Semaphorins act as attractive and repulsive guidance signals during the development of cortical projections

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SUMMARY

Members of the semaphorin family have been implicated in mediating axonal guidance in the nervous system by their ability to collapse growth cones and to function as chemorepellents. The present findings show that recombinant Semaphorin D has similar effects on cortical axons and, in addition, inhibits axonal branching. In contrast, semaphorin E acts as an attractive guidance signal for cortical axons. Attractive effects were only observed when growth cones encountered increasing concentrations or a patterned distribution of Semaphorin E, but not when they were exposed to uniform concentrations of this molecule. Specific binding sites for Semaphorin D and Semaphorin E were present on cortical fibers both in vitro and in vivo at the time when corticofugal projections are established. In situ hybridization analysis revealed that the population of cortical neurons used in our experiments express neuropilin-1 and neuropilin-2, which are essential components of receptors for the class III semaphorins. Moreover, semD mRNA was detected in the ventricular zone of the neocortex whereas semE mRNA was restricted to the subventricular zone. Taken together, these results indicate that semaphorins are bifunctional molecules whose effects depend on their spatial distribution. The coordinated expression of different semaphorins, together with their specific activities on cortical axons, suggests that multiple guidance signals contribute to the formation of precise corticofugal pathways.

Key words: Semaphorins, Cortex, Wiring molecules, Axonal guidance

INTRODUCTION

Over the past few years significant progress has been made in understanding the molecular basis of axonal guidance in vertebrates by the identification of several families of molecules that can act as substratum-bound, short-range or long-range diffusible signals (for reviews, see Tessier-Lavigne and Goodman, 1996; Varela-Echavarria and Guthrie, 1997). The purification of a chemoattractant for commissural axons of the spinal cord led to the cloning of members of the netrin family, which can also act as chemorepellents for trochlear motoneurons (Kennedy et al., 1994; Serafini et al., 1994; Colamarino and Tessier-Lavigne, 1995). Members of the Eph receptor tyrosine kinase family and their ligands have been implicated in the formation of topographic projections in the retinotectal (Cheng et al., 1995; Drescher et al., 1995) and hippocamposeptal system (Gao et al., 1996) and the formation of local cortical circuits (Castellani et al., 1998). The largest family of signaling molecules implicated in axonal guidance are the semaphorins. They are characterized by the presence of a conserved semaphorin domain of 500 amino acids that is found in both invertebrate and vertebrate proteins (for reviews, see Kolodkin, 1996; Tessier-Lavigne and Goodman, 1996; Mark et al., 1997). The vertebrate semaphorins can be subdivided into at least four distinct classes, which contain both secreted and membrane-bound proteins (Adams et al., 1996; Zhou et al., 1997). The secreted class III semaphorins Semaphorin A, Semaphorin D (SemD) and Semaphorin E (SemE) are synthesized as pro-proteins whose activity is regulated by proteolytic processing and have repulsive effects on sympathetic axons (Adams et al., 1997). Exposure of sensory neurites to SemD or its chick homologue collapsin-1 induces a rapid but reversible collapse of their growth cones (Kapfhammer and Raper, 1987a,b; Luo et al., 1993). In contrast, a limited contact of growth cones with immobilized collapsin can redirect the trajectories of growing axons (Fan and Raper, 1995). These effects are mediated, at least in part, by neuropilin-1 (npn-1) and neuropilin-2 (npn-2) which bind the secreted class III semaphorins with high affinity (Kolodkin et al., 1997; He and Tessier-Lavigne, 1997; Feiner et al., 1997; Chen et al., 1997). Homozygous mice in which the npn-1 gene has been disrupted by targeted deletion (npn-1−/− mice) exhibit
a phenotype that is very similar to that of semaIII/D−/− mice (Kitsukawa et al., 1997) supporting the idea that npn-1 is an essential component of the SemD receptor.

The repulsive properties of semaphorins suggest that they represent guidance signals that prevent axons from entering inappropriate tissues. SemD/SemaIII has been implicated in the guidance of sensory neurons in the spinal cord (Messersmith et al., 1995; Püschel et al., 1995, 1996; Behar et al., 1996) and the innervation of peripheral targets by sensory axons (Kitsukawa et al., 1997; Taniguchi et al., 1997). Mutation of the semaIII/D−/− gene results in severe defects in the peripheral nervous system with a large number of aberrant projections (Taniguchi et al., 1997). The phenotype of semaIII/D−/− mice is consistent with the hypothesis that one function of the semaphorins is to restrict the choice of pathways available to axons. Alternative explanations are that semaphorins regulate the motility of growth cones or act as chemotropic signals that steer axons by providing directional information as gradients.

The semaphorins display developmentally regulated expression patterns not only in the spinal cord but also in the brain which suggests an involvement of semaphorins in guiding cortical axons (Giger et al., 1996, 1998; Püschel et al., 1996; Skalaia et al., 1998). In the developing cerebral cortex, many neurons send an axon along stereotyped pathways and establish precise connections with remote regions of the brain. For example, all subcortically projecting axons first grow laterally towards the internal capsule, the gateway between cortical and subcortical structures, and thereafter turn caudally in order to reach their target. Other populations of cortical neurons send their axons towards the dorsal midline across the corpus callosum to innervate the contralateral cortex. In addition, collaterals of all efferent axons establish precise local connections within the different cortical layers. Recent in vitro studies provided evidence for the existence of both diffusible and membrane-associated guidance factors that might assist cortical axons in establishing local cortical circuits and in navigating toward distant targets (Bolz et al., 1990; Heffner et al., 1990; Novak and Bolz, 1993; Bolz and Castellani, 1997; Castellani and Bolz, 1997; Ménét et al., 1997; Richards et al., 1997; Castellani et al., 1998). However, little is known about the molecular nature of the signals that control the directed growth of cortical axons and which regulate target recognition and synapse formation. Here we show that the semaphorins SemD and SemE have opposite effects on cortical axons in vitro. Whereas SemD is a chemoattractive signal for cortical axons, SemE acts as a chemorepulsive molecule. Using probes to detect semd, semE, npn-1 and npn-2 mRNAs and binding sites for SemD and SemE in situ, we could show that these semaphorins and their receptors are present in the developing brain at the time when cortical projections are established in vivo.

MATERIALS AND METHODS

Generation of cell lines
Human embryonic kidney 293 cells (ATCC CRL 1573) were transfected with expression vectors for either SemD or SemE (pBKSemD-AP, pBKSemE-AP, pBKFlagSemD1b and pBKFlagSemE1b; Adams et al., 1997) by calcium phosphate coprecipitation (Sambrook et al., 1989) and stable cell lines selected in 1 mg/ml G418 (GIBCO-BRL). Expression levels of the recombinant fusion proteins were determined with the Phospholight chemiluminescence assay (Tropix) or by western blot analysis using the anti-Flag M2 antibody (Kodak) as described previously (Adams et al., 1997). The cell lines (D3.5, E3.2, E1.5, D602.77, E626.38) with the highest expression level were chosen for the experiments. Consistent with previous results (Luo et al., 1993), western blots of membranes preparations from lines D and E confirmed that they contained comparable amounts of recombinant proteins. Independent cell lines expressing SemD or SemE had qualitatively similar effects on cortical axons and lines D3.5 and E3.2 were chosen to quantify these responses.

Coculture assay
Human 293 cells were cultured in MEM with 5000 u/ml Penicillin, 5 mg/ml Streptomycin, 200 mM L-glutamine, 10% FCS and 1 mg/ml G418 (Gibco-BRL). Aggregates of cells stably expressing recombinant semaphorins were formed as described previously (Püschel et al., 1995). E16 rat embryos were obtained from Cesarean section of pregnant Lewis rats (the day of sperm detection was designated as E1). Brains were removed and dissected in cold Gey’s balanced salt solution supplemented with glucose (6.5 mg/ml). Blocks of neocortex were then placed on a McIlwain tissue chopper and cut in cubes of 200 μm³. Explants were maintained in 2 ml coculture medium consisting of 50% Eagle’s basal medium, 25% Hank’s balanced salt solution and 25% horse serum supplemented with 0.1 mM glutamine and 6.5 mg/ml glucose (all from Gibco). The same procedure was used to prepare explants for all assays. Aggregates and cortical explants were placed in 20 μl chicken plasma on a glass coverslips. During coagulation of the clot with 20 μl thrombin, explants were arranged around aggregates at 100-900 μm distances. After 30-45 minutes, coverslips were placed in 30 mm dishes containing 2 ml of culture medium and transferred to the incubator (37°C, 5% CO₂). The plasma clot provides a good substrate for growing cortical axons and at the same time appears to stabilize diffusible molecules released by cell aggregates (Bolz et al., 1990). After 48 hours in vitro, outgrowth was sufficient and cultures were fixed in 4% paraformaldehyde (3% sucrose) and then analyzed. In order to quantify effects of semaphorins, we determined number of axons by counting individual fibers. Using a grid ocular and a ×20 objective, we measured the length of clearly identifiable fibers. For each explant, a minimum of 10 axons was used to determine mean axonal length both on the side of the explant facing the aggregates and on the side distal to the aggregates.

Collapse assay
Cortical explants were cultured on glass coverslip coated with laminin (1 mg/ml) / poly-L-lysine (1 mg/ml) in GBSS. After 2 days in vitro, axons emerged radially from the explants and it was possible to analyze individual fibers and their growth cones. Aliquots of conditioned media (400 μl) were added to the cultures and growth cone morphology was examined under live conditions 4 hours after incubation in a microscope chamber at 37°C and 5% CO₂. Neurite tips without lamellipodia or filopodia were scored as ‘collapsed’ (Raper and Kapfhammer, 1990).

Membrane preparation
Cortical membranes were prepared as described before (Götz et al., 1992). Briefly, blocks of cortex prepared in GBSS/glucose from postnatal animals (P0-P4) were placed in a homogenization buffer (10 mM Tris-HCl, 1.5 mM CaCl₂, 1 mM spermidine, 25 μg/ml aprotinin, 25 μg/ml leupeptin [all from Serva] and 15 μg/ml 2,3-dehydro-2-desoxy-N-acetylneuraminic acid [Sigma]), pH 7.4. The homogenate was centrifuged for 10 minutes at 50000 g in a sucrose step gradient (upper phase 150 μl 5% sucrose, lower phase 500 μl 50% sucrose). The interband containing the membranes fraction was washed twice in PBS without Ca²⁺ and Mg²⁺ at 14000 revs/minute in an Eppendorf biofuge. After resuspension, the concentration of the purified membranes was determined by its optical density at 220 nm with a
spectrophotometer. To prepare membranes from 293 cells, medium from confluent dishes were removed and cells were washed in 1 ml PBS before cell scrapping. Cells were resuspended in the same homogenization buffer and processed as cortical tissue.

**Preparation of membrane substrata**

To prepare uniform membrane carpets, pairs of laminin/poly-L-lysine coated glass coverslips were incubated as a ‘sandwich’ with 100 μl of membrane suspension (50% postnatal membranes, 50% cell membranes; optical density at 220 nm = 0.1 after 15-fold dilution in 2% SDS) for 3 hours at 37°C. After separation, the coverslips were placed in a Petriperm dish and covered with 750 μl of culture medium. Membrane stripes were prepared according to the technique of Walter et al. (1987). Tissue was explanted on coverslips and culture medium was adjusted to 2 ml. Cultures were kept for 2 days at 37°C under 5% CO2 in air atmosphere, and then fixed with PFA 4% and 3% sucrose for microscopic analysis. Number and length of axons extending from the explants were determined using the grid ocular of a ×20 objective. Axonal branching was quantified using a ×40 phase-contrast objective in combination with additional lenses (×1.6 Optovar). The number of side branches was determined along individual axons excluding crossing fibers and fascicles. Axonal branching was defined as the ratio between the total number of axonal branches and the total segment length analyzed. Axon branching was normalized to 100% in control condition. Statistical analysis was performed using a permutation test.

**Receptor affinity probes**

The coding sequence of the secreted alkaline phosphatase (AP, Flanagan and Leder, 1990) was fused to the amino terminus of SemDP1b or SemEP1b (Adams et al., 1997) by PCR to create the vectors BKAP-SemDP1b and BKAP-SemEP1b (detailed protocols and primer sequences are available upon request from A. W. P.). 293 cells were transfected with these expression vectors and conditioned media were concentrated after 3 days of culture as described previously (Adams et al., 1997). In order to characterize binding sites of semaphorins in the developing cortex, mouse embryos were sectioned on a cryostat and fixed with methanol at −80°C for 5 minutes. After rehydrating the sections in PBS, they were equilibrated in Hanks Balanced Salt Solution (HBSS) without Ca2+/Mg2+ for 5 minutes and incubated in HBSS supplemented with 20% FCS for 2 hours. The sections were then overlaid with concentrated conditioned medium containing the recombinant protein diluted in HBSS + 20% FCS for 90 minutes. After one wash with HBSS and three washes with TBS (20 mM Tris-HCl, 135 mM NaCl, pH 7.5) for 5 minutes each, they were equilibrated with PBS for 5 minutes and fixed in 3.7% formaldehyde in PBS for 5 minutes. After one wash with PBS, endogenous phosphatases were heat-inactivated at 65°C for 50 minutes. After equilibrating with AP-Buffer (100 mM Tris, 100 mM NaCl, 5 mM MgCl2, pH 9.5), bound AP fusion proteins were visualized with a staining solution containing 34 mg/ml Nitro-blue-tetrazolium and 18 mg/ml 5-bromo-4-chloro-3-indolylphosphate (Boehringer Mannheim) in AP buffer.

Using the same probes, binding experiments were performed on cortical explants after 2 days in cultures. Explants were overlaid with culture medium collected from untransfected cell cultures (Control), AP-SemD or AP-SemE-producing 293 cells for 90 minutes after three washes in PBS. Cultures were fixed in 4% paraformaldehyde for 5 minutes and binding sites visualized as described above for sections.

**In situ hybridization**

NMRI mice were purchased from Harlan-Winkelmann Ltd (Borchern, FRG). The day of detection of the vaginal plug was designated as E0. The day of detection of the vaginal plug was designated the day of 8.5% of the number of cells that secreted SemD or SemE. Aggregates of untransfected 293 cells cultured cortical explants next to aggregates of 293 cells that secreted SemD or SemE. Aggregates of untransfected 293 cells served as control. 293 cells were plated in a Petriperm dish and covered with 750 μl of culture medium.

**RESULTS**

**Soluble SemD induces growth cone collapse of cortical axons**

Previous studies have established that soluble SemD induces the collapse of sensory growth cones, consistent with its possible role in guiding sensory afferents in the spinal cord (Luo et al., 1993). Analyses of the distribution of semaphorin transcripts during the development of rodents revealed complex and highly dynamic expression patterns in the embryonic brain suggestive of a role in guiding cortical axons (Adams et al., 1996; Püschel et al., 1996; Skaliora et al., 1998). This led us to test the effect of diffusible recombinant semaphorins on the morphology of cortical growth cones. As depicted in Table 1, addition of SemD led to a significant increase in the number of collapsed growth cones compared to control medium (P<0.001, n=526 growth cones examined, χ² test). In contrast, SemE did not lead to an increased incidence of growth cone collapse above background level (P>0.05, n=297 growth cones examined, χ² test).

**Secreted Semaphorins orient cortical axons**

SemD and SemE are members of the class III semaphorins of secreted proteins. Earlier work has shown that sensory neurites extending from dorsal root ganglia (DRGs) were repelled by co-cultured cell aggregates secreting SemD (Mersersmith et al., 1995; Püschel et al., 1995). To test whether secreted semaphorins also influence the growth of cortical axons, we cultured cortical explants next to aggregates of 293 cells that secreted SemD or SemE. Aggregates of untransfected 293 cells served as control. As illustrated in Fig. 1, axons extending from SemD-secreting cell aggregates were significantly shorter than those growing away from the aggregates (Fig. 1A). Comparing the number and length of axons extending toward SemD-secreting cell aggregates to those extending away from the aggregates showed that the repulsion by SemD was highly significant (P<0.001 for length, P<0.005 for number, n=51 explants, Student’s t-test; see Fig. 1D,E). The opposite effect was observed with SemE-expressing cells: axons were more numerous and they were considerably longer on the side of the explant directed towards SemE-secreting cells than those growing away from them (44% increase in number, P<0.001, 31% increase in length, P<0.001; Student’s t-test; Fig. 1C-E). The decrease in the number of axonal processes and the length of cortical axons emerging from the explants on the side facing

**Table 1. Quantitative analysis of the collapse assay**

<table>
<thead>
<tr>
<th>Cultures conditions</th>
<th>Normal</th>
<th>Collapsed</th>
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<tbody>
<tr>
<td>Sem D</td>
<td>45.1% (n=237)</td>
<td>54.9% (n=289)*</td>
</tr>
<tr>
<td>Control</td>
<td>80.3% (n=387)</td>
<td>19.7% (n=95)</td>
</tr>
<tr>
<td>Sem E</td>
<td>76.1% (n=226)</td>
<td>23.9% (n=71)*</td>
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Percentage (and absolute number) of growth cones with ‘normal’ and with ‘collapsed’ morphology after the addition of conditioned medium from SemD, SemE and control 293 cells. After addition of SemD medium, the number of collapsed growth cones is higher than under control conditions (P<0.05, χ² test) (*), SemE has no significant effect (ns).
SemD-producing 293 cells could be due to a localized growth-inhibiting effect of SemD. Likewise, the effects observed with SemE could reflect a local growth-promoting effect on cortical axons. Alternatively, semaphorins could confer a directional information to these fibers. To address whether semaphorins can exert a tropic influence on growing cortical fibers, we analyzed the trajectories of individual axons emerging parallel to the cell aggregates in several explants. Most fibers emerging parallel to control cell aggregates grew straight, 39 out of 42 axons examined deviated by less than 10° from a linear course. However, 40 out of 43 axons emerging parallel to SemD-secreting cell aggregates and 35 out of 41 axons emerging parallel to SemE-secreting cells deviated significantly from a straight course by more than 10°. As illustrated in Fig. 2, in the case of SemD, axons were deflected away and for SemE, axons were deflected towards the semaphorin-secreting source. Thus, cortical axons exhibit a tropic response to SemD and SemE, with SemD acting as a chemorepulsive and SemE as a chemoattractive signal.

Substratum-bound semaphorins affect cortical axons outgrowth

Collapsin/SemD was originally isolated from brain membranes, suggesting that semaphorins bind very tightly to...
the cell surface and/or extracellular matrix (ECM). The structure of secreted semaphorins indicates that they have a carboxy terminal domain, which is rich in basic amino acids, which might mediate the binding of semaphorins to various membranes or ECM components. Therefore, depending on the composition and spatial arrangement of the extracellular environment, secreted semaphorins might not be distributed uniformly and, instead, may form gradients or sharp boundaries in vivo. This prompted us to test the growth behavior of cortical axons on substratum-bound SemD and SemE, with the semaphorins either presented as a homogeneous or as a patterned substratum. To this end, we prepared membranes from SemD- and SemE-producing cells and supplemented these membranes with cortical membranes. Previous work has indicated that cortical membrane preparations contain unknown growth-promoting molecules for cortical axons (Götz et al., 1992; Henke-Fahle et al., 1996) and, in the absence of these additional factors, outgrowth of cortical axons on membranes from untransfected 293 cells was rather poor. Thus it was possible to evaluate the effects of SemD and SemE in a more native and complex molecular context, as present in the cortical membranes.

When cortical explants were placed on a substratum consisting of a 1:1 mixture of cortical membranes with control 293 cell membranes, they extended numerous axons, which reached an average length of 637±8.5 μm (n=65 explants) after 2 days in vitro. Using membranes from SemD-producing cells instead of control membranes, the number of axons decreased by 33% and axonal length by 28% (P<0.0001 for number, P<0.0001 for length, n=74 explants, Student’s t-test; see Fig. 3A,B). SemD also influenced the formation of collaterals from cortical axons. On substrata containing membranes from SemD-expressing cells, cortical fibers exhibited only about half as many branches as on substrata containing membranes from control or SemE-expressing cells (P<0.001, permutation test; Fig. 3C). Membranes from SemE-secreting cells led to an increased amount of axon outgrowth, but unexpectedly at the same time also led to a reduction in axonal length. Thus, when growing on a substratum containing a uniform concentration of SemD, outgrowth, axonal length and branching were all reduced. In contrast, a uniform distribution of SemE has no influence on the branching of cortical axons, but it increases the outgrowth from cortical explants and at the same time decreases axonal length (see Fig. 3A-C).

Spatial distribution of semaphorins influences axonal trajectories

To examine whether the trajectories of cortical fibers can be oriented by the spatially restricted presentation of semaphorins, we used the ‘stripe assay’ developed by Bonhoeffer and colleagues (Walter et al., 1987). We first assessed the growth pattern on equidistant membrane stripes on laminin/poly-L-lysine-coated glass coverslips. When the membranes were prepared from postnatal cortex, axons grew preferentially on the membrane stripes and avoided lanes containing laminin/poly-L-lysine alone (data not shown). This is consistent with previous observations that axons can be guided by growth-promoting components in cortical membrane preparations (Götz et al., 1992; Hübener et al., 1995; Tuttle et al., 1995). However, when membranes from SemD-expressing 293 cells were added to the cortical membranes, cortical axons avoided the membrane stripes and extended preferentially on the laminin/poly-L-lysine stripes (Fig. 4B). In contrast, addition of control membranes (Fig. 4A) or membranes from SemE-expressing cells (Fig. 4C) did not interfere with the preference of cortical axons for the membrane stripes. These experiments show that SemD can convert a permissive substratum into one that is repellent for cortical axons, thereby preventing these fibers entering specific regions delineated by this molecule.

If SemE is acting as a chemoattractant for cortical axons, as indicated by our co-culture experiments, then the stripe assay described above is not suitable to test for an attractive guidance function of SemE, because cortical fibers already show a clear preference for cortical membranes alone. We therefore tested the growth pattern of cortical axons on alternating membrane stripes, one set of stripes contained a 1:1 mixture of cortical membranes with membranes from untransfected 293 cells, the other a 1:1 mixture of cortical membranes with membranes...
from either SemD or SemE transfected 293 cells. As illustrated in Fig. 4D, in this test situation 78% of the cortical fibers preferentially grew on membranes containing SemE and only 22% on the control membranes (P<0.001). The converse was true with SemD: 82% of the cortical axons avoided the membrane stripes containing SemD and rather grew on stripes containing membranes from untransfected 293 cells (P<0.001). Thus SemD and SemE have opposing effects on cortical axons, with SemD being a repulsive and SemE being an attractive guidance signal (Fig. 4E).

**Binding and expression patterns of SemD and SemE in the developing cortex**

In order to examine the distributions of cells expressing semD and semE...
and semE and of binding sites for the encoded semaphorins in the developing cortex, we used in situ hybridization and receptor affinity probes consisting of fusion proteins of SemD and SemE with the secreted alkaline phosphatase (Flanagan and Leder, 1990). We examined sections from E14.5 mouse brains, the stage when corticofugal axons start to grow towards the internal capsule, and from E16.5 brains, when cortical fibers passed through the internal capsule to extend towards subcortical targets (De Carlos and O’Leary, 1992). At E14.5 semD and semE transcripts were found in a complementary lateromedial gradients in the ventricular zones (semD) and the subventricular zone (semE), respectively (Fig. 5A,F). Binding sites for both AP-SemD and AP-SemE were predominantly detected in the intermediate zone (Fig. 5B,G) where also npn-1 and npn-2 mRNAs were found (Fig. 5C,H). Binding sites corresponded to a large extent, but not completely, to sites of npn-1/-2 expression. In accordance with several previous studies (Adams et al., 1996; Giger et al., 1996; Püschel et al., 1996; Skaliora et al., 1998), semD mRNA was predominantly expressed in the ventricular and subventricular zones (Fig. 5A,E) while semE transcripts were restricted to the subventricular zone (Fig. 5F,J) in the E16.5 neocortex. The AP-SemD and AP-SemE fusion proteins detected binding sites for both proteins predominantly in the intermediate zone, complementary to the distribution of their transcripts (Fig. 5B,D,G,I). In addition, fiber bundles passing through the internal capsule were clearly stained with both AP-SemD and AP-SemE (Fig. 6A,B). AP-SemD and AP-SemE detected specific binding sites as these could be competed by the addition of untagged SemD and SemE, respectively, and no staining was observed when AP was used alone (data not shown). Thus, cortical axons in vivo exhibit binding sites for SemD and SemE at the time when cortical projections are established.

Binding sites were also detected on axons extended by explanted neocortical neurons which were used for our in vitro experiments. All fibers extending from cortical explants were heavily labeled with both, AP-SemD and AP-SemE probes (Fig. 7). These results suggest that all cortical axons bind

Fig. 6. Fibers in the internal capsule express semaphorin-binding sites. Frontal sections of a E14.5 mouse brain were incubated with receptor affinity probes for SemD (A) or SemE (B). Binding sites for both semaphorins were observed in the internal capsule (IC), the pathway of corticofugal axons.

Fig. 7. Cortical axons express specific binding sites for SemD and SemE in vitro. Explants were cultured for 2 days in vitro before application of culture medium from untransfected 293 cells (control A,B), AP-SemD-producing 293 cells (visualization of AP-SemD-binding sites C,D) and AP-SemE-expressing 293 cells (visualization of AP-SemE-binding sites E,F). A,C,E are phase-contrast photographs and B,D,F are the corresponding bright-field illuminations. Note that axons emerging from the explants were heavily stained by both AP-SemD and AP-SemE.

Fig. 8. Possible function of semaphorins for the specification of corticofugal pathways. (left) The distribution of semD, semE and neuropilin-expressing cells is shown schematically at the time corresponding to the initial extension of corticofugal projections. Cortical neurons (CP) express neuropilin-1 (npn-1) and neuropilin-2 (npn-2), components of the receptors for SemD and SemE respectively. The attractant SemE is produced by cells in the subventricular zone (SVZ) whereas the repulsive SemD is produced within the ventricular zone (VZ) and subventricular zone (SVZ). (right) Because semaphorins are diffusible molecules, it is assumed that two partially overlapping antagonistic gradients are formed. As a consequence, cortical axons are attracted towards the IZ by SemE whereas SemD prevents the fibers from entering the SVZ and VZ.
SemD and SemE and that these binding sites correspond to the expression of specific receptors. This notion is supported by the detection of npn-1 and npn-2 mRNA in the intermediate zone of the cortex (Fig. 5C,H) as detected by in situ hybridization and in the cortical plate in more caudal regions of the brain (data not shown). These expression patterns are similar to those reported by Chen et al. (1997).

DISCUSSION

SemD is a repellent signal for cortical axons

Collapsin, the chicken homologue of SemD, was originally identified by its ability to induce the rapid but reversible collapse of growth cones (Kapfhammer and Raper, 1987a,b; Raper and Kapfhammer, 1990; Luo et al., 1993). Using the same in vitro assay, we found that SemD also induced growth cone collapse of cortical axons. In coculture experiments, the outgrowth of cortical explants was strongly diminished on the proximal side of SemD-secreting cell aggregates compared to the outgrowth on the distal side. One possible explanation for this effect is that SemD acts as a growth suppressor for cortical axons. Analysis of the trajectories of axons extending parallel to the source of SemD indicated that these fibers were deflected away from the aggregates. Moreover, in time-lapse recordings, we observed that cortical axons extend at the same growth speed on a membrane substratum with SemD-secreting cells as on a membrane substratum from control cells, the only difference was that axons exhibited an increased frequency of growth cone collapse on the substratum containing SemD (D. B. and J. B., unpublished observations). These findings could account for the apparent reduction of axonal length and number of fibers growing towards the aggregates. Thus, in the co-culture assay, SemD might act as a growth cone collapsing factor rather than an outgrowth inhibitor and the deflection of cortical axons by gradients of secreted SemD would be the consequence of a partial collapse of their growth cones, as suggested previously by Raper and colleagues (Fan and Raper, 1995).

SemD is also capable of patterning axonal projections when presented as a substratum-bound signal. The stripe assay demonstrated that this molecule can transform a permissive substratum into a non-permissive one as has been shown previously for sensory axons (Messersmith et al., 1995; Püschel et al., 1995, 1996; Püschel, 1996). Therefore SemD might restrict the regions accessible to specific axonal populations and perform a function for cortical fibers similar to that shown for sensory axons (Taniguchi et al., 1997). On the contrary, cortical fibers extending in SemD-containing territories exhibited a strong reduction in axonal branching, similarly to the effects described for G-Semal and D-Semal in the insect nervous system (Kolodkin et al., 1992; Matthes et al., 1995). Moreover, preliminary imaging data suggested that SemD also increases the rate of fasciculation of cortical axons (Bagnard and Bolz, unpublished observations). Thus, a repulsive axonal guidance molecule can have additional functions for fibers already growing in regions expressing this signal. In the case of SemD, such functions include regulation of axonal branching and modulation of axonal fasciculation.

SemE is an attractant signal for cortical axons

Cortical axons extending towards a source secreting SemE are longer than fibers growing away from it. Moreover, cortical fibers are deflected towards SemE-producing cell aggregates and, in the stripe assay, they exhibit a clear preference for the lanes containing SemE. Chemoattraction has been proposed as an axonal guidance mechanism since the end of the last century by Ramón y Cajal (1892). However, experimental evidence for the existence of diffusible target-derived guidance signals came only in the late 1980s (Lumdsden and Davies, 1983, Tessier-Lavigne et al., 1988; Bolz et al., 1990). The identification of the molecular nature of these chemoattractant factors started with the isolation of netrin-1 and netrin-2 from chicken brains (Serafini et al., 1994). Strikingly, netrin-1 turned out to be a bifunctional molecule when it was shown that it is a chemoattractant factor for commissural axons, but also a chemorepellent for trochlear axons (Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995). Only repulsive effects have been reported so far for semaphorins (Luo et al., 1993; Messersmith et al., 1995, Püschel et al., 1995; Matthes et al., 1995; Adams et al., 1997; Varela-Echavarría et al., 1997; Yu et al., 1998) although a recent work of Wong et al. (1997) suggested that grasshopper semaphorin I has a permissive role for growth cones of the subgenual organ. Previous work by Adams et al. (1997) indicated that SemE is a repulsive signal for sympathetic axons. The data presented here demonstrate that SemE is an attractive signal for cortical neurons both as a gradient of soluble protein and when bound to the substratum. Thus, like the netrins, semaphorins exhibit a dual and cell-type-specific chemotrophic function.

Npn-2 has been suggested as an essential component of the SemE receptor (Chen et al., 1997). Two splice variants have been described that differ in their cytoplasmic tail (Chen et al., 1997). The functional significance of this observation is not clear at present, but it is tempting to speculate that it could be connected to the bifunctional activity of SemE. Alternatively, additional components of a SemE receptor or intracellular signaling mechanism could determine the response of axons towards SemE gradients as has been reported for netrin-1 (Ming et al., 1997).

Possible roles of semaphorins during cortical development

In situ hybridization confirmed that semD and semE mRNAs are detected in the cortex at the time when corticofugal projections are established. Using AP fusion proteins as probes, we found that the cortical axons in situ exhibit binding sites for both SemD and SemE at these developmental stages. The presence of binding sites is not necessarily an indicator for the activation of a signaling pathway (Koppel et al., 1997). However, our data also demonstrate that mRNA transcripts of npn-1 and npn-2, coding for components of the SemD and SemE receptor, respectively, are present in the developing cortex. The high expression of neuropilin mRNA in the intermediate zone might be located in migrating neurons and/or axons that extend through this region. Such an accumulation of receptor mRNA in axons has been observed previously in olfactory receptor neurons (Ressler et al., 1994). Taken together, our results suggest that SemD and SemE are present in the cortex and that cortical neurons express receptors for these semaphorins at the time when in vivo cortical axons project to distant targets and when in vitro they respond to these molecules in a highly specific manner.

What might be the functional role of the attractive and
repulsive guidance effects of semaphorins in the developing cortex? During the establishment of cortical connections, thalamic afferents extend in the subplate zone, whereas axons leaving the cortex travel deep in the intermediate zone (De Carlos and O’Leary, 1992). It has been suggested that efferent cortical fibers are attracted towards the internal capsule by netrin-1 (Richards et al., 1997; Métin et al., 1997). However, the identity of the factors directing the initial outgrowth of cortical axons remains unknown. Growing corticofugal axons might require attractant signals driving them deep into the intermediate zone, in combination with repulsive molecules that prevent these fibers from growing into the ventricular zone. Because SemE mRNA can be detected in subventricular zone and SemD transcripts are expressed in the ventricular zone the in vitro data presented here suggest that cortical axons are directed by a gradient of SemE into the intermediate zone until they encounter SemD, a repulsive cue that keeps them out of the ventricular and subventricular regions. As illustrated in Fig. 8, the coordinated expression of an attractant and a repellent gradient detected by cortical axons via specific receptors might then be crucial for the initial formation of a precise efferent cortical pathway. However, this model does not explain how cortical axons, once they are in the intermediate zone, find their way towards the internal capsule. The studies of Richards et al. (1997) and Métin et al. (1997) suggest that yet another guidance signal, netrin-1 released from the internal capsule, might be an important cue for cortical axons to reach this intermediate target.

According to the model outlined above, the targeting of corticofugal axons is based on the coordinated expression of multiple guidance signals along their pathway, some acting sequentially and some acting simultaneously. The balance of positive and negative signals then determines the behavior of a growth cone and the trajectory of a cortical axons results from the integration of these guidance cues as already has been suggested for the pathfinding of motor axons in Drosophila (Winberg et al., 1998). What might then be the consequence of the elimination of a single guidance molecule for the development of corticofugal pathways? In mutant mice lacking SemD, Taniguchi et al. (1997) did not report cortical abnormalities. In another independently generated semIII/D mutant, Behar et al. (1996) described an abnormal differentiation of the cortex with misoriented processes of cortical neurons. Recently, a closer examination of the phenotype of these mice revealed that many axon pathways in the CNS, including thalamocortical and corticotectal projections, developed apparently normal (Catalano et al., 1998). From the in vitro activities of semaphorins observed in the present study, one would not expect to find coarse defects on corticofugal fiber tracts in SemD-deficient animals. Experiments with the stripe assay indicated that the repulsive and attractive functions of SemD and SemE, respectively, are equally effective in guiding cortical axons. Thus, in SemD knock-out animals, the pathway of corticofugal axons might still be centered on the subventricular zone, where SemE is expressed. However, this pathway might be less strictly confined to this zone and/or corticofugal axons might not be as tightly fasciculated as in wild-type animals. Such more subtle effects have indeed been reported in mutants for the projection from the basal forebrain to the cortex (Catalano et al., 1998).

### Semaphorins are cortical wiring signals

There is now increasing evidence that, at least in the developing cortex, molecules classified as ‘axonal guidance signals’ have a wide and complex range of action. For instance, ephrin-A5, a ligand for Eph receptor kinases, acts as a ‘repulsive axonal guidance signal’ for neurons from the upper cortical layers. However, ephrin-A5 has no axonal guidance function for cells from the deep cortical layers, instead it promotes axonal branching for this population of cortical neurons (Castellani et al., 1998). Likewise, results obtained with the neurotrophic factor NT-3 indicated that this factor acts as an ‘attractive axonal guidance signal’ for cells from the deep cortical layers and as a ‘repulsive axonal guidance signal’ for cells from the upper cortical layers (Bolz and Castellani, 1997). In the present study, cortical explants were prepared from E16 rats, at a time when cells destined for the upper cortical layers are not yet generated in this species (Bayer and Altman, 1991). Thus, it remains to be determined whether neurons from the superficial layers respond in a similar way to SemD and SemE as their counterparts from the deep cortical layers. However, our results illustrate the concept that, depending on the spatial distribution, a given molecule can have very different effects on a specific population of cortical neurons. On homogenous membrane carpets prepared from SemE-producing cells, cortical axons were shorter than on membranes from control cells. Based on this observation alone one might classify SemE as a ‘repulsive’ or ‘growth inhibiting’ signal. However, our experiments with SemE gradients and stripe assays indicated that, under these conditions, SemE acts as an ‘attractive axonal guidance signal’. Thus the modulation of the spatial and temporal expression patterns of a single molecule during development or during evolution allows to specify axonal projections by either attractive or repellent mechanisms. Taken together with previous reports, the present findings indicate that, at least in the developing cortex, molecules that influence axon behavior should not be classified according to their action on a single cell population or in a single assay. Rather, as proposed previously (Bolz and Castellani, 1997), such molecules should be called collectively ‘wiring molecules’: although they can function in multiple and alternative ways, they serve as signals for assembling the intricate network of cortical connections.

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