Characterization of Factors Regulating Lamina-Specific Growth of Thalamocortical Axons

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ABSTRACT: During development, most thalamocortical axons extend through the deep layers to terminate in layer 4 of neocortex. To elucidate the molecular mechanisms that underlie the formation of layer-specific thalamocortical projections, axon outgrowth from embryonic rat thalamus onto postnatal neocortical slices which had been fixed chemically was used as an experimental model system. When the thalamic explant was juxtaposed to the lateral edge of fixed cortical slice, thalamic axons extended farther in the deep layers than the upper layers. Correspondingly, thalamic axons entering from the ventricular side extended farther than those from the pial side. In contrast, axons from cortical explants cultured next to fixed cortical slices tended to grow nearly as well in the upper as in the deep layers. Biochemical aspects of lamina-specific thalamic axon growth were studied by applying several enzymatic treatments to the cortical slices prior to culturing. Phosphatidylinositol phospholipase C treatment increased elongation of thalamic axons in the upper layers without influencing growth in the deep layers. Neither chondroitinase, heparitinase, nor neuraminidase treatment influenced the overall projection pattern, although neuraminidase slightly decreased axonal elongation in the deep layers. These findings suggest that glycosylphosphatidylinositol-linked molecules in the cortex may contribute to the laminar specificity of thalamocortical projections by suppressing thalamic axon growth in the upper cortical layers.


Keywords: layer specificity; cortical development; thalamocortical projection; target recognition; growth-inhibitory molecule

An axonal behavior that accompanies target recognition is the cessation of forward growth. The thalamocortical projection is one of the best systems in which to study the mechanisms that are responsible for axonal termination, since the thalamic afferents project to layer 4 of the neocortex, a discrete target zone which is distinguishable by cytoarchitectonic structure (Garey and Powell, 1971; Ribak and Peters, 1975; Jones, 1981; Gilbert, 1983). Several developmental studies have demonstrated that most thalamic axons grow through the deep layers to terminate in layer 4 without invading the more superficial layers (Lund and Mustari, 1977; Ghosh and Shatz, 1992; Agmon et al., 1993; Miller et al., 1993; Kageyama and Robertson, 1993; Molnár and Blakemore, 1995; Catalano et al., 1996). In vitro studies using coculture preparations of the thalamus and cortex have indicated that this laminar specificity is based on target recognition by afferent axons (Yamamoto et al., 1989, 1992; Molnár and Blakemore, 1991; Bolz et al., 1992), although it may be modified additionally by neuronal activity (Herrmann and Shatz, 1995; Dantzker and Callaway, 1998). Our previous time-lapse
study in vitro has further shown that thalamic axons stop growing upon reaching the target layer (Yamamoto et al., 1997). These findings suggest that molecules which regulate axonal growth are distributed in a lamina-specific manner. Indeed, it has been shown that a few transcriptional factors, some cadherin members, and a class of proteoglycans are expressed in specific cortical layers (Litwack et al., 1994; Frantz et al., 1996; Maeda and Noda, 1996; Suzuki et al. 1997), but their roles in the formation of cortical connections are not evident.

On the other hand, biochemical investigations have demonstrated that the developing cortex contains not only growth-promoting factors for thalamic axons (Götz et al., 1992; Tuttle et al., 1995), but also growth-inhibitory activity (Emerling and Lander, 1996). In addition, the possibility has been raised that some collapsin/semaphorins or ligands of receptor tyrosine kinases regulate the patterning of specific cortical connections (Castellani et al., 1998; Gao et al., 1998; Polleux et al., 1998). However, the molecular mechanisms for the formation of layer-specific thalamocortical projections remain unknown.

In the present study, we investigated characteristics of possible factors that regulate layer specificity of thalamic axon growth, by culturing living thalamic explants with cortical slices that were lightly fixed with paraformaldehyde. Under these conditions, membrane-associated components in the cortex can be analyzed by the application of various enzymatic treatments while preserving cortical lamination. In fact, specificity of axonal projections has been examined in other systems using chemically fixed tissues or cryosections (Yamagata and Sanes, 1995; Halloran and Kalil, 1996). The results reported here demonstrate that growth-inhibitory components for thalamic axons are distributed primarily in the upper layers of neocortex and may contribute to the formation of lamina-specific thalamocortical projections.

**MATERIALS AND METHODS**

**Preparation of Fixed Cortical Slices**

Timed-pregnant Sprague-Dawley rats were used in the present study, and the day of birth was referred to as postnatal day (P) 0. The fixation of cortical slices was carried out according to the method of Yamagata and Sanes (1995). The whole brain was dissected from P1 or P7 pups and then cut into 250-μm-thick coronal slices with a vibratome (Dosaka). These slices were immediately immersed in Hepes (20 mM)-Hank’s solution containing 3.5% paraformaldehyde. After 3 h of incubation in the fixative at 4°C, the slices were washed several times with Hepes–Hank’s solution overnight at 4°C, and cortical portions (occipital region) were taken from the coronal slices and kept in the washing solution until culturing with thalamic explant or incubating with enzymatic solutions (see below). The 3-h incubation for fixation was determined by attempting a variety of periods. In fact, a short incubation (<1 h) resulted in slices being quite fragile, but longer incubation times (e.g., overnight) permitted little growth and invasion of thalamic axons.

**Biochemical Treatments of Cortical Slices**

Heparitinase, chondroitinase, and neuraminidase were applied to P7 fixed cortical slices. Heparitinase (2.5–5 U/mL; Seikagaku Corp.), chondroitinase ABC (5 U/mL; Seikagaku Corp.), and neuraminidase (1 U/mL; Seikagaku Corp.) were dissolved in appropriate buffers as described below, containing protease inhibitors (protease inhibitor cocktail; Sigma, P2714). Heparitinase buffer was composed of 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 4 mM CaCl₂, 0.01% bovine serum albumin (BSA; Wako Chemical). Chondroitinase buffer consisted of 33 mM Tris-HCl, pH 8.0, 154 mM NaCl, 33 mM CH₃COONa, 0.025% BSA. Neuraminidase buffer consisted of 150 mM CH₃COONa, pH 6.5, 154 mM NaCl, 9 mM CaCl₂, 0.05% BSA. Fixed cortical slices were incubated in the heparitinase solution at 25°C (according to the instruction for the enzymatic reaction) for 9 h, and in the chondroitinase or neuraminidase solution at 37°C for 9 h. After incubation with each enzyme, the slices were washed intensively with Hank’s solution several times until culturing with the thalamic explant. Phosphatidylinositol phospholipase C (PI-PLC) was applied to P7 living cortical slices, as glycosylphosphatidylinositol (GPI)-anchored molecules could not be removed by this treatment after fixation. PI-PLC (0.5–1 U/mL; Boehringer Manheim) was dissolved in Hank’s solution containing the protease inhibitors and applied to living cortical slices for 15 min at 37°C. Then, treated slices were fixed as described above and washed with Hank’s solution several times until culturing. Conversely, heparitinase, chondroitinase, and neuraminidase treatments were not applied to living cortical slices, since long incubation and distinct buffer conditions were necessary for these reactions. The immunohistochemical experiment described below confirmed that these enzymatic treatments were effective.

**Immunohistochemistry and Immunoblotting**

To assess the performance of enzymatic treatments, antibody staining was carried out in enzyme-treated and untreated slices. As the background staining was increased in thicker slices, thin sections (20 μm thickness) of separated cortical tissue were subjected to the same enzymatic treatments applied to slices used for culturing and stained by immunohistochemistry. After enzymatic treatments, cortical slices or sections were washed three times with phosphate-
buffered saline (PBS) and incubated in the monoclonal antibodies (mAbs) overnight at 4°C. mAB 12E3 (1:5000; a generous gift from Dr. T. Seki) against highly sialylated neural cell adhesion molecule (NCAMH), mAB against chondroitin sulfates (CS56, 1:100-200: Seikagaku Corp.), and mAB against TAG-1 (4D7, 1:2; a gift from Dr. M. Yamamoto) were used to examine the efficacy of neuraminidase, chondroitinase, and PI-PLC, respectively. After three washes with PBS, slices or sections were incubated for 2 h at room temperature in the secondary antibody conjugated with fluorescein isothiocyanate (FITC) (1:50-200; Organon Teknika Corp.) or Cy3 (Chemicon International). After three washes, they were mounted with glycerol and observed under an epifluorescence microscope. In this immunohistochemical experiment, P7 fixed cortex was used for chondroitinase, heparitinase, and neuraminidase treatments, whereas P1 cortex was used for PI-PLC treatment because TAG-1 staining was almost absent at P7 cortex.

As available antibodies against heparan sulfates did not produce a reliable staining pattern, Western blotting analysis was performed with polyclonal antibodies against the core protein of N-syndecan, a heparan sulfate proteoglycan (HSPG) (Watanabe et al., 1997). P7 neocortex was homogenized in ice-cold PBS containing protease inhibitors. The pellet was collected by centrifugation and washed two to three times with the heparitinase buffer. The pellet was resuspended and incubated in an enzyme-containing buffer (5 U/mL) with protease inhibitors for 9 h at 25°C. The pellet was centrifuged and dissolved in a sample buffer for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The dissolved material was applied to SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was incubated in polyclonal antibodies against N-syndecan (a generous gift from Dr. A. Oohira), followed by alkaline phosphatase-conjugated secondary antibody, and visualized by alkaline phosphatase reaction.

Axonal Labeling and Quantitative Analysis of Axonal Growth

After a week in vitro, cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, and a small crystal of a fluorescent lipophilic dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarboxyamine perchlorate (DiI; Molecular Probes) was implanted into the thalamic explant (Godement et al., 1987). To label thalamic axons entirely, small crystals of DiI were placed at roughly 150-μm intervals along the boundary between the cortical and thalamic slices.

One to two weeks after DiI implantation, these samples were observed by confocal microscopy. The ×10 objective lens and a filter set for rhodamine were used for DiI-labeled axons. A series of images was collected at different depths (5- to 10-μm intervals) and superimposed. Each image was averaged 10 times to increase the signal-to-noise ratio. Transmitted light images were also collected with ×10 and ×4 objective lenses to locate the pial surface of the cortical slice and the boundary between the thalamic explant and cortical slice. After confocal microscopy, the slices were stained in a bisbenzimide solution (0.001–0.002% in PBS) and laminar organization was observed with an epifluorescence microscope. The collected images obtained by confocal microscopy were stored on magneto-optical disks for the following analysis. All measurements of axonal growth were performed with NIH Image software macros (generously made by Dr. E. S. Ruthazer). First, the boundary [curve in Fig. 1(B)] between the thalamic explant and cortical slice was drawn using the transmitted light image. To assess axonal growth, the distance from the most distant axonal tip to the boundary was measured in every 50-μm strip parallel to the cortical layers [Fig. 1(B)], and then the average length of thalamic axon growth was calculated for each layer, the borders of which were determined by bisbenzamide staining. Layers 2/3 and 4 were not easily distinguished in thick slices of P7 cortex [Fig. 1(C)] because cortical lamination is not fully developed at this stage. Therefore, the upper and lower halves of the cell dense cortical plate were defined as layers 2/3 and 4, respectively. In each 50-μm strip, the axons which traveled obliquely with an angle of more than 45° off the strip orientation were excluded from analysis. The bin size of 50 μm was small enough to determine average axonal length in each layer.

Student t test was used for all statistical analyses except for the comparison between untreated and enzymatically treated slices where Dunnett’s method, one of multiple comparison analyses, was adopted.

RESULTS

Laminar Difference in Thalamic Axon Growth on Fixed Cortical Slice

To test directly for a laminar difference in axonal growth, thalamic explants were placed at the lateral edge of fixed cortical slices dissected from P7 rats. At

Culture of Living Thalamic or Cortical Explant with the Fixed Cortical Slice

The day of vaginal plug detection was designated as embryonic day (E) 0. The dorsal thalamic region containing the lateral geniculate nucleus was dissected from E15 rat embryos. Cortical slices (300–400 μm thick) were dissected from P1–2 occipital region for control experiments. The thalamic block or cortical slices were cultured with fixed cortical slices or with enzyme-treated fixed slices on collagen-coated membrane in serum-free, hormone-supplemented medium (Yamamoto et al., 1989, 1992). The thalamic block was placed at either the ventricular side, pial side, or lateral edge of the fixed cortical slice. Cultures were maintained at 37°C in an environment of humidified 95% air and 5% CO₂.
this developmental stage, cortical lamination of the occipital region is just established [Fig. 1(C)] and most thalamic axons terminate in layer 4 (Lund and Mustari, 1977; Kageyama and Robertson, 1993; Molnár et al., 1998) although they continue to grow with the expansion of the cortical plate (Miller, 1981). After a week in vitro, a number of thalamic axons had elongated on the fixed tissue and exhibited a tendency to travel parallel to the layer structure [Fig. 1(A)]. The most interesting feature was that thalamic axons ap-

Figure 1 Thalamic axon growth on fixed cortical slices. (A) Living thalamic explant (left side) was cultured at the lateral edge of a fixed P7 cortical slice. After 7 days in vitro, cultures were fixed, and thalamic axons were labeled with DiI and observed by confocal microscopy. The contrast is reversed. (B) Axonal tip distance from the boundary (curve) between the thalamic explant and cortical slices is measured for each 50-μm strip (short lines) which is parallel to the laminar structure. (C) Cortical lamination is revealed by bisbenzamide staining. In (B,C) the pial surface and the laminar boundaries between layers 1, 2/3–4, 5, 6, and the WM are shown to the right side (black and white arrows). The upper and lower halves of the cell dense layer were defined as layers 2/3 and 4, respectively. (D) Statistical analysis of axonal growth in each layer was performed from a total of 18 cultures. Error bars represent S.E.M. Asterisks indicate significant difference from axonal growth in layer 2/3 or 4 (p < .005, Student t test). Bar in (A,C) represents 200 μm.
The above results showed a laminar difference in thalamic axon growth on fixed cortical slices, indicating an unequal distribution of cortical substratum components that regulate thalamic axon growth. There are at least two ways that growth-regulating molecules could be distributed to produce this effect. One possibility is that a growth-promoting factor is expressed more in the deep layers than in the upper layers. Another possibility is that a growth-inhibitory factor in the upper layers suppresses axon ingrowth in these layers. To distinguish between these two possibilities, enzymatic treatments were applied to P7 cortical tissues before culturing with living thalamic explants, and projection patterns of thalamic axons in these treated slices were compared to those in untreated slices. PI-PLC, neuraminidase, chondroitinase, and heparitinase, which remove GPI-linked molecules or sugar moieties of glycoproteins or proteoglycans, were used in this study, because these membrane-associated molecules could influence axonal growth (see Discussion).

First, the efficacy of these enzymatic treatments was tested by immunohistochemical or immunoblotting methods (see Materials and Methods). Immunohistochemistry with an mAB against chondroitin sulfates demonstrated that the antigen was predominantly found in layer 1 and the boundary between layer 6 and the WM [Fig. 4(A)]. After chondroitinase treatment, this immunoreactivity was almost completely removed [Fig. 4(B)]. Likewise, NCAMH immunoreactivity was expressed more in the deep layers and was fully eliminated after neuraminidase treatment [compare Fig. 4(C,D)]. The action of PI-PLC treatment was confirmed by an mAB against TAG-1 [Fig. 4(E,F)], which is a typical GPI-anchored protein. The effect of heparitinase was analyzed by Western blotting with an antibody against the core protein of N-syndecan, one form of HSPGs [Carey et al., 1992; Watanabe et al., 1996]. The result clearly showed that the smeared band found in un-
treated tissue was reduced to a sharp band in treated slices, indicating that heparan sulfates binding to the core protein are almost entirely digested under heparitinase treatment [Fig. 4(G)]. Therefore, all of the enzyme treatments were effective for removing their specific substrate molecules from the cortical slices.

Among these treatments, PI-PLC produced a considerable change in the layer-specific pattern of thalamic ingrowth. This enzyme produced more uniform axonal extension across all layers, although axonal elongation was still smaller in the upper layers than in the deep layers. Average distances in layers 2/3, 4, and 5 in PI-PLC-treated slices (n = 12) were approximately 1.4- to 1.6-fold longer than those in untreated slices, without a substantial change in layer 6 and the WM [compare Fig. 5(A,B)]. Statistical analysis clearly demonstrated that the increase was significant (Dunnett’s test, p < .01 for layer 4/5, and p < .05 for layer 2/3), when axonal length was compared with untreated slices in each layer [Fig. 6(A,B)]. By contrast, neither chondroitinase (n = 8) nor heparitinase (n = 8) treatments altered the overall growth pattern [Fig. 5(C,D)]. In these cases, there was no significant difference in any layers from untreated cortical slices, although axonal growth in all layers tended to decrease (5–25%) in chondroitinase treatment [Fig. 6(C,D)]. In neuraminidase treatment (n = 9), the

Figure 2  Specificity of axonal growth on fixed cortical slices. (A) Thalamic explant (left side) is placed at the lateral edge of a fixed P1 cortical slice. Bar represents 500 μm. (B) Quantitative analysis of thalamic axon growth on P1 cortical slices (n = 4). Error bars represent S.E.M. Asterisks indicate significant difference from axonal growth in the cortical plate (p < .01, t test). (C) Cortical axon growth on fixed cortical slice. Bar represents 200 μm. Living cortical explant was cultured to the left side of the fixed P7 cortex. (D) Quantitative analysis of cortical axon growth (n = 10). Asterisks indicate significant difference from axonal growth in layer 2/3 (p < .01, t test). Arrows in (A,C) indicate the pial surface and the laminar boundaries between layers 1, 2/3–4, 5, 6, and the WM.
laminar pattern was also similar to that in the untreated group [Fig. 5(E)], but axonal growth decreased with statistical significance (Dunnett’s test, $p < .05$) in layer 5 [Fig. 6(E)].

**DISCUSSION**

The present study demonstrated that thalamic axons extended less in the upper layers than in the deep layers of fixed cortical slices, and that this laminar difference was mostly due to membrane-associated growth-inhibitory activity distributed in the upper layers. This inhibitory activity appeared to be selective for thalamic axons, as outgrowth of axons from control cortical explants was hardly inhibited in the upper layers. The evidence further suggested that GPI-anchored molecules are primarily responsible for this activity.

The main finding in this study is that growth-inhibitory activity for thalamic axons is present in the cortex in a layer-specific manner. Previous studies of growth-regulating molecules by biochemical fractionation and enzymatic application have demonstrated the existence of growth-inhibitory activity in the cortex (Emerling and Lander, 1996; Ghosh and David, 1997). In these investigations, however, the developmental time course and spatial distribution of these possible factors were not clear, and could not account for the formation of layer-specific thalamocortical projections. Moreover, the fact that the growth-inhibitory activity we observed was severely diminished by PI-PLC treatment suggests that the activity is not likely to be attributable to the classic collapsin/semia...
Figure 4  Removal of membrane-associated molecules by enzymatic treatment. (A) The immunohistochemistry was carried out for untreated (A,C,E) and enzymatically treated cortical slices (B,D,F). P7 cortical sections were stained with an antibody against chondroitin sulfate in (A,B) and anti-NCAMH in (C,D), respectively. P1 slices were stained with anti-TAG-1 in (E,F). Cortical sections or slices were subjected to chondroitinase (B), neuraminidase (D), and PI-PLC (F) treatment before immunohistochemistry. Upper and lower arrows in (A–D) indicate the pial surface and the boundary between layer 6 and the WM, respectively. In (E,F), arrows and dashed lines represent the pial surface and the boundary between layer 6 and the WM, respectively. Bar in (F) represents 200 μm and also applies to (A–E). In (A) layer 1 and the boundary between layer 6 and the MW are primarily stained with anti-CS. In (B) the deep layers and the WM are stained with anti-NCAMH. In (C) radially oriented processes are stained with anti-TAG-1. Note that the immunoreactivity found in untreated tissues almost completely disappears after each enzymatic treatment (B,D,F). (G) Western blot analysis with an antibody against the core protein of N-syndecan. The pellet obtained from untreated (left) or heparitinase-treated fixed P7 cortical slices (right) was examined by Western blotting analysis. The positions of molecular markers (78, 121, and 204 kD) are indicated to the right. Upper and lower arrows in (G) indicate a smeared band from untreated slices and sharper band from heparitinase-treated slices, respectively.
phorin family, which consists of secreted and transmembrane molecules (Kolodkin et al., 1993), although members of this family have recently been implicated in the navigation of cortical afferents and efferents through an inhibitory action (Bagnard, 1998; Polleux et al., 1998; Skaliora et al., 1998).

Various types of GPI-linked molecules are present in the developing cortex. Ligands for the Eph family of receptor tyrosine kinases are GPI-anchored molecules and are involved in axonal guidance by inhibitory activity (Drescher, 1997). In particular, ephrin-A5 (RAGS or AL-1) is expressed in the primary sensory cortices (Winslow et al., 1995; Gao et al., 1998) and has been suggested to play a role in forming intracortical connections (Castellani and Bolz, 1997; Castellani et al., 1998) and area-specific connections (Gao et al., 1998). However, whether this expression pattern matches the laminar distribution of the inhibitory activity shown in this study is not yet clear. Further efforts are necessary to characterize members of this family in the developing neocortex. TAG-1 and a form of NCAM are also GPI-anchored adhesion molecules which appear in the neocortex (Yamamoto et al., 1986; Chung et al., 1991; Seki and Arai, 1991), but neither of their distributions is consistent with the growth-inhibitory activity we have observed (Fig. 4). Another candidate may be GPI-anchored proteoglycans, some of which are expressed

Figure 5 Thalamic axon growth in enzymatically treated cortical slices. The living thalamic explant is juxtaposed to an untreated slice (A) or slice treated with PI-PLC (B), chondroitinase (C), heparitinase (D), or neuraminidase (E). P7 cortical slices were used for all treatments. In every case, axonal growth was assayed by confocal microscopy after a week in vitro. White lines indicate the boundary between thalamic explants and fixed cortical slices. Arrows indicate the pial surface and the laminar boundaries between layers 1, 2/3–4, 5, 6, and the WM. Bar in (A) represents 200 μm and also applies to (B–E).
in a layer-specific manner in the postnatal cortex (Litwack et al., 1994; Oohira et al., 1994; Maeda and Noda, 1996). Their effects on thalamic axon growth are not obvious at present, but it is possible that these types of molecules could be responsible for the present inhibitory activity. However, the fact that neither chondroitinase nor heparitinase treatments influenced thalamic axonal growth suggests that at least the sugar residues of the proteoglycans are not associated with the inhibitory activity in the postnatal cortex, although some molecules that bind to chondroitin sulfates can inhibit axon growth in the early stages of the development of the cortical plate (Emerling and Lander, 1996) and in the peripheral nervous system (Snow et al., 1990).

The existence of growth-promoting activity has already been demonstrated in membrane fractions from the developing cortex (Götz et al., 1992; Hübener et al., 1995; Tuttle et al., 1995). Götz et al. (1992) further showed that thalamic axons grow better on membranes from the deep layers than the middle layer of cortex. It has also been reported that adhesion of thalamic neurons to cryosections of the cortex is much more robust in the deep layers (Emerling and Lander, 1994). The present finding is consistent with these reports. The growth-inhibitory factor could possibly overlie the growth-permissive or promoting factors. Moreover, the finding that neuraminidase treatment reduced axonal growth in layer 5 suggests that part of the growth in the deep layer may be attributable to sialic acid–containing molecules such as NCAMH (Rutishauser and Landmesser, 1991). In fact, the immunostaining with anti-NCAMH was stronger in the deep layers of the early postnatal cortex (Fig. 4) (Seki and Arai, 1991).

A previous study has demonstrated that the growth-promoting activity of the developing cortex includes PI-PLC sensitive components, which are less

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**Figure 6** Quantitative analysis of thalamic axon growth in untreated and enzymatically treated slices. Axonal growth was analyzed in untreated slices (A) \( (n = 18) \) or slices treated with PI-PLC (B) \( (n = 12) \), chondroitinase (C) \( (n = 8) \), heparitinase (D) \( (n = 8) \), or neuraminidase (E) \( (n = 9) \). Error bars represent S.E.M. Double asterisks indicate highly significant difference (Dunnett’s test, \( p < .01 \)) from the value in the same layer of untreated slices. Single asterisk indicates less significant difference (Dunnett’s test, \( p < .05 \)).
prominent in postnatal than in embryonic stages (Tuttle et al., 1995). GPI-anchored growth-promoting factors may be negligible by P7 since PI-PLC treatment did not decrease axonal growth in any layers. It has been suggested that chondroitin sulfate proteoglycans in the intermediate zone but not in the cortical plate promote thalamic axon growth in embryonic stages (Bicknese et al., 1994; Emerling and Lander, 1996; Fukuda et al., 1997). HSPGs have been also suggested to play some role in axonal growth in the cortex (Watanabe et al., 1996). The present result excludes the possibility that sugar moieties of these proteoglycans possess growth-promoting activity for thalamic axons, as chondroitinase and heparitinase treatments did not influence thalamic axon growth, although it is possible that some factors enhance axon growth by binding to them (Emerling and Lander, 1996; Walz et al., 1997).

Our present results provide some insights into the mechanisms for the formation of layer-specific thalamocortical connections in vivo. First, the growth-inhibitory factor is distributed predominantly in the upper layer while growth is permissive in the deep layers, where thalamic axons invade the cortex. Second, it is likely that the laminar difference continues to be present from the time (E20–P2 in rat visual cortex) when thalamic axons are invading the cortical plate to the time (P3–7) when they innervate the target layer (Lund and Mustari, 1977; Kageyama and Roberts, 1993; Molnár et al., 1998). Third, the inhibitory activity was selective for thalamic axons over cortical axons, perhaps accounting in part for the difference between the laminar pattern of thalamocortical and corticocortical connections (Jacobson, 1970; Olavarria and Van Sluyters, 1985; Yamamoto et al., 1992; Barbe and Levitt, 1995). The above findings lead to the following scenario. Thalamic axons, possibly under the influence of cortical growth-promoting activity, extend in the WM and layer 6, and are gradually suppressed in layer 5 where the growth-inhibitory components begin to emerge. Once thalamic axons enter layer 4 where the increase of the inhibitory activity and the decrease of the promoting activity take place simultaneously, they may not only slow down but also stop growing. Such spatial change in the substratum could even account for the stop behavior of axons entering from the pial surface as shown in our previous time-lapse study (Yamamoto et al., 1997) as well as in this study (Fig. 3), as it has been shown that axons tend to stop at the boundary between two different substrata regardless of the orientation of ingrowth (Burden-Gulley et al., 1995). Therefore, the differential distribution of growth-regulating factors may contribute to axonal termination in the target layer. A similar mechanism also might be responsible for the formation of other layer-specific connections (Yamagata and Sanes, 1995; Yamagata et al., 1995; Forster et al., 1998; Zhang and Mason, 1998).

There is a discrepancy between thalamic axon behavior in vivo and in the present conditions, in that thalamic axons did not show a preference for layer 4 in fixed cortical slices. In cocultures of living thalamus and cortex, thalamic axons exhibited preferential growth in layer 4, as they do in vivo (Yamamoto et al., 1992, 1997). It is therefore likely that some membrane-bound molecules which are responsible for axonal growth are inactive in the fixed tissue. However, most other aspects of axonal growth seemed not to have been disrupted (Yamagata and Sanes, 1995). Indeed, the growth profile on fixed cortical slices is compatible with the previous findings that thalamic axons grow fast in the deep layers and exhibit the stop behavior in layer 4, as described above. Moreover, the fact that PI-PLC treatment altered the growth pattern reveals the existence of at least one of growth-regulating mechanisms in the cortex, even if some other molecules may become inactive in fixed conditions. An obvious difference from the living cortex is the lack of soluble factors which are released from living cortical cells. Preferential growth in the target layer found in vivo and living coculture preparations might be explained by action of soluble factors in the cortex. Neurotrophins are plausible candidates, since evidence has accumulated suggesting that neurotrophins are involved in axonal extension as well as dendritic growth (Cabelli et al., 1995; McAllister et al., 1995; Cohen-Cory and Fraser, 1995). Our preliminary results show that neurotrophin-3 (NT-3) application increases thalamic axon elongation in fixed cortex (data not shown). The laminar distributions of the GPI-linked growth-inhibitory component and some growth-promoting factor(s), such as NT-3, may together be sufficient to account for the selective termination and extension of thalamocortical axons in the target layer.

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