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Commissural axon pathfinding on the contralateral side of the floor plate: a role for B-class ephrins in specifying the dorsoventral position of longitudinally projecting commissural axons

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SUMMARY

In both invertebrate and lower vertebrate species, decussated commissural axons travel away from the midline and assume positions within distinct longitudinal tracts. We demonstrate that in the developing chick and mouse spinal cord, most dorsally situated commissural neuron populations extend axons across the ventral midline and through the ventral white matter along an arcuate trajectory on the contralateral side of the floor plate. Within the dorsal (chick) and intermediate (mouse) marginal zone, commissural axons turn at a conserved boundary of transmembrane ephrin expression, adjacent to which they form a discrete ascending fiber tract. In vitro perturbation of endogenous EphB-ephrinB interactions results in the failure of commissural axons to turn at the appropriate dorsoventral position on the contralateral side of the spinal cord; consequently, axons inappropriately invade more dorsal regions of B-class ephrin expression in the dorsal spinal cord. Taken together, these observations suggest that B-class ephrins act locally during a late phase of commissural axon pathfinding to specify the dorsoventral position at which decussated commissural axons turn into the longitudinal axis.

Key words: Commissural axons, Spinal cord, B-class ephrins, B-class Eph receptors, Boundary, Contralateral pathway, Longitudinal fiber tract, Chick, Mouse

INTRODUCTION

In a wide variety of bilaterally symmetric organisms, sensory information is transferred from one side of the body to the other through axon commissures formed by interneurons (i.e. commissural interneurons) that extend axons across the ventral midline (Tear, 1999). In vertebrates, commissural axons located within a dorsal region of the developing spinal cord initially project axons along a stereotypic pathway on the ipsilateral side of the spinal cord (Bovolenta and Dodd, 1990; Colamarino and Tessier-Lavigne, 1995). This segment of their trajectory is relatively simple and is characterized by circumferential/transverse growth toward the ventral midline (Bovolenta and Dodd, 1990). The roof plate and the floor plate, specialized structures that are situated at the dorsal and ventral midline, respectively, appear to play reciprocal roles in the ventral migration of commissural axons/growth cones (Kaprielian et al., 2001). Netrin 1, a soluble chemoattractant secreted by floor plate cells (and by cells situated in the ventral ventricular zone) and presumably distributed along a dorsoventral (DV) gradient, guides DCC-expressing commissural axons ventrally (Fazeli et al., 1997; Serafini et al., 1996). In complementary fashion, the ability of bone morphogenetic protein (BMP) 7, a TGFβ superfamily member secreted by cells comprising the roof plate, to repel commissural axons in vitro suggests a possible role for this structure in orienting these axons away from dorsal regions of the spinal cord during an earlier phase of axon outgrowth (Augsburger et al., 1999).

Upon reaching the ventral midline, commissural axons cross over to the contralateral side of the spinal cord by navigating through the floor plate. After exiting this structure, these axons execute an orthogonal turn and join other types of axons traveling within the ventral funiculus, a longitudinal fiber tract that projects alongside the floor plate (Colamarino and Tessier-Lavigne, 1995; Kaprielian et al., 2001). Analyses of commissural axon pathfinding in the spinal cords of *Sd* (Bovolenta and Dodd, 1991) and *Gli2* (Matise et al., 1999) - deficient mouse embryos, which lack floor plate cells (and immediately adjacent interneurons in the case of *Gli2*$^{-/-}$ mutants), suggest that short-range, contact-mediated interactions between commissural axons/growth cones and floor plate cells are required for axons to cross the midline and subsequently turn appropriately into the longitudinal axis. Support for this interpretation is provided by the finding that a direct interaction between commissural axons expressing axonin 1 (the avian ortholog of rodent TAG1), and floor plate cells expressing Nr-CAM, regulates the entry of commissural axons into the floor

plate (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997) through a mechanism that apparently occurs independently of axon elongation (Fitzli et al., 2000). Similarly, abrogation of the function of F-spondin, an extracellular-matrix molecule secreted by floor plate cells, promotes premature turning of commissural axons on the ipsilateral side of the floor plate and may thus prevent the passage of these axons across the floor plate in chick embryos (Burstyn-Cohen et al., 1999). More recent studies have demonstrated that commissural axons exhibit a variety of midline pathfinding defects, including stalling within the floor plate and rostrocaudal polarity errors at the contralateral floor plate margin in mice deficient in *neuropilin 2* (*npn2*) (Zou et al., 2000). These observations suggest that npn2 expressed by commissural axons mediates the effects of local cues distributed at the ventral midline (e.g. class 3 semaphorins and possibly one or more slits) to control floor plate exit and/or entry of growth cones into the white matter (Zou et al., 2000).

Very little is known about the pathfinding behavior of commissural axons on the contralateral side of the floor plate. Studies of commissural axon pathfinding in the spinal cords of developing chick (Yaginuma et al., 1991) and rat (Bovolenta and Dodd, 1990) embryos have carefully noted that newly crossed axons turn abruptly to join an established longitudinal fiber tract that projects in close proximity to the floor plate. However, it is not clear from these studies whether this behavior is representative of a small population of axons that extend from cell bodies situated within a circumscribed region of the dorsal spinal cord, and whether decussated commissural axons remain associated with the contralateral margin of the floor plate throughout the remainder of their trajectory. Interestingly, in lower vertebrates, commissural axons exhibit a complex pattern of axon outgrowth that consists of a turn at the ventral midline, oblique/diagonal growth away from the floor plate and through ventral and intermediate regions, and a final turn in the dorsal spinal cord that results in the formation of an ascending longitudinal pathway within this region (Bernhardt et al., 1990; Kuwada et al., 1990; Roberts et al., 1987). These observations prompted us to perform detailed DiI analyses to re-examine commissural axon pathfinding during later stages of mouse and chick embryogenesis when these axons have crossed the ventral midline and extended for considerable distances on the contralateral side of the spinal cord. We find that most decussated commissural axons in both the mouse and chick spinal cord follow a contralateral pathway that is similar to their zebrafish and *Xenopus* counterparts and which culminates in the execution of a longitudinally directed turn at an intermediate (mouse) or dorsal (chick) position within the spinal cord white matter.

Taken together, these observations represent a major extension of current models of vertebrate midline guidance and provide a framework for future studies aimed at elucidating roles for known or putative short- and long-range guidance cues and their receptors in commissural axon pathfinding. Given their transmembrane structure and their demonstrated ability to mediate axonal patterning and pathway selection in a number of neural systems, B-class ephrins are particularly well suited to act as tightly localized, contact-dependent guidance molecules (Flanagan and Vanderhaeghen, 1998; Wilkinson, 2000). We have previously demonstrated that B-class ephrins are expressed in the dorsal spinal cord and floor plate, and that the corresponding receptor(s) is reciprocally expressed on axons that project longitudinally between these ephrin domains in the developing mouse spinal cord. Moreover, we have shown that B-class ephrins mediate the collapse of commissural growth cones (Imondi et al., 2000). We now extend these findings and show that the complementary expression of transmembrane ephrins and their receptors is conserved in the chick spinal cord. Furthermore, we demonstrate that both mouse and chick commissural axons execute their final turn at the ventralmost boundary of ephrinB expression in the intermediate and dorsal spinal cord, respectively. In vitro perturbation of endogenous EphB/ephrinB interactions in intact spinal cord explants resulted in the inappropriate growth of decussated commissural axons into dorsal regions of ephrinB expression. These findings suggest that repulsive interactions between commissural axons expressing EphB receptors, and one or more dorsal cell types that express the corresponding B-class ephrins, may influence the dorsoventral position of an ascending longitudinal tract formed by decussated commissural axons on the contralateral side of the spinal cord.

MATERIALS AND METHODS

DiI tracing

Commissural neuron cell bodies were retrogradely labeled with DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine; Molecular Probes) by placing a low-resistance electrode (5 MΩ) containing 1.0% DiI in methylene chloride into the ventral commissure in 100-150 μm transverse vibratome sections obtained from fixed E4.5 chick and E12 mouse embryos. After placement of the electrode into the ventral commissure, approximately 3.0 μA of current was applied for 15-30 seconds to iontophoretically deposit a small crystal of DiI directly into the injection site. Anterograde labeling of commissural axons was performed essentially as described above, except that the electrodes were placed into dorsal spinal cord regions determined to contain commissural neuron cell bodies in E4-E5.5 chick or E11-E13 mouse open-book spinal cord preparations. Following DiI applications, the tissue was immersion fixed in 4% paraformaldehyde (PFA) and stored in the dark at room temperature for a maximum of 72 hours. These conditions were sufficient to permit diffusion of the dye to the terminal ends of axons (as determined by the robust labeling of growth cones), and minimized or eliminated dye transfer to neighboring cell bodies and axons. DiI-labeled commissural axons and growth cones were visualized exclusively from the marginal (versus ventricular) surface of flat-mounted explants, within the spinal cord white matter. To quantify growth cone morphology, we examined digitally captured images of individual growth cones and scored the number and corresponding lengths of filopodial extensions. Area calculations were performed as previously described (Bovolenta and Dodd, 1990) and represent the product of maximum growth cone length and width. To classify the types of projection patterns exhibited by decussated commissural axons, we made focal DiI applications within the dorsal spinal cord at various distances away from the roof plate (i.e. injections were made adjacent to the roof plate and at approximately 20-30 μm intervals ventral to this structure); we then scored, for each axon cohort labeled in this manner, the proportion of axons that followed distinct contralateral pathways.

In vivo binding analyses

For chick binding analyses, E3-E5 White Leghorn chick embryos (Spafas) were exposed by boring-out a 20 mm diameter window

through the egg shell with a dental drill (Dremel). A pressure injection system (Nanoject II; Drummond) was then used to deliver 200-300 nl of 1.0 mg/ml Fc alone (Jackson Immunoresearch), recombinant mouse ephrinB1-Fc or EphB3-Fc (R&D Systems) through a glass micropipette and into the lumbar region of the central canal. Open-book spinal cord explants were obtained from embryos 6-12 hours post-injection and were fixed for 12-24 hours in 4% PFA. Transverse vibratome sections (100-150 μm) were taken from injected embryos that were first fixed for 12-24 hours in 4% PFA. Tissue was then washed three times for 10 minutes in phosphate-buffered saline (PBS; 150 mM Na_2HPO_4, 20 mM NaH_2PO_4, 150 mM NaCl) and incubated at 65°C for 3 hours to destroy endogenous alkaline phosphatase activity. After heat inactivation, the tissue was blocked in BTX (1.0% heat inactivated goat serum (HIGS), 1.0% bovine serum albumin (BSA), and 0.01% sodium azide in PBS), incubated for 12 hours at 4°C in AP-conjugated goat anti-human IgG (Promega) diluted 1:1000 in BTX, and washed five times for 1 hour at 4°C in BTX. ephrinB1- or EphB3-Fc binding was visualized as previously described (Imondi et al., 2000). Receptor-Fc binding to transverse and open-book spinal cord preparations obtained from mouse embryos was performed exactly as described (Imondi et al., 2000). Immunolabeling of transverse vibratome sections with mAb 8D9 (anti-chick L1; mouse IgG; Developmental Studies Hybridoma Bank) and anti-mouse L1 (rat IgG; Boehringer-Mannheim) was used to show the position of post-commissural axons within the marginal zone of the chick and mouse spinal cord, respectively. These analyses were performed essentially as described (Dodd et al., 1988), except that an AP-conjugated secondary was used to visualize binding.

Cultured mouse open-book spinal cord explants

All dissections were performed in ice-cold Dulbecco's Modified Eagle Medium (DMEM)/F12 (Gibco-BRL) containing 10% heat-inactivated fetal bovine serum (FBS; Gemini Bio-products). Mouse spinal cord explants in the open-book configuration were individually embedded in a matrix composed of rat tail collagen, 10× MEM (Gibco-BRL) and 0.26 M $NaHCO_3$ (100:10:1). Embedded explants were subsequently cultured for 48 hours in DMEM/F12 supplemented with 10% FBS, Bottenstein's N2 supplement (Gibco-BRL), penicillin/streptomycin/glutamine (Gibco-BRL), and containing Fc alone, recombinant rat EphA1-Fc, recombinant mouse ephrinB1-Fc or recombinant rat EphB3-Fc (all were used at 50 μg/ml). In explants cultured under control conditions and subsequently labeled with EphB1- or EphB3-Fc, ephrinB protein localized to the floor plate and to a dorsal domain that terminated at a point approximately midway between the roof plate and the floor plate; we used this distance as a reference boundary to score pathfinding defects in explants cultured under the conditions described (at present, technical limitations preclude DiI labeling of axons in explants processed for receptor- or ligand-Fc labeling). Focal DiI applications were made in each explant at an average distance of 30 μm ventral to the roof plate, within the dorsal spinal cord proper. To quantify pathfinding errors, we scored for each condition the proportion of decussated axons within a cohort of DiI labeled axons that extended across the dorsal ephrin boundary. The total number of axon cohorts examined in each condition are presented in Table 2.

Photodocumentation

DiI-labeled axons in open-book explants and transverse vibratome sections were visualized under epifluorescence optics (Nikon Eclipse TE300) using a Cy3/DiI optical filter (Chroma Technology). Ligand- and receptor-Fc binding to transverse and open-book spinal cord preparations was visualized with a Zeiss Stemi-2000C stereo dissecting scope. Color slides were digitally captured through an Agfa Duoscan flatbed scanner; composites were constructed and subsequently annotated using the Adobe Photoshop software program.

RESULTS

Commissural axons follow a complex pathway on the contralateral side of the floor plate

Previous studies in both chick (Yaginuma et al., 1991) and rat (Bovolenta and Dodd, 1990) have demonstrated that spinal commissural axons project ventrally and circumferentially towards the floor plate. Upon reaching the ventral midline, these axons enter and traverse the floor plate, and subsequently turn into the contralateral ventral funiculus. In chick, the previous use of antibodies to label commissural axons at a time when longitudinal fiber tracts are already established in the ventral spinal cord obscured visualization of decussated commissural axon segments on the contralateral side of the floor plate (Yaginuma et al., 1990). Furthermore, although the lipophilic carbocyanine dye, DiI, has been successfully used to unambiguously determine the initial trajectory of rat commissural axons after they execute a turn at the contralateral floor plate boundary, the subsequent pathway followed by these axons was not examined (Bovolenta and Dodd, 1990). To delineate the trajectory of commissural axons on the contralateral side of the floor plate, we examined individual or small groups of axons in fixed chick and mouse open-book spinal cord preparations obtained from embryos at different developmental stages. This was achieved by the iontophoretic application of minute crystals of DiI into dorsal spinal cord regions shown to contain commissural neuron cell bodies (Fig. 1A). As our analyses were confined to the contralateral commissural pathway, we show in most whole-mount preparations only that region of the spinal cord which lies opposite (contralateral) to the side in which DiI was applied [for a detailed description of the initial segment of the commissural axon pathway in rat, see Bovolenta and Dodd (Bovolenta and Dodd, 1990)].

We began our analysis at embryonic day (E) 4 in the chick spinal cord (Fig. 1B), after commissural axons have crossed through the floor plate and executed orthogonal turns into the anteroposterior axis. Consistent with previous findings in the rat (Bovolenta and Dodd, 1990), most axons maintained close association with the contralateral floor plate margin for approximately 100-150 μm after executing a rostrally directed turn into the ventral funiculus. Interestingly, however, most commissural axons did not remain within the ventral funiculus for distances exceeding 150 μm. Rather, they turned gradually away from the floor plate and projected obliquely through the ventral and intermediate white matter (Fig. 1C).

At E5.5 (Fig. 1D), many axons had extended over distances of 600-800 μm on the contralateral side of the floor plate. At this age, many leading axons executed a final rostral turn in the dorsal marginal zone and projected for distances greater than 500 μm along a strict longitudinal pathway to form an ascending fiber tract that lies ventral to the dorsal funiculus (Fig. 1D and data not shown).

Taken together, these observations suggest that the majority of commissural axons in the chick spinal cord follow a complex contralateral pathway consisting of: (1) a rostral turn and transient growth adjacent to the floor plate; (2) a gradual deflection away from the floor plate and oblique growth through ventral and into intermediate and dorsal white matter; (3) a final rostral turn into the longitudinal axis within the

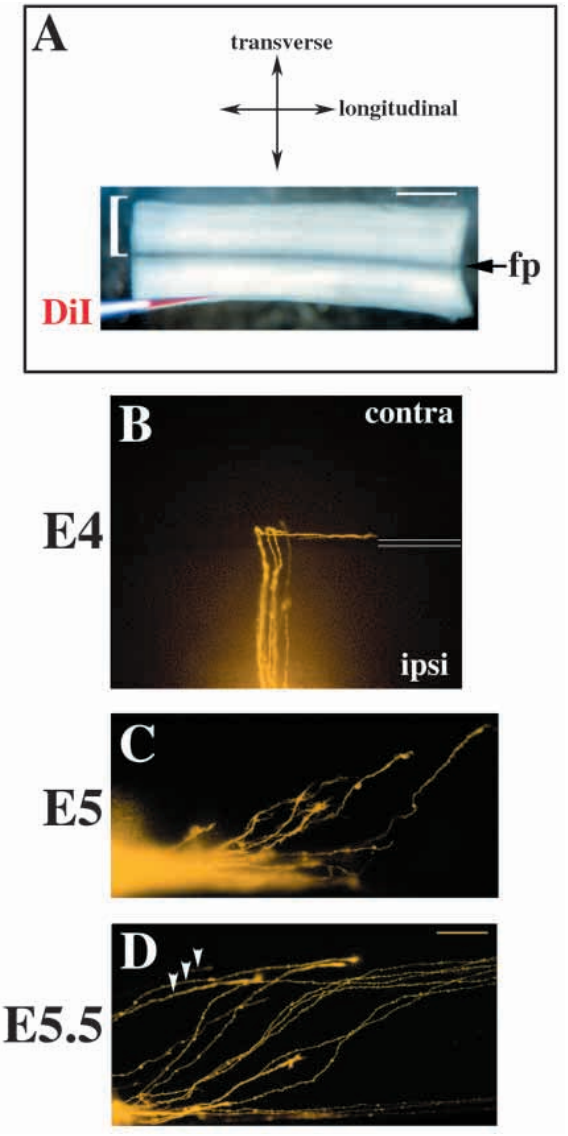

Fig. 1. Chick commissural axons follow a complex pathway on the contralateral side of the floor plate. (A) Small crystals of DiI were iontophoretically applied to dorsal spinal cord regions containing commissural neuron cell bodies in fixed open-book spinal cord preparations obtained from E4-E5.5 chick embryos. The microelectrode used to deliver the dye is shown in the lower left corner of the micrograph. (B) At E4, commissural axons anterogradely labeled with DiI extend across the floor plate (indicated by the white lines) in the transverse plane. At the contralateral floor plate margin, axons turn rostrally and extend for approximately 100 μm alongside the floor plate in the longitudinal plane. (C,D) Labeled axons extending on only the contralateral side of the spinal cord (indicated by the white bracket in A; dorsal is upwards and rostral is towards the right). (C) At E5, axons turn away from the floor plate (after extending for 100 μm adjacent to this structure) and project obliquely into more dorsal regions of the white matter. (D) At E5.5, commissural axons execute a final rostral turn and grow longitudinally within the dorsal marginal zone (the white arrowheads indicate decussated axons labeled by an adjacent DiI application). A small number of commissural axons are shown at the bottom of this panel traveling adjacent to the floor plate. The dorsal limit of the explant shown in this panel extends beyond the top edge of this micrograph. Scale bars: in A, 500 μm for A; in D, 50 μm for B-D. contra, contralateral; fp, floor plate; ipsi, ipsilateral.

dorsal marginal zone; and (4) strict longitudinal growth over distances greater than 500 μm.

Commissural axons exhibit three distinct projection patterns on the contralateral side of the floor plate

Commissural neurons comprise a large, heterogeneous class of sensory interneurons that can be distinguished on the basis of morphology (Silos-Santiago and Snider, 1992), position along the dorsoventral (DV) and mediolateral (ML) axes (Silos-Santiago and Snider, 1992), and gene expression (DeFelipe et al., 1995; Helms and Johnson, 1998; Lee and Jessell, 1999; Mansouri and Gruss, 1998; Matise et al., 1999; Phelps et al., 1999; Tran and Phelps, 2000). Consistent with these observations, our analyses revealed discrete projection patterns exhibited by commissural axons emanating from cell bodies located in the dorsal spinal cord (Fig. 2; also see Fig. 5). As

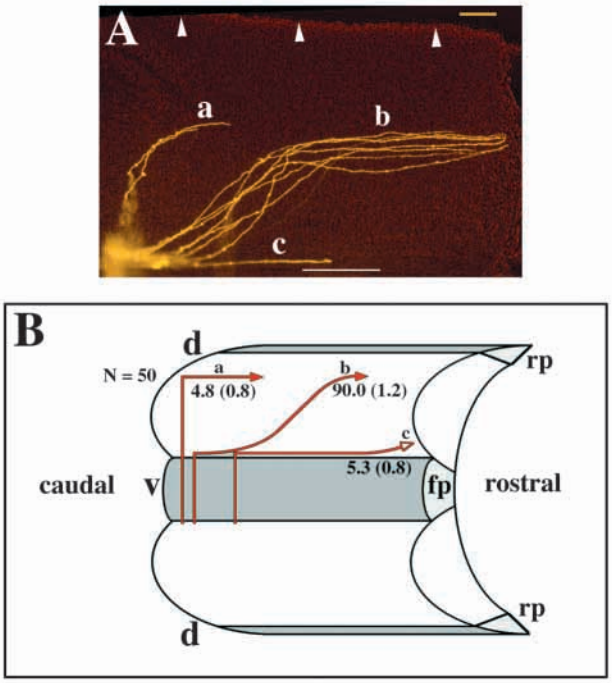

Fig. 2. Chick commissural axons follow three distinct contralateral pathways. (A) Combined phase and fluorescent micrograph showing three distinct trajectories followed by DiI labeled commissural axons on the contralateral side of an E5.5 chick open-book spinal cord explant (dorsal is upwards and rostral is towards the right). (a) A small population of axons crosses the floor plate and extends directly into more dorsal regions of the white matter before initiating a rostral turn into the longitudinal axis. (b) The major class of axons exhibits an arcuate trajectory that consists of a rostral turn at the contralateral floor plate boundary, followed by growth alongside the floor plate for ~100 μm, diagonal growth away from the floor plate, and then a rostral turn into the longitudinal axis within the dorsal marginal zone. (c) A third, small population of axons turns orthogonally at the contralateral floor plate boundary and projects rostrally adjacent to this structure for distances greater than 100 μm. (B) Schematic representation of an open-book spinal cord preparation (marginal surface facing upwards) showing each contralateral pathway depicted in A. For each application of DiI into the dorsal spinal cord (see Materials and Methods for details), the relative proportion of axons within a cohort of DiI labeled axons (n=50) that followed each pathway was calculated. Scale bar: 100 μm in A. d, dorsal; fp, floor plate; rp, roof plate; v, ventral.

noted above, the majority of commissural axons followed an arcuate contralateral pathway consisting of several growth transitions (transverse to longitudinal, longitudinal to diagonal and diagonal to longitudinal) and culminating in the formation of a commissural axon tract in the dorsal marginal zone (Fig. 2A(b),B). Ninety percent of labeled axons assumed this trajectory at all axial levels examined (Fig. 2B). A significantly smaller proportion of axons (approximately 5%) exhibited either of two distinct projection patterns (Fig. 2A(a,c),B) Consistent with previous observations in rat (Bovolenta and Dodd, 1990), one class of axons (4.8%) did not turn at the contralateral floor plate boundary, but instead projected directly through the contralateral marginal zone (Fig. 2A(a),B). These axons either turned orthogonally or at a more gradual angle in the dorsal white matter at the same DV position as their more predominant cohorts. A second minor class of axons (5.3%) projected within the ventral funiculus in close association with the floor plate for distances up to 500 μm (Fig. 2A(c),B). Although we have not yet determined whether these axons ultimately project into the dorsal white matter and execute a final turn within this region, many of these axons were observed to eventually exit the ventral funiculus (after growth within the fiber tract for distances greater than 500 μm) and gradually project diagonally away from the floor plate (data not shown).

Commissural neurons are widely distributed along the dorsoventral axis of the developing rodent spinal cord (Silos-Santiago and Snider, 1992) (data not shown). The focal application of DiI into extreme dorsal regions (i.e. adjacent to the roof plate) will result in anterograde labeling of axons emanating from cells occupying only those regions. By contrast, a more ventral placement of DiI will not only label axons extending from cell bodies located at the site of application, but those originating from more dorsally situated cell bodies that pass through the site of dye application. Thus, by comparing the projection patterns of axons labeled by the placement of dye at different DV levels, it is possible to determine the relative location of cell bodies that give rise to distinct projections. To determine if the three projection patterns we observed were the result of labeling small populations of commissural neurons residing within a defined location in the dorsal spinal cord, we varied the DV placement of DiI crystals within a broad dorsal region (see Materials and Methods). In over 50 axon cohorts labeled at each DV position, we observed the same classes of projections in the same relative proportions regardless of the placement of DiI (data not shown). Although our labeling method precludes the precise placement of DiI along the ML axis, the reproducibility of the labeling patterns that we observed, despite a random placement of dye in this axis, suggests that commissural neurons distributed within a fairly broad dorsal domain give rise to axons that follow one of three contralateral pathways. It should be noted here that our analyses did not include projections emanating from neurons located in the ventral spinal cord proper. It is thus possible that ventral commissural neurons give rise to axons that follow a pathway distinct from those described here.

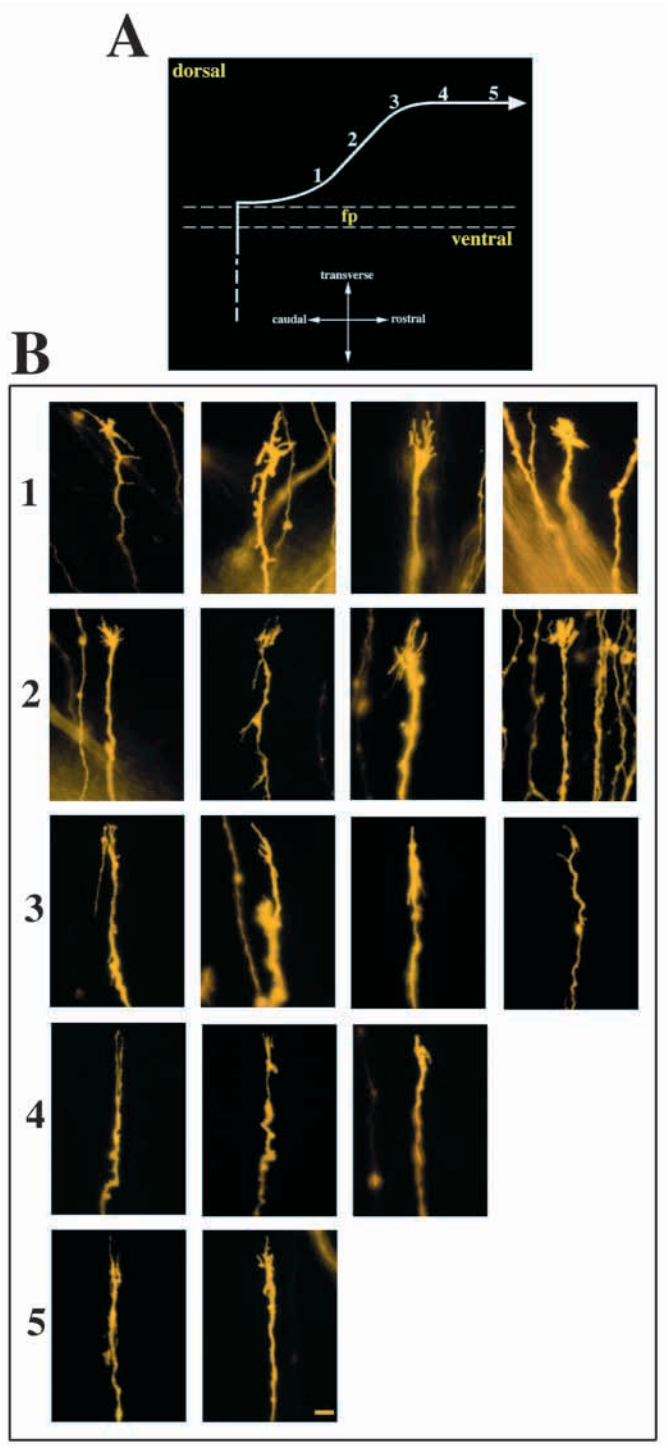

Fig. 3. Chick commissural growth cones decrease in complexity as they execute their final turn. (A) Schematic representation of the most predominant contralateral commissural pathway. The numbers indicate the positions along the pathway at which growth cone morphology was examined. In B, rows 1-5 show the morphology of growth cones at the corresponding regions of the contralateral pathway depicted in A. Commissural growth cones are large and highly elaborated with numerous filopodial extensions and well-spread lamellipodia within more proximal regions of the contralateral pathway (rows 1 and 2). By contrast, upon reaching the dorsal limit of their extension (row 3), axons terminate in growth cones that are simple and elongated. This simple morphology is maintained in more distal segments of the pathway (rows 4 and 5), as axons project rostrally in the longitudinal plane. Scale bar: 100 μm.

Table 1. Growth cone and filopodia dimensions

	Proximal (*n*=10)	Distal (*n*=12)
Growth cone width (μm)	6.9±1.0	1.6±0.3
Growth cone length (μm)	11.8±1.6	7.1±1.6
Growth cone area (μm^2)	79.9±12.9	14.0±4.6
Filopodia/growth cone	5.9±0.8	2.1±0.3
Filopodial length (μm)	6.6±0.6	11.8±2.0

DiI-labeled growth cones occupying positions within proximal (1, 2 in Fig. 3A; *n*=10) or distal (3-5 in Fig. 3A; *n*=12) regions of the contralateral pathway were examined along the indicated dimensions. Data are expressed as mean±s.e.m.

Commissural growth cones decrease in complexity as they approach their final turn

Although commissural growth cones exhibited a wide range of morphologies on the contralateral side of the floor plate, clear distinctions could be made among growth cones navigating through different regions of this pathway. As the morphology of growth cones situated within the ventral funiculus have been described elsewhere (Bovolenta and Dodd, 1990; Yaginuma et al., 1991), we restricted our analysis to more distal segments of the contralateral trajectory (Fig. 3A, Table 1). Axons traveling within the diagonal segment of the contralateral pathway [Fig. 3A (positions 1 and 2), B (rows 1 and 2)] terminated in large growth cones (average area 79.9 μm^2±12.9, *n*=10) that were highly elaborated with well-spread lamellipodia and numerous filopodia (average number of filopodia/growth cone 5.9±0.8, *n*=10). Filopodial extensions were variable in length (1.4-23.4 μm, *n*=59) and were often oriented diagonally in the direction of the turn; some processes emanating from the main body of the growth cones were observed to project radially from the sides of the growth cones (data not shown). Filopodia were also observed to extend from the proximal segment of the axon, or from the trailing edge of the growth cone in this region of the pathway. A dramatic transition in growth cone morphology occurred within the dorsal region of the diagonal segment, as axons executed a final turn into the longitudinal axis [Fig. 3A (position 3), B (row 3)]. Growth cones in this and more distal regions of the contralateral pathway [Fig. 3A (positions 3-5), B (rows 3-5)] underwent a marked reduction in area (average area 14.0 μm^2±4.6, *n*=12) and assumed a simple, elongated morphology. Furthermore, growth cones projected fewer filopodia (average number of filopodia/growth cone 2.1±0.3, *n*=12) and, in many cases, long filopodial processes (average length 11.8 μm±2.0, *n*=25) extended both rostrally and caudally from the leading edge of the growth cone. In the most distal segments of the pathway [Fig. 3A (positions 4 and 5), B (rows 4,5)], caudally projecting filopodia were retracted, and rostrally directed filopodia extended longitudinally at variable lengths (1.7-42.1 μm, *n*=25) from the tips of the growth cones, parallel to the forming dorsal commissural tract.

Mouse commissural axons follow the same complex pathway as their chick analogs

To determine if the trajectory followed by decussated chick commissural axons is conserved in mammals, we analyzed the contralateral commissural pathway in whole-mount spinal cord preparations obtained from E11-E13 mouse embryos. As observed in chick, most commissural axons followed a complex contralateral pathway that began with a rostral turn at the contralateral floor plate boundary (Fig. 4A), followed by extension within the ventral funiculus for approximately 100 μm, a deflection away from the floor plate, and diagonal growth into and through the ventral white matter (Fig. 4B). A second rostral turn into the longitudinal axis was also executed by these axons, although the position of this turn occurred at a more intermediate position than that observed in chick (Fig. 4C). By varying the placement of DiI in the dorsal spinal cord (indicated by lower bracket in Fig. 5A, positions 1-3), we were able to unambiguously identify interneuron populations that could be distinguished by their axonal projection patterns (Fig. 5A,B). Axons extending from cell bodies located immediately adjacent to the roof plate projected ventrally and then turned rostrally on the ipsilateral side of the spinal cord [Fig. 5B (panel 1)]. These axons correspond to a previously undescribed population of ipsilaterally projecting axons. A smaller population of axons originating from cell bodies approximately 30 μm ventral to the roof plate projected within the ventral

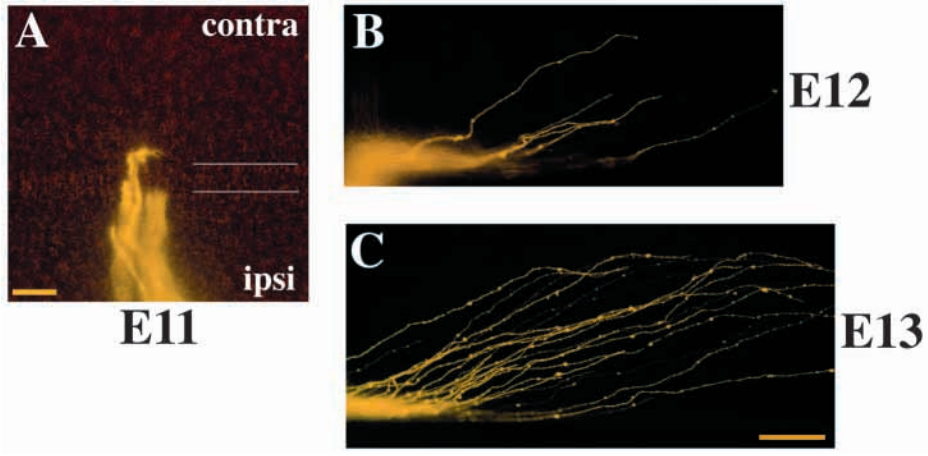

Fig. 4. Mouse commissural axons extend along the major contralateral pathway followed by their chick counterparts. (A-C) Commissural axons were anterogradely labeled with DiI in open-book spinal cord explants obtained from E11-E13 mouse embryos as described in Material and Methods. (A) At E11, leading commissural axons extend ventrally and are found within, or just on the contralateral side of, the floor plate (region between the white lines). This preparation was visualized under both fluorescence and phase-contrast optics and shows a single growth cone emerging from the floor plate and executing a rostral turn (as well as other axons that have just entered the floor plate). (B,C) Decussated commissural axons projecting on only the contralateral side of the floor plate between E12 and E13 (dorsal is up and rostral is to the right). (B) As observed in the chick spinal cord, most crossed commissural axons extend alongside the floor plate for approximately 100 μm before turning diagonally into more dorsal regions of the white matter. (C) By E13, most commissural axons have executed a final rostral turn into the longitudinal axis within an intermediate region of the marginal zone. Scale bars: in A, 100 μm; in C, 100 μm for B,C.

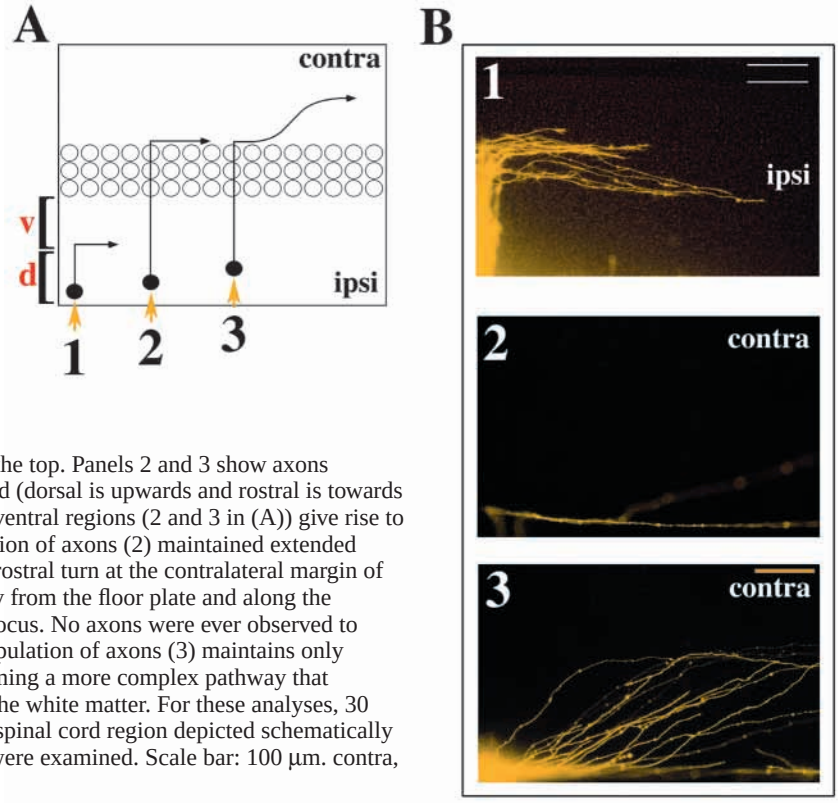

Fig. 5. Mouse commissural axons exhibit distinct contralateral trajectories. (A) Schematic diagram of an open-book spinal cord explant in which the placement of DiI was varied within the dorsal spinal cord (the dorsal and ventral regions of the spinal cord are indicated by brackets). The numbers in A correspond to the positions of three separate DiI applications that labeled ventrally directed axons whose trajectories are shown in panels 1-3 in B. (B) Cell bodies immediately adjacent to the roof plate (1) extend axons that project ventrally and subsequently turn rostrally on the ipsilateral side of the spinal cord for distances up to 450-500 μm. In this micrograph, the floor plate (indicated by the white lines) is shown at the top. Panels 2 and 3 show axons projecting on only the contralateral side of the spinal cord (dorsal is upwards and rostral is towards the right). Cell bodies located within successively more ventral regions (2 and 3 in (A)) give rise to axons that cross through the floor plate. A minor population of axons (2) maintained extended (>100 μm) contact with the floor plate after executing a rostral turn at the contralateral margin of this structure. An axon that is projecting diagonally away from the floor plate and along the pathway depicted in C is shown outside of the plane of focus. No axons were ever observed to recross the floor plate in these studies. A much larger population of axons (3) maintains only transient (~100 μm) contact with floor plate before assuming a more complex pathway that culminates in a rostral turn in an intermediate region of the white matter. For these analyses, 30 axon cohorts labeled by the application of DiI into each spinal cord region depicted schematically in A (see Materials and Methods for additional details) were examined. Scale bar: 100 μm. contra, contralateral; d, dorsal; ipsi, ipsilateral; v, ventral.

funiculus for distances exceeding 100-150 μm after crossing the floor plate [Fig. 5B (panel 2)]. As noted in chick, this class of commissural axons also ultimately departed from the ventral funiculus and subsequently extended through the ventral white matter (data not shown). The application of DiI into successively more ventral positions within the dorsal spinal cord resulted in the labeling of commissural axons that assumed a complex trajectory which culminated in a rostral turn in an intermediate region of the spinal cord [Fig. 5 (panel 3)]. As observed in chick, ~90% of dorsal commissural neuron populations extended axons that followed this particular pathway.

Mouse and chick commissural axons execute their final contralateral turn at a boundary of B-class ephrin expression

In the DiI studies described above, chick and mouse commissural neurons were visualized along their entire extent at the marginal (versus ventricular) surface of open-book spinal cord preparations, within the spinal cord white matter. To confirm that these axons do not re-enter the gray matter after crossing through the floor plate, we used anti-L1 antibodies as independent markers to label decussated commissural axons in transverse vibratome sections obtained from E5.5 chick (Fig. 6A) and E13 mouse (Fig. 6B) embryos. Consistent with previous observations (Imondi et al., 2000), L1-positive postcommissural axons were confined to fiber tracts situated within the marginal zone (indicated by red arrowheads in Fig. 6A,B). Our use of ephrinB1-Fc (to detect EphB receptor expression) labeled postcommissural axons in a pattern strikingly similar to that observed with anti-L1 antibodies (Imondi et al., 2000). Taken together with our DiI analyses, these findings demonstrate that upon exiting the floor plate, commissural axons are excluded from the gray matter and navigate exclusively within surrounding fiber tracts throughout their contralateral pathway. These data are summarized schematically in Fig. 6C.

The recent demonstration that decussated commissural axons acquire responsiveness to a repellent activity made by the gray matter that involves slit and semaphorin proteins (Zou et al., 2000) is consistent with the behavior exhibited by commissural axons on the contralateral side of the midline. As previously suggested (Zou et al., 2000), these repellent activities may not only propel newly crossed axons into the ventral funiculus, but prevent these axons from re-entering the gray matter as they depart from this tract and navigate along more distal segments of their contralateral pathway. The dramatic change in growth cone morphology and trajectory observed as commissural axons assemble into an ascending fiber tract in the dorsal (chick) and intermediate (mouse) marginal zone suggests that additional repulsive guidance forces influence the contralateral commissural pathway. B-class Eph receptors represent particularly good candidates for mediating commissural axon pathfinding on the contralateral side of the floor plate, as they are expressed exclusively on crossed segments of these axons in mouse (Imondi et al., 2000). In chick, B-class Eph receptors are expressed on commissural axons in vitro, and within spinal cord regions containing crossed commissural axon segments in vivo (data not shown). Because contact between cells expressing B-class ephrins, and axons expressing the corresponding receptor(s), is required for a functional interaction to occur (Flanagan and Vanderhaeghen, 1998), we examined the relative positions of decussated commissural axons and B-class ephrin expression

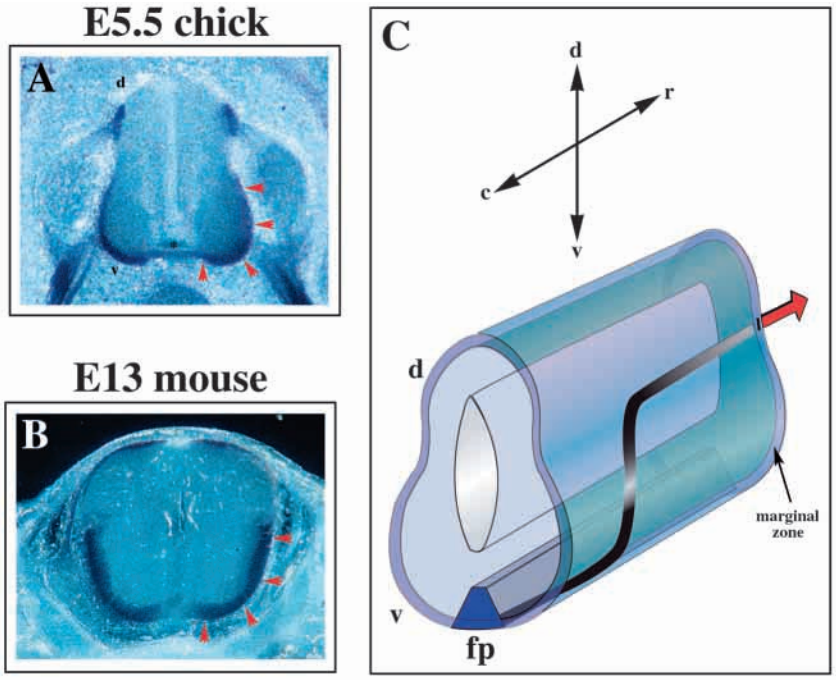

Fig. 6. Postcommissural axons do not re-enter the spinal cord gray matter. Transverse vibratome sections taken from E5.5 chick (A) and E13 mouse (B) embryos were labeled with anti-L1 antibodies to reveal the position of postcommissural axons within the M/L axis of the spinal cord. In both chick and mouse embryos, decussated commissural axons are excluded from the gray matter and are confined to fiber tracts within the marginal zone of the spinal cord (indicated by red arrowheads in A,B). (C) The contralateral trajectory of commissural axons in a schematic representation of a spinal cord cross-section. After exiting the floor plate, most commissural axons navigate along a complex pathway within the spinal cord white matter and assemble into an ascending tract within the dorsal (chick) or intermediate (mouse) marginal zone. In C, an axon is shown exiting the marginal zone (indicated by red arrow) to emphasize the observation that crossed commissural axons do not re-enter the gray matter. c, caudal; d, dorsal; fp, floor plate; r, rostral; v, ventral.

domains in the mouse and chick spinal cord. As a first step toward this goal, we examined the distribution of B-class ephrins in transverse and whole-mount open-book spinal cord preparations obtained from E5 chick and E13 mouse embryos. Consistent with previous findings (Imondi et al., 2000), B-class ephrin protein localized to the dorsal spinal cord and floor plate in both chick (Fig. 7A,B) and mouse (Fig. 7E,F) embryos. Importantly, the ventralmost limit of ephrinB protein expression in dorsal regions of the chick and mouse spinal cord extended across the mediolateral axis and into the marginal zone, where EphB-expressing commissural axons navigate (Fig. 7A,E) (Imondi et al., 2000).

We next examined the position of crossed commissural axons in relation to the dorsal and ventral domains of ephrin expression by anterogradely labeling commissural axons with DiI in both the chick and mouse spinal cord (Fig. 7D,H).

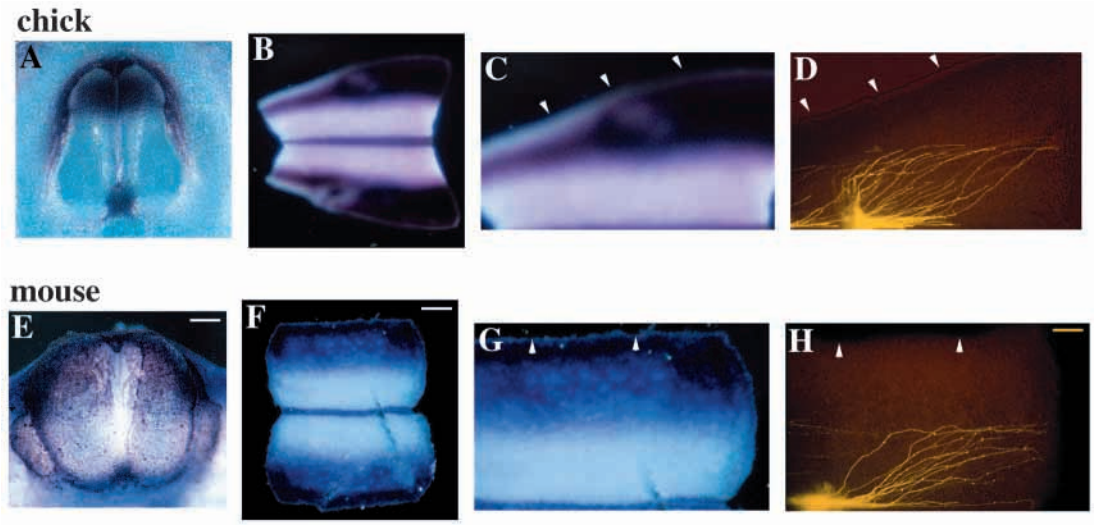

Fig. 7. Commissural axons execute their final turn and project alongside a B-class ephrin boundary in both the chick and mouse spinal cord. EphB3-Fc detects B-class ephrin protein in the floor plate and in dorsal regions of E5.5 chick and E13 mouse transverse (A,E) and open-book (B,F) spinal cord preparations. In the dorsal spinal cord, ephrinB protein is expressed within a broad domain that extends from the ventricular zone to the marginal zone (A,E). (C,G) At high magnification, the top halves of the explants shown in B,F, respectively (the dorsal edge of each explant is indicated by the white arrowheads). (D,H) Combined fluorescence and phase-contrast micrographs of separate preparations (taken at the same AP level and shown at the same magnification as the explants in C and G) reveal the position of DiI-labeled commissural axons relative to B-class ephrin expression domains. Decussated commissural axons in both chick and mouse spinal cord explants initiate a final turn and project longitudinally adjacent to a B-class ephrin boundary in the dorsal (chick) or intermediate (mouse) marginal zone. Scale bars: in E, 100 μm for A,E; in F, 250 μm for B,F; in H, 125 μm for C,D,G,H.

Strikingly, commissural axons execute their final turn, and project longitudinally for extended distances alongside the ventralmost boundary of ephrin expression in the dorsal spinal cord (compare Fig. 7C,D with 7G,H). A cervical region of the chick spinal cord (Fig. 7C,D) is shown to emphasize the observation that commissural axons do not turn and project longitudinally at a fixed distance from the dorsal (lateral) limit of these spinal cord preparations; rather, axons reorient their growth from diagonal to longitudinal at a dorsal (chick) or intermediate (mouse) boundary of B-class ephrin expression. Importantly, mouse commissural axons make their final turn at a slightly more ventral position than do their chick counterparts (compare Fig. 7D with 7H). Accordingly, this turn coincides with a boundary of ephrin expression that extends into more ventral regions than it does in chick (compare Fig. 7A,B with 7E,F).

Collectively, these observations suggest that decussated commissural axons in chick and mouse initiate two rostral turns at boundaries of B-class ephrin expression: the first occurs at the contralateral floor plate margin, as axons exit the floor plate and turn orthogonally into the ventral funiculus; the second occurs at a more dorsal position, as axons undergo a transition from diagonal to longitudinal growth.

Perturbation of endogenous B-class Eph receptor-ligand interactions results in the aberrant projection of commissural axons into dorsal regions of ephrin expression in cultured mouse spinal cord explants

B-class ephrins are expressed at points along the contralateral commissural pathway where obvious alterations in trajectory are observed. These correlative findings suggest a contact-dependent role for these repulsive ligands in establishing barriers to axon growth and/or in specifying or maintaining the rostrocaudal polarity of axons at these decision regions. To explore this possibility in vitro, we cultured collagen-embedded mouse open-book spinal cord preparations for 48 hours in the presence of ephrinB1-Fc (to block endogenous receptor sites on commissural axons), EphB3-Fc (to block endogenous ephrin sites in intermediate and dorsal regions of the spinal cord), EphA1-Fc or Fc alone (Fig. 8, Table 2). In uncultured explants obtained from E11-E11.5 littermates (Fig. 8A), the earliest commissural axons (i.e. those that had

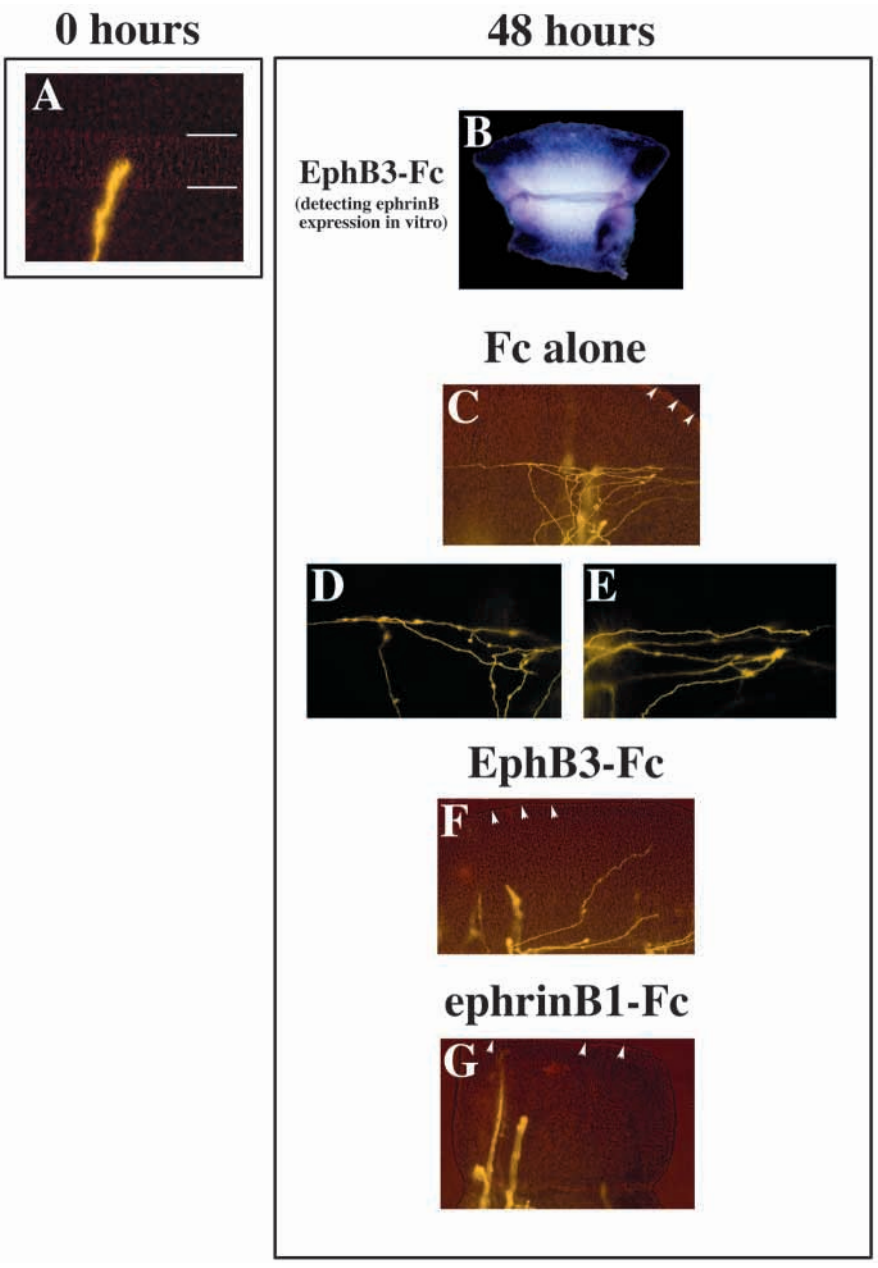

Fig. 8. In vitro blockade of EphB-ephrinB interactions results in the misprojection of commissural axons into dorsal regions of the spinal cord that express B-class ephrins. (A) A combined fluorescence and phase-contrast micrograph of an E11 mouse open-book spinal cord explant in which commissural axons were anterogradely labeled with DiI at 0 hours in culture. In this preparation, a single axon is shown within the floor plate (bounded by white lines). (B) EphB3-Fc appropriately detects B-class ephrin expression in the floor plate and in a dorsal region of cultured E11.5 open-book spinal cord explants. (C-G) Commissural axons were anterogradely labeled with DiI in open-book spinal cord preparations obtained from E11-E11.5 mouse embryos and cultured for 48 hours in the presence of 50 μg/ml Fc alone (C-E), EphB3-Fc (F) or ephrinB1-Fc (G). Each panel depicts only the trajectory followed by commissural axons on the contralateral side of the spinal cord. (C) In explants cultured in the presence of Fc alone, commissural axons grew a considerable distance away from the floor plate and turned at an appropriate position within an intermediate region of the spinal cord white matter. (D,E) At high magnification, the turns executed by decussated commissural axons in C. In explants cultured in the presence of EphB3-Fc (F) or ephrinB1-Fc (G), commissural axons aberrantly projected into more dorsal regions of the spinal cord where B-class ephrins are expressed.

Table 2. Spinal cord explant analysis

Condition (50 μg ml^{-1})	Number of axon cohorts analyzed	Percentage of crossed axons that misprojected into dorsal ephrin boundary
Fc alone	18	4.0
EphA1-Fc	7	3.1
EphrinB1-Fc	27	41.4
EphB3-Fc	26	26.9

Intact open-book spinal cord explants were cultured for 48 hours in a collagen gel and subsequently examined for commissural pathfinding defects. Focal DiI applications were made in the dorsal spinal cord of fixed preparations approximately 30 μm ventral to the roof plate. The percentage of crossed axons that extended into the dorsal ephrin boundary (see Materials and Methods) was calculated in DiI-labeled cohorts (one axon cohort was examined in each explant cultured under the conditions outlined above). The values indicated represent the mean scores obtained from three independent experiments.

extended the furthest distance) were found within the floor plate. To determine if B-class ephrin expression is maintained in vitro along the same spatial dimensions as those observed in vivo, we labeled explants cultured for 48 hours with EphB3-Fc. As expected, ephrinB protein localized to the intermediate and dorsal spinal cord, as well as to the floor plate, in a manner consistent with that observed in vivo (Fig. 8B). Furthermore, in identical in vitro preparations, the ventral boundary of this domain demarcated the turn executed by commissural axons within this region (data not shown). In control explants cultured for 48 hours in the presence of Fc alone or EphA1-Fc (Fig. 8C-E, Table 2), axons successfully crossed the midline and extended over distances in excess of 1 mm. More importantly, although the growth of these axons deviated somewhat from the stereotypic diagonal trajectory observed in vivo, they nevertheless projected away from the floor plate and into a more dorsal position. Here, axons turned into the AP axis at an appropriate distance from the floor plate, although roughly half of these axons turned in both rostral and caudal directions; whether this is a secondary consequence of rostrocaudal polarity errors similarly observed at the contralateral floor plate boundary is presently unclear. Strikingly, in explants cultured for 48 hours in the presence of either EphB3-Fc (Fig. 8F) or ephrinB1-Fc (Fig. 8G), decussated commissural axons failed to turn at the ephrin boundary and instead inappropriately projected dorsally into regions of ephrinB expression. In many cases, axons approached the dorsal (lateral) edge of these spinal cord preparations (Fig. 8G). These observations support a role for B-class ephrins in defining a barrier to commissural axon growth in the dorsal marginal zone.

DISCUSSION

We provide, for the first time, a detailed characterization of the contralateral commissural pathway in the developing mouse and chick spinal cord. Commissural neuron cell bodies located within a broad dorsal domain of the spinal cord extend axons that assume one of three distinct trajectories on the contralateral side of the floor plate. Consistent with previous findings, commissural axons execute a rostral turn at the contralateral floor plate margin and extend for a short distance within the ventral funiculus, a longitudinal fiber tract that forms in close apposition to the floor plate (Bovolenta and Dodd, 1990). Subsequently, most of these axons depart from the ventral funiculus and project diagonally away from the ventral midline and into a more dorsal position, where they initiate a final rostral turn into the longitudinal axis. This turn occurs at a conserved boundary of B-class ephrin expression, which may prohibit continued growth into more dorsal regions and thereby specify the dorsoventral position of an ascending commissural axon tract. In support of this interpretation, perturbation of endogenous EphB-ephrinB interactions in vitro results in the misprojection of commissural axons into dorsal regions of B-class ephrin expression.

Virtually all commissural axons follow the same initial post-crossing trajectory, which consists of a rostral turn at the contralateral floor plate margin and growth within the ventral funiculus for distances of at least 100 μm. Although we observed a population of axons that projects longitudinally in close proximity to the floor plate for extended distances, these represented a very minor proportion of the total number of axons that crossed the floor plate. Indeed, the vast majority of decussated axons maintained only transient association with the floor plate before continuing along a complex pathway that culminates in the formation of a longitudinally projecting commissural axon bundle in a more dorsal region of the spinal cord white matter. A remarkably similar pattern of commissural axon outgrowth has been described in zebrafish (Bernhardt et al., 1990; Kuwada et al., 1990) and *Xenopus* (Roberts et al., 1987) embryos, suggesting that the contralateral pathway is likely to be conserved across all vertebrate species.

Within a given cohort of DiI-labeled axons, we could distinguish individual commissural axons that differed in their extent of growth along the contralateral pathway in both the chick and mouse spinal cord. After leading axons had initiated a final turn into the longitudinal axis within the dorsal white matter, follower axons (in the same cohort of axons) were observed within the ventral funiculus or at various points along the diagonal segment of the pathway. Although most commissural axons, upon exiting the floor plate, transiently fasciculate with other axons extending within the ventral funiculus, they eventually depart from this fiber tract and extend obliquely into more dorsal (chick) and intermediate (mouse) positions. In these regions, early and late commissural axons extend at varying angles and are loosely apposed to one another. While these observations suggest that commissural growth cones respond independently of one another to cell- or substratum-associated cues in distal segments of the contralateral pathway, we cannot rule out the possibility that fasciculation with other types of early-projecting axons influences their trajectory.

The observation that most commissural axons ultimately turn away from the floor plate is interesting in light of a recent study in which cultured rat commissural axons were shown to require trophic support from the floor plate at developmental stages that are roughly equivalent to E11-E15 in mouse (Wang and Tessier-Lavigne, 1999). In the present study, the earliest mouse commissural axons were observed to cross through the floor plate between E10 and E11; longitudinal growth alongside this structure occurred for roughly 12-15 hours, after which time axons turned into more intermediate regions of the white matter. Although we cannot directly compare the age-dependency of trophic support observed in vitro with the

pathfinding behavior of commissural axons observed in the present study, it seems reasonable to suggest that in vivo, trophic support from the floor plate may not be required later than E11.5 in mouse. It should be noted, however, that as decussated commissural growth cones navigate along more distal segments of their contralateral trajectory, they leave behind an axonal segment that lies in close proximity to the floor plate and that may bear receptors capable of responding to a trophic signal originating from adjacent floor plate cells.

Short- and long-range guidance cues expressed by floor plate cells and other ventral cell types play a key role in the guidance of commissural axons along ipsilateral segments of their trajectory (Kaprielian et al., 2001; Tear, 1999). The complex behavior exhibited by these axons on the contralateral side of the floor plate suggests additional roles for ventral cells in the guidance of axons along more distal segments of their pathway. In support of this interpretation, analyses of commissural axon pathfinding in the spinal cords of *Sd*- (Bovolenta and Dodd, 1991) and *Gli2*- (Matise et al., 1999) deficient mouse embryos demonstrate that the floor plate (and/or immediately adjacent interneurons) instructs axons to turn appropriately into the longitudinal axis. More recently, in mice lacking *npn2*, commissural axons were shown to exhibit a variety of defects upon crossing the midline, including rostrocaudal polarity errors and misdirected growth into ventral spinal cord regions (Zou et al., 2000).

In a previous study (Imondi et al., 2000), we demonstrated that B-class transmembrane ephrins are expressed at the lateral margins of the floor plate and in a broad dorsal region of the mouse spinal cord. We further showed that the corresponding receptor was reciprocally expressed on axons that project longitudinally between these ephrin domains. Based on the established role of B-class ephrins as repellent molecules in other systems (Cowan et al., 2000; Flanagan and Vanderhaeghen, 1998; Gale and Yancopoulos, 1997; Holder and Klein, 1999; Nakagawa et al., 2000), and on the ability of soluble forms of ephrins to induce the collapse of a subset of commissural growth cones in vitro (Imondi et al., 2000), we proposed at that time that local B-class Eph/ephrin interactions restrict longitudinal projections to a region of the spinal cord adjacent to, but not overlapping, the dorsal spinal cord or floor plate. We now extend these findings, and show that the complementary expression of transmembrane ephrins and their receptors is conserved in the chick spinal cord. The DiI tracing analyses performed in the present study enabled us to visualize at high resolution the behavior of decussated commissural axons and growth cones at these ephrin expression domains. In the chick spinal cord, abrupt changes in commissural growth cone morphology were observed as axons encountered B-class ephrins in the dorsal marginal zone. Furthermore, both chick and mouse commissural axons underwent stereotypic alterations in trajectory that correlated with boundaries of transmembrane ephrin expression. The first occurred at the contralateral margin of the floor plate, as newly crossed axons exhibited a sharp transition from transverse to longitudinal growth. The second occurred at a dorsal (chick) or an intermediate (mouse) boundary of ephrin expression, where axons underwent a transition from diagonal to longitudinal growth. The identity of the cells that comprise the dorsal ephrinB expression domain is presently unknown. Conceivably, glial end-feet or other non-neuronal cell types that reside within the marginal zone could provide a source of ephrinB protein encountered by commissural axons as they execute their final turn. Regardless of their source, the localization of transmembrane ephrins to regions of the contralateral pathway where axons turn in a longitudinal direction suggests a contact-dependent role for these proteins in demarcating barriers to axon growth and/or in establishing or maintaining the polarity of commissural axons.

To address these possibilities in vitro, we developed an assay system in which intact spinal cord explants, obtained from mouse embryos at an age when the earliest commissural axons have extended into the ventral midline, are embedded within a collagen matrix and cultured over a period of 48 hours. During this culture period, commissural axon pathfinding retains many, but not all, of the characteristics observed in vivo over an equivalent time span. More specifically, commissural axons successfully traverse the ventral midline and turn, but often fail to project rostrally at the contralateral floor plate margin. Nevertheless, crossed axons appropriately project away from the floor plate and into more intermediate regions of the spinal cord, albeit in a less organized fashion than that observed in vivo. These axons ultimately execute a final turn at an appropriate dorsoventral position, and subsequently project longitudinally for distances comparable to those observed in vivo, but do so in both rostral and caudal directions.

Clearly, the inability of many decussated axons to initiate turns in the appropriate directions represents a limitation of this assay system for studies examining the contribution of cues, like ephrins, to rostrocaudal polarity decisions. As the spatial distribution of ephrinB protein is maintained in vitro, these errors are likely to reflect changes in the distribution of other cues introduced by our culture conditions. Alternatively, normal geometric constraints on the diffusion of soluble cues away from their point sources could conceivably be disrupted by the configuration of our culture preparations. Distinguishing between these interesting possibilities is beyond the scope of the present study.

Despite other obvious deviations from their in vivo trajectory, commissural axons in our assay system did not extend into dorsal regions of B-class ephrin expression. Instead, axons turned either rostrally or caudally at, and then projected longitudinally immediately alongside, the ventralmost boundary of ephrin expression in the dorsal marginal zone. Perturbation of interactions between dorsal cell types expressing one or more ephrins, and growth cones/axons expressing the corresponding receptor, resulted in the failure of these axons to execute a turn at the ventralmost limit of ephrin expression in the dorsal spinal cord; rather, axons projected directly through this region of ephrin expression and, in many cases, reached an extreme dorsal position near the margin of the roof plate.

Our findings suggest a role for B-class ephrins in establishing a repulsive barrier to axon growth in the dorsal spinal cord and are consistent with recent data showing a role for B-class ephrins in prohibiting the growth of a subset of retinal ganglion axons across the metamorphic optic chiasm in *Xenopus* embryos (Nakagawa et al., 2000). Surprisingly, we did not observe specific pathfinding defects at the ventral midline, where ephrin expression might be expected to play a contact-dependent role in preventing decussated commissural

axons from re-crossing the floor plate. Although this may simply reflect a limitation of our assay system, several alternative explanations are plausible. Other midline-associated guidance cues may act alone, or in combination with transmembrane ephrins to prohibit commissural axon recrossing. This possibility is consistent with the demonstrated ability of these axons to acquire responsiveness to the repellent activity of floor plate-derived slit in vitro (Zou et al., 2000). Alternatively, ephrins may play a more prominent role in establishing rostrocaudal polarity as axons emerge from the floor plate. We could not reliably assess a potential contribution of ephrins in specifying rostrocaudal polarity at the ventral midline in our assay system as many axons inappropriately projected in both rostral and caudal directions in the absence of function-blocking reagents. Interestingly, in ovo perturbation of endogenous B-class Eph receptor-ephrin interactions results in rostrocaudal polarity defects at the ventral midline of the developing chick spinal cord (R. I. and Z. K., unpublished).

In vertebrates, longitudinal axons corresponding to a given class of neurons form bundles that assume distinct positions along the DV axis of the spinal cord (Kuwada, 1986; Yaginuma et al., 1994). A priori, it seems reasonable to assume that a combination of guidance forces act coordinately to determine the position of these axon tracts. In the *Drosophila* CNS, a presumptive gradient of repulsive slit, which originates from the midline and extends laterally, appears to act a long range to coarsely define the mediolateral regions within which longitudinal axons expressing different combinations of Robo receptors project (i.e. the 'Robo code') (Rajagopalan et al., 2000; Simpson et al., 2000). In light of these observations, it is tempting to speculate that a similar long-range guidance mechanism influences the pathfinding of spinal commissural axons. This possibility is consistent with the expression of slits at or near the ventral midline, and with the expression of Robo1 and Robo2 mRNA by positionally distinct commissural neuron populations (Brose et al., 1999; Kidd et al., 1998). Thus, in addition to excluding commissural axons from the gray matter, slits (and possibly one or more semaphorins) may subserve an additional role in repelling commissural axons away from the ventral midline and into more dorsal positions.

Interestingly, slits are among a growing number of known or putative guidance molecules that are expressed in both the floor plate and the roof plate during the period of commissural axon pathfinding (Kaprielian et al., 2001). These midline structures may thus play complementary roles in shaping different portions of the contralateral commissural trajectory through the deployment of cues that act a different points along the pathway (Fig. 9). Support for this interpretation is provided by the recent identification of BMP7 as a roof plate-derived factor capable of repelling commissural axons/growth cones in vitro (Augsburger et al., 1999). The ability of BMP7 to re-orient axons emanating from dorsal spinal cord explants obtained at an age when commissural axons have crossed the ventral midline (Augsburger et al., 1999) suggests that it may play a role in the later pathfinding of commissural axons within contralateral segments of their trajectory. Thus, in the spinal cord, opposed gradients of soluble repellents originating from the floor plate and the roof plate may operate in combination with the short-range effects of B-class ephrins to precisely determine the position of longitudinal fiber tracts.

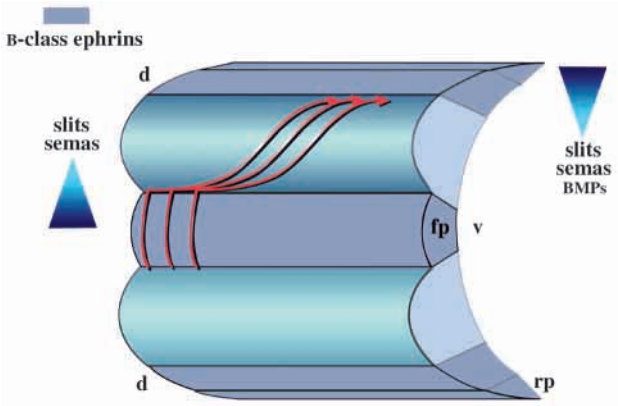

Fig. 9. Repulsive guidance cues that are likely to influence the contralateral commissural pathway. Several repellent guidance cues expressed in the spinal cord are ideally positioned to shape the contralateral trajectory of commissural axons. Slit proteins expressed by both floor plate cells and motoneurons may force newly crossed commissural axons to turn into the longitudinal axis (Li et al., 1999). A graded distribution of soluble repellent cues (e.g. one or more slits or semaphorins) secreted by floor plate cells or other ventral cell types may also act to deflect decussated commissural axons (shown in red) away from the ventral midline and into more dorsal spinal cord regions. In complementary fashion, secreted cues emanating from cells situated at or near the dorsal midline, including one or more slits, semaphorins and BMPs, may act coordinately with the local effects of B-class ephrins to specify the dorsoventral position of the longitudinal commissural tract. d, dorsal; fp, floor plate; rp, roof plate; v, ventral.

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