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Slit1 and Slit2 Cooperate to Prevent Premature Midline Crossing of Retinal Axons in the Mouse Visual System

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Summary

During development, retinal ganglion cell (RGC) axons either cross or avoid the midline at the optic chiasm. In *Drosophila*, the Slit protein regulates midline axon crossing through repulsion. To determine the role of Slit proteins in RGC axon guidance, we disrupted *Slit1* and *Slit2*, two of three known mouse *Slit* genes. Mice defective in either gene alone exhibited few RGC axon guidance defects, but in double mutant mice a large additional chiasm developed anterior to the true chiasm, many retinal axons projected into the contralateral optic nerve, and some extended ectopically—dorsal and lateral to the chiasm. Our results indicate that Slit proteins repel retinal axons in vivo and cooperate to establish a corridor through which the axons are channeled, thereby helping define the site in the ventral diencephalon where the optic chiasm forms.

Introduction

Retinal axons grow from their origin within the retinal ganglion cell layer of the developing retina to the optic disk where they coalesce to form the optic nerve. Early in the development of the visual system, optic nerve axons grow toward the brain, where they enter at the ventral-most aspect of the diencephalon to form the optic chiasm. Here many axons cross to the contralateral hemisphere, becoming the optic tract, which eventually terminates at visual targets in the thalamus and midbrain. Some axons do not cross, projecting to the same targets but ipsilaterally. As with many other brain commissures, formation of the optic chiasm occurs at an invariant position along the antero-posterior axis of the developing forebrain. Mechanisms must therefore exist not only to direct divergence at the midline but also to prevent retinal axons from crossing at inappropriate locations.

While significant progress in recent years has led to a greater understanding of the factors that help establish a topographic map within the retina and its targets (O'Leary et al., 1999), less progress has been made in identifying the rudimentary axon guidance cues that establish the basic trajectories within the vertebrate visual system, particularly those that function at the midline to regulate the positioning and decussation of retinal axons. In mammals, chondroitin sulfate proteoglycans (CSPGs), L1, netrin-1, and specific EphB ligands are important for guidance within the retina (Birgbauer et al., 2000; Brittis et al., 1992, 1995; Deiner et al., 1997; Snow et al., 1991). Perturbation of these molecules results in either misdirected growth of axons within the eye or an inability of axons to enter the optic disk and project into the optic nerve. In addition to controlling entry of axons into the optic nerve head, netrin-1 also helps establish the angle by which these axons enter into the optic chiasm (Deiner and Sretavan, 1999), but does not appear to affect other aspects of retinal axon guidance. In *Xenopus*, an ephrin-B ligand has been implicated in preventing midline crossing of some but not all retinal axons (Nakagawa et al., 2000); however, it is not known whether this mechanism also operates in mammals. CSPGs may also influence midline guidance events in the developing visual system either directly or indirectly as evidenced by the observation that their removal from the region posterior to the chiasm in a semi-intact slice preparation leads to perturbations of axon growth and divergence (Chung et al., 2000).

In mice, *Slit* gene family members (specifically *Slit1* and *Slit2*, but not *Slit3*) have been hypothesized to control retinal axon guidance based on their patterns of expression within the developing visual system, and the ability of Slit2 to inhibit outgrowth and induce collapse of cultured retinal axons (Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000). The Slit proteins are an evolutionarily conserved family of secreted axon guidance cues. *Slit* was identified in *Drosophila* (*dSlit*) (Rothberg et al., 1990) and initially thought to be required for the development of midline glia, but subsequently shown to act as a midline axon repellent (Kidd et al., 1999). In *Drosophila,* Slit functions to prevent ipsilaterally projecting fibers from ever crossing the midline and contralaterally projecting fibers from recrossing, and further helps to determine the distance from the midline that crossed fibers travel, a phenomenon mediated by the combinatorial action of three Robo receptors (all of which are members of the immunoglobulin superfamily) expressed on crossing axons (Rajagopalan et al., 2000; Simpson et al., 2000). Three vertebrate homologs of *Slit* have been identified (Holmes et al., 1998; Itoh et al.,

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1998; Brose et al., 1999; Li et al., 1999) and implicated through in vitro studies as possible mediators of a number of axon guidance events within the developing spinal cord and brain, including the guidance of spinal motor axons, post-crossing spinal commissural axons, and a variety of axons in the forebrain (Brose et al., 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999). In vitro studies have further suggested the involvement of these proteins in a number of other biological processes, including the regulation of neuronal cell migration (Wu et al., 1999; Zhu et al., 1999) and lymphocyte trafficking (Wu et al., 2001). Three *Robo* homologs have been identified in vertebrates as well, and at least two bind Slit proteins (Brose et al., 1999; Kidd et al., 1998; Li et al., 1999; Yuan et al., 1999). Recently, the Robo receptor Robo2 was shown to control multiple aspects of retinal axon guidance in zebrafish (Fricke et al., 2001). Its expression in mammalian RGCs (Erskine et al., 2000), taken together with the expression of the *Slit* genes along the trajectory followed by these axons has suggested that Slit/Robo ligand/receptor complexes may play an important role in regulating retinal axon guidance in higher organisms as well.

Despite extensive in vitro analysis, the role of vertebrate Slits in mediating axon guidance events in vivo has not been defined. As a first step toward determining the in vivo roles of Slit proteins in axon guidance, we have therefore generated mice deficient in *Slit1* and *Slit2*. In *Slit1*-, *Slit2*-, and *Slit1/2*-deficient mice, no obvious defects in commissural axon guidance were observed within the developing spinal cord (data not shown), presumably due to the overlapping expression in the midline floorplate of a third Slit, *Slit3*. In the developing visual system where *Slit3* is not expressed, however, we have found profound defects in retinal axon guidance. Our results document an important role for these proteins in retinal axon guidance through repulsion, particularly with regard to defining where the optic chiasm forms. However, unlike at the midline of the *Drosophila* nervous system, during formation of the vertebrate optic chiasm the Slit proteins do not appear to play a critical role in regulating axon divergence. Rather, Slit1 and Slit2 cooperate in a unique fashion to channel retinal axons along their appropriate pathway and thereby determine the precise position along the neuraxis at which this important commissure develops.

Results

Generation of *Slit1*- and *Slit2*-Deficient Mice

Using gene targeting in embryonic stem (ES) cells, we generated mice deficient in either *Slit1* or *Slit2*. A rat cDNA probe specific for either *Slit1* or *Slit2* was used to screen a mouse bacterial artificial chromosome (BAC) 129 ES cell library (Incyte Genomics). To target *Slit1*, BAC DNA was subcloned into plasmids and a portion of an exon encoding most of the second leucine rich repeat (LRR) was replaced with a cassette containing (in order from 5′ to 3′): an endoplasmic reticulum KDEL retention sequence (Munro and Pelham, 1987), a stop codon, an IRES element, a tauGFP fusion protein, a loxP site, a PGK-1 promoter, a neomycin resistance (neo^r) gene, a PGK-1 polyA tail, and a second loxP site (abbreviated IRES-tauGFP-LNL and referenced in Rodriguez et al., 1999) (Figure 1A). The KDEL element was used to prevent any potential partial peptide translated from the small amount of residual 5′ *Slit1* gene from being secreted. The IRES element was inserted to allow bicistronic expression of the tauGFP reporter from the *Slit1* promoter. The loxP elements were used to allow Cre-mediated excision of the neo^r gene and the flanking PGK-1 regulatory elements. Following Cre-mediated recombination of the loxP flanked neo cassette (using a ubiquitously expressed Cre transgenic mouse line [Lewandoski and Martin, 1997]), GFP expression appeared unchanged, so all analyses were done in the nonrecombined lines.

ES cells containing homologous integrants were screened by using a 5′ flanking probe (Figure 1B) and confirmed by using a neo-specific and a 3′ internal probe. These clones were used to generate chimeric male mice that were then mated to CD-1 or C57Bl6 females to generate germline transmissible *Slit1*-deficient mice on either a CD-1/129Sv or a C57Bl6/129Sv outbred genetic background. Resulting heterozygotes were crossed to generate homozygous deficient mice, and the expected Mendelian ratio was observed among wild-type, heterozygous, and homozygous mutant mice. The homozygous deficient animals appeared grossly normal. In situ analysis of *Slit1* expression showed that a 3′ probe failed to hybridize to E11.5 ventral spinal cord, where intense *Slit1* expression occurs in wild-type mice (Figure 1C). This demonstrated that, at the level of detection by in situ hybridization, significant alternative splicing did not occur around the targeted exon.

To target *Slit2*, a similar approach was taken to that used to disrupt *Slit1*. The *Slit2* mouse genomic locus was subcloned into plasmids and a portion of the putative first and second exons were replaced with the same targeting cassette used with *Slit1*, excluding the KDEL element (Figure 2A). This insertion left the initiation methionine codon intact but disrupted the probable signal sequence, and a portion of the first LRR. Using a 5′ flanking probe, we identified several homologous recombinants in the *Slit2* locus (Figure 2B). The faithfulness of this integration event was confirmed by further Southern blot analysis using a neo-specific and an internal 3′ probe. Correctly targeted ES clones were used to generate several chimeras that were then mated with C57Bl6 females to create homozygous deficient mice on a C57Bl6/129Sv outbred genetic background. Heterozygous intercrosses revealed that *Slit2* homozygous deficiency was lethal. Although a small percentage of *Slit2* homozygous mutants lived for a few days after birth, the majority died within the first day of life, and none lived past 2 weeks. When crossed into the *Slit1*-deficient background, *Slit1/2*-deficient mice also died within the first day of life. At E18, 1 day prior to birth, both *Slit2*- and *Slit1/2*-deficient mice appeared grossly normal and were present in an expected Mendelian ratio, indicating that the lethality was perinatal.

The Optic Chiasm Develops Normally in *Slit1*- or *Slit2*-Deficient Mice

To assess the role of the Slit proteins in axon guidance within the developing visual system, we labeled retinal

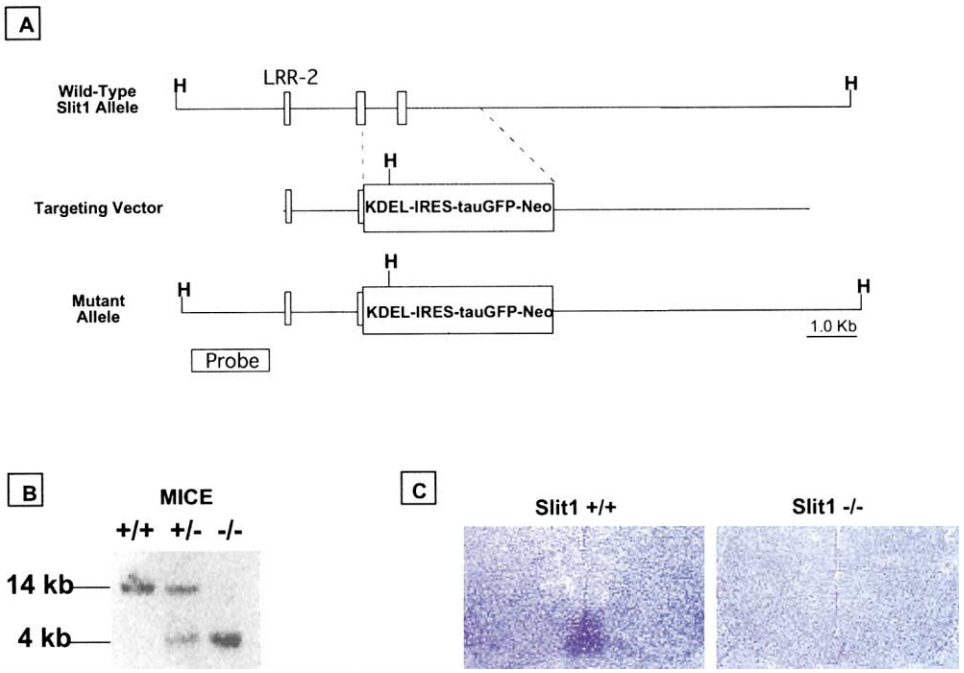


Figure 1. Creation of *Slit1*-Deficient Mice

(A) Targeting strategy. The upper line shows the wild-type *Slit1* locus. The middle line shows the targeting vector. A portion of an exon containing sequence coding part of the second leucine rich repeat (LRR-2), located in the 5′ region of the *Slit1* gene was replaced with a targeting cassette containing an internal ribosome entry site (IRES), a tauGFP fusion protein, and a neomycin resistance gene (neo) flanked by a PGK-1 promoter and polyA tail, and by two loxP sites. A stop codon and the endoplasmic retention sequence, KDEL, was placed in frame in the *Slit1* gene to prevent secretion of any potentially translated partial peptide. The lower line shows the correctly targeted locus. H, HindIII.

(B) Southern blot of HindIII digested genomic DNA hybridized with the 5′ flanking probe shown in the schematic above.

(C) In situ hybridization of a transverse section from an E11.5 thoracic spinal cord using a 3′ *Slit1* antisense probe. Since the *Slit1*-deficient mice are viable, we wanted to verify that the targeted genetic disruption resulted in disruption of *Slit1* mRNA. As shown in the in situ, no detectable message can be seen in the ventral spinal cord of the *Slit1*-deficient mouse, an area of significant expression in normal mice.

axons from the right eye of E15.5 wild-type and *Slit*-deficient mice with the lipophilic dye, DiI. E15.5 is a developmental time point during which the divergence of crossing and noncrossing axons occurs at the optic chiasm (Godement et al., 1990; Sretavan, 1990). In mice deficient for either *Slit1* or *Slit2* individually, the optic chiasm appeared relatively normal (Figures 3A–3D and 3F; Figure 4). In wild-type and each single mutant, the axons extended along their normal pathway toward the midline where the majority crossed to project to targets on the contralateral side of the brain. There also was a small proportion of fibers that projected ipsilaterally and a few fibers that extended into the contralateral optic nerve. This analysis suggested that in general *Slit1* and *Slit2* deficiency alone do not cause significant defects in axon guidance within the developing visual system; however, it is possible that more subtle defects exist that are beyond the sensitivity of these experiments. For example, there could be a shift in the relative proportion of fibers that project ipsilaterally versus contralaterally. Additional detailed analysis would be needed to assess the possibility of quantitative defects of midline axon divergence.

The Optic Chiasm in *Slit1*/*Slit2* (*Slit1/2*) Double Mutant Mice Develops Abnormally

Although the visual projections of *Slit1*- or *Slit2*-deficient mice developed normally, mice deficient for both *Slit1* and *Slit2* developed striking abnormalities (Figures 3E and 3G). The fact that defects were seen only in the double mutants was unexpected given that *Slit1* and *Slit2* are expressed in complementary rather than overlapping domains within the ventral diencephalon (Erskine et al., 2000). Of those observed, the most striking defect was the development of an ectopic commissure in the pre-optic area, anterior and slightly ventral to the normal optic chiasm. Some of the fibers that crossed in this ectopic commissure extended into the contralateral optic nerve while others appeared to travel ventral to the optic nerve to join the contralateral optic tract. There was also a significant increase in the number of fibers that, after crossing in the normal optic chiasm, projected into the contralateral optic nerve. We also found that in all *Slit1/2* double mutant mice examined ($>$20) a significant proportion of fibers projected ipsilaterally (in the example shown in Figure 3E, the appearance of the ipsilateral tract is not well visualized because of the plane of imaging). The existence of an ipsilateral optic tract in the double mutants suggests that the Slit proteins alone do not regulate axon divergence at the developing optic chiasm. The possibility that the Slits act to affect divergence in a more subtle way, however, cannot be ruled out. Due to the large number of fibers that projected ectopically we were unable to determine in a more quantitative fashion (for example, by retrograde labeling)

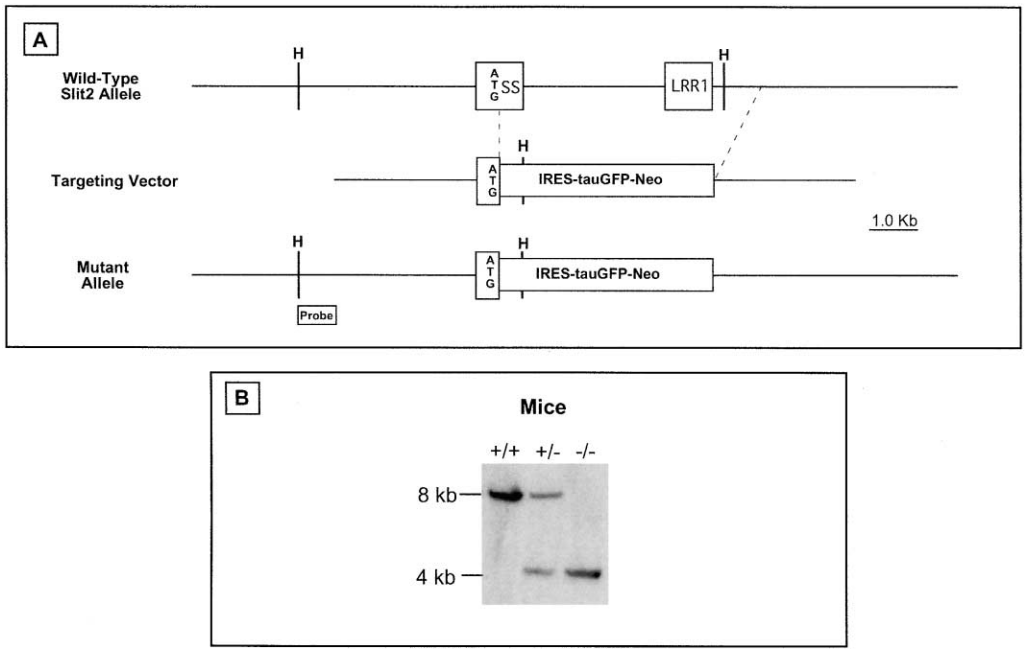


Figure 2. Creation of *Slit2*-Deficient Mice

(A) Targeting strategy for *Slit2* using a similar approach as that used for *Slit1*. A more 5′ portion of the gene was targeted using the same cassette as the one employed for *Slit1* (without the KDEL element). The cassette replaced the likely signal sequence (SS) and part of the first leucine rich repeat (LRR1).

(B) Southern blot of HindIII digested genomic DNA hybridized with the 5′ flanking probe shown in (A). As noted in the text, the *Slit2*-deficient mice die at birth.

whether the size of the ipsilateral projection was affected in these mice. Similar but less severe defects were also found in *Slit1*$^{+/-}$; *Slit2*$^{-/-}$ mice but not in mice from any of the other genotypic combination (Figure 4; data not shown).

Pioneer Retinal Axons Project Ectopically and the Defects Persist at Least Until Birth

We next examined the time course over which the ectopic chiasm developed to address two questions: do pioneer axons stray, or is the defect restricted to later developing axons; and do the defects persist after retinal axons have reached their targets? At E12.5, retinal axons normally enter the brain and project posteriorly and then anteriorly, avoiding the pre-optic region (Marcus et al., 1995) (Figure 5A). In *Slit1/2* mutant mice, many axons projected normally; however, we consistently found that there was a subset of axons that no longer avoided the pre-optic area but instead projected in an aberrant anterior direction toward the midline (Figure 5B). A day later (E13.5), as more axons grew into the brain, the number of fibers projecting anteriorly had increased and the ectopic second commissure could clearly be seen (Figures 5C and 5D). The increased number of fibers projecting into the contralateral optic nerve also became apparent at this age. As seen by tracings performed in P0 mice, the latest age we were able to examine (the double mutants die around P0), we found that many of the observed defects persisted (Figures 5E and 5F). Although a considerable amount of plasticity exists in the visual system in the first few weeks after birth, by P0 many RGC axons have normally reached their targets in the superior colliculus and lateral geniculate nucleus. At this time point, the chiasm appeared abnormally thick around the midline and the axons that had crossed in the ectopic commissure appeared to loop around to merge with fibers in the contralateral optic tract.

Ectopically Projecting Fibers in *Slit1/2* Mutant Mice Are Distributed throughout the Retina

In the adult mouse retina, approximately 97% of axons cross the midline at the chiasm to innervate contralateral targets (Drager, 1985). The cell bodies for these fibers are found throughout the retina, whereas the cell bodies of axons that do not cross the midline at the optic chiasm are for the most part restricted to the ventrotemporal quadrant. To determine whether the ectopically projecting RGC axons were restricted to a particular region of the retina, we used small crystals of DiI to selectively label axons from each of the quadrants. Using this approach, we found that axons from all four retinal quadrants projected aberrantly (Figures 5H and 5J, and data not shown). We also found that, as in wild-type mice, ipsilaterally projecting axons arose specifically from the ventrotemporal region of the retina (see supplemental figure at http://www.neuron.org/cgi/content/full/33/2/219/DC1 and data not shown), providing additional evidence that Slit proteins are not critical for retinal axon divergence.

The quadrant fills further demonstrated that RGC axons are responsive to Slit prior to crossing the midline. Axons coming from regions of the retina that contain

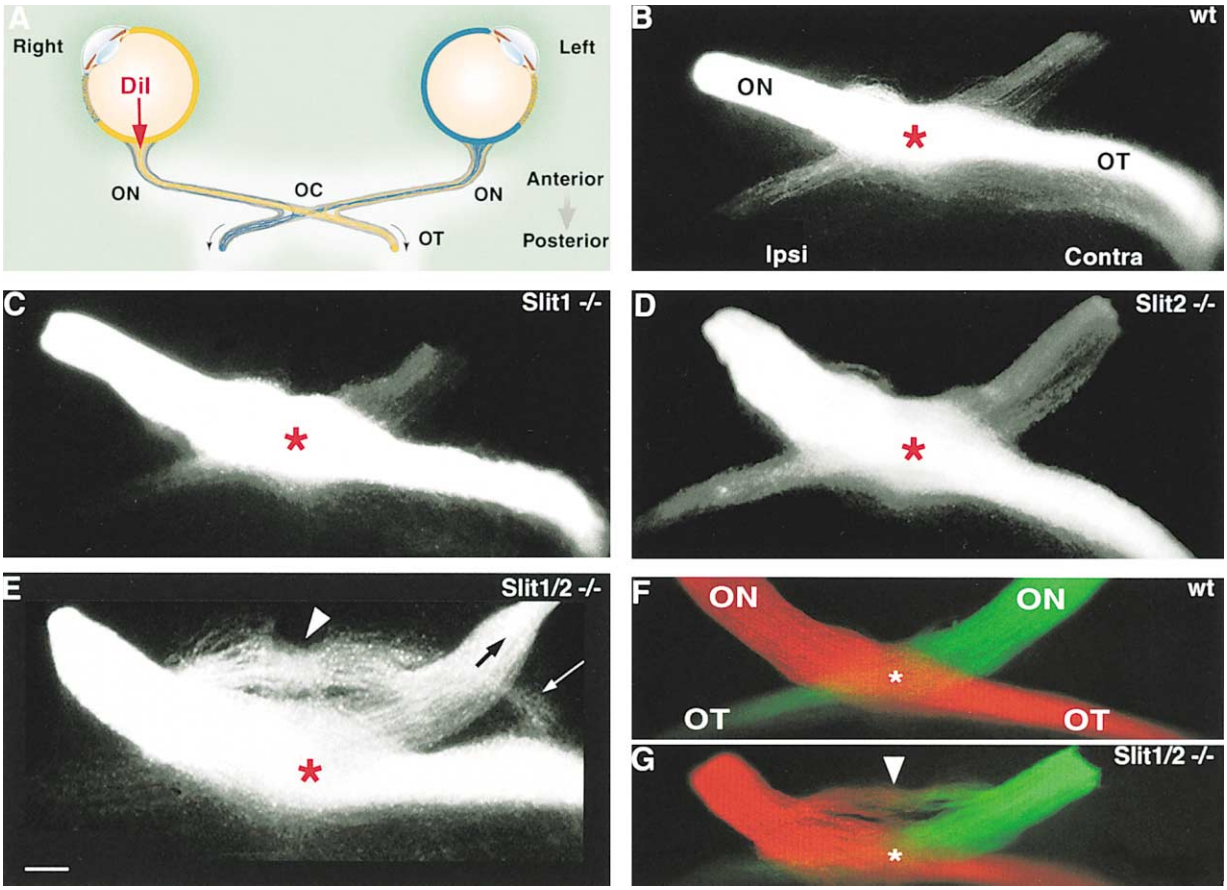


Figure 3. Defects in Retinal Axon Projections in *Slit* Single and Double Mutant Mice at E15.5

Lipophilic dye tracings of the proximal visual pathway in E15.5 mice. Crystals of DiI (A–E) were placed unilaterally in the right optic disk, and for (F) and (G), in addition to DiI, DiO crystals were placed in the left optic disk, to create complete retinal axon fills. After diffusion of the dye, brains were dissected out and the ventral surface imaged with a fluorescent dissecting microscope.

(A) Schematic of the visual system looking up at the ventral surface of the brain. Anterior is to the top, posterior to the bottom. The ventrotemporal quadrant of each retina, as shown in speckled blue and yellow, contains both ipsi- and contralaterally projecting fibers. The arrows denote the path of the optic tracts as they dive dorsally or into the plane of the page.

(B) Wild-type. ON, optic nerve; OT, optic tract; Ipsi, ipsilateral pathway; Contra, contralateral pathway; the red asterisk marks the location of the normal optic chiasm.

(C) *Slit1*-deficient.

(D) *Slit2*-deficient.

(E) *Slit1/2*-deficient. The arrowhead shows the ectopic optic chiasm and the thin arrow highlights wandering fibers that have crossed in the aberrant commissure and then righted themselves by traveling toward the contralateral optic tract. Note also the high percentage of axons that travel alongside axons of the contralateral optic nerve and toward the contralateral eye (black arrow).

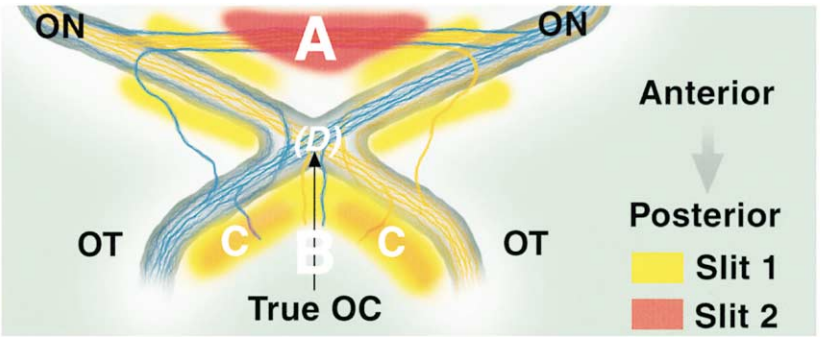
(F) Wild-type. Double label of the visual system with DiI (right) and DiO (left). White asterisk marks the normal optic chiasm.

(G) *Slit1/2*-deficient. Arrowhead marks the ectopic optic chiasm. Scale bar, 100 μm.

predominately contralaterally projecting cells (for example, the dorsonasal quadrant; Figure 5H) projected into the ectopic chiasm. This strongly suggests that contralaterally projecting RGC axons are normally responsive to Slit before they have crossed the midline, contrasting markedly with what has been found in *Drosophila* and in the vertebrate spinal cord. In these two settings, contralaterally projecting axons only normally become Slit-responsive after they have crossed the midline (Kidd et al., 1999; Zou et al., 2000). Because ipsilaterally projecting fibers constitute such a small percentage of total RGC axons and are mixed together with contralaterally projecting cells within the ventrotemporal retina, we could not specifically assess the behavior of the ipsilaterally projecting axons. Nevertheless, these data strongly suggest that the functions of Slit proteins in the vertebrate visual system are fundamentally different from those previously described in *Drosophila*.

Additional Axon Guidance Defects in the Visual System of Slit-Deficient Mice

When we sectioned the DiI-labeled brains of the Slit-deficient mice, a number of additional axon guidance defects were found in the region of the developing optic chiasm. As seen in coronal sections, the normal retinal axon projection through the optic chiasm is a single tightly bundled fascicle (Figures 6A and 6B). In *Slit1/2*-deficient mice, ectopically projecting axons were consistently found in two distinct locations. First, a small number of axons appeared to be impeded at the midline,



Genetic Background	A Ectopic Chiasm	B Anterior-Posterior Midline Wandering	C Ventrolateral Wandering	D Ventral-Dorsal Midline Wandering
Slit1 -/-	-	+	-	-
Slit2 -/-	-	-	-	-
Slit1 -/- ; Slit2 +/-	-	+	-	-
Slit1 +/- ; Slit2 -/-	++	NA	NA	NA
Slit1 -/- ; Slit2-/-	++++	+	+	+

Figure 4. Summary of Axon Guidance Defects in *Slit1*- and *Slit2*-Deficient Mice

The top panel shows the anatomy of the midline region of the mouse visual system with anterior to the top and posterior the bottom. Highlighted are the approximate expression patterns of *Slit1* (yellow) and *Slit2* (red). (A–D) label the location of the axon guidance defects seen in these mice. Not schematized are the ventral-dorsal midline wandering axons labeled by (D) since these grow into the plane of the paper. In the lower panel, the relative extent of the various axon guidance defects are annotated qualitatively. In each case, the defect was fully penetrant with minimal variability in expressivity (i.e., seen in all animals of a particular genotype to approximately the same degree in each one). At least three mice, and in many cases more than ten, were evaluated for each genotype. ON, optic nerve; OT, optic tract; OC, optic chiasm; NA, not analyzed.

projecting in an aberrant dorsal direction (Figure 6C and 6D). This is a phenotype reminiscent of the midline wandering seen in *Drosophila Slit* mutants. Secondly, in the region where the optic tract turns as it projects dorsally, there is another subset of axons that have grown inappropriately into a dorsolateral portion of the diencephalon (Figure 6D). This region normally expresses *Slit1* and *Slit2* and in vitro possesses an inhibitory activity toward retinal axon growth (Tuttle et al., 1998). Lastly, horizontal sections through the ventral diencephalon of *Slit1/2*-deficient mice revealed a third group of fibers that had extended in an ectopic direction (Figures 6E–6H). These fibers grew posteriorly along the border of the third ventricle, a domain where *Slit1* but not *Slit2* is normally expressed. As might be predicted based on this pattern of expression, similar straying fibers were found in the *Slit1* but not *Slit2* singly deficient mice (Figure 4; data not shown).

Immunohistochemistry using an anti-neurofilament antibody further revealed that retinal fibers projected abnormally within the chiasm. In the *Slit1/2*-deficient mice, two distinct axon fascicles could be seen as opposed to the single fascicle in wild-type mice (Figures 7A and 7B). One of these axon bundles followed the normal pathway along the pial surface, whereas the other extended dorsally toward the midline. This suggests that for a subpopulation of retinal axons the Slit proteins control the position at which the axons travel along the dorsoventral axis. Furthermore, these observations indicate that the Slit proteins play an important role in mediating the tight fasciculation of axons within the chiasm as has been suggested in vitro for retinal axon projections within more distal portions of the optic tract (Ringstedt et al., 2000). The finding that there are defasciculation and axon pathfinding defects within the normal optic chiasm was unexpected since neither *Slit1* nor *Slit2* are expressed in this region of the ventral diencephalon (Niclou et al., 2000; our unpublished data).

The Ventral Diencephalon of Slit-Deficient Mice Appears to Develop Normally

One potential explanation for the retinal axon pathfinding errors that we have found in the Slit-deficient mice would be that the brains of these mice develop abnormally. We do not believe that this is the case. Firstly, the gross morphology of the brains of each of the Slit-deficient mice is indistinguishable from wild-type (data not shown). Secondly when sectioned, the diencephalon appears relatively normal (see companion paper, Bagri et al., 2002, in this issue of *Neuron*). Thirdly, the CD44/SSEA-1 neurons located posterior to the chiasm and implicated in playing a role in its development (Sretavan

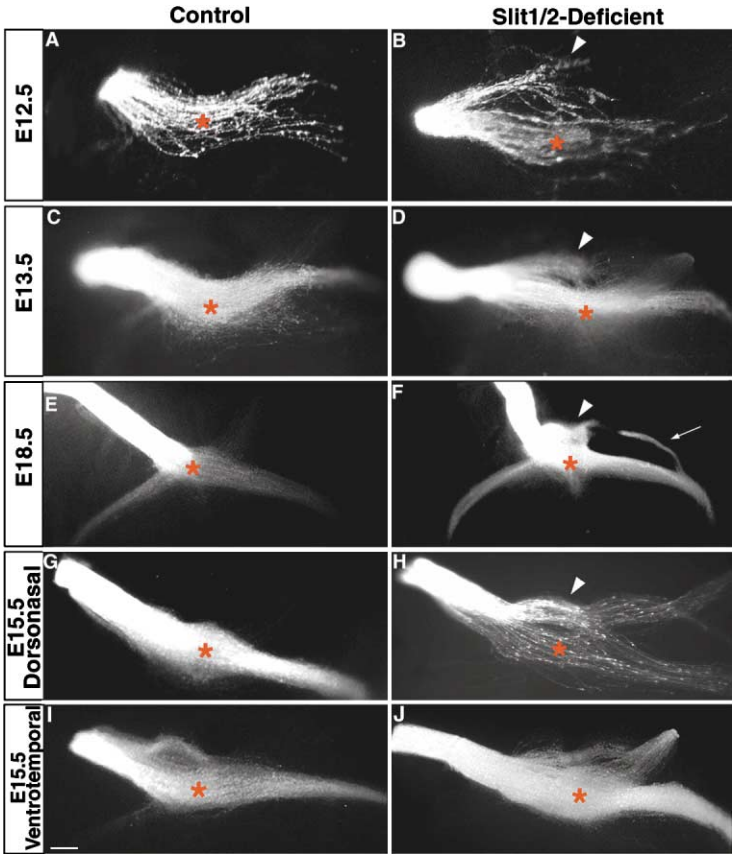


Figure 5. Development of Defects in Retinal Axon Projections in *Slit* Mutant Mice

Time course study of retinal axon pathfinding defects at the level of the optic chiasm following complete DiI fills of the right retina (A–F), and analysis of dorsonasal and ventrotemporal retinal quadrant fills in E15.5 mice (G and H, I and J, respectively). Images from control mice are on the left and *Slit1/2*-deficient mice on the right. (A and B) E12.5. (C and D) E13.5. (E and F) E18.5. The red asterisk shows the position of the normal optic chiasm. In all *Slit1/2*-deficient images the arrowhead shows the ectopic chiasm. In (F), the thin arrow highlights wandering axons traveling toward the contralateral optic tract. At E12.5 in the *Slit1/2* mutant (B), as the pioneer retinal axons reach the pre-optic area, they begin to grow toward the site where the ectopic chiasm later develops. At E13.5 (D), there is a sense that in addition to the major commissural defect, the true chiasm appears to form at slightly less of an angle. For the E18.5 images (E and F), the contralateral optic nerve was removed during the dissection and is not shown. Note in (F) the presence of a small group of abnormal posteriorly projecting fibers, as well as the presence of a normal appearing ipsilateral optic tract. (G and H) Tracings from a dorsonasal retinal quadrant fill from a control (G), and a *Slit1/2*-deficient (H) mouse. (I and J) Tracings from a ventrotemporal quadrant fill from a control (I), and a *Slit1/2*-deficient mouse (J). Note that in such whole-mount views, the ipsilateral projection arising from the ventrotemporal retina is out of the plane of focus. As shown in Supplemental Figure 1 at http://www.neuron.org/cgi/content/full/33/2/219/DC1; however, there is a well-formed ipsilateral tract in the double mutant that appears qualitatively similar to that formed in the control mouse. Scale bar, 100 μm.

et al., 1994) are positioned in the same manner as those in control mice (Figures 7C and 7D).

Slit1 Inhibits Outgrowth of Retinal Ganglion Cell Axons

These results strongly suggest that Slit1 and Slit2 play a critical role in channeling retinal axons toward their appropriate midline crossing point, serving as inhibitors for growth into inappropriate regions of the brain. This model is supported by the fact that Slit2 can function as a repellent for these axons in vitro (Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000) (Figure 8E). To test whether Slit1 also can inhibit retinal axon outgrowth, we cocultured explants of retina from E14.5 mice in collagen gels with aggregates of COS cells expressing Slit1 or Slit2, or transfected with the empty expression plasmid (used as a control). Because the ventrotemporal retina contains mixed populations of ipsilaterally and contralaterally projecting axons, we excluded this quadrant from the analysis and used pooled tissue from the three remaining quadrants. Since *Slit1* is expressed by retinal ganglion cells, in preliminary experiments, we also tested the response of *Slit1*$^{-/-}$ retina to Slit1 and Slit2 and found it to be similar to wild-type retina, so the data in Figure 8E are presented as pooled samples of wild-type and *Slit1*$^{-/-}$ retinae. We found that Slit1 is a potent inhibitor of retinal axon growth, inducing a similar decrease in the extent of outgrowth to that seen in the presence of Slit2 (Figures 8A, 8B, and 8E). Compared to that seen in the presence of control transfected cells, outgrowth was reduced by 80% in cultures containing Slit1-expressing cells and 71% in the presence of Slit2-expressing cells.

The Pre-Optic Area of *Slit1/2*-Deficient Mice Is Less Inhibitory to Retinal Axon Outgrowth than Wild-Type Pre-Optic Area

We next tested whether the region of the ventral diencephalon where the ectopic chiasm forms (the pre-optic area, POA) has an inhibitory effect on retinal axon outgrowth. We cultured retinal explants in collagen gels either alone or with pre-optic area tissue taken from wild-type or *Slit1/2*-deficient mice (Figures 8C and 8D). Explants of pre-optic area tissue from wild-type mice had a potent inhibitory effect on retinal axons, inducing an approximately 85% decrease in the amount of outgrowth observed in the absence of this tissue. By comparison, in parallel experiments pre-optic area tissue from *Slit1/2*-deficient mice was found to have lost some but not all of this activity and only induced an approximately 40% decrease in the extent of retinal axon outgrowth (Figure 8F). Both the number and the length of the axons extending into the collagen were increased. Inhibitory cellular cues anterior to the chiasm have been described (Silver et al., 1987). The fact that some inhibitory activity remains in the *Slit1/2*-deficient pre-optic

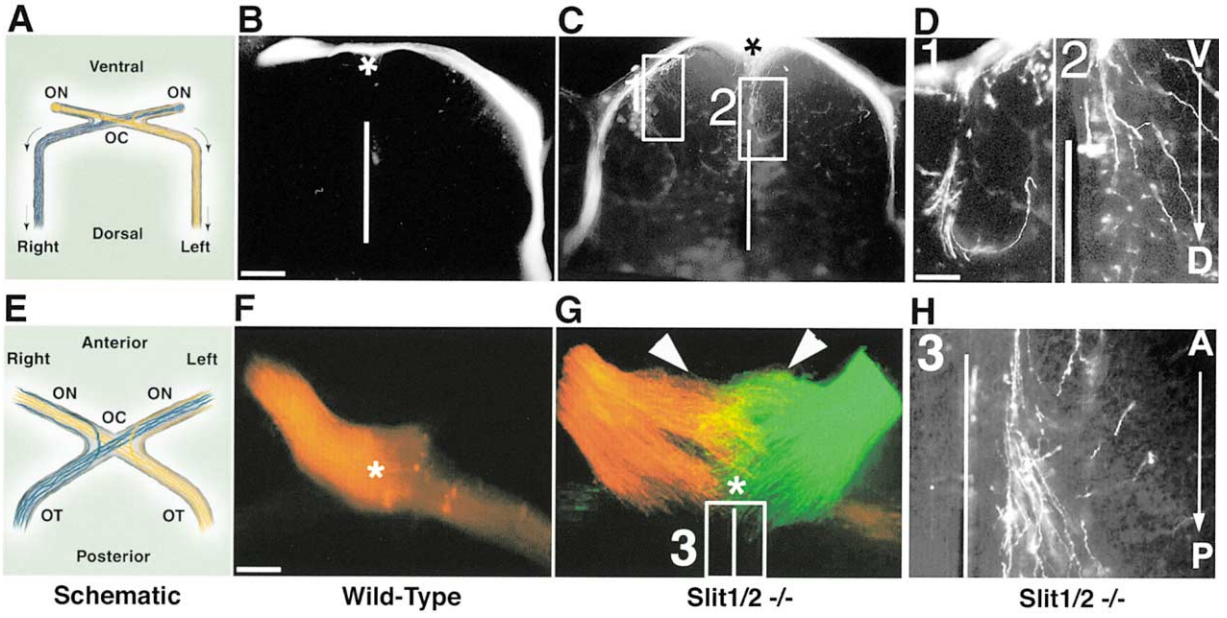


Figure 6. Axonal Wandering in *Slit* Mutant Mice

Sections of DiI tracings of the visual pathway in E15.5 mice.

(A) Schematic of a coronal section taken through the level of the optic chiasm showing the orientation of the sections shown in (B–D).

(B) Coronal section from a control mouse. Vertical line shows the midline as defined by the third ventricle. The asterisk denotes the optic chiasm.

(C) Coronal section from a *Slit1/2*-deficient mouse. Note the two regions where axons wander from their normal trajectory: in the ventrolateral diencephalon as the right optic tract turns dorsally (Box 1) and in the midline (Box 2).

(D) High magnification of the region boxed in (C), but imaged from a different section. As noted ventral is up and dorsal down, with axons wandering in the direction shown by the arrow.

(E) Schematic of a horizontal section through the optic chiasm, showing the orientation of (F)–(H). Anterior is to the top and posterior the bottom.

(F) Horizontal section of a control mouse with the DiI tracing shown in color for comparison to (G).

(G) Horizontal section of a *Slit1/2*-deficient mouse labeled with DiI (right eye) and DiO (left eye). Note the mixing of green and red at the midline in the ectopic commissure (arrowheads) and in the true chiasm posterior to it.

(H) High magnification of the boxed area in (G). Anterior is up and posterior down, with axons wandering in the direction shown by the arrow.

area explants suggests that there are other diffusible cues inhibitory to retinal axon outgrowth and may explain why not all retinal axons extend ectopically into this region.

Discussion

The guidance of RGC axons from the retina to their targets is essential for the establishment of a normal visual circuit. In this study, we provide evidence that the mammalian Slit1 and Slit2 proteins are potent regulators of retinal axon growth in vivo and are critical for the establishment of a normal visual pathway. Using gene targeting in ES cells, *Slit1*- and *Slit2*-deficient mice were created. *Slit1/2* double mutants developed severe and persistent axon guidance defects in the visual system, including the formation of a second, ectopic optic chiasm, aberrant growth of retinal axons into the contralateral optic nerve, and axon wandering defects in the ventral diencephalon. Taken together with the fact that only subtle or no anomalies existed in the singly-deficient mice, these findings suggest that these two family members act synergistically to control retinal axon guidance, particularly around the region of the developing optic chiasm. In addition to revealing the molecular identity of two molecules essential for visual system development, these results, in parallel with those of a companion study demonstrating severe defects in several cortical and thalamic axon tracts in the forebrain of *Slit2*- and *Slit1/2*-deficient mice (Bagri et al., 2002), provide the first evidence in vertebrates that the evolutionarily conserved Slit proteins can act as repulsive axon guidance cues in vivo.

Slit1 and *Slit2* Define Zones Inhibitory to Retinal Axon Extension

Within the ventral diencephalon, *Slit1* and *Slit2* are expressed in complementary domains surrounding the path of the ingrowing retinal axons (Erskine et al., 2000) (Figure 9A). *Slit1* is expressed dorsal to and around the junction of the optic nerve and the brain, whereas *Slit2* is strongly expressed at the ventral midline of the diencephalon in a region directly dorsal and slightly anterior to the site where the optic chiasm develops (pre-optic area). These zones of *Slit* gene expression appear to establish a corridor through which retinal axons can travel, resulting in the correct positioning of the optic chiasm within the brain. In the absence of both *Slit1* and *Slit2*, we have found that retinal axons are no longer restricted to their normal pathway but instead can pro-

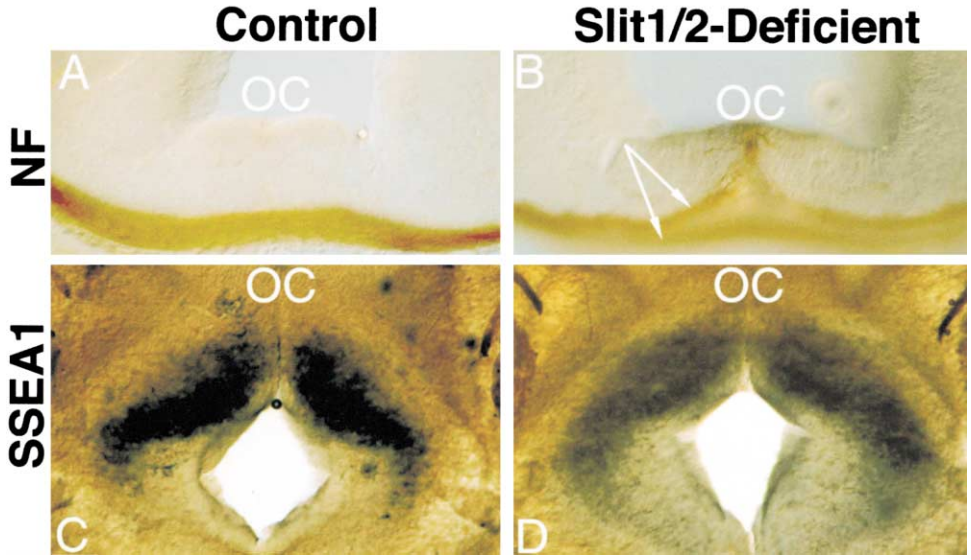


Figure 7. Defects in Axonal Projections at the Optic Chiasm in *Slit* Mutant Mice

Neurofilament and SSEA-1 immunohistochemistry at the level of the optic chiasm in E13.5 mice. Control sections are to the left, and *Slit1/2*-deficient sections to the right.

(A and B) Neurofilament immunostaining of coronal sections. The arrows denote the defasciculation that occurs within the mutant chiasm. Dorsal is to the top, ventral the bottom.

(C and D) SSEA-1 immunostaining of horizontal sections. Just above the area of SSEA-1 positive neurons in the ventral diencephalon is the optic chiasm. Anterior is to the top, posterior the bottom. OC, optic chiasm.

ject ectopically into the pre-optic area, a brain region inhibitory for these axons. This results in the formation of a second optic commissure anterior to the normal chiasm, the inappropriate extension of the axons into the contralateral optic nerve, and the wandering of retinal axons along the midline and at the junction of the optic tract.

With regard to chiasm development, three features of the Slit-deficient mice are noteworthy: the somewhat unique mechanism by which the Slits appear to channel RGC axons, the severity of the observed defects, and the fact that the ectopic commissure seems to develop in an invariant position along the antero-posterior axis. Regarding the first two, there are few examples of what appears to be an axon "cordoning" mechanism existing in other CNS systems, and none so dramatic as that for the Slits in the visual system. The severity of these defects is further highlighted by the fact that many of these pathfinding errors persist late into development. The fact that an ectopic commissure only forms anterior to the normal chiasm suggests that additional inhibitory cues exist caudal to the chiasm. In vitro, the pre-optic area of wild-type mice has a potent inhibitory effect on retinal axon guidance, whereas the pre-optic area of *Slit1/2*-deficient mice has lost most, but not all, of this activity (Figure 8). Together with the finding that Slit1 and Slit2 can inhibit outgrowth and induce collapse of RGC axon outgrowth (Figure 9) (Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000), this strongly suggests that Slit1 and Slit2 normally function in vivo in an inhibitory manner to prevent retinal axons from extending into the pre-optic area and are one critical component of the mechanism regulating the positioning of the optic chiasm along the antero-posterior axis. The fact that some inhibitory activity persists in the *Slit1/2*-deficient pre-optic area explants may explain why only a subset of axons project ectopically into this region and further suggests that inhibitory guidance cues other than Slits are involved in determining the anterior boundary of the chiasm.

Slit1 and Slit2 Regulate Axon Fasciculation

In addition to being inhibitors of retinal axon outgrowth, Slit1 and Slit2 also have been implicated in maintaining the proper fasciculation of retinal axons (Ringstedt et al., 2000). In the *Slit1/2*-deficient mice, we found clear evidence for fasciculation errors within the chiasm itself (Figure 7) where extensive reorganizing of the retinal axons normally occurs (Chan and Chung, 1999; Silver, 1984). This suggests that Slit proteins may act not only to define the antero-posterior position at which the retinal axons cross the midline but also to control the organization of axons within the true chiasm, an unexpected observation given the lack of *Slit* gene expression at this location. In *Drosophila*, Slit can act at a distance from its site of expression to regulate the relative position of axon tracts (Rajagopalan et al., 2000; Simpson et al., 2000). Thus, one possibility is that Slit proteins might influence the organization of axons within the optic chiasm either by inward diffusion or presentation of Slit into this region by nearby expressing radial glial cells (Mason and Erskine, 2000). The ability of Slit proteins to diffuse away from their site of expression and/or localize on radial glia processes will require Slit antibodies to assess experimentally. Changes in the degree of fasciculation of retinal axons also could contribute to the formation of the ectopic commissure and other axon pathfinding defects in the Slit-deficient mice. Whether

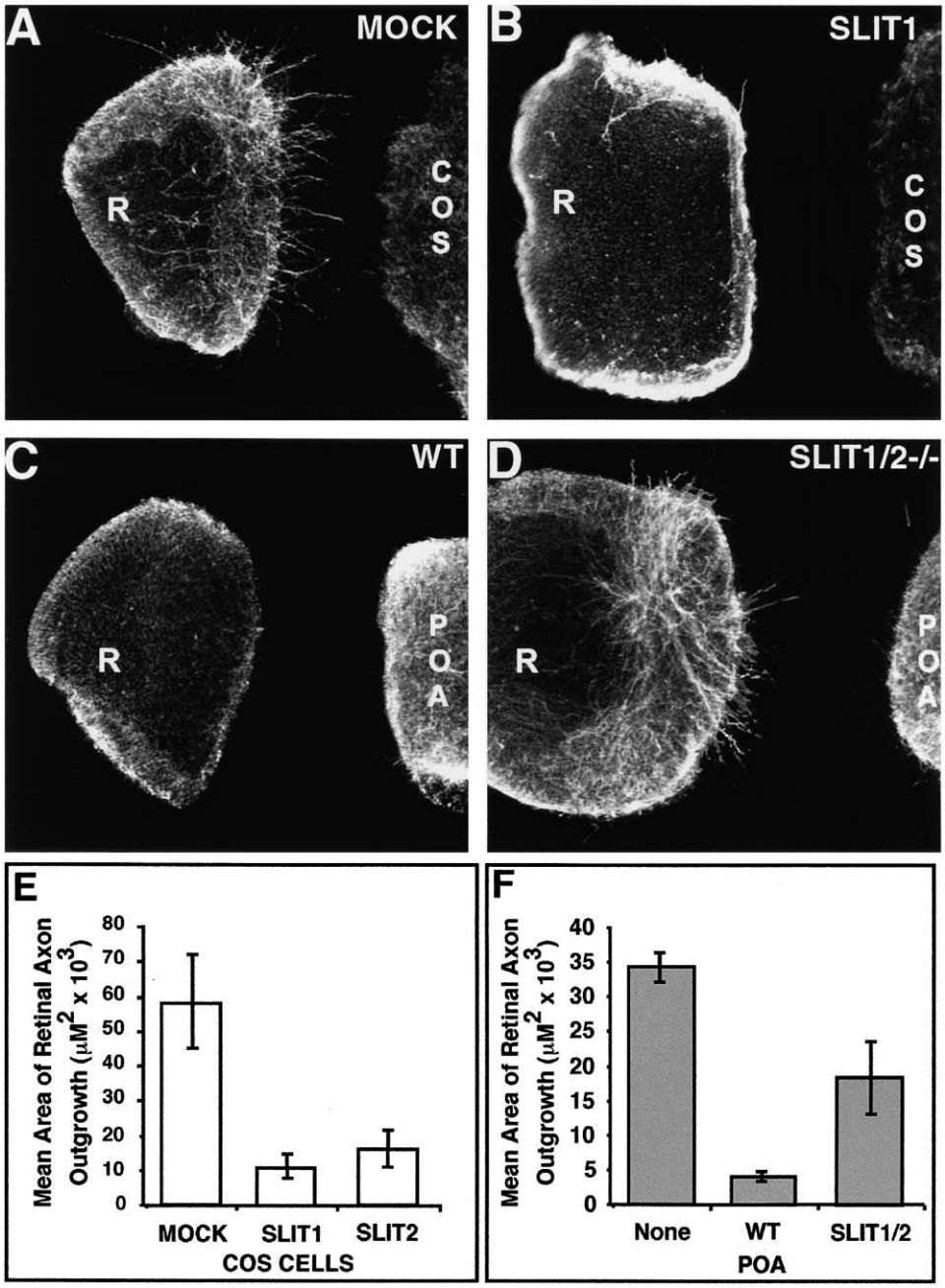


Figure 8. Slit Proteins Mediate Repulsion by Pre-Optic Area Tissue

Inhibition of retinal ganglion cell explants by Slit1 and pre-optic tissue. Retinal explants (R) from contralaterally projecting quadrants (all but the ventrotemporal quadrant) were taken from E14.5 mice and cultured in collagen adjacent to either transfected COS cell aggregates or a piece of E14.5 pre-optic tissue (POA).

(A) Mock transfected COS cells.

(B) *Slit1* transfected COS cells.

(C) POA from wild-type mice.

(D) POA from *Slit1/2*-deficient mice.

(E) Outgrowth from retinal explants was quantified and data expressed as mean area of retinal axon outgrowth ± SEM. The data graphed is from a single experiment, with similar results obtained on three separate occasions. The retinal explants were derived from wild-type, *Slit1*$^{+/-}$, and *Slit1*$^{-/-}$ mice. There were no differences in outgrowth seen between wild-type and the *Slit1*-deficient retina, and the data presented represents pooled samples. Retinal axon outgrowth when exposed to: mock transfection, $59 \pm 28 \times 10^3$ μm^2 ($n = 19$); Slit1, $12 \pm 8 \times 10^3$ μm^2 ($n = 18$); or Slit2, $17 \pm 11 \times 10^3$ μm^2 ($n = 14$). Compared to mock transfected cells, Slit1 and Slit2 inhibited outgrowth with a $p < 0.00001$. In these experiments, Western blots demonstrated equivalent levels of Slit1 and Slit2 protein (data not shown).

(F) Quantification of retinal axon outgrowth when placed adjacent to POA from control or *Slit1/2*-deficient mice. Wild-type POA was 3.8-fold more potent at inhibiting retinal axon outgrowth than *Slit1/2*-deficient POA, $5 \pm 1 \times 10^3$ μm^2 ($n = 6$) versus $19 \pm 6 \times 10^3$ μm^2 ($n = 33$) ($p < 0.05$, Student's t test). Compared to untreated retinal explants ($35 \pm 2 \times 10^3$ μm^2, $n = 3$) both wild-type ($p < 0.0001$) and *Slit1/2*-deficient POA ($p < 0.05$) significantly inhibited outgrowth.

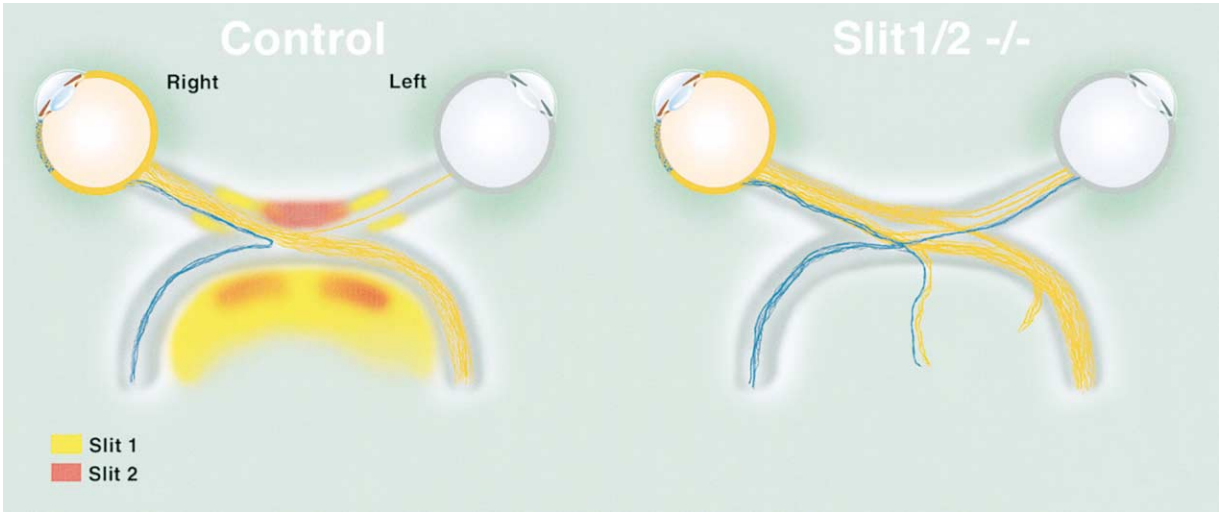


Figure 9. Schematic Diagram Showing the Location of Retinal Axon Guidance Defects in the *Slit1/2*-Deficient Mice with Respect to Areas of *Slit1* (Yellow) and *Slit2* (Red) Expression

Retinal axons are depicted as orange or blue depending on whether they will end up crossing (orange) or not crossing (blue) at the optic chiasm. The areas of Slit gene expression were schematized based on known patterns of expression from previous mRNA in situ hybridization analysis. We have simplified the three-dimensional space in which the retinal axons travel into a two-dimensional schematic. In doing this, the anterior area where the ectopic chiasm forms (the pre-optic area), which normally sits at a slightly more posterior location, appears inaccurately at the level of the chiasm in the diagram. (Left) Schematic of a unilateral optic pathway in a control mouse. (Right) Schematic of a unilateral pathway in a *Slit1/2*-deficient mouse. Note the three major defects observed in the mutant: the ectopic chiasm, growth of fibers into the contralateral nerve, and the peri- and postchiasmatic wandering axons.

the defasciculation of the axons from their normal pathway is secondary to the loss of inhibition in the surrounding regions or the primary cause of the pathfinding errors is currently not clear.

Slit1 and Slit2 Act Together to Regulate Axon Pathfinding in the Visual System

Although slight wandering defects were seen in *Slit1*-deficient mice, only the *Slit1/2* double knockout mice developed an aberrant midline chiasm. This was a surprising observation since the two *Slit* genes are expressed in a complementary rather than overlapping fashion in the region of the developing chiasm (Figure 9). The simplest explanation for this result is that Slit1 and Slit2 cooperate to regulate retinal axon guidance. The optic nerve enters the diencephalon at the level of the pre-optic area and must dive ventrally and posteriorly to reach its normal site of decussation at the optic chiasm (Colello and Guillery, 1990). In doing so, RGC axons avoid an area previously termed the "glial knot," thought to prevent these fibers from entering the olfactory region (Silver, 1984). It is likely that Slit1 facilitates initial RGC axon growth by creating a repulsive environment around the nerve to funnel its fibers toward the chiasm (Figure 9). In the absence of *Slit1*, the intense expression of *Slit2* in the POA appears to act in a compensatory fashion. This may result from axons initially misrouting but then correcting their trajectories upon encountering Slit2, or alternatively from diffusion of Slit2 into the region that normally expresses *Slit1*. Time-lapse imaging of retinal axon growth within the optic chiasm has, in fact, demonstrated that their growth cones can sample the environment and often grow into inhibitory regions before righting themselves and rerouting toward their proper destination (Godement et al., 1994). In either case, no observable defects are seen in the pre-optic area of *Slit1*-deficient mice. The absence of observable defects in the *Slit2*-deficient mice likely results from the fact that retinal axons cannot escape from the immediate barrier created by Slit1. The formation of an ectopic chiasm in the $Slit1^{+/-}$, $Slit2^{-/-}$ but not in the $Slit1^{-/-}$, $Slit2^{+/-}$ mice further suggests that the higher levels of *Slit2* expression within this region allows it to play a more dominant role.

While our data support a non-cell-autonomous function for the Slits in retinal axon guidance, the expression of *Slit1* by retinal ganglion cells in a dorsoventral gradient (Erskine et al., 2000; Ringstedt et al., 2000) suggest that it could also act in a cell-autonomous fashion. Coexpression of ephrinA2 and ephrinA5 with their Eph receptors in the retina has been implicated in modulating the response of the retinal axons to these same ligands when encountered along their pathway (Dutting et al., 1999; Hornberger et al., 1999). The fact that only slight defects are seen in the singly *Slit1*-deficient mice perhaps argues against a major cell autonomous role for Slit1 in retinal axon guidance. Furthermore, our initial experiments suggest that *Slit1*-deficient retina do not respond differently to aggregates of Slit expressing COS cells; however, these studies do not test for subtle changes in responsiveness, for example in the threshold of inhibition, and cannot rule out in vivo axon guidance effects. Additional studies will therefore be required to more fully assess a potential cell-autonomous function of Slit1 in mediating other pathfinding choices such as more subtle midline axon divergence decisions or even the establishment of the topographic maps within the superior colliculus.

Vertebrate Slits Act in a Manner Distinct from that of *dSlit* in Regulating Midline Axon Guidance

Slit expression at the midline of the *Drosophila* nervous system acts to prevent ipsilaterally projecting axons from ever crossing the midline while allowing contralaterally projecting axons to cross (but only once). Our results suggest that in the mouse visual system Slit proteins are not critical for retinal axon divergence. In the *Slit1/2*-deficient mice, both an ipsilateral and contralateral projection develop. Furthermore, tracings from the different quadrants of the retina demonstrated that, as in wild-type mice, the uncrossed axons in the Slit mutant mice arise specifically from the ventrotemporal crescent. The in vivo phenotype and in vitro data together thus appear to identify retinal ganglion cells as a set of axons that are normally Slit-responsive prior to crossing, and for which Slit proteins play a previously undescribed role of helping channel axons across the appropriate region of the midline rather than directing the crossing/noncrossing decision per se.

Slit-Robo Interactions in Vertebrates

In *Drosophila*, Slit repels postcrossing commissural and precrossing ipsilaterally projecting axons by signaling through the Robo family of transmembrane receptors (Kidd et al., 1999) and acts through a combination of the three Robo receptors to establish the distance that individual axons extend away from the midline before turning to project longitudinally (Rajagopalan et al., 2000; Simpson et al., 2000). Vertebrate Slit proteins also bind Robo proteins (Brose et al., 1999; Li et al., 1999), and Slit2 binding to Robo1 does have functional significance as this interaction can silence the signaling of netrin-1 through DCC (Stein and Tessier-Lavigne, 2001). However, no direct loss-of-function evidence currently exists to prove the idea that they signal through Robo receptors.

Two pieces of evidence suggest that Robo2 is the likely RGC Slit receptor. First, *Robo2* is expressed at high levels by RGCs from the time that the first retinal axons begin to grow into the brain (Erskine et al., 2000; Ringstedt et al., 2000). Second, zebrafish carrying a mutation in the *astray/robo2* gene have profound defects in retinal axon pathfinding (Fricke et al., 2001). Although there are differences in the phenotypes of the *astray* zebrafish and the *Slit1/2*-deficient mice, there are also some striking similarities. The differences could reflect either the anatomical differences between these species or the existence of additional Slit receptors and/or Robo ligands. Although Robo2 is thus the likely RGC receptor for Slit proteins, further studies will be required to determine the specific mechanism of Slit signaling in retinal ganglion cells.

Concluding Remarks

We initially hypothesized two potential major roles for the Slit proteins in retinal axon guidance: regulating the divergence of crossing versus noncrossing axons, or controlling formation of the optic chiasm itself. The present studies in the mouse visual system suggest that the Slits do not act in an obvious manner to control divergence. Instead, Slit1 and Slit2 act in a synergistic fashion to regulate axon guidance by promoting fasciculation and by creating a repulsive barrier for retinal axons. By acting together, these molecules establish a repellent tract which helps channel retinal axons toward the optic chiasm, thereby helping to establish the position at which the optic decussation forms within the brain.

Experimental Procedures

Generation of Slit-Deficient Mice

Genomic DNA containing portions of either the *Slit1* or *Slit2* genes was isolated by screening a BAC library (Incyte Genomics) with a *Slit1* or *Slit2* specific cDNA probe. BAC DNA was then used to generate the targeting vectors shown in Figures 1A and 2A using standard recombinant DNA techniques. For specific details regarding the generation of each targeting vector, refer to the respective figure legends. Southern blot and in situ analyses were performed using standard techniques. For both targeting events, genomic DNA was digested with HindIII and hybridized with a DNA probe external to the targeting vector as noted in the targeting figures. For in situ analysis of *Slit1* expression, RT PCR was used to amplify a 3′ portion of the gene from embryonic brain RNA that was then used as a template for digoxin labeled riboprobe synthesis. ES cell culture and generation of mice was carried out as previously described (Mombaerts et al., 1996). For genotyping, a PCR based screen was developed for each gene: *Slit1*: wild-type allele-forward primer 5′-AAG ATGCCTCCTCTGACTTC-3′; reverse primer 5′-ACCCTTAGCTTC TACCAACC-3′; mutant allele-forward primer 5′-TCTCCTTTGATCT GAGACCG-3′; reverse primer 5′-AGGTTTCTCGAGCGTCATAG-3′. *Slit2*: wild-type allele-forward primer 5′-AAGACCTGTCGCTTCTGT CAG-3′; reverse primer 5′-AAACAGGTTTCTACCGCACG-3′; mutant allele-forward primer 5′- AAGACCTGTCGCTTCTGTCAG -3′; reverse primer 5′-AAGTCTAGTAGAGTCGAGCG-3′. PCR was carried out in the "Green" buffer (67 mM tris [pH 8.8], 6.7 mM $MgCl_2$, 17 mg/ml BSA, 16.6 mM $[NH4]_2SO_4$) using the following program: 95°C × 5′ with hot start then 30 cycles of 95°C × 30 s, 60°C × 30 s, and 72°C × 30 s. *Slit1*-deficient mice are in a mixed CD-1/129Sv and C57Bl6/129Sv background, and *Slit2*-deficient mice in a mixed C57Bl6/129Sv background.

Lipophilic Dye Tracing

For complete optic nerve labeling, embryonic mouse tissue was fixed in 4% paraformaldehyde in PBS, one or both eyes enucleated, and crystals of DiI (and in some cases DiO) implanted unilaterally into the optic disk (and in some cases bilaterally). Heads were incubated at 37°C in PBS containing 0.1% sodium azide for 2–10 days depending on the embryonic age. Brains with the optic nerve intact were then carefully removed, and the proximal visual system imaged en face with a fluorescence dissecting microscope. The tissue was then sectioned with a vibratome at 100 μm thickness to allow higher resolution visualization for identification of more subtle wandering defects. For quadrant labeling, after enucleation a small crystal of DiI was placed into a specific peripheral location to label cells within the ventrotemporal, ventronasal, dorsonasal, or dorsotemporal retina.

Immunostaining

Fixed heads from E13.5 embryos were sectioned with a vibratome and immunostained with Mab 480-1.1 to label the early born CD44/SSEA-1 neurons posterior to the chiasm (Erskine et al., 2000), or with a neurofilament specific antibody to label axon tracts (Lee et al., 1987). Immunostaining was performed as previously described (Erskine et al., 2000).

Explant Assays

Retinal explant assays were performed as previously described (Erskine et al., 2000). For isolation of POA tissue, E14.5 heads were embedded in 4% low melting temperature agarose in PBS and sectioned at 250 μm on a vibratome in cold L15 medium. The section containing the pre-optic area was identified as the one just anterior to the optic chiasm, and a portion of this region was further isolated using tungsten dissecting pins to generate a final piece of pre-optic tissue that was approximately 300 μm^2 centered around the midline.

Though not shown in the figures, Western blot analysis was used to assess the reproducibility of transfections and was performed using standard techniques with an anti-Myc antibody (9E10; gift of J.M. Bishop) that recognizes a C-terminal myc epitope tag on each of the Slits.

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