

Netrin-DCC Signaling Regulates Corpus Callosum Formation Through Attraction of Pioneering Axons and by Modulating Slit2-Mediated Repulsion

Thomas Fothergill^{1,5}, Amber-Lee S. Donahoo¹, Amelia Douglass¹, Oressia Zalucki¹, Jiajia Yuan¹, Tianzhi Shu⁴, Geoffrey J. Goodhill^{1,3} and Linda J. Richards^{1,2,6}

¹The University of Queensland, Queensland Brain Institute, Brisbane, Qld., Australia ²School of Biomedical Sciences, Brisbane, Qld. 4072, Australia ³School of Mathematics and Physics, Brisbane, Qld. 4072, Australia ⁴Department of Anatomy and Neurobiology, School of Medicine, The University of Maryland, Baltimore, MD 21250, USA and ⁵Current address: Department of Neuroscience, University of Wisconsin-Madison, Madison, WI 53706, USA

T.F. and A.-L.S.D. contributed equally to this work

Address correspondence to Linda J. Richards, The University of Queensland, Queensland Brain Institute, Building 79 St Lucia Campus, Brisbane, Qld. 4072, Australia. Email: richards@uq.edu.au.

⁶Some of this work was completed in the Department of Anatomy and Neurobiology, School of Medicine, The University of Maryland, Baltimore, MD, 21250, USA.

The left and right sides of the nervous system communicate via commissural axons that cross the midline during development using evolutionarily conserved molecules. These guidance cues have been particularly well studied in the mammalian spinal cord, but it remains unclear whether these guidance mechanisms for commissural axons are similar in the developing forebrain, in particular for the corpus callosum, the largest and most important commissure for cortical function. Here, we show that Netrin1 initially attracts callosal pioneering axons derived from the cingulate cortex, but surprisingly is not attractive for the neocortical callosal axons that make up the bulk of the projection. Instead, we show that Netrin-deleted in colorectal cancer signaling acts in a fundamentally different manner, to prevent the Slit2-mediated repulsion of precrossing axons thereby allowing them to approach and cross the midline. These results provide the first evidence for how callosal axons integrate multiple guidance cues to navigate the midline.

Keywords: axon guidance, commissure formation, corpus callosum, cortical development, DCC, neocortex, Robo1, silencing

Introduction

During nervous system development, navigating axons sense and respond to attractive and repulsive guidance cues presented by the surrounding environment. A fruitful model for investigating mechanisms of axon navigation has been the guidance of commissural axons toward and across the midline. In the developing spinal cord, it is well established that Netrin1, expressed by the floorplate, acts via its receptor deleted in colorectal cancer (DCC) to attract commissural axons to the midline, and that a gradient of Slit, acting via its receptor Robo, subsequently repels postcrossing axons away from this region (reviewed in Evans and Bashaw 2010). However, far less is understood about how axons are guided across the midline in forebrain axon tracts. Recent data have demonstrated that Slit-Robo and DCC-Netrin signaling systems interact in the brain to modulate the response of axons to their environment. In the mouse thalamocortical projection, Slit1 and Netrin1 are expressed in opposing gradients (Bielle et al. 2011). Alone, Netrin1 is not attractive for thalamocortical axons, but in the presence of a sufficient concentration of Slit1, Netrin1-mediated attraction is revealed (Bielle

et al. 2011). In the zebrafish forebrain, Robo2 responds to Slit while suppressing the attractive effects of Netrin1 (Zhang et al. 2012). These results suggest that the interaction between both Slit-Robo and DCC-Netrin signaling systems may be crucially important for axon tract formation in the brain. Here, we investigate this in the context of the corpus callosum, the largest commissural projection in placental mammals.

The principal function of the corpus callosum is the inter-hemispheric transfer of cerebral cortical information. Agenesis of the corpus callosum in humans is associated with a large number of different neurological syndromes with a diverse range of symptoms, including language dysfunction, abnormalities in social interaction, attention deficits, and poor personal insight (reviewed in Paul et al. 2007). Thus, understanding the developmental basis for how defects in the formation of this major commissure occur may provide significant benefit to patients by identifying the cause of these disorders, leading to better prognosis and management, as well as elucidating how the brain is wired during development.

At the cortical midline, developing callosal axons grow dorsal to the glial wedge, a bilaterally symmetrical glial structure that expresses the repulsive molecules Slit2, Draxin, and Wnt5a (Shu and Richards 2001; Bagri et al. 2002; Marillat et al. 2002; Shu, Sundaresan, et al. 2003; Keeble et al. 2006; Islam et al. 2009). In the lateral cortex, Sema3A acts to repel callosal axons toward the midline (Zhao et al. 2011); thus, most of the currently demonstrated guidance of callosal axons at the midline of forebrain is repulsive. To date, only Sema3C has been identified as an attractant for callosal axons (Niquille et al. 2009; Piper et al. 2009), although one obvious possibility is that, analogous to commissural axons in the spinal cord, callosal axons are attracted to the midline by Netrin1 acting via DCC. Support for this hypothesis comes from a number of lines of evidence. First, Netrin1 and DCC are both highly evolutionarily conserved (reviewed in Rajasekharan and Kennedy 2009) and usually mediate attraction. Second, Netrin1 is expressed at the cortical midline both dorsal and ventral to the corpus callosum (Serafini et al. 1996), and third, Netrin1 and DCC mutant mice both display agenesis of the corpus callosum (Serafini et al. 1996; Fazeli et al. 1997).

Despite the evidence suggesting that Netrin1 attracts commissural axons throughout the nervous system, this has

never been formally tested in the corpus callosum. Here, we examined the response of cortical axons at different stages of development in both rat and mouse tissue for ease of confirmation of previously published rat data and to facilitate the use of mouse knockout tissue for some of our experiments. The results demonstrated the surprising finding that Netrin1 function differs between callosal axons derived from the cingulate cortex versus the neocortex, and that these effects are temporally specific to stages when axons are growing across the midline. In contrast to our expectation, Netrin1 did not attract callosal axons from the neocortex, which make up the vast majority of the axons of the corpus callosum.

This result prompted us to test a novel hypothesis for Netrin1 function in neocortical callosal axon guidance: that Netrin1-DCC, acting through the direct interaction of DCC and Robo1 (Stein and Tessier-Lavigne 2001), modulates the repulsive effects of Slit-Robo signaling. Unlike in the developing spinal cord, where precrossing silencing of repulsion is mediated either by Robo3 (Sabatier et al. 2004) or through *Comm* regulation of Robo in *Drosophila* (Seeger et al. 1993; Keleman et al. 2002), we show that for callosal axons, this modulation is achieved through Netrin1-DCC attenuation of Slit-Robo repulsion. Together, these data provide critical insight into how the 2 cerebral hemispheres become connected during development, and highlight mechanisms via which multiple axon guidance cues can function together during nervous system formation in general.

Materials and Methods

Animals and Tissue Collection

Original breeding pairs of *Netrin1* and *DCC* heterozygous founders were obtained from Dr Marc Tessier-Lavigne (Genentech, Stanford, CA; Serafini et al. 1996; Fazeli et al. 1997). *Netrin1* and *DCC* mutant mice were received on a CD1 and 129sv/B6 APC^{min/+} background, respectively. Backcrossing onto the C57Bl/6 strain was performed for >10 generations to eliminate strain-dependent variations in the phenotype (Magara et al. 1999). *Netrin1* mutant mice are severe hypomorphs, and were generated in a gene trap screen where the *lacZ* gene was inserted into the *Netrin1* gene (Skarnes et al. 1995; Serafini et al. 1996). *DCC* knockout mice were generated by the targeted insertion of the neomycin resistance cassette into exon 3 by homologous recombination, resulting in a complete functional deletion (Fazeli et al. 1997). *DCC*^{Kanga} mice carry a spontaneous mutation and were maintained on a AKR^{C3H} background. *Robo1* knockout mice were obtained from Drs Vasi Sunderasan and Bill Andrews (Kings College, London). These mice were generated by the deletion of exon 5, which was floxed with a neomycin resistance gene, and are complete nulls for *Robo1* (Andrews et al. 2006). All experiments were approved by the University of Queensland Animal Ethics Committee and were performed according to the National Health and Medical Research Council's code of practice for animal experimentation.

Heterozygous *Netrin1*, *DCC*, *DCC*^{Kanga}, or *Robo1* mice were time-mated to obtain litters of wild-type, heterozygote, and knockout embryos. The morning of the vaginal plug was designated as embryonic day (E) 0. Pregnant dams were anesthetized with an intraperitoneal injection of 100 mg/kg sodium pentobarbitone (Virbac). Embryos were sequentially removed from the uterus and transcardially perfused with 0.9% saline solution containing 0.9% w/v NaCl in water, followed by 4% w/v paraformaldehyde (PFA; ProSciTech) in Dulbecco's phosphate-buffered solution (DPBS; Lonza) at pH 7.4. For immunohistochemistry using antibodies against neuropilin-1, 2.5% acrolein (Sigma-Aldrich) was added to the 4% PFA. The brains were then postfixed in 4% PFA at 4 °C for 24 h before being stored in DPBS at 4 °C.

Immunohistochemistry

Fixed brains were embedded in 3% w/v Difco™ Noble agar (Becton, Dickinson and Company) in water, and then sectioned coronally at 45 μm thickness using a vibratome (Leica). All incubations were performed at room temperature on a laboratory rotator (Heidolph, John Morris Scientific). Sections were washed in DPBS for 30 min and then incubated for 2 h in blocking solution containing 2% v/v normal goat serum (Vector Laboratories) or normal donkey serum (Jackson Laboratories), and 0.2% v/v Triton X-100 (Sigma-Aldrich) in DPBS. Sections were then incubated overnight with primary antibody diluted in blocking solution. Primary antibodies included: mouse antigrowth-associated protein (Gap)-43 (used at a concentration of 1:100 000 for nickel-diaminobenzidine (DAB) immunohistochemistry; Chemicon), rat anti-glial fibrillary acidic protein (used at a concentration of 1:30 000 for nickel-DAB immunohistochemistry; DAKO), rabbit polyclonal anti-DCC ((2744) 1:5000; a gift from Dr Helen Cooper, The University of Queensland, Australia), and rabbit anti-neuropilin-1 (1:50 000; a gift from Prof. David Ginty, Johns Hopkins University, USA). Sections were washed in DPBS for 1 h before incubating with secondary antibody (1 h for nickel-DAB immunohistochemistry and 2 h for immunofluorescence). The secondary antibodies used were biotinylated goat anti-rabbit (1:500; Vector Laboratories), biotinylated donkey anti-mouse (1:500; Jackson Laboratories), goat anti-rabbit Alexa 488 (1:1000; Invitrogen), and goat anti-mouse Alexa 594 (1:1000; Invitrogen) diluted in DPBS containing 0.2% v/v Triton X-100 (Sigma-Aldrich). Sections for fluorescence were incubated in 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) before being washed, mounted, and coverslipped (using 2.5% PVA/DABCO; Fluka). Sections for nickel-DAB immunohistochemistry were washed for 1 h and then incubated for 1 h in an avidin-biotin complex solution consisting of 0.2% v/v Triton X-100, avidin (1:500), and biotin (1:500) (Vectastain™ Elite ABC kit, Vector Laboratories) in DPBS. After a 30-min wash in DPBS, sections were transferred to a nickel-DAB chromogen solution consisting of 95 mM NiSO₄, 175 mM sodium acetate, 0.56 mM DAB, and 0.00075% hydrogen peroxide. Sections were observed until staining was visible, after which the color reaction was stopped by returning them to DPBS. After the final wash, sections were mounted in serial order onto gelatinized glass slides and dried at room temperature. They were then dehydrated through an ethanol series (70–100%) and cleared with Histo-clear™ (National Diagnostics) for at least 1 min each. Slides were coverslipped with DPX neutral mounting medium (Ajax Finechem).

In Situ Hybridization

Fixed brains (E15 and E19 Wistar rat and E15.5 and E17 C57Bl/6 mouse; *n* = 3 per age) were blocked and sectioned as described above. All steps used RNase/DNase-free DPBS and water. Sections were mounted onto Superfrost slides (Menzel-Glaser) and allowed to dry at room temperature. The in situ hybridization protocol was as described previously (Christiansen et al. 1995), with minor modifications. All hybridization steps were performed at 68 °C. Digoxigenin-labeled antisense riboprobe for human *Netrin1* (1 μg/mL) revealed the expression pattern with the use of the color substrate BM Purple (Roche).

Dissociated Primary Neuron Cultures

The neocortex and cingulate cortex were dissected from E13 CD1 mouse embryos and suspended in 0.25% trypsin-EDTA (Invitrogen). After trituration of the tissue, 10% fetal bovine serum (FBS; Invitrogen) was added to the suspension, which was spun at 5000 rpm for 5 min. The cell pellet was resuspended in Neurobasal medium (minus l-glutamine with phenol red) (Invitrogen), containing 0.1% penicillin streptomycin (Invitrogen), 10% FBS, 2% B27 Supplement (Invitrogen), and 0.25% l-glutamine (Invitrogen). The cell suspension was placed into a 24-well plate, with one poly-D-lysine (Sigma-Aldrich)-coated coverslip per well, at a density of 50 000 cells per well. Cells were incubated for 2.5 days (cingulate cortex) or 4 days (neocortex) at 37 °C in 5% CO₂. Each day, half the medium was replaced with fresh medium. After incubation, coverslips were washed in phosphate-buffered saline (PBS), fixed in 4% PFA/4% sucrose for

15 min and washed in PBS for 45 min. Coverslips were incubated for 10 min in PBS containing 0.1% TritonX-100 and 1% bovine serum albumin (BSA) to permeabilize the cell membranes, then blocked in 1% BSA in PBS for 30 min. Coverslips were incubated in primary DCC A-20 antibody (1:100; Santa Cruz) for 1 h, washed in PBS, and incubated in secondary antibody (Alexa Fluor donkey anti-goat 546; 1:500; Invitrogen) for 1 h. After washes in DPBS, coverslips were incubated in DAPI and mounted with Prolong Gold (Invitrogen).

Growth Cone Turning Assay

The neocortex was dissected from E19 Wistar rat embryos, triturated, and plated as above, then grown overnight. The growth cone turning assays were carried out at 37 °C on a heated microscope stage (Fryer Co.). Growth cones with a straight trailing axon of >10 μm were selected for the assay. The pipette was loaded with either recombinant mouse Netrin1 (10 μg/mL; R&D Systems) or PBS and mixed with 70-kDa dextran labeled with fluorescent tetramethylrhodamine (Molecular Probes Inc.) to monitor the gradient produced. Steep gradients with a 10–15% change in concentration across 10 μm were generated using the pulsatile ejection method (Pujic et al. 2008). Images of the growing axon were taken using the 20× objective of a Zeiss Axio Observer inverted microscope and AxioVision 4 software at 60 s intervals for 1 h. The trace of each axon, its turning angle, and the distance of growth were calculated as described in Thompson et al. (2011). The center of the growth cone was manually located in each frame, and the turning angle was defined as the angle between the original direction of growth and the direction defined by the average position of the growth cone in the final 5 frames in the trace. Only growth cones with >10 μm of net growth over the period of the assay were included in the analysis.

In Vitro Collagen-Gel Assay

Pregnant wild-type Wistar rat (E15 and E19) or C57Bl/6 mouse (E15.5, E17, and P0) dams were anesthetized with 100 mg/kg sodium pentobarbitone, and placed on a heating pad. Embryos were removed sequentially from the uterus into Leibovitz's L-15 medium (Invitrogen), the brain removed, and the neocortex or cingulate cortex carefully dissected. Explants were then immediately cut from the dissected tissue at 250–350 μm using a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co.).

Agar cubes (~500 μm; 2% w/v low-melting point agar in DPBS) were either soaked in 10 μg/mL of recombinant mouse Netrin1 (R&D Systems) in plating medium consisting of OptiMEMI with B27 supplement (Invitrogen) and 1% v/v penicillin/streptomycin/fungizone (Invitrogen), or soaked in plating medium alone for at least 1 h. Slit2-expressing cell blocks were made by preparing a suspension of HEK-Slit2 stable cells or control HEK cells in 100-μL plating medium and setting in 2% low-melting point agar as previously described (Richards et al. 1997). To create Netrin1 + Slit2-expressing cubes, Slit2 (or control) cell blocks were soaked in recombinant Netrin1 protein as above. A bottom layer of 250 μL of 0.2% collagen mix consisting of OptiMEMI, 0.09% v/v NaHCO₃ (Biowhittaker), 1% v/v penicillin/streptomycin/fungizone, and 58% v/v Type-1 rat-tail collagen (BD Biosciences) in sterile distilled water was added to each well of a 4-well plate (Nunc) and allowed to set. Explants were then removed from the medium and washed briefly in collagen before plating in a 50-μL top layer volume of collagen. Agar cubes soaked in recombinant Netrin1 or control agar cubes were positioned ~300–500 μm from the explants. The plates were incubated at 37 °C in a 5% CO₂ humidified incubator for 2 days. No liquid medium was added to the cultures.

After growth, the explants were fixed overnight in 10% formalin with 0.1% Triton X-100 in DPBS, after which the fixative was thoroughly removed from the collagen with no fewer than 5 1 h DPBS washes. Neurons were then labeled by incubating overnight at room temperature with mouse anti-neuronal-specific βIII-tubulin (TuJ1 clone, at a concentration of 1 μg/mL; R&D Systems) in DPBS containing 0.01% gelatin. The explants were given 5 1 h washes with DPBS before being incubated with goat anti-mouse Alexa488

(Invitrogen) at a concentration of 1:1000 in DPBS and 0.01% gelatin overnight at room temperature in the dark. They were then imaged at ×5 magnification with deconvolution using an upright Axio-Imager Z1 (Carl Zeiss) fitted with Apotome (Carl Zeiss) and an AxioCam HRm camera (Carl Zeiss). Optical sections were acquired at 20 μm across the explant to capture all neurites in focus before flattening into a multiple image projection.

A red arrow was placed on each explant during imaging to indicate the position of the agar cube relative to the center of the explant, and images cropped to the same size. Pixels representing neurites were identified using a ridge-tracing algorithm (modified from Weaver et al. 2003). Neurite growth up versus down the gradient was quantified using the “guidance ratio” (Rosoff et al. 2004; Mortimer et al. 2009). Briefly, using Matlab (The Mathworks Inc.) a line was defined through the center of the explant (center of mass of pixels representing the explant body) orthogonal to the direction of the gradient. The number of pixels U representing neurites on the upgradient side of this line was then counted, as was the number of pixels D representing neurites on the down gradient side of this line. The guidance ratio was defined as $(U - D)/(U + D)$. A positive guidance ratio indicates attraction (more neurite growth from the upgradient side of the explant), whereas a negative ratio indicates repulsion. “Outgrowth” was defined as total neurite pixels $(U + D)$ divided by the total number of pixels representing the neurite body. These measures provide control for growth-related effects and specifically allow measurement of directional guidance (Rosoff et al. 2004; Mortimer et al. 2009).

Explants displaying poor growth (those below one standard deviation of the mean level of outgrowth for all experiments across all conditions) were eliminated from the experiment. About 83–153 explants per condition, per experiment were plated to ensure a sufficient number of explants for statistical significance. The data were then analyzed using an unpaired 2-tailed Student's t -test. Data are presented as average values ± standard error of the mean (SEM), for all samples pooled from at least 3 independent experiments per tissue. Differences were considered significant when $P < 0.05$.

Coimmunoprecipitation of Endogenous Robo1 and DCC

Control supernatant, recombinant Netrin1, concentrated supernatant from Slit2-expressing HEK cells, or a combination of both were overlaid, and the explants allowed to grow for 2 days to extend axons. Explants, together with collagen gels, were then transferred to cold lysis buffer with protease inhibitors to allow extraction of membrane proteins, after which they were incubated for 30 min on ice. Lysates were clarified by centrifugation at 5000 × g for 10 min to pellet insoluble material (largely collagen). After clarification, the solubilized receptors were immunoprecipitated from the lysates. Antibodies, or a control preimmune serum, were first bound to Protein-G Dynabeads (Invitrogen) for 30 min at room temperature as per the manufacturer's directions. The cleared lysates were then incubated with 20 μL beads at 4 °C overnight with end-over-end rotation. Receptor-bound beads were extracted from the lysates and washed in citrate buffer as per the manufacturer's guidelines. The protein was then solubilized in 1× electrophoresis loading buffer, and analyzed by gel electrophoresis and western blot using antibodies against Robo1 (goat polyclonal), DCC (PAb 2744), 6xHis monoclonal (to detect R&D Systems recombinant Netrin1), or Myc (to detect recombinant Slit2).

Western Blot

Neocortex was removed from fresh C57Bl/6 and DCC^{Kanga} embryonic brains and snap frozen in liquid nitrogen. Postnatal pups were anesthetized on ice for 10 min before dissecting the neocortex. The tissue was solubilized in loading buffer and the proteins separated by gel electrophoresis. Western blot was performed to detect DCC expression levels using an anti-DCC antibody (G97 monoclonal, 1:1000 or 2744, 1:1000 overnight). The blots were then reprobed with anti-β-III tubulin (TuJ1, 1:1000) or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000; Imagenex) to control for protein loading and neuronal number.

Results

Netrin1 Does Not Act as a Long-Range Guidance Molecule for Neocortical Callosal Axons

The cerebral cortex contains neurons with long-range projections to subcortical structures (corticofugal axons) via the internal capsule and to the contralateral cortical hemisphere via the corpus callosum. Corticofugal projection neurons arise predominantly in the subplate and Layers 5 and 6, and project early in development from E12, whereas callosal projections are formed later in development and arise predominantly from upper layer neurons. Netrin1 is a secreted protein (Serafini et al. 1994) that can exert chemoattractive or chemorepulsive effects depending on the repertoire of receptors expressed on the growth cone (reviewed in Baker et al. 2006). Previous studies have shown that Netrin1, expressed within the internal capsule, acts as a chemoattractant for corticofugal axons early in development (Métin et al. 1997; Richards et al. 1997; Braisted et al. 2000; Bonnin et al. 2007; Powell et al. 2008). Callosal axons have been shown to express DCC (Shu et al. 2000), which commonly elicits an attractive response to Netrin1 binding, although the long-range effect of Netrin1 on these axons has yet to be investigated. In order to examine this, the following series of experiments were performed in which cortical explants were cocultured alongside a source of Netrin1 in collagen gels, and the preferential growth of axons toward or away from this source was quantified using a neurite-tracing algorithm (see Materials and Methods section).

To confirm the biological activity of the commercially available recombinant Netrin1 available (R&D Systems), we used the well-established rat cortical explant collagen-gel assay in which Netrin1, secreted from transfected cells, has previously been shown to attract corticofugal axons toward the internal capsule (Métin et al. 1997; Richards et al. 1997). To investigate whether Netrin1 could act as an attractant for rat callosal axons which develop later than corticofugal axons, explants were taken at a specific time-point in rat cortical development (E19, equivalent to mouse age E17) to enrich for callosal projections; these explants were then compared with those taken earlier in development (E15) when laterally projecting corticofugal axons are known to be responsive to Netrin1 (Fig. 1A; Métin et al. 1997; Richards et al. 1997). As previously described, Netrin1 promoted outgrowth and attraction of axons from E15 rat neocortical explants (Fig. 1B,C,J,K), confirming the biological activity of recombinant Netrin1 used in the experiment. In contrast, E19 rat neocortical axons did not show a preference for directed growth when presented with Netrin1 (Fig. 1D,E,J). We did not assess turning in the collagen gel assay as we have previously shown that the asymmetric outgrowth from explants we observed here is a robust indicator of a guidance response even without turning (Mortimer et al. 2010). Nevertheless, to confirm these findings, we repeated this experiment using the growth cone turning assay (Lohof et al. 1992). This assay utilizes dissociated cells and examines their turning in a local gradient of factor produced from a pipette positioned next to the growth cone. In this assay, E15 axons were attracted to Netrin1 whereas E19 axons were unresponsive to this cue (Fig. 1F,G vs. Fig. 1H,I, and Fig. 1P), thereby confirming the temporal specificity of the attractive effect mediated by Netrin1. To confirm the expression of both Netrin1 and DCC in rat at these developmental stages, we performed *in situ* hybridization for Netrin1

and immunohistochemistry for DCC at E15 and E19. At both stages of development, Netrin1 was expressed at the cortical midline, and DCC was expressed by callosal axons (Fig. 1L–O).

These data suggest that, although earlier neocortical lateral projections are attracted to Netrin1, it is unlikely that the Netrin1 source at the telencephalic midline acts as a long-range attractant for the later medially projecting axons from the neocortex that form the majority of the corpus callosum.

As mouse mutants for both Netrin1 and DCC display callosal defects (Serafini et al. 1996; Fazeli et al. 1997; Ren et al. 2007; see Fig. 4 later for a comparison of these phenotypes), we next performed the same collagen-gel experiment at the equivalent stage (E17) in mouse when callosal axons are pathfinding so that we could utilize mouse mutants for DCC in our experiments. When wild-type E17 mouse neocortex was paired with Netrin1, no effect on guidance or outgrowth was observed (Fig. 2A–D). In some experiments, rat and mouse tissue were investigated in the same experiment, using E15 rat tissue as a positive control for Netrin1 attraction, thus demonstrating that Netrin1 does not act as an attractant for neocortical axons at stages when callosal axons from the neocortex target the midline in either species. We also examined whether neocortical axons at slightly younger stages respond to Netrin1. In this experiment, we paired neocortical axons at E15.5, just prior to when significant numbers of neocortical callosal axons cross the midline, but at a stage when callosal axons from the cingulate cortex are crossing the midline. We again observed that neocortical axons at E15.5 were not guided by Netrin1 (Fig. 2C,D). Taken together, these results suggest that at a stage when the neocortex produces predominantly deeper layer neurons Netrin1 guides their axons to the internal capsule, but this response is downregulated by E15.5, coincident with the generation of upper layer cortical neurons.

Netrin1 Attracts Callosal Pioneering Axons From the Cingulate Cortex

As the corpus callosum comprises axons from both the neocortex and the cingulate cortex, we wanted to investigate whether guidance responses to Netrin1 were area- or stage-specific, by repeating the *in vitro* explant coculture assay experiments with cingulate cortical axons at E15.5 and E17. These ages allowed us to compare cingulate axon guidance at the same developmental stages as those we had studied in the neocortex, with E15.5 being when cingulate pioneering axons are known to cross the midline in mice (Rash and Richards 2001).

In contrast to neocortical axons, axons from E15.5 cingulate cortex explants were attracted to Netrin1 (compare Fig. 2A–C and Fig. 2E–G), and displayed increased outgrowth compared with controls (Fig. 2H). This effect was only observed at E15.5, as cingulate cortical explants dissected at E17 were not responsive to Netrin1 (Fig. 2G). Together these findings demonstrate a previously unknown spatial and temporal specificity in the function of Netrin1 in mediating the attractive guidance of cortical axons. Moreover, immunohistochemical analyses of DCC expression in both mouse and rat (Figs. 1L,N and 2I,K) showed higher levels of staining in the dorsal and most ventral regions of the corpus callosum where

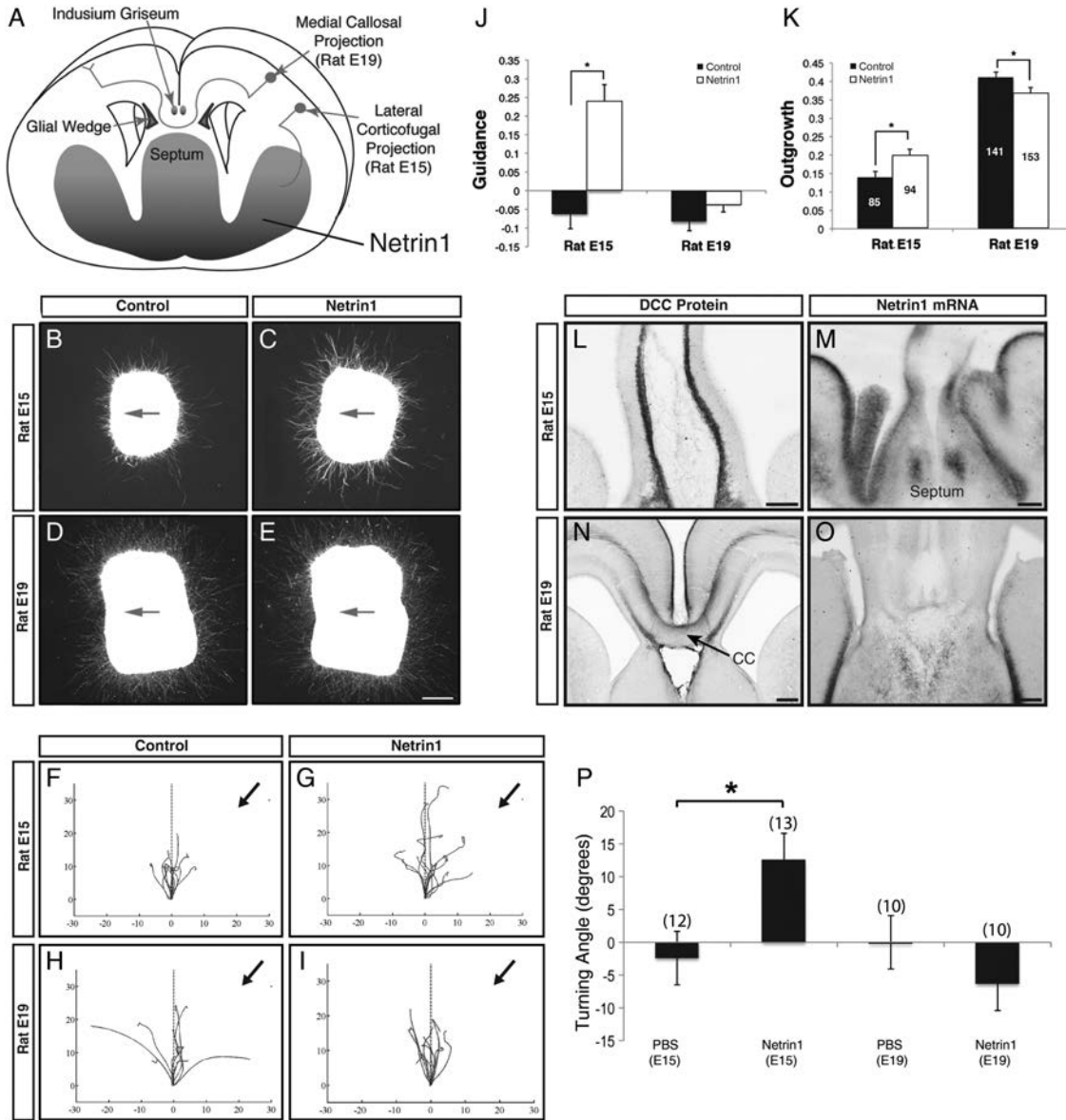


Figure 1. Netrin1 does not guide E19 rat neocortical axons in vitro. (A) Schematic of a coronally sectioned rodent brain demonstrating the trajectories of the laterally projecting corticofugal axons and the medially projecting callosal axons, together with the expression pattern of Netrin1 (green) and DCC (red). As these axonal populations both originate from the neocortex, this tissue was collected at different temporal stages to optimize for either corticofugal axons or callosal axons. (B–E) Neocortical explants were taken from E15 rat (as a positive control; B and C) for corticofugal axons, and E19 rat (D and E) for callosal axons, and were cocultured with control (B and D) or recombinant Netrin1-soaked (C and E) agar cubes (positioned $\geq 250 \mu\text{m}$ from the explant in the direction of the arrow). (J and K) The laterally projecting axons (E15) were attracted toward the Netrin1 source (J; $P < 0.001$), and the explants also displayed more outgrowth than seen in the control condition (K; $P = 0.004$, $n = 85$ control explants and $n = 94$ Netrin1 explants). However, axons from E19 explants were neither attracted nor repelled by Netrin1 (J; $P = 0.072$), and outgrowth was slightly decreased (K; $P = 0.021$, $n = 141$ control explants, $n = 153$ Netrin1 explants). Explants were pooled from 4 separate experiments; total explant n values are in columns in panel K. Asterisk indicates a statistically significant difference ($P < 0.05$). (L–O) DCC and Netrin1 expression were analyzed at E15 and E19 in rat. DCC protein was expressed on axons approaching the midline at E15 and E19 (L and arrow in N, $n = 3$ per stage). Netrin1 mRNA was expressed in the septum and ventral forebrain at both stages of rat development as assessed by in situ hybridization (M and O). (F–I) Growth cones of dissociated rat neocortical axons were subjected to a gradient of Netrin1 (G and I) or PBS as a control (F and H) in the growth cone turning assay. Growth cones of neurons dissociated at E15 were attracted to Netrin1 (G) whereas growth cones of neurons dissociated at E19 were unresponsive (I). Traces shown indicate the growth cone trajectories. (P) Summary of the turning angles in response to PBS and Netrin1. Error bars = SEM, numbers in brackets are growth cones per condition, Asterisk indicates $P < 0.05$, Kolmogorov–Smirnov test. All scale bars = $200 \mu\text{m}$.

cingulate pioneering axons reside, correlating with their attractive response. To confirm expression of DCC in both neocortex and cingulate cortex we also performed immunohistochemistry on dissociated cells from each of these regions. DCC was found to be expressed on the growth cones of neurons arising from both regions at the time these axon populations are crossing the midline (Fig. 2M,N).

Netrin1 Modulates the Repulsion of E17 Neocortical Axons by Slit2

Our experiments thus far had demonstrated that Netrin1 did not act as an attractant for neocortical axons at later stages. We therefore sought to determine how Netrin-DCC might be functioning to guide these axons. Previous studies using in vitro-translated protein assays have reported that Robo and

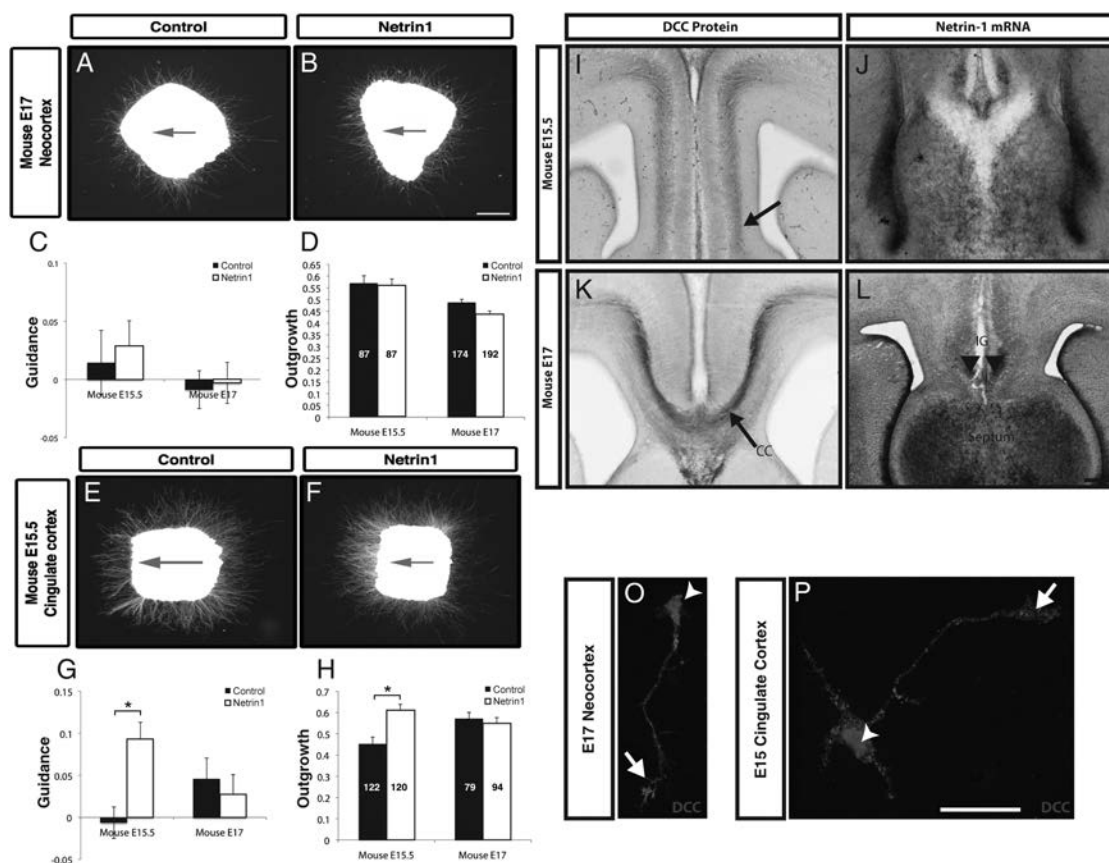


Figure 2. Netrin1 attracts callosal pioneering axons projecting from the cingulate cortex but not neocortical callosal axons in vitro. (A–D) To examine the guidance of cingulate callosal axons by Netrin1, cingulate explants were dissected from E15.5 and E17 (explants shown only for E15.5) mouse and plated next to control or Netrin1-soaked agar cubes (the direction of the red arrow in A and B indicates the position of the agar cube). Callosal pioneering axons from E15.5 cingulate cortex were attracted toward the Netrin1 source (C; $P = 0.00003$) and also displayed more outgrowth compared with control conditions (D; $P = 10^{-5}$). Axons from E17 cingulate cortex were neither attracted nor repelled by Netrin1 (C; $P = 0.59$) and outgrowth was unaffected (D; $P = 0.56$). (E–H) Neocortical explants from E15.5 and E17 (explants shown only for E17) mouse neocortex were dissected and plated next to control or Netrin1-soaked agar cubes. Neocortical explants at both ages were neither attracted nor repelled by Netrin1 (G) and axon outgrowth was also unaffected. Explants were pooled from 3 to 6 separate experiments; total explant n values are in columns in panel D and H. (I–L) Netrin1 and DCC expression was investigated in mouse coronal brain sections. The DCC antibody labeled callosal pioneering axons at E15.5 in mouse (arrow in I; $n = 3$) and later callosal axons as they crossed the midline (E17 in mouse, arrow in K; $n = 3$). Netrin1 mRNA (J and L) was expressed in the ventrolateral septum and the indusium griseum (IG); $n = 3$ per stage. (M and N) To examine DCC expression on growth cones, neurons were stained for DCC and DAPI by immunofluorescence. DCC is expressed in neurons of both the cingulate cortex and the neocortex at stages equivalent to when axons arising in these areas are navigating the corpus callosum. DCC expression was present throughout the cell body (arrowhead in M and N), axon and growth cone (arrow in M and N). Scale bar in F = 200 μ m for A and B and E and F; bar in I–L = 200 μ m and M and N = 20 μ m.

DCC can molecularly interact via their P3 and CC1 intracellular domains respectively (Stein and Tessier-Lavigne 2001; Yu et al. 2002), and as a number of repellents but few attractants have been identified at the cortical midline, we hypothesized that instead of acting as an attractant, Netrin1 may function to modulate Slit2-mediated repulsion of callosal axons, thereby allowing them to approach and cross the midline. Mice with functional deletions in Netrin1, DCC, Slit2, or Robo1 have all been shown to display malformations in corpus callosum formation (Serafini et al. 1996; Fazeli et al. 1997; Bagri et al. 2002; Andrews et al. 2006; López-Bendito et al. 2007; Unni et al. 2012). Netrin1 (Fig. 2J,L) and Slit2 (Shu and Richards 2001) are both expressed at the cortical midline when callosal axons are crossing, and Slit2 regulates the guidance of both precrossing and postcrossing callosal axons by axonal repulsion (Shu and Richards 2001; Shu, Sundaresan et al. 2003). Protein expression of Robo1 and DCC in the telencephalon appears to be largely localized to axons comprising the 3 major forebrain commissures, as well as subcortical

projections (Shu et al. 2000; Bagri et al. 2002; Figure 2I,K). To examine the coexpression of DCC and Robo1, we used thin (4 μ m) paraffin sections of E17 mouse brain to allow resolution of individual axon bundles, and then double-labeled for DCC and Robo1 by fluorescence immunohistochemistry. Both receptors were coexpressed on callosal axons (arrows, Fig. 3H–M), whereas subpopulations of the fornix showed differential staining for the 2 receptors (arrowheads, Fig. 3J). No specific labeling was observed in preimmune serum controls (data not shown).

Given these lines of evidence, we cocultured mouse neocortical explants at E17 with Slit2 and Netrin1, either alone or in combination to investigate whether Netrin1-DCC may interact with Slit2-Robo1 to guide callosal axons. Explants cocultured with control medium or with Netrin1 alone showed no guidance preference as described above, and explants cultured with Slit2 alone were repelled as previously described (Shu and Richards 2001). However, when Netrin1 and Slit2 were combined, the repulsion of axons by Slit2 was

abolished, and there was no significant difference between the guidance of these explants compared with the response to either Netrin1 alone or control medium (Fig. 3D,E; $P > 0.05$). The outgrowth of these axons did not differ significantly between conditions (Fig. 3F; $P > 0.05$ in all cases) suggesting that Netrin1 was not modulating repulsion by increasing axonal outgrowth.

It has previously been shown that Slit is able to bind directly to Netrin (Brose et al. 1999). To examine whether this was occurring in our explant coculture assay, we immunoprecipitated Slit2 from concentrated supernatant obtained from Slit2-expressing HEK cells, in the presence or absence of recombinant Netrin1, and then examined the immunoprecipitates for Netrin1 and Slit2 by western blot. Netrin1 did not coimmunoprecipitate with Slit2 (Fig. 3G). This suggests that the 2 recombinant ligands do not interact in our assay, and that the Netrin1-mediated silencing observed in our explant coculture experiment (Fig. 3E) is due to Netrin1 and Slit2 interacting through binding to their specific receptors.

Slit2-Mediated Repulsion of Neocortical Axons is Transduced by Robo1

As there are 3 Slit molecules and 4 Robo receptors expressed in mammals (Unni et al. 2012 and for reviews see Hohenester et al. 2006; Chédotal 2007), understanding the key family member(s) involved in callosal axon guidance is critical. In mice, the predominant ligand involved in callosal development is Slit2, although other Slits also make a contribution, as evidenced by an increase in the severity of the phenotype in double knockout mice in comparison to Slit2 single knockout mice (Bagri et al. 2002; Unni et al. 2012). Of the 4 Robo receptors, only Robos 1–3 are expressed in the brain, with the expression of Robo4 being restricted to endothelial cells (Huminiński et al. 2002). Robo1 protein is highly expressed on callosal axons, with much lower levels of Robo2 being present (Andrews et al. 2006). *Robo1*, but not *Robo2*, single knockout mice display callosal abnormalities (Andrews et al. 2006), and *Robo1/2* double knockout mice display a similar, but more severe, phenotype (López-Bendito et al. 2007). A requirement for *Robo1* in callosal axon guidance has not been tested in vitro. To examine this, we cultured E17 neocortical explants from *Robo1* knockout and wild-type littermates with Slit2-expressing or control cell blocks for 2 days in a collagen matrix. While neurites from E17 wild-type neocortical tissue were repelled by Slit2, as previously observed (Shu, Puche et al. 2003; Supplementary Fig. 1A; $P < 0.05$), those from *Robo1* knockout mice were not significantly affected (Supplementary Fig. 1A; $P > 0.05$). No significant changes in overall neurite outgrowth were observed between conditions for either of the genotypes (Supplementary Fig. 1B; $P > 0.05$). This result, together with the fact that *Robo1*, but not *Robo2*, single mutants display callosal axon guidance defects, suggests that although Robo2 plays a role in callosal formation, Robo1 is sufficient to mediate Slit2-regulated repulsion in callosal axons.

In the mammalian spinal cord, Robo3 has been shown to attenuate *Robo1*-mediated repulsion (Sabatier et al. 2004) and to mediate interneuron development (Barber et al. 2009); however, its expression in later forebrain development has not previously been investigated. To address this, we performed in situ hybridization for Robo3 and found that it was

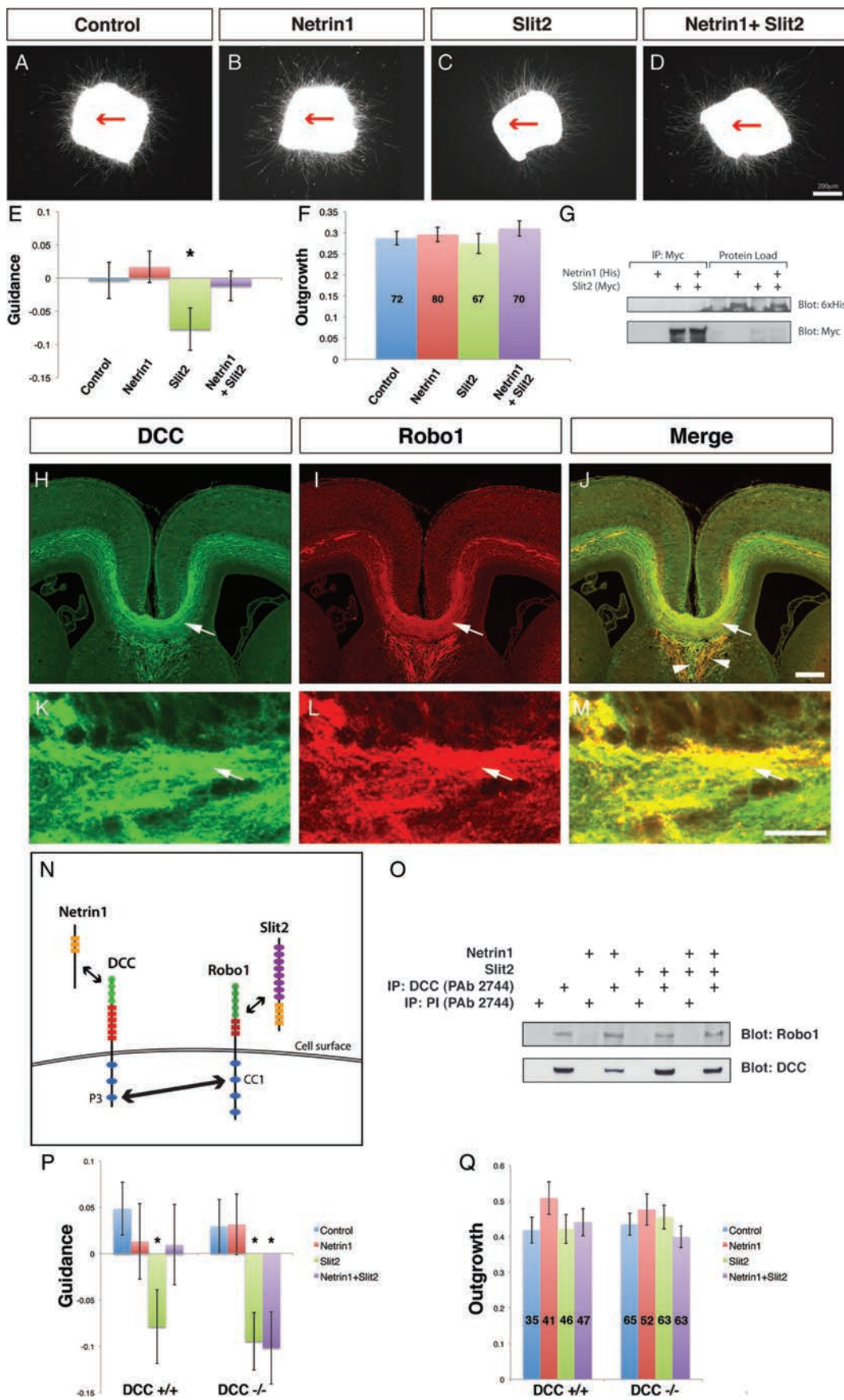
not expressed on neocortical callosal axons at E17 or P0 (Supplementary Fig. 1). However, Robo3 was expressed by the medial septum and habenula at both ages (arrows in Supplementary Fig. 1C–F), and by the lateral septum at E17 (arrowheads in Supplementary Fig. 1C). These data suggest that, although Robo3 plays a major role in spinal commissural development, it is not significantly involved in callosal axon guidance. Taken together with our recent evaluation of the function of the Slit ligands in callosal axon guidance (Unni et al. 2012), these data indicate that Slit2 and Robo1 are the predominant family members involved in corpus callosum development.

Endogenous DCC and Robo1 Receptors Expressed by Neocortical Axons Associate with Each Other

Having confirmed that neocortical callosal axons respond to Slit2 repulsion via Robo1, we wanted to examine whether DCC and Robo1 endogenously interact in neocortical axons. To do this, we performed coimmunoprecipitation experiments with extracts taken from E17 neocortical explants cultured for 2 days in semidry collagen gels treated with either Slit2, Netrin1 or a combination of both ligands, after which DCC was immunoprecipitated from explant extracts. Preimmune serum was used as a negative control for the immunoprecipitations. A western blot was then performed on the immunoprecipitates to determine the presence of Robo1 and DCC. Robo1 was observed to associate with DCC in either the presence or absence of Netrin1 and/or Slit2 (Fig. 3O). This provides the first evidence that these 2 receptors may interact in vivo, and suggests that they always form complexes on neocortical axons at E17, independent of activation by Slit2 and/or Netrin1.

Netrin1 Attenuates Slit2 Repulsion via Its Receptor, DCC

Based on the finding that endogenous DCC binds to Robo1 (Fig. 3O), Netrin1-mediated modulation of Slit2 repulsion (Fig. 3D,E) might occur in a DCC-dependent manner. To investigate this hypothesis, we performed an explant coculture assay combining both Netrin1 and Slit2, similar to the experiment illustrated in Figure 3A–F, but instead using neocortical explants taken from E17 *DCC* knockout mice to assess the guidance activity of Slit2/Robo1 in the absence of DCC. When explants were challenged with Slit2 alone, wild-type and knockout neurites were significantly repelled compared with controls (Fig. 3P; $P < 0.05$), indicating that Slit2-mediated repulsion is functional in *DCC* knockout mice. Wild-type and knockout explants that were challenged with Netrin1 displayed no significant axon guidance response when compared with controls (Fig. 3P; $P > 0.05$), demonstrating that Netrin1 did not guide cortical axons independently of DCC. When Slit2 and Netrin1 were combined, wild-type-littermate explants produced axons that were neither attracted nor repelled compared with controls (Fig. 3P; $P > 0.05$). However, when *DCC* knockout explants were challenged with a combination of Slit2 and Netrin1, the axons were repelled (Fig. 3P; when compared with control $P < 0.05$). This repulsion was significant when compared with the response to Netrin1 alone, and the wild-type paired-condition equivalent (Fig. 3P; $P < 0.05$), suggesting that in the *DCC* knockout axons, Netrin1 had lost its ability to silence Slit2-mediated repulsion. No significant difference in outgrowth was observed between



conditions for either phenotype (Fig. 3Q). These data indicate that Netrin1-mediated attenuation of Slit2-regulated repulsion requires signaling via DCC. Furthermore, we examined the expression of both Slit2 and Robo1 in *DCC* knockout brains using in situ hybridization and immunohistochemistry. No significant differences were observed between genotypes in terms of Slit2 mRNA expression, or Robo1 protein and mRNA expression (Supplementary Fig. 1G–L), demonstrating that Slit2/Robo1 are still expressed in the *DCC* knockout tissue and could mediate these effects in vivo.

DCC^{kanga} Mutants Display an Identical Callosal Phenotype to DCC Knockout Animals

Our results demonstrate that the intracellular interaction of DCC and Robo1 may be crucial for the Netrin1-Slit2 regulation of axon guidance in the corpus callosum. To examine this in vivo, we utilized a naturally occurring mouse mutation in DCC called *DCC^{kanga}*, which has been reported to lack the exon encoding the P3 intracellular domain (Finger et al. 2002) which is known to interact with Robo1 (Stein and Tessier-Lavigne 2001; Figure 4S,T). The *DCC^{kanga}* mouse has previously been reported to have a malformation of the corpus callosum, but this has not been investigated in detail (Finger et al. 2002). To investigate whether this mouse expresses DCC protein lacking the P3 domain, we performed western blot analysis using antibodies directed against either the C-terminal or N-terminal domain of DCC in protein derived from E17 neocortex (Fig. 4T). This revealed that DCC protein is expressed but lacks the C-terminal domain that encodes the P3 Robo1 interaction domain (Fig. 4U). We then examined the callosal phenotype of these mice and compared this to the *DCC* knockout phenotype. We observed an identical callosal phenotype in the *DCC^{kanga}* mutants compared with *DCC* knockout animals (compare Fig. 4M–R vs. Fig. 4A–F), providing conclusive evidence that the P3–Robo1 interaction domain of DCC is required for corpus callosum formation.

Robo1 Does Not Silence Netrin1 Attraction of Neocortical Axons

Previous work using *Xenopus* spinal neurons (Stein and Tessier-Lavigne 2001) and zebrafish forebrain commissural axons (Zhang et al. 2012) has shown that Netrin1 attraction can be silenced by DCC–Robo interactions, which are dependent on the presence of Slit. As both sets of receptors and ligands are also present during callosal development, we wanted to determine whether Robo1 might be responsible for inhibiting Netrin1-mediated attraction via an interaction with DCC. In order to investigate this, we cocultured neocortical explants from *Robo1* knockout and wild-type-littermate E17 mice with a localized source of recombinant Netrin1 or control medium. No significant difference in the guidance of axons between wild-type and *Robo1* knockout tissue was observed (Supplementary Fig. 1M; $P > 0.05$ for all genotypes). A slight decrease in outgrowth was observed in wild types between control and Netrin1-treated explants but no difference in outgrowth was observed in tissue from *Robo1* knockout mice (Supplementary Fig. 1N). These data demonstrate that the lack of attraction by Netrin1 in this system does not occur through Robo1-mediated silencing of Netrin1.

Downregulation of DCC Allows Postcrossing Callosal Axons to be Repelled by Slit2

If Slit2-mediated repulsion is abrogated by Netrin-DCC signaling, how are axons then able to leave the midline? A reduction in the level of DCC protein on callosal axons occurs during development (Shu et al. 2000). If Robo and DCC interact to modulate Slit-mediated repulsion, then reduced DCC expression levels might promote Slit-regulated repulsion of postcrossing axons, thereby repelling them from the midline and into the contralateral hemisphere. To address this possibility, we quantified the level of neocortical DCC expression from ages before midline crossing, up to P0, when a large majority of neocortical axons have already projected across the midline. Western blot analysis was performed using

Figure 3. Netrin1 abolishes Slit2-mediated repulsion of E17 neocortical axons. (A–F) Explants of E17 wild-type mouse neocortex were cocultured in semidry collagen for 2 days alongside control agar cell blocks containing HEK cells alone (A), or a source of guidance factor consisting of HEK cell blocks soaked in recombinant Netrin1 (B), HEK-Slit2-expressing cell blocks (C), or HEK-Slit2 cell blocks soaked in recombinant Netrin1 (D). A red arrow shows the direction of the cell block in relation to the explant. In the presence of Slit2 (C) axons were repelled compared with controls (E; $P = 0.04$, Student's *t*-test, $n = 67$ – 80), but in the presence of Netrin1 (B), axons showed no guidance preference compared with controls. When Netrin1 and Slit2 were combined, Slit2-mediated repulsion was abolished and no significant guidance was observed (E; $P = 0.41$ vs. Control, $P = 0.047$ vs. Slit2, Student's *t*-test, $n = 70$). No significant differences in outgrowth were observed between conditions (F). Explants were pooled from at least 3 independent experiments. Total explants per condition are shown on the bars in panel F. Asterisk indicates a significant (1-tailed Student's *t*-test) difference from all other conditions. (G) Recombinant Netrin1 and Slit2 from HEK-Slit2 cell supernatants do not coimmunoprecipitate from collagen cultures. Explants were cultured with an overlay of HEK-Slit2-conditioned medium, control HEK medium, Netrin1-containing medium, or Netrin1-containing Slit2-conditioned medium. Immunoprecipitates and total protein lysates were analyzed by western blot. Slit2 was detected with anti-Myc antibody, and Netrin1 with anti-6xHis antibody. (H–M) DCC and Robo1 are colocalized on callosal axons at E17. Immunohistochemistry on thin (4 μ m) paraffin coronal sections of an E17 wild-type mouse brain stained with DCC (H and K), or Robo1 (I and L). Both receptors are located on callosal axons with a largely overlapping pattern at the cerebral midline (arrows in H–M), while some fibers in the fornix label with either DCC or Robo1 alone (J, arrowheads). (N) Model diagram adapted from Stein and Tessier-Lavigne (2001), demonstrating receptor and ligand interactions on callosal axons. (O) Endogenous DCC and Robo1 coimmunoprecipitate from explants grown in the presence or absence of Netrin1 and Slit2 ligands. E17 neocortical explants were cultured for 2 days in a semidry collagen matrix with an overlay of control-conditioned medium, control medium with added recombinant Netrin1, HEK-Slit2-conditioned medium, or HEK-Slit2 medium-containing Netrin1. Proteins were immunoprecipitated from lysates with anti-DCC (PAb 2744) or control preimmune antiserum (PI), and analyzed by western blot with antibodies against Robo1 or DCC. DCC was successfully immunoprecipitated in all conditions with anti-DCC antibody, but not the preimmune serum. Robo1 was detected in the presence or absence of Netrin1 and/or Slit2. (P and Q) DCC is required for Netrin1-mediated silencing of Slit2-mediated repulsion. E17 neocortical explants from wild-type, heterozygote, or *DCC* knockout mice were cultured for 2 days in semidry collagen alongside control cell blocks, or a source of Netrin1, Slit2, or Netrin1 and Slit2 combined. Slit2 significantly repelled neocortical axons from wild-type tissue as previously shown (P ; $P = 0.016$, 2-tailed Student's *t*-test), from *DCC* heterozygous tissue (P ; $P = 0.019$, 2-tailed Student's *t*-test), and from *DCC* knockout tissue (P ; $P = 0.004$, 2-tailed Student's *t*-test). However, whereas neocortical axons were not responsive to a combination of Netrin1 and Slit2 in the wild type (P ; $P = 0.49$, 2-tailed Student's *t*-test), and heterozygous conditions (P ; $P = 0.16$, 2-tailed Student's *t*-test), *DCC* knockout axons were significantly repelled by a combination of these 2 ligands (P ; $P = 0.008$, 2-tailed Student's *t*-test). Asterisks denote significant levels of repulsion compared with control and Netrin1 conditions per genotype ($P < 0.05$, 2-tailed Student's *t*-test). No significant differences in outgrowth were observed between conditions in either wild-type or *DCC* knockout tissue (Q; $P > 0.05$, 2-tailed Student's *t*-test). Explants were pooled from 4 separate experiments. The total numbers of explants used per condition are shown on bars in panel Q. Scale bar in H–J = 200 μ m; scale bar in K–M = 20 μ m in D–F.

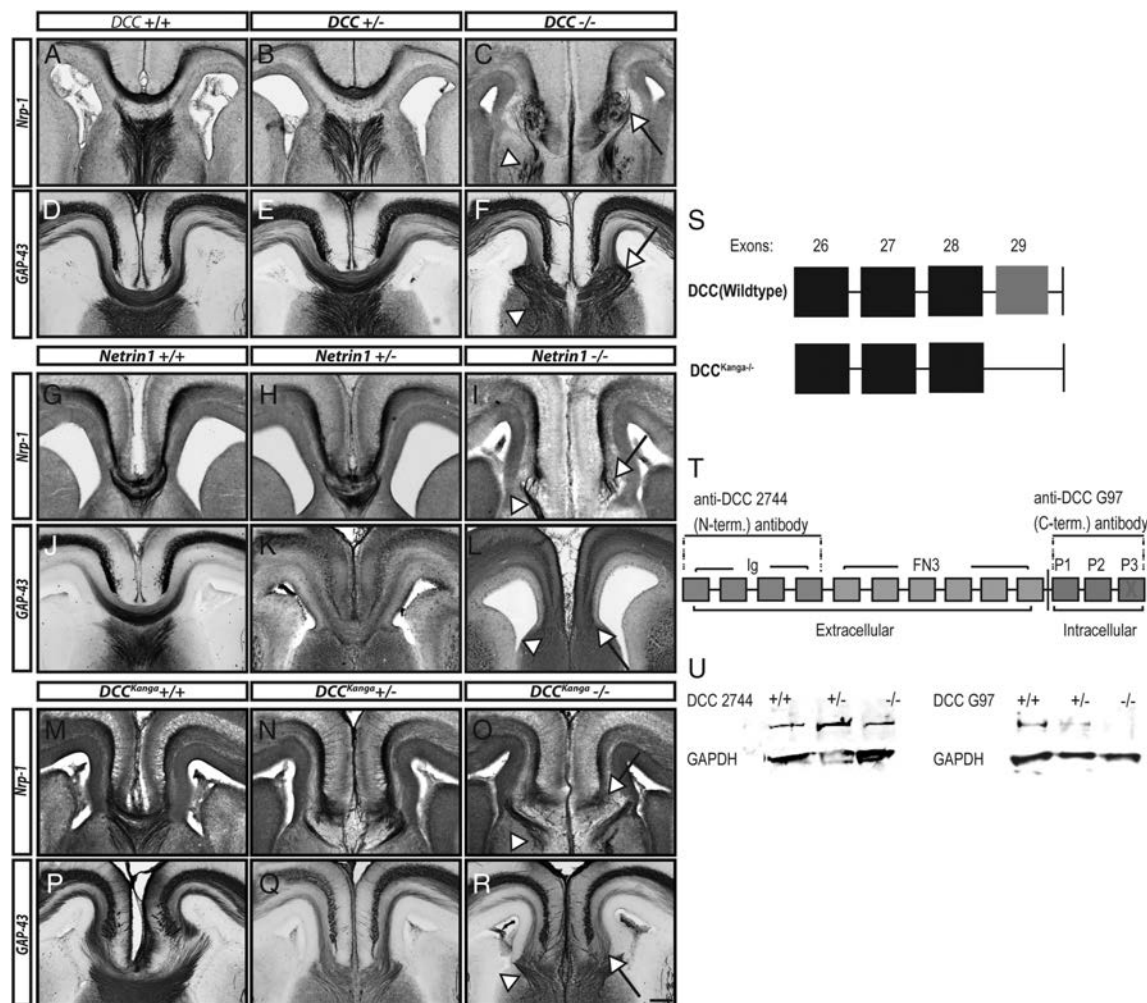


Figure 4. Comparison of the callosal malformation phenotypes in *Netrin1* and *DCC* knockout mice and *DCC^{Kanga}* mutant mice. (A–R) Coronal brain sections of E17 *Netrin1*, *DCC*, and *DCC^{Kanga}* mutant mice ($n > 3$ per genotype) were stained for GAP-43, a marker of growing axons and Neuropilin-1 (Nrp-1), a marker of cingulate pioneering axons (Piper et al. 2009). The corpus callosum formed normally in both wild-type (+/+; A and D, G and J, and M and P) and heterozygous (+/-; B and E, H and K, and N and Q) *Netrin1*, *DCC*, and *DCC^{Kanga}* mice, with GAP-43- and Nrp-1-positive axons having crossed the telencephalic midline within the callosal tract as well as within the fornix and the hippocampal commissure (not shown). In contrast, GAP-43- and Nrp-1-positive axons were disrupted in the *Netrin1*, *DCC*, and *DCC^{Kanga}* knockout animals and failed to cross the midline (C and F, I and L, and O and R). Instead, these axons misprojected within ipsilateral Probst bundles (arrow in C and F, I and L, and O and R). Some GAP-43- and Neuropilin-1-positive axons projected ventrally into the septum; these were likely to be axons of the fornix (arrowhead in C and F, I and L, and O and R). (S–U) In *DCC^{Kanga}* mice, the final exon of the *DCC* gene, exon 29, that encodes the P3 intracellular domain (T), is deleted, such that a truncated mRNA is expressed (S). To determine whether a truncated protein is expressed, western blots were performed on neocortical protein lysates of *DCC^{Kanga}* E17 mice using an antiserum raised against a DCC N-terminal peptide (2744) and an antibody raised against a DCC C-terminal peptide (G97) (T). (2744: $n = 1$ for all genotypes, G97: $n = 3$ for all genotypes). In *DCC^{Kanga}* +/+ animals, DCC protein was detected using the N-terminal antiserum and the C-terminal antibody, while lower levels of protein were present in heterozygous mice (U). In homozygous animals, DCC protein was detected using the N-terminal antiserum but was undetectable using the C-terminal antibody (U). Scale bar in R = 200 μ m for A–R.

neocortical tissue taken between E16 and P0, and DCC protein expression was determined (Fig. 5A). Band intensities were quantified, and the protein level was normalized against neuron numbers by probing the same blots with β -III tubulin antibody (Fig. 5B). This analysis revealed that DCC expression significantly declined over time from E16 to P0 (Fig. 5B, $P < 0.05$), consistent with the hypothesis that DCC expression is high precrossing and during the time that axons are targeting the midline, but then declines postcrossing.

We next used P0 neocortical explants to test the hypothesis that reduced levels of DCC at ages after E17 (postcrossing axons) result in an increase in Slit2-mediated repulsion. Explants were dissected from wild-type mice and cultured for 2 days alongside a source of Netrin1, Slit2, or a combination of

both ligands. Netrin1 did not attract or repel axons, as previously observed for E17 neocortical explants (Fig. 5C; $P > 0.05$), and Slit2 repelled neocortical axons in a similar manner to that observed for E17 neocortical explants (Fig. 5C; $P < 0.05$). Crucially, however, when Netrin1 and Slit2 were presented simultaneously, the axons were repelled to a similar extent as the Slit2-challenged explants (Fig. 5D; $P > 0.05$ compared with control), suggesting that Netrin1 signaling was no longer able to block Slit2-mediated repulsion. Overall, the response of the P0 neocortical explants was similar to that observed for E17 neocortical explants lacking the DCC receptor (Fig. 3P). This result suggests that, after midline crossing, Netrin-DCC-mediated attenuation of Slit-Robo-mediated repulsion no longer occurs, allowing the axons to be repelled away from the midline.

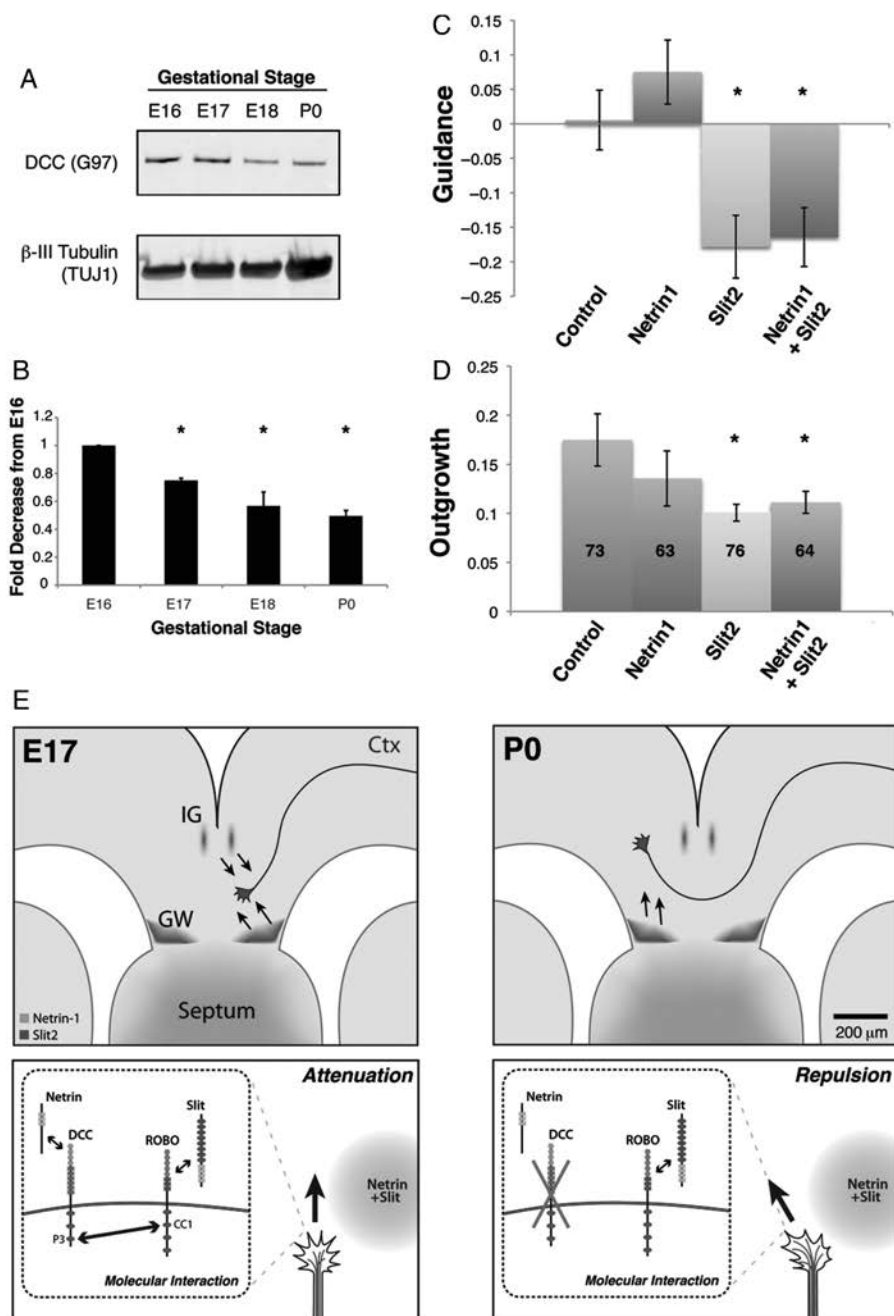


Figure 5. Downregulation of DCC allows postcrossing axons to be repelled from the midline by Slit2. (*A* and *B*) DCC protein is progressively downregulated in the neocortex from E16 through to P0. Neocortical tissue was taken from E16, E17, E18, and P0 mice and DCC protein levels were analyzed by western blot (*A*). An approximate 2-fold progressive reduction in expression was observed between E16 and P0, which was significant at all ages tested (*B*, asterisks, $P < 0.05$, Student's *t*-test). Data are representative of tissue collected from 3 separate litters per age. (*C* and *D*) Explants of P0 wild-type mouse neocortex were cocultured for 2 days in semidry collagen alongside control HEK cell blocks, or a source of Netrin1, Slit2, or Netrin1 and Slit2 combined. Explants projected neurites that were neither attracted nor repelled by control and Netrin1 cell blocks, but were repelled by Slit2-expressing cell blocks (*C*; $P = 0.009$, 2-tailed Student's *t*-test). When Netrin1 and Slit2 were combined, similar levels of repulsion were observed to those seen with Slit2 alone (*C*; $P < 0.001$ vs. Control, $P = 0.322$ vs. Slit2, 2-tailed Student's *t*-test). (*D*) A significant reduction in outgrowth was observed in the Slit2 alone condition ($P = 0.012$, 2-tailed Student's *t*-test) and with Netrin1/Slit2 combined ($P = 0.041$, 2-tailed Student's *t*-test). Explants were pooled from 4 independent experiments. Total explants per condition are shown on the bars in panel *D*. Asterisk indicates a significant difference ($P < 0.05$, 2-tailed Student's *t*-test) from control and Netrin1 conditions. (*E*) Schematic diagram depicting callosal axons as they traverse the midline at E17 and P0 in mouse. At E17, callosal axons express both Robo1 and DCC. As they approach, the midline the increasing level of surrounding Netrin1 attenuates the Slit2-mediated repulsion encountered from the glial wedge and indusium griseum, allowing axons to approach the midline while helping to "channel" them into a narrow tract (arrows). At P0, DCC is downregulated, thus allowing Slit2-mediated repulsion as axons leave the midline and enter the contralateral hemisphere.

Discussion

The proper guidance of commissural axons throughout the nervous system is essential for the functional wiring of the nervous system. Netrin-DCC signaling has been shown to

regulate commissure formation throughout the neuraxis, and its mechanism of action has been elucidated in a number of systems (Lai Wing Sun et al. 2011). However, the exact role of Netrin-DCC signaling in the formation of brain commissures

in mammals has not been defined, even though mouse mutants for both genes lack all forebrain commissures, and both genes are expressed in a temporally and spatially relevant manner to guide callosal axons. Based on data in other systems, including the mouse corticofugal system, we hypothesized that Netrin1 would attract callosal axons toward the midline; however, surprisingly, our results demonstrated a lack of guidance by Netrin1 for callosal axons derived from the neocortex at stages when callosal axons are growing toward and across the midline. This result was temporally and spatially specific, as cingulate pioneering callosal axons at E15.5 were attracted to Netrin1, identifying an important difference in the guidance of these 2 populations of callosal axons. Furthermore, given that corticofugal axons from the neocortex were attracted to Netrin1 at early stages, Netrin-DCC signaling appeared to function in a fundamentally different manner for neocortical callosal axons.

A potential mechanism for the differential attraction of cingulate versus neocortical axons at E15.5 is that DCC expression is higher on cingulate axons than on neocortical axons at this stage. The immunohistochemical data presented here (Figs. 1 and 2) and previously (Shu et al. 2000) demonstrate that DCC is not expressed ubiquitously throughout the callosal tract, but is expressed more highly on axons in the most dorsal and ventral regions of the corpus callosum where cingulate axons cross the midline. A difference in the level of DCC expression may explain the differences in guidance between the neocortex and the cingulate cortex but this is yet to be tested. Another possible explanation for the lack of attraction of neocortical axons is that DCC is unable to transduce an attractive signal under these circumstances due to receptor silencing. In this model, DCC-mediated attractive signaling would be inhibited by other receptors that are present on callosal axons, such as Robo1 (Andrews et al. 2006; López-Bendito et al. 2007). This has previously been demonstrated for postcrossing spinal cord commissural axons, whereby a ligand-gated interaction between Robo1 and DCC acts to silence Netrin1-mediated attraction (Stein and Tessier-Lavigne 2001; Zhang et al. 2012). However, when we cultured neocortical explants from Robo1 knockout mice in the presence of Netrin1, no increase in attraction was observed (Supplementary Fig. 1), making it unlikely that DCC is being silenced by this mechanism. An alternative explanation is that Netrin1 is interacting with another ligand, which modulates its effect. Recently, thalamic axons were shown to be nonresponsive to Netrin1 alone, whereas in the presence of a combination of Netrin1 and Slit1, they were attracted to these guidance factors (Bielle et al. 2011). Our results demonstrate that a combination of Slit2 and Netrin1 does not induce attraction of callosal axons but rather modulates their repulsion by Slit2.

Further experiments showed that this effect is not due to an interaction between these ligands but rather is receptor dependent. Previously, it has been shown that Robo1 and DCC receptors interact via their CC1 and P3 intracellular domains, respectively, and that a genetic interaction between the receptors exists in *C. elegans* (Stein and Tessier-Lavigne 2001; Yu et al. 2002). Indeed, although Stein and Tessier-Lavigne (2001) showed that a Slit2 ligand-gated interaction silences the responsiveness of postcrossing *Xenopus* spinal commissural axons to Netrin1, they also hypothesized that a Robo-DCC complex could potentially signal in the reverse manner, thereby silencing Slit-Robo repulsion. However, due

to the expression of Robo3 and low levels of Robo1/2 in *Xenopus* spinal neurons, this mechanism could not be addressed because the precrossing spinal commissural axons are not responsive to Slit2. Our data support the hypothesis that Netrin-DCC attenuates Slit-Robo-mediated repulsion in the corpus callosum, where *precrossing* axons are repelled by Slit.

The data presented here suggest 3 distinct differences between the guidance of commissural axons in the spinal cord and the brain of mice. First, unlike developing spinal cord axons, neocortical callosal axons are not attracted to the midline by Netrin1. Second, Robo1 is expressed on precrossing dorsal spinal commissural axons, but does not transduce a repulsive signal because it is attenuated by the presence of a specific isoform of Robo3. In contrast, callosal axons utilize DCC to attenuate Robo1-mediated repulsion. Third, in the spinal cord, Netrin1-mediated attraction must be overcome to allow axons to leave the midline, and this occurs by Robo-mediated attenuation of DCC-mediated attraction. In contrast, DCC expression appears to be downregulated on postcrossing callosal axons to levels that no longer attenuate Slit2-mediated repulsion, thereby allowing the axons to be repelled by Slit2 and away from the cortical midline.

Another aspect of callosal axon guidance that is unique compared with the formation of other central nervous system commissures is that both precrossing and postcrossing axons are responsive to midline Slit2 (Shu, Sundaresan et al. 2003). In other vertebrate and invertebrate commissural systems, responses to Slit are tightly regulated, particularly in precrossing commissural axons. For example in *Drosophila*, *Comm* regulates the presentation of Robo receptors on the cell surface, thereby regulating responsiveness to Slit2 (Seeger et al. 1993; Keleman et al. 2002). Recently, signaling via Frazzled/DCC has been shown to activate *Comm* transcription in the *Drosophila* nerve cord, and consequently to modulate Slit/Robo-responsiveness (Yang et al. 2009). This is a similar mechanism to that which occurs in the corpus callosum, with the difference being that callosal axons utilize a direct interaction between DCC and Robo, whereas in *Drosophila* DCC indirectly regulates Robo through its activation of *Comm*. In contrast, Slit-mediated repulsion of precrossing commissural axons in the mammalian spinal cord is modulated by Robo3 (Sabatier et al. 2004).

As axons cross the midline, they must somehow upregulate repulsive axon guidance to grow away from the midline and into the contralateral hemisphere. In the vertebrate spinal cord, postcrossing axons express both Robo1 and 2, as well as an alternatively spliced form of Robo3 that contributes to Slit-mediated repulsion (Long et al. 2004; Chen et al. 2008), thus promoting commissural axon growth away from the midline. During callosal axon guidance, Ryk/Wnt5a signaling provides an additional mechanism to promote the repulsion of axons from the midline, acting only on postcrossing axons (Keeble et al. 2006). In addition, postcrossing callosal axons are also repelled by Slit2 in vivo (Shu, Sundaresan et al. 2003). The results from the present study indicate that this occurs by a developmental downregulation of DCC expression from E16 to P0, to levels where Netrin1 can no longer attenuate Slit2-mediated repulsion.

Finally, our data show that rather than acting as an attractant or repellent, as has been shown in other systems (reviewed in Rajasekharan and Kennedy 2009), Netrin1

functions by modulating the activity of another guidance molecule, namely, Slit2, in callosal axons. This study presents the first evidence of a Netrin-DCC-based mechanism to silence Slit-mediated repulsion of axons at the cerebral midline, and adds to the emerging complexity of interactions between guidance receptors by showing that these receptors interact *in vivo*. It is clear that a tight regulation of attractive and repulsive forces is required to modulate axonal guidance at critical choice points. Our findings demonstrate a mechanism of attenuating repulsive cues, dependent on receptor crosstalk, which is modulated through the regulation of receptor expression level as development proceeds. They also reveal that the precise control of navigating commissural axons by multiple guidance cues is distinctly different from that which occurs in the developing spinal cord.

Supplementary Material

Supplementary material can be found at: <http://www.cercor.oxfordjournals.org/>.

Funding

This study was funded by the March of Dimes Foundation for Birth Defects, grant #1-FY05-119 and project grants #APP1043045 and #631446 from the National Health and Medical Research Council of Australia.

Notes

We thank the following colleagues who generously provided mice and reagents; Marc Tessier-Lavigne, Bill Andrews, and Vasi Sundaresan for the DCC and Robo1 knockout mice, respectively, Yi Rao for the Slit2-expressing cells, Alain Chédotal for the Robo3 *in-situ* probe, and Helen Cooper for the DCC antibody. We thank John Baisden for his assistance with the genotyping of mice, and Jonathan Hunt, Tim Vaughn, Stanley Chan, and Duncan Mortimer for their assistance with quantitative analysis. We are grateful to Rowan Tweedale, Helen Cooper, and Massimo Hilliard for their critical reading of the manuscript. *Conflict of Interest*: None declared.

References

Andrews W, Liapi A, Plachez C, Camurri L, Zhang J, Mori S, Murakami F, Parnavelas JG, Sundaresan V, Richards LJ. 2006. Robo1 regulates the development of major axon tracts and interneuron migration in the forebrain. *Development*. 133:2243–2252.

Bagri A, Marín O, Plump AS, Mak J, Pleasure SJ, Rubenstein JLR, Tessier-Lavigne M. 2002. Slit proteins prevent midline crossing and determine the dorsoventral position of major axonal pathways in the mammalian forebrain. *Neuron*. 33:233–248.

Baker KA, Moore SW, Jarjour AA, Kennedy TE. 2006. When a diffusible axon guidance cue stops diffusing: roles for netrins in adhesion and morphogenesis. *Curr Opin Neurobiol*. 16:529–534.

Barber M, Di Meglio T, Andrews WD, Hernandez-Miranda LR, Murakami F, Chédotal A, Parnavelas JG. 2009. The role of Robo3 in the development of cortical interneurons. *Cereb Cortex*. 19(Suppl 1): i22–i31.

Bielle F, Marcos-Mondéjar P, Leyva-Díaz E, Lokmane L, Mire E, Mailhes C, Keita M, García N, Tessier-Lavigne M, Garel S et al. 2011. Emergent growth cone responses to combinations of Slit1 and Netrin 1 in thalamocortical axon topography. *Curr Biol*. 21:1748–1755.

Bonnin A, Torii M, Wang L, Rakic P, Levitt P. 2007. Serotonin modulates the response of embryonic thalamocortical axons to netrin-1. *Nat Neurosci*. 10:588–597.

Braisted JE, Catalano SM, Stimac R, Kennedy TE, Tessier-Lavigne M, Shatz CJ, O'Leary DD. 2000. Netrin-1 promotes thalamic axon growth and is required for proper development of the thalamocortical projection. *J Neurosci*. 20:5792–5801.

Brose K, Bland KS, Wang KH, Arnott D, Henzel W, Goodman CS, Tessier-Lavigne M, Kidd T. 1999. Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell*. 96:795–806.

Chédotal A. 2007. Slits and their receptors. *Adv Exp Med Biol*. 621:65–80.

Chen Z, Gore BB, Long H, Ma L, Tessier-Lavigne M. 2008. Alternative splicing of the Robo3 axon guidance receptor governs the midline switch from attraction to repulsion. *Neuron*. 58:325–332.

Christiansen JH, Dennis CL, Wicking CA, Monkley SJ, Wilkinson DG, Wainwright BJ. 1995. Murine Wnt-11 and Wnt-12 have temporally and spatially restricted expression patterns during embryonic development. *Mech Dev*. 51:341–350.

Evans TA, Bashaw GJ. 2010. Axon guidance at the midline: of mice and flies. *Curr Opin Neurobiol*. 20:79–85.

Fazeli A, Dickinson SL, Hermiston ML, Tighe RV, Steen RG, Small CG, Stoeckli ET, Keino-Masu K, Masu M, Rayburn H et al. 1997. Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. *Nature*. 386:796–804.

Finger JH, Bronson RT, Harris B, Johnson K, Przyborski SA, Ackerman SL. 2002. The netrin1 receptors Unc5h3 and Dcc are necessary at multiple choice points for the guidance of corticospinal tract axons. *J Neurosci*. 23:10346–10356.

Hohenester E, Hussain S, Howitt JA. 2006. Interaction of the guidance molecule Slit with cellular receptors. *Biochem Soc Trans*. 34:418–421.

Huminiecki L, Gorn M, Suchting S, Poulson R, Bicknell R. 2002. Magic roundabout is a new member of the roundabout receptor family that is endothelial specific and expressed at sites of active angiogenesis. *Genomics*. 79:547–552.

Islam SM, Shinmyo Y, Okafuji T, Su Y, Naser IB, Ahmed G, Zhang S, Chen S, Ohta K, Kiyonari H et al. 2009. Draxin, a repulsive guidance protein for spinal cord and forebrain commissures. *Science*. 323:388–393.

Keeble TR, Halford MM, Seaman C, Kee N, Macheda M, Anderson RB, Stacker SA, Cooper HM. 2006. The Wnt receptor Ryk is required for Wnt5a-mediated axon guidance on the contralateral side of the corpus callosum. *J Neurosci*. 26:5840–5848.

Keleman K, Rajagopalan S, Cleppien D, Teis D, Paiha K, Huber LA, Technau GM, Dickson BJ. 2002. Comm sorts robo to control axon guidance at the Drosophila midline. *Cell*. 110:415–427.

Lai Wing Sun K, Correia JP, Kennedy TE. 2011. Netrins: versatile extracellular cues with diverse functions. *Development*. 138:2153–2169.

Lohof AM, Quillan M, Dan Y, Poo MM. 1992. Asymmetric modulation of cytosolic cAMP activity induces growth cone turning. *J Neurosci*. 12:1253–1261.

Long H, Sabatier C, Ma L, Plump A, Yuan W, Ornitz DM, Tamada A, Murakami F, Goodman CS, Tessier-Lavigne M. 2004. Conserved roles for Slit and Robo proteins in midline commissural axon guidance. *Neuron*. 42:213–223.

López-Bendito G, Flames N, Ma L, Fouquet C, Di Meglio T, Chédotal A, Tessier-Lavigne M, Marín O. 2007. Robo1 and Robo2 cooperate to control the guidance of major axonal tracts in the mammalian forebrain. *J Neurosci*. 27:3395–3407.

Magara F, Muller U, Li ZW, Lipp HP, Weissmann C, Stajlar M, Wolfer DP. 1999. Genetic background changes the pattern of forebrain commissure defects in transgenic mice underexpressing the β -amyloid-precursor protein. *Proc Natl Acad Sci USA*. 96:4656–4661.

Marillat V, Cases O, Nguyen-Ba-Charvet KT, Tessier-Lavigne M, Sotelo C, Chédotal A. 2002. Spatiotemporal expression patterns of slit and robo genes in the rat brain. *J Comp Neurol*. 442:130–155.

Métin C, Deléglise D, Serafini T, Kennedy TE, Tessier-Lavigne M. 1997. A role for netrin-1 in the guidance of cortical efferents. *Development*. 124:5063–5074.

Mortimer D, Pujic Z, Vaughan T, Thompson AW, Feldner J, Vetter I, Goodhill GJ. 2010. Axon guidance by growth-rate modulation. *Proc Natl Acad Sci USA*. 107:5202–5207.

- Mortimer D, Feldner J, Vaughan T, Vetter I, Pujic Z, Rosoff WJ, Burrage K, Dayan P, Richards LJ, Goodhill GJ. 2009. Bayesian model predicts the response of axons to molecular gradients. *Proc Natl Acad Sci USA*. 106:10296–10301.
- Niquille M, Garel S, Mann F, Hornung JP, Otsmane B, Chevalley S, Parras C, Guillemot F, Gaspar P, Yanagawa Y et al. 2009. Transient neuronal populations are required to guide callosal axons: a role for semaphorin 3C. *PLoS Biol*. 7:e1000230.
- Paul LK, Brown WS, Adolphs R, Tyska JM, Richards LJ, Mukherjee P, Sherr EH. 2007. Agenesis of the corpus callosum: genetic, developmental and functional aspects of connectivity. *Nat Rev Neurosci*. 8:287–299.
- Piper M, Plachez C, Zalucki O, Fothergill T, Goudreau G, Erzurumlu R, Gu C, Richards LJ. 2009. Neuropilin 1-Sema signaling regulates crossing of cingulate pioneering axons during development of the corpus callosum. *Cereb Cortex*. 19 Suppl 1:i11–i21.
- Powell AW, Sassa T, Wu Y, Tessier-Lavigne M, Polleux F. 2008. Topography of thalamic projections requires attractive and repulsive functions of Netrin-1 in the ventral telencephalon. *PLoS Biol*. 6:e116.
- Pujic Z, Giacomantonio CE, Unni D, Rosoff WJ, Goodhill GJ. 2008. Analysis of the growth cone turning assay for studying axon guidance. *J Neurosci Methods*. 170:220–228.
- Rajasekharan S, Kennedy TE. 2009. The netrin protein family. *Genome Biol*. 10:239.
- Rash BG, Richards LJ. 2001. A role for cingulate pioneering axons in the development of the corpus callosum. *J Comp Neurol*. 434:147–157.
- Ren T, Zhang J, Plachez C, Mori S, Richards LJ. 2007. Diffusion tensor magnetic resonance imaging and tract-tracing analysis of Probst bundle structure in Netrin1- and DCC-deficient mice. *J Neurosci*. 27:10345–10349.
- Richards LJ, Koester SE, Tuttle R, O'Leary DDM. 1997. Directed growth of early cortical axons is influenced by a chemoattractant released from an intermediate target. *J Neurosci*. 17:2445–2458.
- Rosoff WJ, Urbach JS, Esrick MA, McAllister RG, Richards LJ, Goodhill GJ. 2004. A new chemotaxis assay shows the extreme sensitivity of axons to molecular gradients. *Nat Neurosci*. 7:678–682.
- Sabatier C, Plump AS, Ma L, Brose K, Tamada A, Murakami F, Lee EY, Tessier-Lavigne M. 2004. The divergent Robo family protein rig-1/Robo3 is a negative regulator of slit responsiveness required for midline crossing by commissural axons. *Cell*. 117:157–169.
- Seeger M, Tear G, Ferres-Marco D, Goodman CS. 1993. Mutations affecting growth cone guidance in *Drosophila*: genes necessary for guidance toward or away from the midline. *Neuron*. 10:409–426.
- Serafini T, Colamarino SA, Leonardo ED, Wang H, Beddington R, Skarnes WC, Tessier-Lavigne M. 1996. Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell*. 87:1001–1014.
- Serafini T, Kennedy TE, Galko MJ, Mirzayan C, Jessell TM, Tessier-Lavigne M. 1994. The netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans* UNC-6. *Cell*. 78:409–424.
- Shu T, Puche AC, Richards LJ. 2003. Development of midline glial populations at the corticoseptal boundary. *J Neurobiol*. 57:81–94.
- Shu T, Richards LJ. 2001. Cortical axon guidance by the glial wedge during the development of the corpus callosum. *J Neurosci*. 21:2749–2758.
- Shu T, Sundaresan V, McCarthy MM, Richards LJ. 2003. Slit2 guides both precrossing and postcrossing callosal axons at the midline in vivo. *J Neurosci*. 23:8176–8184.
- Shu T, Valentino KM, Seaman C, Cooper HM, Richards LJ. 2000. Expression of the netrin-1 receptor, deleted in colorectal cancer (DCC), is largely confined to projecting neurons in the developing forebrain. *J Comp Neurol*. 416:201–212.
- Skarnes WC, Moss JE, Hurtley SM, Beddington RS. 1995. Capturing genes encoding membrane and secreted proteins important for mouse development. *Proc Natl Acad Sci USA*. 92:6592–6596.
- Stein E, Tessier-Lavigne M. 2001. Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a Robo/DCC receptor complex. *Science*. 291:1928–1938.
- Thompson AW, Pujic Z, Richards LJ, Goodhill GJ. 2011. Cyclic nucleotide-dependent switching of mammalian axon guidance depends on gradient steepness. *Mol Cell Neurosci*. 47:45–52.
- Unni DK, Piper M, Moldrich RX, Gobius I, Liu S, Fothergill T, Donahoo AL, Baisden JM, Cooper HM, Richards LJ. 2012. Multiple Slits regulate the development of midline glial populations and the corpus callosum. *Dev Biol*. 365:36–49.
- Weaver CM, Pinezich JD, Lindquist WB, Vazquez ME. 2003. An algorithm for neurite outgrowth reconstruction. *J Neurosci Methods*. 124:197–205.
- Yang L, Garbe DS, Bashaw GJ. 2009. A frazzled/DCC-dependent transcriptional switch regulates midline axon guidance. *Science*. 324:944–947.
- Yu TW, Hao JC, Lim W, Tessier-Lavigne M, Bargmann CI. 2002. Shared receptors in axon guidance: SAX-3/Robo signals via UNC-34/enabled and a Netrin-independent UNC-40/DCC function. *Nat Neurosci*. 5:1147–1154.
- Zhang C, Gao J, Zhang H, Sun L, Peng G. 2012. Robo2-Slit and Dcc-Netrin1 coordinate neuron axonal pathfinding within the embryonic axon tracts. *J Neurosci*. 32:12589–12602.
- Zhao H, Maruyama T, Hattori Y, Sugo N, Takamatsu H, Kumanogoh A, Shirasaki R, Yamamoto N. 2011. A molecular mechanism that regulates medially oriented axonal growth of upper layer neurons in the developing neocortex. *J Comp Neurol*. 519:834–848.