Development/Plasticity/Repair

Robo1 and Robo2 Cooperate to Control the Guidance of Major Axonal Tracts in the Mammalian Forebrain

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The function of the nervous system depends on the precision of axon wiring during development. Previous studies have demonstrated that Slits, a family of secreted chemorepellent proteins, are crucial for the proper development of several major forebrain tracts. Mice deficient in Slit2 or, even more so, in both Slit1 and Slit2 have defects in multiple axonal pathways, including corticofugal, thalamocortical, and callosal connections. In the spinal cord, members of the Robo family of proteins help mediate the function of Slits, but the relative contribution of these receptors to the guidance of forebrain projections remains to be determined. In the present study, we addressed the function of Robo1 and Robo2 in the guidance of forebrain projections by analyzing Robo1−/−, Robo2−/−, and Robo1;Robo2−/− mice. Mice deficient in Robo2 and, more dramatically, in both Robo1 and Robo2, display prominent axon guidance errors in the development of corticofugal, thalamocortical, and corticocortical callosal connections. Our results demonstrate that Robo1 and Robo2 mostly cooperate to mediate the function of Slit proteins in guiding the major forebrain projections.

Key words: axon guidance; cortex; repulsion; Robo; Slit; midline; thalamocortical

Introduction

The development of the major axonal projections in the mammalian forebrain has been extensively studied in the recent years (for review, see López-Bendito and Molnár, 2003; Richards et al., 2004; Price et al., 2006). Among them, the reciprocal connections between the cortex and the thalamus, corticocortical projections linking both cerebral hemispheres through the corpus callosum, and corticofugal projections directed toward the mesencephalon, pons, and spinal cord have received much attention. These connections underlie some of the most essential functions of the mammalian cerebral cortex, such as perception and motor behavior.

Several guidance molecules have been shown to influence the development of these major forebrain connections. For example, Netrin1 is required for the formation of the corpus callosum (Serafini et al., 1996), and it has also been shown to influence the development of corticofugal (Métil et al., 1997; Richards et al., 1997) and thalamocortical projections (Braisted et al., 2000). Similarly, EphA/ephrinA signaling appears to underlie the topographic organization of thalamocortical projections (Dufour et al., 2003; Torii and Levitt, 2005). In addition, Slits have been shown to play a fundamental role in the guidance of all three major forebrain connections (corticocortical, corticofugal, and thalamocortical) in the mammalian forebrain (Bagri et al., 2002).

Slit proteins have been implicated in axon guidance of insects, nematodes, and vertebrates, contributing through different mechanisms to the development of multiple axonal projections. In addition to playing a major role in regulating axon crossing at the midline (Kidd et al., 1999; Long et al., 2004), Slits specify the lateral and dorsoventral positioning of longitudinal axonal pathways (Rajagopalan et al., 2000; Simpson et al., 2000; Bagri et al., 2002; Nguyen Ba-Charvet et al., 2002; Long et al., 2004) and contribute to the formation of commissures by channeling axons into particular regions (Bagri et al., 2002; Hutson and Chien, 2002; Plump et al., 2002; Shu et al., 2003).

As in Drosophila (Kidd et al., 1999) and Caenorhabditis elegans (Hao et al., 2001), the function of Slit proteins in the vertebrate nervous system is primarily mediated by Robo receptors (Brose et al., 1999; Li et al., 1999; Yuan et al., 1999; Hutson and Chien, 2002; Long et al., 2004; Sabatier et al., 2004). In the mammalian spinal cord, Robo1, Robo2, and the Robo family protein Rig1/Robo3 cooperate to mediate the function of Slit proteins in the
guidance of commissural axons (Long et al., 2004; Sabatier et al., 2004). In the developing forebrain, the expression pattern of Robo1 and Robo2 strongly suggest their involvement in the guidance of corticocortical, corticothalamic, and corticofugal projections (Marillat et al., 2001; Bagri et al., 2002; Whitford et al., 2002; Sundaresan et al., 2004). Accordingly, a recent study has shown that Robo1 plays a role in the development of some of these pathways, most notably in the formation of corticocortical callosal projections (Andrews et al., 2006).

Here, we investigated the function of Robo1 and Robo2 in the formation of forebrain connections by analyzing mice carrying severe loss-of-function alleles for Robo1, Robo2, or both Robo1 and Robo2 receptors. Our results demonstrate that Robo1 and Robo2 mostly cooperate to control the development of the major axonal tracts in the mammalian forebrain. The resemblance of the axonal defects found in Robo1;Robo2 and Slit1;Slit2 double mutants strongly suggests that Robo1 and Robo2 mediate the function of Slit1 and Slit2 in the formation of these connections.

Materials and Methods

Animals. Mice were treated according to protocols approved by the Committee on Animal Research at the University Miguel Hernández, following Spanish and European Union regulations. Embryonic day 13.5 (E13.5), E14.5, and E18.5 embryos were obtained by mating Robo1−/−, Robo2−/−, or Robo1−/−/Robo2−/− mice, which were maintained in CD1, C57BL/6, and mixed CD1–C57BL/6 backgrounds, respectively. Genotyping was performed by PCR as described previously (Grieshammer et al., 2004; Long et al., 2004). Because the Robo1 and Robo2 genes are linked (being separated by only 1.8 Mb), the Robo1−/−/Robo2−/− colony was generated through meiotic recombination of the mutant alleles (Z. Chen et al., unpublished observations).

Immunohistochemistry and in situ hybridization. Embryos were obtained by cesarean section, anesthetized by cooling, perfused with 4%

Results

Expression of Robo1 and Robo2 receptors in the developing forebrain

To investigate the role of Robo receptors in axon guidance in the forebrain, we first analyzed their expression pattern focusing on regions and developmental stages where the major axonal tracts navigate and develop. Consistent with previous studies (Marillat et al., 2001; Bagri et al., 2002; Whitford et al., 2002; Sundaresan et al., 2004), Robo1 and Robo2 mRNA are highly expressed in the dorsal thalamus and the cerebral cortex at the time when corticofugal and thalamocortical projections form (Fig. 1A, D). Interestingly, the pattern of expression of Robo1 and Robo2 mRNA appears mostly complementary, both in the developing cortex (Fig. 1B, E) and dorsal thalamus (Fig. 1C, F). Specifically, cells at the intermediate zone of the developing cortex appear to express Robo2 (Fig. 1E), whereas neurons that begin to accumulate in the
corticospinal axons in tissue obtained from Robo1;Robo2 double mutants (Fig. 2H) (data not shown), whereas Slit1–AP probes revealed only slight staining in some of these fibers tracts (Fig. 2G) (data not shown).

In summary, the temporal and spatial pattern of Robo1 and Robo2 expression in cortical and dorsal thalamic axons suggest that these receptors may play a role in the guidance of the major forebrain axonal tracts, probably mediating the function of Slit proteins in this process.

Slit2 repels neocortical and dorsal thalamic axons in vitro

The previous results strongly suggest that cortical and thalamic axons respond to Slit proteins through both Robo1 and Robo2 receptors. In agreement with this view, it has previously been shown that cortical axons are repelled by Slit2 in vitro (Shu and Richards, 2001) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). However, the effect of Slits on thalamic axons has not been tested. To examine the role of Slit proteins on thalamic axons, we cocultured E13.5 dTh explants with COS cells aggregates expressing Slit2 in three-dimensional collagen gels (Fig. 3). After 4 d in vitro, axons extended radially from explants in control experiments (Fig. 3A, D) (n = 19). In contrast, axon length was significantly shorter on the side of explants facing COS cells aggregates expressing Slit2 (Fig. 3B, D) (n = 22). Moreover, we observed many axons turning away from COS cells aggregates expressing Slit2 (Fig. 3B, arrow).

Abnormal corticospinal and thalamocortical projections in Robo2 but not Robo1 mutants

The mostly complementary protein expression pattern of Robo1 and Robo2 in the developing forebrain axons suggests that these receptors may coordinate their activity in the guidance of cortico- and thalamocortical projections. To directly address the role of individual Robo receptors in the guidance of these axonal tracts, we first examined the organization of these axons in mice carrying loss-of-function alleles of Robo1 or Robo2. At E18.5, when most forebrain connections have been established, immunohistochemistry for the cell adhesion molecule L1 labels both corticothalamic and thalamocortical axons as they course through the internal capsule in the basal ganglia (Fig. 4A) (Jones et al., 2002; López-Bendito et al., 2002). The distribution of L1+ fibers in the telencephalon of these results suggest that, as in the case of cortical axons, Slits proteins also repel the growth of dorsal thalamic axons.

binding was also detected in the absence of either one of the Robo receptors, reinforcing the notion that Robo1 and Robo2 are mostly coexpressed in these projections (Fig. 2E, F) (data not shown). In contrast, Slit2–AP probes did not stain callosal, thalamocortical, corticothalamic, or corticospinal axons in tissue obtained from Robo1;Robo2 double mutants (Fig. 2H) (data not shown), whereas Slit1–AP probes revealed only slight staining in some of these fibers tracts (Fig. 2G) (data not shown).
Robo1 or Robo2 single mutants was mostly indistinguishable from control mice \( (n = 8 \text{ for each genotype}) \) (Fig. 4A–C). Moreover, labeling of axons with DiI crystals placed in the neocortex of E18.5 Robo1 or Robo2 single mutants did not reveal major differences in the distribution of corticofugal axons as they course through the internal capsule (Fig. 5A–C). In some Robo2 mutants, however, a few corticofugal axons were found ventrally displaced, abnormally reaching the ventral midline at the level of the anterior commissure \( (n = 4 \text{ of 8 brains}) \) (Fig. 5C).

Immunohistochemistry for NPY, a transient marker of corticothalamic projections (Bagri et al., 2002), did not reveal major differences in the distribution of corticofugal axons in either Robo1 or Robo2 single mutants \( (n = 4 \text{ for each genotype}) \) (Fig. 4D–F). Similar results were obtained from the analysis of corticofugal axons after DiI placements in the neocortex \( (n = 8 \text{ for each genotype}) \) (Fig. 5D,E) (data not shown). Occasionally, some abnormal bundles of cortical fibers were found in the dorsal thalamus of Robo2 mutants \( (n = 4 \text{ of 8 brains}) \) (Fig. 5F), suggesting the existence of targeting defects in a subset of mice lacking Robo2 function.

Because the development of corticofugal and thalamocortical axons is highly coordinated in time and space (for review, see López-Bendito and Molnár, 2003), we next analyzed thalamocortical projections in Robo1 and Robo2 single mutants. Analysis of thalamocortical projections using immunohistochemistry against calretinin, a marker of thalamic axons, revealed a normal distribution of thalamocortical axons in Robo1 mutants compared with controls \( (n = 4) \) (Fig. 4G,H) (data not shown). In contrast, in Robo2 mutants, some thalamic fibers were observed to fail reaching the telencephalon, and instead growing abnormally toward the ventral diencephalon, a region normally nonpermissive for thalamocortical axon outgrowth \( (n = 4) \) (Fig. 4I) (Braisted et al., 1999). DiI injections in the dorsal thalamus of control, Robo1 and Robo2 mutants confirmed that numerous thalamocortical axons failed to enter the telencephalon and instead invaded the hypothalamus in Robo2 mutants (Fig. 5I), whereas no major defects were observed in the guidance of thalamocortical axons in Robo1 mutants compared with wild-type control brains \( (n = 4) \) (Fig. 5G,H).

We next studied the organization of other corticofugal projections, such as those contributing to cerebral peduncle or the corpus callosum. Analysis of the cerebral peduncle at the level of the diencephalon demonstrated that corticospinal axons develop normally in the absence of Robo1 function compared with wild-type controls \( (n = 4) \) (Figs. 4J,K, 5J,K). In contrast, the cerebral peduncle appears to be ventrally displaced in all Robo2 mutants examined \( (n = 4) \) (Figs. 4L, 5L). Moreover, DiI-labeled corticofugal axons were observed to abnormally defasciculate from the cerebral peduncle in some Robo2 mutants \( (n = 3 \text{ of 4 brains}) \) (data not shown).

Previous studies have shown that Slit2 is required for the guidance of corticocortical projections at the corpus callosum (Bagri et al., 2002; Shu et al., 2003). However, analysis of Slit2–AP binding assays revealed only minor defects in the development of the corpus callosum in Robo1 mutant embryos, and no apparent abnormalities in Robo2 mutant embryos compared with controls \( (n = 3) \) (Fig. 2E,F). Immunohistochemistry against NPY \( (n = 4) \) (Fig. 4M–O) or DiI tracing from the neocortex \( (n = 4) \) (Fig. 5M–O) reinforced this view, because both methods failed to reveal major defects in the development of callosal projections in Robo1 or Robo2 single mutants.

**Corticofugal axon guidance is severely disrupted in Robo1; Robo2 double mutants**

The mild defects seen in the single mutants suggested two possibilities: (1) Robo receptors do not mediate the function of Slit proteins during the development of forebrain connections, or (2) Robo1 and Robo2 receptors are mostly coexpressed and function cooperatively in corticofugal and thalamocortical axons. In the latter case, each Robo receptor may functionally compensate for

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**Figure 2.** Coronal sections through the forebrain of E18.5 wild-type \((A–D)\), Robo1\(^{-/-}\) (E,F), Robo2\(^{-/-}\) (G,H), and Robo1;Robo2 double-mutant (I–L) fetuses showing Slit1–AP binding \((A, B, G, H)\) and Slit2–AP binding \((E, F, I, J)\). AP staining labels corticocortical axons at the corpus callosum (cc) \((A, C)\), corticospinal axons at the cerebral peduncle \((cp)\) \((D)\), and thalamocortical/corticofugal projections \((B, E, F)\). Slit1–AP staining identifies corticocortical callosal projections in Robo1 \((E)\) and Robo2 \((F)\) mutant brains. Some ectopic bundles are abnormally displaced at the corpus callosum \((cc)\) of Robo1 mutants \((E, G)\). H, Slit–AP binding assays do not generally stain any axonal tract in the forebrain of Robo1, Robo2 double mutants. Only in a few cases, very weakly stained fibers could be observed after Slit1–AP binding \((G, I)\). H, hippocampus; Str, striatum; NCx, neocortex; SE, septum; dTh, dorsal thalamus; GP, globus pallidus; Hyp, hypothalamus; HC, hippocampal commissure. Scale bars: \(A, C, E, F, 500 \mu m; B, G, H, 1 \text{ mm}; D, 200 \mu m\).
the loss of the other one, providing a rational explanation to the absence of guidance defects found in either Robo1 or the relatively minor abnormalities present in Robo2 single mutants. To test this hypothesis, we next examined the organization of cortical and thalamic projections in Robo1;Robo2 double mutants.

In contrast to Robo1 and Robo2 single mutants, a simple cytoarchitectonical analysis of the telencephalon of Robo1;Robo2 double mutants revealed the existence of large bundles of ectopic fibers crossing the ventral midline at E18.5 (n = 5) (Fig. 6A–C). This abnormal crossing of fiber tracts at the level of the anterior commissure was also observed on sections stained with antibodies against L1 (n = 5) (Fig. 6D–F). L1 and calbindin immunostaining on sagittal sections revealed even more clearly that, in addition to the anterior commissure, large bundles of axons aberrantly cross the midline in the basal telencephalon of Robo1;Robo2 double mutants (n = 3) (Fig. 6G–I). Because of the presence of the ectopic commissural axons, the anterior commissure is always displaced dorsally in Robo1;Robo2 double mutants (Fig. 6K, L).

To determine the source of the ectopic commissural axons.
found in the basal telencephalon of Robo1; Robo2 double mutants, we next traced the trajectory of cortical axons by placing crystals of Dil in the developing neocortex. At E18.5, Dil injections in the parietal cortex of wild-type mice revealed a thick bundle of labeled axons coursing through the internal capsule as they progress toward the diencephalon (n = 10) (Fig. 7A, B). In contrast, Dil placements in the neocortex of Robo1;Robo2 double mutants showed that most corticofugal axons were diverted toward the midline, which they abnormally crossed (n = 8) (Fig. 7C, D, F). Interestingly, many cortical axons appear to return toward the midline after crossing it (Fig. 7D, F), because only a few axons were found to grow away from the midline toward the contralateral cortex (data not shown). Dil injections in the neocortex, in particular its caudal part, labeled some axons that did not decussate in the ventral telencephalon but followed their normal route toward the diencephalon (Fig. 7E) (data not shown). The abnormal trajectory of corticofugal axons was observed in embryos as early as E14.5 (n = 3) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

The massive number of axons that abnormally cross the ventral telencephalon in Robo1:Robo2 double mutants suggests that both corticothalamic (layer 6) and cerebral peduncle (layer 5) projections are affected in the absence of Robo function. Accordingly, immunohistochemistry for NPY as well as Dil tracing experiments from the parietal cortex revealed that only a few cortical axons reach the dorsal thalamus in Robo1;Robo2 double mutants (n = 8) (Fig. 8A–D,G,H). Moreover, these axons follow a more ventral trajectory than wild-type axons as they enter the diencephalon on their way to the dorsal thalamus (Fig. 8A, B, G, H).

Whereas some corticothalamic axons were consistently found in the absence of Robo1 and Robo2 function, Dil placements in the neocortex revealed that virtually no corticospinal axons reach the diencephalon through the cerebral peduncle in Robo1;Robo2 double mutants (n = 8) (Fig. 8E–H). Thus, Robo1 and Robo2 appear essential for the normal development of layer 5 cortical projections.

Figure 4. Abnormal axonal trajectories in the forebrain of Robo1 and Robo2 single-mutant mice. Coronal sections through the telencephalon of E18.5 embryos showing cell adhesion molecule L1 (L1) (A–C), NPY (D–F), and calretinin (CR) (G–I) immunohistochemistry in wild-type (A, D, G, J, M), Robo1 (B, E, H, K, N), and Robo2 (C, F, I, L, O) mutant mice. A–C. In wild-type embryos, L1+ axons are confined to the intermediate zone of the neocortex (NCx), striatum (Str), and dorsal thalamus (dTh). In Robo1 and Robo2 mutants, L1+ fascicles are observed at the NCx and Str in a similar pattern as in wild-type embryos. D–F. Immunohistochemistry for NPY demonstrates that corticofugal axons reach the diencephalon in wild-type (D), and Robo1 (E) and Robo2 mutant (F) brains. G–I. Coronar sections at the level of the diencephalon showing the trajectory of thalamocortical axons by immunohistochemistry for calretinin (CR) in wild-type (G), and Robo1 (H) and Robo2 (I) mutant brains. At this level, CR+ thalamocortical axons normally turn rostrally to enter the telencephalon, thus leaving the plane of section as observed in wild-type (G) and Robo1 mutants (H). In contrast, abnormal CR+ bundles were observed descending to the hypothalamus in Robo2 mutants (I, arrows). J–L. Abnormal development of the cerebral peduncle (cp) in Robo2 mutant brains (L), as revealed by calretinin immunostaining. M–O. Coronal sections at the level of the corpus callosum (cc) showing corticofugal fibers labeled by NPY immunohistochemistry in wild-type (M), and Robo1 (N) and Robo2 (O) mutant mice. ic, Internal capsule; H, hippocampus; Hb, habenula; Rt, reticular thalamic nucleus; VMH, ventromedial hypothalamic nucleus; MeA, medial amygdala; LA, lateral amygdala; MS, medial septum; LV, lateral ventricle. Scale bars: A–F, 1 mm; G–L, 100 μm; M–O, 200 μm.

Simultaneous loss of Robo1 and Robo2 perturbs corticocortical projections at the corpus callosum

We next analyzed whether the projections of corticocortical axons were also impaired in the absence of both receptors. At
E18.5. Nissl staining revealed that the size of the corpus callosum was reduced in Robo1;Robo2 double mutants compared with wild-type mice (n = 5) (Fig. 9A, B). In addition, two large ectopic bundles of fibers were also found on either side of the corpus callosum of Robo1;Robo2 double mutants, which resemble Probst bundles (Fig. 9B). These ectopic bundles were also observed when sections were stained for NPY (n = 3) (Fig. 9D). Moreover, Dil tracing experiments demonstrated that the ectopic axons were corticocortical axons that normally defasciculated from the corpus callosum and coursed ventrally into the septum (n = 4) (Fig. 9E–H). This defect was consistently observed after Dil placements in different cortical regions (data not shown).

Prominent thalamocortical axon guidance defects in Robo1;Robo2 double mutants

We next investigated the consequences of the simultaneous loss of Robo1 and Robo2 receptors in the guidance of thalamocortical axons. At E18.5, immunohistochemistry against calretinin, which labels thalamocortical projections originating from medial thalamic nuclei, revealed that numerous thalamic axons abnormally invade the hypothalamus, failing to turn rostrally into the telencephalon (n = 3) (Fig. 10A–C, M, N). In agreement with this observation, Dil injections into the dorsal thalamus of E18.5 Robo1;Robo2 double mutants showed that, compared with control mice, the vast majority of thalamocortical projections fail to enter the telencephalon and instead invade the hypothalamus (n = 3) (Fig. 10G–I, M, N), a phenotype that was observed already in E14.5 embryos (n = 3) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

Dil injections in the dorsal thalamus revealed that only some thalamic axons enter the telencephalon in Robo1;Robo2 double mutants. Once in the telencephalon, very few thalamocortical axons were found to grow through the internal capsule into the cerebral cortex (n = 5) (Fig. 10J–L, M, N). Instead, many of the thalamocortical projections that succeeded in entering the telencephalon were abnormally diverted toward the midline, which they cross (Fig. 10D–F, M, N) (data not shown). In summary, thalamocortical projections are severely disrupted in the absence of Robo1 and Robo2 function, with many axons aberrantly projecting toward the hypothalamus or the telencephalic ventral midline.
The midline is indicated by a dotted line in extension through the striatum in the medial preoptic region (MPO) (mice. Coronal (A–C), cell adhesion molecule L1 (D–F, J–L), and calbindin (G–I) immunohistochemistry in wild-type (A, D, G, J) and Robo1;Robo2 (B, C, E, F, H, I, K, L) mutant mice. A–C, Hoechst staining shows ectopic axonal bundles crossing the midline at the level of the medial preoptic region (MPO) (B, C, arrows). The anterior commissure (ac) is severely displaced dorsally. D–F, Immunostaining for L1 confirms that abnormal bundles of fibers cross the midline in Robo1;Robo2 mutants (E, F, arrows). G–I, Immunohistochemistry for calbindin delineates the abnormal crossing of the midline by unstained fibers (H, I). J–L, Ectopic bundles of axons crossing the midline are very evident in sagittal sections. Very few thalamic L1+ axons extend through the striatum in Robo1;Robo2 mutants because they accumulate in the midline (asterisk). The midline is indicated by a dotted line in C and F. NCx, Neocortex; ic, internal capsule; ob, olfactory bulb, RMS, rostral migratory stream; Str, striatum; H, hippocampus. Scale bars: A, B, D, E, G, H, J, K, 1 mm; C, 300 μm; F, I, L, 200 μm.

Figure 6. Abnormal axonal trajectories in the forebrain of Robo1;Robo2 double-mutant mice. Coronal (A–F) and sagittal (G–I) sections through the telencephalon of E18.5 embryos showing Hoechst staining (A–C), cell adhesion molecule L1 (D–F, J–L), and calbindin (G–I) immunohistochemistry in wild-type (A, D, G, J) and Robo1;Robo2 (B, C, E, F, H, I, K, L) mutant mice. A–C, Hoechst staining shows ectopic axonal bundles crossing the midline at the level of the medial preoptic region (MPO) (B, C, arrows). The anterior commissure (ac) is severely displaced dorsally. D–F, Immunostaining for L1 confirms that abnormal bundles of fibers cross the midline in Robo1;Robo2 mutants (E, F, arrows). G–I, Immunohistochemistry for calbindin delineates the abnormal crossing of the midline by unstained fibers (H, I). J–L, Ectopic bundles of axons crossing the midline are very evident in sagittal sections. Very few thalamic L1+ axons extend through the striatum in Robo1;Robo2 mutants because they accumulate in the midline (asterisk). The midline is indicated by a dotted line in C and F. NCx, Neocortex; ic, internal capsule; ob, olfactory bulb, RMS, rostral migratory stream; Str, striatum; H, hippocampus. Scale bars: A, B, D, E, G, H, J, K, 1 mm; C, 300 μm; F, I, L, 200 μm.

Discussion
The functioning of the cerebral cortex relies on several stereotypical long-distance projections, such as the corticofugal, callosal, and thalamocortical connections. Several studies have examined the early development of these projections and have identified pioneering axonal populations as well as potential intermediate targets and choice points for these axons (McConnell et al., 1989; De Carlos and O’Leary, 1992; Météin and Godemment, 1996; Molnár et al., 1998; Braisted et al., 1999; Tuttle et al., 1999; Auladell et al., 2000; López-Bendito et al., 2006). In addition, multiple molecules that participate in the guidance of these connections have been identified, providing a comprehensive framework in which to understand their development (Serafini et al., 1996; Météin et al., 1997; Richards et al., 1997; Braisted et al., 2000; Leighton et al., 2001; Bagri et al., 2002; López-Bendito et al., 2006). Thus, Slits play a fundamental role in the development of corticocortical callosal projections, layer 5 corticofugal projections toward the mesencephalon, pons, and spinal cord, and layer 6 corticothalamic projections (Bagri et al., 2002; Shu et al., 2003). Moreover, the development of the reciprocal thalamocortical projections also depends on Slit function (Bagri et al., 2002). Here, we demonstrate that the function of Slits in the guidance of these connections is mostly mediated by the coordinated activity of Robo1 and Robo2 receptors.

Robo1 and Robo2 have mostly redundant functions in forebrain axon guidance
Our previous analysis of Slit1 and Slit2 mutants led us to suggest that Slit proteins contribute in at least three different ways to the development of the mammalian forebrain: (1) the maintenance of the dorsoventral position of longitudinal axonal tracts by preventing axons from entering into ventral regions; (2) the prevention of axonal extension toward and across the midline; and (3) the channeling of axons into particular regions, such as commissures (Bagri et al., 2002). Slit1 and Slit2, which are expressed in partially overlapping patterns in the forebrain, cooperate to fulfill these functions: Slit2 is mostly responsible of preventing ventral invasion and axon channeling (1 and 3), whereas both Slit1 and Slit2 contribute to prevent midline crossing of ipsilateral tracts (2) (Fig. 11).

The finding that Robo1 and Robo2 proteins are expressed in developing forebrain axons at the time when these connections are formed, led us to hypothesize that these receptors may play an important role in their guidance. However, analysis of mouse mutants for Robo1 or Robo2 revealed very few guidance errors in the absence of either one of these receptors. In the case of Robo2 mutants, our analysis revealed only relatively moderate defects in the dorsoventral position of corticofugal, corticothalamic, and thalamocortical tracts, which enter ventral regions that they normally avoid (Fig. 11). In the case of Robo1 mutants, we could detect only very minor defects in the development of some corticocortical callosal connections (Fig. 2E). In contrast to our findings, a recent analysis of a different mutant allele of Robo1 showed a more severe phenotype, demonstrating that the function of this receptor is essential for the development of callosal projections, the absence of which cannot be compensated by the function of Robo2 (Andrews et al., 2006). We do not know the source of the discrepancy; however, it is possible that, as previously observed with other genes (Zheng et al., 2003), differences in the genetic background of the two mutant strains could explain the different penetrance of the Robo1 mutation. In addition, our Robo1 allele is likely to be a severe hypomorph rather than a complete null (Long et al., 2004); this would be consistent with the observation of weak Slit1–AP binding in Robo1;Robo2 double mutants (Fig. 2G). In any case, our Robo1 allele only appears to behave as a hypomorph in the callosal projection [compared with the Robo1 allele described by Andrews et al. (2006)], because the remaining defects observed in Robo1;Robo2 double mice phenocopy those found in Slit1;Slit2 double mutants.

The absence of severe axon guidance defects in Robo1 and Robo2 single mutants suggests that both receptors cooperate in the guidance of most forebrain projections. However, as described above for the callosal projections in Robo1 mutants (this study; Andrews et al., 2006), there are defects in Robo2 mutants that cannot be compensated by Robo1 function, such as the ventrally projecting axons found in corticofugal and thalamocortical tracts. This suggests that Robo1 and Robo2 functions may not be completely redundant. Interestingly, cortical and thalamic projections also overshoot ventrally in Slit2, but not in Slit1 mutants, which led to the proposal that the range of Slit2 function includes regions more distant from the midline than Slit1 (Fig.
axons abnormally cross the midline, and is unlikely, however, because in the absence of both receptors neurons do not express significant levels of Robo1 receptors. This Robo2 mutants? One possibility is that some cortical and thalamic neurons normally contain functional levels of both receptors in growing axons. This hypothesis is agreement with the analysis of Robo1;Robo2 double mutants, which mostly recapitulate the phenotypes observed in Slit1;Slit2 double mutants (Bagri et al., 2002) (Fig. 11).

In Slit1;Slit2 double mutants, most corticofugal and corticothalamic axons fail to reach the diencephalon, because they massively cross the midline in the ventral telencephalon (Bagri et al., 2002). Thalamocortical axons also make prominent guidance errors in Slit1;Slit2 double mutants; many run into the hypothalamic region, and the few that enter the telencephalon also turn aberrantly toward the midline. Thus, in the simultaneous absence of Slit1 and Slit2 or Robo1 and Robo2, cortical and thalamic ipsilateral projections fail to maintain their ipsilateral condition and instead abnormally invade the midline. Because these prominent defects are not present in either Robo1 or Robo2 single mutants (this study; Andrews et al., 2006), it seems clear that Slit1 and Slit2 prevent midline crossing of ipsilateral projections in the mammalian forebrain through both Robo1 and Robo2 receptors.

In the spinal cord, commissural axons upregulate Robo1 and Robo2 once they have crossed the midline, thereby contributing to their navigation beyond the floor plate (Long et al., 2004). In contrast, the only population of commissural axons examined in this study, the corticocortical callosal axons, is sensitive to Slit proteins before midline crossing. In this region, two glial populations adjacent to the midline, the glial wedge and the indusium griseum, express Slit2 and form a narrow pathway through which callosal axons extend (Shu et al., 2003). It has been recently suggested that Robo1 might be the unique mediator of Slit2 function in the formation of the corpus callosum (Andrews et al., 2006). Our results, however, strongly suggest that Robo2 also contributes to the formation of this commissure, because we observed very prominent defects in Robo1;Robo2 double mutants but no abnormalities in either Robo1 or Robo2 single mutants. Even as-

Figure 7. Corticofugal axons abnormally reach the telencephalic midline in Robo1;Robo2 double-mutant mice. Coronal sections through the telencephalon of E18.5 brains with Dil implanted in the neocortex (NCx), showing computer-generated overlays of Dil-labeled corticofugal axons and Hoechst counterstaining from wild-type (A) and Robo1;Robo2 mutants (C–E). The midline is indicated with a dotted line in D. The schemas summarize the results obtained in control (B) and Robo1;Robo2 mutants (F). A, B, in wild-type mice, labeled axons extend from the cortex into the striatum (Str). C–F, In Robo1;Robo2 mutants, labeled axons from the internal capsule (ic) abnormally approach the midline and cross it (C, arrow). A few axons that reach the midline course ventrally (arrowheads). Most of the axons that crossed the midline at more anterior levels were found in the contralateral side, where they either travel to the base of the telencephalon or extend toward the contralateral cortex (C, D). MPO, Medial preoptic area; H, hippocampus, AH, anterior hypothalamus. Scale bars: A, C, E, 1 mm; D, 300 μm.
assuming that the Robo1 allele analyzed here is only a severe hypomorph, our genetic analysis strongly suggests that Robo2 signaling contributes along with Robo1 in the formation of the corpus callosum.

Interaction of Slit/Robo signaling with other molecules

It has been previously shown that molecules such as cell surface heparan sulfates may modulate the interaction of Slits and Robo receptors. Thus, biochemical experiments have shown that Slits bind the heparan sulfate proteoglycan glypican-1 (Liang et al., 1999; Ronca et al., 2001). Heparan sulfate enhances the affinity of Slit2 for Robo1 receptors in vitro, and removal of cell surface heparan sulfate by heparinase III treatment or addition of saturating amounts of heparan sulfate abolishes the repulsive activity of Slit2 toward olfactory bulb axons in explant cultures (Hu, 1999a).
Moreover, nervous system-specific conditional mutants for EXT1, a glycosyltransferase enzyme required for the synthesis of heparan sulfate, display guidance defects at the optic chiasm that are similar to those found in Slit1;Slit2 double mutants (Plump et al., 2002; Inatani et al., 2003). Furthermore, reduction of one Ext1 allele in Slit2/H11002/H11002 mice, which otherwise have a relatively normal optic chiasm, cause profound retinal axon misguidance similar to those found in conditional Ext1 mutants and Slit1;Slit2 double mutants.

Similarly, zebrafish mutants for both ext2 (dackel, dak) and extl3 (boxer, box), two additional glycosyltransferases implicated in heparan sulfate biosynthesis, show retinal axon guidance defects that phenocopy robo2 mutants (Hutson and Chien, 2002; Lee et al., 2004). Together, these results suggest that heparan sulfate plays a physiologically essential role in Slit/Robo-mediated retinal axon guidance at the optic chiasm.

Although heparan sulfate may function in the extracellular environment, some evidence suggests that it may also or instead function on the surface of Robo-expressing, Slit responding cells, where it may contribute to concentrate Slit protein and promote its binding to Robo receptors. In the telencephalon, thalamic neurons express the heparan sulfate proteoglycan N-syndecan (Kinnunen et al., 1999), and both corticofugal and thalamocortical axons are labeled with antibodies against heparan sulfate (J. A. Sánchez and O. Marín, unpublished observations). Thus, it is conceivable that heparan sulfate may also contribute along with Slit/Robo signaling to the guidance of these major forebrain projections.

Concluding remarks

The present results, along with our previous work on the analysis of Slit mutants (Bagri et al., 2002), demonstrate that Slit/Robo interactions play a crucial role in the guidance of some of the most prominent axonal tracts in the mammalian forebrain. Through a mechanism that involves the repulsion of growing axons (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999), Slits intervene in multiple aspects in the development of major connections in the mammalian forebrain. These functions appear to be mediated mostly or exclusively by Robo1 and Robo2, because the forebrain phenotype of Robo1;Robo2 double mutants looks highly similar, and possibly identical to the Slit1;Slit2 double-mutant phenotype. Furthermore, Robo1 and Robo2 have mostly redundant roles in...
this process. Future studies will aim to reveal how Robo1 and Robo2 receptors are specifically regulated in distinct forebrain axonal tracts and how these receptors may interact with other guidance cues to control their final targeting.

References


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