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Slit Proteins Prevent Midline Crossing and Determine the Dorsoventral Position of Major Axonal Pathways in the Mammalian Forebrain

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Summary

We report that Slit proteins, a family of secreted chemorepellents, are crucial for the proper development of several major forebrain tracts. Mice deficient in *Slit2* and, even more so, mice deficient in both *Slit1* and *Slit2* show significant axon guidance errors in a variety of pathways, including corticofugal, callosal, and thalamocortical tracts. Analysis of multiple pathways suggests several generalizations regarding the functions of Slit proteins in the brain, which appear to contribute to (1) the maintenance of dorsal position by prevention of axonal growth into ventral regions, (2) the prevention of axonal extension toward and across the midline, and (3) the channeling of axons toward particular regions.

Introduction

Within the forebrain, major ascending and descending pathways such as the corticofugal, callosal, and thalamocortical projections establish stereotypical long-distance connections between different regions of the nervous system. Thalamocortical projections relay sensory information into the appropriate processing centers in the cortex, whereas callosal and corticofugal projections allow communication between the two cortical hemispheres and carry all cortical output to subcortical structures. As such, these pathways are essential to higher level information processing in the brain (Jones and Peters, 1996). Several studies have examined the early development of these projections and have identified pioneering axonal populations as well as potential intermediate targets and guidance decision points for these axons (Auladell et al., 2000; Braisted et al., 1999; De Carlos and O'Leary, 1992; Metin and Godement, 1996; Mitrofanis and Baker, 1993; Molnar et al., 1998; Molnar and Blakemore, 1995; Molnar and Cordery, 1999; Murray and Whitington, 1999; Tuttle et al., 1999). However, little is known about the molecular nature of the signals that control the directed growth of these major projections within the forebrain. In vitro studies have implicated Semaphorins, particularly Sema3C and Sema3A, functioning as attractive and repulsive cues, respectively, in drawing axons out of the cortical plate (Bagnard et al., 1998; Polleux et al., 1998). Sema6A has also been shown to play a role in guiding thalamic efferent pathways in vivo (Leighton et al., 2001). Further, netrin-1 has been implicated in regulating the growth of corticofugal and thalamocortical axons in the internal capsule region, an intermediate target for both projections (Braisted et al., 2000; Metin et al., 1997; Richards et al., 1997). Clearly, additional axon guidance cues exist, and many questions remain regarding the nature and role of these cues involved in regulating more distal aspects of the trajectories of both cortical and thalamic efferent tracts. In particular, the role of medial structures in guiding these largely noncrossing, laterally located pathways in the structurally complex environment of the forebrain is unclear. As well, how these longitudinally projecting pathways establish their dorso-ventral and medio-lateral position is poorly understood.

Slit proteins have been implicated in axon guidance in both vertebrates and invertebrates. The *slit* gene was initially identified in *Drosophila*; *slit* mutants are characterized by collapse of axonal tracts toward the midline, which was thought to result from abnormal midline development (Rothberg et al., 1988, 1990). *Slit* was later recognized as an axon guidance mutant in which ipsilateral and commissural axons enter and never leave the midline because of loss of the repellent action of Slit, which prevents midline crossing and/or recrossing (Kidd et al., 1999). In *Drosophila*, Slit signals through a combinatorial code of three Robo receptors (*d-robo*, *d-robo2*, and *d-robo3*) not only to regulate crossing at the midline, but also to specify the distance from the midline where longitudinal pathways are placed—in essence, establishing a code for lateral position (Kidd et al., 1998; Rajagopalan et al., 2000; Simpson et al., 2000). In addition to a similar conserved role in preventing midline crossing, the *C. elegans* Slit homolog, SLT-1, acting through its receptor, sax3/robo, also plays a role in directing dorso-ventral guidance of axons as well as anterior-posterior guidance of some migrating neurons (Zallen et al., 1998; Hao et al., 2001).

In mammals, the three *Slit* genes are expressed in, but not limited to, medial structures, including the floor plate and roof plate of the spinal cord, and the septum, preoptic area, hypothalamus, and hippocampus (Brose et al., 1999; Li et al.,1999; Yuan et al., 1999; Erskine et al., 2000; V. Marillat et al., submitted). In vitro studies have shown that rodent Slit proteins can repel spinal motor, retinal, cortical, olfactory, and hippocampal axons, as well as migrating neurons (Brose et al., 1999; Ringstedt et al., 2000; Chen et al., 2001; Hu, 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Shu and Richards, 2001; Wu et al., 1999); they have also been implicated in negatively regulating lymphocyte chemo-

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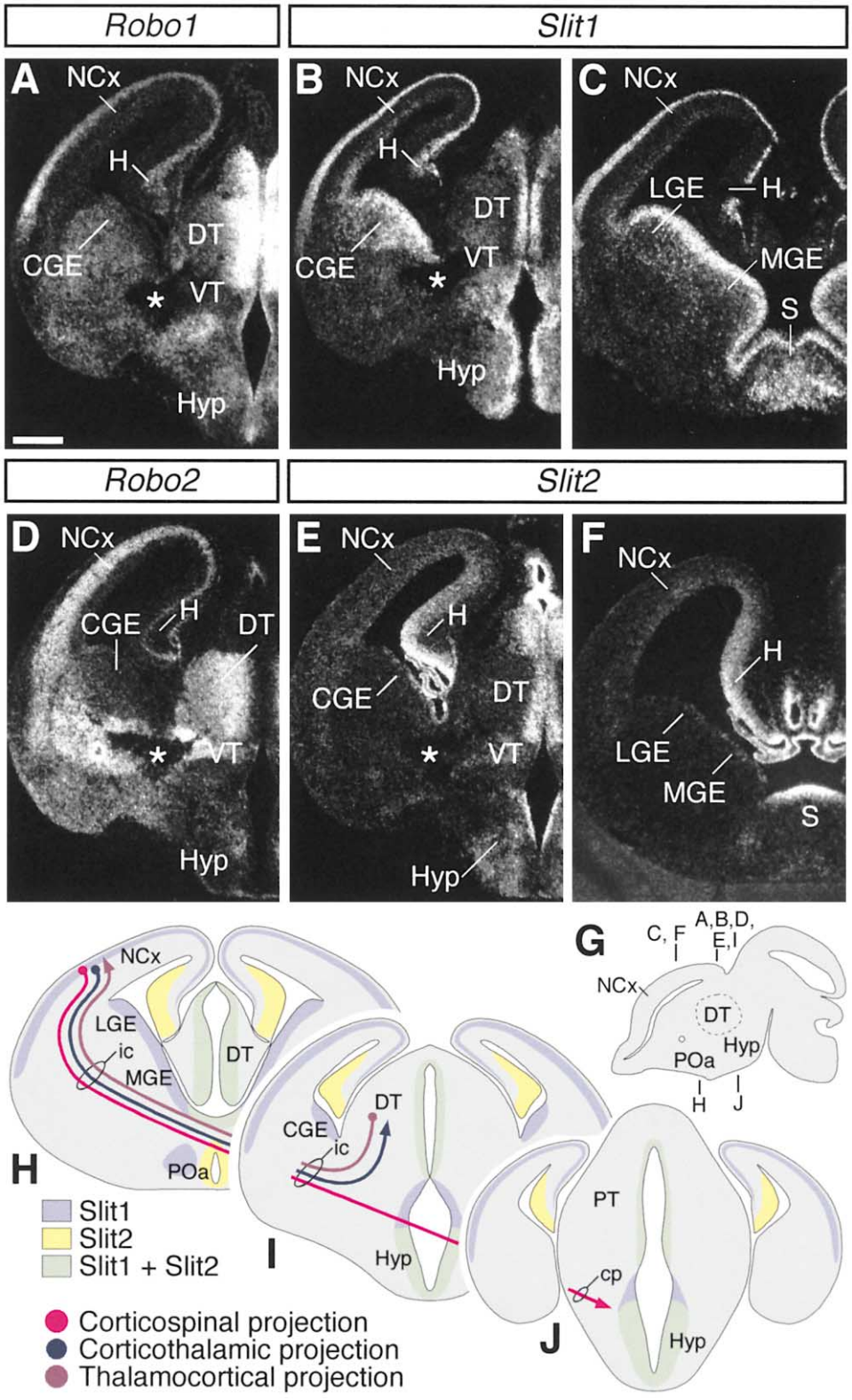

Figure 1. Expression of *Slits* and Their Receptors, *Robo*s, in the Embryonic Mouse Forebrain

Serial coronal sections through caudal telencephalic/rostral diencephalic levels (A, B, D, and E) and midtelencephalic levels (C and F) of an E14.5 embryo showing the expression of *Robo1* (A), *Robo2* (D), *Slit1* (B and C), and *Slit2* (E and F). Dorsal is up, and the midline is to the right in all panels.

(A and D) *Robo1* and *Robo2* are expressed in the developing cortex and thalamus. *Robo1* is expressed in the cortical plate, in a gradient decreasing from lateral to medial cortex, as well as in the dorsal thalamus, in a gradient decreasing from the neuroepithelium to the mantle

taxis (Wu et al., 2001). Taken together, the expression patterns and in vitro observations suggest that Slit proteins are also likely to play important roles as repulsive guidance cues for multiple populations of migrating axons and cells, likely using Robo receptors for their functions as well (Brose et al., 1999). A mammalian Slit protein was also independently identified as a positive regulator of branching and elongation of sensory axons in vitro (Wang et al., 1999), a function recently extended to the control of cortical dendrite branching as well (Whitford et al., 2002). However, despite these extensive in vitro studies, no in vivo data regarding the functions of mammalian Slit proteins have yet been reported.

In a companion paper, we described the generation of mice deficient in two of the three mouse *Slit* genes (*Slit1* and *Slit2*) and show that mice deficient in both have striking defects in the development of the visual system (Plump et al., 2001, in this issue of *Neuron*). In this study, we focus on the role of Slit proteins in regulating the guidance of long projection pathways within the forebrain. *Slit3* was excluded from our analysis because its expression is weak or absent in the regions that could potentially influence the growth of these axons. Our results demonstrate that Slit proteins are crucial for the proper development of several major CNS tracts in the forebrain, including corticofugal, thalamocortical, and callosal fiber tracts.

Results

Robo and Slit Expression in the Forebrain Correlates with the Development of Cortical and Thalamic Projections

Consistent with other studies (Erskine et al., 2000; Ringstedt et al., 2000; Yuan et al., 1999; Marillat et al., 2002), in situ hybridization analysis demonstrated that at E14.5, when cortical and thalamic projections are being established, mRNAs for the Slit receptors, *Robo1* and *Robo2*, are expressed in dorsal thalamus and throughout the rostral-caudal extent of the developing cortical plate, which at this stage consists primarily of layer 5 and 6 neurons (Figures 1A and 1D; additional analysis at E12.5 demonstrated similar expression patterns, data not shown), suggesting that these receptors may be involved in the guidance of these axons. The expression patterns of *Slit* genes have also been previously studied at various stages in the developing mammalian CNS (Erskine et al., 2000; Ringstedt et al., 2000; Yuan et al., 1999; Marillat et al., 2002). Here we provide a systematic and detailed analysis of their expression in the developing forebrain, focusing on regions where cortical and thalamic axon tracts extend and are thought to make critical guidance decisions, and during stages of development when those decisions are occurring (E14.5: Figures 1B, 1C, 1E, and 1F; E12.5: expression was similar, data not shown).

The observed patterns of *Slit* mRNA distribution are consistent with a role in cortical and thalamic axon guidance. As cortical fibers grow toward subcortical targets, they leave the cortical plate through the lower intermediate zone and turn into the developing striatum to form the internal capsule (Figure 1H). When running through the internal capsule, these corticofugal fibers avoid the proliferative regions of the ganglionic eminences, which express high levels of *Slit* mRNA (Figures 1B, 1C, and 1E–1I). At this same time, these axons avoid two other areas of high *Slit* expression: the ventral region of the basal telencephalon and the midline (Figures 1B, 1C, and 1E–1I). At the telencephalic-diencephalic boundary, corticofugal axons destined for the thalamus exit the internal capsule and make a sharp turn, extending dorsally into the thalamus. These axons avoid approaching the proliferative zone of the thalamus, which expresses high levels of *Slit* mRNA (Figures 1B, 1E, 1H, and 1I). Cortical axons directed toward other subcortical structures, such as the spinal cord, maintain their dorsoventral position as they enter the diencephalon and form the cerebral peduncle (Figures 1I and 1J). These axons then travel toward the mesencephalon by following a route roughly parallel to the alar/basal boundary, dorsal to the hypothalamic region—a region that also expresses high levels of *Slit* mRNA (Figures 1B, 1E, 1I, and 1J).

Thalamocortical axons course through a route that is largely reciprocal to that followed by corticothalamic fibers. Initially, they run ventrally to the boundary between the diencephalon and the telencephalon, where they then make a sharp turn to enter the mantle region of the caudal ganglionic eminence (Figure 1I). Thalamocortical axons turn into the telencephalon at the same approximate dorsoventral location, avoiding the hypothalamus, a region of high *Slit* mRNA expression (Figures 1B, 1E, and 1I). Once in the telencephalon, thalamocortical axons grow dorsolaterally and pass through the internal capsule to reach the cortex (Figure 1H). As in the case of corticofugal axons, thalamocortical projections avoid crossing the midline or approaching the progenitor zones of the basal telencephalon, which express high levels of *Slit* mRNA (Figures 1C, 1F, 1H, and 1I).

We took advantage of the expression of GFP from the

(A) *Robo2* is expressed in the subplate and intermediate zone of the cortex, in a pattern complementary to that of *Robo1*, and in the dorsal thalamus (DT), in a decreasing gradient from the neuroepithelium to the mantle (D).

(B and C) *Slit1* is expressed in the cortical plate (B and C) and in the proliferative zone of the dorsal thalamus (B), hypothalamus (Hyp, [B]), lateral and medial ganglionic eminences (LGE and MGE, respectively, [C]) and septum (S, [C]). *Slit1* is also expressed in the mantle of the septum (C) and preoptic area (not shown).

(E and F) *Slit2* is expressed in the neuroepithelium of the medial cortex (E and F) and in the proliferative zone of dorsal thalamus (E), hypothalamus (E), preoptic area (not shown), and septum (F). *Slit2* is also expressed in the mantle of the developing hypothalamus (E). The asterisk indicates the approximate location of fibers connecting the cortex and the thalamus.

(G–J) Schematic diagrams of coronal sections through the developing forebrain showing the trajectory of cortico-spinal (red), corticothalamic (blue), and thalamocortical (purple) axons in relation to *Slit1* (blue) and *Slit2* (yellow) expressing regions at selected levels to summarize and complement data shown in (A)–(I). Regions depicted in green express both *Slit1* and *Slit2*. The rostro-caudal levels of the other panels in this figure are indicated in (G).

CGE, caudal ganglionic eminence; H, hippocampus; ic, internal capsule; NCx, neocortex; VT, ventral thalamus. Scale bar, 500 μm.

Slit2 locus in mice with a targeted mutation for this gene (Plump et al., 2001) to establish the identity of the *Slit2*-expressing cells in the preoptic area and hypothalamus. To this end, we performed double immunofluorescence for GFP and the radial glial markers nestin or vimentin in *Slit2* heterozygous tissue. In the preoptic area and hypothalamus, *Slit2* is expressed in radial glia (data not shown; see supplemental data at http://www.neuron.org/cgi/content/full/33/2/233/DC1), raising the possibility that Slit2 protein may be transported laterally by the processes of these glia.

In summary, the complementary pattern of expression of *Robo* and *Slit* genes in the developing forebrain suggests that these molecules may play a role in the guidance of corticofugal and thalamocortical projections, potentially in preventing axons from entering ventral or medial regions where Slit proteins are likely to be present. Furthermore, in vitro experiments have demonstrated that cortical axons are repelled by both Slit2 (Shu and Richards, 2001) and Slit1 (K. Whitford and A. Ghosh, personal communication) and that thalamocortical axons are also repelled by Slit2 (D. O'Leary, submitted), strengthening the possibility that Slit proteins may play important roles in corticofugal and thalamocortical development.

Abnormal Axonal Trajectories in the Telencephalon of Slit Mutants

To directly address the role of Slit proteins in the guidance of corticofugal and thalamocortical projections, we analyzed the development of these connections in mice carrying loss-of-function alleles of *Slit1*, *Slit2*, or both (Plump et al., 2001). We first examined the gross morphology of the major forebrain axon pathways at early points in development when these projections are being established, taking advantage of the fact that the neurofilament (NF) protein antibody RMO270 allows detection of a subset of mature axons and axonal bundles within the developing CNS (Lee et al., 1987). In wild-type mice (n = 3), a few fascicles of cortical axons had reached the internal capsule at E14.5 (Figure 2A). Perhaps surprisingly, given the pattern of *Slit1* expression, NF staining showed roughly normal axonal pathways in the telencephalon of *Slit1* mutant mice (n = 4) at E14.5 (data not shown). In contrast, similar analysis of NF staining in *Slit2* mutant mice (n = 6) revealed severe abnormalities in the trajectories of axons within the internal capsule. NF+ axons in the internal capsule of *Slit2* mutants were displaced ventrally when compared to control littermates, and thick fascicles of NF+ axons were found in an abnormal position close to the ventral surface of the basal telencephalon (Figures 2A and 2B). In addition, some NF+ fibers inappropriately approached the midline in *Slit2* mutants (Figure 2B and data not shown). To assess whether these defects persisted at later stages, we analyzed the expression of NF and other axonal markers at E18.5 (n = 5; *Slit2* mutants die at birth). Immunohistochemistry for NF (data not shown) and calbindin (Figures 2D and 2E) revealed that numerous axons coursing through the internal capsule descended abnormally in a ventromedial direction, crossing the midline at the level of the hippocampal commissure, then traveling ventral to the anterior commissure, which was displaced dorsally. This defect is most prominent at levels caudal to the hippocampal commissure (Figure 2E). In addition, analysis of sections stained with antibodies against calretinin (n = 4) revealed that numerous unstained fibers aberrantly leave the internal capsule at more caudal levels, coursing toward the ventral surface of the telencephalon (Figure 2G and 2H).

Since *Slit1* and *Slit2* have distinct patterns of expression in the developing forebrain, we asked whether loss of both *Slit1* and *Slit2* could result in additional axon guidance defects. As in *Slit2* mutants, NF staining demonstrated that fibers in the internal capsule of *Slit1*;*Slit2* double mutants run more ventral than normal at E14.5 (n = 5). In *Slit1*;*Slit2* mutants, however, most NF+ fibers were dramatically directed toward the midline, and only some NF+ axons projected ventrally (Figures 2A and 2C). In line with these observations, large fascicles of fibers crossed the midline at the level of the anterior commissure at E18.5 (n = 7; Figures 2D and 2F), whereas few fibers were observed to course ventrally at more caudal telencephalic levels (Figures 2G and 2I). As with the *Slit2* mutant, the axon bundle crossing the midline was most prominent at levels caudal to the hippocampal commissure. In sum, loss of *Slit2* or simultaneous loss of *Slit1* and *Slit2* results in abnormal axonal trajectories in the ventral telencephalon. Since the affected axons course through the internal capsule, they likely represent the developing corticofugal projections.

Guidance of Corticofugal Projections Is Impaired in the Telencephalon of *Slit2* and *Slit1;Slit2* Double Mutants

To verify that these abnormally projecting axons represent corticofugal axons, we traced the trajectory of cortical axons by placing crystals of DiI in the developing cortex. At E14.5, DiI injections in the parietal cortex of wild-type mice (n = 6) revealed a thick bundle of labeled axons grouped in the internal capsule, which transverse the developing striatum while progressing caudally toward the diencephalon (Figure 3A). As expected from NF staining, DiI placements in the cortex of *Slit1* mutants (n = 4) revealed no prominent defects in corticofugal projections (data not shown). On the other hand, whereas DiI injections in the cortex of *Slit2* mutants (n = 6) at E14.5 revealed that cortical projections extend normally to the internal capsule (Figure 3D), two major guidance defects were observed as the corticofugal axons grew past the internal capsule toward the diencephalon. First, some cortical fibers abnormally approached the midline and crossed it at the approximate level of the prospective anterior commissure (Figure 3D). Second, at more caudal telencephalic levels, a large bundle of cortical axons coursed toward the ventral surface to the base of the brain (Figure 3G). Injections of DiI crystals placed in the parietal cortex of *Slit2* mutant mice at E18.5 (n = 8) revealed even more dramatic defects. As observed at E14.5, many labeled cortical axons aberrantly projected ventrally into the medial preoptic region (Figures 3H and 3I) and other axons, at the level of the anterior commissure, ran ventrally and crossed the midline (Figures 3E and 3F). After crossing the midline, these cortical axons either fasciculated with the contra-

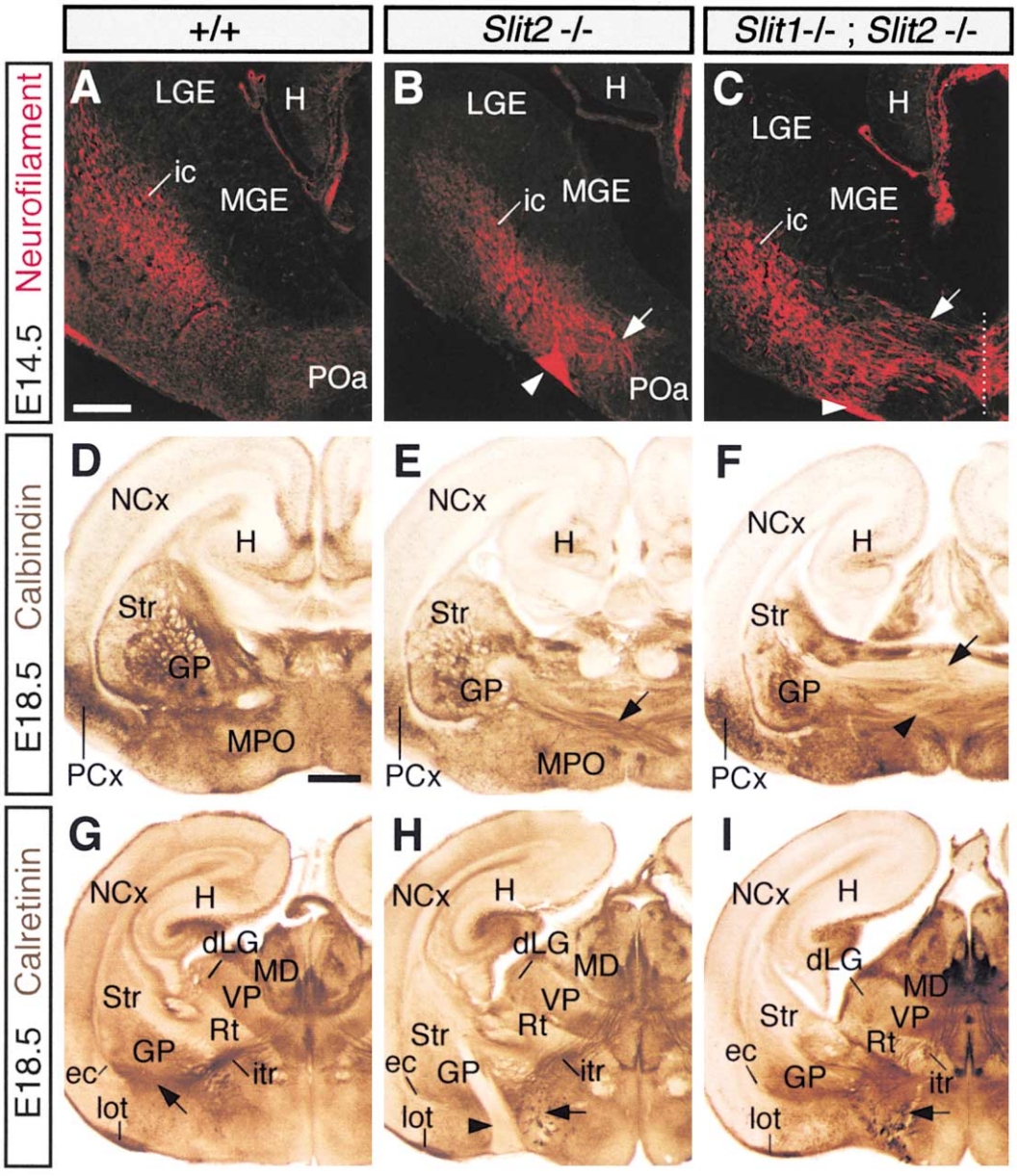

Figure 2. Abnormal Axonal Trajectories in the Telencephalon of *Slit* Mutant Mice

Coronal sections through the telencephalon of E14.5 embryos (A–C) and E18.5 fetuses (D–I) showing neurofilament (NF) (A–C), calbindin (D–F), and calretinin (G–I) immunohistochemistry in wild-type (A, D, and G), *Slit2* (B, E, and H), and *Slit1;Slit2* (C, F, and I) mutant mice.

(A–C) In wild-type embryos, NF+ axons are confined to the internal capsule (ic, [A]). In *Slit2* (B) and *Slit1;Slit2* (C) mutants, the internal capsule is ventrally displaced. Additionally, NF+ fascicles approach the ventral surface of the telencephalon in *Slit2* mutants ([B], arrowhead) or cross the midline in *Slit1;Slit2* mutants ([C], arrow). Some NF+ axons are also directed toward the midline in *Slit2* mutants ([B], arrow). The midline is indicated with a doted line in (C).

(D–F) Immunohistochemistry for calbindin (D–F) demonstrates abnormal bundles of fibers crossing the midline at the level of the hippocampal commissure (HC) (D–F) and at the level of the preoptic region (POa) in *Slit2* mutants ([E], arrows) and in *Slit1;Slit2* mutants ([F], arrows). This defect is very prominent in the preoptic region of *Slit1;Slit2* mutants, where a large ectopic commissure containing calbindin+ fibers (arrowhead) and unstained fibers (arrow) is found.

(G–I) Immunohistochemistry for calretinin shows the wild-type (G) trajectory of the inferior thalamic radiation (itr) as it travels ventro-laterally from the dorsal thalamus, to the globus pallidus (GP), turning to laterally project through the external capsule (ec), and the abnormal trajectory in axons of the itr as they course ventrally in *Slit2* mutants ([H], arrow) and in *Slit1;Slit2* mutants ([I], arrow). A large bundle of unstained fibers is also observed descending to the ventral surface of the telencephalon in *Slit2* mutants ([H], arrowhead). Note that the position of the lateral olfactory tract (LOT) appears to be roughly comparable in all three genotypes (G–I).

H, hippocampus; MGE and LGE, medial and lateral ganglionic eminence; MPO, medial preoptic area; NCx, neocortex; PCx, piriform cortex; Rt, reticular thalamic nucleus; Str, striatum; VP, ventral posterolateral thalamic nucleus. Scale bar, 100 μm (A–C), 500 μm (D–L).

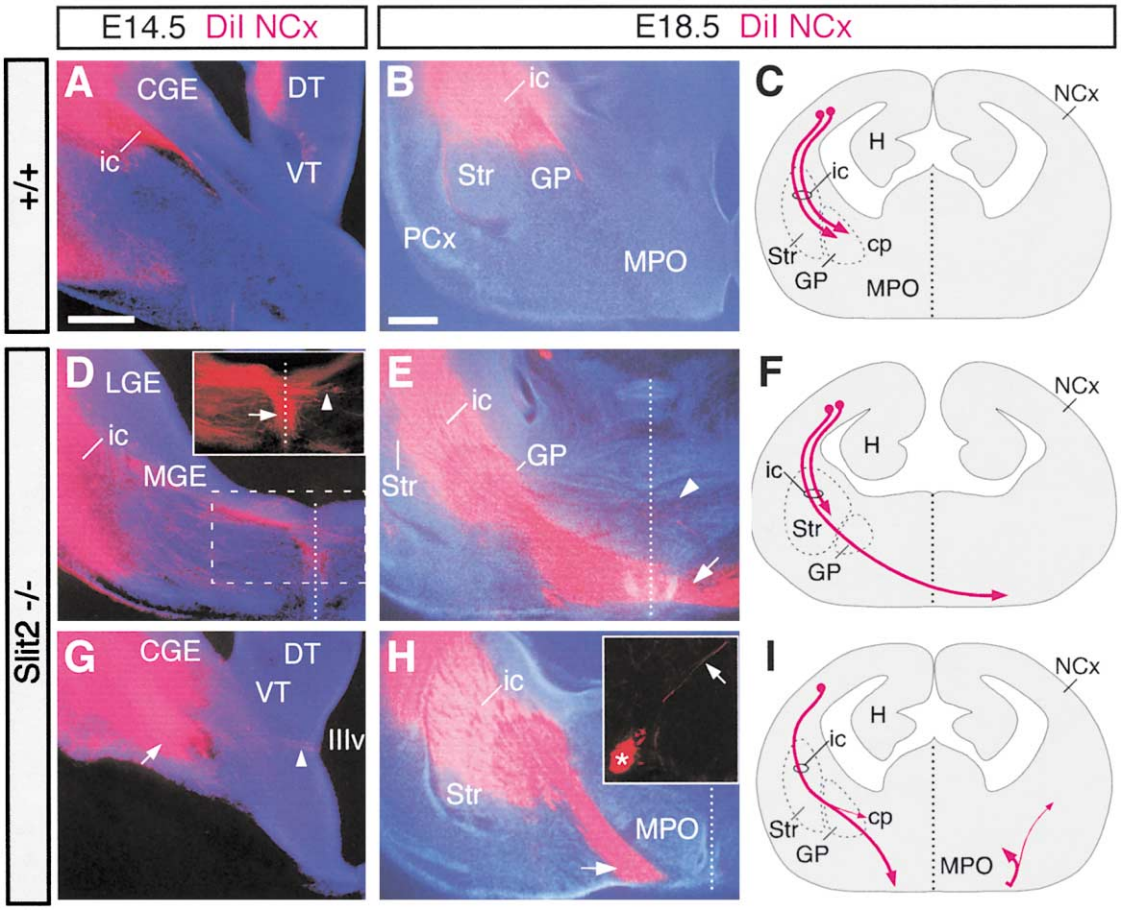

Figure 3. Corticofugal Projections Follow Abnormal Paths in the Telencephalon of *Slit2* Mutants

Coronal sections through the telencephalon of E14.5 embryos (A, D, and G) and E18.5 fetuses (B, E, and H) with DiI implanted in the neocortex (NCx), showing computer-generated overlays of DiI labeled corticofugal axons and bisbenzimide counterstain from wild-type (A and B) and *Slit2* mutants (D, E, G, and H). The midline is indicated with a dotted line. The schemas summarize the results obtained in control (C) and *Slit2* mutants (F and I).

(A–C) In wild-type mice, labeled axons extend from the cortex into the internal capsule (ic).

(D and G) In *Slit2* mutants at E14.5, labeled axons from the internal capsule (ic) abnormally approach the midline and cross it ([D], arrowhead in inset). A few axons that reach the midline course ventrally ([D], arrow). Caudally, a large bundle of labeled fibers project ventrally to the base of the telencephalon ([G], arrow), whereas some axons approach the midline of the diencephalon ([G], arrowhead).

(E, F, H, and I) Similar axonal defects are found at E18.5, with corticofugal axons either crossing the midline ([E], arrow) or descending to the ventral surface of the telencephalon ([H], arrow). Some of the axons that crossed the midline at more anterior levels are found in the contralateral side ([H], inset), where they either travel to the base of the telencephalon or extend toward the contralateral cortex ([H], arrowhead in inset).

CGE, MGE, and LGE, caudal, medial, and lateral ganglionic eminence; cp, cerebral peduncle; DT, dorsal thalamus; GP, globus pallidus; MPO, medial preoptic area; PCx, piriform cortex; Str, striatum; VT, ventral thalamus; IIIv, third ventricle. Scale bars, 300 μm (A, D, and G), 500 μm (B, E, and H).

lateral internal capsule and traveled caudally toward the diencephalon in the cerebral peduncle, or grew dorsally toward the contralateral cortex (Figures 3E, 3H, and 3I). Nevertheless, in all cases, some cortical axons were found to leave the telencephalon on both the ipsilateral and contralateral sides of the brain.

DiI injections into the cortex of *Slit1*;*Slit2* double mutants (n = 8) also revealed prominent guidance defects in corticofugal axons within the telencephalon (Figure 4 and data not shown). As in the case of *Slit2* mutants, cortical axons projected normally to the internal capsule in *Slit1*;*Slit2* double mutants. As predicted from the analysis of NF staining (Figure 2), most cortical axons were subsequently directed toward the midline, which they abnormally crossed (Figure 4C). However, a small component of the aberrant fibers projected to the ventral surface of the brain (Figure 4E). Interestingly, after crossing the midline, many DiI labeled axons appeared to turn around and return to the midline (Figure 4D). DiI injections in the cortex also labeled a few axons that did not decussate in the telencephalon and followed their normal course toward the diencephalon (Figure 4E, arrowhead).

Abnormal Cerebral Peduncle and Corticothalamic Projections in *Slit2* and *Slit1*;*Slit2* Mutants

Despite the major axon guidance defects observed in the telencephalon, DiI experiments revealed that some corticofugal fibers within the internal capsule extended caudally into the diencephalon in both *Slit2* and

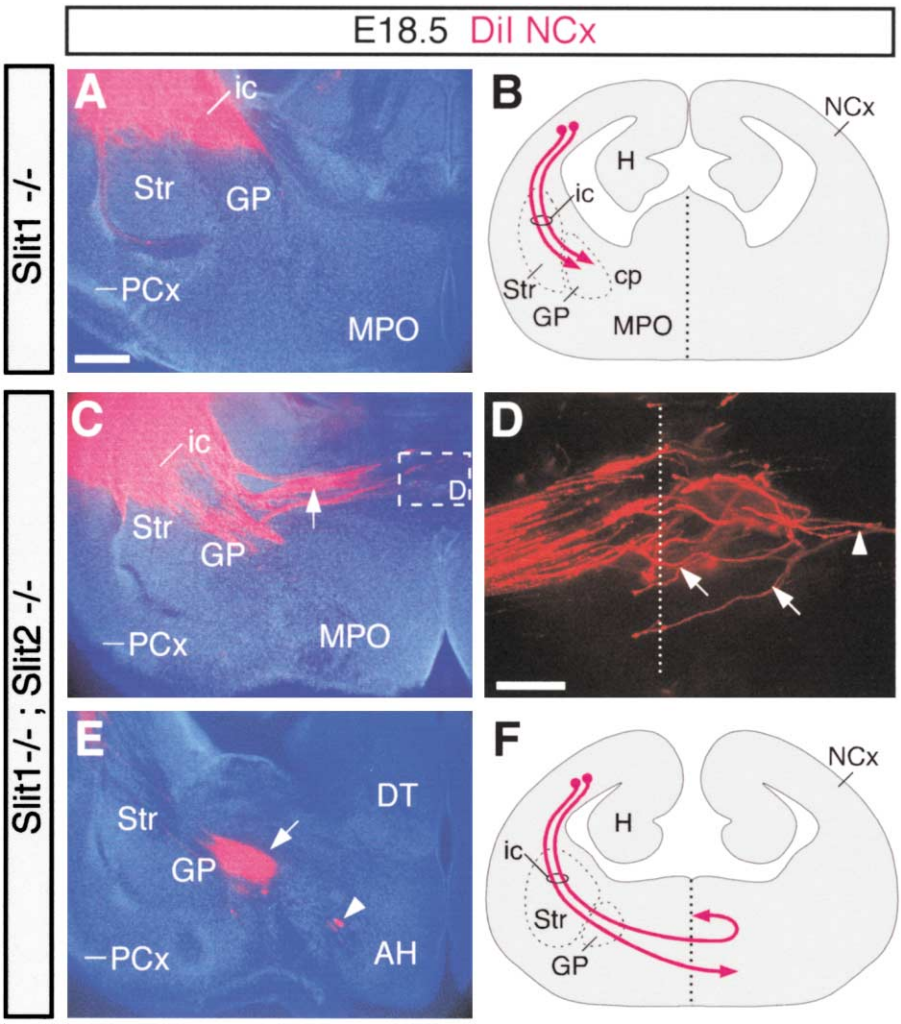

Figure 4. Corticofugal Projections Follow Abnormal Paths in the Telencephalon of *Slit1;Slit2* Mutants

Coronal sections through the telencephalon of E18.5 fetuses (A, C, and E) with DiI implanted in the neocortex (NCx), showing computer-generated overlays of DiI labeled corticofugal axons and bisbenzimide counterstain from *Slit1* (A) and *Slit1;Slit2* mutants (C and E). The midline is indicated with a dotted line. The schemas summarize the results obtained in *Slit1* (B) and *Slit1;Slit2* mutants (F). (A) In *Slit1* mutant mice, labeled axons extend from the cortex into the internal capsule (ic) normally. (C and E) In *Slit1;Slit2* mutants, most labeled axons abnormally approach the midline and cross it in the ventral regions ([C], arrow). Many axons that cross the midline turn around soon after crossing and return toward the midline ([D], arrows), whereas a few axons continue extending away from the midline ([D], arrowhead). Some labeled axons proceed normally to the telencephalic/diencephalic boundary ([E], arrow), where a few abnormally enter the anterior hypothalamus (AH) ([E], arrowhead).

DT, dorsal thalamus; GP, globus pallidus; H, hippocampus; MPO, medial preoptic area; PCx, piriform cortex; Str, striatum. Scale bar, 500 μm (A, C, and E), 100 μm (D).

Slit1;*Slit2* mutant mice (data not shown). Immunohistochemistry against NF, however, demonstrated that corticothalamic projections in *Slit2* mutants follow a trajectory more ventral than normal (Figures 5A, 5B, 5G, and 5H). A similar conclusion was reached from the analysis of sections stained with antibodies against NPY (wild-type, n = 2; *Slit2* mutant, n = 3), a transient marker of a subset of corticothalamic axons (Figures 5D, 5E, 5G, and 5H).

The cerebral peduncle in *Slit2* mutants was always displaced ventrally when compared to that of wild-type mice (Figures 7A, 7B, 7D, and 7E and data not shown). In some cases, NF+ axons were observed to leave the cerebral peduncle and abnormally travel ventrally into the hypothalamus (data not shown). DiI labeling was never seen at levels caudal to the hypothalamus (n = 8), suggesting that all corticospinal axons exited the cerebral peduncle by the level of the hypothalamus.

Analysis of the destination of corticofugal projections in *Slit1*;*Slit2* mutants (n = 3) revealed similar but more dramatic phenotypes than those found in *Slit2* mutants. Immunohistochemistry against NF or NPY revealed that the cortical projections that reached the thalamus followed an unusual caudal and ventral trajectory before they turned into the dorsal thalamus (Figures 5C, 5F, and 5I). The small number of fibers exiting the telencephalon precluded analysis of fibers in the cerebral peduncle of these double mutants.

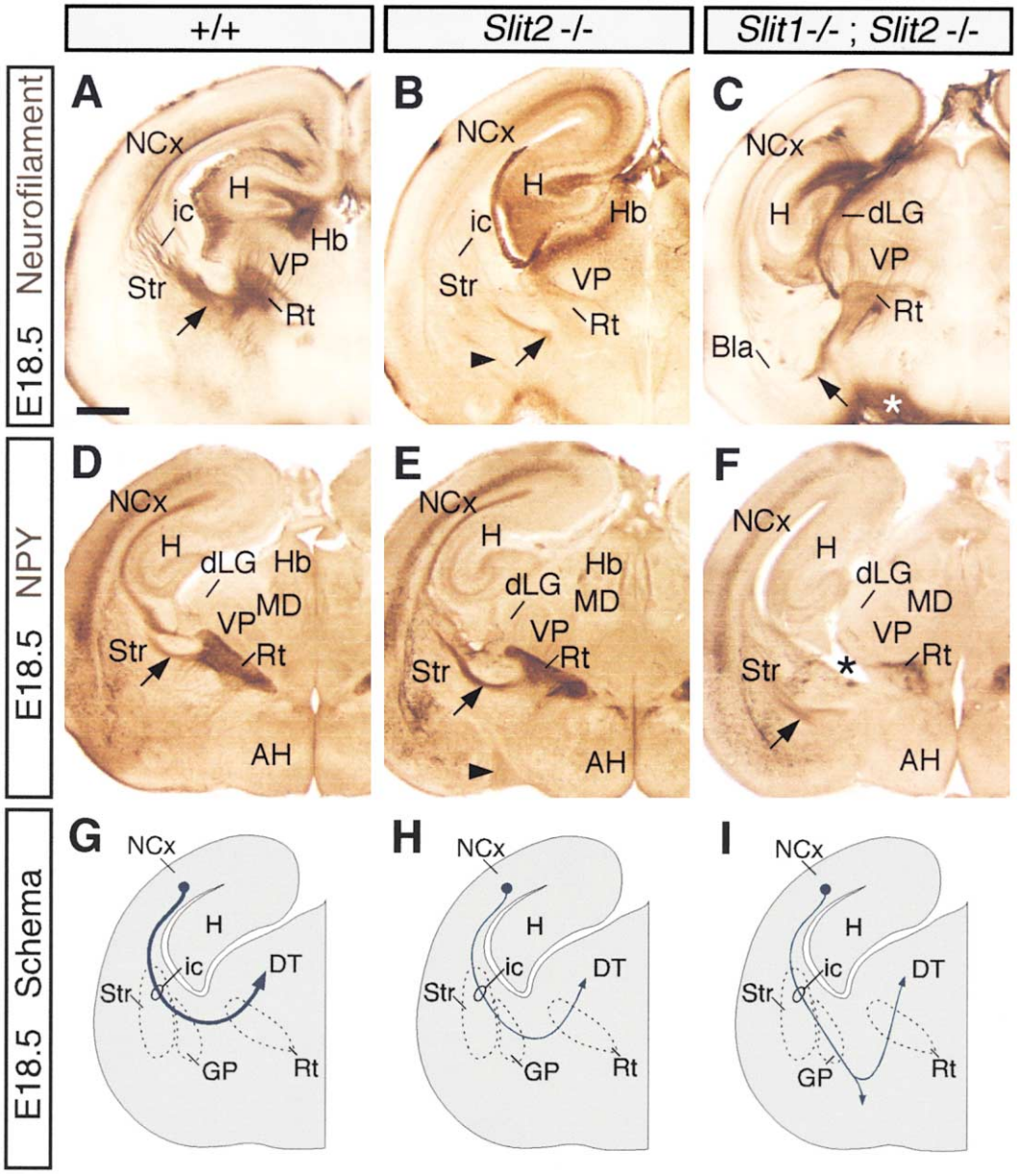

Figure 5. Corticothalamic Projections Follow Abnormal Paths in Slit Mutants

(A–F) Coronal sections through the dorsal thalamus of E18.5 fetuses showing neurofilament (NF) (A–C) and neuropeptide Y (NPY) (D–F) immunohistochemistry in wild-type (A and D), *Slit2* (B and E), and *Slit1;Slit2* (C and F) mutant mice. Both NF and NPY immunohistochemistry demonstrate that some cortical axons reach the dorsal thalamus in both *Slit2* (B and E) and *Slit1;Slit2* (C and F) mutants, although the paths they follow are abnormally ventral and caudally extended (arrows). Furthermore, in *Slit2* mutants (E) the ventrally projecting bundle of axons is weakly NPY+ (arrowhead). In *Slit1;Slit2* (C and F) mutants, this defect is very prominent, and corticothalamic axons reach very caudal and ventral telencephalic levels before most turn into the diencephalon (C and F). Additionally, some fibers fail to turn dorsally and instead project into the anterior hypothalamus (AH) ([F], arrow).

(G–I) The schemas summarize the pathway followed by corticothalamic axons in wild-type (G), *Slit2* (H), and *Slit1;Slit2* (I) mutant mice.

Cp, cerebral peduncle; dLG and vLG, dorsal and ventral lateral geniculate nucleus; H, hippocampus; Hb, habenula; ic, internal capsule; Mea, medial amygdaloid nucleus; Po, posterior thalamic nucleus; RT, reticular thalamic nucleus; Str, striatum; VP, ventral posterolateral thalamic nucleus. Scale bar, 500 μm.

Slit2 Is Required for Guidance of Corticocortical Projections at the Corpus Callosum

We next examined whether Slit proteins, particularly *Slit2*, might also influence the guidance of corticocortical projections, specifically those coursing through the corpus callosum. *Slit2* is expressed in two glial populations (glial wedge, GW; indusium griseum, IG) in the region where the corpus callosum forms (Shu and Richards, 2001, and data not shown). Further, in vitro studies have shown that *Slit2* can repel outgrowing cortical axons, leading to the suggestion that *Slit2* secreted from the GW and IG may play a role in the guidance of cortico-

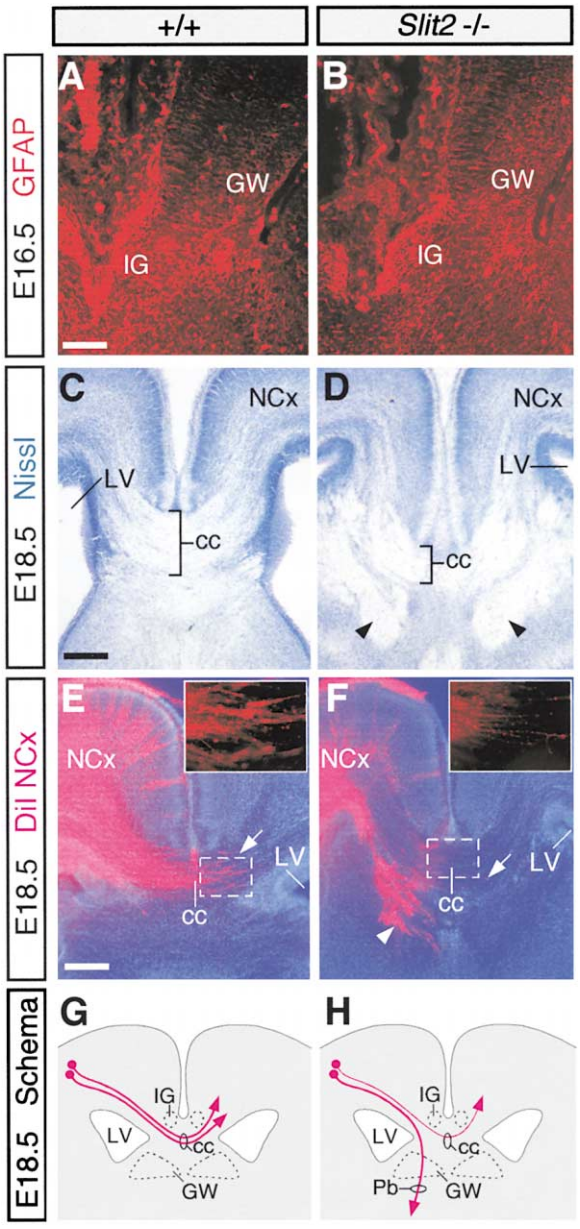

Figure 6. Abnormal Development of the Corpus Callosum in *Slit2* Mutants

Coronal sections through the telencephalon of E16.5 (A and B) and E18.5 (C and D) fetuses showing GFAP (A and B) and neurofilament (C and D) immunohistochemistry in wild-type (A and C) and *Slit2* (B and D) mutant mice.

(A and B) Organization of the glial wedge (GW) and the indusium griseum (IG), is normal in *Slit2* mutants (B).

(C and D) Nissl staining demonstrates that *Slit2* mutant mice have a very small corpus callosum (cc) and that large ectopic bundles of axons form on either side of it, resembling Probst bundles (Pb; arrowheads).

(E and F) Coronal sections through the telencephalon of E18.5 fetuses with DiI implanted in the neocortex (NCx), showing DiI labeled corticocortical axons extending through the corpus callosum in wild-type (E) and *Slit2* (F) mutants. Note that the number of labeled axons crossing in the corpus callosum is dramatically reduced in *Slit2* mutants ([D], arrow), and that most labeled axons are abnormally directed ventrally before they reach the midline ([D], arrowhead). The insets in (C) and (D) show a high magnification view of the axons crossing the midline. The schemas summarize the pathways followed by corticocortical axons through the corpus callosum in wild-type (G) and *Slit2* (H) mutant mice.

LV, lateral ventricle. Scale bar, 150 μm (A and B), 300 μm (C–F).

cortical axons across the corpus callosum (Shu and Richards, 2001). Since these glial populations may also constitute a physical barrier that helps to direct the trajectory of corticocortical axons across the corpus callosum, we first examined whether there were any gross abnormalities in their location or organization. GFAP immunohistochemistry at E16.5 (when the corpus callosum is forming) revealed indistinguishable position, size and structure of these glial populations between wild-type and *Slit2* mutants (Figures 6A and 6B), suggesting that their development is not sensitive to the loss of *Slit2*. To analyze the development of the corpus callosum, we next examined Nissl stained sections. At E18.5, the size of the corpus callosum was severely reduced in *Slit2* mutant mice compared to wild-type mice (n = 6; Figures 6C and 6D). In addition, two large ectopic bundles of fibers were also found at either side of the corpus callosum, resembling Probst bundles (Figure 6D). These ectopic bundles were also observed in sections stained with antibodies against NF, which normally labels a subpopulation of axons in the corpus callosum (data not shown). DiI injections in the cortex confirmed these observations. In wild-type mice (n = 4), DiI-labeled cortical axons formed a single bundle that crossed toward the contralateral cortex in the corpus callosum. In their pathway, corticocortical axons avoided the GW or the IG (Figures 6E and 6G). In contrast, only a few labeled axons were found to cross the midline through the corpus callosum in *Slit2* mutant mice (n = 5). Instead, most DiI labeled axons approached the midline but failed to maintain their normal position, traversing the GW and coursing ventrally into Probst bundles (Figures 6F and 6H). DiI placements in different cortical regions of *Slit2* mutants consistently labeled a few fibers crossing the corpus callosum, suggesting that the abnormal corpus callosum contains axons from all cortical regions (data not shown).

Guidance Defects in Thalamocortical Projections of *Slit2* and *Slit1*;*Slit2* Mutants

Since the development of corticothalamic and thalamocortical projections is highly coordinated (Adams et al., 1997; Miller et al., 1993; Metin and Godement, 1996; Auladell et al., 2000; Molnar et al., 1998; Skaliora et al., 2000; Kim et al., 1991; McConnell et al., 1989), we next analyzed thalamocortical axon trajectories in the *Slit* mutant mice. In agreement with previous reports, DiI injections in the parietal cortex of wild-type mice at E18.5 resulted in the labeling of the somatosensory nuclei of the dorsal thalamus (Figure 7A; n = 4). The labeling observed in the dorsal thalamic nuclei corresponds to both retrogradely labeled thalamic cells (thalamocortical projecting neurons) and anterogradely labeled fibers (corticothalamic projections). Similar results were obtained after DiI placement in the cortex of *Slit1* mutants (n = 3; data not shown). In contrast, comparable DiI injections in the parietal cortex of *Slit2* (n = 8) and

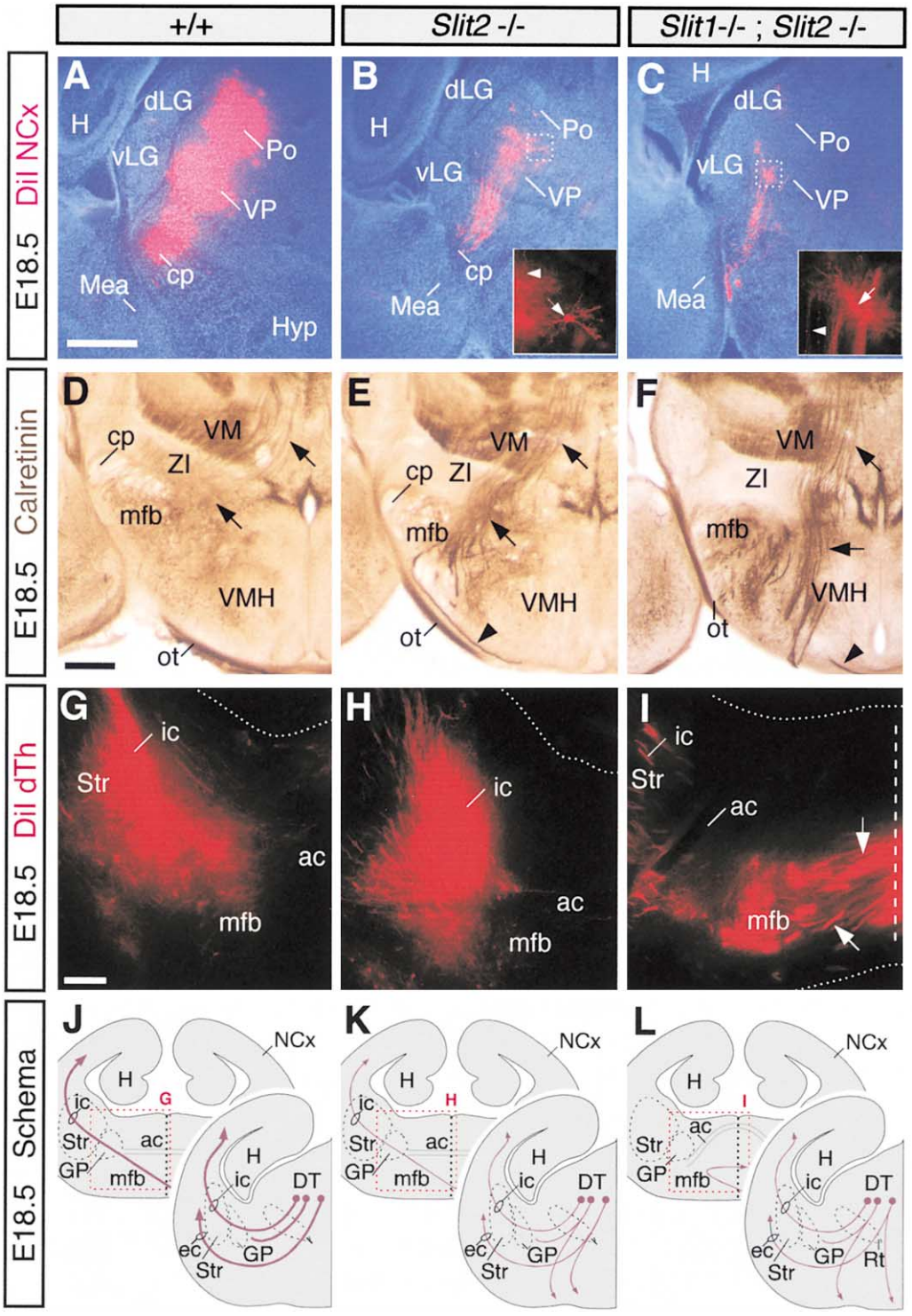

Figure 7. Thalamocortical Axons Follow Abnormal Paths in *Slit* Mutants

(A–C) Coronal sections through the dorsal thalamus of E18.5 embryos with DiI implanted in the neocortex (NCx), showing computer-generated overlays of DiI retrogradely labeled cells and bisbenzimide counterstain from wild-type (A), *Slit2* (B), and *Slit1;Slit2* mutants (C). The number of retrogradely labeled cells found in the dorsal thalamus after injection of DiI in the neocortex is greatly reduced in both *Slit2* and *Slit1;Slit2* mutants. Examples of some of these cells are shown in the insets in (B) and (C).

(D–F) Coronal sections through the diencephalon of E18.5 fetuses showing calretinin immunohistochemistry in wild-type (D), *Slit2* (E), and *Slit1;Slit2* (F) mutant mice. (D) In wild-type mice, axons of the inferior thalamic radiation (arrows) course laterally and then leave the plane of the section to travel rostrally into the telencephalon. In *Slit2* mutants (E) and, more prominently, in *Slit1;Slit2* mutants (F), axons proceed along a more ventral course, abnormally enter the hypothalamus (arrows) and turn toward the midline (arrowheads).

(G–I) Coronal sections through the telencephalon of E18.5 fetuses with DiI implanted in the dorsal thalamus, showing DiI labeled thalamocortical

Slit1;*Slit2* (n = 3) mutants resulted in a prominent reduction in the labeling of somatosensory nuclei in the dorsal thalamus (Figures 7B and 7C). This phenotype is likely to be due to a reduction in both anterogradely labeled fibers (corticothalamic projections) and retrogradely labeled cells (thalamocortical projecting neurons), although limitations in the ability to quantify DiI tracing experiments prevent a more definitive analysis of this phenotype.

Further analysis using calretinin immunohistochemistry to label thalamocortical projections from midline thalamic nuclei confirmed and extended these observations (Figures 2G–2I). In control mice (n = 3), midline thalamic nuclei give rise to the inferior thalamic radiation, which carries fibers that reach the cortex through both the external and internal capsules (Figures 2G and 7J). In *Slit2* mutants (n = 4), numerous calretinin positive fibers traveling in the inferior thalamic radiation failed to appropriately enter the telencephalon, and never reached the cortex (Figure 2H). Instead, most of these fibers turned ventrally and caudally to enter the hypothalamus (Figures 7E and 7K), a region normally inhibitory to thalamic axon outgrowth (Braisted et al., 1999). This defect was more prominent in *Slit1*;*Slit2* double mutants (n = 4), where a large number of calretinin-positive fibers project ventrally, directly from the thalamus into the hypothalamus (Figures 2I, 7F, and 7L). Further, once in the hypothalamus, many of these fibers turned medially and approached the midline (Figure 7F).

DiI injections in the dorsal thalamus in both *Slit2* and *Slit1*;*Slit2* mutants confirmed that a large number of thalamocortical projections failed to enter the telencephalon and instead descended into the hypothalamus (data not shown). Nevertheless, DiI placement in the dorsal thalamus of *Slit2* mutants demonstrated that some thalamocortical projections were directed appropriately toward the internal capsule and from there into the cortex (Figures 7H and 7K). DiI injections into the dorsal thalamus of *Slit1*;*Slit2* mutants demonstrated that a large number of thalamocortical fibers were also able to reach the telencephalon. However, once in the telencephalon, most of these fibers failed to reach the internal capsule, turning instead abruptly toward the midline (Figures 7I and 7L).

In summary, loss of *Slit2* and, more so, *Slit1* and *Slit2*, results in a smaller connection between the thalamus and the cortex when compared to the wild-type thalamocortical pathway. In the *Slit2* mutant, thalamocortical axons proceed along a more ventral course to enter the cortex. In *Slit1*;*Slit2* double mutants, axons not only follow a more ventral trajectory, but also travel toward the midline.

Abnormal Guidance of Ascending Serotonergic and Dopaminergic Fiber Systems in the Forebrain of *Slit2* and *Slit1*;*Slit2* Mutants

Our previous experiments have demonstrated that a large number of corticofugal and thalamocortical axons fail to follow their normal paths in mice lacking Slit proteins. Interestingly, pathfinding errors are seen in both ascending (i.e., thalamocortical) and descending (i.e., corticofugal) projections at similar anatomic locations. These observations raised the question whether expression of Slit proteins in these regions might also affect the trajectories of other axon tracts, in particular those whose origins lie outside the forebrain but whose tracts course longitudinally through forebrain regions. To address this question, we focused our analysis on two of the major ascending fiber systems that innervate the forebrain, the serotonergic (5-HT) projections from the raphe nuclei and the dopaminergic projections from the substantia nigra/ventral tegmental area complex (Nieuwenhuys, 1985). To analyze the distribution of these fiber systems during development, we used antibodies against 5-HT or against tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis, which primarily labels dopaminergic fibers. In normal mice, fibers from both ascending systems coursed through the medial forebrain bundle, which lies slightly medial to the cerebral peduncle. Once in the telencephalon, serotonergic and dopaminergic projections remain largely ipsilateral and diverge to innervate multiple subcortical and cortical targets. When compared to control mice (n = 5; Figures 8A and 8C and data not shown), both 5-HT+ and TH+ fibers in the medial forebrain bundle of *Slit2* mutants (n = 4) were displaced ventrally as they coursed through the diencephalon (data not shown). In *Slit1*;*Slit2* double mutants (n = 4), the medial forebrain bundle was commonly split in two components and numerous fibers descended ventrally into the hypothalamus, approaching the midline (Figure 8D and data not shown). Although many 5-HT+ and TH+ fibers entered the telencephalon normally, in *Slit1*;*Slit2* mutants a significant percentage abnormally crossed the midline in the basal telencephalon (Figure 8B and data not shown). These defects were readily apparent at E14.5, suggesting that loss of Slit function affects the development of the serotonergic and dopaminergic systems as they course rostrally into the forebrain (Figures 8A and 8B and data not shown).

Discussion

The cortex makes connections with subcortical regions and the contralateral cortex via the corticofugal and

axons from wild-type (G), *Slit2* (H), and *Slit1;Slit2* mutants (I). Note that whereas in *Slit2* mutants thalamocortical axons normally extend through the internal capsule (ic) on their way to the cortex (G), many labeled axons fail to do so in *Slit1;Slit2* mutants (I), abnormally crossing the midline (arrows). The location of (G)–(I) is indicated with red-dashed boxes in the schemas shown in (J)–(L), respectively. Furthermore, the anterior commissure ([I], ac) is dorsally displaced due to the abnormal crossing of multiple fiber systems at this level. The schemas summarize the pathways followed by thalamocortical axons in wild-type (J), *Slit2* (K), and *Slit1;Slit2* (L) mutant mice. The caudal level schematized in (J)–(L) represent the level shown in (D)–(F), whereas the rostral level schematized in (J)–(L) represent the level shown in (G)–(I).

Ac, anterior commissure; cp, cerebral peduncle; mfb, medial forebrain bundle; ot, optic tract; Str, striatum; VH, ventral hypothalamus; VMH, ventromedial hypothalamic nucleus; ZI, zona incerta. Scale bar, 300 μm (A–C), 200 μm (D–F).

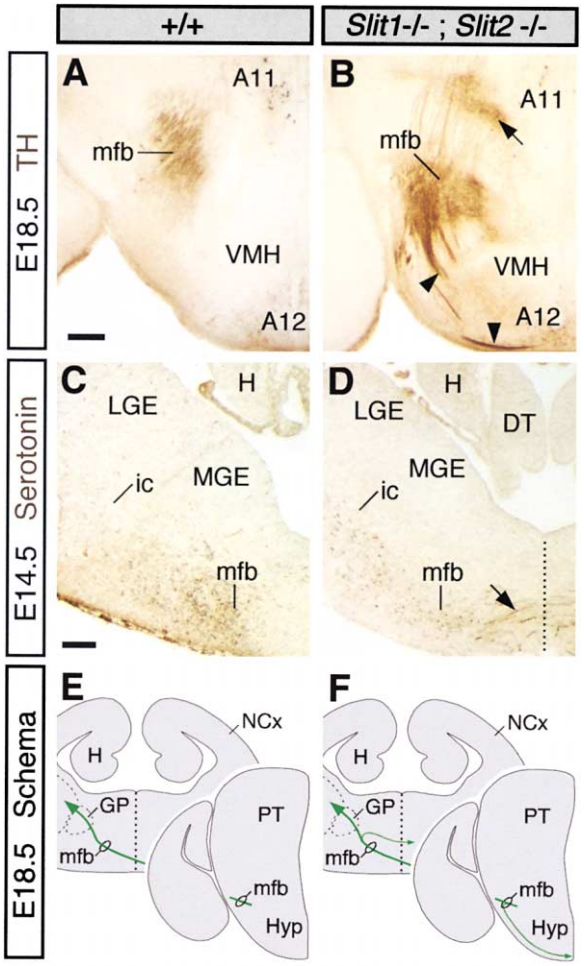

Figure 8. Ascending Serotonergic and Dopaminergic Fiber Systems Follow Abnormal Paths in the Forebrain of Slit Mutants

Coronal sections through the diencephalon of E18.5 fetuses showing tyrosine hydroxylase (TH) immunohistochemistry (A and B) and through the caudal telencephalon of E14.5 embryos showing serotonin immunohistochemistry (C and D) in wild-type (A and D) and *Slit1;Slit2* (B and D) mutant mice.

(A and B) TH immunohistochemistry to label the ascending dopaminergic pathway shows fibers coursing through the medial forebrain bundle (mfb), which lies slightly medial to the cerebral peduncle in normal mice (A). TH+ fibers in the medial forebrain bundle of *Slit1*;*Slit2* double mutants were displaced ventrally as they coursed through the diencephalon (B). Additionally, the medial forebrain bundle was commonly split in two components and numerous fibers traveled ventrally into the hypothalamus.

(C and D) Serotonin staining in the telencephalon shows that ascending serotonergic projections remained largely ipsilateral and diverged to innervate multiple subcortical and cortical targets (C). In *Slit1*;*Slit2* mutants a significant percentage abnormally crossed the midline in the basal telencephalon (D).

(E and F) The schemas summarize the pathway followed by these ascending axons in wild-type (E) and *Slit1;Slit2* (F) mutant mice.

A11 and A12, A11 and A12 dopaminergic cell groups; DT, dorsal thalamus; GP, globus pallidus; H, hippocampus; Hyp, hypothalamus; ic, internal capsule; MGE and LGE, medial and lateral ganglionic eminence; VMH, ventromedial hypothalamic nucleus. Scale bar, 300 μm.

callosal projections and receives its main input from the thalamus via the thalamocortical projections. Due to the functional importance of these major CNS axonal pathways, the mechanisms underlying the development of these connections are of significant interest. Although several studies have examined the early development of these projections and have identified potential intermediate targets, guidance decision points and pioneering axonal populations (Auladell et al., 2000; Metin and Godement, 1996; Mitrofanis and Baker,1993; Molnar et al., 1998; Molnar and Blakemore, 1995; Molnar and Cordery, 1999; Murray and Whitington, 1999; McConnell et al., 1989), little is known about the molecular nature of the cues that guide these axons as they form their complex trajectories. In vitro experiments have demonstrated that cortical axons are repelled by both *Slit2* (Shu and Richards, 2001) and *Slit1* (K. Whitford and A. Ghosh, personal communication) and that thalamocortical axons are also repelled by *Slit2* (D. O'Leary, submitted), raising the possibility that *Slits* play important roles in corticofugal and thalamocortical development. Here we provide in vivo evidence that Slit proteins are indeed key regulators of guidance of corticofugal, callosal, thalamocortical, serotonergic, and dopaminergic projections in the embryonic forebrain. The similarities in the types of defects observed in several disparate pathways leads us to propose that Slit proteins are used in at least three different ways in the mammalian forebrain: (1) in the maintenance of dorsal position by the prevention of axonal entry into ventral regions; (2) in the prevention of axonal extension toward and across the midline; and (3) in channeling axons into particular regions.

Maintenance of Dorsal Position by Prevention of Axonal Entry into Ventral Regions

Analysis of *Slit2* mutant mice revealed striking defects in the position of numerous tracts, which project into ventral regions that they normally avoid. This phenotype is most easily illustrated using the corticospinal tract. In comparing *Slit2* mutants with wild-type animals, the internal capsule, normally located in the developing striatum, is ventrally displaced. Many axons leave the internal capsule abnormally to descend to the ventral surface of the caudal telencephalon rather than entering the cerebral peduncle, which is also displaced ventrally. Additionally, axons exit the cerebral peduncle to enter the hypothalamus. A similar constellation of abnormal ventral projections can also be seen in corticothalamic, thalamocortical, and the ascending serotonergic and dopaminergic projections.

It is unlikely that the abnormal ventral projection of axons occurs simply due to a loss in the ability of axons to remain fasciculated with the main bundle, since it can be seen at very early development times (E14.5), when the tracts are beginning to form and have not yet become highly fasciculated. Furthermore, the axons that leave their tracts to descend ventrally do so in large, tightly fasciculated bundles, not as single fibers. Many different pioneer axonal populations have been implicated in the establishment of these pathways (Auladell et al., 2000; Metin and Godement, 1996; Mitrofanis and Baker, 1993; Molnar and Cordery, 1999; Tuttle et al., 1999; McConnell et al., 1989), and since some of the pioneer neurons express Robo receptors (Figure 1 and data not shown), we cannot entirely rule out the possibility that the defects seen in the tracts may occur second-

arily to the improper differentiation and/or migration of one or more of these pioneer populations. However, our data (calbindin immunohistochemistry and DiI tracing at E14.5) suggest that at least some of the pioneer populations are present and develop normally. A more detailed analysis of these populations will be necessary to rule out this possibility for all the fiber tracts discussed. Despite this residual uncertainty, we favor a model in which Slit2 sets the dorsal position of axons by preventing their entry into ventral regions. It is important to note that *Slit1* must also be able to serve this function at least partly, since some thalamocortical axons in *Slit1*;*Slit2* double mutant mice travel even further ventrally than in the *Slit2* mutant mice before turning to enter the internal capsule. Finally, by analogy with the graded function of *Slit* in positioning longitudinal axonal tracts in *Drosophila* (Rajagopalan et al., 2000; Simpson et al., 2000), it is tempting to speculate that the different dorso-ventral positions normally adopted by the different brain tracts that are affected in mammalian *Slit* mutants could result from differential sensitivities of these fibers to a *Slit2* gradient, a possibility for which we do not, however, have any direct support at this time.

Slit2 is expressed in the ventricular zone that is present at and near the midline. However, our results indicate that the site of axonal defects in *Slit2* mutants is in more lateral and ventro-lateral regions. Our results would most easily be explained if the Slit2 protein is localized in those regions, despite expression of its mRNA in more medial regions; the development of antibodies to Slit2 would make it possible to test this directly. If Slit2 is indeed found at a distance from its site of mRNA expression, how would that occur? One possibility is that *Slit2* diffuses into these areas. A number of lines of evidence support the idea that Slits are diffusible molecules that can form functional gradients at a distance from their site of expression. In *Drosophila,* the diffusion of Slit proteins has been demonstrated both immunohistochemically (Kidd et al., 1999; Rothberg et al., 1990) and also by virtue of demonstrated functional effects of the Slit protein at a distance from the midline (Kidd et al., 1999). Diffusion of Slit proteins is also evident from their ability to regulate axonal growth at a distance in vitro in collagen gel coculture assays (Brose et al., 1999; Chen et al., 2001; Hu, 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Shu and Richards, 2001; Wu et al., 1999). In addition to diffusion, however, Slit2 protein may become localized to regions lateral and ventral of the sites of its mRNA expression simply by virtue of being expressed by radial glial cells, the long processes of which may deliver Slit2 protein to these sites.

Prevention of Axonal Extension toward and across the Midline

Despite strong *Slit1* expression in the preoptic area and hypothalamic regions, loss of *Slit1* does not have a significant impact on the trajectory of corticofugal or thalamocortical axons. However, the dramatic differences in phenotypes between the *Slit1*;*Slit2* double mutant and the *Slit2* mutant suggests that *Slit1* does in fact have the ability to affect the pathways under study. These differences illustrate another way in which Slit proteins are used to pattern these axonal connections, through

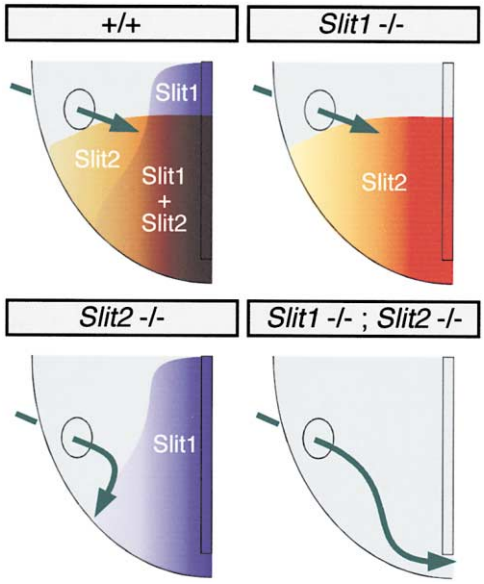

Figure 9. Schematic Summarizing Axonal Defects in the Slit Mutants in Relation to Regions of Slit Action

Slit2 acts in ventral and ventro-lateral domains and Slit1 action is limited to medial areas. In wild-type animals, Slit2 prevents axons from entering ventral regions and also prevents the axons from coursing medially. Loss of Slit1 does not have an impact on the axonal trajectories due to the continued presence of Slit2. However, loss of Slit2 allows the axons to project ventrally and medially, potentially in response to positive cues from these areas. These axons project medially until they encounter Slit1. They then project ventrally along the border of this Slit1 zone. Loss of Slit1 and Slit2 allows axons to travel ventrally and medially and to approach and cross the midline.

short-range guidance at the midline. In contrast to *Slit2* mutants, *Slit1*;*Slit2* double mutant corticospinal axons that enter the internal capsule are directed toward the midline, where they cross in a large ectopic commissure. This crossing occurs at the expense of ventrally projecting axons, suggesting that Slit1 normally inhibits the axons from approaching the midline. This abnormal crossing can also be seen in the double mutants in other pathways, including corticothalamic and ascending serotonergic and dopaminergic axons. The fact that the axons course medially in the double mutants suggests that the axons are directed to the midline, perhaps by a midline attractive activity that is normally masked by *Slit1*. One component of this putative midline attractive activity may be netrin-1, which is expressed in the midline region and has previously been shown to act as an attractant for cortical and thalamic axons (Braisted et al., 2000; Metin et al., 1997; Richards et al., 1997).

Thus, we hypothesize that *Slit2* normally prevents axons from entering ventral regions, and in the absence of *Slit2* most axons that enter the ventral area then approach the midline, at least partly due to midline attraction, until they encounter Slit1 protein. They then travel along the border of this repulsive Slit1 domain to the ventral surface (Figure 9). Why do the axons not simply wander in the ventral region of the telencephalon in the absence of repulsion from Slit2? One possibility is that there is an attractive cue for cortical axons in the

ventral region that is being unmasked by removing the Slit proteins. Interestingly, netrin-1 and Sema3C, which have both been shown to act as an attractive cues for some cortical axons in vitro (Braisted et al., 2000; Metin et al., 1997; Richards et al., 1997; Bagnard et al., 1998), are strongly expressed along with their receptors in appropriate positions to function in this role (data not shown). Further studies will help define the cues that function with Slit1 and Slit2 to guide these axons.

The fact that loss of *Slit2* activity is required to uncover a function of *Slit1* indicates a hierarchy of action of these molecules in the guidance of these axons. The simplest explanation for this hierarchy is that *Slit2* acts in more lateral domains and that *Slit1* acts more medially. In this model, the role of *Slit1* becomes apparent only when *Slit2* is lost, thereby allowing axons to approach the region of *Slit1* function.

One exception to this rule is provided by an abnormal midline crossing event that occurs in the *Slit2* single mutants. This defect occurs at the level of the medial preoptic area, where some descending corticofugal (but not any ascending thalamocortical, serotonergic or dopaminergic axons) cross the midline, despite the presence of the *Slit1* expression in the *Slit2* single mutant. We do not know why these particular axons are not prevented from crossing. Among other possibilities, there might be a Slit1-insensitive subpopulation of corticofugal axons. This insensitivity may occur at or downstream of the level of the receptor. Alternatively, loss of Slit2 may result in the disruption of the balance of repulsive and attractive (e.g., from midline netrin) forces acting upon the axons, resulting in inappropriate midline crossing. Further studies are required to address these possibilities. Once these axons cross the midline in *Slit2* mutant mice, they do one of three things: join the large descending fascicle of the contralateral side, enter the opposite internal capsule to travel to the contralateral cerebral peduncle, or ascend dorsally into the contralateral cortex. Interestingly, many corticofugal axons that abnormally cross the midline in *Slit1*;*Slit2* double mutants turn back toward the midline to re-cross it, suggesting that some but not all midline repulsion is absent, as is the case in the *Drosophila robo* mutant.

In *Drosophila*, *slit* mutant embryos display an extreme phenotype where both ipsilateral and commissural axons enter the midline and never leave it. This suggests another function for Slit in the *Drosophila* system: preventing axons from lingering at the midline, where attractive cues are likely to be at their highest concentrations. It is interesting to note that many axons that reach the midline of *Slit1*;*Slit2* mice do not remain there, but instead readily cross to the contralateral side, suggesting the presence of yet additional molecules that drive axons from the midline in mice. Such molecules might include semaphorins, which have been proposed to prevent midline recrossing in the spinal cord (Zou et al., 2000).

Channeling of Axons

The role of Slit proteins in channeling axons to form a specific pathway can be best illustrated using the example of corpus callosum development. Previous work has provided evidence implicating Slit2 in corpus callosum formation (Shu and Richards, 2001). Interestingly, in this region, *Slit2* is not expressed in the midline, but in two glial populations, the IG and GW, that lie adjacent to the midline. The callosal axons extend into a narrow pathway that forms between these two populations. In the *Slit2* mutant, callosal axons crossed the GW, which appears normal morphologically, to enter Probst bundles that form on either side of the midline. These results suggest that these populations of glial cells direct callosal axons at least in part through chemical repulsion provided by Slit2, rather than simply by creating a physical barrier. Slit2 derived from the GW appears to prevent callosal axons from entering the septum, whereas Slit2 from the IG may prevent the axons from traveling dorsally back into the ipsilateral cortex, instead channeling them across the midline to the contralateral side.

It is interesting to note that callosal axons are a population of contralaterally projecting axons that are sensitive to Slit proteins prior to midline crossing. This contrasts with contralaterally projecting axons in *Drosophila* (Kidd et al., 1999) and in the vertebrate spinal cord (Zou et al., 2000), which appear to become Slit-responsive only after midline crossing. Thus, in this region Slit proteins appear to regulate midline crossing by channeling axons into an appropriate crossing site, rather than by regulating crossing or recrossing at the midline per se. This function is analogous to the one we observed for Slit proteins in regulating the channeling of retinal ganglion cells toward an appropriate crossing site at the optic chiasm midline (Plump et al., 2002, this issue of *Neuron*). Thalamocortical and corticothalamic axons also appear to employ Slit in a similar channeling function. As they exit or enter the thalamus, these axons are surrounded by Slit1 and Slit2, which appear to force the axons to make a sharp turn to enter or exit the mantle region of the caudal ganglionic eminence. Furthermore, it is interesting to note that two other major contralaterally projecting tracts within the forebrain, the anterior commissure and the hippocampal commissure appear grossly normal in *Slit2* mutant mice, suggesting that the role of *Slit2* in channeling axons does not extend to all major commissural pathways in the forebrain.

In summary, analysis of major forebrain tracts in *Slit1*, *Slit2,* and *Slit1*;*Slit2* mutants demonstrates that Slit proteins play a major role in the guidance of these tracts during development. Additionally, several generalizations emerge from this analysis. Slits appear to act similarly on distinct axon tracts through repulsion to maintain dorsal position by the preventing axonal entry into ventral regions, to prevent axonal extension toward and across the midline, and to channel axons into particular regions. Further analysis will reveal how these cues interact with other types of guidance cues, both short and long range, to direct development of the final complex trajectories of these major forebrain axonal tracts.

Experimental Procedures

Mouse Breeding, Genotyping, and Tissue Preparation

All animals were treated according to protocols approved by the Committee on Animal Research at the University of California, San Francisco. E14.5 embryos and E18.5 fetuses were obtained from matings between two *Slit1*$^{-/-}$, *Slit2*$^{+/-}$, or *Slit1*$^{-/-}$;*Slit2*$^{+/-}$ animals. At 14.5 or 18.5 days of gestation, embryos were obtained by Caesarian

section, anesthetized by cooling, perfused with 4% PFA in PBS, and postfixed in PFA for 2–8 hr. Mutant animals were initially recognized by detection of bright GFP fluorescence due to GFP transgene insertion in the *Slit* locus during generation of the mutant allele (Plump et al., 2002, this issue of *Neuron*). Additional genotyping was performed by PCR as described elsewhere (Plump et al., 2002). Following postfixation, brains were cryoprotected in 30% sucrose and either frozen in embedding medium and cut in a cryostat (E14.5) or frozen and cut in a freezing sliding microtome (E18.5).

Immunohistochemistry

Immunohistochemistry was performed on 40 μm thick free floating sections (E18.5) or on 12 μm thick cryostat sections mounted onto glass slides (E14.5). Free-floating sections were preincubated in 5% normal serum of the species in which the secondary antibody was raised, 1% BSA and 0.3% TX in PBS for 1 hr at room temperature, and subsequently incubated with the primary antisera for 24–36 hr at 4°C in 2% normal serum and 0.3% TX in PBS. The following antibodies were used: mouse monoclonal antibody RMO270 against neurofilament (gift of V. Lee; diluted 1:2000), rabbit anti-calbindin (Swant, Bellinzona, Switzerland; diluted 1:5000), rabbit anti-calretinin (Chemicon; diluted 1:5000), rabbit anti-NPY (Incstar; diluted 1:3000), mouse monoclonal antibody 4D7 against Tag-1 (Developmental Studies Hybridoma Bank, DSHB; diluted 1:100), rat anti-GFAP (gift of V. Lee; diluted 1:10), rabbit anti-GFP (Molecular Probes; diluted 1:2000), mouse anti-nestin (gift of R. McKay; diluted 1:1000), rabbit anti-serotonin (Diasorin; diluted 1:5000), and rabbit anti-tyrosine hydroxylase (Pel-Freeze; diluted 1:1000). Sections were then incubated in biotinylated secondary antibodies (Vector; diluted 1:200) and processed by the ABC histochemical method (Vector). The sections were then mounted onto Superfrost Plus slides (Fisher), dried, dehydrated, and coverslipped with Permount (Fisher). In each experiment, sections from homozygous mutants and their wild-type or heterozygous littermates were processed together. Primary antiserum omission controls and normal mouse, rabbit, and goat serum controls were used to further confirm the specificity of the immunohistochemical labeling. Immunohistochemistry was performed on cryostat sections on glass slides using essentially the same protocol.

Additionally, single and double immunofluorescence labeling using fluorochrome conjugated secondary antibodies was performed on 12 μm sections on glass slides as described (Parent et al., 1997). DNA counterstaining was performed with Sytox Green (Molecular Probes; diluted 1:25,000), and the slides were mounted with Prolong Antifade (Molecular Probes).

In Situ Hybridization

^{35}S-riboprobes were used for in situ hybridization as described previously (Marin et al., 2000). Probes used for *Slit1*, *Slit2*, *Robo1*, and *Robo2* have been previously described (Brose et al., 1999).

Axon Tracing

Axon tracing was performed on E14.5 and E18.5 mouse brains following perfusion. The brains were postfixed by immersion for 2–4 weeks in cold (4°C) fixative solution containing 4% paraformaldehyde, 4% sucrose, and 0.1M sodium phosphate (pH 7.3). For neocortical injections, large crystals of DiI (Molecular Probes) were placed into the parietal cortex of E14 and E18.5 brains. For thalamic injections, the brains were bisected into hemispheres, each of which was used for a separate experiment. Small crystals of DiI were injected into the medial face of dorsal thalamus with an insect pin. The brains were then incubated in fixative solution for 4–8 weeks, rinsed in 0.1M sodium phosphate (pH 7.3), and embedded in 4% low-melt agarose, and sections were cut on a vibrating microtome in the coronal plane. Sections were counterstained with DAPI (Molecular Probes), coverslipped, and examined with a fluorescence microscope.

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