was affinity-purified from HeLa cells with a monoclonal antibody (mAb) to ERCC3 as described by G. LeRoy *et al.* [*J. Biol. Chem.* **273**, 7134 (1998)]. TFIID was affinity-purified from HeLa cells expressing hemagglutinin-tagged TATA binding protein with an mAb to hemagglutinin as described by Q. Zhou *et al.* [*Genes. Dev.* **6**, 1964 (1992)]. RNAPII was affinity-purified from HeLa cells with an mAb to RNAPII as described in (*29*). PC4 was expressed in *E. coli* and purified as described by H. Ge *et al.* [*Cell* **78**, 513 (1994)]. $GAL4_{1-94}$ and $GAL4_{1-94}$-VP16 were expressed in *E. coli* and purified as described by R. J. Reece *et al.* [*Gene* **126**, 105 (1993)]. FACT was purified from HeLa cells up to the phosphocellulose step as described in (*26*).

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Patterning of Cortical Efferent Projections by Semaphorin-Neuropilin Interactions

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Cortical neurons communicate with various cortical and subcortical targets by way of stereotyped axon projections through the white matter. Slice overlay experiments indicate that the initial growth of cortical axons toward the white matter is regulated by a diffusible chemorepulsive signal localized near the marginal zone. Semaphorin III is a major component of this diffusible signal, and cortical neurons transduce this signal by way of the neuropilin-1 receptor. These observations indicate that semaphorin-neuropilin interactions play a critical role in the initial patterning of projections in the developing cortex.

The directed growth of cortical efferent axons toward the white matter suggests that they might be guided by extracellular signals (*1*). To detect the presence and distribution of such signals, we developed a slice overlay assay that would allow us to assess the effect of local extracellular signals on axon growth and guidance. In this assay DiI-labeled cortical neurons were plated onto slices of cortex that provided a substrate for axon growth (*2*). When embryonic day 18 (E18) neurons were plated onto E18 or postnatal day 3 (P3) cortical slices, over 85% of the labeled cells had extended axons by 3 hours after plating that could be clearly scored for length and orientation (*3*). This analysis revealed marked differences in the pattern of axon outgrowth across the cerebral wall. Whereas labeled cells plated over the intermediate zone of E18 slices and over the white matter of the P3 slices did not show oriented axon outgrowth, virtually all of the cells plated over the cortical plate had an axon that was directed toward the ventricular surface (Fig. 1, A and B) (*4*). Quantitative analysis of the axons growing over the cortical plate indicated that almost 80% were directed toward the ventricular surface (ventricle) and less than 10% were directed toward the pia (Fig. 1C). Thus, an extracellular signal locally detectable in the cortical plate is responsible for the directed outgrowth of cortical axons on cortical slices.

Although the slice overlay experiments revealed that the cortical plate contains a signal that directs axon outgrowth, they did not reveal the nature and source of the signal. To determine if this signal could act at a distance to influence the cortical axon orientation, we examined whether a second slice of cortex placed next to the white matter could affect the behavior of labeled cells plated over the white matter. Cells plated over the white matter normally do not show oriented axon growth (Fig. 2, A, F, and G). When a second slice of cortex was placed immediately adjacent to the white matter, the cells plated over the white matter extended axons directed away from the second slice (Fig. 2, A, C, and G). This result was particularly striking because cells on either side of the marginal zone of the second slice had axons directed away from the marginal zone (Fig. 2, C and D). Therefore, cortical neurons are repelled by a diffusible signal present at high concentrations near the marginal zone.

Because semaphorin III (SemaIII) and its orthologs (semaphorin D and collapsin-1) have been extensively characterized as diffusible chemorepulsive molecules (*5*), and because SemaIII is expressed in the cortical plate and marginal zone both at E18 and P3 (*6*), we examined the role of SemaIII in cortical axon guidance. If SemaIII is involved in orienting axon growth in the cortical plate, then perturbation of a putative SemaIII gradient should lead to disoriented axon growth. To test this possibility, we incubated cortical slices with 293T cell–derived recombinant SemaIII (*7*) to reduce or abolish any SemaIII gradient before plating labeled cortical neurons onto the slices. In control slices (incubated with conditioned media from untrans-

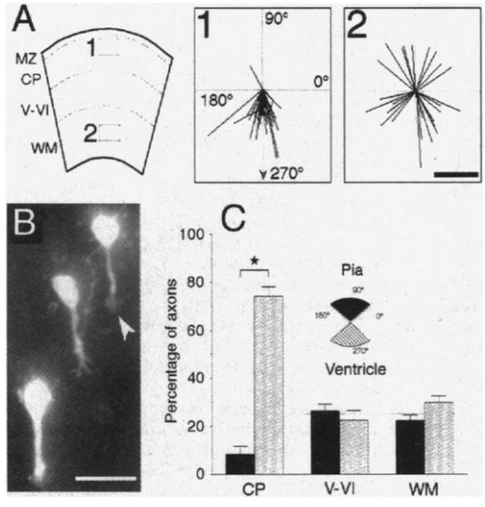

Fig. 1. A signal in the cortical plate (CP) directs axons toward the white matter (WM). (**A**) Axon orientations of E18 cortical neurons plated over the CP (box 1) or WM (box 2) of P3 cortical slices. MZ, marginal zone; V-VI, cortical layers V and VI. Bar, 25 μm. (**B**) Video image of E18 neurons growing over the CP of a P3 slice. The growth cones are directed toward the WM (arrowhead). Bar, 10 μm. (**C**) Axon orientation histograms of E18 neurons plated over P3 slices. The horizontal line in this and other histograms indicates expected distribution for random orientation. Statistically significant differences ($P < 0.001$) are indicated by an asterisk (*3*).

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fected 293T cells) axon growth in the cortical plate was clearly directed toward the ventricular surface (Fig. 3, A1 and B). In contrast, preincubation of slices with recombinant SemaIII led to disoriented axon growth (Fig. 3, A2 and B), suggesting that cortical axons normally respond to a gradient of endogenous SemaIII or a related molecule.

To determine if SemaIII could orient cortical axons, we placed aggregates of 293T cells expressing recombinant SemaIII next to the white matter of a cortical slice for 2 to 3 hours before E18 cells were plated onto the slices (*8*). In control experiments cortical axons growing near the 293T cell aggregates showed no preferred orientation (Fig. 3, C1 and D). In contrast, cortical axons growing near aggregates of SemaIII-expressing 293T cells grew away from the SemaIII source (Fig. 3, C2 and D). Thus, SemaIII can act as a diffusible chemorepellant for cortical axons.

To test directly whether endogenous SemaIII was involved in directing cortical axons, we plated cortical neurons on slices obtained from wild-type mice or mice carrying a targeted disruption of the semaphorin III gene (*semaIII*) (*9*). When E18 cortical cells were plated on slices derived from E20 wild-type mice (*10*), about 60% had axons oriented toward the ventricle and only about 10% had axons that were directed toward the pia (ratio of 6:1) (Fig. 4, A and B). In contrast, when cortical cells were plated on slices derived from *semaIII* null mice, only about 40% had axons that were directed toward the ventricle and about 20% had axons directed toward the pia (ratio of 2:1) (Fig. 4, A and B). This reduction in the extent of oriented cortical axon outgrowth indicates that endogenous SemaIII contributes to the directed growth of cortical axons.

To determine if SemaIII was required for the development of cortical axons in vivo, we labeled cortical projection neurons by placing DiA into the white matter of cortical slices obtained from E20 wild-type and *semaIII* null mice (*10*). After such injections, projection neurons in both the superficial and deep cortical

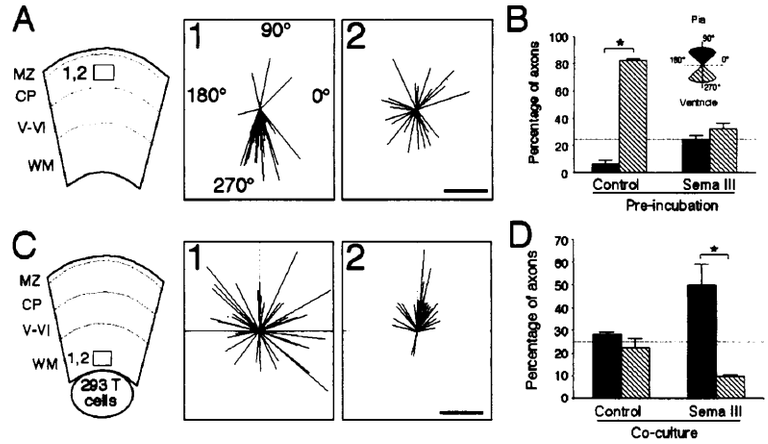

Fig. 3. Cortical neurons are responsive to SemaIII. (**A**) Axon orientations of E18 neurons plated over the CP of P3 slices after control (box 1) or SemaIII (box 2) preincubation of the slice. (**B**) Axon orientation histograms for experiment shown in (A). (**C**) Axon orientations of E18 neurons plated over the WM of P3 slices placed next to control (box 1) or SemaIII-expressing (box 2) 293T cells. (**D**) Axon orientation histograms for experiment shown in (C). Bar, 40 μm.

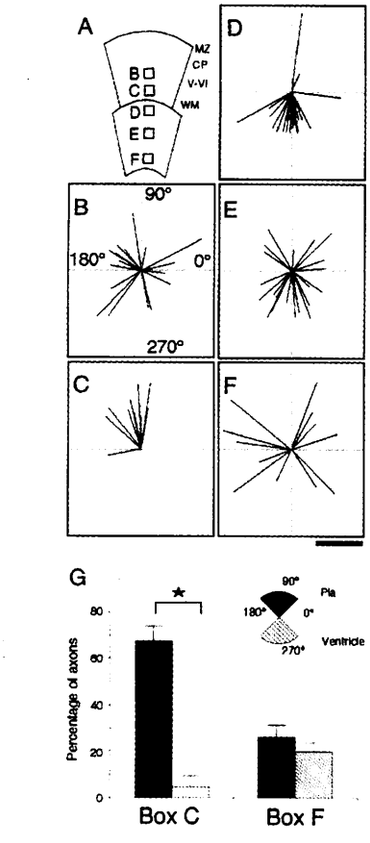

Fig. 2. Cortical axons are repelled by a diffusible signal present near the MZ. (**A**) Configuration of P3 slice coculture to determine the nature of the cortical axon guidance signal. (**B** to **F**) Axon orientations of E18 neurons plated at various locations indicated in (A). Bar, 40 μm. (**G**) Axon orientation histograms for indicated locations.

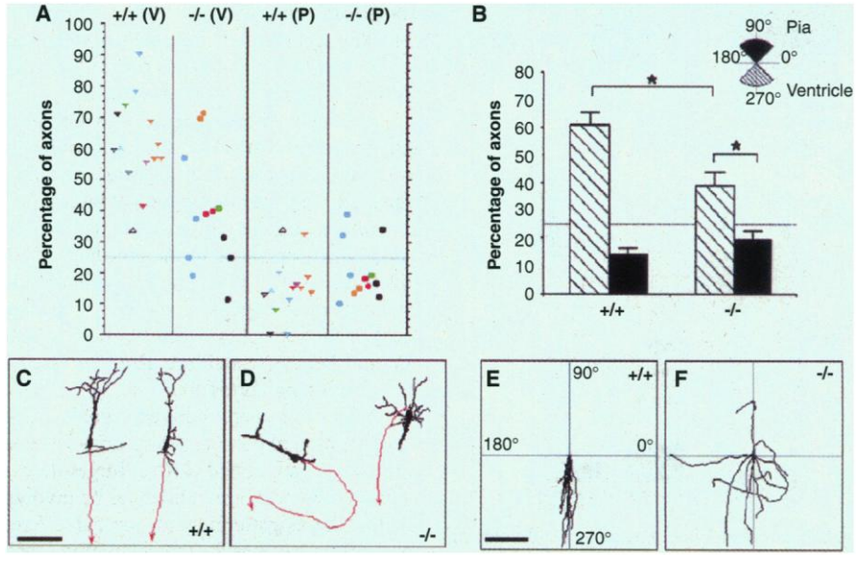

Fig. 4. Endogenous SemaIII contributes to cortical axon guidance. (**A**) Axon orientations of E18 rat cortical neurons plated over the CP of slices obtained from E20 wild-type (+/+) and *semaIII* null (−/−) mice. Each point represents the percentage of axons that are oriented toward the ventricle (V) or the pia (P) in a given slice, and slices obtained from the same animal are indicated by the same color or symbol. Data points are spread in the *x* axis to allow visualization of individual points. (**B**) Axon orientation histograms for experiment shown in (A). (**C** and **D**) Examples of cortical neuron morphologies in layers V-VI of (+/+) or (−/−) mice labeled by white matter DiA injections. The pia is to the top; axons are indicated in red. (**E** and **F**) Summary of the initial axon trajectory of cortical projection neurons in E20 (+/+) or (−/−) mice. Bar, 100 μm.

layers become retrogradely labeled via their efferent axons. Whereas retrogradely labeled neurons in wild-type mice had typical pyramidal morphologies and an axon directed toward the white matter, about 50% of the neurons in *semaIII* null mice had abnormal morphologies (Fig. 4, C and D). Many of the labeled neurons in *semaIII* null mice had aberrantly oriented apical dendrites and axons (Fig. 4, C to F). Thus, SemaIII is required for proper morphological development of cortical neurons and is specifically required for proper orientation of cortical axons in vivo.

It has recently been shown that neuropilin-1 (*11*) is a component of the SemaIII receptor (*12, 13*). Immunohistochemical localization studies indicated that the neuropilin-1 protein was present in the developing cortical plate and the intermediate zone (IZ)–subventricular zone (SVZ) border where the subcortically projecting axons travel (Fig. 5A) (*1, 11*). Binding of an alkaline phosphatase–SemaIII (AP-SemaIII) fusion protein to slices of cortex was also restricted to these zones (Fig. 5B) (*14*). Consistent with this pattern of labeling, neuropilin-1 immunofluorescence was associated with the axon shaft and growth cones of dissociated cortical neurons (*4*). To determine whether neuropilin-1 mediates the directed growth of cortical axons toward the white matter, we preincubated dissociated cortical neurons with antibodies to neuropilin-1 (anti–neuropilin-1) or control antibodies before plating them over cortical slices (*15*). When cortical neurons were preincubated with a nonimmune serum, the cells showed clearly oriented axon outgrowth in the cortical plate (Fig. 5, C and F). In contrast, preincubation with anti–neuropilin-1 completely disrupted directed growth of cortical axons toward the white matter without affecting axon length (*16*) (Fig. 5, D and F). Anti–neuropilin-1 preadsorbed with recombinant neuropilin-1 was no longer effective in disorienting the directed growth of axons within the cortical plate (Fig. 5, E and F), indicating that the effect of anti–neuropilin-1 on directed axon outgrowth was due to its ability to bind and inhibit neuropilin-1 function.

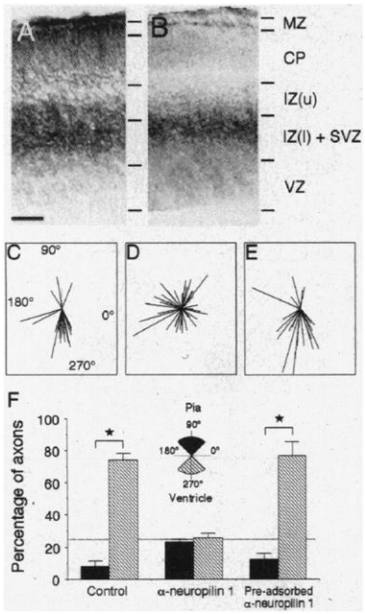

Fig. 5. Neuropilin-1 is expressed in the developing cortex and is required for oriented axon outgrowth. (**A** and **B**) Immunocytochemistry for neuropilin-1 (A) and AP–Sema-III binding activity (B) in E18 rat cortex (*14*). Bar, 100 μm. (**C** to **E**) Axon orientations of E18 neurons incubated with nonimmune serum (C), anti–neuropilin-1 serum (D), or anti–neuropilin-1 preadsorbed with recombinant neuropilin-1 (E), plated over the CP of P3 slices. (**F**) Axon orientation histograms for experiment shown in (C) to (E).

These experiments provide significant insight into the mechanisms that direct growth of nascent cortical axons toward the white matter. The slice overlay experiments indicate that although the entire thickness of the developing cerebral wall is growth permissive, local cues present in the cortical plate direct cortical axons toward the white matter. The slice coculture experiments indicate that the extracellular ligand that directs this growth is a chemorepellant that is present at high levels near the marginal zone. The recombinant SemaIII experiments indicate that SemaIII can act as a repulsive guidance cue for cortical axons, and experiments with *semaIII* null mice indicate that endogenous SemaIII contributes to the guidance of cortical axons in vivo. Finally, the neuropilin-1 blocking experiments indicate that the directed growth of cortical axons toward the white matter is mediated by neuropilin-1.

Although morphological abnormalities are present in cortical neurons in *semaIII* null mice, at least some cortical axons are able to navigate their way to subcortical targets (*17*). Therefore, signals in addition to SemaIII might also contribute to the guidance of cortical axons. The most likely candidates for such alternate signals would be other members of the semaphorin family, such as Sema IV and Sem E, which are also expressed in the developing cortex (*6*).

A role for target-derived signals in the specification of cortical connections has been previously suggested (*18*). Target-derived signals, however, are unlikely to be involved in the initial patterning of cortical efferent projections because the target structures, such as the tectum and thalamus, are located well beyond distances over which diffusible signals are thought to act, and at the time of initial axon outgrowth the target structures have not yet differentiated. Our observations reveal that the initial patterning of cortical projections is regulated by locally produced semaphorins, and suggest that semaphorin–neuropilin-1 interactions may be widely involved in defining the initial trajectory of axon projections in the developing brain.

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2. Embryonic day 18 (E18) or postnatal day 3 (P3) rat brains were dissected in cold Hanks' balanced salt solution (HBSS), embedded in 2.5% low–melting point agarose in HBSS, and sectioned (coronal) on a vibratome at 300 μm. Slices were cultured on a transparent porous membrane (1 μm pore size, Becton Dickinson) (*19*) in a 35-mm well containing media (70% basal medium Eagle, 25% HBSS, 20 mM glucose, 1 mM glutamine, 1 mM penicillin-streptomycin, 5% horse serum). Dissociated cells (*20*) were labeled for 10 min in media containing DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine percholate, 10 mg/ml, Molecular Probes), washed, and resuspended in media at 5×10^5 cells per milliliter before being plated onto cortical slices. Live DiI-labeled cells were imaged and analyzed with IPLab Spectrum 3.1.1 (Scanalytics).
3. To generate axon orientation plots, we assigned individual labeled neurons a vector to indicate axon orientation and length. Each experiment was repeated 6 to 12 times; 100 to 200 axons were analyzed, and scored as being directed toward the pia (45° to 135°) or toward the ventricle (225° to 315°) to generate axon orientation histograms. Percentages are indicated as the mean ± SE. Statistically significant differences [chi-square test (StatView 4.1), $P < 0.001$] are indicated by an asterisk.
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7. Slices were incubated with 5 nM of AP-SemaIII for 30 min before plating E18 cells.
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14. Performed on E18 cryostat sections (*13, 21*).
15. Forty-five minute incubation (last 15 min in presence of DiI); immunoglobulin G concentration, 100 μg/ml.
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