Commentary & View

Leaving the midline

How Robo receptors regulate the guidance of post-crossing spinal commissural axons

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Abbreviations: VM, ventral midline; FP, floor plate; MLC, medial longitudinal commissural; ILC, intermediate longitudinal commissural; Robo1/2, Robo 1 and 2; CNS, central nervous system; FL, full length; SCF, stem cell factor

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In the developing nervous system, pathfinding axons navigate through a series of intermediate targets in order to form synaptic connections. Vertebrate spinal commissural axons extend toward and across the floor plate (FP), a key intermediate target located at the ventral midline (VM). Subsequently, post-crossing commissural axons grow either alongside or significant distances away from the floor plate (FP), but never re-cross the VM. Consistent with this behavior, post-crossing commissural axons lose responsiveness to the FP-associated chemoattractants, Netrin-1 and SHH, and gain responsiveness to Slits, which are potent midline repellents, in vitro. In addition, the results of several in vivo studies suggest that the upregulation of Slit-binding repulsive Robo receptors, Robo1/2, alters the responsiveness of decussated commissural axons to midline guidance cues. Nevertheless, in vertebrates, it is unclear whether Robo1/2 are the sole or major repellent receptors responsible for driving these commissural axons away from the VM and preventing their re-entry into the FP. We recently re-visited these issues in the chick spinal cord by assessing the consequences of manipulating Robo expression with this behavior, post-crossing commissural axons lose responsiveness to the FP-associated chemoattractants, Netrin-1 and SHH, and gain responsiveness to Slits, which are potent midline repellents, in vitro. In addition, the results of several in vivo studies suggest that the upregulation of Slit-binding repulsive Robo receptors, Robo1/2, alters the responsiveness of decussated commissural axons to midline guidance cues. Nevertheless, in vertebrates, it is unclear whether Robo1/2 are the sole or major repellent receptors responsible for driving these commissural axons away from the VM and preventing their re-entry into the FP. We recently re-visited these issues in the chick spinal cord by assessing the consequences of manipulating Robo expression by crossing the midline, represent an ideal model system for elucidating mechanisms that control guidance decisions at intermediate targets. In vertebrate embryos, commissural neurons are widely distributed along the dorsoventral axis of the spinal cord and project their axons ventrally in the transverse/circumferential direction to, and across, the floor plate (FP), a key intermediate target located at the ventral midline (VM).1 Whereas pre-crossing commissural axons follow a relatively simple route toward the ventral midline, post-crossing axons extend along a variety of complex trajectories on the contralateral side of the FP. We previously used focal applications of DiI, in both chick and mouse embryos, to characterize the post-crossing behavior of several distinct classes of spinal commissural axons on the contralateral side of the VM. This revealed that most decussated commissural axons turn rostrally into the longitudinal plane and then either project alongside the FP for significant distances (Medial Longitudinal Commissural; MLC) or along a sigmoidal trajectory away from the VM and into an intermediate/dorsal region of the spinal cord where they make a second turn into the longitudinal plane (Intermediate Longitudinal Commissural; ILC).2,3 These findings raise the possibility that post-crossing MLC and ILC axons express different complements of guidance receptors, which may account for the distinct pathfinding behaviors these axons display on the contralateral side of the VM. On the other hand, since both MLC and ILC axons never re-cross the VM, a common molecular mechanism may prevent both types of axons from re-entering the FP. The FP is a rich source of both positively acting chemoattractants and inhibitory chemorepellents. Accordingly, in order to ultimately leave the VM and move on to the next intermediate (or their final) target, post-crossing commissural axons must lose responsiveness to the midline-associated attractants that initially

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Figure 1. Model for the roles of Robo receptors in regulating commissural axon guidance within the developing vertebrate spinal cord. Pre-crossing spinal commissural axons express high levels of Robo3.1 and low levels of Robo1, Robo2 and Robo3.2. Robo3.1 is thought to repress the premature responsiveness of pre-crossing axons to midline-associated Slits so that these axons fail to perceive the VM as an inhibitory environment and are attracted to the FP by Netrin. After crossing the VM, dI1 and dI2 axons extend along both MLC and ILC trajectories on the contralateral side of the FP. Post-crossing commissural axons downregulate the expression of Robo3.1 and upregulate Robo1, Robo2 and Robo3.2 (highlighted in red). The upregulation of repulsive Robos alters the responsiveness of decussated axons to midline cues and facilitates their expulsion from, but does not bar their re-entry into, the VM. Concomitantly, a Slit dependent interaction between the cytoplasmic domains of Robo and DCC is likely to silence Netrin attraction on post-crossing axons.

The results of loss-of-function studies carried out in mouse and chick embryos provide strong in vivo support for key roles of Robo-Slit signaling in regulating spinal commissural axon guidance. For example, in mice lacking Robo1, Robo2, both Robo1 and Robo2 or all three Slits, some commissural axons temporarily stall within the confines of the FP and a subset of post-crossing axons inappropriately re-cross the FP in Slit triple knockout mice. These phenotypes are consistent with repulsive Robo-Slit interactions expelling post-crossing commissural axons away from the VM and preventing these axons from re-entering the FP.

Several years ago, a key study reported the existence of a third Robo, referred to as Robo3, that is expressed at high levels on pre-crossing commissural axons and at low levels on the decussated portions of these axons and that likely regulates repulsive Robo-Slit signaling. Strikingly, essentially all commissural axons fail to cross the VM and inappropriately turn into the longitudinal plane on the ipsilateral side of the FP in Robo3-lacking mice, mimicking the Commissiless (Comm) mutant phenotype in Drosophila. Consistent with this observation, pre-crossing commissural axons derived from Robo3 knockout spinal cords are prematurely responsive to Slits in vitro and the loss of Slit(s) or Robo1 in a Robo3 null background partially rescues the lack of crossing phenotype. The precise mechanism through which Robo3 inhibits Robo function

Guided them to the VM and gain responsiveness to midline repellents, which presumably block their re-entry into the FP. The results of several key in vitro studies have provided support for an altered responsiveness model of midline guidance that enables post-crossing commissural axons to perceive the once attractive VM as a repulsive environment. First, decussated, rodent hindbrain-associated commissural axons fail to grow towards an ectopically positioned FP or a source of Netrin-1, the chemoattractant that guides most commissural axons to the midline, despite the fact that these axons continue to express DCC, an attractive Netrin receptor. Second, post-crossing commissural axons selectively gain responsiveness to midline-associated Slits (Slits1-3) that function as repulsive ligands for the Robo receptors, Robo1 and Robo2, which are dynamically upregulated on these axons as they cross the VM in the mouse and chick spinal cord, just as they are in the Drosophila ventral nerve cord (Fig. 1). Finally, the results of a particularly ingenious set of experiments utilizing the Xenopus turning assay, in which the responsiveness profile of individual neurons/axons to exogenously applied guidance cues can be readily assessed in vitro, has provided an elegant model for how guidance receptors may operate in a hierarchical manner to drive commissural axons away from the VM. These studies were based on the observation that spinal cord neurons/axons derived from Xenopus embryos at early stages of development are attracted to a source of Netrin-1, but that later stage neurons/axons are indifferent to Netrin. By performing a comprehensive array of turning assays, some of which involved the mis-expression of a variety of chimeric receptors in Xenopus neurons, and binding experiments, these investigators showed that, at least in vitro, midline-associated Slits induce an interaction between the cytoplasmic domains of Robo and DCC, which effectively silences Netrin-mediated attraction of the older axons (Fig. 1). This presumably ensures that post-crossing commissural axons selectively lose their responsiveness to Netrin-1, preventing a tug-of-war between the attractive and inhibitory forces that these axons encounter at the VM. Despite the ground breaking nature of this work, whether commissural neurons or even the same types of neurons were assayed at both early and late stages of Xenopus development, and whether Robo silences DCC in vivo remain important questions that will need to be addressed in follow-up studies (see also Dickson B.J., and Gilestro G.F., 2006). It is also unclear how midline-derived Slits can induce a Robo-DCC interaction, which presumably has a major role in expelling post-crossing commissural axons away from the VM, if only low levels of Robo receptors are expressed on these axons as they cross the midline. Furthermore, why DCC expression is maintained on post-crossing segments of commissural axons is a key open question.

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is unclear. In Drosophila, Comm prevents Robo receptors from reaching the surface by sorting them into intracellular compartments and in Comm mutants Robos are aberrantly expressed on pre-crossing commissural axons. However, Robo3 does not appear to regulate Robo trafficking in the same way as Drosophila Comm, since the levels of Robo1 and Robo2 protein appear unchanged in Robo3-4 embryos. Taken together with the observation that Robo3 is likely to bind Slits, these findings support a model in which Robo3 normally suppresses the responsiveness of pre-crossing commissural axons to midline-associated Slits. More recently, two different isoforms of Robo3 were identified in both chick and mouse embryos. The mouse Robo3 isoforms, Robo3.1 and Robo3.2, arise from alternative splicing of Robo pre-mRNA and are segregated to pre- and post-crossing segments of commissural axons, respectively (Fig. 1). Surprisingly, the results of loss-of-function and gain-of-function, as well as rescue, experiments conducted in mouse embryos reveal functionally antagonistic roles for these isoforms. Specifically, it appears that Robo3.1 on pre-crossing commissural axons ensures (through an unknown mechanism) that these axons are unresponsive to midline repellents. In contrast, the upregulation of Robo3.2 on post-crossing commissural axons facilitates the expulsion of these axons from the VM and prevents them from re-entering the FP (Fig. 1). In addition to a clear role in regulating commissural axon guidance at the VM, Robo-Slit signaling may also facilitate the lateral positioning of decussated commissural axons through the actions of a so-called Robo code. Despite these observations, it is important to note that a significant number of commissural axons elaborate appropriate contralateral projections in mice lacking Robo1 and Robo2 or all three Slits, raising the possibility that there may exist functionally-distinct populations of commissural axons that do not require or are not influenced by Robo/Slit signaling.

Taken together, the compelling findings described above implicate key roles for Robos and Slits in regulating commissural axon guidance within the vertebrate spinal cord. However, these studies leave open several important questions. For example, (1) does the upregulation of repulsive Robos (Robo1/2) on post-crossing commissural axons facilitate the expulsion of these axons away from the VM as well as prevent them from re-entering the FP? (2) Is Robo-Slit signaling required to regulate the post-crossing behavior of different classes of commissural axons that elaborate distinct contralateral projections and is it consistent with the current model? (3) Will the precocious expression of Robo1/2 on pre-crossing commissural axons in vivo prevent these axons from crossing the VM? These critical questions have previously been addressed in Drosophila, but have only begun to be carefully and explicitly considered in the mouse and chick spinal cord.

We have recently re-investigated these issues by using electroporation-based strategies to visualize and manipulate receptor expression on commissural axons in the developing chick spinal cord. As a first step towards assessing the role(s) of repulsive Robo receptors in chick embryos, we developed a genetic labeling system that can be used to reliably and reproducibly mark subsets of commissural axons in an in ovo setting. The basic helix-loop-helix (bHLH) transcription factors, Atoh1 and Neurog1 define non-overlapping progenitor populations that differentiate into genetically distinct dl1 and dl2 commissural neurons, respectively. Since it had previously been shown that Atoh1 and Neurog1 enhancers can direct reporter gene expression to dl1 and dl2 neurons/axons in transgenic mice, we reasoned that these regulatory elements may allow us to reproducibly label commissural axons in electroporated chick embryos. Accordingly, we unilaterally electroporated chick embryos with Atoh1 and Neurog1 reporter constructs to label dl1 and dl2 commissural axons as they extend toward and across the VM, and as they elaborate contralateral projections. These analyses revealed that murine Atoh1 and Neurog1 enhancers effectively direct reporter gene expression to pathfinding commissural neurons/axons in the avian spinal cord, and that both dl1 and dl2 axons elaborate rostrally-directed MLC trajectories on the contralateral side of the FP (Fig. 1). We next developed new anti-Robo1/2 antibodies and showed, consistent with what has been described in the fly and mouse spinal cord, that these receptors are preferentially expressed on post-crossing commissural axons. Notably, however, Robo 1 and 2 were not segregated to distinct longitudinal tracts within the chick spinal cord, as has been elegantly shown in the Drosophila ventral nerve cord and as is likely to be the case in the mouse spinal cord. A possible explanation for this discrepancy is that the Robo/Slit guidance system may not regulate the pathfinding of commissural axons in precisely the same manner across species. A non-mutually exclusive possibility is that ventral and lateral funiculi in the chick spinal cord may be composed of a more heterogeneous population of post-crossing commissural axons than their mouse counterparts.

To elucidate in vivo roles for Robo receptors in the chick spinal cord we disabled Robo signaling by mis-expressing cytoplasmic truncations of Robo1 and Robo2 on commissural axons and assessed the consequences of these manipulations on the pathfinding of dl1 and dl2 axons. Initially, we designed constructs that use the Atoh1 enhancer to drive truncated forms of Robo1 or Robo2 to dl1 neurons/axons, but this resulted in essentially undetectable levels of ectopic expression. Therefore, we co-electroporated chick embryos with Atoh1 and Neurog1 reporter constructs and CMV-Robo1Δ-GFP and CMV-Robo2Δ-GFP expression constructs, which encode the extracellular and transmembrane domains of human Robo1 or Robo2. The use of dl1 and dl2 enhancers has allowed us to reliably and reproducibly label, and assess the consequences of manipulating guidance receptor expression on, the same subsets of commissural axons in each embryo. Whereas the forced expression of truncated Robos did not impair the ability of dl1 and dl2 axons to cross the midline, the post-crossing segments of these axons failed to project away from the FP along ILC trajectories and, instead, adopted exclusively MLC trajectories, projecting alongside the FP for significant distances (Fig. 2A). Importantly, none of these axons stalled within the FP or re-entered the VM. Surprisingly, we observed that even those post-crossing axons emanating from the non-electroporated side of the spinal cord and which do not express truncated Robos failed to project away from the FP. Given that mis-expressed cytoplasmic truncations of Robo likely bind all available Robo ligands,
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The results of our chick studies and these previously published observations in knockout mouse embryos suggest that, unlike in the Drosophila ventral nerve cord, other guidance systems are likely to have a more significant role than Robo/Slit signaling in ensuring that commissural axons cross the midline only once in the vertebrate spinal cord.

The genetic labeling strategy that we have developed could, in principle, be used in conjunction with loss-of-function and gain-of-function approaches to provide a model for how the collective actions of multiple molecular systems establish a contralateral navigational program that controls the guidance of relatively homogeneous populations of commissural axons. Importantly, the Atoh1 and Neurog1 enhancer elements may even make it possible to analyze the effects of selectively mis-expressing guidance receptors on dI1 or dI2 axons in order to determine whether the guidance of genetically-distinct commissural axons is controlled by different molecular programs. Our recent findings indicate that, at least in chick embryos, the upregulation of Robo1/2 on decussated commissural axons facilitates the expulsion of these axons from the VM, but is unlikely to bar their re-entry into the FP.10 Accordingly, other receptors and cues are likely to operate (potentially in concert with Robo1/2) to ensure that MLC and ILC axons never re-cross the FP in the chick spinal cord. Good candidates include the Robo3 isoform, Robo3.2, that is selectively expressed on post-crossing segments of mouse commissural axons13 as well as the midline-associated repulsive Npn2-Sema and EphB-ephrin-B2 guidance systems. By utilizing the genetic labeling system and strategies for manipulating receptor expression on commissural axons that we have recently described,10 it should be possible to identify whether these molecules have key roles in ensuring that commissural axons never re-cross the midline. We have also recently shown that following the forced expression of truncated or full-length Robos, mis-guided longitudinally-oriented axons re-cross the midline.

Figure 2. Manipulating Robo1/2 expression in ovo perturbs the guidance of dI1 and dI2 commissural axons in the chick spinal cord. (A) In response to mis-expressing a cytoplasmic truncation of Robo1 or Robo2 (RoboΔ), dI1 and dI2 axons successfully cross the VM, turn into the longitudinal plane and project adjacent to the FP along MLC trajectories (solid lines), but do not grow away from the FP along ILC trajectories (dashed lines represent wild-type ILC trajectories). (B) Following mis-expression of full-length (FL) Robo, many dI1 and dI2 labeled axons fail to cross the FP and inappropriately turn into the longitudinal plane on the ipsilateral side of the VM (solid lines). Dashed lines represent wild-type ILC and MLC trajectories. Lightning bolts denote unilateral electroporation of RoboΔ-GFP and RoboFL-GFP; α = anterior, p = posterior, rp = roof plate; fp = floor plate.

this intriguing finding suggests that unilaterally mis-expressed truncated Robos block the expulsion of post-crossing axons on both sides of the spinal cord through a cell non-autonomous mechanism by presumably sequestering all midline-derived Slits. Notably, this model is consistent with a Slit-dependent, Robo1/2-mediated silencing mechanism normally facilitating the expulsion of post-crossing axons from the FP in the chick spinal cord.10

In a complementary set of experiments, designed to determine whether the presumed upregulation of Robo1/2 switches the responsivity of commissural axons from attraction to repulsion at the midline in vivo, we mis-expressed full length (FL) Robo on pre-crossing commissural axons as they project towards the FP. In response to this manipulation, most dI1 and dI2 axons failed to cross the FP and inappropriately turned into the longitudinal plane on the ipsilateral side of the VM (Fig. 2B).10 This robust phenotype is consistent with FP contact and the upregulation of Robo1, 2 and 3,2 altering the responsiveness of commissural axons to midline repellents7 and demonstrates that mis-expression of FL Robo is capable of overcoming the positive actions of Robo3/3.1, which normally facilitates midline crossing.13,14 Taken together, these findings suggest that, at least in the chick spinal cord, the upregulation of repulsive Robos on post-crossing commissural axons alters the responsiveness of these axons to midline guidance cues and facilitates their expulsion from, but are not likely to have a major role in preventing their re-entry into, the VM (Fig. 1). These chick findings contrast previous studies carried out in Drosophila, and to some extent, in the mouse spinal cord. Notably, Long et al. failed to detect midline re-crossing events in Robo1 and Robo2 single knockout mice, and this raised the possibility that these Robos function redundantly to bar the re-entry of decussated commissural axons into the FP. Also, midline re-crossing was not observed in Robo1/2 double mutants13 and only a small number of axons re-crossed the FP in Robo triple knockout embryos.13

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The genetic labeling strategy that we have developed could, in principle, be used in conjunction with loss-of-function and gain-of-function approaches to provide a model for how the collective actions of multiple molecular systems establish a contralateral navigational program that controls the guidance of relatively homogeneous populations of commissural axons. Importantly, the Atoh1 and Neurog1 enhancer elements may even make it possible to analyze the effects of selectively mis-expressing guidance receptors on dI1 or dI2 axons in order to determine whether the guidance of genetically-distinct commissural axons is controlled by different molecular programs. Our recent findings indicate that, at least in chick embryos, the upregulation of Robo1/2 on decussated commissural axons facilitates the expulsion of these axons from the VM, but is unlikely to bar their re-entry into the FP.10 Accordingly, other receptors and cues are likely to operate (potentially in concert with Robo1/2) to ensure that MLC and ILC axons never re-cross the FP in the chick spinal cord. Good candidates include the Robo3 isoform, Robo3.2, that is selectively expressed on post-crossing segments of mouse commissural axons as well as the midline-associated repulsive Npn2-Sema and EphB-ephrin-B2 guidance systems. By utilizing the genetic labeling system and strategies for manipulating receptor expression on commissural axons that we have recently described,10 it should be possible to identify whether these molecules have key roles in ensuring that commissural axons never re-cross the midline. We have also recently shown that following the forced expression of truncated or full-length Robos, mis-guided longitudinally-oriented axons re-cross the midline.
Commissural axons mainly project in the rostral direction on the contralateral or ipsilateral side of the VM, respectively. These observations suggest that neither manipulation interferes with the responsiveness of these axons to Wnts and Shh, which likely control axon guidance along the anterior-posterior axis of vertebrate embryos. Given that post-crossing segments of dl1 and dl2 axons primarily elaborate rostrally-directed projections, it should be possible to directly determine whether gradients of Wnts and Shh are key regulators of commissural axon guidance along the rostrocaudal axis of the chick spinal cord. Notably, a very recent study has identified Stem Cell Factor (SCF, also known as Steel Factor) as a positively acting axon outgrowth factor required for the expulsion of post-crossing commissural axons from the VM. Thus, it should be possible to assess the relative contributions of repulsive Robo signaling and the positive actions of SCF, in driving decussated commissural axons away from the VM. In addition, a key remaining challenge is to identify, and determine exactly how, mechanisms other than alternative splicing, such as local protein synthesis, might underlie the upregulation of repulsive guidance receptors (e.g., Robo1/2, EphB) on decussated segments of commissural axons. The results of these particular studies should provide exciting new insights into the mechanisms that facilitate a switch in the responsiveness of post-crossing axons to midline guidance cues in vivo. Each of these goals will need to be achieved in order to construct an integrated view of how multiple guidance systems operate in concert to regulate the pathfinding of spinal commissural axons.

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