Development/Plasticity/Repair

The Wnt Receptor Ryk Is Required for Wnt5a-Mediated Axon Guidance on the Contralateral Side of the Corpus Callosum

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Ryk (receptor related to tyrosine kinase) has been shown to be a novel Wnt receptor in both *Caenorhabditis elegans* and *Drosophila melanogaster*. Recently, Ryk–Wnt interactions were shown to guide corticospinal axons down the embryonic mouse spinal cord. Here we show that, in *Ryk*-deficient mice, cortical axons project aberrantly across the major forebrain commissure, the corpus callosum. Many mouse mutants have been described in which loss-of-function mutations result in the inability of callosal axons to cross the midline, thereby forming Probst bundles on the ipsilateral side. In contrast, loss of Ryk does not interfere with the ability of callosal axons to cross the midline but impedes their escape from the midline into the contralateral side. Therefore, $Ryk^{-/-}$ mice display a novel callosal guidance phenotype. We also show that Wnt5a acts as a chemorepulsive ligand for Ryk, driving callosal axons toward the contralateral hemisphere after crossing the midline. In addition, whereas callosal axons do cross the midline in $Ryk^{-/-}$ embryos, they are defasciculated on the ipsilateral side, indicating that Ryk also promotes fasciculation of axons before midline crossing. In summary, this study expands the emerging role for Wnts in axon guidance and identifies Ryk as a key guidance receptor in the establishment of the corpus callosum. Our analysis of Ryk function further advances our understanding of the molecular mechanisms underlying the formation of this important commissure.

Key words: **axon guidance; corpus callosum; Ryk receptor; loss-of-function; Wnt5a; chemorepulsion**

Introduction

Members of the Wnt family are key regulators of pivotal developmental processes, including embryonic patterning, specification of cell fate, and determination of tissue polarity (for review, see Ciani and Salinas, 2005). The versatility of Wnt signaling is further highlighted by their role in establishing the complex architecture of the mammalian CNS by regulating axon guidance, dendritogenesis, and synaptogenesis (Hall et al., 2000; Packard et al., 2002; Lyuksytova et al., 2003; Liu et al., 2005; Rosso et al., 2005). Three distinct but interconnected signaling pathways (the canonical, noncanonical, and Wnt/calcium pathways) have been shown to mediate the diverse developmental outcomes triggered by Wnt–Frizzled interactions (Ciani and Salinas, 2005). Moreover, each signaling pathway is activated by only a subset of ligand–receptor pairs, indicating that the biological outcome of Wnt signaling is tightly regulated at the level of ligand–receptor interactions.

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An additional complexity in Wnt signaling is now emerging with the identification of a novel family of Wnt receptors, the Ryk (receptor related to tyrosine kinase) family (Hovens et al., 1992), which is likely to signal independently of the Frizzled-activated pathways (Bonkowsky et al., 1999; Yoshikawa et al., 2003; Inoue et al., 2004). Derailed, the Ryk ortholog in *Drosophila*, is expressed on axons that cross the anterior commissure at the midline of the ventral nerve cord (Callahan et al., 1995). Loss- and gain-of-function studies have shown that Derailed is a repulsive guidance receptor for these axons and that Wnt5 is the Derailed ligand (Bonkowsky et al., 1999; Yoshikawa et al., 2003). Liu et al. (2005) demonstrated that Ryk is required for corticospinal axon navigation down the dorsal funiculus of the developing mouse spinal cord. In this context, Ryk transduces a repulsive guidance signal during ligation of Wnt1 or Wnt5a, both of which are expressed in an anterior-high, posterior-low gradient around the corticospinal tract within the dorsal neural tube. Lyuksytova et al. (2003) demonstrated previously that Wnt4–Frizzled3 signaling promotes the anterior trajectory of spinal commissural axons toward the brain via a chemoattractive mechanism after they have crossed the ventral midline of the neural tube. A complex interplay between Ryk and Frizzled receptors has also been revealed in the mapping of retinal ganglion cell (RGC) axons (Schmitt et al., 2006). In this instance, ventral RGC axons are repelled from tectal domains expressing high levels of Wnt3 in a Ryk-dependent manner, whereas Frizzled3 is required for the

attraction of dorsal RGC axons to areas of low Wnt3 concentration. Together, these studies indicate that Wnt–Frizzled interactions drive chemoattraction, whereas Wnt–Ryk interactions promote chemorepulsion.

Here we show that Ryk is a key guidance receptor in the establishment of the corpus callosum. We also show that Wnt5a is a chemorepulsive ligand for Ryk. Loss of Ryk does not interfere with the ability of callosal axons to cross the midline but impedes their escape from the midline into the contralateral side. Therefore, this study provides novel insights into the guidance mechanisms governing the projection of callosal axons into the contralateral hemisphere.

Materials and Methods

Embryos. $Ryk^{-/-}$ mice were generated from brother–sister matings of $Ryk^{+/-}$ animals (129/Sv × C57BL/6j background) and genotyped as described previously (Halford et al., 2000). Embryos were derived from timed matings, with embryonic day 1 (E1) set at 24 h after the dark period when the animals were initially mated. The subsequent developmental stages were determined according to Kaufman (1992). The use of animals as described here was approved by the Animal Ethics Committee of the University of Queensland in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Histology and immunohistochemistry. Embryos were harvested by cesarean section, anesthetized by inducing hypothermia at 4°C, and then intracardially perfused with ice-cold PBS, pH 7.5, followed by ice-cold 4% paraformaldehyde (PFA), pH 7.5, and postfixed in 4% PFA at 4°C. Serial paraffin-embedded sections, 8 μm, were used for Nissl substance or hematoxylin and eosin staining. For L1 CAM and GFAP immunohistochemistry, 50 μm vibratome sections were incubated for 2 h in blocking solution [2% fetal calf serum (FCS), 2% goat serum, and 0.2% Triton X-100 in PBS] and incubated overnight at 4°C with rat anti-L1 CAM antibody (1:1000; Chemicon, Temecula, CA) or rabbit anti-GFAP antibody (1:1000; DakoCytomation, Carpinteria, CA) in blocking solution. L1 CAM immunostaining was amplified with a biotinylated mouse anti-rat IgG antibody (1:1000; Vector Laboratories, Burlingame, CA) and visualized with streptavidin conjugated to AlexaFluor 594 (1:1000; Invitrogen, Carlsbad, CA). GFAP immunostaining was visualized directly with a goat anti-rabbit IgG conjugated to AlexaFluor 488 (1:1000; Invitrogen). Ryk immunohistochemistry was performed on 10 μm frozen cryostat sections using either a rabbit anti-Ryk antiserum (1:100; a kind gift from Dr. Yimin Zou, University of Chicago, Chicago, IL) (Liu et al., 2005) or a commercial antibody (Abgent, San Diego, CA). Ryk immunostaining was amplified with a biotinylated mouse anti-rat IgG antibody (1:500; Vector Laboratories) and visualized with streptavidin conjugated to AlexaFluor 488 (1:500; Invitrogen). Slides were mounted in DakoCytomation Fluorescent Mounting Medium. Light microscope images were acquired on an Olympus (Tokyo, Japan) IX81 microscope using AnalySIS software.

DiI axon tracing. DiI (Invitrogen) was used to label axons as they crossed the corpus callosum. E18 embryos were perfusion fixed as described above. The skull overlying the cortex was removed, and the brain was postfixed overnight in 4% PFA. Ten microliters of DiI (10% w/v in dimethylformamide) was unilaterally injected into the dorsomedial cortex in a rostrocaudal series using a glass micropipette attached to a Picospritzer II (General Valve, Fairfield, NJ) with the aid of a dissecting microscope and stereotaxic frame. After injection, brains were postfixed in 4% PFA for up to 6 weeks before 100 μm vibratome sections (Leica, Nussloch, Germany) were cut. DiI-positive axons were visualized with a Axioplan fluorescence microscope (Zeiss, Oberkochen, Germany). Higher-resolution images were obtained using a Zeiss LSM laser scanning confocal microscope. *Z*-series images were merged using the appropriate software from Zeiss (Oberkochen, Germany).

Immunoprecipitation and Western blotting. HEK293T cells were transiently transfected with Fugene 6 (Roche Diagnostics, Mannheim, Germany) and plasmid pcDNA3 (Invitrogen). Wnt5a–Myc5 (full-length mouse Wnt5a tagged at the C terminus with 5× Myc), pApex3–Ryk-ECD–Fc–Flag (full-length mouse Ryk extracellular domain fused to the Fc portion of human IgG, tagged with Flag at the C terminus), or pApex3–RykDWD–Fc–Flag [mouse Ryk extracellular domain with deleted WIF (Wnt inhibitory factor) domain (DWD) fused to the Fc portion of human IgG, tagged with Flag at the C terminus]. Cells were lysed 24 h after transfection [lysis buffer containing 150 mM NaCl, 50 mm Tris, pH 7.5, 1% Triton X-100, complete protease inhibitor cocktail (Roche Diagnostics), and 1 mM Na_3VO_4]. Immunoprecipitations were performed from 400 μg of lysate protein, using 9E10-coupled Protein A Sepharose beads (Amersham Biosciences, Uppsala, Sweden) for anti-Myc or anti-Flag M2 affinity gel (Sigma, St. Louis, MO) for anti-Flag. Proteins were separated on 4–12% Bis-Tris NuPAGE gels (Invitrogen) under reducing conditions and electroblotted to polyvinylidene difluoride (PVDF). The anti-Flag M2 monoclonal antibody was used for immunoblotting at 100 ng/ml as an HRP conjugate (Sigma). For anti-Myc immunoprecipitates electroblotted to PVDF, the membrane was stripped and then reprobed with the anti-Myc 9E10 monoclonal antibody at 200 ng/ml as an HRP conjugate (Roche Diagnostics). Bound primary antibodies were visualized using Super Signal West Pico chemiluminescence reagents (Pierce, Rockford, IL).

In situ *hybridization.* Full-length *Wnt5a* cDNA was used as template for the synthesis of the specific antisense and control sense riboprobes. The digoxigenin (DIG) RNA Labeling kit (Roche Diagnostics) was used to synthesize the DIG-labeled antisense or sense riboprobes according to the instructions of the manufacturer. All riboprobes were hydrolyzed to an average length of 100 nucleotides by random alkaline hydrolysis. E16–E18 embryos were perfusion fixed with 4% PFA as described above, and their brains were removed and postfixed overnight with 4% PFA. Heads from E16 embryos were submersion fixed overnight with 4% PFA. *In situ* hybridization was performed on 100 μm vibratome sections as described in the DIG Application Manual for Nonradioactive *In Situ* Hybridization (Roche Diagnostics). Hybridization was performed overnight at 65°C by the addition of 0.5–2 μg/ml riboprobe in hybridization buffer (50% formamide, 2.5× SSC, 10% dextran sulfate, 0.1% Triton X-100, 1 mg/ml yeast tRNA, 0.05% heparin, and 5 mM EDTA). Bound riboprobe was visualized using an anti-DIG-alkaline phosphatase Fab fragment (Roche Diagnostics), followed by the BM Purple chromatogenic reaction (Roche Diagnostics) according to the instructions of the manufacturer.

Quantitative PCR. Cortical tissue from E16–E18 embryos was snap frozen before processing for RNA extraction using the RNeasy Mini kit (Qiagen, Hilden, Germany). Single-stranded cDNA was reverse transcribed from random-hexamer-primed RNA using the SuperScript III kit (Invitrogen). Quantitative PCR (Q-PCR) was performed using the following Ryk primer pair: forward, 5′-cgctctgtcctttaacctgc-3′; reverse, 5′-ccagttcaatccttttcatgc-3′. 18S rRNA primers were as follows: forward, 5′-ccatccaatcggtagtagcg-3′; reverse, 5′-tggccgttcttagttggtgg-3′. Q-PCR was performed in triplicate on a Rotogene 2000 Quantitative PCR machine (Corbett, Townsville, Queensland, Australia) using the SYBR Green PCR mix (Invitrogen). The relative levels of *Ryk* mRNA were standardized to 18S rRNA controls for each time point and then expressed as the ratio of the level of *Ryk* mRNA at E16–E17 or E18–E17.

Cortical explants assays. Explant assays were performed as described previously (Shu et al., 2003). Briefly, COS cells were transfected with cDNAs encoding *Wnt5a*– internal ribosomal entry site (*IRES*)–green fluorescent protein (*GFP*) in the pIRES–hrGFP–1α expression vector (Stratagene, La Jolla, CA) or pEF–Myc–cyto–GFP expression vector (Invitrogen). The Wnt5a plasmid was a generous gift from Dr. Jinling Wu (University of Pennsylvania, Philadelphia, PA) (Yang et al., 2003). Transfection efficiency was monitored by the level of GFP protein expressed 48 h after transfection. Forty-eight hours after transfection, cells were mixed with 80 μl of 2% low-melting-point agarose (Sigma) in DMEM/F-12 (Invitrogen) and cultured overnight. Explants from embryonic cortex (300 μm^3) were placed in 30 μl of collagen matrix (Chemicon) adjacent to agarose blocks (200 μm^3) containing COS cells expressing either Wnt5a–GFP or GFP alone. After the collagen had set, explants were cultured (DMEM/F-12, 0.1% penicillin–streptomycin, 3.6% D-glucose, 2 mM glutamine, and 15% FCS) for 72 h and then fixed with 4% PFA. Axons were visualized by immunostaining with mouse anti-βIII tubulin (1:2000; Promega, Madison, WI), followed by a goat anti-mouse IgG conjugated to AlexaFluor 596 (1:1000; Invitrogen). *Z*-stack

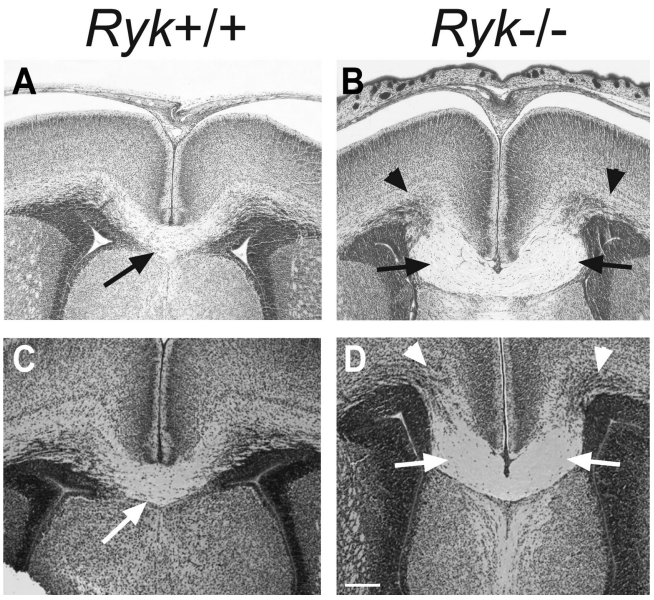

Figure 1. The corpus callosum in *Ryk*$^{-/-}$ embryos form axon bundles on the contralateral side of the midline. Coronal sections of E18 forebrain from wild-type (***A***, ***C***) and *Ryk*$^{-/-}$ (***B***, ***D***) embryos stained for Nissl substance (***A***, ***B***) or with hematoxylin and eosin (***C***, ***D***). In wild-type cortex, callosal axons cross the midline in a tightly fasciculated tract (***A***, ***C***, arrows). In *Ryk*$^{-/-}$ cortex (***B***, ***D***), callosal axons cross the midline and form axon bundles (arrows) on the contralateral side of the midline. The intermediate zone in the *Ryk*$^{-/-}$ cortex (arrowheads) contains fewer axons than that in wild-type brain. Scale bar, 200 μm.

images of the explants were captured on an Olympus Optical IX50 microscope using AnalySIS software. The images were then reconstructed with the AnalySIS deconvolution module. The number of axons entering the agarose block was counted and expressed as the percentage of total axons projecting from the surface of the explant facing the block. Three to five experiments were performed for each embryonic age in Figure 8*A*. For Figure 8*B*, four independent experiments were performed. Statistical analysis was performed using the nonparametric Mann–Whitney two-tailed test.

Results

Ryk$^{-/-}$ mice have an abnormal corpus callosum

The first report on the phenotypic consequences of deleting the mouse *Ryk* gene described craniofacial abnormalities (including cleft palate) and a shortening of the forelimbs and hindlimbs (Halford et al., 2000). Because loss-of-function mutations in the *Drosophila* ortholog of *Ryk* (*derailed*) resulted in guidance defects in axons crossing the anterior commissure of the developing ventral nerve cord (Callahan et al., 1995), we predicted that loss of Ryk protein within the developing mouse nervous system would also result in commissural axon guidance defects.

We initially analyzed serial paraffin-embedded coronal sections of wild-type and *Ryk*$^{-/-}$ E18 brains stained for Nissl substance or with hematoxylin and eosin. The gross architecture of the *Ryk*$^{-/-}$ brains appeared normal, and craniofacial defects were noted as described previously (Halford et al., 2000). The size of the ventricles and the thickness of the neocortex in the *Ryk*$^{-/-}$ brains were equivalent to those of wild-type littermates, indicating that neurogenesis, gliogenesis, and neural migration were not overtly disrupted in the mutant mice (data not shown). Although the overall cytoarchitecture of the *Ryk*$^{-/-}$ brain was normal, we observed a significant abnormality in the major midline commissure of the mammalian forebrain, the corpus callosum. Figure 1 shows that, although a thick axon tract crossed the midline, large bundles of axons have accumulated on either side of the midline

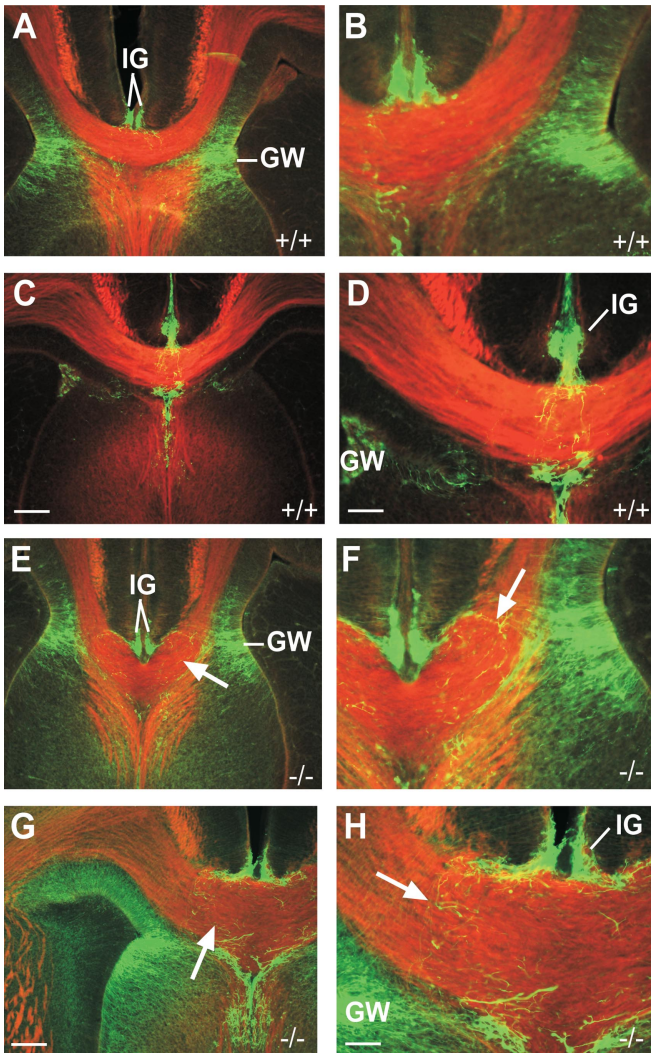

Figure 2. The glial wedge and indusium griseum are not affected by loss of Ryk activity. Coimmunostaining of coronal sections of wild-type (***A–D***; 2 individual E18 embryos) and *Ryk*$^{-/-}$ (***E–H***; 2 individual E18 embryos) forebrains with antibodies specific for GFAP (green) and L1 CAM (red). Both the GW and IG are formed normally in *Ryk*$^{-/-}$ embryos. L1 CAM-expressing contralateral axon bundles can been seen in *Ryk*$^{-/-}$ forebrains (***E–H***; arrows). ***B***, ***D***, ***F***, and ***H*** are higher-magnification images of ***A***, ***C***, ***E***, and ***G***, respectively. Scale bars: ***A***, ***C***, ***E***, ***G***, 200 μm; ***B***, ***D***, ***F***, ***H***, 100 μm.

within the corpus callosum of *Ryk*$^{-/-}$ embryos (Fig. 1*B*,*D*, arrows). We also noted that the width of the corpus callosum at the midline of the *Ryk*$^{-/-}$ forebrain was significantly greater than in wild-type littermates (Fig. 1*A*,*C*, arrows). These observations suggest that, in the absence of Ryk, callosal axons cross but are unable to project away from the midline once on the contralateral side. In support of this hypothesis, the density of axons within the intermediate zone, directly adjacent to the bundles, was notably decreased (Fig. 1*B*,*D*, arrowheads), indicating that fewer axons had traversed this region. We observed this phenotype in 25% of *Ryk*$^{-/-}$ embryos. The contralateral axon bundles were never observed in wild-type (Fig. 1*A*,*C*) or *Ryk*$^{+/-}$ heterozygous animals. A complete survey throughout the *Ryk*$^{-/-}$ mouse brain revealed no obvious perturbations in other midline commissures.

To visualize the callosal axons in the *Ryk*$^{-/-}$ forebrain more clearly, 50 μm vibratome sections of fixed E18 brains were stained with an antibody specific for the axonal cell adhesion molecule, L1 CAM (Fig. 2, red), present on callosal axons as they

project across the midline. The L1 CAM staining confirmed that, although callosal axons in $Ryk^{-/-}$ embryos were able to cross the midline successfully, they formed axon bundles on either side of the midline (Fig. 2*E–H*, arrows). In contrast, wild-type axons crossed the midline in a tightly fasciculated tract and projected away from the contralateral side into the intermediate zone (Fig. 2*A–D*).

Two populations of midline glia, the indusium griseum (IG) and the glial wedge (GW), are crucial for the accurate guidance of callosal axons across the midline. These glial populations have been shown to express the soluble guidance factor Slit2, which directs both pre-crossing callosal axons across the midline and post-crossing axons away from the midline (Shu and Richards, 2001; Shu et al., 2003). To determine whether the IG and GW were affected by the loss of Ryk, we coimmunostained with an antibody specific for the glial marker GFAP (Fig. 2, green). When compared with wild-type littermates (Fig. 2*A–D*), the IG was positioned correctly in the $Ryk^{-/-}$ forebrain, in which it was located dorsal to the corpus callosum and on either side of the midline (Fig. 2*E–H*). Similarly, no abnormalities were observed in the $Ryk^{-/-}$ GW. The GW could be seen spanning the region on either side of the midline adjacent to the lateral ventricle in both wild-type (Fig. 2*A–D*) and mutant (Fig. 2*E–H*) brains. Therefore, the aberrant axonal projection observed in the $Ryk^{-/-}$ corpus callosum cannot be attributed to the loss or abnormal morphology of the IG or GW.

Callosal axons fail to escape from the contralateral side of the midline in $Ryk^{-/-}$ forebrains

To clearly identify the source of the axons contributing to the axon bundles in the $Ryk^{-/-}$ forebrain, we injected DiI into one cortical hemisphere and observed the trajectory of labeled axons as they crossed the midline of the corpus callosum in the E18 brain using confocal microscopy. Figure 3 shows axons within the corpus callosum of $Ryk^{+/-}$ (Fig. 3*A*) and $Ryk^{-/-}$ brains (Fig. 3*B*) as they cross the midline (indicated by the dashed white line). Axons in heterozygote embryos approached the midline in a tightly fasciculated tract, turned appropriately into the corpus callosum, crossed the midline, and then projected away from the midline into the contralateral hemisphere. Although $Ryk^{-/-}$ callosal axons also successfully crossed the midline, they approached and crossed the midline in a loosely fasciculated manner when compared with axons in heterozygote littermates. Furthermore, in contrast to $Ryk^{+/-}$ callosal axons, $Ryk^{-/-}$ axons failed to project away from the midline once on the contralateral side and could be seen making abrupt turns back toward the midline (Fig. 3*B*, arrows). The axon tract projecting ventrally at the rostrocaudal position seen in Figure 3*B* represents the ipsilateral perforating pathway connecting the cingulate cortex with the medial septum and the diagonal band of Broca complex (Shu et al., 2001). This pathway did not appear to be affected by loss of Ryk. Figure 3*C* shows a higher-magnification image of $Ryk^{+/-}$ callosal axons as they project away from the midline. The axons remained within a tightly fasciculated tract as they projected toward the contralateral hemisphere. In contrast, $Ryk^{-/-}$ axons (Fig. 3*D–F*, three individual $Ryk^{-/-}$ embryos) were clearly defasciculated at both the midline and on the contralateral side. Moreover, individual axons followed aberrant paths on the contralateral side, either turning within the tract (Fig. 3*D–F*, arrows) or projecting ventrally, sometimes at right angles, away from the appropriate callosal trajectory (Fig. 3*D*,*F*, arrowheads). Ventrally projecting axons could be seen aberrantly projecting toward the septum. Interestingly, growth cones could be seen on some of these misguided axons (Fig. 3*E*, asterisk), suggesting that a chemoattractive cue was now directing these axons ventrally.

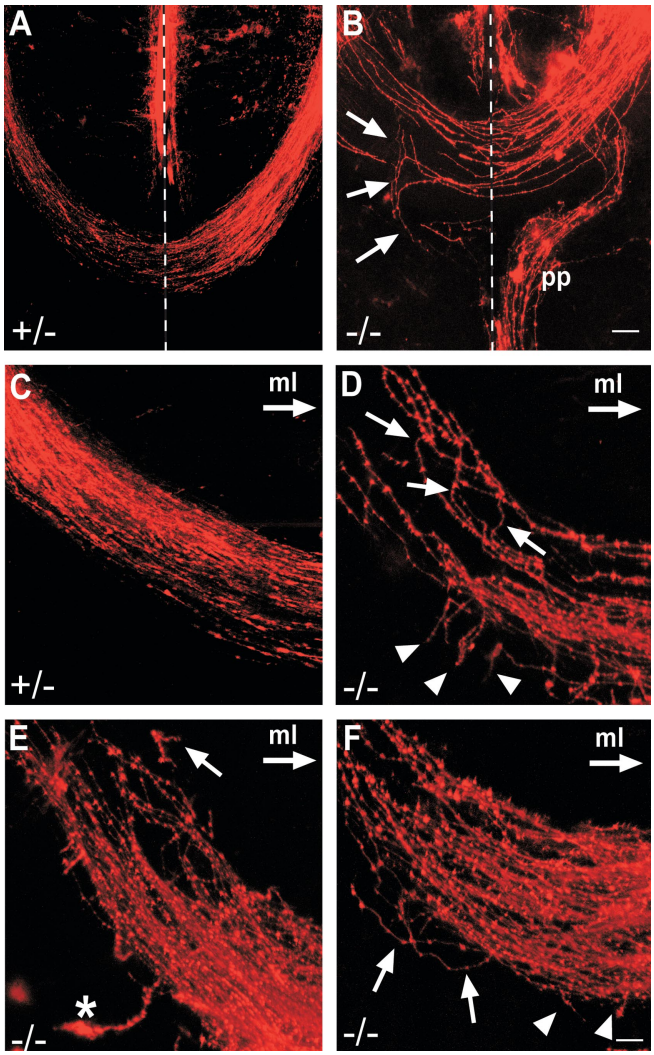

Figure 3. DiI tracing demonstrates that $Ryk^{-/-}$ callosal axons follow aberrant trajectories after crossing the midline. ***A***, ***C***, $Ryk^{+/-}$ axons cross the midline and project to the contralateral cortex in a tightly fasciculated tract ($n = 21$). ***B***, ***D–F***, $Ryk^{-/-}$ axons cross the midline in a defasciculated manner and project back toward the midline (arrows) or toward the septum (arrowheads) once on the contralateral side This phenotype was seen in 5 of 22 (23%) $Ryk^{-/-}$ forebrains. ***C*** is a higher-magnification image of ***A***. ***B*** and ***D–F*** are four individual $Ryk^{-/-}$ embryos. The dotted lines in ***A*** and ***B*** indicate the midline. ml⟶ indicates that the midline is to the right of the image. The asterisk indicates a growth cone migrating toward the septum. pp, Perforating pathway. Scale bars: ***A***, ***B***, 200 μm; ***C–F***, 100 μm.

In summary, we have shown that the Ryk receptor is required for two distinct aspects of callosal axon guidance at the midline. Initially, Ryk is required for the tight fasciculation of callosal axons as they approach the midline on the ipsilateral side and as they subsequently cross the midline. The thickening of the corpus callosum at the midline of the $Ryk^{-/-}$ forebrain observed in Figures 1 and 2 is explained by the defasciculation of these axons. We also demonstrated that the bundles of callosal axons situated on either side of the $Ryk^{-/-}$ midline arise as a result of aberrant axonal trajectories on the contralateral side of the midline. Therefore, these studies have revealed a role for Ryk in driving axonal projections away from the midline and into the contralateral hemisphere.

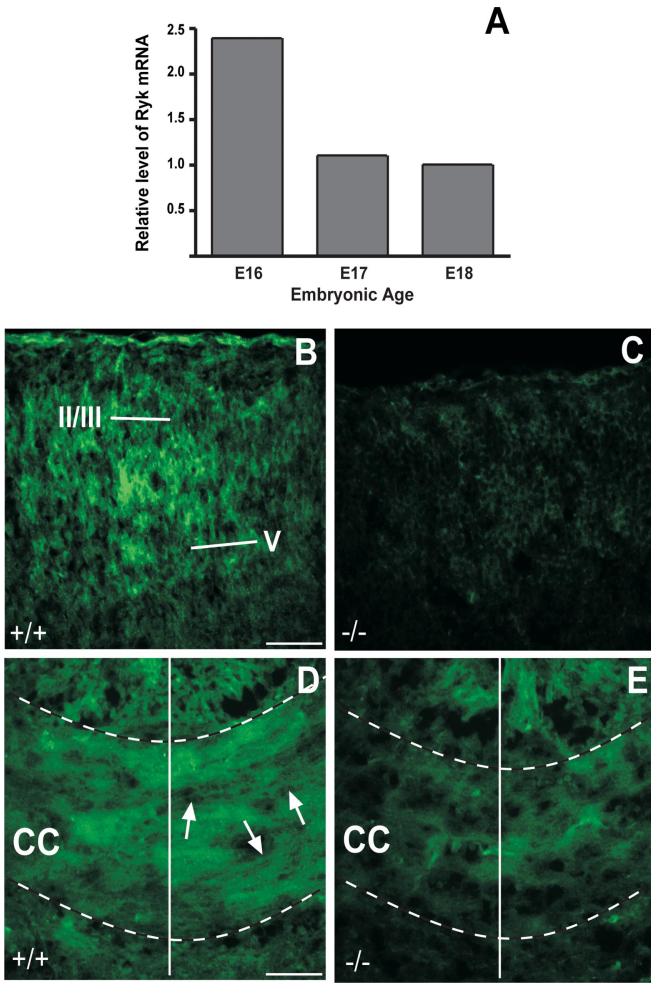

Figure 4. Ryk is present on callosal axons as they cross the midline and project into the contralateral hemisphere. ***A***, Q-PCR shows that *Ryk* mRNA is present in the embryonic cortex between E16 and E18. ***B***, Immunohistochemistry using a rabbit anti-Ryk antiserum demonstrates that Ryk protein is present at low levels in all layers of the E18 $Ryk^{+/+}$ cortex. ***D***, Ryk is also localized to callosal axons as they cross the midline and enter the contralateral hemisphere. Ryk protein can be seen on single axons and small fascicles (arrows). ***C***, ***E***, Specific immunostaining was not observed on cortical neurons or callosal axons in E18 $Ryk^{-/-}$ embryos. The solid lines indicate the midline, and dotted lines outline the corpus callosum. CC, Corpus callosum; II/III, cortical layers II/III; V, cortical layer V. Scale bars, 50 μm.

Ryk is present on callosal axons as they cross the midline and enter the contralateral hemisphere

Axons from pyramidal neurons in layers 2/3 and 5 of the mouse embryonic cortex first approach the midline between E15 and E16. By E17, they have crossed the midline, projecting into the contralateral hemisphere at approximately E18 (Wahlsten, 1981; Shu and Richards, 2001; Richards et al., 2004). The temporal expression of *Ryk* mRNA was analyzed by Q-PCR on RNA samples derived from cortical tissue containing all layers of the E16–E18 embryonic cortex (Fig. 4*A*). The levels of *Ryk* mRNA detected in each sample were normalized against 18S RNA levels. The expression of *Ryk* mRNA was highest at E16 when callosal axons are beginning to cross the midline. At E17–E18, when the majority of axons are projecting away from the midline, *Ryk* mRNA was still expressed, albeit at a lower level. Immunohistochemistry using a rabbit polyclonal anti-Ryk antiserum (Liu et al., 2005) demonstrated that Ryk is present on the cell bodies of neurons in all $Ryk^{+/+}$ cortical layers (Fig. 4*B*) at E18. In addition, Ryk protein was present at low levels on axons traversing the $Ryk^{+/+}$ corpus callosum at this age (Fig. 4*D*). Arrows in Figure 4*D* indicate single axons, or small fascicles that are Ryk positive. Although some nonspecific staining could be seen in the $Ryk^{-/-}$ corpus callosum, no Ryk-positive callosal axons were observed in these embryos (Fig. 4*E*). Equivalent Ryk immunostaining was observed using a second commercial rabbit anti-Ryk antiserum (data not shown). Therefore, Ryk is present on callosal axons when they are actively crossing the midline and projecting into the contralateral hemisphere. Recently, Liu et al. (2005) have shown by *in situ* hybridization that *Ryk* mRNA is present in layer 2/3 cortical neurons and at lower levels in layers 5 and 6 of the E18 cortex.

Wnt5a is a ligand for Ryk

The chemorepulsive ligand responsible for the Derailed-dependent guidance of axons across the anterior commissure within the *Drosophila* nerve cord has been identified as Wnt5 (Yoshikawa et al., 2003). Therefore, because Ryk is required for correct axon guidance away from the midline of the corpus callosum in the mouse embryonic forebrain, we postulated that Wnt5a (the mouse ortholog of *Drosophila* Wnt5) is likely to be the ligand recognized by Ryk in this context. To determine whether *Wnt5a* is expressed in, or around, the midline of the corpus callosum when axons are traversing this point, we performed *in situ* hybridization using a *Wnt5a* riboprobe (Fig. 5). Before callosal axons reaching the midline at E16, *Wnt5a* is expressed in the subventricular zone adjacent to the lateral ventricles and within the early GW and IG (Fig. 5*A*). At E17 and E18, when callosal axons have crossed the midline and are projecting toward the contralateral cortex, *Wnt5a* mRNA was strongly expressed in the GW on either side of the midline and in cells surrounding the IG and to a lesser extent within the IG itself (Fig. 5*B*,*C*,*E*). Moreover, *Wnt5a* was expressed in a medial-high, lateral-low gradient in the E17 subventricular zone directly ventral to the intermediate zone of the cortex (Fig. 5*B*, arrowheads). Both the IG and GW are required for correct guidance of callosal axons across the midline (Shu and Richards, 2001; Shu et al., 2003). Therefore, the spatial and temporal expression pattern of *Wnt5a* at E17 and E18 indicates that it is likely to influence the guidance of callosal axons on the contralateral side of the midline. Furthermore, the mediolateral expression gradient suggests that Wnt5a may also steer post-crossing axons through the intermediate zone on reaching the contralateral hemisphere. No significant expression of the closely related *Wntb* gene was observed in this region of the forebrain at E17 and E18 (data not shown).

To test whether Ryk could specifically bind Wnt5a, we performed coimmunoprecipitation experiments on lysates from HEK293T cells cotransfected with cDNAs encoding Myc-tagged Wnt5a (Wnt5a–Myc) and the Flag-tagged Ryk extracellular domain (RykECD) or the Flag-tagged Ryk extracellular domain in which the WIF domain was deleted (RykDWD) (Fig. 6). Immunoprecipitation of Wnt5a–Myc with an anti-Myc antibody and Western blotting with an antibody directed to the Flag tag revealed that RykECD but not RykDWD could interact with Wnt5a–Myc (Fig. 6*A*). Figure 6*B* demonstrates that an equivalent concentration of Wnt5a was present in each lysate. In addition, significantly more RykDWD than RykECD protein was produced by the HEK293T cells (Fig. 6*C*). Therefore, Wnt5a is a ligand for Ryk and binds in an WIF domain-dependent manner to the extracellular domain of Ryk.

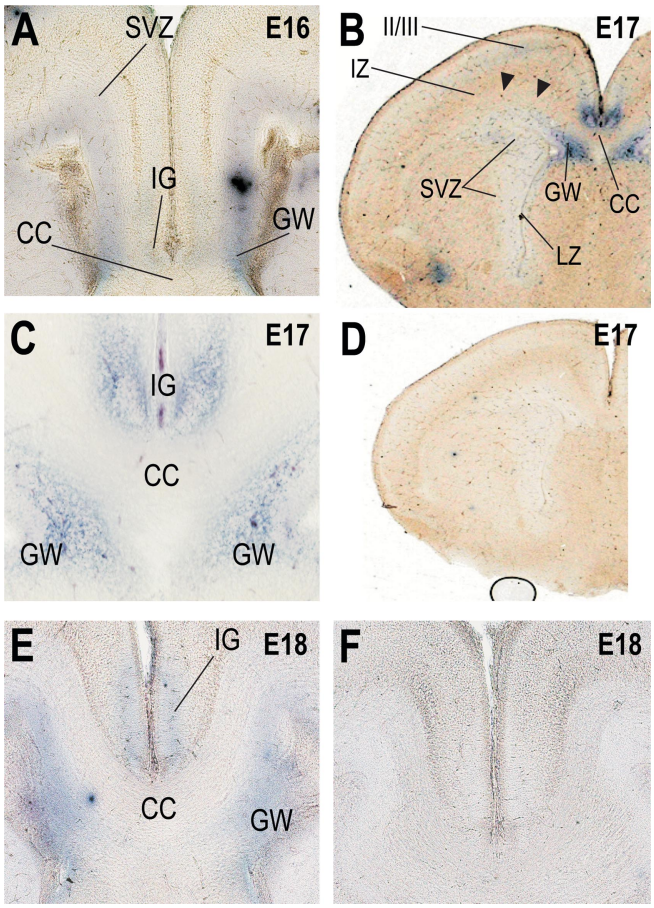

Figure 5. *In situ* hybridization showing that the *Wnt5a* expression domain surrounds the corpus callosum. At E16 (***A***), E17 (***B***, ***C***), and E18 (***E***) *Wnt5a* is expressed in the GW and IG surrounding the midline of the corpus callosum (CC). ***B***, At E17, *Wnt5a* mRNA is also expressed at lower levels in the subventricular zone (SVZ) surrounding the lateral ventricles (LZ) and can be seen in a medial-high, lateral-low gradient (arrowheads) adjacent to the intermediate zone (IZ) of the embryonic cortex. Expression can also be seen in layers 2 and 3 (II/III) of the cortex. ***D***, ***F***, No specific hybridization signal was observed with the *Wnt5a* sense riboprobe.

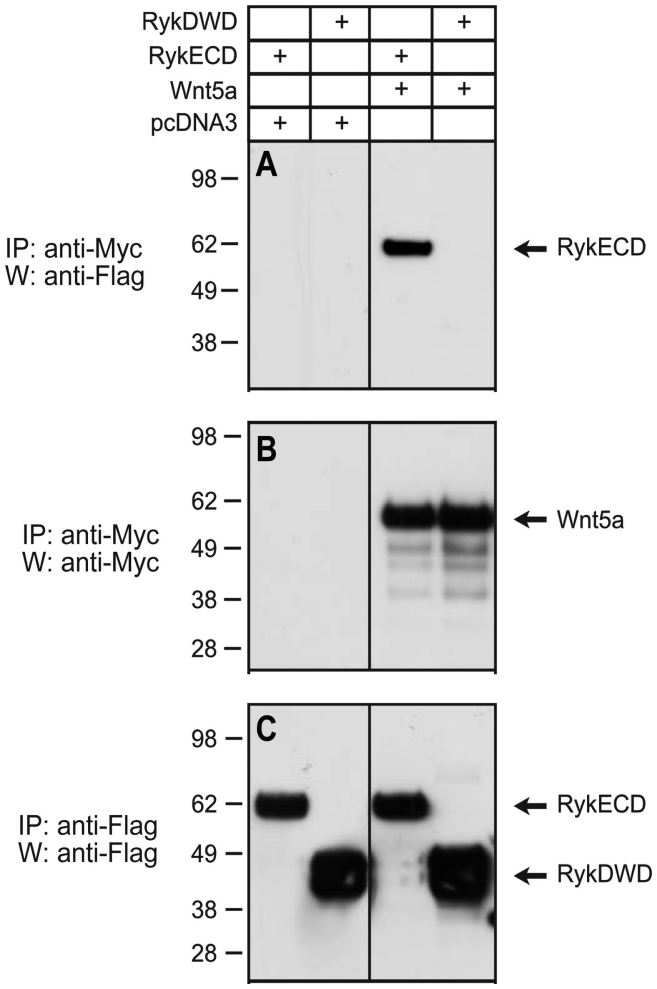

Figure 6. Wnt5a interacts with Ryk through the WIF domain. cDNAs encoding Wnt5a (Myc-tagged) and the complete extracellular domain of Ryk (RykECD) or the Ryk extracellular domain lacking the WIF domain (RykDWD) (both tagged with the Flag epitope) were cotransfected into HEK293T cells. The empty pcDNA3 vector was used as the control for Wnt5a. ***A***, RykECD but not RykDWD was detected by Western blotting (W) with anti-Flag after coimmunoprecipitating (IP) with Wnt5a. ***B***, Equivalent levels of Wnt5a were detected in both lysates by immunoprecipitation and Western blotting with anti-Myc. ***C***, High levels of RykECD and RykDWD were detected in all lysates by immunoprecipitation and Western blotting with anti-Flag.

Wnt5a is a chemorepulsive guidance cue for Ryk on cortical axons

To determine whether Wnt5a is the ligand recognized by Ryk on cortical axons, we performed *in vitro* experiments in which explants of embryonic cortex were apposed to blocks of agarose containing COS cells producing Wnt5a or control cells producing GFP. Explants were allowed to grow for 72 h before being fixed in 4% PFA and immunostained with an antibody to βIII-tubulin to visualize the projecting axons. Figure 7*A* shows axons projecting from an E18 wild-type explant (placed on the right) into an agarose block (outlined by the dotted line) containing GFP-expressing cells. In contrast, very few E18 cortical axons entered the agarose block containing Wnt5a-producing cells (Fig. 7*B*). Therefore, the presence of Wnt5a inhibited the extension of E18 cortical axons into the agarose block. It should be noted that the cortical explants contained neurons from all layers of the cortex. Because cortical neurons in layers 5 and 6 project through the internal capsule, we would not expect 100% of axons growing out of these explants to respond to Wnt5a. Recently, Liu et al. (2005) performed similar explant assays demonstrating that, although cortical explants from postnatal day 0 pups were repulsed by *Wnt5a*-expressing cells, no repulsive response was observed for E18.5 cortical explants. It should be noted that these experiments were performed selectively on frontal motor cortex, whereas our data were generated from explants taken from all regions of the E18 cortex.

We have shown that the loss of Ryk activity results in the inability of callosal axons to project away from the midline on reaching the contralateral side after E17. However, $Ryk^{-/-}$ axons do approach and cross the midline, implying that these axons are not responsive to the Ryk ligand until after crossing the midline at E17. Therefore, we also tested the ability of cortical axons from E16 and E17 explants to respond to Wnt5a. The number of axons that grew into the GFP or Wnt5a blocks was quantitated and expressed as a percentage of the total number of axons projecting from the surface of the explant facing the block (Fig. 8*A*). These experiments were repeated a minimum of three times, and the statistical significance was determined using the nonparametric Mann–Whitney two-tailed test. The total number of explants assayed in each condition is shown on the top of each column in the histogram. Figure 8*A* demonstrates that axons projecting from the E16 and E17 explants entered the Wnt5a block as frequently as they entered the control GFP block, indicating that

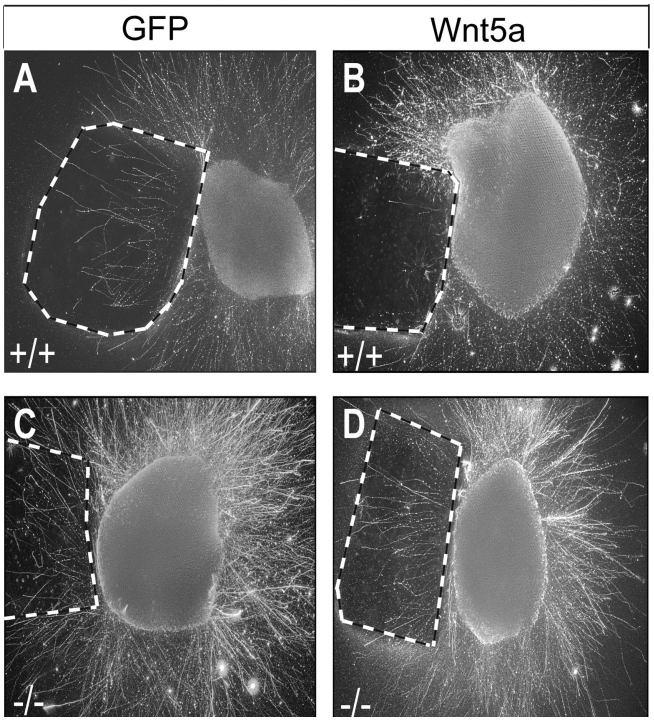

Figure 7. Wnt5a inhibits the extension of E18 cortical axons. Cocultures of E18 cortical explants with agarose blocks containing *GFP*-expressing cells (***A***, ***C***) or *Wnt5a*-expressing cells (***B***, ***D***). $Ryk^{+/+}$ axons grow into the agarose block containing *GFP*-expressing cells (***A***) but do not enter the block in the presence of Wnt5a (***B***). $Ryk^{-/-}$ axons grow into the GFP agarose block (***C***). In contrast to wild-type axons, $Ryk^{-/-}$ axons also grow vigorously into the agarose block containing Wnt5a (***D***).

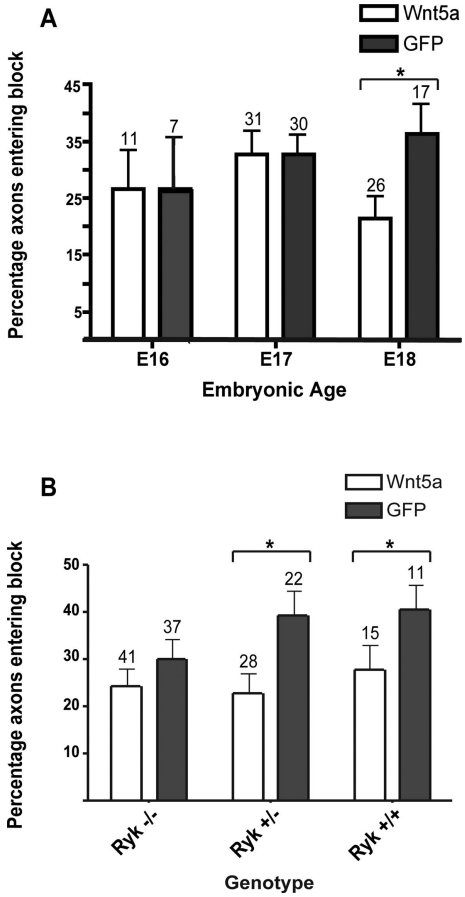

Figure 8. ***A***, E18 but not E16 or E17 cortical axons are responsive to Wnt5a. E16–E18 cortical explants were apposed to agarose blocks containing COS cells expressing *Wnt5a* or *GFP*. The number of E16 or E17 axons growing into the Wnt5a block was not significantly different from that entering the GFP block ($p = 1.00$, $p = 0.79$, respectively). In contrast, the number of E18 axons entering the Wnt5a block was significantly reduced compared with the number entering the GFP block ($p = 0.015$). ***B***, E18 cortical axons respond to Wnt5a in a Ryk-dependent manner. E18 cortical explants from $Ryk^{+/+}$, $Ryk^{+/-}$, or $Ryk^{-/-}$ embryos were apposed to agarose blocks containing COS cells expressing *Wnt5a* or *GFP*. Although the number of $Ryk^{+/+}$ or $Ryk^{+/-}$ axons entering the Wnt5a block was significantly reduced ($p = 0.05$, $p = 0.03$, respectively) when compared with the number entering the GFP block, there was no significant difference in the number of $Ryk^{-/-}$ axons growing into the Wnt5a or GFP blocks ($p = 0.32$). $^*p \leq 0.05$ indicates statistical significance. The total number of explants assayed in each condition is shown on the top of each column.

they are not responsive to Wnt5a. In contrast, we observed that significantly fewer axons from E18 cortical explants entered the Wnt5a block when compared with the number growing into the GFP block. These experiments demonstrate that the ability of cortical axons to respond to Wnt5a is temporally regulated. Cortical axons from E16 and E17 explants are unresponsive to Wnt5a but become responsive at E18. In wild-type and $Ryk^{-/-}$ embryos, axons are approaching and crossing the midline at E16–E17. After E17, callosal axons are projecting away from the midline. We demonstrated here that E18 cortical axons cannot grow into domains of *Wnt5a* expression. Because the contralateral axon bundles form in the $Ryk^{-/-}$ forebrain at approximately E18, it is likely that Wnt5a is the ligand for Ryk on the contralateral side of the midline and that the Ryk–Wnt5a interaction promotes chemorepulsion away from the midline. Wnt1 and Wnt3 have also been shown to be chemorepulsive ligands for Ryk (Liu et al., 2005; Schmitt et al., 2006). We are now investigating whether these or other Wnts are present at the callosal midline and are ligands for Ryk.

To further demonstrate that Wnt5a is a repulsive guidance cue for the Ryk receptor on cortical axons, we performed assays using explants from wild-type, heterozygous, and $Ryk^{-/-}$ E18 cortex. Figure 7*D* shows that, in contrast to wild-type E18 cortical axons (Fig. 7*B*), axons from E18 $Ryk^{-/-}$ explants grew vigorously into the agarose block containing Wnt5a (Fig. 7*D*). Quantification of axon growth revealed that there was no significant difference between the number of $Ryk^{-/-}$ axons entering the Wnt5a and GFP blocks (Fig. 8*B*). As expected, the number of axons from the $Ryk^{+/-}$ and $Ryk^{+/+}$ explants entering the Wnt5a block was significantly reduced when compared with that entering the GFP block. Therefore, these experiments demonstrate that, in the absence of the Ryk receptor, cortical axons are significantly less responsive to Wnt5a, thereby confirming that Wnt5a is a repulsive ligand for Ryk in the context of callosal axon guidance.

Discussion

The corpus callosum is the major interhemispheric commissure in the human brain, comprising ~3 million myelinated fibers that connect homologous regions in the neocortex. To date, >50 different human congenital syndromes have been described (for review, see Richards et al., 2004) in which dysgenesis or partial agenesis of the corpus callosum occurs. In these syndromes, callosal axons approach but are unable to cross the midline at the corticoseptal boundary and instead form disorganized bundles of axons (Probst bundles) on the ipsilateral side. Failure of callosal midline crossing and Probst bundle formation have also been seen in mice lacking a variety of axon guidance receptors, including the netrin receptor DCC (deleted in colorectal cancer), the

repulsive guidance receptors EphB2 and EphB3, and the semaphorin receptor neuropilin-1 (for review, see Richards et al., 2004). Thus, a variety of molecular navigational systems govern the axon pathfinding of callosal axons as they approach and cross the midline. In contrast, loss of Ryk leads to the formation of contralateral axon bundles, a novel callosal guidance phenotype not observed previously. Therefore, this study is the first to identify a guidance receptor that acts on the contralateral side of the midline to promote callosal axon escape from the midline into the contralateral hemisphere.

In this study, we have shown that loss of Ryk does not interfere with the ability of callosal axons to cross the midline but impedes their escape from the contralateral side, suggesting that Ryk is detecting a repulsive guidance cue within the contralateral environment. *In situ* hybridization studies revealed that *Wnt5a* is expressed in the IG and GW surrounding the callosal trajectory at the midline. Thus, Wnt5a is perfectly positioned to influence callosal axon guidance. In support of this hypothesis, we demonstrated that Wnt5a binds the WIF module within the extracellular domain of Ryk. Our *in vitro* explant experiments demonstrated that E16 and E17 cortical axons do not respond to Wnt5a, whereas a subpopulation of axons are repulsed by Wnt5a only at E18, a time coincident with the embryonic age after which callosal axons have crossed the midline and are actively projecting into the contralateral hemisphere. We also demonstrated that $Ryk^{-/-}$ E18 cortical axons are no longer sensitive to Wnt5a, confirming that Wnt5a is a repulsive guidance ligand for Ryk on E18 cortical axons.

The IG and GW are essential for the guidance of callosal axons through the midline (Shu and Richards, 2001). Slit2 has been identified as one of the chemorepulsive guidance cues produced by the IG and GW (Shu et al., 2003). Slit2–Robo repulsion is required to channel ipsilateral axons into the midline in a tightly fasciculated tract, preventing them from projecting ventrally into the septum while crossing the midline (Shu et al., 2003). In addition, Slit2 has been shown to repel callosal axons away from the midline after crossing (Shu et al., 2003). That Slits are required for midline crossing was confirmed in compound mouse mutants lacking both Slit1 and Slit2 in which callosal axons are unable to project across the midline, resulting in the formation of Probst bundles (Bagri et al., 2002). Here we identify a second repulsive guidance system that must work in concert with the Slit–Robo system to ensure the correct formation of the corpus callosum. Like Slit2, both the IG and GW also express Wnt5a. However, in contrast to the Slit–Robo system, callosal axons are only sensitive to Wnt5a repulsion once on the contralateral side. The formation of the contralateral axon bundles in $Ryk^{-/-}$ embryos strongly agues that Ryk activity is required for the projection of axons into the contralateral hemisphere. Furthermore, *Wnt5a* is expressed in a decreasing mediolateral gradient directly ventral to the corpus callosum and the medial half of the intermediate zone. Thus, Wnt5a-mediated repulsion is likely to provide a long-distance guidance cue, steering axons through the intermediate zone within the contralateral hemisphere. In contrast, Slit2 expression is restricted to the IG and GW, ensuring that Slit2–Robo repulsion acts only over a short distance to confine axons within the corpus callosum and to push axons away from the midline. That Wnt–Ryk signaling is used for the guidance of long-distance axonal trajectories has also been observed in the corticospinal tract. Liu et al. (2005) demonstrated that Wnt–Ryk chemorepulsive signaling is required for the anterior-to-posterior projection of corticospinal axons down the dorsal funiculus of the developing mouse spinal cord. In this context, both the Ryk ligands Wnt1 and Wnt5a are expressed in an anterior-high, posterior-low gradient surrounding the axon tract.

That the Wnt5a–Ryk guidance system works in concert with other guidance systems at the callosal midline is further suggested by our observation that the severe contralateral axon bundle phenotype occurs in only 25% of $Ryk^{-/-}$ forebrains. In accordance with this observation, we found that explants from some $Ryk^{-/-}$ embryos were able to respond to Wnt5a in a manner indistinguishable from that seen for wild-type and heterozygous axons. Thus, it is likely that there is one or more modifier genes in the 129/Sv × C57BL/6j genetic background affecting the penetrance of the callosal $Ryk^{-/-}$ phenotype. Intriguingly, the *in vitro* phenotype (i.e., the lack of response to Wnt5a by $Ryk^{-/-}$ explants) (Fig. 8*B*) is significantly more robust than the *in vivo* phenotype. This is possibly because the Ryk–Wnt5a guidance system is working in parallel with other axon guidance systems (e.g., DCC/netrin and/or Slit/Robo) *in vivo* to guide callosal axons across the midline and into the contralateral cortex. We would hypothesize that, in the 25% of $Ryk^{-/-}$ embryos exhibiting the phenotype, Ryk–Wnt5a is the predominant guidance system. In contrast, in the 75% of $Ryk^{-/-}$ embryos that do not exhibit the phenotype, the DCC/netrin and/or the Slit/Robo system may be more predominant, thereby rescuing the phenotype. This may be achieved by variation in receptor or ligand concentrations in individual embryos. In the *in vitro* assays, we are exposing the explants to high concentrations of Wnt5a (relative to the *in vivo* situation) in the absence of other guidance cues. Because other guidance receptors would not be activated under these conditions, we would predict that the Ryk response would predominate. We are currently investigating this hypothesis.

Despite the fact that *Wnt5a* expression is bilateral, wild-type callosal axons are not sensitive to the chemorepulsive activity of Wnt5a before crossing the midline. We demonstrated that axons projecting from E18 cortical explants are more sensitive to Wnt5a repulsion than either E16 or E17 axons. Lack of repulsive activity, however, cannot be attributed to the absence of Ryk on ipsilateral axons because the $Ryk^{-/-}$ callosal axons exhibit a defasciculation phenotype as they approach the midline, indicating that Ryk is present on the ipsilateral axons. In the neural tube, DCC promotes commissural axon migration toward the floor plate, the source of both netrin and Slit. While on the ipsilateral side, commissural axons do not express Robo. However after crossing the midline, Robo is upregulated, leading to a direct interaction between the cytoplasmic domains of DCC and Robo, thereby silencing DCC signal transduction (Stein and Tessier-Lavigne, 2001). This receptor crosstalk allows axons to escape the high netrin concentration and simultaneously become responsive to Slit. Thus, guidance receptor crosstalk provides a precise temporal and spatial mechanism that accurately controls growth cone responses to conflicting directional information. Halford et al. (2000) have shown that Ryk can be coimmunoprecipitated and tyrosine phosphorylated by EphB2 and EphB3, suggesting the Eph receptors may be able to modulate Ryk signaling on the ipsilateral side.

To navigate through Wnt5a expression domains, pre-crossing axons may also reduce the density of Ryk receptors on their plasma membrane. The absolute levels of Ryk may be modulated such that only a low concentration of Ryk protein is present on callosal axons at E15–E17. In support of this contention, the level of Derailed protein is tightly regulated on *Drosophila* commissural axons. Derailed is only present on axons as they cross the anterior commissure and is rapidly removed once the axons have

reached the contralateral side, allowing them to escape the domain of *Wnt5* expression (Callahan et al., 1995). Therefore, low abundance of Ryk on pre-crossing callosal axons may make these axons insensitive to the high levels of Wnt5a in the ipsilateral callosum, perhaps by allowing other guidance receptors to more effectively silence Ryk signaling. Once on the contralateral side, Ryk may be upregulated, thereby eliciting a chemorepulsive response to Wnt5a. Ryk has been shown to be a high-affinity receptor for Wnt5a (Liu et al., 2005). Therefore, formation of Ryk–Wnt5a complexes may overcome possible silencing mediated by other receptors. We observed growth cones projecting into the contralateral septum in $Ryk^{-/-}$ embryos, suggesting that a chemoattractive guidance receptor is present on post-crossing axons that is overridden when Ryk is present.

Frizzled3 is expressed by cortical neurons and is required for the outgrowth of callosal axons (Wang et al., 2002). Therefore, it is possible that Wnt–Frizzled interactions could also modulate the Wnt5a–Ryk signaling pathway. In the *Drosophila* nerve cord, Derailed/Ryk has been shown to signal independently of the Frizzled receptors and does not require Disheveled to promote the Wnt5-dependent chemorepulsive response (Yoshikawa et al., 2003). Because Disheveled is a key downstream component of both the canonical and noncanonical Wnt signaling pathways, it is unlikely that Ryk activates these signaling cascades. In contrast, a recent study has proposed that mammalian Ryk acts as a coreceptor with Frizzled8, to bind Wnt1 and Wnt3a, and that Disheveled can bind to the PDZ (postsynaptic density-95/Discs large/zona occludens 1)-binding motif in the Ryk cytoplasmic domain (Lu et al., 2004). However, the relevance of these interactions in terms of generating chemoattractive or chemorepulsive guidance responses was not addressed. During vulval morphogenesis in the *Caenorhabditis elegans* embryo, LIN-18/Ryk has also been shown to be a Wnt receptor that acts independently of the classical Wnt receptor LIN-17/Frizzled (Inoue et al., 2004). In this instance, each receptor recognizes a distinct subset of Wnt ligands that trigger parallel signaling pathways that intersect somewhere upstream of POP-1/TCF (T-cell factor), the downstream target of the canonical Wnt signaling pathway. Because Wnt signaling pathways are highly conserved across all vertebrates and invertebrates, it is likely that Frizzled and Ryk pathways also intersect in mammals, providing a precise temporal and spatial mechanism for accurate control of growth cone responses at a given choice point.

In summary, this study identifies Ryk as a key guidance receptor in the establishment of the corpus callosum. In humans, there have been >50 syndromes described in which the formation of the corpus callosum is perturbed. Our analysis of Ryk function further advances our understanding of the molecular mechanisms underlying the formation of this important commissure.

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