Semaphorin 5B Is a Novel Inhibitory Cue for Corticofugal Axons

Neuronal connectivity is generated by the precise guidance of neuronal growth cones in response to the spatiotemporal distribution of molecular guidance cues in the developing embryo. Here we show that the class 5 semaphorin, Semaphorin 5B, is expressed in regions of the cortex and subcortex flanking the projection of and avoided by descending cortical axons, suggesting a role as a repulsive guidance cue in the formation of the internal capsule. Axons from cortical explants cultured in vitro with Semaphorin 5B-expressing cells exhibited characteristic avoidance behaviors. In organotypic slices, ectopic Semaphorin 5B expression along the presumptive internal capsule was sufficient to cause cortical axons to avoid their normal trajectory, resulting in either stalling at the boundary of Semaphorin 5B or turning into inappropriate areas of the cortex. In contrast, thalamocortical axons were not inhibited either in vitro or in slice culture by ectopic Semaphorin 5B. To further examine the function of Semaphorin 5B in situ, we knocked down its expression in the ventricular zone (VZ) at the corticostriatal angle. We found that labeled cortical fibers navigating toward and within subcortical regions. Therefore, we show that Semaphorin 5B functions to prevent corticofugal axons from abnormally projecting into germinal regions as they project to their subcortical targets.

Keywords: axon guidance, corticofugal, development, semaphorin, thalamocortical

Introduction

The reciprocal connections between cortex and thalamus are among the longest of the mammalian forebrain, requiring many intermediate targets and guidance decisions. The pioneer projections to the thalamus and other subcortical targets are formed by axons of subplate neurons, subsequently followed by axons from layers VI and V of the newly forming cortical plate (McConnell et al. 1989; De Carlos and O’Leary 1992; Clascá et al. 1995). The initial targeting of subplate axons is toward the corticostriatal boundary (CSB). Axons are repelled from the cortical plate by Sema3A while being attracted to the lower intermediate zone (IZ)/upper subventricular zone (SVZ) by the semaphorin, Sema3C (Bagnard et al. 1998; Skalióra et al. 1998; Polleux et al. 2000; Bagnard et al. 2001). Cortical axons also avoid the lower SVZ and ventricular zone (VZ), a repulsion originally thought to be mediated by Sema3A (Bagnard et al. 2000), although this remains controversial (Polleux et al. 2000). In addition, a medial to lateral gradient of slit proteins is thought to be responsible for directing subplate axon outgrowth in a lateral direction away from medial cortical structures (Bagri et al. 2002; López-Bendito et al. 2007).

Dorsal cortical axons are the first to reach the CSB around E13.5, pausing approximately 24 h as axons accumulate (Molnár and Cordery 1999; Bellion et al. 2003; Bellion and Ménin 2005; Jacobs et al. 2007). Having accumulated, cortical projections coordinate the lateral ganglionic eminence (LGE), in part due to netrin-1 attraction (Ménin et al. 1997; Richards et al. 1997), wherein it has been proposed that they physically interact with thalamocortical axons that may serve as a scaffold for cortical axons as they pathfind into subcortical targets (Molnár and Blakemore 1991; Braisted et al. 2000; Hevner et al. 2002; López-Bendito et al. 2006). Likewise, axons from the cortex are also believed to serve as scaffolding for thalamic axons as they target layer IV (Molnár and Cordery 1999).

Semaphorins are known to play a role in nervous system development generally (Fiore and Püschel 2003) and axon guidance specifically (Luo et al. 1995; Püschel 1996; Nakamura et al. 2000). They are characterized by the presence of a conserved “sema” domain, and individual members are grouped into classes based upon other domain specializations (Committee 1999). The sema domain conveys the inhibitory activity of the molecule, whereas additional domains may modulate this activity (Koppel et al. 1997; Gherardi et al. 2004; Kantor et al. 2004). Class 5 semaphorins are transmembrane proteins that in addition to the typically inhibitory semaphorin domain possess 7 thrombospondin repeats (Adams et al. 1996; Püschel et al. 1996). This is intriguing as thrombospondin repeats are permissive for axon outgrowth and can modulate synaptogenesis (Christopherson et al. 2005), suggesting a complex developmental role for class 5 semaphorins (Adams and Tucker 2000). Indeed, Sema5A has shown to be permissive or inhibitory to axon outgrowth depending on whether matrix proteoglycans are present (Oster et al. 2003; Goldberg et al. 2004; Kantor et al. 2004), and it has been reported from glycosaminoglycan microarray studies that Semaphorin 5B (Sema5B) also possesses the ability to interact with both heparan and chondroitin sulfate proteoglycans (Shipp and Hsieh-Wilson 2007).

Previous observations in the developing rat indicated that Sema5B is expressed in regions of the basal telencephalon coincident with the corticofugal projection into subcortical regions (Skalióra et al. 1998). We were therefore interested in the function of Sema5B during corticofugal development in the mouse. In our examination of the developing mouse cortex, we found that Sema5B expression flanks the presumptive internal capsule during its formation. Through gain-of-function experiments, we show that Sema5B is repulsive to cortical axons, but not to dorsal thalamic axons. In addition, using short hairpin RNA (shRNA) to knock down Sema5B, we show that it is necessary as a source of repulsion for the appropriate guidance of cortical axons away from structures such as the VZ as they navigate toward and within subcortical regions.
Materials and Methods

Animals
Timed pregnant CD1 mice were lethally anesthetized by chloral hydrate injection (0.25 mg/kg, intraperitoneally). E10-E18 CD1 mouse embryos were dissected into ice-cold phosphate-buffered saline (PBS). For protocols requiring fixed tissue, mouse embryos (whole or dissected, depending on their size) were placed in 4% paraformaldehyde (PFA; in PBS, pH 7.0) and rocked at 4 °C overnight. Fixed tissue was cryoprotected in 15%, then 30% sucrose/PBS, for at least 4 h on a rocker at 4 °C. Cryoprotected tissue was then embedded in optimal cutting temperature compound (TissueTek) and stored at -80 °C until sectioning.

Stable Cell Lines
Mouse semaphorin 5B (Adams et al. 1996; Püschel et al. 1996) was subcloned into pDisplay (Invitrogen, Burlington, Canada) for the purpose of stably expressing Sema5B in cell lines. This full-length semaphorin 5B was transfected into HEK293 cells with Lipofectamine 2000 (Invitrogen). Clonally derived stable cell lines were maintained with Geneticin (Invitrogen) selection in the culture medium (Dulbecco's modified Eagle's medium [DMEM]-F12 Ham supplemented with 5% fetal bovine serum [FBS]). Expression of Sema5B was confirmed by immunocytochemistry (with and without detergent; Fig. 3A-C) using a monoclonal antibody (Sigma, Oakville, Canada) against the hemagglutinin (HA) tag located at the N-terminus of Sema5B.

In Situ Hybridization
Semaphorin 5B in pGEM-T (Promega, Madison, WI) was linearized by restriction enzyme digestion with Sall (antisense direction) or SalI (sense direction), and the probe was transcribed from the SP6 (antisense) and T7 (sense) promoters using the Roche DIG RNA labeling kit. Probes were boiled at 80 °C for 10 min to fragment the full-length transcripts and cooled in ice water for 5 min prior to their dilution to 1 µg/ml in hybridization solution. Smaller embryos (E10-E13) were processed for whole-mount in situ hybridization (ISH) (as per Shen et al. 1997), and animals from E11-E18 were sectioned at 20 µm on a cryostat at -20 °C. ISH of frozen sections was performed with some minor modifications of the Roche ISH manual (Dijkman et al. 1995).

ISH on electroporated slice cultures was conducted as per De Leecea et al. (1997), with some modifications. In brief, slices were fixed in 4% PFA for 48 h at 4 °C, then embedded in grades IV-V bovine albumin (Fisher Scientific, Ottawa, Canada), mounted on a solution of grades I-II chicken albumin and gold gelatin, then resliced on a vibratome at speed 1 g/mL heat-denatured salmon sperm DNA, and 250 µM/L-glutamine, 0.06%D-glucose, and 50 µg/mL gentamicin. This media was replaced with neurobasal medium and supplemented with B27 (Invitrogen), 0.1%/ pen/strep, 2 mM L-glutamine, 0.06% D-glucose, and 50 µg/mL gentamicin. Descending projections in both hemispheres of a slice were labeled with a crystal of 1,1'-dioctodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes). Confluent dishes of Sema5B-expressing or control HEK293 cells were labeled with cell tracker green (Molecular Probes) and centrifuged into a pellet. The pellet was washed twice with DMEM and centrifuged a final time at 4000 r.p.m. for 5 min. Approximately 0.5 µL of condensed labeled cells was pipetted ipsilaterally directly onto the slice over the ganglionic eminences to cover the route of the internal capsule. Slices were cultured with the cells for approximately 48 h and examined with an inverted Nikon fluorescence microscope. DiI and cell tracker green images were captured in the exact same field using Metaview digital imaging software and then merged into a single red/green image using Adobe Photoshop. To quantify the effects of Sema5B on cortical axon pathfinding in slice cultures, merged images of DiI-labeled and cell islands were selected as groupings of HEK293 cells and were only scored if they were within the reach of axon outgrowth from the explant. Growth cone collapse was identified with phalloidin staining as the compaction of actin-based structures into a compact tip (see Fig. 3M,N). Percent growth cone collapse was determined as the number of collapsed growth cones over the total number of axons growing within close proximity of cell islands. Significance of the main effects of cell type, explant type, or their combined effect upon percent contact and percent collapse was determined by analysis of variance (ANOVA-SPPS).

Organotypic Slice Cultures
In order to assay the effects of Sema5B on developing tracts of the telencephalon, organotypic slice cultures were employed, as per Marin et al. (2001) and Flames et al. (2004), with some modifications. Briefly, E11-I brains were embedded in bacterial-grade agar (Fisher), sectioned at a 45 degree angle into 250-µm slices, and placed on polycarbonate organotypic culture supports (Biosciences, Mississauga, Canada) that fit into a 6-well plate. Organotypic slices were placed in a culture dish containing 5% fetal bovine serum [FBS]. Expression of Sema5B was confirmed by immunocytochemistry (with and without detergent; Fig. 3A-C) using a monoclonal antibody (Sigma, Oakville, Canada) against the hemagglutinin (HA) tag located at the N-terminus of Sema5B.

In Vitro Cocultures
E13.5-E14 brains were dissected into neurobasal media containing B27 (Invitrogen), 0.1% pen/strep, 50 mM L-glutamine, 0.06% D-glucose, and 50 µg/mL gentamicin. This media was replaced with neurobasal medium and supplemented with B27 (Invitrogen), 0.1% pen/strep, 2 mM L-glutamine, 0.06% D-glucose, and 50 µg/mL gentamicin. Descending projections in both hemispheres of a slice were labeled with a crystal of 1,1'-dioctodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes). Confluent dishes of Sema5B-expressing or control HEK293 cells were labeled with cell tracker green (Molecular Probes) and centrifuged into a pellet. The pellet was washed twice with DMEM and centrifuged a final time at 4000 r.p.m. for 5 min. Approximately 0.5 µL of condensed labeled cells was pipetted ipsilaterally directly onto the slice over the ganglionic eminences to cover the route of the internal capsule. Slices were cultured with the cells for approximately 48 h and examined with an inverted Nikon fluorescence microscope. DiI and cell tracker green images were captured in the exact same field using Metaview digital imaging software and then merged into a single red/green image using Adobe Photoshop. To quantify the effects of Sema5B on cortical axon pathfinding in slice cultures, merged images of individual organotypic slices (examples of which are seen in Fig. 4) were assessed and grouped as normal or abnormal using the following criteria. Abnormal axon growth included growth into inappropriate cortical regions (ventrolateral cortex) and halting or outgrowth at the border of HEK293 cells compared with that of untreated and control slice cultures. One image was taken per organotypic slice, and slices were pooled by experiment, and the number of slices displaying abnormal pathfinding was compared with the total number of slices to produce a percent error value. Significance of the differences in error rate was determined statistically using t-test for independent means (SPSS). An FV1000 confocal microscope was used to image examples of both control and experimental slices. To assess the guidance of thalamocortical axons in slice culture, the DiI label was instead placed in the dorsal thalamus; cells were labeled with cell tracker blue. Slices were cultured for 4 days prior to fixation to ensure growth of the thalamocortical fibers into the cortex, then mounted in Mowiol (Sigma), and imaged with an FV1000 confocal microscope. Figure 5FG shows organotypic slices under a single channel to clearly visualize the DiI-labeled fibers. The area of cell placement was circumscribed by a green outline.
**Results**

Previous studies examining early expression of semaphorins in the nervous system have suggested that Sema5B may play a role in telencephalic development (Adams et al. 1996; Skaliora et al. 1998). To explore its potential functions, we first examined Sema5B expression throughout cortical development and particularly during the establishment of telencephalic connectivity.

**Sema5B Expression during Telencephalic Development**

Sema5B is expressed throughout the entire embryonic neuraxis as early as E10.5. When hybridization was performed in whole-mount embryos (Fig. 1A–C), it was apparent that the pattern of expression was greatest in the central nervous system (CNS) and levels of expression in other tissues were below detection. At E10.5, Sema5B appeared to be expressed homogenously throughout the CNS, and on closer examination of cryosections, cells expressing Sema5B were found spanning the width of the neuroepithelium (Fig. 1D). Ventricular Sema5B expression was retained along the length of the CNS at E12.0 but appeared to be stronger and more defined within the forebrain (Fig. 1B). Examinations of cryosections at this stage revealed that Sema5B was expressed in the VZ where the cell bodies of radial glia are located, but not in the emerging preplate (Fig. 1E, black arrowheads). Expression was also seen in the VZs of the ganglionic eminences and the septum, which appeared as a stripe of increased staining in the forebrain of the whole-mount preparations (Fig. 1B, arrow). At E13.5, Sema5B signal along the spinal cord had diminished (Fig. 1C and data not shown), and the emergence of expression in the developing olfactory bulbs (Fig. 1C, black arrowhead; Fig. 1F, black arrow) and ventrolateral cortex was observed (Fig. 1C).

In sagittal sections, strong Sema5B expression was apparent within the VZs of the ganglionic eminences and septum and the developing hippocampus (Fig. 1F, white asterisk), whereas

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**Figure 1**. Early embryonic expression of Sema5B. (A) At E10.5, expression is evident along the length of the neuraxis. (A') Sense control at E10.5. (B) Expression of Sema5B at E12.0. The ganglionic eminences (GEs) can be seen through the cortical wall as a dense stripe of label (black arrow) and expression remains fairly robust within the spinal cord (arrowheads). (C) At E13.5, in addition to the GE, there are patches of staining along the ventrolateral aspect of the telencephalon, best seen caudally (white arrowhead), and a developing layer of staining in the newly emerging olfactory bulbs (black arrowhead). (D–F) ISH labeling on sagittal sections corresponding to ages of the embryos in (A–C). The ventricular epithelium is intensely labeled, whereas the developing cortical plate is negative (black arrowheads; E, F). Expression in the VZ of the hippocampus (white asterisk, F) and within the developing olfactory bulb is also evident (black arrow, F).
little expression was detected in the emerging cortical plate (Fig. 1F, arrowheads).

Examination of coronal sections of E14.5 brains showed strong labeling of Sema5B throughout the entire rostral-caudal extent of the VZ, with a slight reduction in intensity at the medial and caudal hippocampal VZ (Fig. 2). Rostrally, the olfactory bulb mantle and the VZ exhibit robust Sema5B expression (Fig. 2A–B). By this stage, regions of Sema5B expression have expanded to include the piriform cortex, particularly in the vicinity of the lateral olfactory tract, which is surrounded by Sema5B expression (Fig. 2B–C). The SVZ of the medial ganglionic eminence (MGE), and the differentiating field of the MGE-derived globus pallidus (Fig. 2C–D), and the dentate migratory path in the hippocampus (Fig. 2E). In the ventrolateral regions of the telencephalon, Sema5B was expressed in the endopiriform nucleus (EN), the basolateral amygdalar complex, in addition to the expression in the piriform and insular cortices (Fig. 2D–E). Also, some Sema5B expression was apparent along several boundary regions of the forebrain, the lateral migratory stream (or CSB; arrows in Fig. 2C–E), the border between the LGE and the MGE (Fig. 2C), and the thalamic reticular nucleus at the border between the dorsal and the ventral thalamus (Fig. 2F).

A similar pattern of expression was maintained over the period of cortical neurogenesis (E13.5 until E16.5), but by E17 Sema5B expression in the dorsal cortex was sharply down-regulated to a very restricted region at the VZ. Sema5B continued to be expressed robustly in ventrolateral cortex (including the piriform cortex) and hippocampus, at diminished levels in the olfactory bulb, and was generally no longer expressed in any of the subcortical regions or migratory streams as seen during neurogenesis. At postnatal ages, Sema5B expression is only maintained in the hippocampus and piriform cortex (data not shown). At the time corresponding to the establishment of connectivity between the cortex and the thalamus, Sema5B was highly expressed in regions of the dorsal and ventral telencephalon that are avoided by corticothalamic axons and largely absent from the route of the internal capsule (between the arrowheads; Fig. 2D–G). This suggested a possible role for Sema5B in corticothalamic axon guidance through the ventral telencephalon.

Sema5B Is Repulsive to Cortical Projections In Vitro

Pioneer axons extending from the cortical plate follow a trajectory that avoids and/or pauses at several Sema5B-expressing regions in the developing telencephalon. First, they avoid the strongly Sema5B-expressing neocortical SVZ and VZ and stop short of the ventrolateral cortical domains that also express Sema5B. When they reach the CSB, a region where a subset of cells express Sema5B (Fig. 2C–E), axons from the dorsal cortex pause until more lateral cortical axons arrive (Molnár and Cordery 1999; Bellion et al. 2003; Bellion and Mékin 2005). Cortical axons then coordinately extend through subcortical regions bordered by Sema5B expression in the MGE and its derivative, the globus pallidus (Fig. 2C–D). We therefore sought to determine whether Sema5B might constrain corticothalamic axons to their correct projection by functioning as an inhibitory cue to cortical axons. In addition, because dorsal cortical axons pause at the CSB for longer than lateral cortical axons, we wished to separately examine dorsal and lateral cortical axons to determine whether there was a differential response to Sema5B depending on the location of the cortical axon origin.

Dorsal and lateral cortical explants from an early stage of corticofugal development (E13.5) were cultured in the presence of Sema5B-expressing or control HEK293 cells. Axons growing from dorsal cortical explants displayed a marked avoidance of Sema5B-expressing cell islands (Fig. 3C–D, 24.7 ± 2.6% contact SEM, n = 102 cell islands surrounding 28 explants) compared with those that encountered vector-only control cells (Fig. 3A–B, O) creating a “halo” of axons around a group of cells. Of the axons that were growing toward control cell islands, 79.8 ± 9.5% (SEM) grew into or upon islands (n = 72 islands, 26 explants; ANOVA: main effect of cell type, P < 0.001). Similarly, axons from lateral cortical explants displayed a significant avoidance of Sema5B-expressing cells (Fig. 3G–H, O) compared with those grown with control cells (Fig. 3E–F, O). Lateral cortical axons contacted and grew upon control cells (80.4 ± 8.5% SEM; n = 91 islands, 38 explants), whereas approximately 75% avoided contact with Sema5B-expressing cells (24.7 ± 2.6% contact SEM; n = 92 islands, 21 explants; ANOVA: main effect of cell type, P < 0.001). The expression of Sema5B at the plasma membrane of HEK293 cells was confirmed by labeling for HA with and without detergent (Fig. 3J, K vector only-transfected cells do not express the HA tag [Fig. 3I]). Sema5B was therefore inhibitory to axon growth upon heterologous cells, but no increase in inhibitory response was seen with dorsally derived axons compared with laterally derived axons.

Growth cones are enriched in polymerized bundles of actin and a dense actin filament network, which underlie filopodia and lamellipodia, respectively. When a growth cone encounters an inhibitory cue, part of the repulsive response is to disassemble and collapse these actin-based structures into a compact tip. Approximately 54.25 ± 5.40% (SEM) of the dorsal cortical growth cones were collapsed at the border of Sema5B cell islands (Fig. 3N; n = 102 islands, 28 explants; graph, Fig. 3P; red arrows highlight compact actin at axon tips), whereas significantly less collapse occurred in growth cones contacting Sema5B cells (4.98 ± 0.59% SEM; Fig. 3M; white arrow; graph, Fig. 3P; ANOVA: main effect of cell type, P < 0.001). Growth cones of laterally derived cortical explants contacting Sema5B cells were similarly significantly more collapsed (58.11 ± 6.09% collapse SEM; n = 92 islands, 21 explants) compared with control conditions (4.39 ± 0.46% SEM; n = 91 islands, 38 explants), at a similar rate to dorsal growth cones (Fig. 3P).

Although qualitatively the avoidance of Sema5B cell islands by dorsal cortical axons appeared more marked, quantitative comparison between the 2 types of explants encountering either cell type revealed no statistically significant difference for percent contact (Fig. 3Q, ANOVA: no main effect of explant type, P = 0.748; no effect of interaction between cell type and explant type, P = 0.933) or for percent growth cone collapse (Fig. 3P, ANOVA: no main effect of explant type, P = 0.458; no effect of interaction between cell type and explant type, P = 0.627). Sema5B is significantly repulsive to all cortical axons and not specifically more inhibitory to dorsal cortical axons.

Sema5B Is Repulsive to Descending Cortical Projections within Organotypic Slices

Sema5B is expressed in a number of regions along the pathway navigated by corticofugal fibers, suggesting a guidance role for Sema5B in establishing this tract. Having
Figure 2. Sema5B is expressed in regions of the telencephalon avoided by corticothalamic axons. (A–F) Rostral to caudal series of coronal cryosections of a representative E14.5 brain. (A) Sema5B expression is evident in the VZ and a layer within the mantle of olfactory bulbs (OBs), whereas there is little expression in the olfactory epithelium (OE). (B) Sema5B expression is evident in the VZ of anterior forebrain and the piriform cortex (Pir). The lateral olfactory tract can be seen surrounded by Sema5B label (arrowheads in B and C). (C) Sema5B expression can be seen in the VZ of the dorsal cortex, LGE (L), septum (S), and piriform cortex; (D) Expression of Sema5B is also apparent in the thalamic VZ (at V3—the third ventricle) and hippocampus (H). Also labeled is the piriform cortex (Pir), the EN, basolateral portions of the amygdalar complex (BLC in D, E), the lateral migratory stream/corticostriatal boundary (arrow in C and E), SVZ of the MGE (M), and the globus pallidus (*). Of note is the lack of Sema5B expression in a corridor between the SVZ of the MGE and the globus pallidus (arrowheads). This corridor connects the dorsal thalamus (dT) with the differentiating field of the striatum (Str), neither of which express Sema5B. (E) LMS/CSB (arrow) labeling is more evident, the ventrolateral label continues, as well as the caudal (c) GE and caudal MGE (*) and the migratory path of the dentate gyrus (dmp) and the thalamic reticular nucleus (TRN). (F) Cortical VZ label appears less intense than at rostral levels, and strong VZ expression continues caudally through the brainstem toward the spinal cord. (G) Schematic of Sema5B expression as seen in (D). Arrowheads are to indicate the routes of the internal capsule, which lacks Sema5B expression. (H) Schematic illustrating the approximate rostral-caudal location of coronal sections shown in (A–F).
shown that Sema5B is avoided by cortical axons in vitro, we hypothesized that Sema5B expression in the cortical SVZ/VZ, ventrolateral cortices, claustrum and amygdaloid complex, and parts of the emergent basal ganglia may serve to inhibit axons from invading these areas during pathfinding. To test this, organotypic slice cultures from E14.5 mice were employed to determine whether Sema5B can perturb the pathfinding of descending cortical axons in their normal environment.

Sema5B-expressing or control cells were placed directly on slices along the pathway normally established by cortical axons in the subcortex. E14.5/15 brains were chosen in order that axons would have already encountered the CSB (which does express Sema5B at lower levels) and would have begun penetrating the LGE. In this manner, we ensured that any effect we observed was due to the exogenous Sema5B added. Corticofugal axons were unaffected by the presence of control cells, growing in a normal trajectory via the prospective internal capsule (Fig. 4D–F, L, L'). In contrast, descending axons grown in the presence of Sema5B-expressing cells did not grow along their normal trajectory (Fig. 4G–K, M, M'); they instead avoided entering regions where the cells were added at the border of the bolus, regardless of proximity to endogenous histogenic boundaries. A variety of repulsive behaviors were seen among these axons. Most slices had axons that ceased growing in the region of cells expressing Sema5B (Fig. 4G–LK), whereas some axons turned away and began to grow inappropriately toward lateral areas (Fig. 4J, MM'). Confocal microscopy further demonstrated that Dil-labeled cortical axons grew normally into the region overlaid with control cells (red arrows, Fig. 4L; merged in L'), whereas those encountering Sema5B-expressing cells make deviations from normal pathfinding (red arrowheads, Fig. 4M; merged in M'). Overall, a significant proportion of slices (76.8 ± 4.4% SEM; n = 97 slice cultures) contained abnormal cortical projections in the presence of Sema5B-expressing cells compared with 19.4%...
Sema5B Is Not Repulsive to the Dorsal Thalamocortical Projection

Sema5B is expressed within specific ventrolateral regions, such as piriform cortex, EN, and basolateral portions of the claustrum/amygdaloid complex, and subcortical regions including the VZ of the LGE, throughout the MGE, and its derivative, the globus pallidus. As we have demonstrated that Sema5B is inhibitory to descending cortical axons, it was attractive to consider that Sema5B may be the unknown inhibitory cue expressed in MGE and MGE-derived territories that have been found to block thalamocortical axon invasion of the subcortex (López-Bendito et al. 2006). As with cortex, explants of E13.5 dorsal thalamus were cocultured in vitro with Sema5B-expressing or control HEK293 cells. Compared with control (Fig. 5A,B,E; 77.3 ± 1.4% contact SEM, n = 41), axons derived from thalamic explants had no increase in avoidance of Sema5B-expressing cells (Fig. 5C,D,E; 77.5 ± 1.3% contact SEM, n = 47; t-test for equality of means, P < 0.001). To further examine whether dorsal thalamic axons were truly nonresponsive to Sema5B, we made E13.5/14 organotypic slice cultures, labeling the dorsal thalamus with DiI. Aggregates of cell tracker-labeled control or Sema5B-expressing HEK293 cells (shown outlined in green in Fig. 5F,G, respectively) were then mixed with DiI-labeled cortical axons. If Sema5B was truly nonrepulsive to dorsal thalamocortical axons, we expected to see no differences between control and experimental slice cultures. As we have demonstrated (López-Bendito et al. 2006), Sema5B-expressing cells inhibited descending cortical axons from the dorsal cortex, and this inhibition was effectively blocked by DiI-labeled cortical axons (Fig. 5H). As with cortex, we observed no increase in avoidance of Sema5B-expressing cells in dorsal thalamocortical axons, as indicated by merged images of Sema5B-expressing cells and DiI-labeled cortical axons (Fig. 5I). As we have demonstrated, Sema5B-expressing cells inhibited cortical axons from the dorsal cortex, and this inhibition was effectively blocked by DiI-labeled cortical axons (Fig. 5H). As with cortex, we observed no increase in avoidance of Sema5B-expressing cells in dorsal thalamocortical axons, as indicated by merged images of Sema5B-expressing cells and DiI-labeled cortical axons (Fig. 5I). As we have demonstrated, Sema5B-expressing cells inhibited cortical axons from the dorsal cortex, and this inhibition was effectively blocked by DiI-labeled cortical axons (Fig. 5H). As with cortex, we observed no increase in avoidance of Sema5B-expressing cells in dorsal thalamocortical axons, as indicated by merged images of Sema5B-expressing cells and DiI-labeled cortical axons (Fig. 5I).
placed as previously over the presumptive internal capsule within the subcortex. We then monitored the guidance of thalamic axons en route to their targets in the cortex. We found that the thalamocortical projection in ex vivo slice culture was established normally and reached the IZ and cortical plate in the presence of ectopic Sema5B in subcortical regions (Fig. 5G). Thus, although corticofugal axons were significantly repelled (as shown in Fig. 4), thalamocortical axons were unaffected by recombinant Sema5B (Fig. 5).

Loss of Sema5B Expression from the Neocortical VZ Results in Corticofugal Axon Pathfinding Errors

Having determined that Sema5B is sufficient to inhibit cortical, though not thalamocortical, axons, we wished to investigate whether Sema5B does inhibit axons in situ. We chose to assay this possibility by ex vivo electroporation of shRNA-expressing plasmids followed by immediate slice culture and DiI labeling of cortical axons (Hand et al. 2005).

Three target sequences within the semaphorin 5B gene were chosen for RNA interference (Reynolds et al. 2004). shRNA1, shRNA2, and shRNA3 were each confirmed as properly inserted into pLentilox 3.7 with the correct desired sequence. A fourth insert containing a nonsense sequence with no similarity to the Sema5B gene and a construct designed toward the luciferase gene were chosen as controls for potential off-target effects due to the other Sema5B-shRNA vectors. Each of the 3 shRNA vectors was tested for knockdown capability by transfection into the stable Sema5B-expressing cell line. Positive transfecants were identified by GFP fluorescence, encoded by an internal ribosome entry site-GFP sequence on pLL3.7 downstream of the shRNA insert. Transfected cultures were also stained for HA, which tags the recombinant version of Sema5B. Cells that were positive for GFP and negative for Sema5B-HA were considered as knocked down (white arrowheads, Fig. 6A), and each of the 3 constructs knocked Sema5B expression down in nearly every cell into which they were successfully transfected (Fig. 6A,B), where 1.0% (±0.4 SEM), 0.7% (±0.4 SEM), and 0.3% (±0.3 SEM) of cells coexpress Sema5B-HA and GFP (red arrows, Fig. 6A, upper 3 panels) indicating a high degree of knockdown. On the contrary, transfection of either control shRNA plasmid had no effect on Sema5B-HA levels (95.0 ± 5.5% of cells coexpress HA and GFP). We were therefore confident in having 3 shRNA vectors effective in producing knock down of Sema5B and valid control vectors that do not result in Sema5B knockdown (quantification; Fig. 6B). Western analysis was also performed on knockdown cultures to show quantitatively that Sema5B expression was reduced compared with controls (Fig. 6C). Not all cells were transfected with Sema5B-targeting shRNA; therefore, HA remained detectable in the cell lysate but was significantly reduced compared with control shRNA-transfected or nontransfected cells.

For the purposes of shRNA-mediated knock down of Sema5B, E13.0/E13.5 mice were chosen because at this stage, axons are still growing within the IZ in proximity to the Sema5B-expressing VZ. We chose shRNA1 and shRNA3 for these experiments. Plasmids were injected unilaterally into the left lateral ventricle and electroporated into the lateral VZ at or near the CSB (see Fig. 7A for schematic of endogenous expression, electroporation, and DiI labeling). After electroporation with control or shRNA plasmids, brains were sliced by vibratome and labeled with DiI in the cortical plate. Some control and shRNA-electroporated slices were not DiI labeled but were cultured for ~24 h and then reembedded in gelatin blocks and resliced at 50 μm for Sema5B ISH (Fig. 6D-G). Control shRNA-electroporated cortex showed no decrease in Sema5B expression in regions positive for GFP expression (bracketed areas, Fig. 6D,E, respectively). In contrast, cortices electroporated with shRNA3 had demonstrable reductions in Sema5B RNA expression within the GFP-positive region (bracketed areas, Fig. 6F,G, respectively), whereas the remaining cortical VZs expressed Sema5B as usual (red arrows,
Therefore, in addition to their effects on the recombinant form, our shRNA constructs were effective in the knock down of endogenous Sema5B.

After a 24-h culture period, DiI-labeled slices were examined for the presence of labeled axons among transfectected VZ cells expressing GFP. Control constructs expressed within the VZ (GFP expression only—Fig. 7B) had no effect on the pathfinding of cortical axons (DiI labeling—Fig. 7C), which did not enter the region of GFP expression (Fig. 7D, H), but rather remained within their proper course in the IZ (red arrows in Fig. 7C, H). In comparison, when the VZ was electroporated with shRNA1 (Fig. 7E–G) or shRNA3 (Fig. 7I), cortical axons were found traversing the normally well-maintained boundary between the IZ and the SVZ/NZ.
and were found within the regions of Sema5B knockdown (Fig. 7F, I; arrowheads in Fig. 7F, I). Overall, knock down of Sema5B by shRNA electroporation caused a significant increase in the number of labeled axons per slice that entered the VZ (15.2 ± 1.5 axons—shRNA1; 11.5 ± 1.3—shRNA3) compared with control constructs (2.2 ± 0.7 axons; 1-way ANOVA: main effect of plasmid type, P < 0.001). Similarly, when analyzing Sema5B loss of function in broader terms, we found that shRNAs against Sema5B increased the percentage of slices exhibiting guidance errors (±SEM; K). By either measure, both shRNA1 and shRNA3 produce a significant increase in the numbers of VZ-penetrating axons compared with control. Scale bar in (B) represents 250 μm for (B–G). Scale bar in (H) represents 100 μm for (H and I).
cortical axon guidance defects from 8.33 ± 0.7% in control electroporations to 88.5 ± 2.4% (shRNA1) and 75.0 ± 3.3% (shRNA3). This increase in the rate of guidance defects is significant between control and experimental conditions (Fig. 7K; 1-way ANOVA: main effect of plasmid type, \( P < 0.001 \)). By either measure, shRNA3 appears to be somewhat less effective in Sema5B knockdown as shRNA1; however, both shRNAs produce guidance defects in descending cortical axons consistent with a loss of repulsion from the VZ/SVZ. This supports our hypothesis that Sema5B mediates repulsion of descending cortical axons from the germinal zones of the developing telencephalon.

**Discussion**

In this study, we show that the class 5 semaphorin Sema5B is expressed in a spatial and temporal pattern consistent with a role in maintaining the trajectory of corticofugal axons toward subcortical structures such as the thalamus. We provide evidence showing that Sema5B is both sufficient and necessary to inhibit cortical axons from entering specific territories such as the VZ during their guidance to subcortical targets.

**Exogenous Sema5B Is Inhibitory to Cortical Axons In Vitro**

Sema5B is found in regions of the developing telencephalon that are specifically avoided by corticofugal axons. The strongest expression of Sema5B is within the VZ and SVZ, particularly in the dorsal pallium, but it is also expressed in moderate levels along the CSB, both of which are a pattern reminiscent of Pax6 (Puelles et al. 2000, Jones et al. 2002). The CSB is an intermediate target for cortical axons as they navigate into the subcortical regions (Močna and Cordery 1999), so we postulated that dorsal cortical axons arriving earliest to the CSB might have a stronger response to Sema5B than lateral cortical axons. The pause of axon outgrowth at boundary regions is a common occurrence during nervous system development, also being found in thalamic axons and centrally projecting sensory axons, and thus is likely an important developmental process although the underlying mechanism is not known. The CSB may provide inhibition that prevents or slows further outgrowth of cortical axons until this accumulation of cortical axons has occurred.

We found that both groups showed significant avoidance responses to Sema5B in vitro, including growth cone collapse; however, there was no difference in the degree of this repulsion. Thus, mechanisms other than degree of responsiveness to Sema5B likely account for the differential pause exhibited at the CSB in vivo. For example, it is possible that cortical axons only cross the CSB once there are sufficient homophilic interactions among corticothalamic axons to overcome narrow inhibitory barriers and possibly to permit heterophilic interactions between the growth cones of cortical and thalamic axons and promote their subsequent fasciculation (Močna 2000). It has also been argued that no interaction exists between thalamic and cortical axons; that they actually travel in mutually exclusive domains within the internal capsule (Bagnard et al. 2001). Alternatively, this pause at a major histogenetic boundary and subsequent accumulation of descending cortical fibers may be important for the continuity of topographical organization as they grow toward their final targets.

**Sema5B Is Sufficient to Inhibit Cortical Axons In Situ**

Although we have shown that Sema5B is indeed capable of inhibiting cortical axons in vitro, this does not necessarily reflect the endogenous function, as the context of modifying factors found in extracellular spaces and on cell surfaces along an axon's trajectory, including proteoglycans, adhesion molecules, and other membrane-bound or secreted guidance cues, can influence the ultimate behavior elicited by a particular guidance cue. This has been demonstrated most importantly with Sema5A, which can act as either a permissive or a inhibitory substrate depending upon local coexpression of heparin or chondroitin sulfate proteoglycans (Kantar et al. 2004). It has been suggested that the same is true at least in vitro for Sema5B (Shipp and Hsieh-Wilson 2007). Our gain-of-function organotypic cell overlay assay was employed to assess whether there exists such a modulation of the demonstrated in vitro inhibitory behavior of Sema5B. Although this may reflect a complete "overwhelming" of endogenous local cues, the creation of ectopias in brain slice culture are often used and are generally assumed to be acting in an endogenous fashion (Marín et al. 2001; Polleux and Ghosh 2002; Flames et al. 2004; López-Bendito et al. 2006). We chose to make our cell overlay on the presumptive internal capsule, a region of the brain along the pathway of descending cortical axons that does not express Sema5B. By adding these ectopias, we showed that the addition of Sema5B can block the entry of axons into a normally permissive zone. In some instances, the ectopic Sema5B placement actually resulted in aberrant turning of axons into regions of endogenous Sema5B expression along the CSB (Fig. 4I). This presumably reflects a guidance phenomenon where an axon challenged by multiple nonpermissive substrates will opt for the less repulsive of the 2 (Bagnard et al. 2000). The inhibition of cortical axons by a gain of Sema5B expression in the internal capsule demonstrates a sufficiency of inhibition by Sema5B, not only in vitro but even in the physiologically relevant environment provided by the ex vivo slice culture system.

**Thalamic Axons Are Nonresponsive to Sema5B**

Much in the same manner as was performed to examine the descending cortical projection to the internal capsule, we also examined the reciprocal thalamic projection. This fiber set also encounters regions of Sema5B expression flanking its path through the subcortex and is also thought to pause at the CSB (López-Bendito and Molnár 2003). We speculated that the developing nervous system would make repeated use of a cue that creates inhibitory domains flanking an equivalent, though reversed, guidance path. In addition, it has also been shown that a permissive bridge of cells from the LGE migrates between the MGE and the globus pallidus and is absolutely necessary in order to permit thalamic axons entry to the subcortex (López-Bendito et al. 2006). The factor within the MGE and globus pallidus that is responsible for the inhibition of thalamic axons has not been identified, and we postulated that Sema5B might subserve that role. It was therefore surprising to discover that this is not the case; dorsal thalamic axons were not inhibited by the presence of Sema5B expressed in islands of HEK293 cells in vitro or by overlays of cells creating ectopic regions of Sema5B expression within the internal capsule.

What is most interesting is that not only are thalamic axons not inhibited by the same cue that inhibits cortical axons but...
also they appear to grow normally and find their cortical targets in spite of an environment where cortical axon guidance has been disrupted. This contrasts with the argument that the interaction of descending and ascending projections near the CSB is a crucial event for the guidance of each fiber set (Vanderhaeghen and Polleux 2004). In further support against the “handshake” hypothesis, thalamic and cortical axons have been shown to segregate from each other in culture, whereas Sema3A expressed within the internal capsule (Skaliora et al. 1998) amplifies these homotypic fasciculations (Bagnard et al. 1998, 2001). In vivo, a disjunct between cortical and thalamic axon guidance has been demonstrated in the chicken ovalbumin upstream promoter-transcription factor 1 mouse mutant, where thalamic projections are defective in their guidance to the cortex although cortical projections reach and pass the CSB normally (Zhou et al. 1999). Similarly, thalamic axons find their way normally in a cortex-specific Pax6 mutant independent of deviations in cortical pathfinding (Pinñon et al. 2008). If the integrity of both axon sets was necessary for the guidance of each as has been suggested (Hevner et al. 2002), we would expect that the lack of cortical axons in the internal capsule of our slice culture assays would disable the guidance of the thalamic axons therein. Our results that thalamocortical fibers are not affected by Sema5B and grow properly into the cortex while corticofugal fibers are deflected by Sema5B would suggest that the thalamocortical projection cannot rescue the cortical projection, supporting previous evidence that the 2 fiber sets are not necessarily codependent for their proper targeting.

Sema5B Is a Necessary Inhibitory Component of the Descending Cortical Projection

In order to demonstrate that Sema5B was not only sufficient to inhibit the descending cortical projection but also functioned as an inhibitory factor along the corticofugal projection in situ, we employed ex vivo electroporation of shRNA-expressing plasmids with subsequent slice culture. This allowed us to determine the degree of Sema5B loss of function guidance defects, if any, in the same type of system with which we analyzed the Sema5B gain of function.

In our gain-of-function experiments, we created ectopic domains of Sema5B expression within the normally nonexpressing presumptive striatum and internal capsule. In order to electroporate shRNAs from a ventricular injection, it was required that we examine the sema5B-expressing tissue that it was actually feasible to access, that is, the VZ of the dorsal/lateral pallium, a region that strongly expresses Sema5B within the developing mouse nervous system (see Fig. 2). Therefore, if Sema5B acts endogenously to create inhibitory domains for the descending cortical projection, we would expect that the loss of Sema5B at the appropriate stage of development would result in cortical axon guidance errors. We found that where shRNAs against Sema5B were expressed, cortical axons no longer maintained their trajectory within the IZ of the cortex but could be found invading the VZ that they would normally avoid.

Inhibitory guidance cues other than Sema5B are also present in the developing cortical VZ, including slit1 and slit2. Slit1 is expressed in the cortical plate and the germinal zones of the LGE, MGE, and diencephalon (Bagri et al. 2002). Slit2 is present in a medial high to lateral low gradient and has been shown to direct cortical axons away from midline structures (Lopez-Bendito et al. 2007). It is therefore important to note that although cortical axons displayed defective axon guidance in the vicinity of Sema5B-negative VZ, it appears that the slit2 gradient was maintained as cortical axons maintained their overall lateral trajectory. If the electroporation was fully penetrant and more axons were seen to deviate into the VZ, we would expect that eventually the tract would develop relatively normally due to the slit gradient. It is likely, however, that a full knockout would have a more significant corticofugal pathfinding defect extending from the dorsal cortex into the subcortical regions. It is purely speculative as to whether such a mutant would ultimately retain appropriate connectivity between the deep layer cortical neurons and its various subcortical targets.

Based upon ISHs showing Sema3A expression within the VZ, in conjunction with in vitro repulsion studies, it has been argued previously that Sema3A might be the inhibitory factor keeping the VZ free of cortical axons (Bagnard et al. 1998). Subsequent studies, however, have demonstrated that the Sema3A protein is active within the cortical plate and although it does repel cortical axons, it is attractive to the dendrites of the same cortical neurons. Interestingly, Sema3A exogenously overexpressed at the VZ actually reverses the polarity of cortical neurons, such that their dendrites grew toward the source of Sema3A, whereas the axons were erroneously repelled toward the cortical plate (Polleux et al. 2000). Furthermore, as the leading process of a migrating neuron is analogous to the apical dendrite, it is not surprising that Sema3A has also been shown to attract radially migrating neurons to the cortical plate (Chen et al. 2008). Together, these 2 findings exemplify that Sema3A is not likely present in the VZ to repel axons, therefore leaving the possibility open for another inhibitory guidance cue, such as Sema5B. In the absence of immunohistochemistry, it was crucial that we demonstrate the Sema5B expression in the VZ corresponded to a real activity. By knocking Sema5B down within the VZ and showing the aberrant invasion of cortical axons into the GFP-positive Sema5B-negative regions, we showed that Sema5B is normally active in the VZ where the transcript is found and that this activity corresponds to the creation of domains inhibitory to corticofugal axons projecting toward the subcortex.

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