgrowth was observed on cortical membranes prepared at later developmental stages. This effect on axonal growth rate was specific for axons from the thalamus since axons from other regions of the brain grew equally well on membranes prepared from embryonic and postnatal cortex. Thus, either distinct guidance cues for different axonal subsets are present in the cortex or, alternatively, the same cues act differently on axonal subpopulations, which should then possess different receptors for these molecules (Reichardt and Tomaselli, 1991; Neugebauer and Reichardt, 1991; DeCurtis, 1991). This selectivity is not surprising in view of the large variety of adhesion and extracellular matrix molecules in the developing cortex (Stewart and Pearlman, 1987; Chun and Shatz, 1988; Nakaniishi, 1983; Bignami and Delpech, 1985; Sheppard et al., 1991). A recent study combining fiber-tracking with immunocytochemistry showed that thalamic and cortical axons differ in their growth along the chondroitin sulfate proteoglycan (CSPG)-containing subplate layer, in that axons labeled from the thalamus were found to be restricted to this region, whereas cortical axons crossed the CSPG-rich subplate and performed sharp turns beneath it (Bicknese et al., 1991). In contrast to the increase and spread of PNA-staining from the subplate zone into the developing cortical layers, the CSPG-staining disappeared during further cortical development, similar to the developmental expression of fibronectin in the cortex (Bicknese et al., 1991; Stewart and Pearlman, 1987; Chun and Shatz, 1988). In addition to such guidance molecules for the specific growth of axonal subpopulations, differential fiber-fiber interaction might also play a role in the establishment of separate afferent and efferent paths in the cortical white matter (Woodward et al., 1990). In our thalamocortical cocultures, however, we could not detect interactions such as axonal fasciculation or axonal avoidance. Moreover, the presence of an efferent corticothalamic projection in vitro was neither necessary nor sufficient for the ingrowth and termination of thalamic fibers in cortical explants.

Axonal populations from the cortex and the hindbrain grew equally well on embryonic and postnatal cortical membranes in vitro, in contrast to the lower growth rate of thalamic axons on embryonic compared to their growth rate on postnatal cortical membranes. This in vitro observation corresponds to the behaviour of these axonal subpopulations in the cortex in vivo: both cortical axons and fibers from the hindbrain extend through the cortical thickness in vivo during early development (Üylings et al., 1990; Bicknese et al., 1991), whereas the growth of thalamic axons is still confined to the subplate zone (Fig. 1B; Catalano et al., 1991). The low growth rate of thalamic axons on embryonic cortical membranes in vitro indicates that most of the cortex does not permit the growth of thalamic axons at early developmental stages. Thus, the confinement of thalamic fibers to a narrow band in the cortex during early development seems to be achieved by the non-permissive substratum properties of the surrounding tissue. Consistent with this idea is the observation that thalamic fibers reorient their growth as they encounter less permissive substrata in vitro. As development progresses, the growth rate of thalamic fibers on cortical membranes increases and reaches a peak at early postnatal ages. This increase in the substratum quality for thalamic fiber growth coincides with the invasion of thalamic fibers into the cortical layers in vivo, suggesting
that substratum properties in the cortex regulate the ingrowth of thalamic fibers during development. Enzyme treatment of the cortical membranes and experiments where cortical membranes from different developmental stages were mixed show no indication of inhibitory molecules on embryonic cortical membranes. These experiments reveal the presence of growth-supporting molecules for thalamic axons on postnatal cortical membranes that are rare or absent on membranes prepared from E16 cortex. Thus, molecules that specifically support the growth of thalamic afferents appear to be up-regulated during cortical development, and establish a growth-supportive substratum that allows thalamic fiber ingrowth only when layer 4 is about to be formed. Since layer 4 cells themselves constitute only a small percentage of the membrane fractions, it seems unlikely that the growth-supporting molecules are located only on the thalamic target cells. On the contrary, thalamic fiber growth was reduced on cortical membranes prepared from layer 4 cells alone. Taken together, this suggests that those molecules that establish a growth-promoting substratum for thalamic fibers are widely distributed in early postnatal cortex.

Which molecules that are up-regulated during cortical development could be candidates for these membrane-bound proteins that support the growth of thalamic fibers? Staining with peanut agglutinin (PNA) and some other lectins revealed a close association between the lectin-staining pattern and the cortical invasion of thalamic fibers, both in vivo and in slice cultures. Furthermore, the growth rate of thalamic fibers on postnatal cortical membranes was reduced by the addition of PNA to the membranes. At least a portion of these PNA-binding molecules must be of cortical origin, since they are expressed and up-regulated in isolated cortical slice cultures. A staining pattern similar to that obtained with lectins has also been observed for various extracellular matrix (ECM) molecules (Godfraind et al., 1988; Sheppard et al., 1991). For example, staining for undefined glycosaminoglycans, tenascin and L2 showed a similar distribution during early cortical development (Nakanishi, 1983; Godfraind et al., 1988; Sheppard et al., 1991). Initially, these ECM components are expressed in the subplate zone, but later they gradually spread from the white matter zone towards the pial side of the cortex (Nakanishi, 1983; Godfraind et al., 1988; Sheppard et al., 1991). Thus, a variety of glycosylated molecules develop in concert with the sequential maturation of the cortical layers and the invasion of thalamic fibers. Since glycoproteins and proteoglycans are varied both in their protein core and their glycosylated side chains (Esko, 1991; Gallagher, 1989), future studies will have to characterize the exact nature of the glycosylated proteins that regulate the invasion of the cortex by thalamic afferents. For further isolation and characterization of these growth-promoting molecules in the cortex, their PNA-binding properties should serve as a convenient tool (Davies et al., 1990).

In the light of their axon guidance function, the mechanisms that regulate the expression of adhesion molecules and ECM components during development become of particular interest. Since PNA-binding increases in isolated cortical slice cultures, intrinsic mechanisms in the cortex seem to be involved in the spread of lectin-binding molecules. However, this development of lectin-binding was only observed in slice cultures prepared towards the end of cortical neurogenesis, but not in slice cultures prepared earlier. These results suggest that influences that appear later during cortical development are crucial for the spread of lectin-binding molecules in the cortex. From these experiments, it is not possible to distinguish whether these factors are of intrinsic cortical or extrinsic origin. It has been shown, however, that cortical cells are at least required for the developmental regulation of ECM molecules, since lesioning of the subplate cells prevents the expression of some ECM molecules (Chun and Shatz, 1988). Moreover, Ghosh et al. (1990) reported that lesion of the subplate during early development in cat cortex prevented thalamic fibers from entering the cortex, suggesting that subplate cells interfere with the regulation of molecules that support the ingrowth of thalamic fibers. Thus, cells situated in the cortex are at least required, if not alone responsible, for the developmental regulation of these guidance cues.

Layer-specific termination of thalamic fibers in the cortex

Following the increasing expression of growth-promoting molecules in the cortex, thalamic fibers invade the cortical layers and terminate specifically in layer 4. In co-cultures with slices from postnatal cortex, where cells of layer 4 are located at the appropriate position, axons from thalamic explants are able to terminate in their correct target layer (Yamamoto et al., 1989, Götz et al., 1990; Molnár and Blakemore, 1991; Bolz et al., 1992). This appropriate projection to layer 4 is established in vitro irrespective of whether thalamic fibers invaded the cortex from the pia or the white matter side (Götz et al., 1990; Molnár and Blakemore, 1991; Bolz et al., 1992). The earliest thalamic axons emanating from explants isolated at E16, i.e. shortly after neurogenesis is finished in the thalamus (Jones, 1985), recognized their cortical target cells in vitro (Yamamoto et al., 1989; Götz et al., 1990; Molnár and Blakemore, 1991). These fibers had neither made contacts in the subplate zone nor experienced bypassing layer 4 cells. Thus, thalamic fibers do not need external influences to find their way through the cortex; rather they seem to have an intrinsic mechanism that allows them to recognize their target cells in cortex.

Here we have shown that membrane-bound molecules differentially distributed in the cortical layers seem to be involved in the layer-specific termination of thalamic fibers. The growth rate of thalamic axons was reduced on membranes from layer 4 compared to the growth rate on membranes prepared from the deeper cortical layers through which thalamic fibers have to grow in vivo. This decrease in the growth of thalamic axons could be due to a reduced amount of growth-supporting molecules in layer 4. Alternatively, since thalamic outgrowth on membranes prepared from all cortical layers including layer 4 was also less than the growth rate on membranes prepared from the deep layers, the presence of inhibitory molecules might explain the low growth rate on cortical membranes including layer 4 compared to tissue excluding it. Thus, a membrane-bound stop-signal for growing thalamic axons in layer 4 might initiate their termination. The question remains whether these molecules are located on cellular membranes of the target
neurons in layer 4, or if other components, for example processes of neuronal or glial cells that pass through this layer, are responsible for the reduced growth rate of thalamic fibers. Norris and Kalil (1991) have recently presented evidence that axonal growth cones extend along radial glial fibers in the cortex. Moreover, this mechanism could explain the ordered vertical ingrowth of both thalamic and callosal axons into the cortical layers. On the other hand, results obtained in dissociated cell cultures of the cerebellum have shown that contact-mediated stop-signals for afferent mossy fibers are located on the target cells themselves (Baird et al., 1992). Future studies have to address whether these putative target-related stop-signals act in the same way as guidance molecules that induce growth cone collapses of growing axons (Schwab, 1991; Davies et al., 1991; Cox et al., 1990) or whether there are different mechanisms that initiate the termination of elongating fibers.

In summary, the present study revealed that the invasion of cortical layers by thalamic afferents is controlled by membrane-bound molecules in the cortex. The differential expression of these molecules appears to prevent thalamic fibers from entering the cortical plate early in development when their target cells are not yet generated and allows thalamic ingrowth only after layer 4 is about to be formed. Thus, the cortex orchestrates the timing of thalamic fiber ingrowth by the regulation of growth-permissive molecules for thalamic axons, and thereby solves the problem of the temporal mismatch between the arrival of afferents and the generation of their target cells during development. These molecules are specifically recognized by axons from the thalamus. Other axonal populations whose target cells are already present do not recognize developmental changes in the cortical substratum. After layer 4 has been established, membrane-bound molecules specifically located in this target layer reduce the growth of thalamic fibers and might be involved in their termination in the cortex. The developmental status of thalamic fibers does not seem to play a significant role in the establishment of thalamocortical connections, because thalamic fibers of all ages recognize the different membrane-bound properties of cortical tissue and terminate in their cortical target layer. Thus, guidance cues in the cortex are responsible for the proper innervation and termination of thalamic fibers.

We thank Sandra Poesdorf and Renate Thanos for excellent technical assistance, Iris Kehrer for help with the illustrations and Mary Behan, Ysander v.Boxberg and Mark Hübener for valuable comments on the manuscript.

References


(Accepted 14 August 1992)