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Retinal Ganglion Cell Axon Guidance in the Mouse Optic Chiasm: Expression and Function of Robos and Slits

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The ventral midline of the nervous system is an important choice point at which growing axons decide whether to cross and project contralaterally or remain on the same side of the brain. In *Drosophila*, the decision to cross or avoid the CNS midline is controlled, at least in part, by the Roundabout (Robo) receptor on the axons and its ligand, Slit, an inhibitory extracellular matrix molecule secreted by the midline glia. Vertebrate homologs of these molecules have been cloned and have also been implicated in regulating axon guidance. Using *in situ* hybridization, we have determined the expression patterns of *robo1,2* and *slit1,2,3* in the mouse retina and in the region of the developing optic chiasm, a ventral midline structure in which retinal ganglion cell (RGC) axons diverge to either side of the brain. The receptors and ligands are expressed at the appropriate time and place, in both the retina and the ventral diencephalon, to be able to influence RGC axon guidance. *In vitro*, *slit2* is inhibitory to RGC axons, with outgrowth of both ipsilaterally and contralaterally projecting axons being strongly affected. Overall, these results indicate that Robos and Slits alone do not directly control RGC axon divergence at the optic chiasm and may additionally function as a general inhibitory guidance system involved in determining the relative position of the optic chiasm at the ventral midline of the developing hypothalamus.

Key words: axon guidance; diencephalon; hypothalamus; optic chiasm; Robo; retinal ganglion cell; Slit

In the mouse visual system, retinal ganglion cell (RGC) axons from the two optic nerves grow toward one another and either cross the ventral midline of the diencephalon (developing hypothalamus) or turn away from it, forming an x-shaped fiber pathway, the optic chiasm. Contralaterally projecting RGCs are distributed throughout the retina, whereas ipsilaterally projecting cells are found exclusively in the ventrotemporal crescent (for review, see Guillery et al., 1995). In addition to being essential for the establishment of normal binocular vision, this system provides an excellent model for studying axon guidance at the ventral midline of the CNS.

Using dye tracing and video microscopy, the trajectory and dynamic behavior of RGC axons as they navigate through the mouse optic chiasm has been described and, in the midline region in which the axons diverge, specialized populations of radial glia and neurons (CD44/SSEA neurons) have been identified (Sretavan and Reichardt, 1993; Godement et al., 1994; Sretavan et al., 1994; Marcus and Mason, 1995; Marcus et al., 1995; Mason and Wang, 1997) (for review, see Mason and Sretavan, 1997). Interactions between these cells and the RGC axons have been implicated in both directing axon divergence and determining the position of the chiasm on the developing hypothalamus (Wizenmann et al., 1993; Sretavan et al., 1994, 1995; Wang et al., 1995, 1996). However, the molecular nature of the underlying guidance signals remains primarily unknown.

In *Drosophila* Roundabout *robo* mutants, axons that normally grow ipsilaterally project across the midline, and all axons aberrantly cross and recross multiple times (Seeger et al., 1993). Roundabout (Robo) defines a novel family of highly conserved guidance receptors (Kidd et al., 1998a; Zallen et al., 1998). Genetic and biochemical evidence has demonstrated that Slit, an inhibitory extracellular matrix molecule secreted by the midline glia, is a ligand for Robo (Battye et al., 1999; Brose et al., 1999; Kidd et al., 1999; Li et al., 1999). To date, three *robo* and three *slit* mammalian homologs have been identified and shown to be expressed within the developing nervous system. *In vitro*, Slits both inhibit CNS axon outgrowth and neuronal migration and promote sensory axon growth and branching (Holmes et al., 1998; Itoh et al., 1998; Kidd et al., 1998a; Ba-Charvet et al., 1999; Brose et al., 1999; Hu, 1999; Li et al., 1999; Wang et al., 1999; Wu et al., 1999; S. Yuan et al., 1999; W. Yuan et al., 1999; Zhu et al., 1999).

To determine whether these molecules play a role in chiasm formation, we examined the expression and potential function of Robos and Slits in the developing mouse visual system. Both the receptors and ligands are expressed in dynamic patterns in the retina and ventral diencephalon. *In vitro*, Slit2 is inhibitory to RGC axon outgrowth. However, no differential effect on ipsilat-

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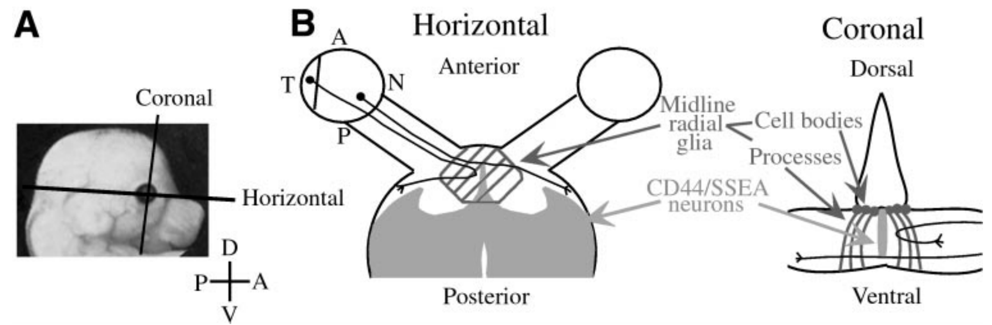

Figure 1. Anatomy of the region of the developing optic chiasm during the major period of RGC divergence. *A*, Head of an E14.5 mouse embryo indicating the planes of section used in this study. *B*, Schematic diagrams of horizontal and coronal sections through the optic chiasm of an E14.5 mouse embryo. The path of crossed and uncrossed RGC axons is shown in relation to the specialized cell types (radial glia, *dark gray hatching*; CD44/SSEA neurons, *light gray*) present in this region. All axons grow into and contact the radial glia before either crossing the midline, at the tip of the SSEA-1 positive region, or turning back into the ipsilateral optic tract (Marcus et al., 1995). *D*, Dorsal; *V*, ventral; *A*, anterior; *P*, posterior; *N*, nasal; *T*, temporal.

erally and contralaterally projecting axons was found. One function of Robos and Slits may be to prevent RGC axons from growing into particular regions of the brain, thereby determining the position on the diencephalon at which the optic chiasm develops.

MATERIALS AND METHODS

Embryos. All experiments were performed using C57BL/6J mice maintained in a timed-pregnancy breeding colony. Noon of the day on which a plug was found was considered embryonic day 0.5 (E0.5). Pregnant mothers were anesthetized with a mixture of ketamine and xylazine, and the embryos were removed by cesarean section. Embryonic age was confirmed by comparing external appearance and crown-rump length with the criteria given by Theiler (1972). For DiI labeling, immunohistochemistry, or *in situ* hybridization, embryos were fixed overnight in 4% paraformaldehyde in PBS.

DiI labeling of RGC axons. To label fully the optic nerve and tract, a small crystal of DiI (D282; Molecular Probes, Eugene OR) was placed over the optic disk of one eye, and the embryos were stored at 37°C for 4 d in PBS containing 0.1% sodium azide. Labeled heads were sectioned horizontally or coronally at 100 μm on a vibratome, and the DiI was photoconverted to a permanent brown reaction product as described previously (Marcus et al., 1995).

Immunohistochemistry. Fixed heads of embryos at E12.5–E17.5 were sectioned horizontally or coronally at 100 μm on a vibratome (Fig. 1) and immunostained as described previously (Marcus et al., 1995). Monoclonal antibody RC2 labels specialized midline radial glia at the optic chiasm (Marcus et al., 1995; Marcus and Mason, 1995) (Fig. 1*B*), Mab 480–1.1, against stage-specific embryonic antigen 1 (SSEA-1), labels the early born CD44/SSEA-1 neurons posterior to the optic chiasm (Marcus et al., 1995; Marcus and Mason, 1995) (Fig. 1*B*), and K4 (guinea pig polyclonal against Islet1/2) marks the RGC layer in embryonic retinas (Thor et al., 1991; R. Rachel, L. Erskine, and C. A. Mason, unpublished observations). Antibodies were a gift of Dr. T. M. Jessell (Columbia University, New York, NY) and were used at a final concentration of 1:3 (RC2 and 480–1.1) or 1:10 000 (K4).

In situ *hybridization*. *In situ* hybridization, using digoxigenin-labeled riboprobes, was performed on 100 μm vibratome sections according to the method of Laufer et al. (1997). Rat cDNAs encoding *robo1,2* and *slit1,2,3* (Kidd et al., 1998a; Brose et al., 1999) were used as templates for riboprobe synthesis.

Photography. Sections were mounted in 90% glycerol in PBS and photographed using Kodak 64T or Ektachrome 400 color slide film on a Zeiss Axioplan microscope. Slides were scanned into a Macintosh computer, and the figures were prepared using Adobe Photoshop.

Collagen gel cultures. Retinal explants from E14.5 mouse embryos were cultured as described previously (Wang et al., 1996), except that a 50:50 mix of bovine dermis and rat tail collagen (Collaborative Research, Bedford, MA) was used. Explants were cocultured, 100–300 μm apart, with aggregates of untransfected COS cells or COS cells transfected, using Lipofectamine Plus (Life Technologies, Grand Island NY), with the vector plasmid or with human Slit2 (hSlit2) fused at its C terminus to a myc-tag (Brose et al., 1999). Heparin (50 ng/ml; Sigma, St. Louis, MO) was added to the medium because this has been reported to augment the release of Slit from the plasma membrane (Brose et al., 1999). Heparin alone had no effect on RGC axon outgrowth (data not shown). After 24 hr, the cultures were fixed with 4% paraformaldehyde in PBS, and the neuronal processes were visualized using an anti-β-tubulin monoclonal antibody (Sigma), followed by a Cy3-conjugated goat anti-mouse antibody. Labeled cultures were mounted on slides in Gelmount and photographed using T-max 400 film on a Zeiss Axioplan microscope.

The extent of axon outgrowth was quantified using the public domain NIH Image analysis system to measure the area covered by the RGC axons. This measurement takes into account both the number and length of the axons, parameters that could not be accurately measured individually in these three-dimensional cultures. Because outgrowth was not radial, care was taken to ensure the correct orientation of the explants (see Fig. 6), and only the outgrowth originating from the half of the explant facing the cells was quantified.

RESULTS

The expression of *robos* and *slits* in the developing mouse retina and ventral diencephalon was determined at four ages, each of which represents a significant time point during the development of the optic chiasm: E12.5, shortly after the onset of RGC genesis and the age at which the first axons begin to grow into the diencephalon; E14.5 and E15.5, the principle period of RGC genesis and axon divergence; and E17.5, after ipsilaterally projecting RGCs have ceased to be born. Generation of contralaterally projecting RGCs continues until birth (Dräger, 1985; Colello and Guillery, 1990; Marcus and Mason, 1995; Marcus et al., 1995). Identical expression patterns were found at E14.5 and E15.5; thus, data from only one of these ages (E14.5) is presented. At all ages examined, no *slit3* expression was detected in either the retina or the region of the developing optic chiasm (data not shown). This is in contrast to the findings of W. Yuan et al. (1999), who reported expression of *slit3* in the developing lens and pigmented epithelium of the mouse retina.

Expression patterns of *robos* and *slits* in the mouse retina

At E12.5, *robo2* and *slit1* are expressed in the dorsocentral region of the retina, the position in which the first RGCs to be born are located. *Robo1* and *slit2* are not expressed at this age (Fig. 2*A–E*). By E14.5, in addition to *robo2* and *slit1*, *robo1* and *slit2* also are expressed throughout the RGC layer (Fig. 2*F–J*). The mRNA for *robo1* is restricted to a scattered subpopulation of cells, whereas *robo2* and the *slits* appear to be expressed by most cells in the RGC layer (Fig. 2, compare *P*, *Q*). The *robo1*-positive cells are distributed throughout the retina (Fig. 2*G*) and therefore are unlikely to represent cells with a particular projection phenotype at the optic chiasm (ipsilaterally projecting RGCs are found exclusively in the ventrotemporal crescent of the retina). At this age, *slit1* mRNA is expressed in a high ventral, low dorsal gradient

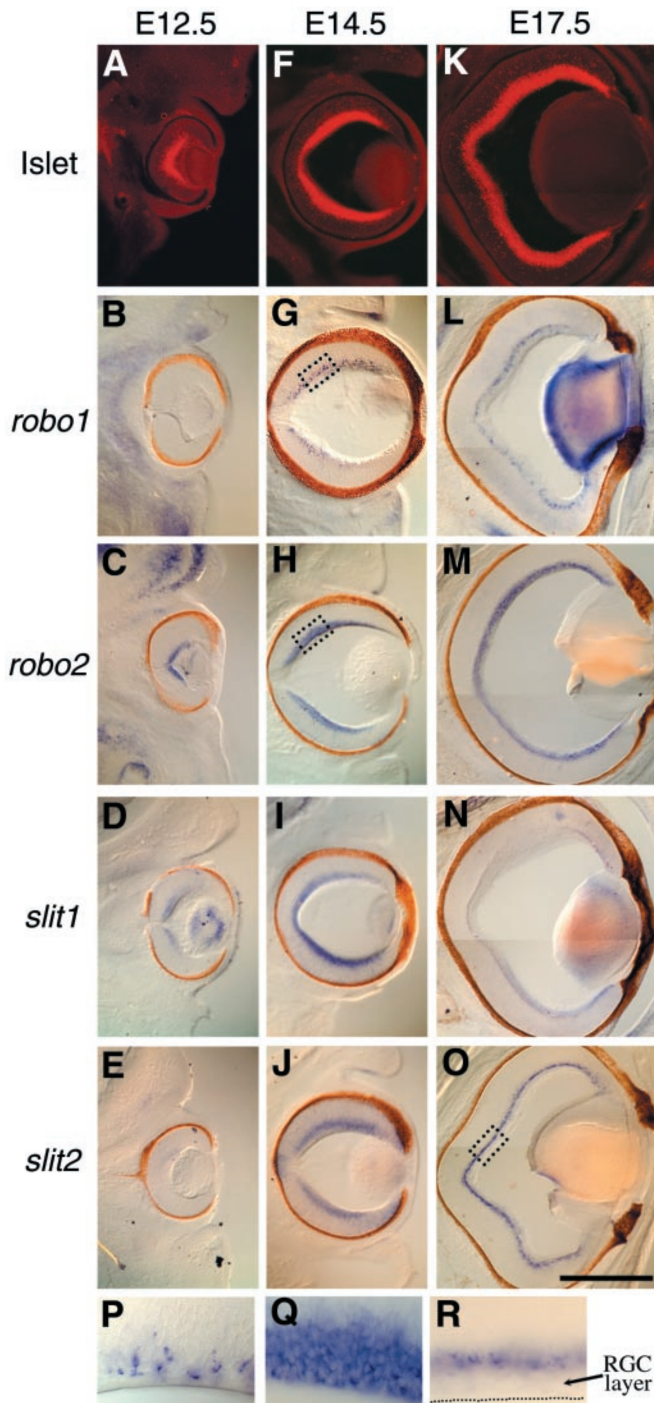

Figure 2. Expression patterns of *robos* and *slits* in the developing mouse retina. Coronal sections of E12.5 (*A–E*), E14.5 (*F–J*), and E17.5 (*K–O*) retinas stained with an antibody against the LIM homeodomain proteins, Islet 1/2 (an early RGC marker; *A*, *F*, *K*) or by *in situ* hybridization using digoxigenin-labeled riboprobes for *robo1* (*B*, *G*, *L*), *robo2* (*C*, *H*, *M*) *slit1* (*D*, *I*, *N*), or *slit2* (*E*, *J*, *O*). *Boxed regions* in *G*, *H*, and *O* are shown at higher power in *P*, *Q*, and *R* respectively. *Dotted line* in *R* marks the central lumen of the retina. Scale bar: *A–O*, 500 μm; *P*, *R*, 80 μm. Dorsal, *Top*; ventral, *bottom*.

(Fig. 2*I*). No nasal-temporal gradients were detected (data not shown). At E17.5, *robo2* expression is maintained within the RGC layer, whereas *slit1* is, at best, only very weakly expressed (Fig. 2*K*,*M*,*N*). *Slit2* is clearly restricted to the inner nuclear layer (Fig. 2*O*,*R*), as is *robo1* at this age (Fig. 2*L*).

Expression patterns of *robos* and *slits* in the region of the developing optic chiasm

At E12.5, the first RGC axons enter the brain in which they establish the correct position and shape of the optic chiasm. These early axons grow into the diencephalon and then course ventrally before extending toward the midline along the border of the CD44/SSEA neurons (Marcus and Mason, 1995) (Fig. 3*A–D*). Later in development, RGC axons make distinct pathway choices at the midline, thereby projecting to targets on both sides of the brain (Figs. 4*A–D*, 5*A*).

At E12.5, *robo1* is expressed in a domain posterior and medial to the CD44/SSEA neurons (Fig. 3*E–H*), which express *robo2* (Fig. 3*I–L*). *Slit1* is expressed around the junction of the optic nerve and the brain (Fig. 3*M*,*N*), with strongest expression dorsal to the site at which the optic stalk joins the diencephalon (Fig. 3*B*, *arrow*). *Slit1* also is weakly expressed in a subset of the CD44/SSEA neurons (Fig. 3*O*,*P*). In the more dorsal region of the developing optic chiasm, the domain of *slit1* expression lies some distance posterior to the axons (Fig. 3*C*,*O*). However, more ventrally, *slit1* is expressed in a region directly adjacent to the path taken by the RGC axons (Fig. 3*D*,*P*). Slit2 is strongly expressed at the ventral midline of the diencephalon in the region in which the RGC axons enter the brain and turn to grow ventrally (Fig. 3*Q–T*). The domain of *slit2* expression includes the position of the glial knot, a glial structure at the midline of the diencephalon that has been proposed to delimit the pathway of the ingrowing RGC axons (Silver, 1984).

Later in development, *robo1* is more weakly expressed throughout the diencephalon, both posterior to the CD44/SSEA neurons and around the third ventricle (Figs. 4*E–H*, 5*B*). *Robo2* continues to be expressed in the CD44/SSEA neurons (Fig. 4*I–L*), a pattern that is maintained until at least E17.5 (Fig. 5*C*). At E14.5, *slit1* is still expressed around the junction of the optic nerves and the brain (Fig. 4, compare *B*, *N*) and, in a similar pattern to *robo2*, in the CD44/SSEA neurons (Fig. 4*N–P*). By E17.5, *slit1* can no longer be detected at the junction of the brain and optic nerve and is only weakly expressed by the CD44/SSEA neurons (Fig. 5*D*). *Slit2* expression is maintained at the ventral midline of the diencephalon, in a region directly dorsal to the site of axon divergence (Fig. 4*Q–T*). At all ages examined, no *slit2* mRNA was seen at the more ventral level at which the axons cross the midline (Fig. 4*S*,*T*, 5*E*).

The cell bodies of the midline glia resident in the ventral diencephalon lie dorsal to the site at which the retinal axons diverge. Only their radial processes are found among the RGC axons (Marcus et al., 1995, their Fig. 1*E*) (Fig. 1*B*). In coronal sections, the mRNA for *slit2* appears to be expressed by a subset of these specialized midline radial glial cells, suggesting that Slit2 protein may be present on their radial processes and consequently at the site at which the axons diverge (Fig. 4*U–W*). Unfortunately, no good antibodies are currently available to confirm this expression pattern. Overall, these results indicate that Robos and Slits are expressed at the appropriate time and place to be able to regulate RGC axon guidance.

In vitro, Slit2 inhibits RGC axon outgrowth

To test whether Slit2 can regulate RGC axon outgrowth, explants, taken from each of the four retinal quadrants, were cocultured in collagen gels with aggregates of cells expressing hSlit2. Explants were taken from the most peripheral part of E14.5 retinas because, at this age, this region of the ventrotemporal retina contains a high percentage of RGCs whose axons will project ipsilaterally at the optic chiasm; the remainder of the retina is

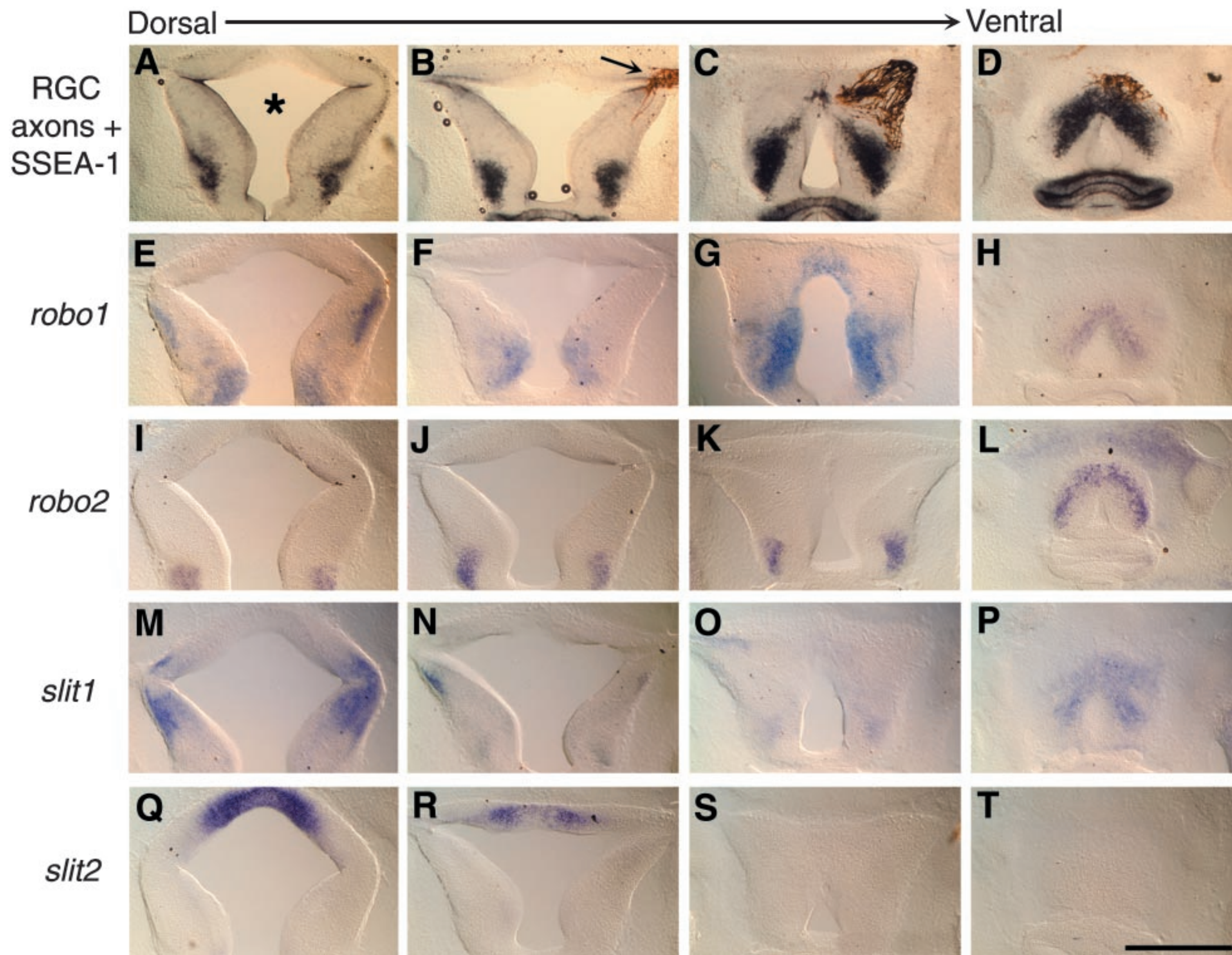

Figure 3. Expression of *robos* and *slits* in the ventral diencephalon of E12.5 mouse embryos. *A–D*, Serial horizontal sections showing both DiI-labeled RGC axons (*brown*) and SSEA-1 (*black*). In the most dorsal section (*A*), no RGC axons are present. *Asterisk* marks the third ventricle. More ventrally (*B*), a few axons can be seen at the junction of the optic stalk and the brain (*arrow*). In *C* and *D*, the region of the future optic chiasm, RGC axons have entered the diencephalon and appear to be growing along the border of the SSEA-1-positive cells but have not yet crossed the midline. *E–T*, Comparable serial sections with those in *A–D* after *in situ* hybridization to show patterns of *robo1* (*E–H*), *robo2* (*I–L*), *slit1* (*M–P*), and *slit2* (*Q-T*) expression. Orientation is the same as in Figure 1*B* (anterior, *top*; posterior, *bottom*). Scale bar, 500 μm.

composed predominately of contralaterally projecting RGCs (Dräger, 1985) (Fig. 6*A*). Thus, a direct comparison could be made of the effect of hSlit2 on ipsilaterally and contralaterally projecting RGC axons.

When cultured alone, outgrowth from these explants was not radial but grew out predominately from the cut edge, with little or no outgrowth emanating from the peripheral side (Fig. 6*B*). The amount of outgrowth from the cut half of the explant was quantified by measuring the area of the dish covered by the RGC axons (Fig. 6*F*; see Materials and Methods). This measurement takes into account both the number and length of the axons growing into the collagen.

Coculturing explants with aggregates of mock-transfected cells had no effect on neurite outgrowth (Fig. 6*C*). However, in cultures containing hSlit2, RGC outgrowth was markedly decreased (Fig. 6*D*). In particular, fewer axons appeared to grow from the explants into the collagen. We refer to this as inhibition rather than repulsion because, particularly in control cultures, we could not measure the angles turned by individual axons (Fig. 6*B*,*C*) and thus definitively show that axons were being repelled by the Slit-expressing cells. Compared with outgrowth in the presence of mock-transfected cells, outgrowth from all four retinal quadrants was significantly decreased (Fig. 6*F*). This was unlikely to be attributable to hSlit2 having a general toxic effect on the explants. When explants were cultured such that growth was directed away from the Slit-expressing cells, Slit2 had no effect on axon outgrowth (Fig. 6*E*; data not shown).

The extent of outgrowth from each retinal quadrant, cultured in the absence of hSlit2, was not identical (Fig. 6*F*, *open bars*). The extent of inhibition induced by hSlit2 therefore was normalized by dividing the amount of outgrowth seen in the presence of hSlit2 by that seen in cultures containing mock-transfected cells (Fig. 6*G*). hSlit2 induced a 47% decrease in the amount of outgrowth from ventrotemporal retina (ipsilaterally projecting RGC axons) and a 51, 40, and 42% decrease in the outgrowth from dorsotemporal, ventronasal, and dorsonasal retina, respectively (all sources of contralalerally projecting RGC axons). These results indicate that hSlit2 is inhibitory to all RGCs and, at least *in vitro*, is not a more potent inhibitor of ipsilaterally than contralaterally projecting axons.

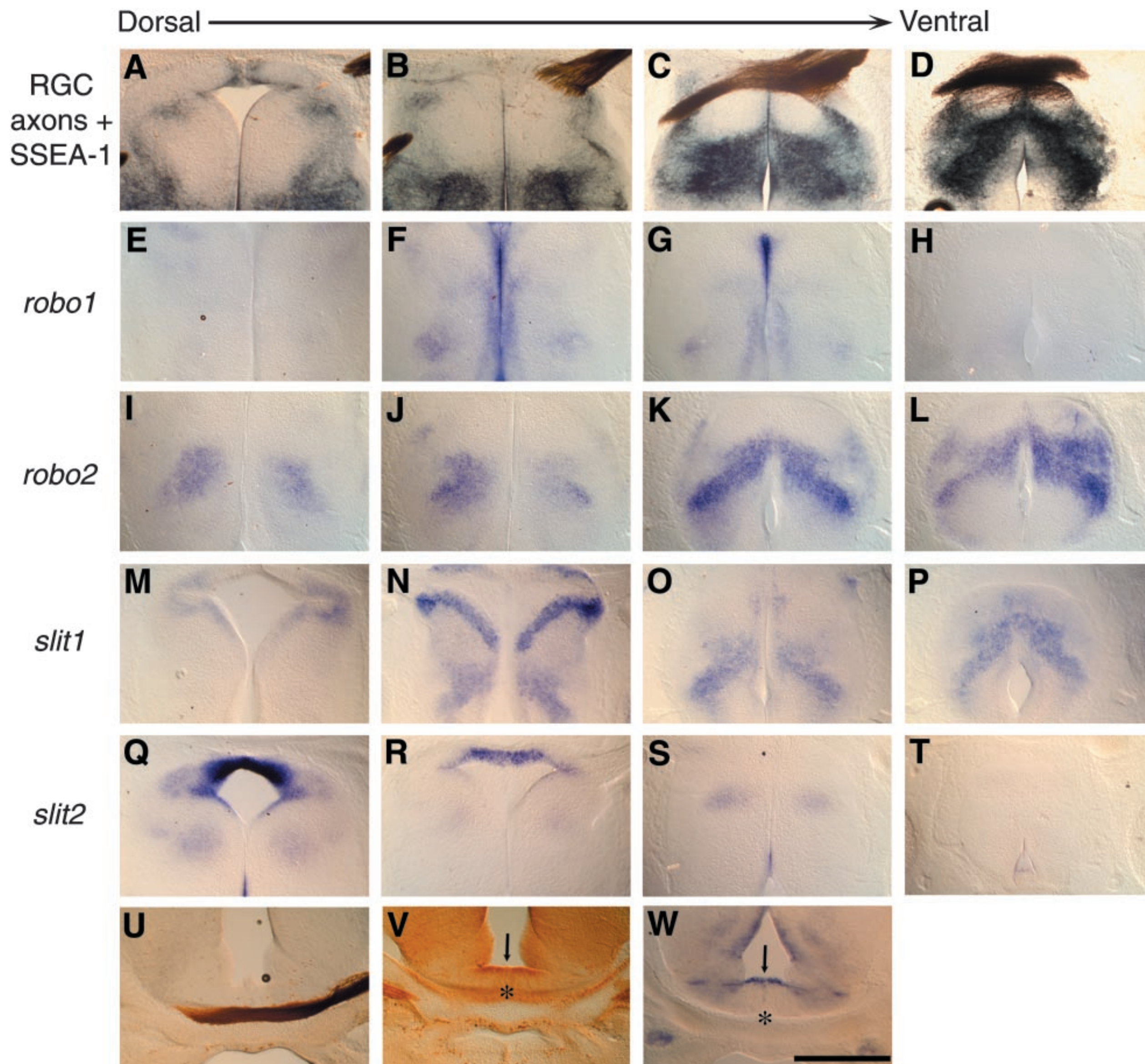

Figure 4. Expression of *robos* and *slits* in the ventral diencephalon of E14.5 mouse embryos. *A–D*, Serial horizontal sections double labeled with DiI to show the RGC axons (*brown*) and SSEA-1 (*black*), which marks the CD44/SSEA neurons posterior to the optic chiasm. In the more dorsal sections (*A*, *B*), RGC axons are present in the optic nerve (*A*) and at the junction of the optic nerve and the brain (*B*). The axons then grow more ventrally before diverging to form the x-shaped optic chiasm (*C*, *D*). The site at which the axons diverge is marked by a thin raphe of the CD44/SSEA neurons. *E–T*, Comparable serial sections with those in *A–D* after *in situ* hybridization to show patterns of *robo1* (*E–H*), *robo2* (*I–L*), *slit1* (*M–P*), or *slit2* (*Q–T*) expression. *U–W*, Coronal sections labeled with photoconverted DiI to show the RGC axons (*U*), the monoclonal antibody RC2 (labels radial glia; *V*), or after *in situ* hybridization for *slit2* (*W*). *Asterisks* in *V* and *W* marks the RGC axons. *Arrows* point to strong staining of RC2 and *slit2* in the radial glial cell bodies. Orientation is the same as in Figure 1*B*. Scale bar, 500 μm.

DISCUSSION

The decision to cross or turn away from the midline is an important intermediate step in the projection of many axons to their targets. One place at which this occurs is the midline of the mammalian diencephalon in which RGC axons from each eye diverge at the optic chiasm. Here we report that Robo receptors and their Slit ligands, molecules important for midline guidance in *Drosophila* (Kidd et al., 1999), are expressed at the appropriate time and place in the mouse retina and diencephalon to be able to influence RGC axon guidance at the optic chiasm. *In vitro*, Slit2 was found to be a potent inhibitor of RGC axon outgrowth. However, no differential effect on ipsilaterally and contralaterally projecting RGCs was evident. Together with the findings of Niclou et al. (2000) and Ringstedt et al. (2000), these results implicate Robos and Slits as important regulators of RGC axon guidance and suggest that, during optic chiasm development, they may play additional roles to controlling guidance across the midline.

Robos and *slits* are expressed in the retina and ventral diencephalon at the time when the optic chiasm is developing

In the retina, *robo2* is expressed by RGCs before any axons have reached the ventral midline of the diencephalon (Figs. 2*C*, 3*C*) and continues to be strongly expressed during later stages of development (Fig. 2*H*,*M*). In contrast, *robo1* is not detected until after a number of axons have started to cross the midline and then

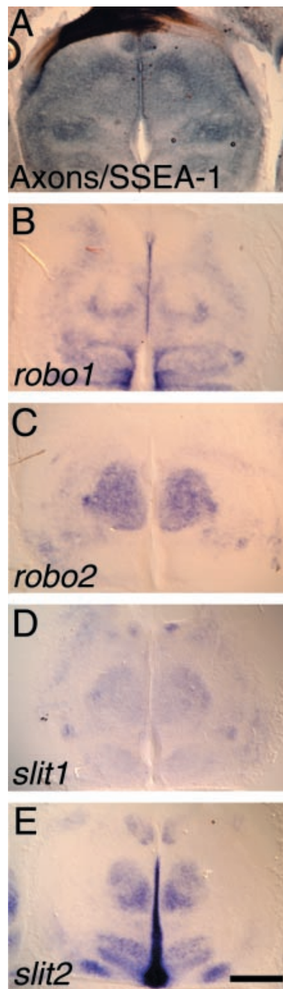

Figure 5. Expression of *robos* and *slits* in the ventral diencephalon of E17.5 mouse embryos. *A*, Horizontal section, at the level of the optic chiasm, double labeled to show both the RGC axons (*brown*) and SSEA-1 (*black*). *B–E*, Comparable sections after *in situ* hybridization to show the patterns of expression of *robo1* (*B*), *robo2* (*C*), *slit1* (*D*), or *slit2* (*E*). Anterior, *Up*; posterior, *down*. Scale bar, 250 μm.

only in a subset of cells (Figs. 2*B*,*G*, 4*C*,*D*). This suggests that Robo2 is likely to be the principle player of these two receptors in terms of RGC axon guidance.

In the diencephalon, the Slits are expressed at three sites, all of which have been identified as regions in which RGC axons make guidance decisions and coincide with zones defined by domains of transcription factor expression (Marcus and Mason, 1995; Marcus et al., 1999). First, *slit1* is expressed around the junction of the optic nerve and the brain, with the strongest expression dorsally (Figs. 3*B*,*M*, 4*B*,*M*,*N*). Netrin, which can promote the growth of RGC axons (Wang et al., 1996), also is expressed at this site (Deiner and Sretavan, 1999). In Vax1 mutants, *netrin* expression is lost from this region, whereas *slit1* expression is maintained, and this is associated with a failure of RGC axons to grow into the diencephalon (Bertuzzi et al., 1999). This indicates that this coexpression of netrin and Slit1 is important for RGC axon guidance and suggests a mechanism by which axons may be directed toward the midline of the diencephalon in which, unlike other regions of the CNS, no chemoattractant activity has been detected (Wang et al., 1996). First, netrin may act to attract the RGC axons, which, upon reaching the brain, lose their ability to respond to this signal (Shirasaki et al., 1998). Slit1 repulsion would then dominate and direct the RGC axons away from the optic nerve, toward the ventral midline.

Slit1 also is expressed in the CD44/SSEA neurons (Fig. 4*N–P*). These neurons define an inhibitory zone posterior to the chiasm into which RGC axons never extend (Sretavan et al., 1994; Marcus and Mason, 1995; Marcus et al., 1995). The first RGC axons appear to grow along the border of these cells thereby establishing the correct position and shape of the forming optic chiasm (Marcus and Mason, 1995). Several molecules inhibitory to RGC outgrowth have been shown to be expressed by these neurons and may act to prevent RGC axons from growing back into this area (Sretavan et al., 1994; Marcus et al., 2000). Whether Slit1 also is involved in preventing axons from growing into this area remains to be determined. Indeed, in some regions, *slit1* is expressed some distance posterior to the front of the growing axons (Fig. 4*C*,*O*) (but see Fig. 4*D*,*P*). This raises the possibility that the Slit1 on the CD44/SSEA neurons may regulate the guidance of only a subset of the RGC axons or is involved in other aspects of hypothalamic development.

Finally, *slit2* is expressed on a subset of the specialized radial glia present at the ventral midline of the diencephalon (Figs. 3*Q*, 4*Q*,*W*). All RGC axons grow into and contact the radial processes of these cells (Marcus et al., 1995), making Slit2 a good candidate for a factor controlling RGC axon divergence at the midline.

Slit2 and RGC axon guidance

Slit2 is expressed at the correct time and place to be able to regulate axon crossing at the developing optic chiasm. However, when presented alone *in vitro*, Slit2 does not have a differential effect on ipsilaterally and contralaterally projecting RGC axons; both are strongly inhibited (Fig. 6). One possibility is that Slit2 alone is not enough to direct divergence but that additional factors are required. Other axon guidance molecules, such as Nr-CAM (M. Lustig, C. A. Mason, M. Grumet, and T. Sakurai, unpublished observations) and Eph/ephrin receptors and ligands (Bertuzzi et al., 1999; Marcus et al., 2000) are expressed on the glial cells present at the ventral midline of the mouse diencephalon. In the future, it will be important to determine whether these molecules can synergize with Slit2 and thereby control RGC axon divergence at the midline.

Another possibility is that the lack of a differential response of crossed and uncrossed RGC axons to Slit2 reflects an inappropriate regulation of Robo expression in our culture system. In *Drosphila* a third molecule, commisureless (Comm), is required for midline guidance (Seeger et al., 1993) Comm is expressed by the midline glia and, as axons approach the midline, downregulates Robo on their growth cones. Depending on their original level of Robo expression, axons can now cross or are still repelled from the midline by Slit (Tear et al., 1996; Kidd et al., 1998b). In the absence of *comm*, all axons are repelled by the midline (Seeger et al., 1993). Thus, appropriate regulation of Robo expression, perhaps by a Comm-like molecule, may be required to induce a differential response of ipsilaterally and contralaterally projecting RGC axons to Slit2.

Alternatively Slit2 could be involved in regulating other aspects of optic chiasm development. RGC axons grow into the diencephalon and then, at the border of the *slit2* domain, a region corresponding to the glial knot (Silver, 1984), turn ventrally before approaching the midline (Figs. 3*B*,*R*, 4*B*,*R*). By preventing axons from growing directly across the midline, Slit2 could determine the exact position along the dorsoventral axis at which the optic

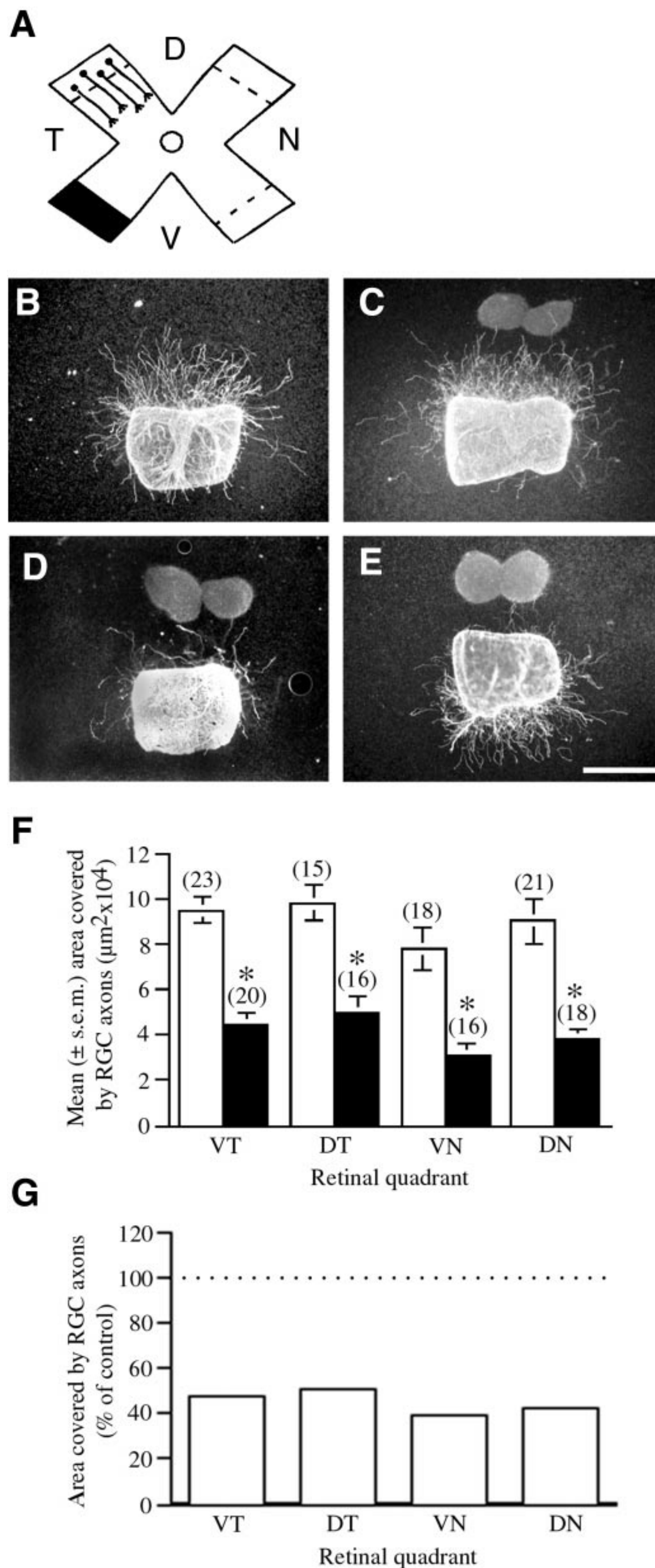

Figure 6. Effect of hSlit2 on RGC axon outgrowth *in vitro*. *A*, Schematic diagram of a flat-mounted E14.5 mouse retina. RGCs that project contralaterally are found throughout the retina, whereas ipsilaterally projecting cells are restricted to the ventrotemporal crescent (*black region*). To enable a comparison of the behavior of crossed and uncrossed RGC axons, explants were prepared only from the most peripheral part of each retinal quadrant. Consequently, growth from these explants was not radial but originated only from the cut edge (indicated by the *dotted lines*). *D*, Dorsal; *V*, ventral; *N*, nasal; *T*, temporal. *B–E*, Explants from dorsotemporal retina cultured alone (*B*), with clusters of mock-transfected cells (*C*), or with clusters of cells transfected with hSlit2 (*D*, *E*). Explant in *E* is oriented such that growth is directed away from the Slit-expressing cells. Scale bar, 500 μm. *F*, Extent of RGC axon outgrowth from explants cocultured with mock-transfected (*open bars*) or hSlit2-expressing COS cells (*filled bars*). *Numbers above bars* indicates number of explants. $^*p <$ 0.001 compared with growth in the presence of the mock-transfected cells (Student's unpaired t test). Data were pooled from five independent experiments. *G*, Extent of RGC axon outgrowth in the presence of hSlit2 expressed as a percentage of the outgrowth seen in cultures containing mock-transfected cells.

chiasm develops and may from part of the molecular basis for the proposed barrier function of the glial knot.

Robos and *slits* colocalize both in the retina and the ventral diencephalon

In addition to their expression in the ventral diencephalon, both *slit1* and *slit2* are expressed within the retina (W. Yuan et al., 1999) (Fig. 2). Colocalization of ephrin ligands with their Eph receptors also occurs within the RGC layer of the chick and mouse retina and may act to modulate RGC axon guidance (Marcus et al., 1996; Dütting et al., 1999; Hornberger et al., 1999). Overexpression of ephrinA ligands within the chick retina leads to the establishment of an ectopic ipsilateral pathway at the optic chiasm and the misrouting of axons within the tectum. In a similar manner, Slit in the retina could function to modulate RGC axon guidance at the optic chiasm. Alternatively, Slit expression in the retina may reflect a role for the Robo–Slit guidance system in intraretinal development or function to prevent RGC axons from extending into deeper layers of the retina. Additional studies will be required to distinguish between these possibilities.

Robo2 and slit1 also colocalize on the CD44/SSEA neurons. These neurons are present in the diencephalon before the first RGC axons grow into the brain and extend their axons dorsally within the diencephalon (Sretavan et al., 1994). Thus, the simplest explanation for the expression of *robo2* by these neurons is that it is required by them to make their own guidance decisions. Slit1 could interact with the Robo2 coexpressed by these cells to modulate their guidance and/or be involved in regulating the development of other axon tracts within the hypothalamus (see above).

Conclusions

We have shown that *robos* and *slits* are expressed in the developing mouse visual system in a manner consistent with their being important regulators of RGC axon guidance and that *in vitro* Slit2 is inhibitory to RGC axon outgrowth. Several different sites at which Slits may influence RGC axon guidance at the optic chiasm were identified: around the junction of the optic nerve and the brain, the CD44/SSEA neurons, and the midline radial glia. These results suggest that Robos and Slits may be involved in regulating the growth of RGC axons from the optic nerve into the brain and in determining the position at which the optic chiasm develops on the ventral midline of the hypothalamus. Additional work will be required to determine whether they are involved in regulating axon divergence at the midline.

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