Axonal Surface Molecules Act in Combination with Semaphorin 3A during the Establishment of Corticothalamic Projections

Interactions between growing axons are considered to play important roles for the establishment of precise neuronal connections during the development of the nervous system. Here we used time-lapse imaging techniques to examine the behavior of neocortical and thalamic axons when they encounter each other in vitro. Results indicate that axonal growth cones are able to respond to specific cues expressed on the surface of fibers. Thalamic growth cones often extended along the surface of other thalamic axons and, likewise, cortical growth cones formed fascicles with cortical axons. In contrast, after contacts between cortical and thalamic fibers, in most cases growth cones collapsed and retracted from the axons. Collapse assays using membrane preparations from cortical or thalamic explants demonstrated the existence of cell-type specific collapsing factors whose activity was enhanced by a member of the semaphorin protein family, Sema3A (expressed in the thalamocortical pathway), as it increased the rate of homotypic fasciculations and at the same time amplified the segregation between cortical and thalamic axons. The interaction between axonal surface molecules and environmental cues might mediate the segregation of afferent and efferent fiber tracts in the neocortical white matter.

Introduction
During embryonic development, projection neurons in the cerebral cortex and relay neurons in the thalamus establish reciprocal connections between distinct cortical regions and specific thalamic nuclei. Anatomical studies using carboxy Flame dyes revealed that pattern formation of afferent and efferent projections in the thalamocortical system is precisely regulated in space and time (McConnell et al., 1989; Catalano et al., 1991, 1996; De Carlos and O’Leary, 1992; Ghosh and Shatz, 1992; Métin and Godement, 1996). In recent years, families of secreted and membrane-associated proteins, the netrins, semaphorins and ephrins, have been discovered which can act as axonal guidance molecules (Kennedy et al., 1994; Serafini et al., 1994; Cheng et al., 1995; ColaMarino and Tessier-Lavigne, 1995; Drescher et al., 1995; Mark et al., 1997; Bagnard et al., 1998). Members of each of these protein families are expressed along the path of thalamic and cortical fibers at the developmental stages when these axons extend towards their targets. Moreover, such molecules are considered as important factors of the genetic control of regionalization and connectivity of the neocortex (Mackarethschian et al., 1999; Rubenstein et al., 1999). Previous work provided evidence that semaphorins can act both as attractive and repellent guidance cues during the initial patterning of corticofugal projections (Bagnard et al., 1996, 2000) and that netrin-1 might secrete fibronectin to the internal capsule, the gateway between cortex and thalamus (Métin et al., 1997; Richards et al., 1997).

Based on observations on the timing of axonal outgrowth, it has been proposed that molecules on axonal membranes contribute to the targeting in the thalamocortical system. These studies revealed that the first axons which extend towards subcortical regions arise from the cortical subplate, a transient structure composed of early generated neurons (Marin-Padilla, 1971; Luskin and Shatz, 1985). Axons of subplate cells might therefore provide a scaffold for the later growing cortical plate neurons (McConnell et al., 1989), analogous to the ‘pioneer axons’ originally described in the invertebrate nervous system (Bastiani et al., 1986). Moreover, because descending cortico-fugal and ascending thalamocortical axons arrive at the internal capsule in synchrony, it has also been suggested that these two fiber populations might fasciculate and thereby guide each other towards their final destination (Molnár and Blakemore, 1991, 1995; Molnár et al., 1998).

While such models are attractive in their simplicity, the formation of axon fascicles among and between cortical and thalamic fibers has not yet been demonstrated. In the present study, we used time-lapse imaging to examine the behavior of thalamic and cortical axons when they encounter each other in vitro. On growth-permissive substrates, fasciculations were only observed between axons of the same type (homotypic fasciculation), whereas in many cases thalamic and cortical axons retracted from each other. In vitro, a repellent axonal guidance molecule, semaphorin 3A (Sema3A), is expressed in the internal capsule when projections between the thalamus and cortex are being formed (Skalli et al., 1998). We found that addition of Sema3A to the substrate not only increased the rate of homotypic fasciculations, but also enhanced heterotypic growth cone retractions. These results are consistent with in vitro observations which indicate that the thalamic and cortical axons form segregated fiber tracts within the white matter.

Materials and Methods
Preparation of Explants
E16 and E18 embryos were obtained after cesarean section of pregnant Lewis rats receiving lethal dose of anesthetics (the day of sperm detection was designated as E1). Embryonic brains were removed and dissected in cold Gey’s balanced salt solution (GBSS). Blocks of neocortical tissue were placed on a MacIwain tissue chopper and cut into 200 µm3 cubes, whereas thalamic tissue was cut into 200 µm-thick slices. Because of their different shapes, it was always possible to distinguish between cortical and thalamic explants in co-cultures. In most experiments explants were prepared from the whole thalamus and the whole neocortex. In some experiments, however, we dissected only the presumptive visual cortex and the lateral geniculate nucleus (LGN) of the thalamus. Explants of the visual cortex were obtained by cutting the posterior part of the neocortical block of tissue. To identify the LGN of the thalamus, we first visualized this nucleus in separate experiments in fixed E18 brains. For this, Dil crystals were injected in the eye of fixed embryos kept at 37°C in 4% paraformaldehyde. After 10–20 days, dye diffusion was sufficient to stain the termination zone in the dorsolateral part of the posterior diencephalon. This region was then dissected in living embryos for time-lapse experiments. Cultures were maintained in a medium consisting of

© Oxford University Press 2001. All rights reserved.
50% Eagle's basal medium, 25% Hank's balanced salt solution, 25% horse serum supplemented with 0.1 mM glutamine and 6.5 mg/ml glucose (all from Gibco). In most experiments, cortical and thalamic explants were cultured on glass coverslips covered with laminin (1 \( \mu g/ml \)), poly-L-lysine (1 \( \mu g/ml \)) and postnatal cortical membranes prepared at postnatal day (P5) with an optical density of 0.1 (see below). Additional experiments were done with substrates consisting of laminin alone, or laminin/poly-L-lysine without postnatal cortical membranes or postnatal cortical membrane enriched with Sema3A-containing membranes.

Membrane Preparations
Postnatal cortical tissues (P5) or E18 embryonic cortex and thalamus were dissected and treated as described previously (Bagnard et al., 1998). Blocks of tissues were transferred in a homogenization buffer (10 mM Tris–HCl, 1.5 mM CaCl\(_2\), 1 mM spermidine, 25 \( \mu g/ml \) aprotinin, 25 \( \mu g/ml \) leupeptin and 15 \( \mu g/ml \) 2,3-dihydro-2-desoxy-N-acetyltalnuraminic acid (Sigma)), pH 7.4. Homogenates were centrifuged in sucrose step gradients (10 min, 50,000 g). The membrane fraction was washed twice in PBS without Ca\(_2^+\) and Mg\(_2^+\) at 14,000 r.p.m. in an Eppendorf biofuge. The same procedure was used to prepare Sema3A-containing membranes after collection of Sema3A-expressing 293 cells from confluent dishes washed in 1 ml PBS. Untransfected HEK293 cells were used to prepare control membranes. Concentrations of membrane solutions were determined by their optical density (OD) at 220 nm with a spectrophotometer after 15-fold dilution in 2% SDS and then adjusted to 0.1 OD.

Time-lapse Procedure
After 24 h in the incubator, there was sufficient outgrowth to start time-lapse recordings. Cultures were transferred into a cell culture chamber on an inverted microscope (Axiovert 135, Zeiss) equipped with phase-contrast optics. In the culture chamber, temperature (37°C) and chamber on an inverted microscope (Axiovert 135, Zeiss) equipped with phase-contrast optics. In the culture chamber, temperature (37°C) and CO\(_2\) concentration (5%) were kept constant. A CCD camera (Imac) was attached to the microscope and connected to a time-lapse video recorder (Panasonic). Single images were recorded every 3 or 5 min under the control of an image processing system (Angus 10, Hammamatsu). An electronic shutter closed the light path each time after an image has been taken. Co-cultures were arranged in such a way that one thalamic slice was surrounded by 4–6 cortical explants; it was therefore always possible to recognize the identity of fibers by following axons back to the explant. Growth cone contacts were analyzed off-line with an image editing system (AG-A570, Panasonic).

Collapse Assays
Explants were grown on glass coverslips covered with laminin (1 \( \mu g/ml \)) and poly-L-lysine (1 \( \mu g/ml \)). In one set of experiments, embryonic cortical or thalamic membrane solutions were added to the explants cultures. After 2 h, membrane particles adhered to the substrate and it was possible to analyze the morphological features of growth cones reacting to the membranes. As a control, experiments were also performed after heat inactivation of the membrane solutions for 30 min at 65°C. In another set of experiments, aliquots of medium containing recombinant Sema3A were added to the cultures of thalamic explants. Growth cone morphology was examined 4 h after incubation in the culture chamber under live conditions. Neurites without lamellipodia and filopodia were scored as ‘collapsed’ (Bagnard et al., 1998).

Generation of Sema3A-expressing Cell Lines
Human embryonic kidney 293 cells (ATCC CRL 1573) were transfected with expression vectors for Sema3A (pBK-Sema3A-AP, pBKFlag-Sema3AAP1b) (Adams et al., 1997) by calcium phosphate co-precipitation (Sambrook et al., 1989). Stable cell lines were selected in 1 \( \mu g/ml \) G418 (Gibco-BRL). Expression level of recombinant fusion protein were determined with the PhospaLight chemiluminescence assay (Tropix) or by Western blot analysis using the anti-Flag M2 antibody (Kodak) as described previously (Pischel et al., 1995). Western blots of membranes obtained from Sema3A-expressing cells confirmed the presence of the recombinant protein in the membranes preparations.

Co-culture Assay
We prepared aggregates of HEK 293 cell stably expressing Sema3A according to the method described previously (Bagnard et al., 1998). Thalamic explants were co-cultured with cell aggregates in 20 \( \mu l \) chicken plasma coagulated with 20 \( \mu l \) thrombin. During coagulation of the clot, explants were disposed around cell aggregates at distances \( \sim 900 \mu m \). After 48 h, cultures were fixed in 4% paraformaldehyde and axonal growth was analyzed using a grid ocular and a \( \times 20 \) phase-contrast objective. Mean axonal length and mean number of axons were determined on the side facing the cell aggregates and on the side distal to the aggregates.

Results
In this study, time-lapse video microscopy was used to examine the behavior of cortical and thalamic growth cones when they encounter each other. In a first set of experiments, explants from cortex and thalamus were prepared from E16 rat embryos, the developmental stage at which afferent and efferent cortical projections meet within the internal capsule (De Carlos and O’Leary, 1992). The substrate consisted of a mixture of laminin and poly-L-lysine enriched with membranes prepared from postnatal cortex, because previous work indicated that cortical membranes promote fiber outgrowth from cortical and thalamic explants (Götz et al., 1992; Hübener et al., 1995). After 1 day in vitro, numerous axons emerged from the explants, and we then started with time-lapse imaging of two or more growth cones, which were close to each other. The recordings lasted for 2–8 h, and all together \( \sim 1000 \) contacts between a growth cone with another growth cone or with an axon have been analyzed. As illustrated in Figure 1, three different types of reactions occurred during such contacts: either axons grew along each other (fasciculation, Fig. 1A); growth cones collapsed and retracted from axons for several minutes before they started to grow again (retraction, Fig. 1B); or fibers continued their growth without changing speed or direction (crossing, Fig. 1C). For the quantitative analysis of growth cone interactions the angle at which growing fibers encountered each other were taken into account. We distinguished between three cases: fibers growing in about the same direction (‘parallel’, contact angle 0–60°); fibers growing in opposite directions (‘anti-parallel’, contact angle 120–180°); and fibers approaching each other at about right angles (‘perpendicular’, contact angle 60–120°).

Crossing was the predominant behavior when growth cones encountered each other almost perpendicularly (angle 60–120°). In this situation, 34/50 cortical growth cones (68%) and 73/87 thalamic growth cones (84%) crossed axons of the same type (homotypic contacts) without alterations of their growth behavior (Fig. 2A). After contacts between cortical and thalamic fibers (heterotypic contacts), in half of the cases axons crossed (27/54 contacts) and in the other half they collapsed. Thus, after ‘perpendicular’ approaches, axonal fasciculation did not occur at all for heterotypic contacts, and for homotypic contacts fasciculation was observed in only two cases with cortical axons (Fig. 2A).

The rate of fasciculation increased strikingly when fibers extending from the same explant encountered each other at a shallow angle (0–60°). After such ‘parallel’ approaches of homotypic fibers, 60/82 cortical growth cones (73%) and 24/100 thalamic growth cones (24%) continued to grow on the surfaces of cortical and thalamic axons respectively (Fig. 2B). Similar growth cone reactions after homotypic contacts were observed when cortical and thalamic axons from different explants were growing towards each other (‘anti-parallel’ approach, contact angle 120–180°; Fig. 2C). There was no statistical difference in
the rate of fasciculation between 'parallel' and 'anti-parallel' approaches ($P > 0.05$, $\chi^2$ test). This suggests that there are no polarity cues for fasciculation on cortical and thalamic axons. Once fasciculation is established, axons are able to extend along their homotypic counterpart in both directions.

In contrast to homotypic contacts, axonal fasciculation was never observed after heterotypic contacts. Obviously, in cortex–thalamus co-cultures heterotypic contacts can only be observed when axons extending from different explants are growing towards each other. When the contact angle was between 120 and 180°, 39/57 growth cones (68%) retracted from the heterotypic axon. In the remaining cases, growth cones crossed, but never fasciculated after axonal contacts. Thus, compared with 'perpendicular' approaches, the rate of retractions after heterotypic contacts increased significantly, from 50 to 68%, for 'anti-parallel' approaches ($P < 0.05$, $\chi^2$ test). The rate of retraction was higher for cortical growth cones contacting thalamic axons (86% retractions, 14% crossings, $n = 37$) than for thalamic growth cones contacting cortical fibers (46% retractions, 54% crossings, $n = 33$; $P < 0.05$, $\chi^2$ test).

**Effects of Substrate Properties**

There is compelling evidence that the rate of axonal fasciculation depends on the properties of the substrate: the less a substrate is permissive for axonal growth, the higher is the tendency of axons to grow on the surfaces of other axons, i.e. to form fascicles (Tessier-Lavigne and Goodman, 1996). The average growth rate of cortical and thalamic axons on postnatal cortical membranes was $-40 \mu$m/h, whereas after fasciculation with homotypic axons the growth rate increased to $-60 \mu$m/h (Table 1). Thus, axons might form fascicles because the surfaces of homotypic axons provide a better substrate for fiber growth than cortical membrane preparations. Likewise, the reason why fasciculation between cortical and thalamic axons did not occur in these *in vitro* experiments might be that cortical membrane substrates are more permissive for axonal growth than the surfaces of heterotypic axons. To address this issue, we examined interactions between cortical and thalamic axons on different substrates and interactions between identified growth cones were recorded by time-lapse videomicroscopy. Images were taken every 3 min for 6–24 h periods. The sequences presented here were recorded over 12 min and illustrate the capacity of axons to fasciculate with each other (A), the induction of retraction and growth cone collapse (B) or the crossing of fibers (C) after axonal contact. Observations were done using a $\times 20$ phase-contrast objective (A,C) or a $\times 40$ phase-contrast objective (B).

**Figure 1.** Sequence of micrographs showing axonal interactions. Explants of embryonic cortex and thalamus were grown on different substrates and interactions between identified growth cones were recorded by time-lapse videomicroscopy. Images were taken every 3 min for 6–24 h periods. The sequences presented here were recorded over 12 min and illustrate the capacity of axons to fasciculate with each other (A), the induction of retraction and growth cone collapse (B) or the crossing of fibers (C) after axonal contact. Observations were done using a $\times 20$ phase-contrast objective (A,C) or a $\times 40$ phase-contrast objective (B).

**Figure 2.** Quantification of axonal interactions. In the co-culture assays, axons approached each other under various angles. We defined three classes called (A) ‘perpendicular’ (contact angle 60–120°), (B) ‘parallel’ (contact angle 0–60°) and (C) ‘anti-parallel’ (contact angle 120–180°). For each class, we determined the percentage of fasciculation (gray), crossing (white) and retraction (black) after interactions between two cortical axons (ctx–ctx), two thalamic axons (thal–thal) or one cortical and one thalamic axon (ctx–thal). $\chi^2$ test analysis revealed no statistical differences between axonal interactions obtained in (B) and (C) ($P > 0.05$).

**Table 1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Growth rate ($\mu$m/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical axons</td>
<td>Thalamic axons</td>
</tr>
<tr>
<td>Cortical membranes</td>
<td>37.2 ± 7.4 ($n = 46$)</td>
</tr>
<tr>
<td>Laminin</td>
<td>18.4 ± 2.4 ($n = 26$)</td>
</tr>
<tr>
<td>Laminin/poly-L-lysine</td>
<td>8.7 ± 4.8 ($n = 13$)*</td>
</tr>
<tr>
<td>Axonal surface</td>
<td>59.2 ± 5.2 ($n = 21$)*</td>
</tr>
<tr>
<td>Sema3A</td>
<td>11.8 ± 11.9 ($n = 24$)*</td>
</tr>
</tbody>
</table>

Cortical and thalamic explants were grown on cortical membranes, laminin, laminin/poly-L-lysine or Sema3A substrates for 24 h. ‘Axonal surface’ corresponds to growth cones fasciculating on axons from the same population. All growth speeds were expressed in $\mu$m/h and were compared to those measured on cortical membrane substrates ($* P < 0.01$, Student’s t-test).
Lack of Regional Specificity during Axonal Interactions

The cortex is composed of distinct areas which are interconnected by specific thalamic nuclei. For example, the somatosensory cortex is connected with the ventrobasal nucleus of the thalamus, whereas the visual cortex receives input from the lateral geniculate nucleus (LGN) of the thalamus. To test whether there exists a regional specificity for axonal interactions, we co-cultured slices from the occipital part of the neocortex, the presumptive visual cortex, with explants from the lateral geniculate nucleus (LGN) of the thalamus. As described in Materials and Methods, the position of the LGN was initially visualized in fixed E18 brains by tracing the termination zone of the optic nerve in the diencephalon with a fluorescent dye. For the culture experiments, we dissected the LGN. As described in Materials and Methods, the position of the LGN was initially visualized in fixed E18 brains by tracing the termination zone of the optic nerve in the diencephalon with a fluorescent dye. For the culture experiments, we dissected the LGN. As described in Materials and Methods, the position of the LGN was initially visualized in fixed E18 brains by tracing the termination zone of the optic nerve in the diencephalon with a fluorescent dye. For the culture experiments, we dissected the LGN.

Modulation of Axonal Interactions by Sema3A

At the time when thalamic and cortical axons traverse the internal capsule, cells in the ganglionic eminence express Sema3A, a member of the semaphorin gene family (Skalli et al., 1998, 2000). In this study, we examined the effects of recombinant Sema3A on thalamic axons. As depicted in Table 5, Sema3A also elicits collapses of thalamic growth cones. Moreover, in co-cultures of different substrates without cortical membranes. On laminin alone, the growth rate was only ~20 µm/h, and on a 1:1 mixture of laminin with poly-L-lysine it was further reduced to ~10 µm/h (Table 1). However, as depicted in Table 2, in 60 contacts examined (contact angle 120–180°), there was not a single case of heterotypic fasciculation.

Effects of Developmental Stages

Surface molecules that mediate or modulate fasciculation of axons are often only expressed during restricted developmental stages. The timing of the expression of such molecules could be disturbed after axons regenerate from explants in vitro. We therefore also prepared co-cultures from E18 embryos and imaged axonal interactions. As illustrated in Table 3, at E18 only 12/36 cortical axons (33%) fasciculated with other cortical axons, significantly less than at E16 (P < 0.001, χ² test). In contrast, homotypic fasciculation of thalamic axons increased almost threefold, from 24% at E16 to 73% (16/22) at E18 (P < 0.001, χ² test). However, as was observed for co-cultures prepared at E16, heterotypic fasciculation did not occur for axons extending from E18 explants (79% retraction, 21% crossing, n = 14). Finally, we prepared heterochronic co-cultures, using E16 cortical and E18 thalamic explants, the time when both fiber populations exhibited a high tendency to form homotypic fascicles in vitro. Again, in the large majority of cases (13/17, 77%), cortical and thalamic axons retracted from each other. In the remaining cases fibers crossed, but they never fasciculated with each other (Table 3).

Co-cultures from visual cortex and LGN and those obtained with whole neocortex and thalamus (P > 0.05, χ² test). Thus, axon fascicles are also not formed between fibers from thalamic and cortical regions which are connected with each other in vitro.

Cortical and Thalamic Neurons Express Membrane-bound Collapse Factors

The results presented so far suggest that repellant signals on the surfaces of cortical and thalamic axons induce cell-type specific retractions of growth cones. To further address this issue, we used membranes from E18 cortex and E18 thalami in collapse assays with cortical and thalamic explants on laminin/poly-L-lysine substrates. As depicted in Table 4, the addition of embryonic cortical membranes induced collapses in 45% of thalamic growth cones, but in only 21% of cortical growth cones. Addition of thalamic membranes elicited the opposite effects, with 52% collapsed cortical growth cones and only 24% collapsed thalamic growth cones. The cell-type-specific growth cone collapse was not observed when cortical and thalamic membranes were heat-inactivated (P > 0.05, χ² test; Table 4). Thus, compared with inactivated membranes, native embryonic cortical membranes possess a specific collapse activity for thalamic growth cones, and conversely, native embryonic thalamic membranes induce growth cone collapse specifically in cortical fibers.

Table 2 Influence of the substrate during axonal interactions

<table>
<thead>
<tr>
<th>Substrate Combination</th>
<th>Fasciculation (%)</th>
<th>Retraction (%)</th>
<th>Crossing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctx–thal</td>
<td>0.0</td>
<td>66.8</td>
<td>31.6</td>
</tr>
<tr>
<td>laminin (n = 43)</td>
<td>0.0</td>
<td>65.0*</td>
<td>35.0*</td>
</tr>
<tr>
<td>Ctx–ctx (n = 17)</td>
<td>0.0</td>
<td>70.0*</td>
<td>29.0*</td>
</tr>
<tr>
<td>Thal–ctx (n = 18)</td>
<td>78.0*</td>
<td>11.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Ctx–ctx (n = 14)</td>
<td>57.0*</td>
<td>28.6</td>
<td>14.4</td>
</tr>
<tr>
<td>Ctx–Thal (n = 16)</td>
<td>0.0</td>
<td>0.0</td>
<td>100</td>
</tr>
</tbody>
</table>

Rate of fasciculation, crossing and retraction are presented for cortex–thalamus interactions on cortical membranes, laminin or laminin/poly-L-lysine substrates. Results obtained on laminin or laminin/poly-L-lysine were not different from those observed on cortical membrane substrates (ns, not significant, P > 0.05, χ² test). Addition of Sema3A resulted in a significant increase in the rate of homotypic fasciculations and heterotypic retractions (*P < 0.05, χ² test).
thagamic explants with Sema3A-producing cell aggregates, thalamic fibers growing towards the source of Sema3A were shorter and less numerous than fibers growing in the opposite direction (Figure 3). Such effects were not observed in control experiments with aggregates from untransfected cells. Thus, Sema3A acts as a repellent signal for both cortical and thalamic axons.

Previous work suggested that semaphorins expressed in the environment of growing axons can induce fiber fasciculation. When Sema1a in the grasshopper limb bud was blocked by antibodies, axons that were normally tightly fasciculated were instead defasciculated (Kolodkin et al., 1992). Because Sema3A is a repellent signal for both cortical and thalamic axons, its presence along the thalamocortical pathway might create a ‘hostile’ environment that pushes these axons together. To test this possibility, we examined the growth of cortical and thalamic axons on a substrate composed of a 1:1 mixture of postnatal cortical membranes and membranes from Sema3A-expressing HEK C293 cells. In the presence of Sema3A the growth speed of axons was almost three times lower than on control substrates (Table 2). In contrast, when thalamic and cortical growth cones encountered heterotypic axons on a Sema3A-containing substrate, in all cases examined (n = 16) they collapsed and retracted from the fibers.

**Discussion**

In this study, time-lapse imaging techniques served to examine the behavior of individual cortical and thalamic growth cones as they encountered each other in *vitro*. Results indicate that growth cones are able to distinguish between specific cues expressed on axonal surfaces. After heterotypic contacts, cortical and thalamic growth cones collapsed and retracted from each other. Collapse assays confirmed that cortical and thalamic membranes possess specific collapse activities for thalamic and cortical growth cones respectively. Axon fasciculation was observed when cortical growth cones encountered cortical fibers or when thalamic growth cones contacted thalamic axons. Strikingly, addition of the repellent guidance molecule Sema3A to the substrate increased the rate of homotypic fasciculations and at the same time intensified the collapse sensitivity of cortical and thalamic growth cones during heterotypic contacts.

For cortical axons, the tendency to form fascicles was highest when explants were prepared at E16 and the fasciculation rate decreased significantly with E18 explants. In the rat, cortical explants prepared at E16 contain subplate cells and layer 6 cells (Miller, 1988). Neurons in layer 6 establish the adult connection from the cortex back to the thalamus, and during development this projection is formed after subplate neurons have already sent their axons into the internal capsule (De Carlos and O’Leary, 1992). The high rate of fasciculation between cortical axons emerging from E16 explants observed in the present experiments is therefore consistent with the proposal that axons of subplate neurons form a scaffold for descending cortical fibers and ‘pioneer’ the corticofugal pathway (McConnell et al., 1989). The decrease of homotypic fascication observed with E18 explants may be due to the presence of layer 5 neurons that project to the contralateral hemisphere via the corpus callosum (Yorke et al., 1975; Wise and Jones, 1976; Lent et al., 1990).

In contrast to cortical fibers, the rate of fasciculation between thalamic fibers was low at E16 but significantly increased at E18. Adhesive properties of developing axons can be tightly regulated in time, and the expression of axonal surface proteins can also be influenced by molecular cues in the environment of growing fibers. For example, subsets of spinal neurons express the cell surface protein TAG-1 only during initial axon extension. After crossing the ventral midline, TAG-1 disappears from these axons and the expression of L1, another surface glycoprotein, is initiated (Rathjen and Schachner, 1984; Dodg et al., 1988). During development of thalamicortical projections, at E18, most thalamic axons have grown past the internal capsule and have reached the upper intermediate zone and the subplate region of the cortex (De Carlos and O’Leary, 1992). During this period, influences from this new environment might trigger the expression of axonal surface molecules that mediate fasciculation between thalamic fibers. Alternatively, the modulation of the fasciculation rate may reflect differences in the survival of subpopulations of thalamic neurons under *in vitro* conditions. We also observed age-dependent variations in the fasciculation rate for cortical axons. At E14, when explants are composed almost exclusively of subplate neurons, cortical axons rarely fasciculate with each other but often cross the encountered fiber. The fasciculation rate increased at E15 and reached its maximum at E16 when explants contain layer 6 neurons (D. Bagnard and J. Bolz, unpublished observations). The lack of fasciculation observed at an early stage may correspond to the behavior of pioneer fibers that explore the environment to detect the correct pathway. For these axons, fasciculation could represent a disadvantage in case of misrouting of individual
fibers. At later stages, however, fasciculation appears as an efficient mechanism to guide the next generation of fibers to the final destination.

When growth cones approached homotypic axons perpendicularly, they usually crossed the fibers without delay or any sign of retractions. In most cases, fasciculations were only observed when growth cones encountered homotypic fibers under a shallow angle, i.e., when the surface contact between the growth cone and the fiber was relatively large. Cortical and thalamic growth cones are able to extend along homotypic axons in both directions, either retrogradely towards the distal end of the axons or anterogradely towards the cell bodies, suggesting that there are no polarity cues for fasciculation on cortical and thalamic axons. The axonal surface molecules that mediate axon-axon fasciculation and that control the formation of homotypic fascicles are not known. From the results of the present experiments it is clear, however, that cortical and thalamic axons must express different surface molecules that promote the selective fasciculation of these two fiber populations.

Previous studies already indicated that axons, in addition to surface molecules that make them attractive for particular growth cones, can also express cues that have negative effects upon growth cone elongation (Kapfhammer and Raper, 1987a,b; Moorman and Hume, 1990). The present results demonstrate that after contact, cortical and thalamic growth cones collapse and retract from each other. This collapse is reversible, and after a short pause a new growth cone is formed and the axon advances again. We often observed that this retraction-advance cycle was repeated several times until axons finally changed their growth direction and thereby avoided new contacts. Retractions between cortical and thalamic fibers occurred at all developmental stages examined as well as in heterochronic co-cultures, when both fiber populations exhibited a high tendency to form homotypic fascicles. Growth cone collapse also took place when axons from defined regions of the thalamus were confronted with axons from their target area in the neocortex. In addition, cell-type-specific repellent activities were observed in the collapse assays with cortical and thalamic membrane preparations. Taken together, these results indicate that neocortical and thalamic fibers express different collapse factors which prevent the formation of heterotypic axon fascicles.

In a recent study, different carboxy cyanine dyes were used to label simultaneously thalamocortical and corticofugal axons in the developing rat brain (Molnár et al., 1998). The authors report that in some instances early thalamic fibers run in close association with axons from subplate cells, and they proposed that thalamic axons navigate along the axons of subplate cells towards their cortical target area—the ‘handshake hypothesis’ (Molnár and Blakemore, 1991). With fiber tracings in fixed brains, it is difficult to prove that axons fasciculate and actually guide other fibers to their targets. In the present in vitro experiments, we sometimes found that the trajectories of thalamic and neocortical axons were adjacent to each other. The time-lapse movies revealed, however, that also in these cases growth cones first retracted from the fibers after contact, but they then re-established their growth and advanced in close vicinity of the heterotypic fiber. The handshake hypothesis requires a very precise regulation of axon outgrowth in both time and space. We tested the existence of a regional specificity by co-culturing explants from a defined region of the thalamus and explants from their target area in the neocortex. In the absence of cell-type-specific markers it is difficult to prove that thalamic explants consist exclusively of LGN neurons. Neurons from adjacent nuclei, which normally project to other cortical areas, might also be present in these cultures. However, if mutual fasciculation between fibers from specific thalamic nuclei and cortical target regions is a robust effect which is essential for axonal targeting, one would expect to observe at least in a few cases the formation of axon fascicles. Instead, in the present experiments all axons examined from presumptive LGN and visual cortex never fasciculated, they either retracted or at best seemed to ignore each other when they met in culture situations. In contrast, using the same culture techniques as in this study, we previously found that limbic thalamic axons do form fascicles with limbic cortical axons. Antibody perturbation experiments indicated that fasciculation was mediated by the limbic system-associated membrane protein (LAMP), which is expressed on both limbic thalamic and limbic cortical axons (Mann et al., 1998). Thus axon fasciculation might contribute to the establishment of axonal connections between limbic thalamic nuclei and the limbic cortex, whereas the formation of reciprocal projections between non-limbic thalamus and neocortical areas appears to be controlled by other axonal guidance mechanisms.

Recent tract tracing studies in mice with mutations of the transcription factors Gbx-2 or Thr-1 indicated that these animals lack contacts between thalamus and neocortex, and that both thalamocortical and corticothalamic fibers stop their growth in the internal capsule (Miyashita-Lin et al., 1999; Rubenstein et al., 1999). One interpretation of these findings is that direct contacts between thalamic and cortical axons are required for the establishment of connections between thalamus and neocortex. Alternatively, mutations in these transcription factors might lead to changes in the expression patterns of downstream genes that cause abnormalities in different brain regions, including the internal capsule, that prevent thalamic and cortical fibers to traverse this structure. Moreover, in COUP-TFI mutants thalamic projections to the internal capsule are normal, but very few thalamic fibers are able to project out of the internal capsule to reach the cortex (Zhou et al., 1999). Subplate cells in COUP-TFI mutants, however, project normally towards the thalamus at the time when thalamic axons fail to exit the internal capsule. It has therefore been concluded that the subplate scaffold alone is not sufficient to guide thalamic axons to their final cortical target (Zhou et al., 1999).

In the developing neocortical system, the pathway of thalamocortical fibers is centered in the subplate zone and upper region of the intermediate zone, whereas corticofugal axons grow deep in the intermediate zone. The present results suggest that homotypic axon fasciculation in combination with heterotypic fiber repulsion leads to segregated tracts for thalamic and cortical axons. However, our experiments do not explain the preference of fibers for a specific compartment within the white matter. Previous studies provided evidence for molecular heterogeneity in the future white matter that, for example, selectively promotes the growth of thalamic axons in the subplate and upper intermediate zone where these fibers grow toward neocortical target areas (Bicknese et al., 1994; Tuttle et al., 1995; Henke-Fahle et al., 1996). On the other hand, Sema3C, which has been shown to attract cortical axons (Bagnard et al., 1998), is expressed deep in the intermediate zone and in the subventricular zone, the pathway of corticofugal axons. Thus, the differential distribution of attractive and repulsive axonal guidance molecules in combination with fiber-fiber interactions might explain the separation of afferent and efferent fiber tracts.
in different zones in the white matter and how these locations are selected. Because the present experiments were done under in vitro conditions, an obvious question is whether thalamic and cortical axons would exhibit similar reactions when they meet each other in vitro. As outlined above, we used explants from defined cortical and thalamic regions, prepared cultures at different developmental stages and also examined heterochronic co-cultures. All these different in vitro situations led to the same results, homotypic fasciculation and heterotypic axon repulsion. One major difference between in vitro assays for axonal targeting and the in vitro situation is that axons are exposed to different environments and grow on different substrates. This seems to be particularly important in the present context, because it has been demonstrated recently that extracellular matrix components which promote axon outgrowth can also modify the response of growth cones to axonal guidance cues (Hopker et al., 1999). This raises the possibility that the nature of the substrate might also influence interactions between growing axons. This is why we tested several combinations of substrates which are more or less growth permissive for axonal growth. Results indicated that whatever substrate was used, and despite a >6-fold variation of the growth speed on different substrates, the rate of homotypic fasciculation and heterotypic contact repulsion did not change significantly. However, the presence of the repulsive guidance molecule Sema3A, which is expressed in vitro along the thalamocortical pathway, not only increased the rate of homotypic fasciculations but also amplified the segregation between cortical and thalamic axons. As previously suggested, one possible mechanism underlying Sema3A-induced axon fascilitation is that this molecule makes the substrate less permissive for fiber growth and therefore forces fibers to extend on axonal surfaces (Kolodkin et al., 1992, 1993; Matthes et al., 1995). The present findings reveal, however, that Sema3A can also influence axon repulsion. The higher rate of retractions between cortical and thalamic fibers in the presence of Sema3A might be explained by an increase of the sensitivity of growth cones to different collapse factors expressed on the surfaces of cortical and thalamic axons. It is tempting to speculate that this mechanism is related to the modulation of intracellular levels of cyclic nucleotides (cGMP and cAMP), which have been implicated as key regulators of axon repulsion (Song et al., 1999). If collapsing factors on axonal surfaces share the final transduction pathway with Sema3A, a reduction of intracellular cGMP levels might push the internal state of growth cones towards the collapse threshold and thereby might favor the induction of collapses during axonal contacts. Our results therefore provide evidence that a guidance molecule not only defines axonal pathways, but also actively influences the reactivity of growth cones to their environment.

Notes
We thank Marion Lohrum for establishment of semaphorin secreting cell lines. This work was supported by the Human Frontiers Science Program (J.B.) and the DFG (A.W.P.).

Address correspondence to Dr Dominique Bagnard, LNDR—Centre de Neurochimie du CNRS, 5 Rue Blaise Pascal, F-67084 Strasbourg cedex, France. Email: bagnard@neurochem.u-strasbg.fr

References


Mann F, Zhukareva V, Pimenta A, Levitt P, Bolz J (1998) Membrane-


