

The Netrin 1 Receptors *Unc5h3* and *Dcc* Are Necessary at Multiple Choice Points for the Guidance of Corticospinal Tract Axons

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Migrating axons require the correct presentation of guidance molecules, often at multiple choice points, to find their target. Netrin 1, a bifunctional cue involved in both attracting and repelling axons, is involved in many cell migration and axon pathfinding processes in the CNS. The netrin 1 receptor DCC and its *Caenorhabditis elegans* homolog UNC-40 have been implicated in directing the guidance of axons toward netrin sources, whereas the *C. elegans* UNC-6 receptor, UNC-5 is necessary for migrations away from UNC-6. However, a role of vertebrate UNC-5 homologs in axonal migration has not been demonstrated. We demonstrate that the *Unc5h3* gene product, shown previously to regulate cerebellar granule cell migrations, also controls the guidance of the corticospinal tract, the major tract responsible for coordination of limb movements. Further-

more, we show that corticospinal tract fibers respond differently to loss of UNC5H3. In addition, we observe corticospinal tract defects in mice homozygous for a spontaneous mutation that truncates the *Dcc* transcript. Postnatal day 0 *netrin 1* mutant mice also demonstrate corticospinal tract abnormalities. Last, interactions between the *Dcc* and *Unc5h3* mutations were observed in gene dosage experiments. This is the first evidence of an involvement in axon guidance for any member of the vertebrate *unc-5* family and confirms that both the cellular and axonal guidance functions of *C. elegans unc-5* have been conserved in vertebrates.

Key words: dorsal funiculus; mouse; spinal cord; pyramidal decussation; rcm; P3 domain

Development of the CNS depends on correct cellular migrations and axonal pathfinding to establish connectivity. Although axons navigate pathways that can be complex and cover large distances, their routes are simplified through intermediate targets, termed choice points (Stoeckli and Landmesser, 1998). As an axon encounters successive choice points during pathfinding, it responds to different sets of molecules. These guidance molecules provide attractive and repulsive signals to specific receptors present on the growth cone. For example, the *Caenorhabditis elegans* bifunctional cue UNC-6 guides axon migrations by attractive or repulsive mechanisms depending on the response of its receptor (Hedgecock et al., 1990; Wadsworth et al., 1996; Culotti and Merz, 1998). Cells and axons expressing the UNC-6 receptor UNC-5 are repelled by an UNC-6 gradient (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992; Wadsworth et al., 1996). In

contrast, UNC-6 is predominantly attractive for cells and axons expressing the UNC-40 receptor (Hedgecock et al., 1990; Chan et al., 1996).

In vertebrates, similar mechanisms are involved in commissure formation. Spinal cord commissural axons expressing the vertebrate *unc-40* homolog *Dcc* are attracted ventrally toward regions of *netrin 1* (*Ntn1*) expression in the floor plate (Kennedy et al., 1994; Keino-Masu et al., 1996). On reaching the midline, additional cues are necessary for axons to cross the midline, such as axonin-1 (contactin 2) and NrCAM (Stoeckli and Landmesser, 1998). Another receptor molecule, Roundabout (Robo), is postulated to be required on the axons to prevent them from recrossing the midline, presumably by repulsion from Slit expressed at the midline (Li et al., 1999). Thus, the migration route of axons appears broken down into steps, each of which requires a particular set of molecules.

Here we show that in addition to its role in controlling the migrations of cerebellar neurons (Ackerman et al., 1997; Leonardo et al., 1997), *Unc5h3* also plays a role in axonal guidance, a role not demonstrated previously for the vertebrate *unc-5* genes *in vivo*. Our results indicate that *Unc5h3* controls the pathfinding of corticospinal tract (CST) axons at two distinct points, the decussation and the final turn into the dorsal funiculus. Also, we describe CST defects in mice homozygous for a newly identified mutant allele of *Dcc*, *Dcc^{kanga}*, which, unlike the targeted mutant allele, survives to adulthood. Furthermore, we demonstrate that aberrant axonal projections in *Unc5h3^{rcm}* mutant mice are modulated by mutations in the *Dcc* gene. Last, we show that *Ntn1* mutant mice have defects in the pyramidal decussation and dorsal funiculus, demonstrating a role for this guidance cue in the control of CST axons.

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MATERIALS AND METHODS

Mice. The *Unc5h3^{rcm}* (*Unc5h3^{rcm}Tg(Ucp)1.23Kz*), *Dcc^{tm1Wbg}*, and *Ntn1* (*Ntn1^{Gt(pGTL1.8TM)629Wcs}*) mutations were maintained on C57BL/6J × SJL/J segregating, 129×1/SvJ, and C57BL/6J backgrounds, respectively (Serafini et al., 1996; Ackerman et al., 1997; Fazeli et al., 1997). *Dcc^{kanga}* mice arose in a C.AKR-Tgn^{coq}/+ research mouse colony at The Jackson Laboratory, and the Tgn^{coq} mutation has been segregated out of the line. Mutant mice were obtained from heterozygous crosses and were identified by either PCR or their abnormal gait; *Ntn1/Ntn1* mice were identified by the absence of corpus callosum and anterior commissure in forebrain sections (Serafini et al., 1996).

Immunohistology. Adult mice were anesthetized with tribromoethanol and intracardially perfused with PBS followed by cold 4% paraformaldehyde (PFA) in PBS. After overnight incubation at 4°C, brains and spinal cords were dissected and rinsed in PBS. Brains and spinal cords of newborn mice were immersed in either 4% PFA (anti-DCC and anti-neurofilament) or Carnoy's fixative (60% methanol, 30% chloroform, and 10% acetic; anti-neurofilament only) for 24 hr. After processing in ethanol or sucrose, the tissue was embedded in either OCT (Tissue-Tek, Torrance, CA) at -20°C or paraffin and sectioned at 7–12 μm.

For immunofluorescence studies with mouse monoclonal anti-calcium/calmodulin (CaM) kinase II antibody (Oncogene Research Products, San Diego, CA), antigen retrieval was performed on paraffin sections by microwaving slides in 0.01 M sodium citrate, pH 6, for 8 min. After boiling, the slides were cooled to room temperature in this solution for 30 min, washed in PBS, and incubated in the anti-CaM kinase II antibody (1:100) overnight at 4°C before detection with Cy3-labeled goat anti-mouse secondary antibody (1:100; Chemicon, Temecula, CA). For studies with mouse monoclonal anti-DCC (Novocastra) and anti-neurofilament antibody 2H3 (Developmental Studies Hybridoma Bank, Iowa City, IA), antigen retrieval was performed as described above. Sections were washed in Tris-buffered saline and incubated in anti-DCC (1:30) or anti-neurofilament (1:50) overnight at 4°C before detection with biotin-labeled goat anti-mouse IgG, secondary antibody (1:500; Southern Biotechnology Associates, Inc., Birmingham, AL) and Alexa Fluor 488-labeled streptavidin (1:500; Molecular Probes, Eugene, OR). For studies with the mouse monoclonal anti-neurofilament antibody 2H3 only, paraffin sections were blocked in PBS, 0.3% Triton X-100, 3% nonfat dried milk, and 5% DMSO (PBS/T-MD) twice for 1 hr each. Primary antibody (1:50 in PBS/T-MD) was applied and incubated for 2 d at 4°C. After washes in PBS and 0.3% Triton X-100 (PBS/T) and PBS/T-MD, Cy3-conjugated goat anti-mouse secondary antibody (1:500 in PBS/T-MD) was applied and incubated overnight at 4°C. Slides were mounted using either Slow Fade Light Anti-Fade media (Molecular Probes) or Fluoromount G (Electron Microscopy Sciences, Ft. Washington, PA). All images were captured from a Leitz (Wetzlar, Germany) DMRXE microscope with the SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI), an Olympus Optical (Tokyo, Japan) BX50 microscope with a Retiga 1300 camera (Optical Analysis Corp., Nashua, NH) or a Leitz TCSNT confocal microscope (Leica, Bannockburn, IL).

Biotin dextran amine tract tracing. All animal procedures were approved by the Animal Care and Use Committee at The Jackson Laboratory. *Unc5h3^{rcm}/Unc5h3^{rcm}* ($n = 5$) and *Dcc^{kanga}/Dcc^{kanga}* ($n = 6$) animals and their corresponding wild-type controls ($n = 6$), were anesthetized with tribromoethanol, and 3 holes were made in the skull over the right cerebral hemisphere ~1–2 mm apart, using a handheld micro-drill with a 1 mm dental drill bit (Fine Science Tools, North Vancouver, British Columbia, Canada). One microliter of 15% biotin dextran amine (BDA; Molecular Probes) was injected at a depth of 1 mm using a 32 gauge needle attached to a 5 μl Hamilton (Reno, NV) syringe. After 7–10 d, the animals were anesthetized and intracardially perfused with 4% PFA in PBS. The dissected brains and spinal cords were fixed overnight, rinsed in PBS, and allowed to sink in 20% sucrose in PBS before embedding in OCT medium at -20°C for cryosectioning.

Serial sections (10 μm) were mounted on slides coated with poly-L-lysine (Sigma, St. Louis, MO). Sections were air-dried at room temperature for 1 hr, fixed in acetone for 10 min, and air-dried again for 15 min. Sections were washed in PBS/T and incubated in blocking solution (PBS/T and 2% BSA) for 30 min. Streptavidin-Cy3 (Jackson ImmunoResearch, West Grove, PA) was applied at a concentration of 0.5 μg/ml and incubated overnight at 4°C. To assess the injection quality, the forebrains of injected animals were cryosectioned (10 μm), and every third cryosection was stained with streptavidin-Cy3. Slides were mounted using Slow Fade Light Anti-Fade media, and images were captured as above.

In situ hybridization. Postnatal day 0 (P0) brains were fixed overnight at 4°C in 4% PFA in PBS. The paraffin-embedded tissues were sectioned and mounted on Plus slides (Fisher Scientific, Springfield, NJ). Prehybridization washes, hybridization, and posthybridization washes were performed as described previously using ³³P-labeled *Unc5h3* and *Ntn1* antisense and sense riboprobes (Przyborski et al., 1998). Sections were counterstained with hematoxylin.

Southern blot analysis and reverse transcription-PCR. Genomic DNA (15 μg) was digested by *Eco*RI and transferred to a nylon filter by standard methods (Osmonics Inc., Minnetonka, MN). Hybridization was performed at 65°C with a [³²P]dCTP-labeled probe corresponding to bp 3149–4266 of the *Dcc* coding region.

Total or poly(A⁺) RNA was isolated from adult brain (1 μg), and cDNA was prepared using random hexamers and Superscript reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For PCR, 2 μl of the reverse transcription (RT) reaction was incubated with buffer, dNTPs, 400 nM primers, and ExTaq polymerase (TaKaRa, Tokyo, Japan) according to the manufacturer's directions. Exon structure was based on the human genomic sequence of *Dcc* (Cho et al., 1994; Cooper et al., 1995). Primer sequences were designed from the *Dcc* coding sequence (GenBank accession number NM_007831 (Cooper et al., 1995): exon 26, 5'-GCCATCCCTGTACCAACACTAGAAAG-3' (bp 3748–3773); exon 27, 5'-CTGGCTGAGACAAAAGCGGT-3' (bp 4111–4092); exon 28, 5'-TGGCTGGATCCTCTGTGGGCT-3' (bp 4252–4232); and exon 29, 5'-TAAAAGGCTGAGCCTGTGATGG-3' (bp 4344–4322). For genomic PCR, 100 ng of DNA was used in standard PCRs using a 400 nM concentration of each primer corresponding to the intron sequence on either side of exon 29: forward primer, 5'-AGCTACCAAGACCTCATCCTGAC-3'; and reverse primer, 5'-GCAGTGACAAAGACCCAGAACTATG-3'.

RESULTS

The CST is absent from the dorsal funiculus of the spinal cord in *Unc5h3^{rcm}/Unc5h3^{rcm}* mice

The *C. elegans* UNC-5 and UNC-40 receptors mediate UNC-6-dependent migrations of both axons and cells during development of the nervous system (Hedgecock et al., 1990). Similarly, the vertebrate homologs of *unc-6* and *unc-40*, *Ntn1* and *Dcc*, are necessary for the migration of axons of several commissures and neurons of the pontine nuclei (Serafini et al., 1996; Fazeli et al., 1997). Although the *unc-5* homolog *Unc5h3* is necessary for proper granule cell migration during cerebellar formation (Ackerman et al., 1997; Przyborski et al., 1998), the role of the vertebrate UNC-5 homologs in the guidance of axons during CNS development has not been established. Thus, we examined several axon tracts known to be under netrin 1 and DCC guidance in *Unc5h3^{rcm}/Unc5h3^{rcm}* mice.

Examination of the corpus callosum and anterior and hippocampal commissures revealed no apparent defects in *Unc5h3^{rcm}/Unc5h3^{rcm}* brains (data not shown), nor were obvious defects in commissural axons observed in transverse sections of spinal cord in mutant mice. However, a dramatic reduction of the size of the dorsal funiculus was seen in *Unc5h3^{rcm}* mutant mice (Fig. 1A,B). The dorsal funiculus is composed of three axonal tracts: the ascending gracilis and cuneatus tracts, which enter the spinal cord from the hindlimbs and forelimbs, respectively, and the descending corticospinal tract, which resides in the ventral-most portion of the dorsal funiculus (Joosten, 1990; Stanfield, 1992). When compared with that of wild-type mice, the dorsal funiculus appeared shorter and thinner at the ventral aspect, suggesting CST defects in *Unc5h3^{rcm}/Unc5h3^{rcm}* mice.

To more closely examine the CST in mutant animals, immunohistochemistry was performed using an antibody against CaM kinase II, which is expressed on the CST but not the ascending tracts, in the dorsal funiculus (Terashima et al., 1994). Compared with the wild type, there were almost no axons expressing CaM kinase II in the *Unc5h3^{rcm}/Unc5h3^{rcm}* dorsal funiculus, confirming the absence of the CST in these animals (Fig. 1C,D). This

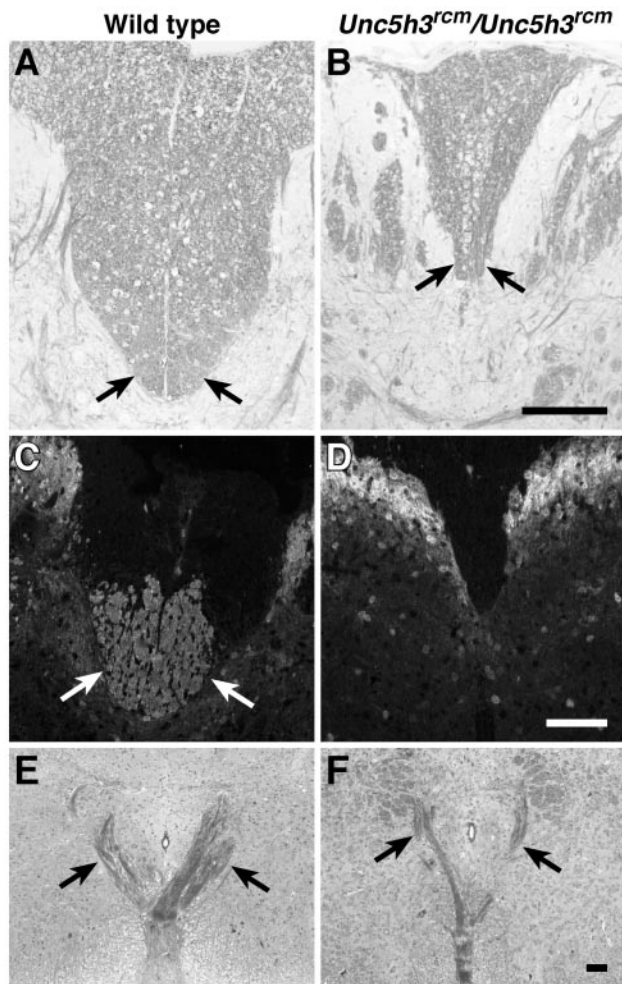


Figure 1. CST abnormalities in *Unc5h3^{rcm}/Unc5h3^{rcm}* mice. The dorsal funiculus (arrows) is shown in transverse sections of cervical spinal cord from wild-type (A, C) and *Unc5h3^{rcm}/Unc5h3^{rcm}* (B, D) mice stained with LFB and counterstained with cresyl violet (A, B) or antibody against CaM kinase II (C, D). The ipsilateral ventral pyramidal tract crosses the midline dorsally, forming the pyramidal decussation (E, wild type; F, *Unc5h3^{rcm}/Unc5h3^{rcm}*). Note the thinner decussation of pyramidal tract fibers (arrows) in *Unc5h3^{rcm}/Unc5h3^{rcm}* brain, and the CST is missing from the dorsal funiculus of the mutant spinal cord. Scale bars, 100 μ m.

defect was also observed in two other mutant alleles of *Unc5h3* (data not shown).

CST axons originate from layer V neurons in the cerebral cortex and pass through the internal capsule and over the pontine nuclei in the pons, becoming the distinct ventral ipsilateral bundles constituting the pyramidal tract in the hindbrain (Stanfield, 1992; Gianino et al., 1999). Just before the spinal cord, the ventral CST fibers cross dorsally over the midline, forming the pyramidal decussation, and enter the spinal cord, continuing down the cord as the ventral-most tract in the dorsal funiculus of the spinal cord. The pyramidal tract was examined in adult wild-type and *Unc5h3^{rcm}/Unc5h3^{rcm}* hindbrains stained with the myelin stain Luxol fast blue (LFB). No apparent differences between wild-type and mutant animals were observed in this tract as it passed through the hindbrain (data not shown). The pyramidal decussation, although thinner than that of the wild type, was also present in *Unc5h3^{rcm}/Unc5h3^{rcm}* mice (Fig. 1E,F), but the CST axons could not be traced further in LFB-stained sections. Therefore,

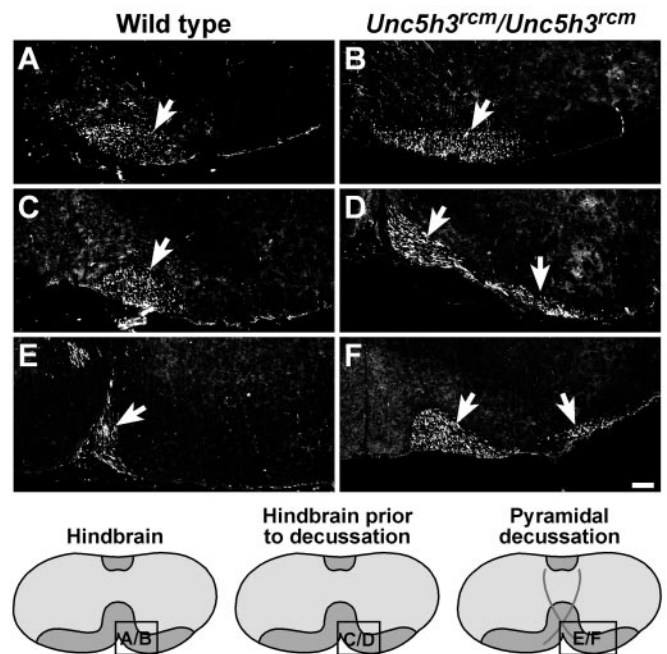


Figure 2. The migration of the CST through the *Unc5h3^{rcm}/Unc5h3^{rcm}* hindbrain. BDA injected into the motor cortex of wild-type (A, C, E) and *Unc5h3^{rcm}/Unc5h3^{rcm}* (B, D, F) animals was visualized with streptavidin-Cy3. The trajectories of the ipsilateral pyramidal tract (arrow) appear similar in both wild-type (A) and *Unc5h3^{rcm}/Unc5h3^{rcm}* (B) mice through the hindbrain before the pyramidal decussation. Just before the pyramidal decussation (C, D), the pyramidal tract broadens (arrows) in *Unc5h3^{rcm}/Unc5h3^{rcm}* mice, whereas the labeled fibers in the wild-type brain remain bundled (arrow). At the pyramidal decussation, the mutant tract splits (arrows) into medial and lateral fiber bundles (F). The relative level of sections is shown in the accompanying diagrams at the bottom. Scale bars, 100 μ m.

the CST disruption in these mice appears to be, at least in part, below the level of the pyramidal decussation.

***Unc5h3^{rcm}* mutant CST axons follow two abnormal trajectories**

To visualize the path of CST axons we performed anterograde labeling of *Unc5h3^{rcm}/Unc5h3^{rcm}* and wild-type control adult animals. BDA was injected unilaterally into the motor cortex, and 1 week after surgery, brains and spinal cords were dissected and serially sectioned. Examination of coronal sections from BDA-injected mutant and wild-type animals confirmed that the pyramidal tract traversed the hindbrain normally in mutant animals (Fig. 2A,B). However, just before the decussation in the ventral region of the hindbrain of *Unc5h3^{rcm}* mutant animals, the pyramidal tract broadened, spreading from medial to lateral (Fig. 2C,D). In wild-type controls, broadening of the pyramidal tract was not observed, and at the level of the pyramidal decussation, the labeled CST axons moved dorsally, crossing the midline (Fig. 2E). In contrast, at the decussation of *Unc5h3^{rcm}* mutant mice, two distinct bundles formed, with one bundle in its normal position close to the midline and another axon bundle located in a slightly lateral position (Fig. 2F).

Once achieving the dorsal contralateral gray matter, decussated fibers curved into the ventral-most portion of the dorsal funiculus of the wild-type spinal cord (Fig. 3A). Tracing of the more medial bundle of axons in the *Unc5h3^{rcm}/Unc5h3^{rcm}* hindbrain confirmed that, as seen in LFB-stained sections, pyramidal tract axons crossed to the contralateral side (data not shown). However, in

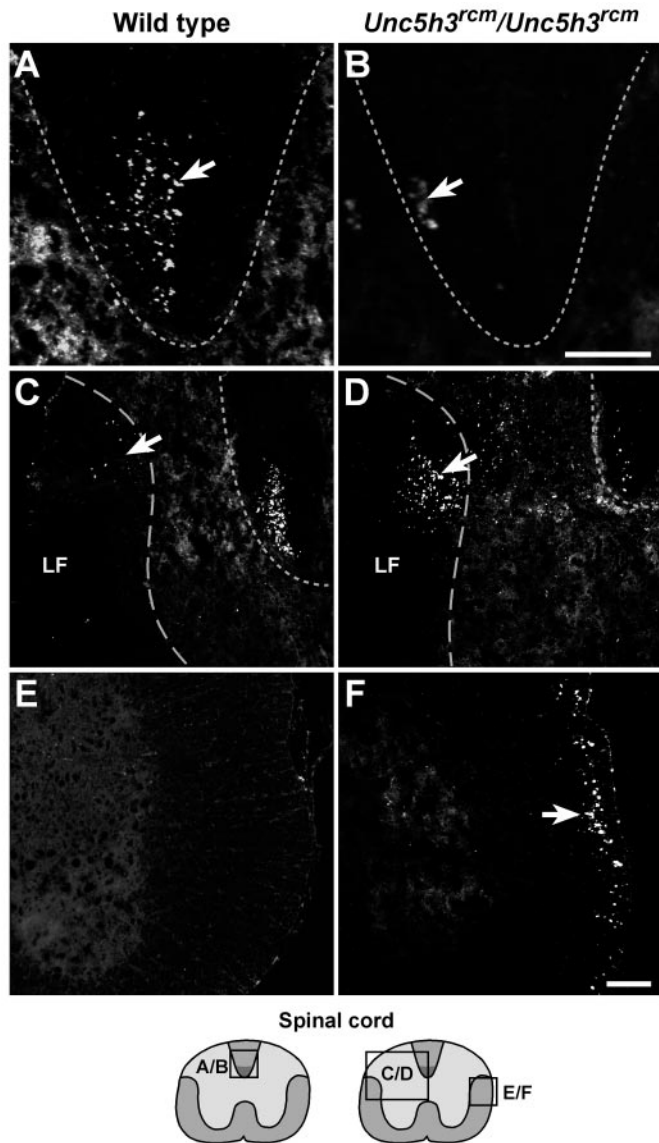


Figure 3. Aberrant CST axon migration in *Unc5h3^{rcm}/Unc5h3^{rcm}* spinal cord. BDA injected into the motor cortex of wild-type (*A*, *C*, *E*) and *Unc5h3^{rcm}/Unc5h3^{rcm}* (*B*, *D*, *F*) animals was visualized with streptavidin-Cy3. Labeled contralateral CST fibers (arrow) are present in the wild-type (*A*) dorsal funiculus (dotted line), whereas only a few labeled fibers are seen in the dorsal funiculus of *Unc5h3^{rcm}/Unc5h3^{rcm}* animals (*B*, arrow). In contrast to the wild-type spinal cord, many labeled axons are visible in the contralateral lateral funiculus (*C*, *D*, elongated dotted line, arrows) and the dorsal gray matter at the thoracic level of the spinal cord of *Unc5h3^{rcm}/Unc5h3^{rcm}* mice. The ipsilateral lateral funiculus of the wild-type cervical spinal cord (*E*) does not contain labeled CST fibers, whereas many labeled ipsilateral CST fibers move from the ventral pyramidal tract into the ipsilateral lateral funiculus of the *Unc5h3^{rcm}/Unc5h3^{rcm}* spinal cord (*F*, arrow). The relative level of sections is shown in the accompanying diagrams at the bottom. LF, Contralateral lateral funiculus. Scale bars, 100 μ m.

contrast to the wild-type CST mice, only a few decussated *Unc5h3^{rcm}/Unc5h3^{rcm}* CST axons were funneled correctly into the contralateral dorsal funiculus, as evidenced by a small amount of staining in the ventral region of the dorsal funiculus of the cervical spinal cord (Fig. 3*B*). Therefore, most CST fibers that cross the midline in these mutant animals do not enter the dorsal funiculus.

Many decussated labeled axons were observed in the dorsal gray matter adjacent to the dorsal funiculus and just into the white matter of the contralateral lateral funiculus of *Unc5h3^{rcm}/Unc5h3^{rcm}* cervical and thoracic spinal cord, whereas only a few labeled fibers were seen in the lateral white matter of cervical and thoracic spinal cord sections of wild-type controls (Fig. 3*C,D*). These axons in wild-type mice likely comprise a minor proportion of CST fibers (<5%) that normally split from the main bundle after decussating and instead of entering the dorsal funiculus enter the gray matter of upper cervical spinal cord sections directly (Gianino et al., 1999). However, it is not clear whether this normal pathway of a small percentage of fibers is acting as a permissive default substrate for the aberrant *Unc5h3^{rcm}* mutant fibers.

As mentioned above, in *Unc5h3^{rcm}/Unc5h3^{rcm}* mice, a significant proportion of labeled CST axons split from the pyramidal tract just before decussating. These fibers continued to follow an ipsilateral trajectory into the cervical spinal cord, whereas in wild-type mice, this split in the pyramidal tract was not seen, and all of the visible fibers crossed the midline (Fig. 3*E,F*). Although the mutant fibers remained ipsilateral, they did not stay ventral but eventually entered the lateral funiculus of the spinal cord, which was not observed in the ipsilateral lateral white matter in wild-type animals (Fig. 3*E,F*). These *Unc5h3^{rcm}/Unc5h3^{rcm}* ipsilateral CST axon fibers were positioned in the outermost region of the lateral funiculus of the spinal cord, away from the spinal gray matter.

To determine whether *Unc5h3* is expressed in cortical neurons that generate CST axons, *in situ* hybridization was performed on the presumptive motor cortex at embryonic day 17.5 (E17.5) and P0 (a time when leading axons have entered the cervical spinal cord). *Unc5h3* mRNA was widely expressed in the wild-type cortex, with highest levels in the cortical plate. Low levels of expression were also noted in other regions of the cortex, including layer V, that were not seen in the sense control (Fig. 4*A–C*; data not shown).

Expression of another netrin 1 receptor, DCC, was also detected throughout the wild-type E17.5 and P0 cortex by immunofluorescence (Fig. 4*D,E*; data not shown). However, we were not able to detect DCC-positive CST fibers as they passed through the hindbrain of E17.5 or P0 animals (Fig. 4*F–H*; data not shown).

Dcc^{kanga}*: a spontaneous mutant allele of *Dcc

Because of the perinatal lethality of the recessive *Dcc*-targeted mutant allele, it has not been possible to study the role of *Dcc* in structures that develop postnatally (Fazeli et al., 1997). However, this difficulty has been circumvented by our identification of a spontaneous recessive mutation of *Dcc* that survives into adulthood.

Adult mice homozygous for a spontaneous mutation that results in the mild to severe inability to maintain an upright position were identified in a production colony. These mice often move their hind legs in a concerted manner, resulting in a somewhat hopping gait; thus this mutation was named *kanga*. Analysis of brains of these mice demonstrated that both the corpus callosum and the anterior commissure were missing (Fig. 5*A,B*). In addition, the pontine nuclei were absent from the hindbrain (data not shown).

A genome scan using polymorphic microsatellite markers was performed on affected F2 animals obtained by intercrossing the F1 progeny from a mapping cross (BALB/cBy.Akr affected \times

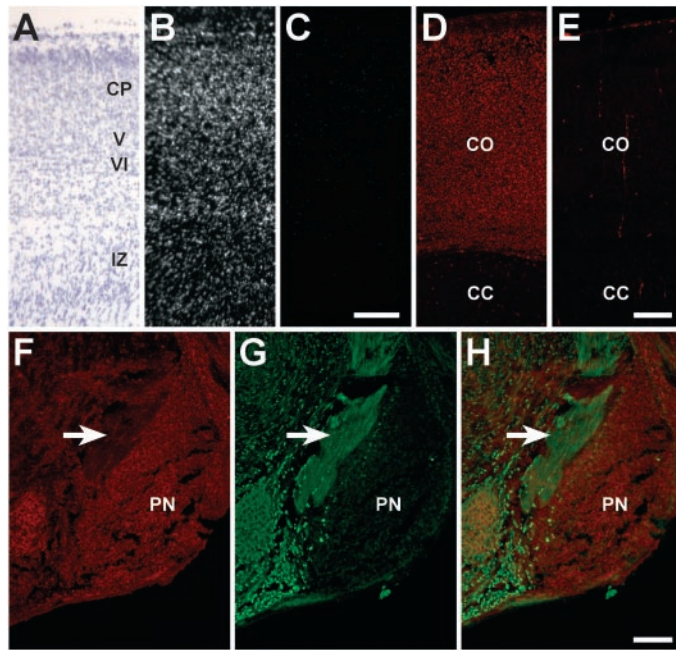


Figure 4. *Unc5h3* and DCC expression at P0. *In situ* hybridization was performed with *Unc5h3*-specific antisense and sense probes on sagittal sections of P0 wild-type forebrain (A–C). At P0, the neurons of the cortical plate and presumptive layers V and VI express the *Unc5h3* transcript (B). A corresponding bright-field photograph of the sections is shown in A, and the *Unc5h3*-specific sense control showing no signal is shown in C. Immunofluorescence with monoclonal anti-DCC antibody and secondary and tertiary antibody control (without primary antibody) on sagittal sections of P0 wild-type forebrain (D, E) shows that DCC is expressed throughout the cortex (CO). DCC immunofluorescence on sagittal sections of P0 wild-type hindbrain (F, H, overlay) revealed very few DCC-positive fibers. The presumptive CST axons (arrow) were detected by immunofluorescence with antibody to the 160 kDa neurofilament on neighboring sections (G, H, overlay). CC, Corpus callosum; CO, cortex; CP, cortical plate; IZ, intermediate zone; PN, pontine nuclei; V, layer V; VI, layer VI. Scale bars, 100 μ m.

C57BL/6J). Analysis of these results demonstrated linkage of the *kanga* gene with markers *D18Mit9* and *D18Mit123* on chromosome 18 near the previously reported position of the *Dcc* gene (Justice et al., 1992; Stassen et al., 1996). The similarity of brain defects in *kanga* mutant animals to those reported for the targeted allele of *Dcc* (Fazeli et al., 1997), combined with the map position of *kanga*, suggested that *kanga* was a mutant allele of *Dcc*. Complementation tests were performed by crossing mice heterozygous for the targeted *Dcc* allele (*Dcc^{tm1Wbg}*) and *kanga* heterozygotes. Offspring were scored for ataxia at weaning. Ataxic animals were obtained at a frequency of 12% (9 of 75), and *kanga* was renamed *Dcc^{kanga}*. Histological analysis of affected animals from the complementation test confirmed that like *Dcc^{tm1Wbg}* mutant mice, the corpus callosum and hippocampal commissure were missing in the forebrain (Fig. 5C).

Southern blot analysis of wild-type and *Dcc^{kanga}/Dcc^{kanga}* genomic DNA revealed restriction fragment length polymorphisms (RFLPs) within the *Dcc* gene, suggesting a rearrangement in the *Dcc^{kanga}* genome (Fig. 5D). To further analyze the mutation in the *Dcc^{kanga}* allele, RT-PCR was performed using *Dcc* coding region-specific primers. No abnormalities in the length or sequence of amplified fragments through exon 26 of the *Dcc* coding sequence were observed in RT-PCR products from *Dcc^{kanga}/Dcc^{kanga}* cDNA (data not shown). However, when reac-

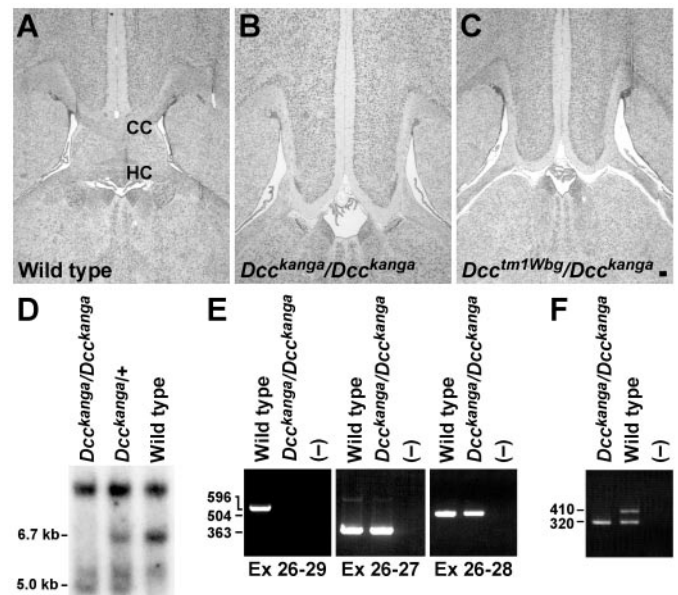


Figure 5. *kanga*, a spontaneous mutant allele of *Dcc*. Luxol fast blue- and cresyl violet-stained frontal sections through the forebrain of wild-type (A), *Dcc^{kanga}/Dcc^{kanga}* (B), and *Dcc^{tm1Wbg}/Dcc^{kanga}* (C) mice are shown. Note the absence of the corpus callosum (CC) and hippocampal commissures (HC) in the *Dcc^{kanga}/Dcc^{kanga}* and *Dcc^{tm1Wbg}/Dcc^{kanga}* forebrains. D, Southern blot of *Eco*RI-digested genomic DNA from wild-type, *Dcc^{kanga}/+*, and *Dcc^{kanga}/Dcc^{kanga}* mice probed with cDNA corresponding to a portion of the *Dcc* coding region (bp 3149–4266). Note the 5.0 and 6.7 kb RFLPs between wild-type and *Dcc^{kanga}* heterozygotes or homozygotes. E, RT-PCR of *Dcc* transcripts in *Dcc^{kanga}* mutants. *Dcc* cDNA between exon 26 through the last exon of coding region (exon 29) does not amplify from *Dcc^{kanga}/Dcc^{kanga}* (*Ex 26–29*). However, primers corresponding to exons 26 and 27 or exons 26 and 28 (*Ex 26–27*, *Ex 26–28*, respectively) do amplify products from mutant cDNA. F, PCR analysis of exon 29 using primers from the surrounding introns (410 bp) demonstrates that exon 29 is deleted in *Dcc^{kanga}/Dcc^{kanga}* genomic DNA. An unrelated fragment from chromosome 3 (320 bp) was amplified as an internal control for the PCR. Scale bar, 100 μ m.

tions were performed using primers corresponding to exons 26 and 29 (the last coding exon), products were not obtained from mutant cDNA (Fig. 5E). In reactions using the exon 26 primer with primers corresponding to exon 27 or 28, amplified products were obtained from mutant cDNA. Combined, these results suggest that *Dcc* transcripts in these mutants do not contain exon 29 (Fig. 5E). In agreement, PCRs using primers corresponding to intron sequence surrounding exon 29 failed to amplify from mutant DNA, demonstrating that this exon is deleted in *Dcc^{kanga}/Dcc^{kanga}* genomic DNA (Fig. 5F).

The CST of *Dcc^{kanga}/Dcc^{kanga}* mice is abnormal at the pyramidal decussation

A hopping gait has been reported previously in mice with CST defects (Dottori et al., 1998). Furthermore, DCC is expressed on the CST axons during extension of this tract (Shu et al., 2000). This prompted us to examine the CST of *Dcc^{tm1Wbg}/Dcc^{kanga}* mice from the complementation test and *Dcc^{kanga}/Dcc^{kanga}* mice. Anti-CaM kinase II immunohistochemical staining of transverse sections of spinal cord from adult *Dcc^{tm1Wbg}/Dcc^{kanga}* and *Dcc^{kanga}/Dcc^{kanga}* mice demonstrated that like *Unc5h3^{rcm}/Unc5h3^{rcm}* mice, the CST is absent from the dorsal funiculus of the spinal cord (Fig. 6). However, in contrast to *Unc5h3^{rcm}/Unc5h3^{rcm}* mice, in which a large number of CST axons crossed

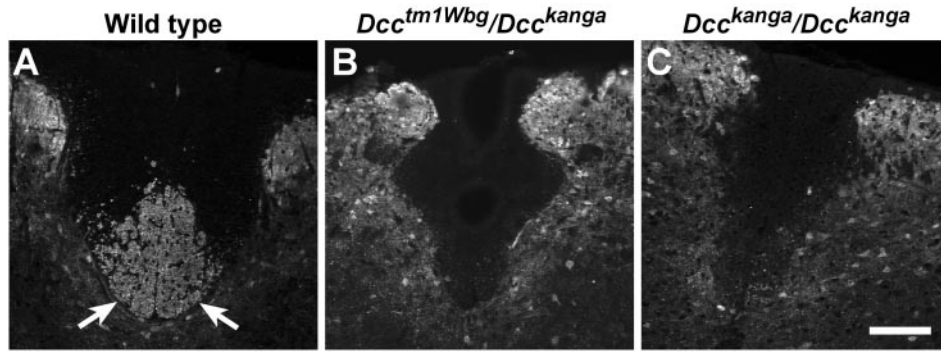


Figure 6. The CST is absent from the dorsal funiculus of the spinal cord in *Dcc^{kanga}/Dcc^{kanga}* mice. The dorsal funiculus is shown in transverse sections of cervical spinal cord from wild-type (**A**), *Dcc^{tm1Wbg}/Dcc^{kanga}* (**B**), and *Dcc^{kanga}/Dcc^{kanga}* (**C**) mice stained with antibody against CaM kinase II. CaM kinase II-positive fibers are present in the wild-type CST (arrows) but absent from the dorsal funiculus of mutant mice. Scale bar, 100 μ m.

the midline, LFB staining did not detect decussating CST fibers in the hindbrains of these mutant mice (data not shown).

Anterograde tracing of the CST was performed by unilateral injection of BDA, as described above. In the pyramidal tract, *Dcc^{kanga}/Dcc^{kanga}*-labeled CST axons resembled the BDA-injected wild-type control mice (Fig. 7*A,B*). However, similar to *Unc5h3^{rcm}* mutants, the labeled fiber bundle spread medially to laterally just before the level expected of the decussation (Fig. 7*C,D*), and this broadened tract split into medial and lateral fiber bundles (Fig. 7*E,F*). However unlike *Unc5h3^{rcm}/Unc5h3^{rcm}* mice, neither axon bundle was observed to decussate in the *Dcc^{kanga}* mutant brain (Fig. 8*A,B*), confirming our LFB staining results. Furthermore, the medial pyramidal tract fiber bundle extended ipsilaterally in the ventral funiculus until at least the upper thoracic spinal cord, and the more lateral fiber bundle moved into the ventral regions of the ipsilateral lateral funiculus in the spinal cord (Fig. 8*C,D*). These lateral labeled fibers were slightly more ventral in the lateral funiculus relative to the nondecussating *Unc5h3^{rcm}* mutant fibers. However, in both *Unc5h3^{rcm}/Unc5h3^{rcm}* and *Dcc^{kanga}/Dcc^{kanga}* mice, CST axons separated from the main CST fiber bundle to project ipsilaterally and laterally into the spinal cord. As expected from our CaM kinase II immunofluorescence results, labeled fibers were not observed in the dorsal funiculus of *Dcc^{kanga}* mutant mice (Fig. 8*E,F*). Thus, mutation of the *Dcc* gene disrupts the decussation of all CST axons, whereas *Unc5h3* mutations only affect the midline crossing of some CST axons.

Dcc modulates the position of nondecussating CST fibers in the *Unc5h3^{rcm}/Unc5h3^{rcm}* spinal cord

The presence of nondecussating CST fibers in both *Unc5h3^{rcm}* and *Dcc^{kanga}* mutant mice suggested that these genes might cooperate in guidance of CST fibers. Gene dosage experiments were performed to further investigate this possibility. Mice with a combination of *Unc5h3^{rcm}* and *Dcc^{tm1Wbg}* mutations were generated, and the position of CST fibers was determined by immunofluorescence analysis with antibodies to CaM kinase II.

In mice homozygous for the *Unc5h3^{rcm}* mutation but wild type at the *Dcc* locus, CST fibers that crossed the midline were found in the contralateral lateral funiculus, whereas those that failed to cross were found in the ipsilateral lateral funiculus but not the ventral funiculus (Figs. 3*D,F*, 9*A*). In contrast, only nondecussating fibers were observed in *Dcc^{kanga}/Dcc^{kanga}* mice in the ventral and the lateral funiculus as two separate bundles (Figs. 8*D*, 9*B*). Mice homozygous for the *Unc5h3^{rcm}* mutation and heterozygous for the *Dcc^{tm1Wbg}* mutation had CaM kinase II-positive fibers in both the lateral and ventral funiculus (Fig. 9*C*; data not shown). A few CaM kinase II-positive axons were also visible in the ventral funiculus of the cervical spinal cord of *Unc5h3^{rcm}/+*;

Dcc^{tm1Wbg}/+ mice (Fig. 9*D*); however, labeled fibers were not seen in mice heterozygous for the *Dcc^{tm1Wbg}* mutation (Fig. 9*E*). Although aberrant CST fibers were found in the ventral funiculus of the cervical spinal cord, they did not continue into the thoracic spinal cord (data not shown). Also, as demonstrated by LFB staining, some CST fibers still crossed the midline at the pyramidal decussation in *Unc5h3^{rcm}/Unc5h3^{rcm};Dcc^{tm1Wbg}/+* mice (Fig. 9*F*). These results demonstrate that *UNC5H3* and *DCC* act synergistically in the guidance of a portion of CST axons.

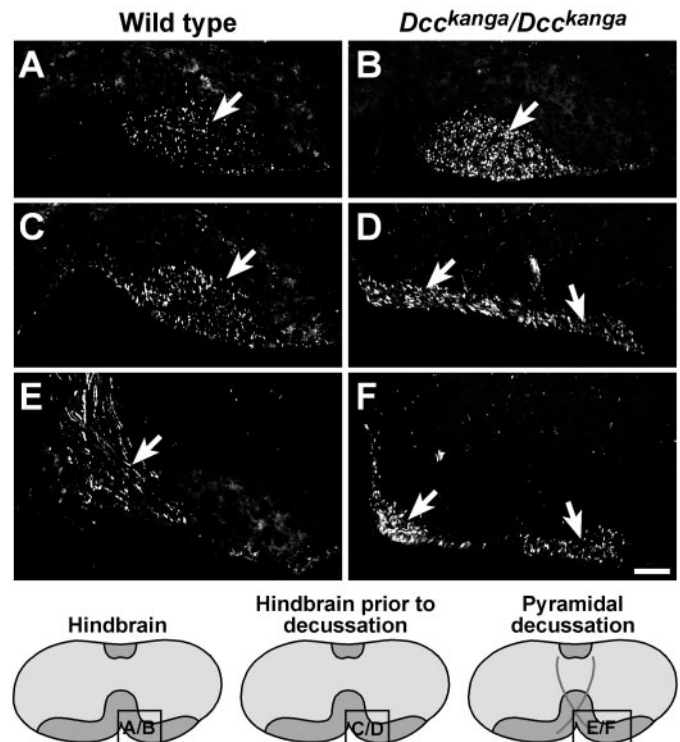


Figure 7. The CST broadens and splits in the hindbrain of *Dcc^{kanga}/Dcc^{kanga}* mice. BDA injected into the motor cortex of wild-type (**A**, **C**, **E**) and *Dcc^{kanga}/Dcc^{kanga}* (**B**, **D**, **F**) animals was visualized with streptavidin-Cy3. No differences in the placement of pyramidal tract fibers (arrow) in the hindbrain before the pyramidal decussation were noted between wild-type and mutant mice (**A**, **B**). Just before the expected level of the pyramidal decussation in *Dcc^{kanga}/Dcc^{kanga}* mice, labeled fibers broaden (arrows), whereas the labeled fibers (arrow) in the wild-type hindbrain remain bundled (**C**, **D**). At the pyramidal decussation, the *Dcc^{kanga}/Dcc^{kanga}* tract splits (arrows) into medial and lateral fiber bundles (**F**). The relative level of sections is shown in the diagrams at the bottom. Scale bars, 100 μ m.

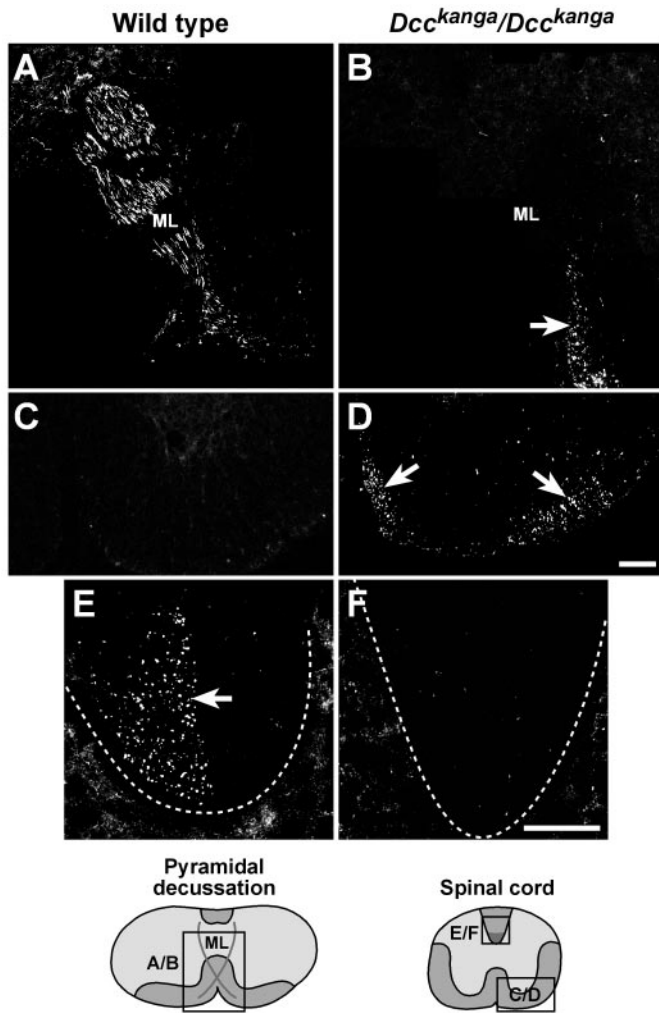


Figure 8. The CST migrates incorrectly in the spinal cord of *Dcc^{kanga}/Dcc^{kanga}* mice. BDA injected into the motor cortex of wild-type (*A*, *C*, *E*) and *Dcc^{kanga}/Dcc^{kanga}* (*B*, *D*, *F*) animals was visualized with streptavidin-Cy3. Labeled fibers cross the midline (*ML*) dorsally in the wild-type pyramidal decussation (*A*) but do not decussate in *Dcc^{kanga}/Dcc^{kanga}* mice (*B*, arrow). Two bundles of ipsilateral fibers were observed in the *Dcc^{kanga}/Dcc^{kanga}* ventral cervical spinal cord (arrows) but not the wild-type spinal cord (*C*, *D*). The dorsal funiculus (dotted lines) in the wild type (arrow) but not mutant spinal cord contains labeled contralateral CST axons (*E*, *F*). The relative level of sections is shown in the diagrams at the bottom. Scale bars, 100 μ m.

Dorsal funiculus defects in *Ntn1/Ntn1* mice

Ligand-binding and other assays suggest that both DCC and UNC5H3 are receptors for the guidance molecule netrin 1 (Keino-Masu et al., 1996; Serafini et al., 1996; Leonardo et al., 1997; Przyborski et al., 1998; Stein et al., 2001). Furthermore, *Dcc* and *Ntn1* homozygous mutant mice have similar commissural defects (Serafini et al., 1996; Fazeli et al., 1997). Thus, we investigated whether netrin 1 also mediates CST axon guidance.

In situ hybridization using an *Ntn1*-specific probe was performed on sections at the level of the pyramidal decussation, which was visualized on an adjacent section with antibody to neurofilament (Fig. 10*A*). *Ntn1* was highly expressed at the midline just ventral to the central canal and adjacent to the point at which the decussating CST axons crossed the midline (Fig. 10*B*).

In addition, we examined the CST of *Ntn1/Ntn1* mice. Because these mice die soon after birth (Serafini et al., 1996), it was not

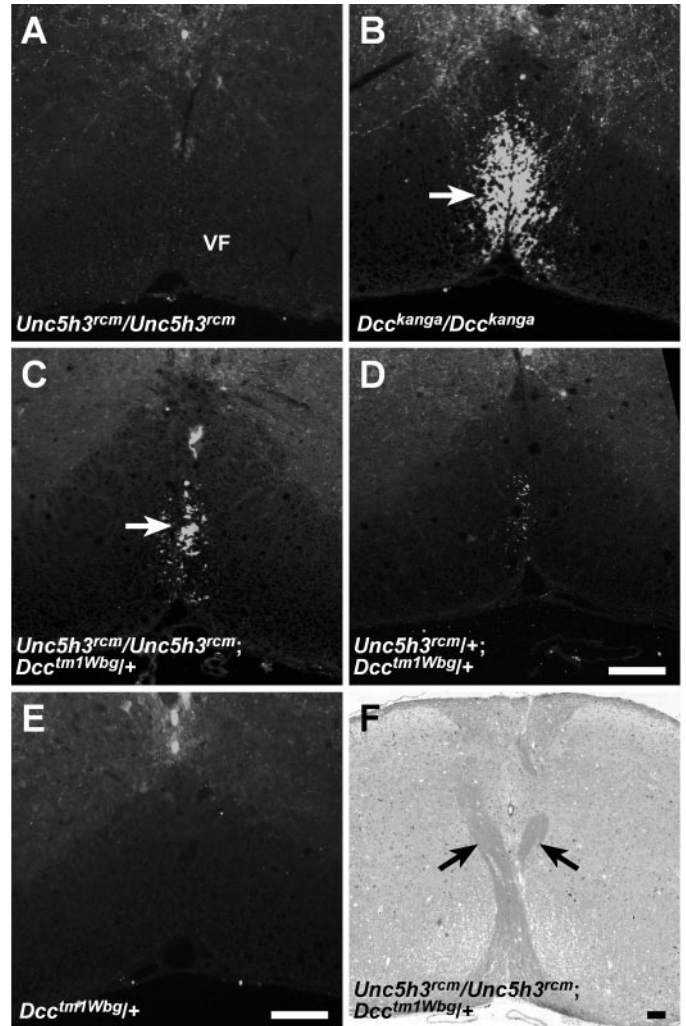


Figure 9. CST fibers are present in the ventral funiculus of *Unc5h3^{rcm}/Unc5h3^{rcm};Dcc^{tm1Wbg/+}* adult mice. Transverse sections of adult cervical spinal cord from *Unc5h3^{rcm}/Unc5h3^{rcm}* (*A*), *Dcc^{kanga}/Dcc^{kanga}* (*B*), *Unc5h3^{rcm}/Unc5h3^{rcm};Dcc^{tm1Wbg/+}* (*C*), *Unc5h3^{rcm}/+;Dcc^{tm1Wbg/+}* (*D*), and *Dcc^{tm1Wbg/+}* (*E*) mice were stained with anti-CaM kinase II antibody. Note that immunopositive fibers are present in the ventral funiculus (*VF*; arrow) of *Dcc^{kanga}/Dcc^{kanga}*, *Unc5h3^{rcm}/Unc5h3^{rcm};Dcc^{tm1Wbg/+}*, and *Unc5h3^{rcm}/+;Dcc^{tm1Wbg/+}* but not *Unc5h3^{rcm}/Unc5h3^{rcm}* and *Dcc^{tm1Wbg/+}* mice. Decussating CST fibers (arrows) are still present in *Unc5h3^{rcm}/Unc5h3^{rcm};Dcc^{tm1Wbg/+}* mice (*F*). Scale bars, 100 μ m.

possible to trace the CST using BDA. Furthermore, CaM kinase II was not expressed in the dorsal funiculus at P0, making immunohistochemistry with this antibody uninformative. Thus, to visualize the pyramidal decussation and the dorsal funiculus of the cervical spinal cord, we performed immunofluorescence staining with a monoclonal antibody to the 160 kDa isoform of neurofilament. This neurofilament isoform is expressed on descending CST fibers, which at birth have reached the midcervical level of the spinal cord (Stanfield, 1992; Gianino et al., 1999), as well as the two ascending tracts of the dorsal funiculus (Fig. 11*A*).

Analysis of hindbrain and spinal cord sections of P0 *Unc5h3^{rcm}/Unc5h3^{rcm}* mice confirmed that pyramidal tract axons decussated, but the dorsal funiculus in the cervical spinal cord appeared abnormal even in P0 animals (Fig. 11*B*). P0 *Dcc^{tm1Wbg}/Dcc^{tm1Wbg}* animals also exhibited abnormalities in the dorsal funiculus, with

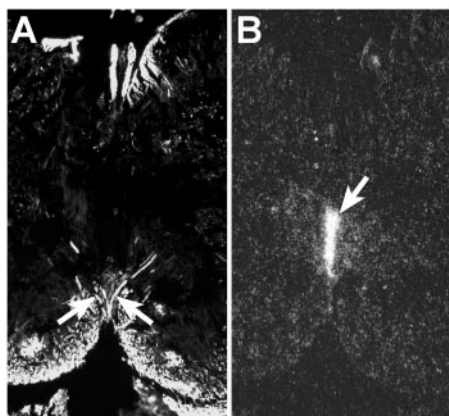


Figure 10. *Ntn1* is expressed adjacent to the midline crossover point of CST fibers at the pyramidal decussation in P0 mice. The pyramidal decussation of wild-type mice at P0 was visualized by anti-2H3 (160 kDa neurofilament isoform) antibody (*A*, arrows). *In situ* hybridization with an *Ntn1*-specific probe is shown on an adjacent section (*B*). At P0, *Ntn1* expression is observed at the midline immediately below the central canal extending ventrally (arrow) toward the pyramidal decussation. Scale bar, 100 μ m.

the expected absence of the pyramidal decussation (Fig. 11*C*; data not shown).

Similar analysis of the upper cervical spinal cord of P0 *Ntn1/Ntn1* mice demonstrated that the dorsal funiculus of these animals was clearly abnormal when compared with the wild-type controls (Fig. 11*D*). Furthermore, the examination of serial sections of the hindbrain demonstrated that the pyramidal decussation was much reduced in these mice (Fig. 11*E,F*). Because neurofilament is widely expressed in the hindbrain and spinal cord white matter, it is not clear whether these mice have the aberrant ipsilateral fibers seen in the tracing studies of *Unc5h3^{rcm}/Unc5h3^{rcm}* and *Dcc^{kanga}/Dcc^{kanga}* adult mice. However, these results demonstrate that netrin 1 also regulates CST development and may act, at least in part, via the UNC5H3 and DCC receptors.

DISCUSSION

As axons move through complicated migration pathways, they arrive at intermediate targets that act as choice points to guide their path. Our results demonstrate that netrin 1 and two of its receptors, UNC5H3 and DCC, are necessary at multiple points along the migration of the longest mammalian axonal tract, the corticospinal tract (Fig. 12). In addition to demonstrating that these molecules are necessary for CST axon guidance, our results reveal the existence of at least two groups of CST axons under different genetic regulation at the pyramidal decussation.

We describe a new mutant allele of the *Dcc* gene, *Dcc^{kanga}*, which we hypothesize is a hypomorphic allele. In contrast to the perinatal death of mice homozygous for the null *Dcc^{tm1Wbg}*-targeted allele (Fazeli et al., 1997), *Dcc^{kanga}* homozygotes survive into adulthood. We have demonstrated that the last coding exon of DCC is deleted in the *Dcc^{kanga}* mutant genome. Interestingly, this exon encodes a region known as the P3 domain that has been shown by *in vitro* axon turning assays to be required for both homodimerization of DCC and netrin 1-dependent attraction of axons (Kolodziej et al., 1996; Stein et al., 2001). In addition, results from similar assays demonstrate that the DCC P3 domain is also the target region for binding of the Slit receptor, Robo, which silences the netrin 1 response of DCC-expressing neurons (Stein and Tessier-Lavigne, 2001). After crossing the midline, it is

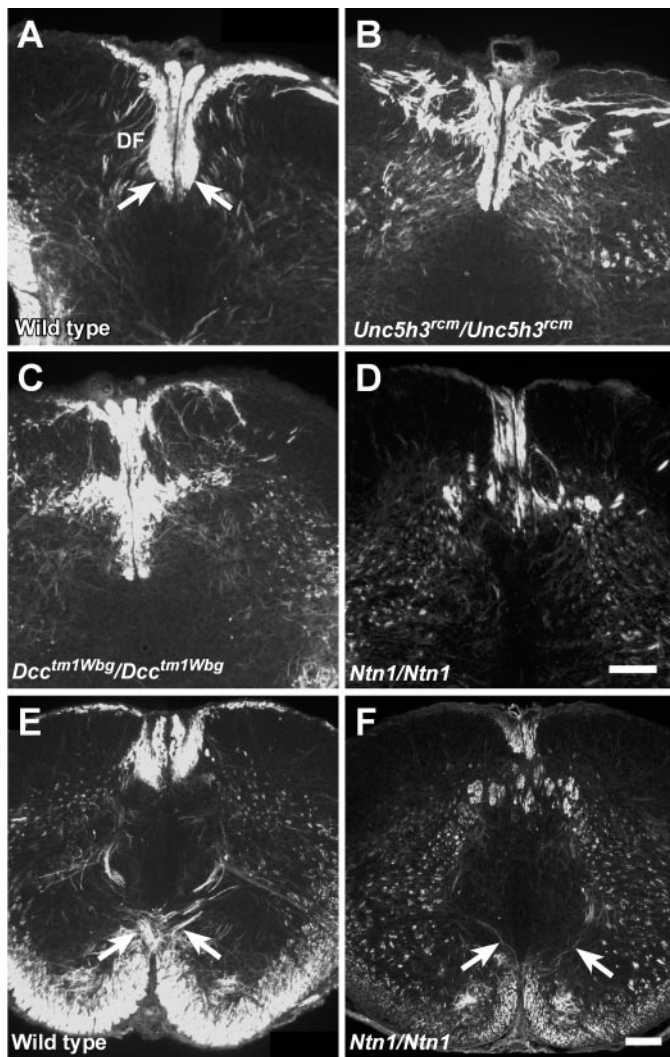


Figure 11. The dorsal funiculus is abnormal in *Unc5h3^{rcm}/Unc5h3^{rcm}*, *Dcc^{tm1Wbg}/Dcc^{tm1Wbg}*, and *Ntn1/Ntn1* newborn mice. Transverse sections of P0 upper cervical spinal cord from wild type (*A*), *Unc5h3^{rcm}/Unc5h3^{rcm}* (*B*), *Dcc^{tm1Wbg}/Dcc^{tm1Wbg}* (*C*), and *Ntn1/Ntn1* (*D*) mice were immunostained with anti-2H3 antibody. Note the abnormal shape of the ventral portion of the dorsal funiculus (*df*; *A*, arrows) is accompanied by many neurofilament-positive misplaced axons throughout the dorsal funicular region of the mutant spinal cords. The pyramidal decussation (arrows) was visualized with anti-2H3 antibody in wild-type mice (*E*) but was much reduced in *Ntn1/Ntn1* newborn mice (*F*). Scale bars, 100 μ m.

hypothesized that axons upregulate Robo, which in turn binds to the P3 domain of DCC. This binding prevents homodimerization of DCC, thus converting the netrin 1-attractive response of these neurons to one of repulsion from the Slit ligand. CST axons do not cross the midline in *Dcc^{kanga}* mutant mice, providing *in vivo* confirmation of the requirement of the P3 DCC domain in netrin 1-mediated attraction to the midline. However, the absence of decussating CST axons in *Dcc^{kanga}* mutant mice precludes the analysis of Robo–DCC interactions in CST development.

The viability of the *Dcc^{kanga}* mutant mice allows analysis of the role of *Dcc* in CST development, which our results show separates into two bundles just before the pyramidal decussation in these mice (Fig. 12). Although both of these bundles move ipsilaterally into the spinal cord, the more medially located bundle approaches but does not cross the midline, whereas the lateral bundle main-

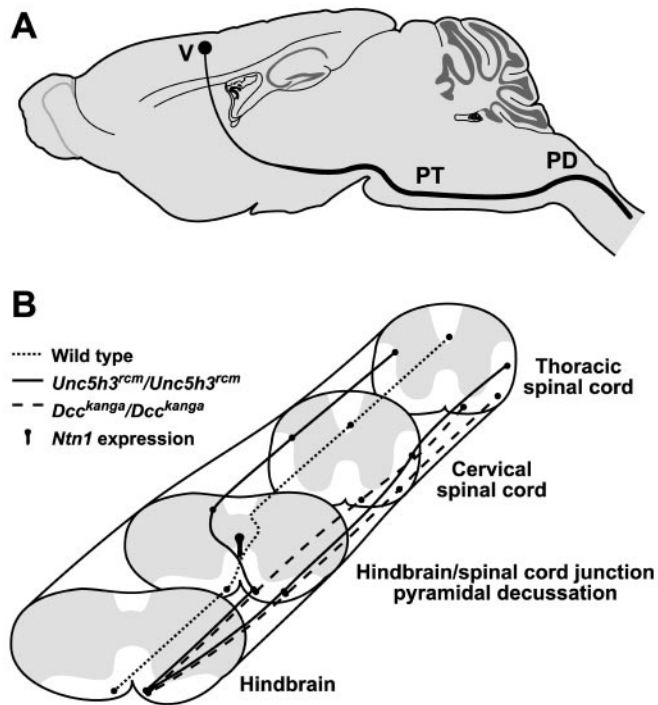


Figure 12. CST pathways in *Unc5h3^{rcm}/Unc5h3^{rcm}* and *Dcc^{kanga}/Dcc^{kanga}* mice. **A**, Normal path of the CST through the brain. Axons from layer V neurons (*V*) in the cerebral cortex migrate through the internal capsule to the ventral aspect of the brain, where they proceed as parallel ipsilateral fiber bundles on either side of midline. In the hindbrain, these fibers form the pyramidal tract (*PT*), which crosses midline at the junction of the hindbrain and spinal cord (*PD*) before entering the dorsal spinal cord. **B**, CST abnormalities in *Unc5h3^{rcm}/Unc5h3^{rcm}* and *Dcc^{kanga}/Dcc^{kanga}* mice. Normally, the pyramidal tract decussates at the hindbrain–spinal cord junction and continues down the spinal cord in the ventral-most portion of the contralateral dorsal funiculus (*dotted line*). In *Unc5h3^{rcm}/Unc5h3^{rcm}* mice, some CST axons in the ipsilateral pyramidal tract separate from the main fiber bundle (*solid lines*). Although the more medial fiber bundle decussates, it does not move into the dorsal funiculus but instead enters and continues in the contralateral lateral funiculus and dorsal gray matter of the spinal cord. The lateral fiber bundle moves further laterally into the ipsilateral lateral funiculus of the spinal cord. Many CST axons in the ipsilateral pyramidal tract of *Dcc^{kanga}/Dcc^{kanga}* mice also separate with neither fiber bundle decussating (*elongated dotted lines*). The medial fiber bundle continues in the ipsilateral ventral funiculus of the spinal cord. As seen in *Unc5h3^{rcm}/Unc5h3^{rcm}* mice, the lateral fiber bundle moves further laterally into the ipsilateral lateral funiculus of the spinal cord. *Ntn1* expression is present dorsal to the crossover point of the pyramidal decussation.

tains its lateral position. This failure of midline crossing is seen in other commissural axons under netrin 1 regulation in the developing brain (Serafini et al., 1996; Fazeli et al., 1997). In agreement, the CST pyramidal decussation is much reduced in *Ntn1/Ntn1* animals. Combined, these data suggest that *Dcc* controls CST formation, in part via the attraction of DCC-expressing axons toward netrin 1 expressed at the midline in the developing brain and spinal cord (Keino-Masu et al., 1996).

We show that DCC is expressed throughout the cortex, and *Unc5h3* is expressed in many layers of the cortex at a time when CST axons are extending. These results are consistent with the cell-autonomous phenotypes seen in mutants of the *C. elegans* homologs *unc-5* and *unc-40*, suggesting that the effect of mutation of *Unc5h3* or *Dcc* on the CST is a primary defect of the axon pathfinding of CST axons. However, we were unable to detect DCC on neurofilament-positive axons likely to be the CST over

the pontine nucleus in the hindbrain. This is consistent with published results showing low levels of DCC expression on CST axons in the forebrain region but not in caudal regions of the brain (Shu et al., 2000). This may indicate that the DCC on these axons is below the levels of detection, as seen previously for EphA4 (Dottori et al., 1998; Leighton et al., 2001). Alternatively, DCC may be expressed only on pioneering axons, which represent only a small minority of the axons in the CST once later-arriving axons have fasciculated. Last, it is possible that this tract, unlike others affected by mutation of *Dcc*, uses DCC in a non-cell-autonomous manner.

Our results further indicate that netrin 1 is a possible ligand for these receptors by its expression pattern adjacent to the pyramidal decussation and by its abnormal phenotype at the decussation and the dorsal funiculus. The extensive disruption of the dorsal funiculus in all of the mutants examined at P0 suggests that the gracilis and cuneatus tracts, in addition to the CST, may also be abnormal, a possibility requiring further study.

UNC5H3 is necessary for correct guidance of CST axons at two choice points: the pyramidal decussation and the turn into the dorsal funiculus (Fig. 12). Like CST axons in the *Dcc^{kanga}* mutant mice, the CST in *Unc5h3^{rcm}/Unc5h3^{rcm}* mice splits into two fiber bundles at the pyramidal decussation. The lateral bundle continues ipsilaterally into the spinal cord. In contrast, the medial bundle decussates, possibly because of netrin 1-mediated attraction of DCC-expressing axons to the midline, but fails to turn into the dorsal funiculus, suggesting that DCC is not sufficient for the dorsal choice point. The final turn of CST axons into the dorsal funiculus takes place from a position lateral and slightly ventral with respect to the dorsal funiculus, suggesting that a repulsive guidance cue for turning axons would be expressed lateral to turning axons. The expression of *Ntn1* transcripts at the ventral midline of the P0 pyramidal decussation makes it likely that UNC5H3-expressing axons would not be repulsed from netrin 1 to turn into the dorsal funiculus and instead use another, as yet unknown, repulsive ligand, which is expressed in the region lateral to migrating axons.

The existence of axons that are independently controlled at two separate choice points, the pyramidal decussation and the turn into the dorsal funiculus, may be indicative of two distinct sets of pioneering axons, perhaps arising from particular regions of the cortex. In agreement, mutations in the *C. elegans unc-40* and *unc-5* genes disrupt the navigation of pioneering axons (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992). Alternatively, the broadening and eventual bifurcation of the CST in *Dcc^{kanga}* and *Unc5h3^{rcm}* mutant mice at the expected level of the decussation suggests a role for DCC and UNC5H3 in fasciculation. This possibility is supported by work in *C. elegans*, which demonstrated that UNC-6 acts as a short-range cue for fasciculating PVP axons (Wadsworth et al., 1996; Culotti and Merz, 1998). Netrin 1 has also been implicated in short-range axonal guidance, directing DCC-expressing retinal ganglion cell axons as they exit the optic disk into the optic nerve (Deiner et al., 1997; de la Torre et al., 1997; Kennedy, 2000). In *Drosophila*, UNC5 can also demonstrate a short-range function by preventing axons from crossing the midline when expressed ectopically (Keleman and Dickson, 2001). Furthermore, UNC5H3 and DCC have immunoglobulin-like domains in the extracellular regions, and DCC also has extracellular fibronectin type III repeats, domains commonly found in cell adhesion molecules such as L1 and neural cell adhesion molecule (Van Vactor, 1998). Thus, in *Unc5h3^{rcm}* mutant and *Dcc^{kanga}* mutant mice, it may be that later-arriving

axons are selectively defasciculating and making pathfinding errors at the choice point of the pyramidal decussation. In addition, *Unc5h3^{rcm}* mutant axons that do cross the midline may defasciculate from pioneering axons at the turn into the dorsal funiculus. Consistent with a role of *Unc5h3* in fasciculation, a few labeled axons that may represent pioneering axons were found in the contralateral dorsal funiculus in *Unc5h3^{rcm}/Unc5h3^{rcm}* mice.

Nonallelic noncomplementation was observed between the *Unc5h3^{rcm}* and *Dcc^{tm1Wbg}* mutations. Although it is not clear whether the fibers in the ventral funiculus of *Unc5h3^{rcm}/Unc5h3^{rcm};Dcc^{tm1Wbg}/+* mice are altered from a normally attractive or repulsive response, a repulsion mechanism is supported by a previous study (Hong et al., 1999). Because both mutations are null alleles, this phenotype is likely attributable to the simultaneous reduction of UNC5H3 and DCC protein levels, not the presence of an altered protein product that binds to and impedes the function of another (Regan and Fuller, 1988; Regan and Fuller, 1990). Although physical interactions of UNC5H3 and DCC have been shown to mediate repulsion when ectopically expressed in *Xenopus* embryos (Hong et al., 1999), and additional results in *C. elegans* and *Drosophila* also suggest interactions (Hamelin et al., 1993; Chan et al., 1996; Colavita and Culotti, 1998; Keleman and Dickson, 2001), the fact that CST axons are not observed in the ventral funiculus of the *Unc5h3^{rcm}* mutant mouse makes it unlikely that *Dcc^{tm1Wbg}* modulation of the *Unc5h3^{rcm}* mutant phenotype is attributable to direct interactions of these protein products. Rather, this result suggests that these molecules may have partially redundant roles in mediating CST guidance at the midline. This suggestion is further strengthened by recent *C. elegans* studies demonstrating that *unc-40* functions in both *unc-5*-independent and -dependent UNC-6-repulsed distal tip cell migrations (Merz et al., 2001).

The CST has a complicated migration path, likely controlled by many genes in addition to those genes in the netrin 1 signaling pathway, some of which have been identified (Cohen et al., 1998; Dottori et al., 1998; Kullander et al., 2001). Our results indicate that, even within the CST, some axons are apparently under the control of separate guidance mechanisms. Netrin 1, which is expressed in the internal capsule, has been shown to stimulate axonal outgrowth from cultured rat cortical explants (Richards et al., 1997). Interestingly, our analysis of *Unc5h3^{rcm}* mutant and *Dcc^{kanga}* mutant mice did not reveal defects in the CST as it traversed the hindbrain until just before the decussation. These results suggest that these two gene products are involved only in guidance of CST fibers into the spinal cord, or that absence of these genes is functionally compensated by other family members during the initial outgrowth stages of the CST.

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