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Cortical Axon Guidance by the Glial Wedge during the Development of the Corpus Callosum

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Growing axons are often guided to their final destination by intermediate targets. In the developing spinal cord and optic nerve, specialized cells at the embryonic midline act as intermediate targets for guiding commissural axons. Here we investigate whether similar intermediate targets may play a role in guiding cortical axons in the developing brain. During the development of the corpus callosum, cortical axons from one cerebral hemisphere cross the midline to reach their targets in the opposite cortical hemisphere. We have identified two early differentiating populations of midline glial cells that may act as intermediate guideposts for callosal axons. The first differentiates directly below the corpus callosum forming a wedge shaped structure (the glial wedge) and the second differentiates directly above the corpus callosum within the indusium griseum. Axons of the corpus callosum avoid both of these populations *in vivo*. This finding is recapitulated *in vitro* in three-dimensional collagen gels. In addition, experimental manipulations in organotypic slices show that callosal axons require the presence and correct orientation of these populations to turn toward the midline. We have also identified one possible candidate for this activity because both glial populations express the chemorepellent molecule *slit-2*, and cortical axons express the *slit-2* receptors *robo-1* and *robo-2*. Furthermore, *slit-2* repels–suppresses cortical axon growth in three-dimensional collagen gel cocultures.

Key words: corpus callosum; axon guidance; glial wedge; cortex development; indusium griseum; slit-2*;* robo*; chemorepulsion; midline*

Developing neocortical axons exit one hemisphere either laterally via the internal capsule or medially via the corpus callosum. The internal capsule acts as an intermediate target for corticofugal axons by secreting the chemotropic molecule netrin-1 (Metin et al., 1997; Richards et al., 1997). However netrin-1 is not expressed within the direct pathway of the corpus callosum (Kennedy et al., 1994), although the cortical axons of the corpus callosum do express the netrin-1 receptor DCC (deleted in colorectal cancer) (Shu et al., 2000), and both netrin-1 and DCC mutant mice are acallosal (Serafini et al., 1996; Fazeli et al., 1997). Recently, a number of other molecules have been identified, which, when mutated in mice, result in an acallosal phenotype (Orioli et al., 1996; Qui et al., 1996; Dahme et al., 1997; Yoshida et al., 1997; Dattani et al., 1999). However, little is known about the critical developmental processes that such molecules might regulate and the guidance mechanisms required for the corpus callosum to form.

Previous studies have identified a population of cells at the cortical midline called the glial sling. The glial sling is glial fibrillary acidic protein (GFAP)-negative (in rodents) and migrates from the lateral ventricular zone to underlie the developing corpus callosum (Silver et al., 1982). Both ablation and rescue experiments (Silver et al., 1982; Silver and Ogawa, 1983) have shown that the glial sling is required for the development of the corpus callosum. Because the glial sling is not fully formed until relatively late in callosal development [at embryonic day (E) 17 in mouse] and other midline glial populations are important for the development of commissural pathways in other regions of the brain (Marcus et al., 1995; Cummings et al., 1997), we investigated the possibility that additional midline glial populations direct axons across the corpus callosum.

In *Drosophila*, midline glia determine which axons cross the midline and which axons remain ipsilateral (Jacobs and Goodman, 1989; Kidd et al., 1999). This guidance function is mediated by a *slit* (ligand)–*roundabout* (receptor) interaction (Kidd et al., 1999). Axons expressing *roundabout* are repelled by *slit*, which is expressed by midline glial cells (Kidd et al., 1998a,b, 1999; Rothberg et al., 1990). A third molecule, commissureless, also secreted by the midline glia, downregulates the expression of *roundabout* and allows axons to cross the midline because they no longer respond to *slit* (Tear et al., 1996; Kidd et al., 1998b, 1999). Recently, the vertebrate homologs of *slit* and *roundabout* (*robo* in vertebrates) were cloned and shown *in vitro* to repel motor, olfactory, hippocampal, and retinal axons and neuronal cell bodies (Ba-Charvet et al., 1999; Brose et al., 1999; Hu, 1999; Li et al., 1999; Wu et al., 1999; Zhu et al., 1999; Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000). Given that *slit-2* is expressed in the septum (Ba-Charvet et al., 1999; Li et al., 1999), a midline forebrain structure, we investigated whether *slit-2* was expressed by the glial wedge and whether it could act as a chemorepellent for cortical axons during the period of callosal development.

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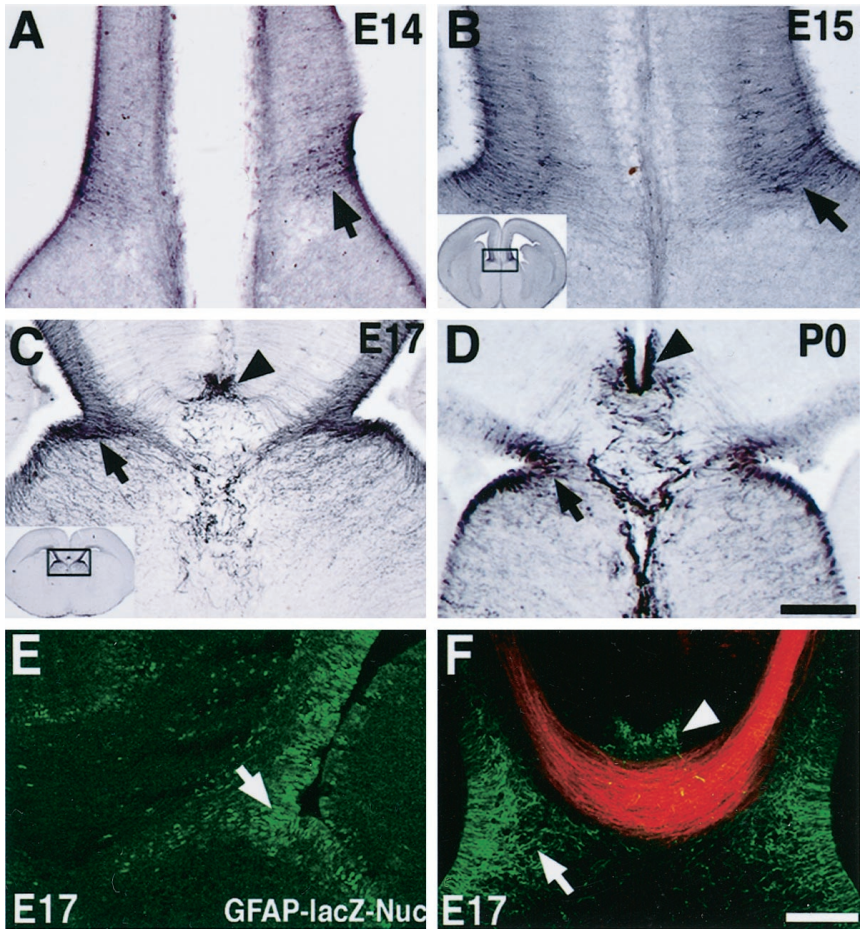

Figure 1. Development of midline glia and the corpus callosum. GFAP-positive cells are present by E14 in mouse cortex (*arrow* in *A*). These glia send long radial-glial-like processes toward the midline, coalescing into a wedge-shaped structure by E15 (*arrow* in *B*). By E17, a second midline glial population arises in the indusium griseum (*arrowhead* in *C*), and the glial wedge is still present (*arrow* in *C*). At P0, both the indusium griseum glia (*arrowhead* in *D*) and the glial wedge (*arrow* in *D*) are present, but the glial wedge remains confined to the ventricular zone of the corticoseptal boundary. Staining of the GFAP-lacZ (nuclear-targeted) mice with an antibody to β-galactosidase shows that the cell bodies of the glial wedge remain within the ventricular–subventricular zone (*arrow* in *E*). *F*, Double-labeling of the glial wedge (*arrow*) and the indusium griseum glia (*arrowhead*) with GFAP antibody (*green*) and the callosal axons with DiI (*red*) show that the callosal axons do not enter these glial structures but pass between them. Scale bars: (in *D*) *A*, *B*, 150 μm; *C*, *D*, 240 μm; (in *F*) *E*, 100 μm; *F*, 200 μm.

Parts of this work have been published previously in abstract form (Shu and Richards, 1999; Richards and Shu, 2000).

MATERIALS AND METHODS

Immunohistochemistry. Immunohistochemistry was performed as described previously (Shu et al., 2000). Primary antibodies used were as follows: (1) rabbit anti-cow GFAP (Dako, Daco, Denmark), 1: 30,000 for nickel-DAB reaction and 1:1000 for fluorescent Cy2 detection, and (2) rabbit anti-*Escherichia coli* β-galactosidase (5 Prime → 3 Prime Inc., Boulder, CO), 1:2000 for fluorescent Cy2 detection. Secondary antibodies used were as follows: (1) biotinylated goat anti-rabbit (Vector Laboratories, Burlingame, CA), 1:600, and (2) Cy2-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA), 1:400.

In situ *hybridization. In situ* hybridization was performed as described previously (Tuttle et al., 1999) on cryostat sections with the addition of dextran sulfate (Sigma, St. Louis, MO) at a concentration of 300 μl/1.5 ml total volume in the both the prehybridization and hybridization steps. For Slit-2–GFAP double-labeling, GFAP immunohistochemistry was performed first as described above, followed by the *in situ* hybridization with two changes: (1) the proteinase K concentration was reduced to 1 μg/μl, and (2) the permeabilization step with DEPC-PBS–Triton X-100 was eliminated.

DiI labeling. Small injections of a 10% solution of DiI (Molecular Probes, Eugene, OR) in dimethylformamide were made using pulled glass pipettes attached to a Picospritzer (General Valve, Fairfield, NJ). Brains were stored in the dark at room temperature for at least 4 weeks to allow DiI transport and then vibratome-sectioned at 45 μm. GFAP immunohistochemistry was performed as described above but without the addition of Triton X-100.

Dissection and culture of explants and transfected cells. Living E17 C57BL/6J mouse brains were blocked in 3% low melting point agar (Sea plaque; FMC Bioproducts, Rockland, ME) and vibratome-sectioned at 350 μm. Explants of cortex and glial wedge were dissected from the regions shown in Figure 2*A* and cocultured in collagen gels (Collaborative Research, Bedford, MA) as described previously (Richards et al., 1997). Collagen gels were cultured in DMEM–F12 medium (Life Technologies, Gaithersburg, MD) supplemented with 1% penicillin-streptomycin, 0.28% glucose, 1% of 200 mM glutamine, 5% rat serum, and 10% fetal bovine serum. After 3 d, the cultures were fixed in 4% paraformaldehyde, and a 10% solution of DiI in dimethylformamide was injected into the cortical explants. Explants were kept in the dark at 37°C for at least 48 hr to allow for DiI diffusion. *slit-2*-expressing cells were prepared by transfecting 10 μg of recombinant human *slit2* DNA or PectagB vector control DNA [a gift from Dr. M. Tessier-Lavigne and Dr. K. Brose (both from University of California, San Francisco, San Francisco, CA)] using LipofectAMINE PLUS reagent (Life Technologies) following a standard protocol. Cell blocks were prepared as described previously (Richards et al., 1997). To quantify the axonal length and number, the mean axon length was derived by measuring the length of the 10 longest axons on each side of the explant. The number of all axons on the proximal side versus the other three sides of the explants were counted. To quantify axon repulsion, we counted the number of axons that grew out straight initially and then turned away from the Slit-2-expressing cells at an angle of at least 30°. Percentages of axons repelled were derived from the number of axons repelled on the proximal side divided by the total number of axons on the proximal side, pooled over all of the explants in one group.

Organotypic slice cultures. Organotypic slices were prepared from living E17 C57BL/6J mouse brains, vibratome-sectioned at 350 μm. Coronal slices were cultured on 30 mm culture plate inserts (Millipore, Bedford, MA) coated with 10 μg/ml poly-L-lysine (Sigma) and 2 μg/ml laminin (Becton Dickinson, Cockeysville, MD), in six-well tissue culture plates. Experimental dissections and manipulations (see schematics in Fig. 3) were performed using a 1 mm blade especially made for these experiments. Slices were cultured in DMEM–F12 medium with 1% penicillin-streptomycin, 0.28% glucose, 1% of 200 mM glutamine, 5% rat serum, 10% FBS, and 10 ng/ml mouse nerve growth factor (2.5S; Alomone Labs, Jerusalem, Israel). After 3 d in culture, the slices were

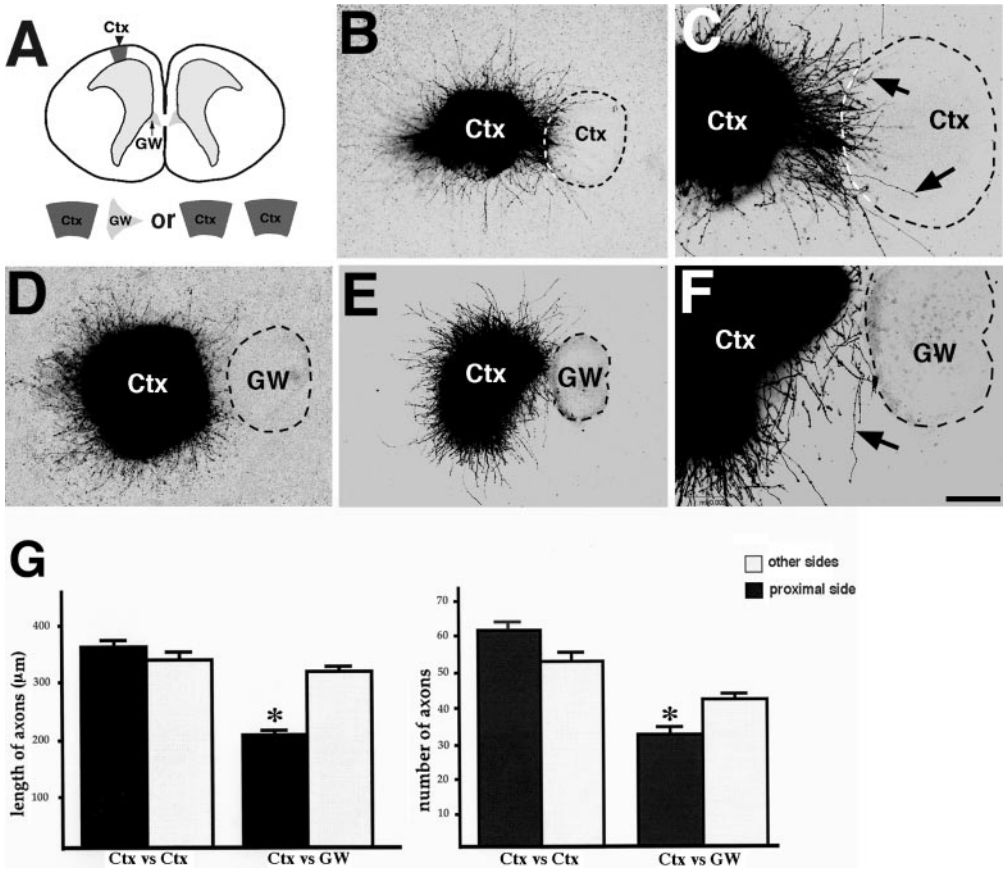

Figure 2. The glial wedge suppresses and repels E17 cortical axons. *A*, Schematic of the experimental paradigm. Cortical explants (*Ctx*) and glial wedge explants (*GW*) were dissected from coronal sections (350 μm in thickness) of E17 mouse brain. Cortical explants were cocultured with either glial wedge explants (*D–F*) or with other cortical explants (*B*, *C*) in three-dimensional collagen gels. After 3 d, the cultures were fixed, and DiI was injected into the cortical explant. When cortex was paired with cortex as a control, the cortical axons projected symmetrically from the explant (*B*), growing into the target cortical explant (*arrow* in *C*; *C* is a higher power view of *B*). When cortex was paired with glial wedge, the length of axons on the proximal side facing the glial wedge was greatly diminished (*D–F*), with many axons turning away (*arrow* in *F*) or stopping at the edge of the glial wedge explant (*D* and *E* are two different examples; *F* is a higher power view of *E*). *G*, Quantification of axonal length (mean axon length was derived by measuring the length of the 10 longest axons on each side of the explant) and number on the proximal side versus the other three sides of the explants. Statistically significant results are labeled (*). Scale bar (in *F*): *B*, *D*, *F*, 300 μm; *C*, *F*, 150 μm.

fixed in 4% paraformaldehyde and then either resectioned at 50 μm for GFAP immunohistochemistry or a DiI crystal (Molecular Probes) was inserted into the cortical plate. Seventy-two hours was allowed for DiI diffusion before the slices were mounted in polyvinyl alcohol–1,4-diazabicyclo-[2.2.2]octane mounting medium for confocal microscopy.

RESULTS

Development of midline glial populations at the corticoseptal boundary

Although GFAP-positive glia were previously thought to arise late in embryonic development (Valentino et al., 1983), more recent reports described GFAP mRNA expression at E15 in rats (Sancho-Tello et al., 1995) and the expression of a reporter transgene under the control of the GFAP promoter at E13.5 in the mouse cortex (Brenner et al., 1994). To investigate the possibility that glial populations may arise early at the cortical midline, we performed a time course of GFAP expression in early embryonic brains. We found two early differentiating populations of midline glial cells. The first differentiates at the dorsomedial aspect of the lateral ventricles at E14, sending long radial processes toward the midline (Fig. 1*A*). By E15, these radial processes coalesce into a wedge-shaped structure on either side of the midline (Fig. 1*B*), which remains evident at both E17 and postnatal day 0 (P0) (Fig. 1*C*,*D*, *arrows*). We term this structure the "glial wedge." Rostrocaudally, the glial wedge is present from the taenia tecta to the hippocampal commissure (a distance of ~500 μm at E17). At the hippocampal commissure, glia can be seen inserting between the corpus callosum and the hippocampal commissure, although the wedge shape is lost in this region. We used a GFAP-lacZ transgenic mouse (Brenner et al., 1994) in which the lacZ was targeted to the nucleus to investigate where the glial wedge cell bodies were located. These cell bodies are located within the ventricular zone (Fig. 1*E*), in contrast to the glial sling cells, which migrate toward the midline (Silver et al., 1982). The second glial population differentiates directly above the corpus callosum in the indusium griseum (Fig. 1*C*,*D*,*F*, *arrowheads*). These three midline glial structures (the glial sling, the glial wedge, and the indusium griseum glia) may coordinate midline cortical axon guidance.

The glial wedge and the indusium griseum glia form a restricted zone within which ventrally projecting callosal axons grow (Fig. 1*F*). The shape of the glial wedge suggests that it may act to deflect the cortical axons medially toward the midline. By labeling cortical axons with DiI and the glial wedge–indusium griseum glia with GFAP, we found that cortical axons make a

Table 1. Quantification of process outgrowth from cortical explants

Experiment	Mean ± SE length of axons (μm)		Mean ± SE number of axons		Total number of explants/total number of experiments
	Proximal side	Other 3 sides combined	Proximal side	Other 3 sides combined	
Cortex vs cortex	364 ± 24	341 ± 27	61 ± 7	52 ± 7	29/7
Cortex vs glial wedge	209 ± 15*	311 ± 14	33 ± 4*	41 ± 3	44/7
Cortex vs control transfected cells	428 ± 21	451 ± 15	89 ± 8	89 ± 7	38/4
Cortex vs Slit-2-transfected cells	234 ± 19*	393 ± 22	22 ± 3*	56 ± 4	51/4

*$p < 0.05$ indicates significant difference; Student's t test.

sharp turn toward the midline when they encounter the glial wedge and extend medially in the channel formed by the glial wedge and the induseum griseum (Fig. 1*F*).

The glial wedge suppresses–repels cortical axon growth

To directly test whether these populations express an axonal guidance activity, we challenged axonal outgrowth from cortical explants with explants of glial wedge in three-dimensional collagen gels (Fig. 2*A*). Because the indusium griseum is so small, it was impossible to dissect this region specifically; therefore, the indusium griseum glia were excluded from this part of the analysis. As a control, cortical explants were also paired with a second cortical explant that did not exhibit guidance activity for cortical axons, as described previously (Richards et al., 1997). After 3 d in culture, DiI was injected into one cortical explant to label axon outgrowth. Because the glial wedge may express either diffusible or bound guidance signals, the distance between the explants may be critical to observing a response. The theoretical distance over which axons may be guided by target-derived diffusible signals is on the order of 1 mm (Goodhill, 1997). However, explants may need to be even closer to observe effects by bound or weakly diffusible molecules. Therefore, from the outset, only cultures in which the inter-explant distance was shorter than the longest axons on the proximal side of the explant were included in the analysis. In cortex–cortex cultures, axons extended radially from the explant (Fig. 2*B*, Table 1), growing into the control cortical explant in 96% of cases (Fig. 2*C*, *arrows*). In cortex–glial wedge cultures, axon length was generally shorter on the side of the explant facing the glial wedge (Fig. 2*D*,*G*). Those axons that did extend on the proximal side of the explant stopped at the edge of the glial wedge explant (Fig. 2*F*), growing into the glial wedge in only 12% of cases. We also observed many axons turning away from the glial wedge explant (Fig. 2*F*, *arrow*). In some explants, cortical axons were able to grow toward the glial wedge but did not enter the glial wedge, although in every case, the inter-explant distance was shorter than the length of the axons on the proximal side (Fig. 2*E*,*F*). These results suggest that the glial wedge expresses a membrane-bound or weakly diffusible molecule that inhibits cortical axons from growing into the glial wedge.

This asymmetric growth could be attributable to a decrease in axonal length or axonal number. Therefore, we quantified the length and number of axons projecting from the cortical explants in each of the conditions. In cortex–cortex cultures, the average length and number of axons on the proximal side was not significantly different from the other three sides combined (Fig. 2*G*, Table 1). In contrast, in cortex–glial wedge cultures, the mean axon length on the proximal side was significantly shorter than that of the other sides (Fig. 2*D–G*, Table 1), and we observed a small but significant difference in the mean number of axons on the proximal side versus the other sides ($p = 0.03$) (Fig. 2*G*, Table 1). The effect on axon length was evident when the explants were <300 μm apart, again indicating that the glial wedge activity was likely to be a membrane-bound or weakly diffusible molecule.

The glial wedge causes turning of callosal axons *in situ*

Callosal axons leave the neocortex and travel ventrally under the subplate of the cingulate cortex. To cross the midline, they must reorient their growth cones by making a sharp turn medially just before crossing. The glial wedge directly underlies this axonal decision point. The axons contact the lateral part of the wedge and grow along it, turning sharply toward the midline. Therefore, both the shape and location of the glial wedge are well suited to direct cortical axons toward the midline and prevent them from entering the septum. In our previous collagen gel experiments, there was no way to determine whether the effect of the glial wedge was on subcortically (laterally) projecting or callosally (medially) projecting axons, because both populations were present in the explants.

To further investigate the ability of the glial wedge to direct medially projecting callosal axon growth, we developed an organotypic cortical slice preparation. Both the glial wedge (Fig. 3*A*) and the corpus callosum formed in these organotypic slices (Fig. 3*B*,*C*). We hypothesized that reorienting the glial wedge might influence callosal axon pathfinding. Thus, the glial wedge was dissected out of slices, rotated 180° (medial to lateral), and implanted back into the slice (Fig. 3*G–I*). Control slices were grown either intact or as sham-operated slices in which the glial wedge was dissected out and reimplanted without being rotated (Fig. 3*D–F*). All of the slices were cultured for 3 d and fixed, and a DiI crystal was inserted into the cortical plate to anterogradely label the callosal axons. Because the glial wedge sits in a notch of the lateral ventricle (also called the septal fork), we were able to identify the position of the glial wedge at the end of the experiment based on the shape of the tissue, the presence of the septal fork, and the cell density difference between the glial wedge and the cell-dense cingulate cortical plate. In both intact ($n = 11$ of 11 cases in six experiments) and sham-operated ($n = 8$ of 8 cases in eight experiments) slices, cortical axons extended normally, making an angular turn at the glial wedge and then crossing the midline (Fig. 3*B–F*). However, when callosal axons encountered the reoriented glial wedge graft, the axons turned away from the midline toward the lateral ventricle ($n = 7$ of 7 cases in six experiments) (Fig. 3*H*,*I*).

In an additional experiment to confirm that the glial wedge was

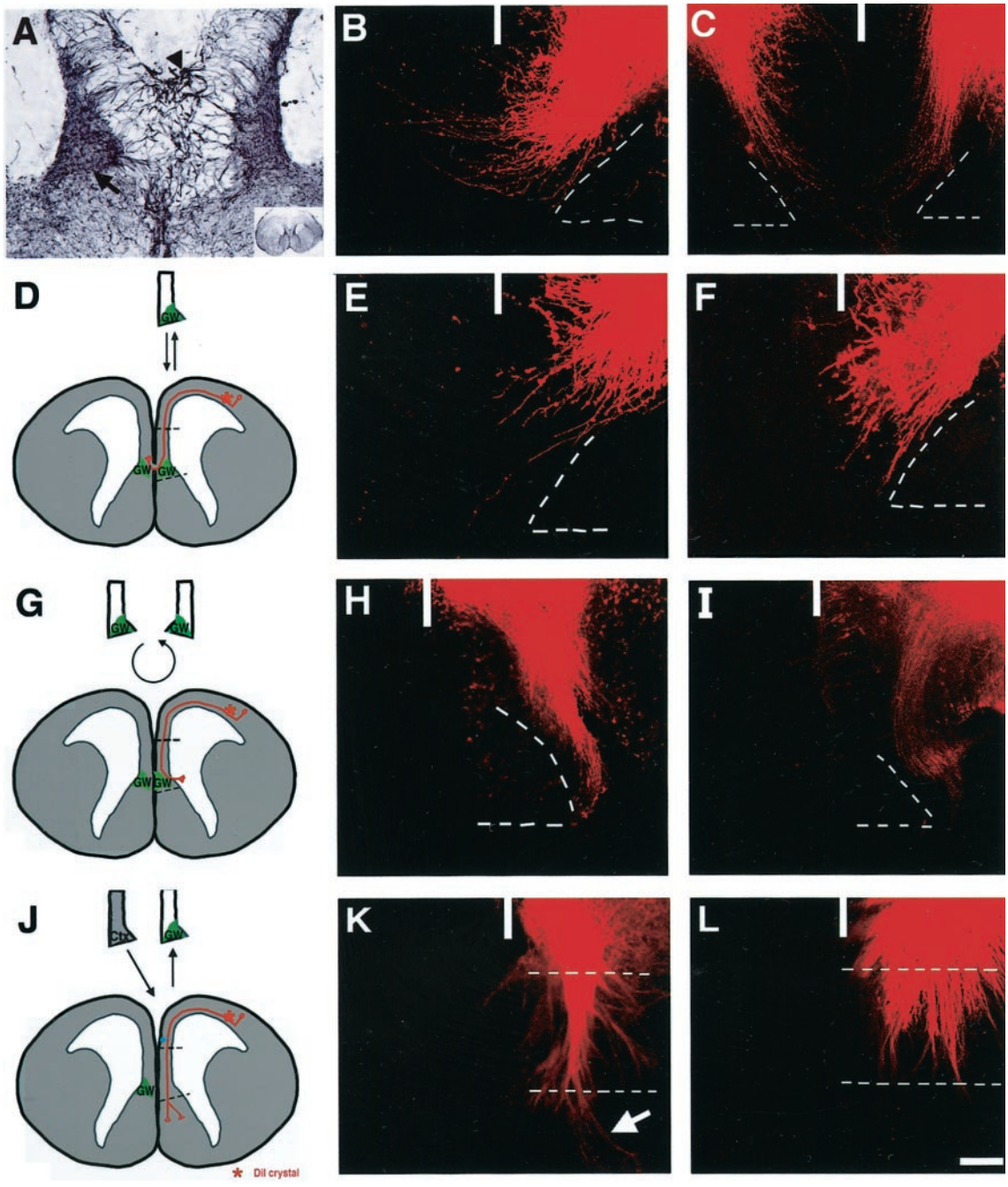

Figure 3. The glial wedge directs the growth of callosal axons *in situ*. *A*, The glial wedge forms in organotypic slices. E17 mouse brains were sectioned at 350 μm, grown for 3 d in culture, fixed, resectioned at 50 μm, and stained with a GFAP antibody. Both the glial wedge (*arrow*) and the indusium griseum glia (*arrowhead*) maintain their *in vivo* morphology after 3 d *in vitro*. *B*, *C*, The corpus callosum forms in organotypic slices. *B* and *C* represent two examples of uncut control slices, cultured for 3 d and fixed, and a crystal of DiI was added to label the callosal axons. In *C*, DiI crystals were placed on both sides of the midline to show that cortical axons from both hemispheres still cross in the same organotypic slice (in all other slices, DiI was added to only one hemisphere). *D–L*, Replacement or reorientation of the midline results in axonal misrouting. *D–F*, The corpus callosum forms normally in sham-operated slices (*D* shows the experimental paradigm, and *E* and *F* are two examples). *G–I*, Reorienting the glial wedge by 180° causes the axons to turn away from the midline (*G* shows the experimental paradigm, and *H* and *I* are two examples). *J–L*, The glial wedge is required for axons to turn toward the midline. When the glial wedge–indusium griseum region is replaced on one side by a piece of cortex, cortical axons fail to turn and instead grow straight through the graft, in many cases entering the septum (*arrow* in *K*; *J* shows the experimental paradigm, and *K* and *L* are two examples). The *white broken lines* in *B*, *C*, *E*, *F*, *H*, and *I* represent the position of the glial wedge; in *K* and *L*, they represent the edges of the cortical graft. The *solid white line* in *B*, *C*, *E*, *F*, *H*, *I*, *K*, and *L* represent the position of the midline. Scale bar (in *L*): *A*, 120 μm; *C*, *E*, *F*, *K*, *L*, 200 μm; *B*, *H*, *I*, 100 μm.

required for cortical axons to turn toward the midline, we removed the glial wedge region and implanted a piece of cortex from another slice. Cortical tissue was used in this experiment because it did not contain significant numbers of GFAP-positive astrocytes at this developmental stage and because axons from cortical explants will grow within other cortical explants in collagen gels. In these experiments ($n = 6$ of 6 cases in two experiments) (Fig. 3*J–L*), axons grew straight into the grafts without

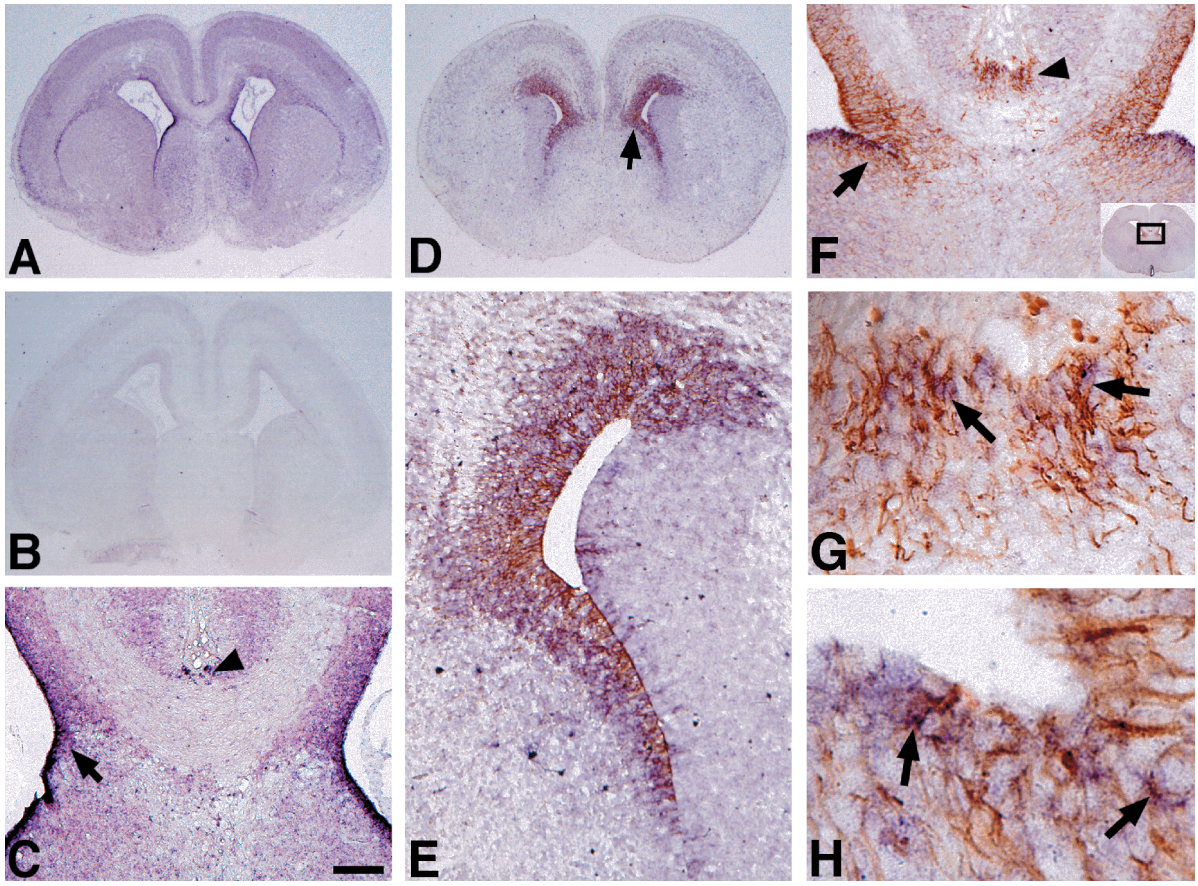

Figure 4. During callosal axon targeting, the glial wedge and the indusim griseum express *slit-2*. *In situ* hybridizations using *slit-2* antisense (*A*, *C*) probes show that *slit-2* is expressed within the ventricular zone of the septum and corticoseptal boundary. The control *slit-2* sense probe shows no specific labeling (*B*). *slit-2* is expressed in both the glial wedge (*arrow* in *C*) and the indusium griseum glia (*arrowhead* in *C*; *C* is a higher power view of *A*). *D*, *E*, Double-labeling of anti-β-galactosidase (driven by the GFAP promoter and targeted to the cytoplasm) immunohistochemistry and *slit-2 in situ* hybridization shows that cells within the glial wedge express both *slit-2* and the lacZ transgene (*E* is a higher power view of *D*). In wild-type embryos, glia within the glial wedge (*arrow* in *F*) and the indusium griseum (*arrowhead* in *F*) are double-labeled with *slit-2* (*purple*) and GFAP (*brown*). *F* is a higher power view of the *boxed* region in the *inset*. Examples of double-labeled cells are shown in the indusium griseum (*arrows* in *G*) and the glia wedge (*arrows* in *H*; *G* and *H* are higher power views of *F*). All sections are from E17 mouse brains. Scale bar (in *C*): *A*, *B*, *D*, 950 μm; *C*, 220; *E*, 110 μm; *F*, 170 μm; *G*, 25 μm; *H*, 20 μm.

making a turn. In some cases, these axons grew through the cortical graft and into the septum (Fig. 3*K*, *arrow*). Together, these results indicate that both the presence and the correct orientation of the glial wedge are required for medially projecting callosal axons to turn toward the midline *in situ*.

The glial wedge and indusium griseum glia express Slit-2

In *Drosophila,* midline glia influence axon growth by expressing *slit*, which repels ipsilaterally projecting axons away from the midline (Jacobs and Goodman, 1989; Kidd et al., 1999). Because vertebrate Slit-2 also acts as membrane-bound weakly diffusible molecule (Brose et al., 1999) and is expressed in the septal fork of the lateral ventricle, a region corresponding to the glial wedge (Ba-Charvet et al., 1999; Li et al., 1999), *slit-2* may be a glial wedge guidance signal. We first determined whether the glial wedge expressed *slit-2* and whether the cortical neurons expressed *robo*. At E17, *slit-2* mRNA was expressed in the medial subventricular zone along the lateral ventricles, in which the glial wedge cell bodies reside, and in the indusium griseum (Fig. 4*A*,*C*) but not in the glial sling. This expression was not observed in sections in which a *slit-2* sense probe was used as a control (Fig. 4*B*). We also performed *in situ* hybridization using *slit-2* antisense probes doubled with GFAP immunohistochemistry on tissue derived from both GFAP-lacZ (cytoplasmic targeted) transgenic mice (Fig. 4*D*,*E*) and C57BL/6J wild-type mice (Fig. 4*F–H*). In each case, *slit-2* expression overlapped with GFAP expression (and lacZ expression), indicating that these glial populations do express *slit-2* (Fig. 4*D–H*). Two other members of the *slit* family, *slit-1* and *slit-3*, have not been shown to be expressed in the region corresponding to the glial wedge (Ba-Charvet et al., 1999; Yuan et al., 1999).

To respond to Slit-2, the cortical axons must express the *slit-2* receptor *robo*. Using *in situ* hybridization, we found that both receptors of *slit-2*, *robo-1* (Fig. 5*A*,*C*) and *robo-2* (Fig. 5*D*,*F*), are highly expressed in cortical neurons at E17. Sections labeled with *robo-1* sense (Fig. 5*B*) or *robo-2* sense (Fig. 5*E*) probes showed no specific labeling. Therefore, the ligand, *slit-2*, is expressed in the glial wedge and the indusium grisuem glia, and the *slit-2* receptors *robo-1* and *robo-2* are expressed in the cortical neurons, at the appropriate time to mediate axon guidance at the midline.

Slit-2 suppresses–repels cortical axon growth

To test the hypothesis that Slit-2 might be an axon guidance molecule expressed by the glial wedge–indusium griseum glia, we cultured cortical explants with agar blocks of 293T cells trans-

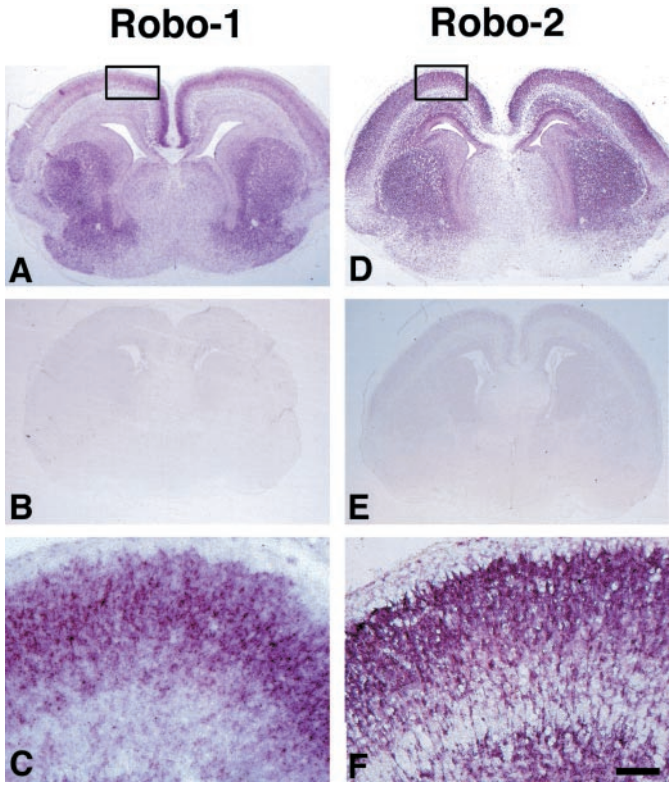

Figure 5. The *slit-2* receptors *robo-1* and *robo-2* are expressed in the neocortex during callosal axon targeting. *In situ* hybridization using antisense probes against *robo-1* (*A*, *C*) or *robo-2* (*D*, *F*) show that both receptors are expressed within the cortical plate at E17 (*C* and *F* are higher power views of the *boxed* regions in *A* and *D*, respectively). Control sense probes for either *robo-1* (*B*) or *robo-2* (*E*) show no specific labeling. Scale bar (in *F*): *A*, *B*, *D*, *E*, 900 μm; *C*, *F*, 80 μm.

fected with either a Slit-2 expression construct or a control vector construct. The transfected cells were cocultured with E17 cortical explants in collagen gels, and DiI was again used to label the axon outgrowth. In controls, cortical axons projected radially (Fig. 6*A*) with axons growing into the cell block in 63% of cases (Fig. 6*D*, *arrow*; Table 1). There was also no significant difference in the mean number or length of axons on the proximal side versus the mean of the other three sides combined (Fig. 6*G*, Table 1). In explants cultured with Slit-2-expressing cells, axons preferentially grew away from the cell block with more axons exiting the explant on the side farthest from the Slit-2-expressing cells (Fig. 6*B*,*C*). In addition, significantly fewer axons extended toward the Slit-2-expressing cells, and those that did were stunted in their growth (Fig. 6*G*, Table 1). Thus, many axons on the proximal side may have been repelled by Slit-2 such that they did not even extend out of the explant, reflecting this decrease in axonal number on the proximal side. In addition, only 6% of these explants extended axons into the Slit-2-expressing 293T cell block, and in most cases, the few axons that did reach the cell block grew along the edge of the cell block without growing into it (Fig. 6*F*, *arrow*). Furthermore, we observed a 10-fold increase in the number of axons turning away from the Slit-2-expressing cells (Fig. 6*E*, *arrow*). Thus, Slit-2-expressing cells repelled cortical axons at a time when the callosal projection is forming.

DISCUSSION

We have shown that early midline glia do play a role in guiding axons at the midline during the formation of the corpus callosum. We have identified the glial wedge and the indusium griseum glia as being important for this guidance. Both collagen gel and organotypic slice experiments show that the glial wedge expresses a guidance activity that causes callosal axons to turn toward the midline. The indusium griseum glia also express Slit-2 and therefore may participate in directing callosal axon growth. Indeed, in organotypic slice cultures, both the glial wedge and the indusium griseum were reoriented, indicating the importance of both populations. Previous reports described GFAP-positive glia in cats as important for midline fusion and radial glia (which may correspond to the glial wedge described here) as a lateral extension of the glial sling (Silver et al., 1993). However, our studies suggest that the glial wedge and the glial sling are independent populations. The glial wedge and the glial sling cells are born at different times and they express different cellular markers (including GFAP and Slit-2; our unpublished observations). Our working hypothesis is that these three populations, the glial wedge, the indusium griseum glia, and the sling, all participate in guiding callosal axons (Fig. 7). The glial wedge and indusium griseum glia participate by repelling callosal axons toward and across the midline through the actions of molecules, such as *slit-2* and *robo*. The sling may participate by providing positive or attractive guidance cues, probably through a contact-mediated mechanism.

Midline glial populations have long been shown to be associated with the formation of commissures and boundaries between neuromeres (Levitt and Rakic, 1980; Van Hartesveldt et al., 1986; Mori et al., 1990; Silver et al., 1993; Marcus and Easter, 1995; Cummings et al., 1997; Fitch and Silver, 1997; Pires-Neto et al., 1998). During the formation of the anterior commissure in mammals, glia develop on either side of the tract, forming a tunnel-like structure as the fibers of the anterior commissure begin to cross the midline (Cummings et al., 1997; Pires-Neto et al., 1998). Their existence and position during anterior commissure formation suggests that these glia may play a role in containing the axons within the tract during development.

In *Drosophila*, midline glia function as guidepost cells that direct axons to project either ipsilaterally or contralaterally to form midline commissures (Jacobs and Goodman, 1989; Hummel et al., 1999a,b; Kidd et al., 1998a,b, 1999). In vertebrates, roof plate glia in the dorsal midline of the spinal cord prevent growing axons from crossing the midline (Snow et al., 1990) through the actions of bone morphogenetic proteins (Augsburger et al., 1999). In the midline raphe of the midbrain, hindbrain, and cervical spinal cord, an extensive radial glial structure exists both during development and in adulthood (Van Hartesveldt et al., 1986; Mori et al., 1990) called the midline raphe glial structure (MRGS). The glial cells that make up the MRGS label with both an antibody against S-100 protein (Van Hartesveldt et al., 1986) and with the monoclonal antibody R2D5 (Mori et al., 1990) but are not GFAP-positive (Van Hartesveldt et al., 1986). The MRGS forms a continuous band of radial glial fibers separating the right and left brainstem but with some interruptions to allow for the passage of decussating fibers. Its structure suggests that the MRGS may be involved in sorting and organizing ipsilaterally and contralaterally projecting axons during development (Mori et al., 1990), as has been shown to be the function of radial glial at the mammalian optic chiasm (Marcus et al., 1995; Erskine et al., 2000).

An additional function of the glial wedge may be to sort out contralaterally projecting callosal axons from ipsilaterally projecting perforating axons (Hankin and Silver, 1988). The perforating pathway consists of axons extending perpendicular to the corpus callosum (Hankin and Silver, 1988). These ipsilaterally projecting

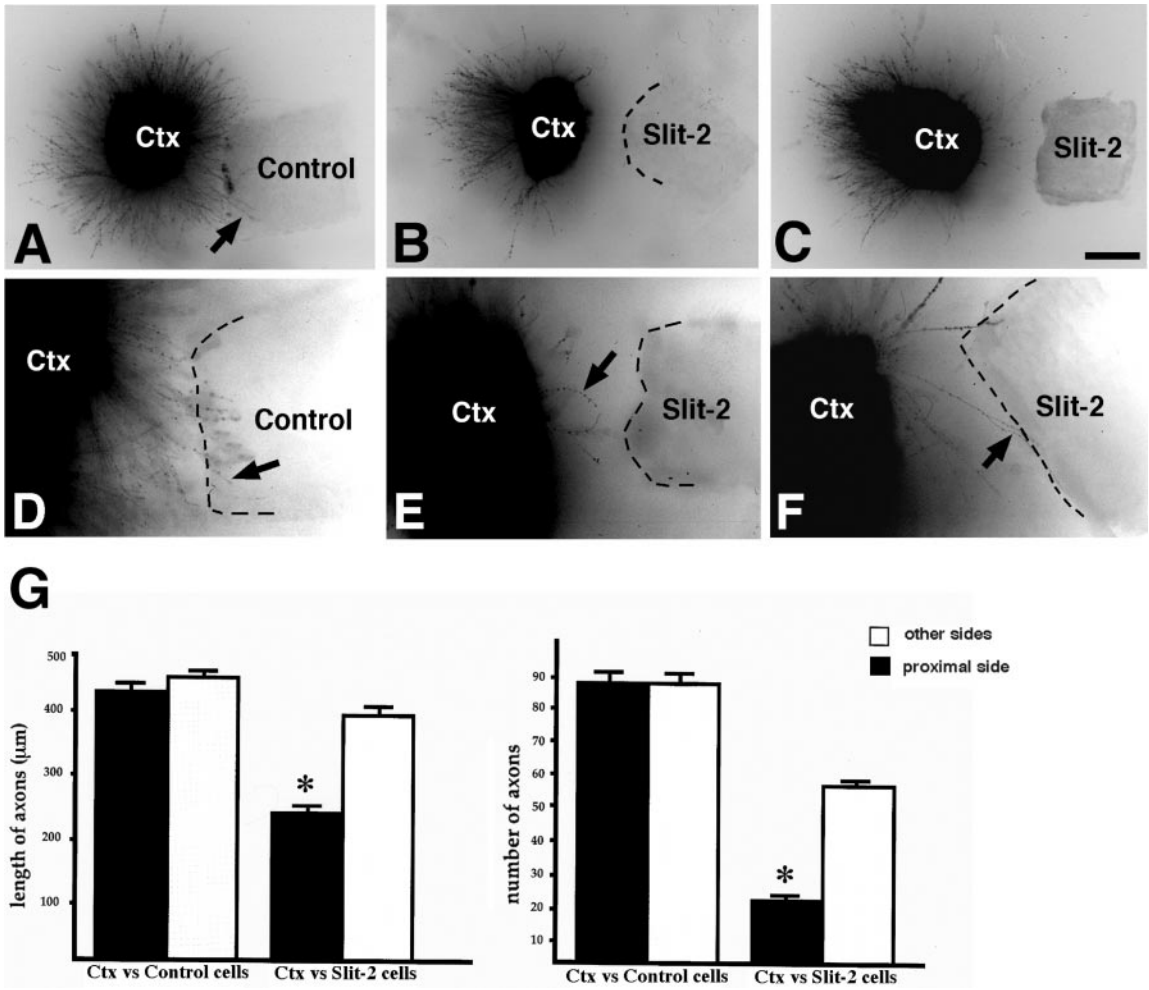

Figure 6. Slit-2 suppresses and repels E17 cortical axons. Cortical explants (*Ctx*) derived from E17 mouse brains were cocultured with 293T cells transfected with either a control (vector alone) construct (*A*, *D*; *D* is a higher power view of *A*) or a Slit-2 expression construct (*B*, *C*, *E*, *F*; 4 different examples). Explants were cultured for 3 d and fixed, and then the cortical explant was injected with DiI to label axonal outgrowth. Cortical explants display a symmetrical growth when cocultured with control transfected cells (*A*), even growing into the transfected cell block (*arrow* in *D*). However, when cocultured with Slit-2-expressing cells, cortical axon outgrowth was severely suppressed in the proximal side facing the cell block (*B*, *C*), with some axons turning away (*arrow* in *E*) and refusing to enter the cell block (*arrow* in *F*). *G*, Quantification of axonal length (mean axon length was derived by measuring the length of the 10 longest axons on each side of the explant) and number on the proximal side versus the other three sides of the explants. Statistically significant results are labeled (*). Scale bar (in *C*): *A–C*, 300 μm; *D–F*, 150 μm.

axons are able to cross the sling, projecting medial to the tips of the glial wedge on either side of the midline (our unpublished observation). Presumably, these ipsilaterally projecting axons are guided by different molecular mechanisms than the contralaterally projecting callosal axons.

The long radial processes of the glial wedge resemble those of radial glia (Rakic, 1972) found within the developing cortex. Radial glia have been shown to assist callosal axons after crossing the midline in finding their correct targets in the contralateral cortical plate (Norris and Kalil, 1991), indicating that glia, including radial glia, may be involved in guiding callosal axons along much of their pathway.

The guidance properties of the glial wedge indicate that callosal axons are guided by a short-range diffusible or membrane–substrate-bound molecule. Slit-2 has been shown to act in a similar manner in a number of different assay systems (Ba-Charvet et al., 1999; Brose et al., 1999; Li et al., 1999). When transfected into cell lines, the Slit-2 molecule is proteolytically cleaved into two fragments: an N-terminal fragment of 140 kDa and a C-terminal fragment of 50–60 kDa (Brose et al., 1999; Wang et al., 1999). Both the N-terminal and full-length molecules remain attached to the cell membrane (and mediate axonal collapse), whereas the C-terminal fragment can diffuse into the media (Brose et al., 1999). Therefore, Slit-2 probably mediates repulsion as a membrane-associated or weakly diffusible molecule in three-dimensional collagen gels. Consistent with this, we observed repulsive influences by the Slit-2-expressing cells when cortical explants were placed <450 μm from the cell blocks, indicating that Slit-2 acts as a short-range guidance molecule identical to the glial wedge guidance activity.

Because callosal axons grow between the glial wedge and the indusium griseum glia, callosal axons may be guided by surround repulsion as has been described for the patterning of sensory axon trajectories from the dorsal root ganglia (Keynes et al., 1997). In this system, axons from neurons in the dorsal root ganglia are channeled into bipolar trajectories when cocultured between explants of notochord and dermomyotome. Slit-2 expression in both the glial wedge and the indusium griseum may provide a surround

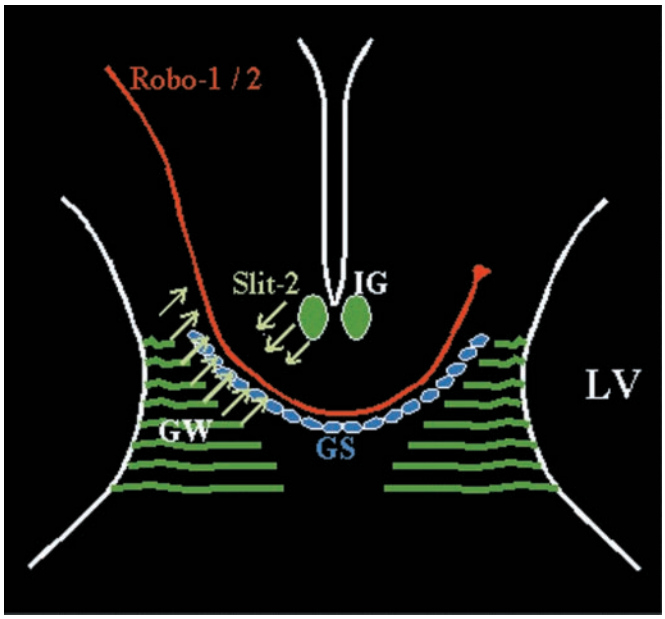

Figure 7. Model of axon guidance across the corpus callosum by midline glial populations. The glial wedge (*GW*) and the indusium griseum glia (*IG*) express Slit-2, repelling axons away from these structures. Because *slit-2* acts as a short-range guidance cue, callosal axons (shown in *red*; expressing the *slit-2* receptors *robo-1* and *robo-2*) are not inhibited from approaching the midline but turn when they encounter the glial wedge. Callosal axons may then use positive guidance signals from the glial sling (*GS*) to cross the midline and may then be deflected dorsally by the glial wedge in the opposite hemisphere. *LV*, Lateral ventricle.

repulsion that defines the pathway of the callosal axons in a similar manner. How callosal axons are then able to leave the midline once they cross the corpus callosum remains to be determined. The results reported here demonstrate that midline glia play a crucial role in the formation of commissures within the mammalian brain.

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