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The role of slits and robos in guiding commissural axons in the vertebrate spinal cord

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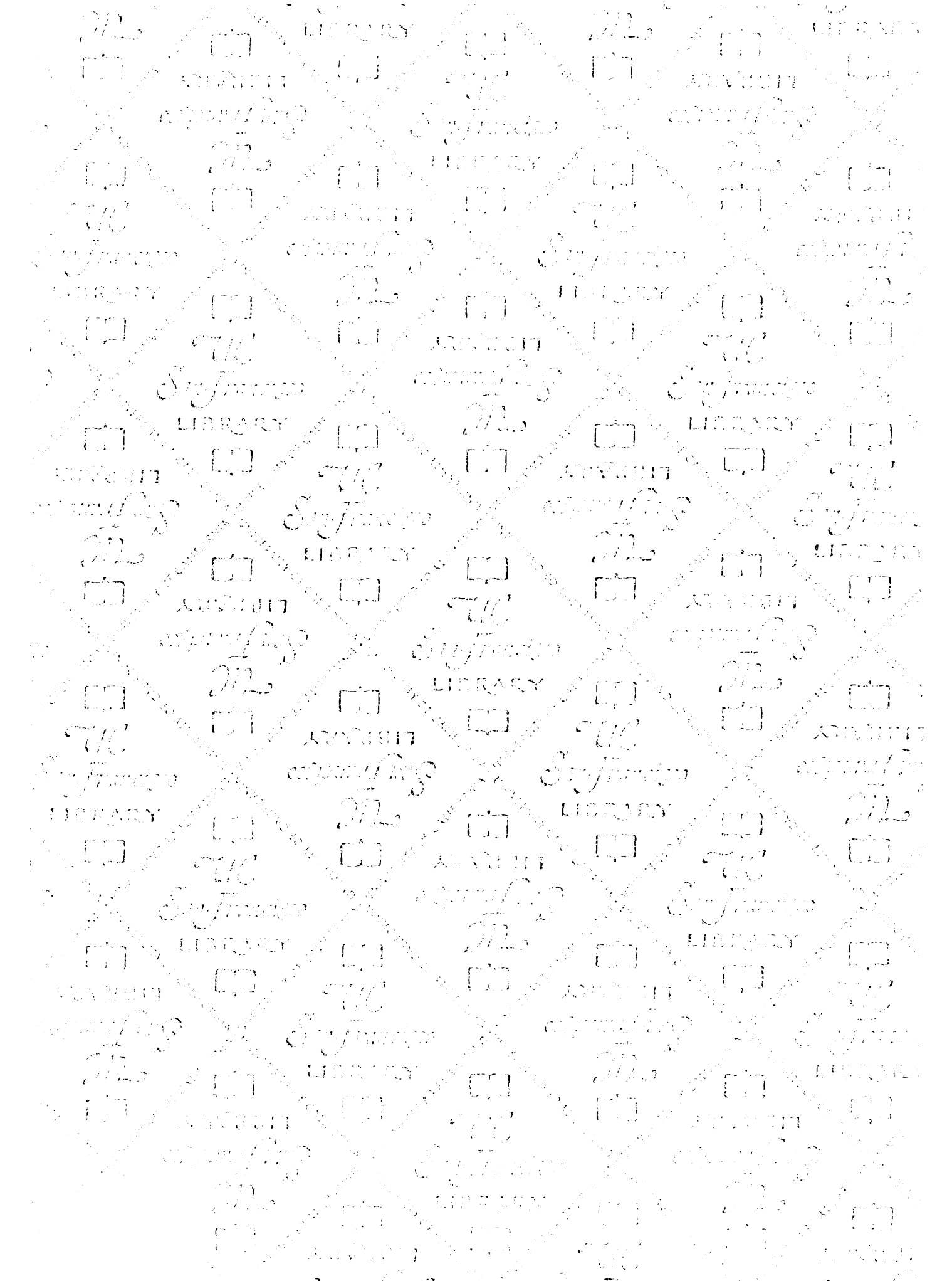
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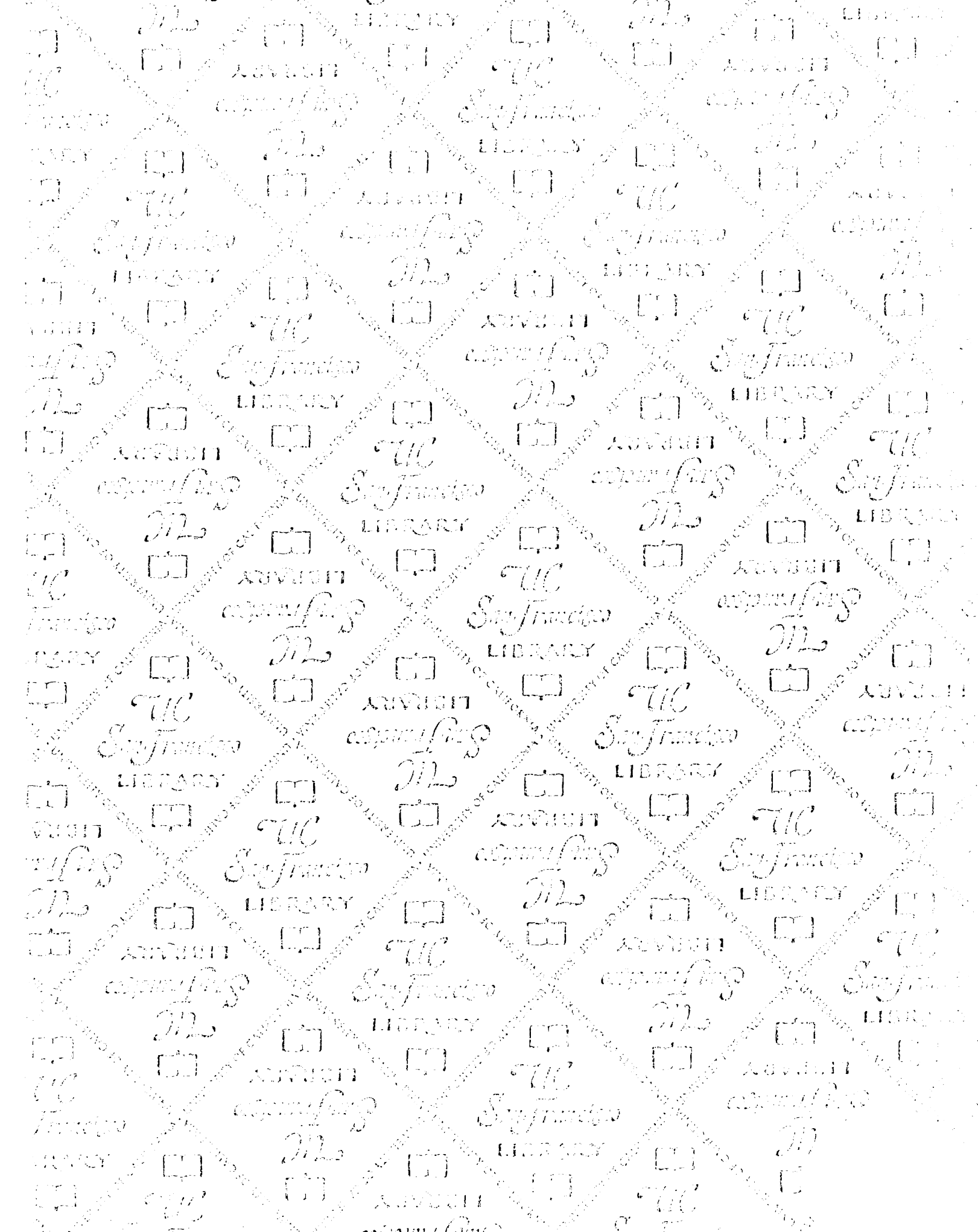
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The Role of Slits and Robos in Guiding Commissural Axons in the Vertebrate Spinal Cord

by

Christelle Sabatier

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

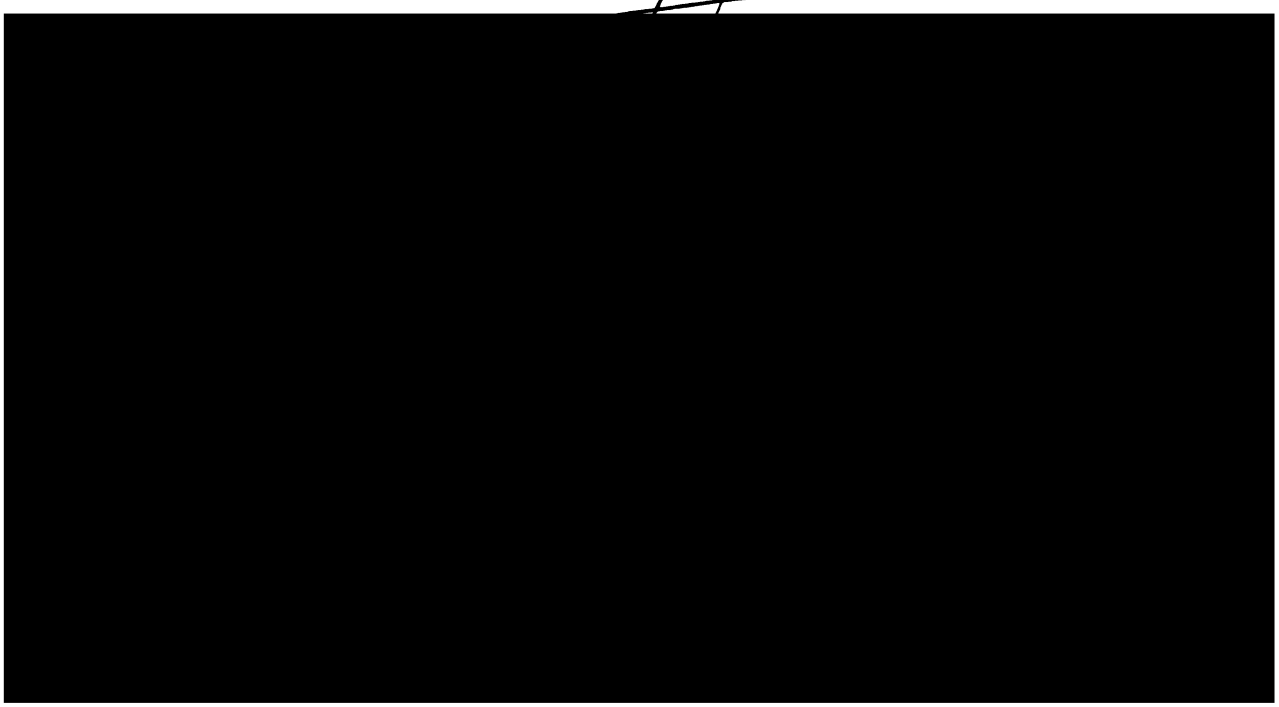
Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO



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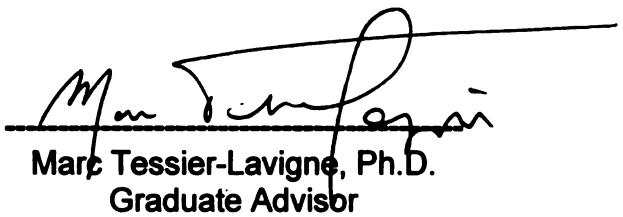

Marc Tessier-Lavigne, Ph.D.
Graduate Advisor

Abstract:

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toward their final targets. Our studies have also uncovered a novel mechanism to regulate commissural axon responsiveness to Slits in the midline through the action of the Robo family member, Rig-1. Together these results have increased our understanding of the molecular basis of the interactions between a well-known intermediate target and the axons that use it.

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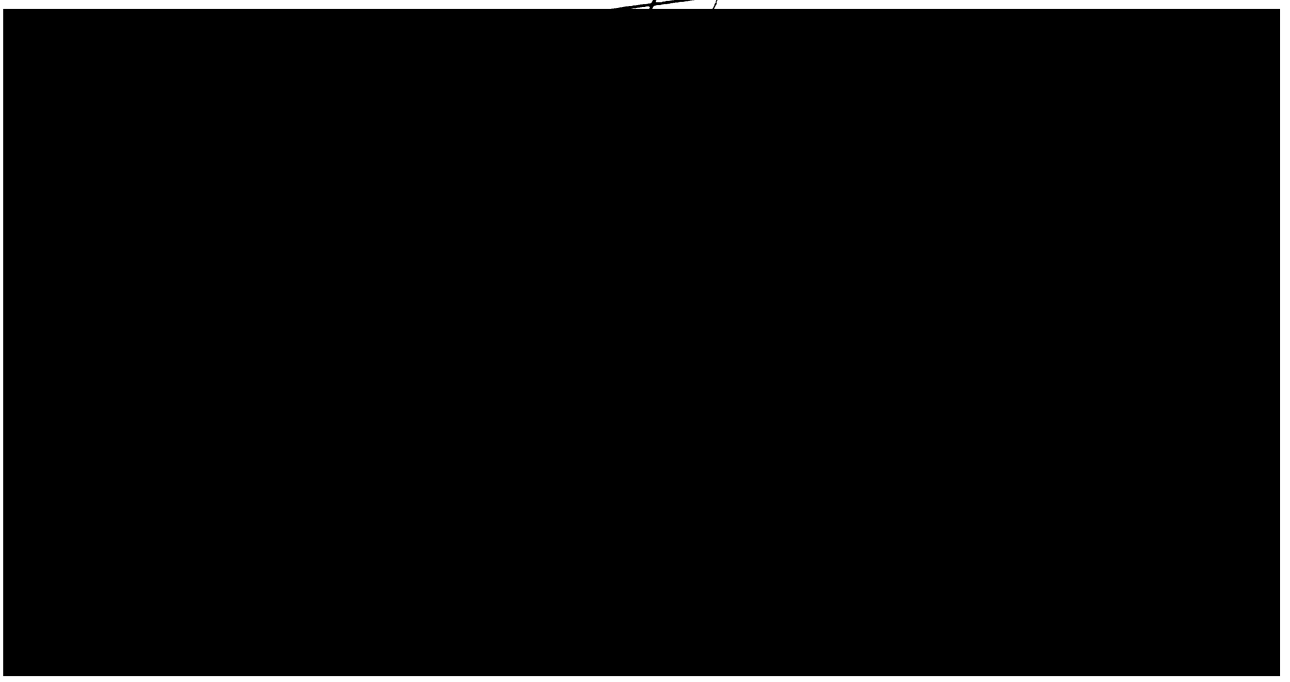

This thesis is dedicated to my family.
My parents, sister and brother for their endless
supply of love and support.
And to my little family, Ryan and Nico, for continually
reminding me of what is most important in life.

Acknowledgements

As one can easily imagine, there are quite a few people that I need to thank for helping me through the past six and a half years in graduate school. It is hard to remember what I was like when I started graduate school at UCSF because so much has changed since then. The incredibly supportive environment there took me from a naïve first year student to a mature, confident scientist.

First and foremost, I would like to thank Marc for teaching me so much both directly and indirectly through the environment that he created in the lab. Marc is an incredibly thorough scientist who always keeps sight of the question at hand and makes sure that the experiments being conducted get to the heart of the problem. I have learned much from him both in terms of keeping focused on interesting questions and in designing proper experiments in order to be able to interpret their outcomes. Marc is also a brilliant conveyor of information. He is able to assess the abilities of his audience and present the material so that they can understand it and learn from it. I hope to have gleaned a tiny bit of his presentation skills from the many lectures I have heard him give and the constructive criticism I have heard him bestow on my own talks as well as those of other people in the lab. Finally, I would also like to thank Marc for making it possible for me to have a family while in graduate school. Without his support and flexibility, raising a child while completing my graduate studies would have been difficult if not impossible. But Marc's complete understanding of what it

takes to have a family life allowed me to easily balance my work and my life at home.

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interpret experiments or simply score phenotypes blind. I will miss that environment terribly as we all move on to better things but I am sure that I will always be able to call up any of these people to chat about an interesting scientific problem or just to gossip. I thank Fan for her wit, her brilliant thoughts and her support over the years. She taught me that it was okay to think big and to shoot for the stars. I thank Bami for his great sense of humor, his need to always play devil's advocate and his interest in anything scientific no matter how esoteric. I thank Zemer for providing a shoulder to cry on and for cheering me on when things got tough. I thank Lisa for being a great friend and providing a good example of how to balance a successful career and family. I thank Hwai Jon for his great insights into so many aspects of my work. I thank all members of the "annex" (especially Jesse, Phil, Shai and Sue) for their friendship and great conversations. I also thank all members of the North wing (Hua, Fred, Bami, Roland, Xiaowei, Joe, Fan and Morgan) for creating such a great environment in which to come to work every day. I also would like to thank Le for his generosity and for his insight into the cell biology of axon guidance.

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that the experiments that I do in lab are unimportant compared to the wonderful and scary task of raising a little boy to be as great as he can possibly be.

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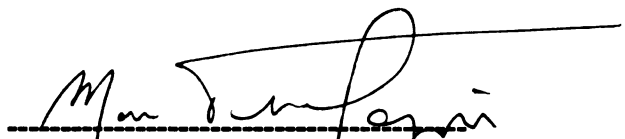

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Chapter One:

Introduction

I. Introduction

To taste, to touch, to hear, to see, or to smell, indeed to be capable of rational thought, we have been endowed with an incredibly complex nervous system. The nervous system is composed of individual cells termed neurons that connect via their axons to targets that are far removed from their cell bodies. The accuracy of these connections must be exquisitely regulated to avoid deleterious effects. Several solutions have been adopted to simplify the complexity of wiring up the nervous system. Many axon guidance events take place in early embryos where the distances axons must travel are not quite as great. Axons also make use of intermediate targets located throughout the developing animal that serve as stepping stones to guide axons to their final targets. Finally, once the initial phase of axon guidance is completed, many corrective mechanisms are engaged that allow only productive connections between axon and target to be maintained in the adult animal. Other reviews have previously discussed the checks and balances of the nervous system that ensure that only properly targeted axons remain in the adult (Kantor and Kolodkin, 2003; Sanes and Lichtman, 1999). In this review, I would like to focus on the choices that axons must make in order to navigate their circuitous paths through the developing embryo. In particular, I will discuss the mechanisms that regulate the response of growth cones to the cues produced by their intermediate targets.

Intermediate Targets in Axon Guidance

Early embryological studies in the grasshopper embryo identified pioneering axons that travel past specific sets of cells, often making 90° turns after each one, in order to reach their final targets (Bate, 1976). One of these so-called guidepost cells was shown to be essential for axon guidance through ablation experiments (Bentley and Caudy, 1983) thus leading to the hypothesis that axons grow toward their final targets in discrete steps using "stepping stones" along the way to simplify their trajectory (Palka et al., 1992). Unfortunately, extensive searches through other organisms failed to reveal isolated guidepost cells that function in the same way as what had been observed in insects. However, there are many examples throughout the animal kingdom of axons using what can be more generally called intermediate targets to pathfind through the complex tissues of an embryo. Intermediate targets are thought to be the source of a tremendous diversity of axon guidance cues that can act over long distances or at short range. Axons are initially attracted to their intermediate targets but once they reach it a switch must take place to allow axons to move on to the next step in their trajectory. This switch may involve a loss of responsiveness to the attractants produced by the intermediate target, a change in their adhesion properties making it more favorable for them to move beyond their intermediate target, or a gain in responsiveness to repellents produced by their intermediate target. Such a switch must be tightly regulated to ensure that axons do not change their responsiveness prematurely.

The central nervous system midline as a key intermediate target

One prominent intermediate target that is utilized from insects to mammals is the central nervous system (CNS) midline (Kaprielian et al., 2001). Several types of CNS neurons can be defined based on their projections relative to the midline. Some neurons such as motor neurons located ventrally in the vertebrate spinal cord project away from the midline. Others, called association neurons in vertebrates, project parallel to the midline on the ipsilateral side of the CNS. Finally, commissural neurons, whose cell bodies are located primarily in the dorsal spinal cord in vertebrates, project across the midline before growing longitudinally, parallel to the midline in the contralateral CNS (Figure 1). Commissural neurons make key connections between the two halves of the body thus allowing for coordinated movement between the left and right sides of bilaterally symmetric organisms. Experiments in a variety of systems have shown that the midline guides commissural axons in a variety of steps as they grow toward their final targets. (1) Commissural axons initially project toward the midline in response to chemoattractants secreted by the midline. (2) Through the effects of short-range guidance cues, they cross to the contralateral side of the CNS. (3) As they cross, commissural axons lose their attraction to the midline such that they can grow away from it. (4) Once they have crossed, they make a dramatic change in direction to grow longitudinally toward their final targets. (5) Finally, repellents expressed by the midline appear to dictate the positions in which commissural axons grow in the contralateral CNS and prevent any

recrossing of the midline. The molecular cues responsible for and the mechanisms of regulation of each of these events will be discussed in turn.

II. The molecular cues that guide axons through the midline

Before discussing the mechanisms that ensure the steps described above take place sequentially at the right time, we will first describe all of the axon guidance cues that have been identified to date and which steps of commissural axon guidance they instruct.

Attraction to the midline

Evidence for a role of the midline in attracting commissural axons initially came from embryological studies of vertebrate embryos. The CNS midline in vertebrate spinal cords is made up of a specialized structure of columnar epithelial cells called the floor plate. Analysis of the Danforth's short-tail (Sd) mutant in which the notochord is absent and the floor plate is missing from the lumbosacral region of the spinal cord indicated that the floor plate was required for proper crossing of the midline. Interestingly, in regions just caudal to areas containing a normal floor plate, commissural axons reoriented to project longitudinally toward the rostrally-located floor plate (Bovolenta and Dodd, 1991). Similarly, floor plate explants were shown to reorient commissural axons in vitro as well as to enable the outgrowth of commissural axons into a collagen matrix (Tessier-Lavigne et al., 1988). The development of such in vitro assays led to the eventual identification of Netrin-1 as a chemoattractant of commissural axons secreted by floor plate cells (Serafini et al., 1994). Netrin-1 homologs in a variety

of other organisms including *Drosophila* and C. elegans were also shown to be crucial to the initial guidance of commissural axons toward the midline (Ishii et al., 1992; Mitchell et al., 1996).

As predicted, animals mutant for either Netrin-1 or its receptor, DCC , exhibit profound defects in the ability of commissural axons to grow ventrally toward the floor plate (Keino-Masu et al., 1996; Serafini et al., 1996). However, a small subset of commissural axons continue to be observed crossing the floor plate in these mutants, indicating that another chemoattractant may play a role in commissural axon guidance. The identity of such a molecule remained a mystery until the recent discovery that the well-known morphogen Sonic Hedgehog (Shh) is able to reorient commissural axons in cultured rat spinal cords. In vivo experiments confirmed that in addition to its role in patterning the spinal cord earlier in development, Shh also serves to guide commissural axons toward the floor plate (Charron et al., 2003). It still remains to be seen whether Hedgehog proteins guide commissural axons in other organisms.

The role of adhesion in midline crossing

Once commissural growth cones approach the ipsilateral side of the midline, short-range guidance cues are thought to take over to drive them across the midline. Short-range axon guidance may involve axon-axon interactions as well as interactions between axons and the midline itself via cell adhesion proteins. Initial experiments characterizing the localization of a subset of cell-adhesion proteins on commissural axons in rat spinal cords fueled great interest in these molecules as being crucial to the switch from pre-crossing axon to post-

crossing axon (Dodd et al., 1988). TAG-1 is a GPI-anchored glycoprotein of the Ig superfamily that is expressed by commissural axons as they grow ventrally toward the floor plate. Once commissural axons enter the contralateral spinal cord, however, TAG-1 protein expression is specifically downregulated from that portion of the axon. Eventually, TAG-1 mRNA expression is also downregulated from commissural neurons. On the other hand, the transmembrane glycoprotein L1 is upregulated only once commissural axons have entered the floor plate and is kept off of pre-crossing commissural axons. Unfortunately, despite their tantalizing expression patterns, it remains unclear what exact roles these cell adhesion proteins play in directing commissural axons across the midline.

The chick orthologs of TAG-1 and L1 (Axonin-1 and Ng-CAM) are also expressed on commissural axons, however, they are found on both the ipsilateral and contralateral portions. This difference suggests that Axonin-1 and Ng-CAM are not true orthologs of TAG-1 and L1 or that chickens have developed a slightly different strategy for guiding commissural axons across the floor plate. Nevertheless, perturbation experiments with antibodies or RNAi specific to either Axonin-1 lead to some commissural axons failing to cross the floor plate and instead growing as an ipsilateral fascicle. This phenotype can also be observed when Nr-CAM, another adhesion protein expressed by floor plate cells, is depleted (Pekarik et al., 2003; Stoeckli and Landmesser, 1995). These results indicate that Axonin-1 may interact with Nr-CAM to pull commissural axons through the floor plate. Depletion of Ng-CAM leads to an increased defasciculation of commissural axons as they grow across the floor plate,

indicating that axon-axon interactions may be an important component to getting across the midline.

Navigating the midline—the role of Derailed

An atypical receptor tyrosine kinase, called Derailed, has been shown in Drosophila to be involved in directing commissural axons to cross the midline in the proper location (Bonkowsky et al., 1999; Yoshikawa et al., 2003). Besides choosing whether or not to cross the midline, axons in the Drosophila CNS can cross in one of two places, the anterior commissure (AC) or the posterior commissure (PC). Derailed is expressed specifically in commissural axons projecting in the AC and is quickly downregulated after commissural axons have crossed, as the Derailed protein is not observed in the longitudinal pathways. Loss-of-function and gain-of-function experiments showed that Derailed is both necessary and sufficient to drive axons to cross in the AC (Bonkowsky et al., 1999). Later experiments identified Wnt5 as being the Derailed ligand. Wnt5 expression on PC-projecting commissural axons repels Derailed-expressing axons, driving them to cross the midline in the AC (Yoshikawa et al., 2003). Although Derailed and Wnt5 have clear vertebrate homologs (RYK and Wnt5a), it remains unclear what role if any these two proteins might play in vertebrate axon guidance. Although there is no exact structure analogous to the PC and AC in the vertebrate spinal cord, commissural axons have been demonstrated to cross in distinct dorsal-ventral regions of the floor plate (Gowan et al., 2001) and it will

be interesting if these commissural pathway choices are controlled in a manner similar to what has been described in Drosophila.

Leaving the midline

In vitro experiments in vertebrates have demonstrated and genetic experiments in *Drosophila* have confirmed that in order to leave the midline, commissural axons downregulate their ability to sense attractants (Shirasaki et al., 1998) while they upregulate their ability to sense repellents in the midline (Kidd et al., 1998a; Zou et al., 2000). Repellents of the Slit, Semaphorin and Ephrin families are expressed in vertebrate spinal cord floor plate while their cognate receptors (Robos, Neuropilins, and Eph receptors) are expressed on commissural axons (Brose et al., 1999; Imondi et al., 2000; Ishii et al., 1992; Zou et al., 2000). The role of Slit proteins in pushing commissural axons out of the midline and into longitudinal tracts in the contralateral side of the CNS have been well characterized in a variety of different organisms. Therefore, I will spend some time discussing the role of Slit repellents and their receptor of the Roundabout (Robo) family in guiding commissural axons in more detail.

The original Robo receptor was initially identified in a screen for mutants with axon guidance defects in the *Drosophila* CNS. *robo* mutants are characterized by an increased tendency for axons in the CNS to cross the midline multiple times (Seeger et al., 1993). Interestingly, another mutant to come out of this same screen exhibited the opposite phenotype, that is a total absence of commissures. This *commissureless* (*comm*) mutant was shown to act upstream of Robo to downregulate Robo expression on commissural axons

prior to crossing the midline (Kidd et al., 1998b). Later experiments that characterized the ligand for Robo, Slit (Kidd et al., 1999), as well as the mechanism by which Comm regulates Robo (Keleman et al., 2002) led to the following model for commissural axon guidance in *Drosophila*. As commissural axons grow toward the midline due to the attraction by Netrins through their receptor Frazzled, Slit responsiveness is kept low by the sequestration of Robo in intercellular compartments by Comm. Once commissural axons have crossed the midline their ability to sense Slit repellents in the midline is dramatically upregulated due to the downregulation of Comm by unknown mechanisms and the subsequent release of Robo onto the plasma membrane. Together these events keep commissural axons from ever recrossing the midline.

Slit has been shown to function as a repellent of Robo-expressing axons in other organisms including C. elegans (Hao et al., 2001). The identification and characterization of vertebrate Slit and Robo homologs has shown these molecules to be expressed in regions of the vertebrate spinal cord analogous to the *Drosophila* CNS. Three vertebrate Robo homologs (Robo1, Robo2, and Rig-1) have been identified in the nervous system and all are expressed by commissural neurons while all three vertebrate Slit homologs (Slit1, Slit2 and Slit3) are expressed in the floor plate (Brose et al., 1999; Yuan et al., 1999). Interestingly, no vertebrate Comm homolog has been identified so far. However, as I will present in Chapters Two and Three of this thesis, Slits and Robos play a crucial role in guiding commissural axons through the floor plate and beyond in

vertebrates using similar but not identical strategies to what has been documented for *Drosophila*.

Growing toward final targets

Once commissural axons have entered the contralateral CNS, they alter their trajectory dramatically and grow parallel to the midline toward their final targets. In this environment, commissural axons must adopt a specific position relative to the midline in which to grow longitudinally. In the Drosophila CNS, ipsilaterally projecting axons pioneer the longitudinal axon pathways. Dynamic expression of the adhesion protein FasciclinII by both pioneers and their follower commissural axons leads to selective fasciculation events that eventually form the distinct longitudinal tracts in the CNS (Lin et al., 1994).

In addition, repellent cues secreted by the midline have also been shown to influence this decision. Genetic studies in Drosophila have identified two members of the Robo family (dRobo2 and dRobo3) that are expressed by commissural neurons. These receptors appear to dictate the positions of longitudinally projecting axons relative to the midline (Rajagopalan et al., 2000a; Rajagopalan et al., 2000b; Simpson et al., 2000a; Simpson et al., 2000b). While Robo is expressed by all commissural axons in Drosophila, Robo2 is only found on axons occupying the most lateral longitudinal positions while Robo3 is expressed in an overlapping subset of axons, some of which tend to grow more medially. This combinatorial code of Robo receptors leads to different subsets of commissural axons having distinct responses to the Slit gradient emanating from

the midline, finding it more or less repulsive. Tracing of commissural axons using the lipophilic dye, DiI, has shown that different subsets of axons display distinct pathfinding behaviors once they have begun to grow longitudinally in the spinal cord (Imondi and Kaprielian, 2001). While commissural axons initially grow adjacent to the floor plate once they have begun their longitudinal growth, they often migrate dorsolaterally after a certain distance. In Chapter Two, we show that this behavior is also dictated by a combinatorial code of Robo receptors expressed by commissural axons.

Previous experiments have also shown a role for ephrinBs in preventing midline crossing. EphrinBs are a group of membrane-bound ligands that typically act to repel axons expressing EphB receptors, members of the receptor tyrosine kinase superfamily. The expression of ephrinB3 and ephrinB1 in the dorsal spinal cord led to the conclusion that these short-range guidance cues stop commissural axons from growing too dorsally in the contralateral spinal cord. This hypothesis was confirmed by the effect of blocking EphB-ephrinB interactions on commissural axon guidance. These results also fit with the observation that loss of Dephrin or Deph in Drosophila leads to the aberrant exit of interneuronal axons from the CNS (Bossing and Brand, 2002). Ephrin expression in the midline itself also implicates this family of repellents in pushing commissural axons out of the midline (Bergemann et al, 1998).

III. Regulated Response to Floor-Plate Derived Signals in Vertebrate Axon Guidance

Now that all of the players have been layed out, we will focus our discussion on the mechanisms that may regulate these axon guidance cues and how they work together to ensure the proper sequence of events takes place to guide commissural axons across the midline. First, we will discuss the evidence that the midline plays an instructive role in regulating the responsiveness of commissural axons to axon guidance cues. Then, we will describe in more detail the regulatory mechanisms for individual sets of axon guidance systems known to guide commissural axons relative to the midline.

Role of the midline in orchestrating the switch in responsiveness

Careful analysis of pioneering commissural axon growth cones as they traverse the floor plate in wildtype animals characterized morphological changes in these growth cones that coincide with their interaction with the midline (Bovolenta and Dodd, 1990). DiI was placed at the dorsal edge of different age spinal cords and the anterogradely labeled commissural axons were then followed and characterized. Prior to interacting with the floor plate, commissural growth cones exhibit simple morphologies and extend very few filopodia. Once they reach the ipsilateral edge of the floor plate, however, commissural growth cones become much more complex and extend a large number of filopodia directed both forward and to the sides. At the contralateral edge of the floor plate, filopodia become directed in a predominantly rostral direction, which is consistent with the fact that most commissural axons turn rostrally after crossing the floor plate. These observations as well as other work analyzing axon pathfinding at the midline in a variety of different organisms (Bernhardt, 1994;

Myers and Bastiani, 1993; Yaginuma et al., 1991) set the ground work for analyzing commissural axon guidance when the midline had been perturbed.

Analyses of commissural axon guidance in embryos lacking midline cells provide the most compelling evidence for the midline playing a role in instructing the coordinated switch that commissural axons undergo as they cross to the contralateral side of the CNS. Besides exhibiting some failures to cross the floor plate, commissural axons in zebrafish *cyc-1* mutants, which lack a floor plate, also make guidance errors in the anterior/posterior axis once they have crossed the midline (Bernhardt et al., 1992a; Bernhardt et al., 1992b). Similar observations were made in the Danforth's short-tail mouse mutant, where the floor plate and notochord are missing from lumbosacral regions of the embryo. Commissural axons in *Sd/Sd* mutants where observed to make multiple types of pathfinding errors after crossing the floor plate, including growing out of the spinal cord along the ventral root or growing back up ventrally projecting axons on the contralateral side of the spinal cord. Interestingly, TAG-1, which as described earlier is normally downregulated on post-crossing commissural axons, appears to be expressed for a greater extent of their trajectory (Bovolenta and Dodd, 1991). Because the *Sd/Sd* mutant mice lack the motor neurons normally born in the ventral spinal cord, it is possible that the effects on commissural axon guidance were not due exclusively to the lack of a floor plate. However, more specific removal of the floor plate with a mutation of the *Gli2* locus, led to similar results (Matisse et al., 1999). Although commissural axons grew normally toward the midline, they made significant pathfinding errors inside

the floor plate and beyond including stalling and aberrant turning. The regulated commissural axon-specific proteins Nr-CAM, TAG-1 and L1 were also misregulated in *Gli2-/-* embryos. Nr-CAM, which is normally localized to the portion of commissural axons growing through the floor plate as well as in floor plate cells themselves, is now seen throughout the commissural axons. TAG-1 is observed on post-crossing commissural axons and L1 is seen weakly on pre-crossing axons.

The midline-dependent upregulation of receptors on commissural axons is not specific to vertebrates. Experiments in grasshopper have shown that interaction of serotonergic commissural axons with the midline is required for the upregulation of SERT, a serotonin transporter that functions to reduce synaptic levels of serotonin (Condron, 1999). A diffusible, FGF-like factor secreted from midline cells was implicated in regulating the midline-dependent upregulation and maintenance of SERT expression by increasing SERT transcription. Later experiments revealed an important general role for transcription in commissural axon guidance at the grasshopper midline (Condron, 2002). Interestingly, commissural axons that had not yet crossed the midline failed to grow in the presence of transcription inhibitors while post-crossing commissural axons continued to grow but made guidance errors. Experiments in vertebrate embryos have shown a similar requirement for transcription for commissural growth cones to make the correct turn after crossing the floor plate (Von Bernhardi and Bastiani, 1995). Although it is difficult to imagine how transcriptional events could act fast enough to account for the rapid change in commissural growth

cone responsiveness that takes place upon midline crossing, they may be required to maintain the changes that take place through other, more rapid mechanisms. Based on all of the observations described above, we can conclude that interaction between midline cells and commissural axons is crucial for enabling a sequence of events to take place within the commissural growth cone that will eventually change its responsiveness to the midline on multiple levels.

Losing Responsiveness to a Chemoattractant: Regulation of the Netrin Response

As described earlier, the chemoattractant Netrin-1, expressed by midline cells, is responsible for guiding commissural axons expressing the Netrin receptor DCC/Frazzled/Unc40 in their trajectory toward the midline. However, due to its role as an intermediate target, the midline can only attract commissural axons transiently and once they have reached the source of the chemoattractant gradient commissural axons must somehow move away from it. One possibility is that this phenomenon is simply achieved through the increased responsiveness to repellents that are also expressed by the midline. However, there is strong evidence that a loss of responsiveness to Netrin also takes place upon crossing the midline. In a set of elegant in vitro experiments, Shirasaki and colleagues showed that commissural axons at hindbrain levels lose the ability to reorient toward an ectopic floor plate once they have crossed the midline (Shirasaki et al., 1998). Although these authors provided further evidence that responsiveness to Netrin was specifically downregulated in post-crossing

commissural axons, their experiments indicate a loss of responsiveness to all attractants in the floor plate. It will also be interesting to assess specifically whether the attraction to Shh is lost upon crossing the floor plate.

Several potential mechanisms by which the Netrin response could be downregulated upon crossing the midline have been characterized in vitro. Although these mechanisms remain to be validated in vivo, they provide a conceptual framework by which we can think about how responses to cues released by intermediates are downregulated upon interaction with that intermediate target. The characterization of metalloprotease inhibitors as potentiators of the Netrin response by commissural axons led to the hypothesis that the receptor for Netrin, DCC, could be a substrate for metalloproteases expressed by commissural axons (Galko and Tessier-Lavigne, 2000). In vitro experiments revealed that a cleaved form of DCC (corresponding to the extracellular domain) was indeed released by commissural neurons, a phenomenon that could be inhibited by inhibitors of ADAM metalloproteases. Although these experiments primarily show an effect of the metalloprotease inhibitors on pre-crossing commissural axons, there is the possibility that such an activity could be upregulated on post-crossing commissural axons thus resulting in a loss of responsiveness to Netrin-1.

Such regulated shedding of receptors has been described in a variety of systems (Black and White, 1998). Interestingly, the GPI-anchored cell adhesion protein, TAG-1, is not detected on commissural axons once they have crossed the floor plate, perhaps due to shedding (Karagogeos et al., 1991). The

expression of TAG-1 on commissural axons is downregulated in vitro with kinetics that are similar with what has been described in vivo, and this coincides with the increased release of soluble TAG-1. Although these in vitro experiments imply that the regulation of TAG-1 is intrinsic to commissural neurons and on some level independent of the floor plate, analysis of TAG-1 expression in mutants lacking a floor plate argue that the floor plate can influence the exact timing of TAG-1 downregulation (Matisse et al., 1999). Although DCC shedding would clearly explain the difference in responsiveness to Netrin between pre-crossing and post-crossing commissural axons, but DCC protein is not downregulated in the ventral and lateral funiculi (Keino-Masu et al., 1996). Therefore, DCC ectodomain shedding may be only part of the explanation for decreased Netrin responsiveness.

Another mechanism by which Netrin responsiveness may be modulated, proteosome-dependent proteolysis, was identified in Xenopus retinal growth cones (Campbell and Holt, 2001). Previous experiments have shown that Xenopus retinal growth cones turn toward a source of Netrin-1 in a DCC-dependent manner in vitro (de la Torre et al., 1997). However, when these growth cones are exposed to proteosome inhibitors, they fail to reorient toward a source of Netrin-1. Similarly, protein synthesis specifically in the growth cone is also required for a proper attraction to Netrin. These results may seem counterintuitive since one might expect that proteolysis would lead to a loss of responsiveness to Netrin. However, since it still remains unclear which components of the Netrin-response are modulated by proteolysis and synthesis,

it is difficult to come up with specific molecular models to account for these results.

Nonetheless, another set of experiments in a different type of Xenopus neuronal culture may give us a clue as to what might be going on. This study shows that as growth cones grow along an increasing Netrin-1 gradient, they undergo consecutive phases of desensitization and resensitization (Ming et al., 2002). Although these authors did not specifically show the effects of proteosome inhibitors on this desensitization and resensitization, they did show that protein synthesis is required for resensitization to Netrin. Therefore, another possible interpretation of the previous experiments with Xenopus retinal growth cones may be that proteosome inhibitors lead to increased desensitization to a Netrin gradient. Although this phenomenon of desensitization and resensitization remains to be shown in other systems, we can still use these concepts to think differently about the regulation of the Netrin response as commissural axons grow through the midline. As commissural axons grow toward the midline they are encountering increasing levels of Netrin-1 and may accordingly become desensitized to its effects. Therefore, the change in responsiveness to Netrin upon crossing of the midline can be thought of as a shift in the regulation of desensitization and resensitization.

Another mechanism by which Netrin responsiveness can be modulated was also identified using culture of Xenopus spinal cord neurons. Since commissural axons are exposed to both attractants (i.e., Netrin) and repellents (i.e., Slit) at the midline, these authors performed experiments to address

whether there is any cross-talk between these pathways (Stein and Tessier-Lavigne, 2001). Growth cones from stage 22 Xenopus spinal neurons turn toward a source of Netrin-1 but are unaffected by a gradient of Slit2. However, when these two ligands are combined, Slit2 inhibits the turning effects of Netrin-1 but not its outgrowth-promoting effects. Biochemical experiments showed that this cross-talk between the two pathways is mediated by a direct interaction between the Slit receptor, Robo1, and the Netrin-1 receptor, DCC, in the presence of the ligand Slit2. Interestingly, when assayed at a later time point (stage 28), Xenopus spinal neuron growth cones are insensitive to a Netrin-1 gradient and repelled by a gradient of Slit2. Therefore, in that system there appear to be two mechanisms by which Netrin-1 responsiveness is modulated, a fast-acting mechanism mediated by protein-protein interaction and a longer-acting effect that may incorporate some of the mechanisms described previously. Although the in vivo significance of this interaction remains to be addressed (See Chapter Four for further discussion), it is gratifying to imagine that the downregulation of Netrin responsiveness is coordinated to the upregulation of Slit responsiveness through direct interaction between these seemingly completely distinct pathways of midline axon guidance.

Gaining Responsiveness to Floor Plate Repellents

As was already introduced in the previous section, as commissural axons cross the floor plate and lose their attraction to Netrin-1 at the midline, they also become repelled by repellents that are also expressed by midline cells. In the vertebrate spinal cord, in vitro experiments have shown that while commissural

axons are insensitive to a number of repellents prior to crossing the midline, they become repelled by Slit, semaphorin and ephrin repellents once they have crossed to the contralateral side (Imondi and Kaprielian, 2001; Zou et al., 2000). A combination of genetic and biochemical experiments have led to a detailed model of how Slit regulation is modulated by midline crossing in Drosophila. As described earlier in this introduction, the upregulation of Robo on the plasma membrane of commissural growth cones accounts for the increased sensitivity to Slit, expressed by the midline, upon crossing (Kidd et al., 1999). Through its direct interaction with Robo, Comm, a type II transmembrane protein expressed by commissural neurons, sequesters Robo in endosomal compartments as commissural axons grow toward and through the midline (Keleman et al., 2002). Comm and Robo interact via their extracellular domains and are sorted to endosomal compartments through a conserved LPSY motif in the cytoplasmic domain of Comm. Through this LPSY motif, Comm also interacts with the ubiquitin ligase, Nedd4, which leads to the ubiquitin-dependent sorting of Comm (Myat et al., 2002).

The mechanism by which Comm downregulates Robo in crossing commissural axons is well understood, however, it simply pushes the regulation issue back one more step. How then is Comm function downregulated after crossing? *Comm* expression is decreased in commissural neurons once their axons have crossed the midline (Keleman et al., 2002). The mechanism by which this takes place is not understood but, in principle, it provides a means of relieving the inhibition of Robo once commissural axons begin to grow

longitudinally in the contralateral CNS. As we show in Chapter Two, vertebrate Robo1 and Robo2 are also localized specifically to the post-crossing region of commissural axons. However, since no clear Comm homolog has yet been identified in vertebrates, it remains unclear whether a similar mechanism exists to regulate Slit sensitivity in the vertebrate spinal cord. As we will discuss in Chapter Three, a separate and not mutually exclusive mechanism to inhibit Slit signaling through Robo1 has been identified in vertebrate commissural axons, thus emphasizing the many layers of regulation that have evolved in order to ensure proper navigation of commissural axons across the midline.

Responsiveness to other repellents besides those of the Slit family is also upregulated upon midline crossing and the mechanisms responsible for these events are just beginning to be worked out. Post-crossing commissural axons are repelled by certain members of the semaphorin family (Sema 3B and Sema3F) (Zou et al., 2000). Consistent with these ligands playing a role in guiding commissural axons through the floor plate, removal of the receptor for these semaphorins, Neuropilin-2, leads to midline crossing errors in the spinal cord. However, very little is known about how the response to Sema 3B and 3F is regulated.

The response to ephrin ligands is also upregulated upon crossing the midline and appears to prevent commissural axons from migrating too laterally (dorsally in vertebrates) as they grow longitudinally in the CNS (Bossing and Brand, 2002; Imondi and Kaprielian, 2001; Imondi et al., 2000). A novel regulatory mechanism for the Eph receptors, which sense the ephrins, was

recently identified (Brittis et al., 2002). EphA2 protein has long been known to be predominantly localized to the ventral funiculus, the spinal cord region in which post-crossing commissural axons course (Magal et al., 1996). The EphA2 gene contains a short 3' UTR sequence part of which is highly conserved across species and contains a cytoplasmic polyadenylation element (CPE). Interestingly, fusion of GFP to this 3'UTR sequence is sufficient to drive GFP expression only on post-crossing commissural axons. Based on these experiments and others, it is thought that EphA2 mRNA is only translated locally within the growth cones of commissural axons once they have crossed the midline potentially through the induction of polyadenylation of the EphA2 mRNA. The signals that drive this RNA-based mechanism for localized regulation are still unidentified.

IV. Intracellular regulators of the response to axon guidance cues

Thus far, we have focused on individual receptor/ligand systems when describing regulatory mechanisms and already we have encountered situations in which cross-talk takes place between seemingly distinct axon guidance cues and their receptors (i.e., between DCC/Netrin and Robo/Slit). However, as the intracellular signaling that occurs downstream of axon guidance receptor becomes better understood (Dickson, 2002), the distinctions between the cytoplasmic elements that are required for a repulsive versus an attractive response become less clear (Schmucker, 2003). What is becoming clearer is that the context in which a axon guidance signal is interpreted is crucial to determine how the growth cone will respond to that signal (as a repellent or an

attractant for example). In this final section, I will describe how signaling molecules that are known to regulate the cytoskeleton are appearing downstream of receptors for both attractants and repellents. And finally, I will describe two molecular pathways that have been shown to alter the context in which gradients of axon guidance cues are interpreted by growth cones.

Signaling to the cytoskeleton

As many axon guidance cues and their receptors have now been identified, many labs have turned to characterizing the signaling pathways downstream of these receptors that link the response to a particular ligand to changes in the cytoskeleton. Surprisingly, a large number of the same cytoplasmic effects appear to function downstream of receptors involved in both attractive and repulsive responses. Ena/VASP proteins have long been implicated in regulating actin dynamics (Gertler et al., 1996). Genetic experiments in C. elegans and Drosophila have implicated Ena/VASP proteins as mediating the signals downstream of both DCC/UNC-40 and Robo/SAX-3 (Bashaw et al, 1998; Colavita et al., 1998; Gitai et al., 2003; Yu et al., 2003). Previous experiments implicating Ena/VASP proteins in promoting actin-dependent motility of the intracellular pathogen *Listeria monocytogenes* led to the model that Ena/VASP proteins promoted actin-dependent outgrowth (reviewed by Cameron et al). However, more recent experiments show that depletion of Ena/VASP from the leading edge of fibroblasts slows their migration (Bear et al., 2000). How do we rationalize such seemingly paradoxical observations? It

appears likely that Ena/VASP proteins affect guidance of axonal growth cones in distinct ways depending on the context in which the signals are received. Recent experiments propose that Ena/VASP proteins lead to the lengthening of actin filaments, thus resulting in an increased number of protrusions at the leading edge (Bear et al., 2002). Whether the presence of additional protrusions promotes or inhibits cell migration or axon outgrowth may depend on whether the protrusions are stabilized or destabilized. For example, in the context of filopodia where actin is not branched and stabilized by actin binding proteins, one would predict that the anti-capping activity of Ena/VASP proteins leads to extension and therefore may promote turning.

Other cytoplasmic effectors of axon guidance signaling appear to be shared between the Robo and DCC pathways including Dock/Nck and Rac. Genetic studies in Drosophila have implicated Dock in mediating part of the signal downstream of the Robo receptor through the recruitment of Pak and subsequent activation of Rac1, resulting in repulsion of the growth cone by Slit (Fan et al, 2003). Similarly, biochemical experiments with commissural neurons have implicated Dock/Nck and subsequent Rac activation in mediating the attractive response to Netrin via DCC (Li et al, 2002). Since these experiments examine global activation and recruitment of particular downstream effectors, it is unclear in which part of the growth cone any of these events are taking place. One way to rationalize these seemingly disparate results may be that activation of Rac occurs on the side of the growth cone growing toward a source of Netrin while it occurs on the side of the growth cone growing away from a source of Slit.

Visualization of the activation of these proteins at the subcellular level will be required to determine when and where these events are taking place. Another possibility is that signaling downstream of Slit and Netrin alters the environment within a growth cone differently such that the activation of Rac has opposite consequences in these two situations. If there are such nodes of axon guidance signaling that are responsive to either attractive or repulsive cues it seems possible that only small changes in the environment of a growth cone may be required to alter the response to a single ligand upon crossing the floor plate.

Regulation of axon guidance receptor signaling by small molecules

Intrinsic changes in the levels of the second messengers cyclic nucleotides within the growth cone itself have been shown to alter the response of an axon to a particular ligand. Experiments using cultured Xenopus neurons have shown that a decrease in intracellular cAMP levels converts the response to Netrin from attraction to repulsion (Ming et al, 1997). These changes in cAMP levels are thought to be responsible for the effect of Laminin-1 on the Netrin response. In the presence of Laminin-1, Xenopus retinal axons are repelled by Netrin rather than attracted to it, as well as exhibiting reduced levels of cAMP in their growth cones (Hopker et al, 1999). Similarly, intracelullar Ca^{2+} levels have also been shown to modulate the responsiveness to Netrin (Hong et al, 2000). Similar experiments using sensory neurons indicate that increasing cGMP levels as well as the presence of Laminin inhibits the ability of these axons to avoid Slit (Nguyen-Ba-Charvet et al, 2001). Genetic experiments in Drosophila also reveal

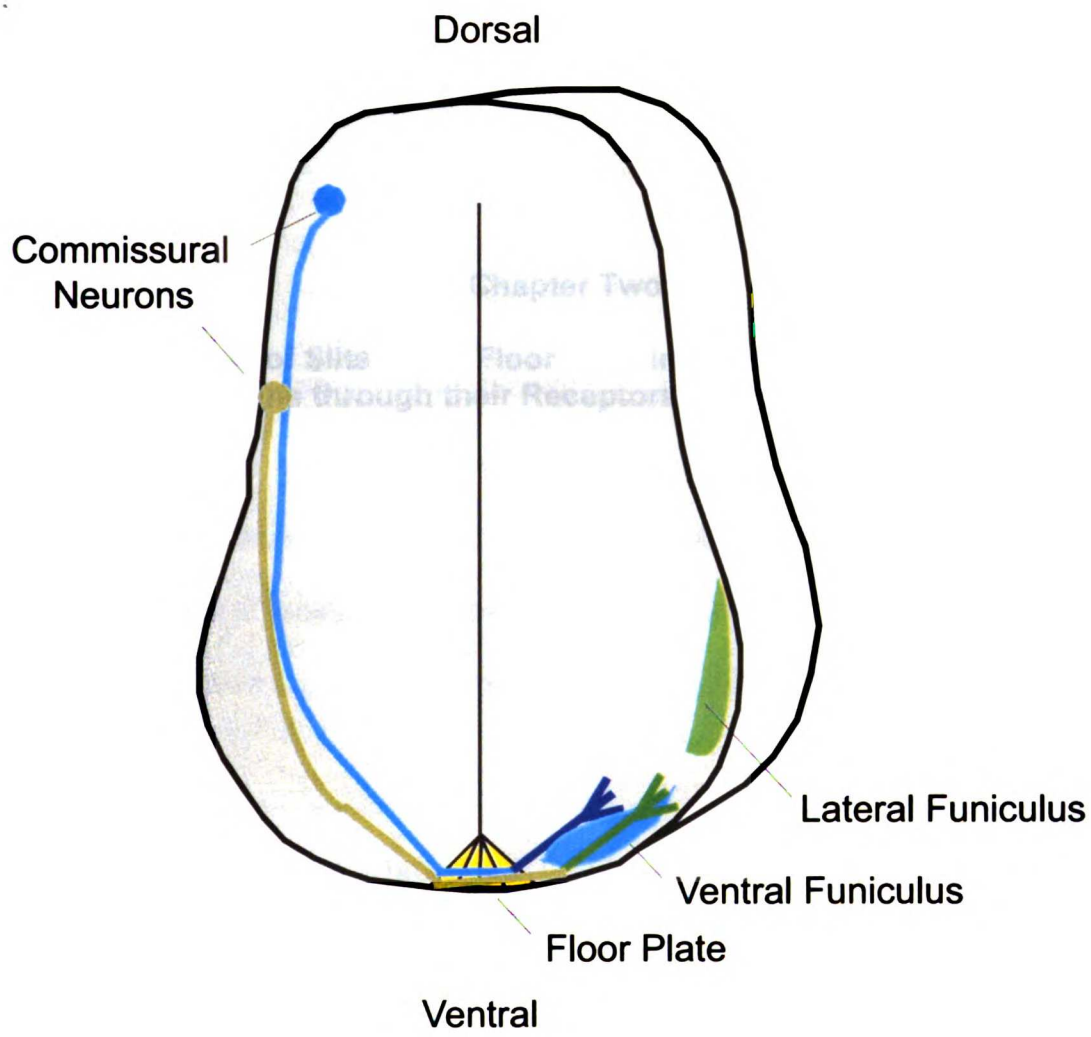
the existence of a cross-talk between integrin receptors and Slit responsiveness. Together these results indicate that alterations in both intracellular and extracellular environments of growth cones can dramatically alter their response to particular axon guidance cues.

Conclusion

In recent years many different axon guidance cues and their receptors have been identified. However, how axonal growth cones integrate their response to the variety of cues that they are exposed to and control their ability to respond to any particular ligand as they grow in the developing embryo remains a mystery. The high interconnectedness between the different pathways responding to the environment is only now becoming apparent. As we continue to understand each signaling pathway in more detail, we can hope to identify the regulatory events that ensure that growth cones only respond to a particular ligand at the right time.

Figure 1.1 Schematic of commissural axon guidance through the spinal cord.

Commissural neurons are born in the dorsal spinal cord and initially extend their axons ventrally toward the floor plate at the ventral midline of the spinal cord. Once they have crossed this intermediate target, commissural axons abruptly change their trajectory to grow longitudinally in the contralateral spinal cord, parallel to the floor plate. Different subsets of commissural axons adopt different lateral positions relative to the floor plate as they grow longitudinally with some making up the ventral funiculus and others that migrate dorso-laterally making up the lateral funiculus as first described by Ramon y Cajal.


Dorsal
Commissural Neurons
Lateral Funiculus
Ventral Funiculus
Floor Plate
Ventral

Chapter Two:

The Role of Slits at the Floor Plate in Guiding Commissural Axons through their Receptors, Robo1 & Robo2

This study was carried out in collaboration with Hua Long, another student in the lab. She carried out all the analysis on the *Slit1;Slit2;Slit3* triple mutant animals while I focused on analyzing the *Robo1* and *Robo2* single mutants. The *Slit1*, *Slit2*, and *Robo1* mutant mice were generated by Dr. Andrew Plump when he was a post-doctoral fellow in the lab. The *Robo2* mutant mice were generated by Dr. Le Ma, currently a post-doctoral fellow in the lab. Dr. David Ornitz provided us with the *Slit3* mutant mice. Dr. Fujio Murakami provided us with antibodies generated against the Robo1 and Robo2 ectodomains.

Abstract

In *Drosophila*, Slit at the midline activates Robo receptors on commissural axons, thereby repelling them out of the midline into distinct longitudinal tracts on the contralateral side of the central nervous system. In the vertebrate spinal cord, *Robo1* and *Robo2* are expressed by commissural neurons, whereas all three *Slit* homologs are expressed at the ventral midline. Previous analysis of *Slit1; Slit2* double mutant spinal cords failed to reveal a defect in commissural axon guidance. We report here that when all six *Slit* alleles are removed, many commissural axons fail to leave the midline, while others recross it. In addition, *Robo1* and *Robo2* single mutants show guidance defects that reveal a role for these two receptors in guiding commissural axons to different positions within the ventral and lateral funiculi. These results demonstrate a key role for Slit/Robo signaling in midline commissural axon guidance in vertebrates.

Introduction

Cells at the midline of the central nervous system (CNS) express many axon guidance cues, both attractive and repulsive, that regulate midline guidance (Tessier-Lavigne and Goodman, 1996). Two different populations of interneurons have been described based on their guidance behavior relative to the CNS midline. Association neurons project axons ipsilaterally, never crossing the midline, whereas commissural neurons send axons contralaterally, forming a commissure across the midline (hence their name). Commissural axons are initially guided to the midline by attractive guidance cues, including netrins, but to

cross the midline and move on to the next leg of their trajectory, they must switch on repulsive responses to other midline guidance cues once they have entered the midline. After crossing the midline, these axons then turn longitudinally, projecting parallel to the midline at specific lateral positions. The same repellents that expel commissural axons out of the midline may also keep commissural axons from recrossing it.

In Drosophilia, the midline repellent that expels commissural axons and prevents them from recrossing is the ligand Slit, which mediates repulsive effects via receptors of the Roundabout (Robo) family (Kidd et al., 1999; Kidd et al., 1998a; Kidd et al., 1998b). When commissural axons grow toward the midline, Robo receptors are kept in intracellular compartments away from the axon surface by the Commissureless (Comm) protein(Keleman et al., 2002). As the axons reach and cross the midline, the inhibition of Robo by Comm is released, Robo proteins are now expressed at the cell surface of commissural growth cones, causing them to sense the Slit repellent and hence expelling them from the midline (Kidd et al., 1998b). It is the tight temporal and spatial regulation of Robo expression that ensures that Slit functions only after commissural axons have crossed the midline. Furthermore, a combinatorial code of Robo receptors controls the lateral positions of commissural axons after they have crossed the midline and turned longitudinally. Axons projecting most medially express only dRobo1, intermediate axons express both dRobo1 and dRobo3, whereas lateral axons

express all three Robo receptors (Rajagopalan et al., 2000b; Simpson et al., 2000a) .

Three mammalian Slit homologs (Slit1, Slit2, and Slit3) and three Robo homologs (Robo1, Robo2, and Rig-1) have been identified, with mRNA expression patterns reminiscent of their Drosophila counterparts. The *Slits* genes are expressed in the floor plate at the ventral midline of the spinal cord and *Robo-1 and -2* in regions that include commissural neuron cell bodies (Brose et al., 1999; Itoh et al., 1998; Kidd et al., 1998a; Li et al., 1999). Rig-1, a divergent member of the Robo family (Yuan et al., 1999), is also expressed in commissural neurons but has an unexpected function of antagonizing Slit responsiveness in pre-crossing axons, as discussed in a companion paper (Sabatier et al., companion manuscript). Here we address whether the more classical Slit and Robo homologues (Slit1-3 and Robo1and 2) play conserved roles in commissural axon guidance. It has been shown that in vitro commissural axons are sensitive to repulsive actions of Slit2 only after they have crossed the floor plate (Zou et al., 2000). However, quite surprisingly in *Slit1;Slit2* double mutant mice, although the formation of several major forebrain tracts (corticofugal, callosal, and the thalamocortical tracts) and the optic chiasm are defective (Bagri et al., 2002; Plump et al., 2002), no obvious commissural axon guidance defects were observed in the spinal cord (Plump et al., 2002). This lack of phenotype raised the possibility that Slit proteins might not, after all, be so important for regulating crossing of the midline in the spinal cord. However, the lack of phenotype could

also be attributed to Slit3, which is also expressed by floor plate cells (Brose et al., 1999). Slit3 is required for diaphragm development (Yuan et al., 2003), but its role in nervous system development has not so far been defined.

As a first step toward determining the in vivo roles of Slit proteins and Robo1 and Robo2 receptors in commissural axon guidance in vertebrates, we have generated mutant mice lacking *Robo1*, *Robo2*, or all three *Slit* genes. Our results support a conserved role for these proteins in vertebrates, with Slit proteins helping expel commissural axons out of the floor plate at least partly by activating Robo1. Robo1 and Robo2 protein localization also appears to specify the lateral positions of the longitudinal tracts adopted by commissural axons as they grow toward their final targets in the contralateral spinal cord.

Results

Commissural axon guidance at the floor plate is defective in *Slit 1, 2, 3* triple mutants

To investigate the role of Slit proteins at the floor plate in commissural axon guidance, we generated *Slit 1, 2, 3* triple mutant mice by crossing *Slit1-/-; Slit2+/-* animals (Plump et al., 2002) to *Slit3-/-* animals (Yuan et al., 2003) and performed TAG-1 immunostaining of E12.5 caudal spinal cord transverse sections from these mice (Figure 1). TAG-1 labels commissural axons as they grow ventrally and as they cross the floor plate, and starts to be downregulated once these axons reach the contralateral side of the spinal cord, although some TAG-1 immunostaining is observed medially in the ventral funiculus (Figure 1A) (Dodd et

al., 1988). Because of the mating strategy, there are no wild type animals among the littermates. However, extensive analysis has shown that *Slit1-/-* embryos resemble wild type animals at the level of commissural axon guidance (Plump et al., 2002). Thus in this study, we used *Slit 1-/-* animals as controls.

In both control and *Slit* triple mutant embryos, commissural axons project ventrally toward the floor plate. However, instead of forming a tightly-bundled commissure at the floor plate as in wild type embryos (Figure 1A', A"), commissural axons in the *Slit* triple mutant are disorganized as they exit the floor plate and appear defasciculated. The TAG-1 signal in the ventral funiculus is largely absent (Figure 1F', F"), suggesting that fewer commissural axons exit the floor plate. Expression of the floor plate marker *Netrin-1* appeared normal in *Slit* triple mutants, suggesting that the observed defects are not a result of altered floor plate cell fate (data not shown) and instead reflect guidance defects.

To further characterize the phenotype at the floor plate, we performed immunostaining using an antibody directly against L1 on transverse sections of E12.5 embryos. Like TAG-1, L1 is expressed by commissural axons, but it is specifically upregulated once these axons start to cross the floor plate (Dodd et al., 1988). Similarly to what we observed with TAG-1 antibodies, commissural axons visualized by L1 immunohistochemistry appear disorganized as they grow through the floor plate in *Slit* triple mutants. As well, more axons are stained by the L1 antibody within the floor plate, presumably another indication that the

axons are stalling or recrossing (Figure 2C, D). In mutant embryos we routinely observed axons projecting dorsally toward the ventral ventricular zone, which is clear of axonal projections in wild type animals (Figure 2E, F arrowhead). A similar disorganization and dorsally projecting axons were observed with an antibody to Neurofilament (NF-M), which labels all axons. (Figure 2A, B). Using the L1 antibody, we also observed that the ventral funiculus still forms in the triple mutants, but the lateral funiculus appears thinner than in wild type, indicating that fewer axons project dorsally after they have crossed the floor plate (Figure 2C, D arrow). Curiously, L1 also appears to be expressed on the pre-crossing portions of some ventrally-projecting axons as they approach the floor plate (Figure 2D, F, asterisk), from which it is normally excluded.

We also examined the expression patterns of the Slit receptors, Robo1 and Robo2 in the triple mutants. As discussed in detail below, in control animals, Robo1 expression starts to be upregulated after the axons have crossed the floor plate and entered the ventral funiculus, and there is no Robo1 expression within the floor plate (Figure 2G and 4A). In Slit triple mutants, in contrast, high Robo1 expression was observed within the floor plate, and the lateral funiculus appeared much thinner than in controls, suggesting that commissural axons linger close to the floor plate. Inappropriate Robo1 expression was also seen on the pre-crossing portions of ventrally-projecting axons approaching the floor plate, similar to what was seen for L1 (Figure 2H, asterisk). The pattern of Robo2 immunostaining was altered in a similar way in the triple mutants, with

higher expression in the floor plate, and inappropriate expression of Robo2 on pre-crossing axons (data not shown). The expression patterns thus support further the finding with TAG-1 and L1 immunohistochemistry of defects in commissural axon guidance in the Slit triple mutants.

We also examined commissural axons in animals in which different numbers of Slit alleles had been removed. Commissural axons visualized by TAG-1 immunohistochemistry in *Slit1-/-; Slit2-/-* animals (Plump et al., 2002), *Slit1-/-;Slit3-/-* animals, and *Slit1-/-;Slit2+/-;Slit3-/-* all appeared normal. However, *Slit1-/-;Slit2-/-;Slit3+/-* animals showed some subtle defects, in that the ventral commissure appeared thicker and the TAG-1 staining in the ventral funiculus appeared reduced (Figure 1B-E).

Midline stalling and recrossing of commissural axons in *Slit* triple mutant embryos

Since this initial evaluation of commissural axon pathfinding was based on analysis of proteins that are highly spatially regulated in commissural axons (with the exception of NF-M), some of our observations could reflect either a direct effect on axon guidance itself or misregulation of marker expression. To directly examine axon guidance defects, we anterogradely labeled commissural axons by DiI injection in the dorsal part of an E12.5 spinal cord open book preparation, which allowed us to visualize the entire axon trajectory. As shown in Figure 3, in control animals commissural axons crossed the floor plate in a well-organized fashion and turned rostrally on the contralateral side of the spinal cord (Bovolenta

and Dodd, 1990); in only around 16% of the DiI injections were axons observed stalled in the floor plate (n=74) (Figure 3A-C, M). In *Slit1,2,3* triple mutants, two highly penetrant phenotypes were observed, and in most cases these phenotypes existed simultaneously in a given axon cohort labeled with a single DiI injection. First, many of the commissural axons were stalled in the floor plate: stalling axons were observed in 90% of the injections (n=91) (Figure 3E-G, M). Second, some commissural axons projected first across the floor plate, made a turn and projected back to the ipsilateral side of the spinal cord, recrossing the floor plate. This phenotype was observed in about one fourth of the injections (n=91) (Figure 3 I-K, M), whereas in control animals, recrossing was never seen (n=74). Note, however, that in mutant animals, there are still axons that crossed the floor plate normally and projected longitudinally upon reaching the contralateral side of the spinal cord (Figure 3G). *Slit1-/-,Slit2-/-,Slit3+/-* animals showed a slight phenotype: in 31% of DiI injections there were axons stalling in the floor plate, and recrossing axons were also observed in 4% of injections (n=49) (Figure 3M). Our immunochemistry and DiI labeling results thus support a significant role for Slit proteins in vivo in guiding commissural axons out of the floor plate, and in preventing commissural axons from recrossing the floor plate once they have crossed.

Robo1 and Robo2 are localized primarily on postcrossing commissural axons

To better understand the mechanisms by which Slits act to influence commissural axon guidance across the floor plate, we characterized the

expression of their cognate receptors of the Robo family in the spinal cord. As previously described, the mRNAs for both *Robo1* and *Robo2* are found in overlapping populations of commissural neurons at a time when their axons are crossing the floor plate (Brose et al., 1999). *Robo2* appears to be expressed by a distinct population of commissural neurons whose cells bodies lie at more lateral positions in the spinal cord compared to *Robo1* (Figure 4D-E). To determine where Robo1 and Robo2 proteins are localized in the developing spinal cord, antibodies were generated against the extracellular domains of either receptor. These antibodies were then used to stain transverse sections of E11.5 mouse spinal cords. Similarly to what is observed in Drosophila commissural axons (Kidd et al., 1998a), both Robo1 and Robo2 appear to be localized primarily to the post-crossing portion of commissural axons (Figures 4A-B), although low levels of protein are clearly observed pre-crossing. Interestingly, Robo1 and Robo2 appear to label overlapping but distinct regions of the ventral and lateral funiculi (Figures 4A-B and 4F). This localization of Robo1 and Robo2 to the post-crossing portion of commissural axons contrasts with the expression of TAG-1, a well-characterized marker of commissural axons as they grow toward and through the floor plate (Figure 4C).

Generation of *Robo1* and *Robo2* mutants

To determine the role of Robo1 and Robo2 in guiding commissural axons, we analyzed mice mutant for either of the two receptors. The generation of the *Robo2* mutant mice was described in a separate study, which focused on a

kidney malformation phenotype (Grieshammer et al., manuscript in preparation; copy attached for review); as described there the mutation is a null mutation. To generate a mutant allele of Robo1, we identified the region corresponding to the first intron of Robo1 on BAC clones from a 129 ES cell library (Incyte Genomics). We then targeted a PLAP secretory trap vector, composed (in order from 5' to 3') of a splice acceptor site, a transmembrane domain fused to β-geo (TM-β-geo), an internal ribosomal entry site (IRES), placental alkaline phosphatase, and a PGK1 polyA tail, to the first intron of Robo1 (Leighton et al., 2001). The *Robo1* targeted allele generates a fusion protein between exon 3 and TM-β-geo that is trapped in an intracellular compartment (Supplementary Figure 1A). Additionally, the PLAP vector allows bicistronic expression of the PLAP reporter from the *Robo1* promoter.

ES cell colonies containing homologous integrants were screened using a 3' flanking probe (Supplementary Figure 1B). These clones were used to generate chimeric male mice that were then mated to CD-1 females to generate germline transmissible *Robo1*-deficient mice. Resulting heterozygotes were crossed to generate homozygous deficient mice, and the expected Mendelian ratio was observed among wild-type, heterozygous, and homozygous mutant mice. The homozygous deficient animals are viable, fertile and appear grossly normal. RNAse protection analysis using a probe directed against a 3' region of *Robo1* confirmed that very little *Robo1* mRNA is expressed downstream of the inserted PLAP cassette (Supplementary Figure 1C), confirming the absence of detectable

Robo1 protein on the postcrossing axons (Figure 7C). Thus, the mutant allele is likely to be a severe hypomorph.

Robo1 regulates midline crossing

TAG-1 immunohistochemistry revealed that commissural axons behave normally as they extend ventrally toward and across the floor plate in E11.5 *Robo1* mutant spinal cords (Figure 5K). However, when labeling most of the axons in the developing neural tube using an anti-NFM antibody, axons were observed entering the dorsal floor plate and growing toward the ventral ventricular zone, similar to the phenotype observed in *Slit1;Slit2;Slit3* triple mutant embryos (Figure 5A-B, 5D-E, 2B and 2F). These axons were also labeled using the L1 antibody but not the TAG-1 antibody, as was also observed for the misplaced axons in the dorsal floor plate in the Slit triple mutant (Figure 5G-H and J-K). At E12.5 when more commissural axons have crossed the midline, NF-M-expressing dorsally-extending axons were no longer observed in the floor plate of *Robo1* mutants (data not shown) suggesting that the misprojecting axons correct their errors or are eliminated in the *Robo1* mutants. Using DiI injections into the dorsal spinal cord, we observed a large number of stalled growth cones in the floor plate in *Robo1* mutants (Figures 6B and 6C). Although some stalled axons are observed in the floor plates of control spinal cords, they are seen in low numbers and usually exhibit thin growth cones (arrowheads in Figure 6A). In the *Robo1* mutant floor plate, the stalled growth cones are large and send out many filopodia (arrowheads in Figure 6B).

Once commissural axons exit the floor plate into the contralateral spinal cord, they dramatically change direction, and course longitudinally in the ventral and lateral funiculi. Although most post-crossing commissural axons initially grow adjacent to the floor plate, they rapidly migrate away from the midline to adopt more dorsolateral positions within the contralateral spinal cord (Imondi and Kaprielian, 2001). In wildtype E11.5 spinal cords, only a few commissural axons are observed in the dorsal part of the lateral funiculus (Figure 7A and 7D). However, in *Robo1* mutants, the lateral funiculus is significantly thicker compared to wildtype (Figure 7B and 7E). This tendency for commissural axons to grow further away from the floor plate in *Robo1* mutant spinal cords can be observed both in transverse sections (using L1 immunohistochemistry or DiI labeling of commissural axons) as well as in the open book configuration (Supplementary Figures 2B and 3D-F). To determine the identity of the axons that could account for this phenotype we stained E11.5 spinal cord transverse sections from wildtype or *Robo1* mutant embryos with the Robo2 antibody. This analysis revealed that Robo2-positive axons appear to account for the increased density observed in the lateral funiculus (Figure 7E).

Robo2 controls the extension of commissural axons away from the floor plate in the contralateral neural tube

Although no phenotype was observed in the Robo2 mutant commissural axons as they extend toward and across the floor plate, we noted that the pattern of TAG-1 expression is truncated in the ventral funiculus of E11.5 *Robo2* mutant

spinal cords (Figure 5I), similar to what is observed in the *Slit1-/-;Slit2-/-;Slit3-/-* triple mutant (Figure 1F). To further characterize the effect of removing *Robo2* on the extension of the ventral and lateral funiculi, we turned to L1 immunostaining. Similar to what we observed with TAG-1 immunohistochemistry, the lateral funiculus appears foreshortened in *Robo2* mutant mice, as more axons seem to course in more medial positions relative to the floor plate rather than more lateral positions (Figure 7C and 7F). Labeling of commissural axons with DiI both in transverse sections and in open-book spinal cords revealed the same phenotype (Supplementary Figure 2C and 3G-I): in E12.5 *Robo2* mutant spinal cords, commissural axons remain tightly apposed to the floor plate once they have entered the contralateral neural tube and fail, for the most part, to move to more dorsal longitudinal tracts in the lateral funiculus.

Discussion

In the journey to their final targets, commissural axons are initially guided by attractive guidance cues to an intermediate target, the floor plate of the spinal cord. Upon reaching it, these axons switch on responsiveness to midline repellents and switch off sensitivity to the attractants, which helps move them on to the next leg of their trajectory. We have provided evidence that Slit proteins expressed by the floor plate are required to repel commissural axons away from their intermediate target at the ventral midline into longitudinal tracts in the contralateral spinal cord. Due to the redundancy of the three vertebrate Slit homologs expressed at the floor plate, all six Slit alleles must be disrupted for a

strong phenotype to be apparent. We have also shown that the vertebrate Robo receptors, Robo1 and Robo2 regulate midline crossing and are required for commissural axons to grow in the proper longitudinal tract within the ventral and lateral funiculi in the contralateral spinal cord.

Slits regulate midline crossing in the vertebrate spinal cord

Analysis of the *Slit* triple mutant by TAG-1 immunohistochemistry revealed that the ventral commissure is defasciculated and disorganized, and the TAG-1 signal normally observed in the medial part of the ventral funiculus is absent (Figure 1F, F', F"). This suggests that TAG-1 positive commissural axons are stalled inside the floor plate, and that few of them are reaching the contralateral side and turning to project longitudinally. To better characterize the behavior of commissural axons after they have crossed the floor plate, we used an anti-L1 antibody, which normally labels commissural axons primarily only after they have entered the floor plate. In *Slit1;Slit2;Slit3* triple mutants, L1-positive axons are observed projecting dorsally in the floor plate. These wandering axons are also observed with an antibody directed against NF-M, but very few express TAG-1. These results suggest that the axons observed in the dorsal floor plate of triple mutants in transverse sections might be stalled or recrossing commissural axons that have begun to downregulate TAG-1 and upregulate L1.

Consistent with this interpretation, when we performed anterograte DiI injections in the dorsal spinal cord to trace commissural axon trajectories directly, we

observed large numbers of stalled axons inside the floor plate. Other commissural axons were seen growing back to the ipsilateral side of the spinal cord after having reached the contralateral side, or even looping back once they had already entered the floor plate. This recrossing phenotype was never seen in controls or in any other mutants characterized before, implying that the mutant phenotypes we see by immunochemistry are due partly or entirely to commissural axon guidance defects. We cannot rule out the possibility that some misguided axons observed in transverse sections in the floor plate of *Slit1;Slit2;Slit3* triple mutants are association axons that are inappropriately crossing the floor plate. That possibility would, however, still be consistent with a repulsive role for midline Slit proteins,

Interestingly, both L1 and Robo1 also appear to be misregulated in *Slit* triple mutants, since commissural axons express both of these proteins on their precrossing portions, prior to entering the floor plate (Figures 2D,F, and J, asterisks). The reason for this altered expression is unclear, but it will be interesting to determine whether Slits are involved in preventing the diffusion of Robo1 and L1 protein back up the pre-crossing axon, e.g. by binding and trapping one or more of them.. Since there is aberrant expression of these two proteins on the pre-crossing axon, it was, however, necessary to consider the possibility that the stalled and recrossing axons observed in the *Slit1;Slit2;Slit3* triple mutants are simply a secondary consequence of L1 and Robo1 misregulation. We believe, however, that this is unlikely, because L1 and Robo1

misregulation is also consistently observed in embryos that continue to express some Slit alleles (i.e., *Slit1-/-;Slit2-/-;Slit3+/+*, *Slit1-/-;Slit2+/-;Slit3-/-*, or *Slit1-/-;Slit2+/-;Slit3+/-* embryos) (data not shown), yet animals with these genotypes do not exhibit defects in commissural axon guidance as assessed by DiI injection (data not shown).

In the same vein, since L1 and Robo1 expression are misregulated in the Slit triple mutants, it was necessary to consider the possibility that the altered TAG-1 pattern at the midline in the triple mutant reflected in part a misregulation of TAG-1 surface expression by commissural axons. We cannot, in fact, exclude that such misregulation contributes to the "stalled" appearance seen in cross sections. However, that significant stalling and recrossing do occur in the triple mutant is established by the DiI injection studies. It is thus reasonable to assume that the altered TAG-1 expression patterns reflects partly (and perhaps largely) a change in the projection patterns of the axons.

In summary, despite our observation of an interesting disruption in the pattern of L1 and Robo1 expression in *Slit* triple mutants and the fact that we cannot exclude that TAG-1 expression is not also altered in these mutants, our data provide overwhelming evidence of significant disruptions in commissural axon midline crossing in the triple mutants that are consistent with a key role of Slit proteins in expelling commissural axons from the midline.

Robo1 and Robo2 are required for commissural axons to navigate beyond the floor plate

Based on genetic studies in Drosophila and C. elegans, and biochemical studies in vertebrates, receptors of the Robo family have been implicated in sensing Slit ligands as repellents (Kidd et al., 1998a; Stein and Tessier-Lavigne, 2001; Zallen et al., 1998). In vertebrates, several such receptors have been identified: Robo1, Robo2 and Rig-1 (Brose et al., 1999; Yuan et al., 1999). In a companion paper, we provide evidence that Rig-1 functions as an inhibitor of Slit responsiveness in commissural axons prior to crossing the floor plate (Sabatier et al., companion paper). Since both Robo1 and Robo2 are also expressed by commissural neurons in the developing spinal cord, they are the primary candidate receptors for mediating repulsion by midline Slit proteins. Such a role is supported by the localization of both Robo1 and Robo2 to the post-crossing portion of commissural axons. Antibodies directed against the Robo1 extracellular domain appear to label both the ventral and lateral funiculi, in which commissural axons course longitudinally toward their final targets in the brain. Interestingly, Robo2-positive axons are found primarily in the lateral funiculus. Although some of these may be the axons of association neurons, it appears that many Robo2-expressing axons are commissural, as assessed by expression of the LacZ reporter in the ventral commissure under the floor plate when expressed from the *Robo2* locus in *Robo2* heterozygous animals (Supplementary Figure 4).

Based on the hypothesis that Robo receptors are required to expel commissural axons out of the floor plate once they have crossed, we would predict that Robo mutants, like Slit triple mutants, would exhibit stalling or recrossing phenotypes. Indeed, in transverse sections of E11.5 *Robo1* mutant embryos (Figure 5E), as in the Slit triple mutants (Figure 2F), L1-positive but TAG-1-negative axons are observed growing aberrantly into the dorsal region of the floor plate. As for the Slit triple mutant, the fact that these wandering axons express L1 but not TAG-1 suggests that might be stalled or recrossing commissural axons. This conclusion is further strengthened by DiI analysis, which revealed an increased number of stalled axons in the floor plate of *Robo1* mutant E11.5 embryos (Figure 6), similar to but less penetrant than the stalling observed in Slit triple mutants. These results support a model in which Slit proteins in the floor plate mediate repulsion of commissural axons at least partly through the receptor Robo1.

Along with errors within the floor plate, *Robo1* mutants exhibit an enlarged lateral funiculus (Figure 6B and 6E), apparently due to commissural axons growing further away from the floor plate in the contralateral spinal cord (Supplemental Figure 3D-F). At first glance, this phenotype might appear to contradict a role for Robo1 in sensing midline Slits as repellents. However, there needn't be a contradiction, if we assume that Robo1 and Robo2 are both repellent receptors, but position within the lateral funiculus is determined by the following rules: (1) axons expressing just Robo1 are positioned more medially and ventrally (closer to the floor plate); (2) axons expressing just Robo2 are located in the most dorso-

lateral positions in the lateral funiculus; and (3) axons co-expressing the two receptors (which are predicted to be present, since some cells appear to coexpress mRNAs for the two Robos (Figure 4D and 4E) are found in between these two extreme positions. This model is consistent with the immunohistochemical data, which shows regions enriched in Robo1 compared to Robo2 next to the floor plate, and enriched in Robo2 compared to Robo1 in the dorsolateral-most positions. It also predicts that removal of Robo1 would cause the population that normally only expresses Robo1 to stay closer to the floor plate, but the population that normally expresses both Robo1 and Robo2, which now would express only Robo2, would then project further dorsally, providing an explanation for the dorsal shift observed in the *Robo1* knock-out. Consistent with this interpretation, the axons in the lateral funiculus in the *Robo1* mutant express Robo2. Also consistent with this hypothesis, when *Robo2* is mutated, the lateral funiculus is truncated and commissural axons generally adopt more medial positions in the contralateral spinal cord relative to the floor plate as assessed by both L1 expression and DiI injection (Figure 6G-I and Supplementary Figure 3). Interestingly, commissural axons do not appear to be misguided within the floor plate of Robo2 mutants as assessed by L1 and NF-M immunohistochemistry or by DiI in E12.5 open-book spinal cords. This observation may indicate that commissural axons that only express Robo2 tend to fasciculate with pioneer, Robo1-expressing commissural axons in order to cross the floor plate. This hypothesis is further supported by the cell non-autonomous effects of the Rig-1 mutation on Robo2-expressing commissural

axons, which fail to cross the floor plate in Rig-1 mutants despite not expressing Rig-1 normally (see discussion in Sabatier et al., companion paper).

The localization of both Robo1 and Robo2 proteins is loosely reminiscent of what has been described in Drosophila where commissural axons have been shown to grow along distinct longitudinal tracks after they have crossed the midline. DRobo is expressed by axons in all longitudinal tracks. However, dRobo3 and dRobo2 are expressed in progressively more lateral populations in distinct but overlapping domains. It has been speculated that the lack of two motifs (CC2 and CC3) in the cytoplasmic domains of dRobo2 and dRobo3 cause these receptors to be more sensitive to a Slit gradient. This is consistent with the *robo2* and *robo3* single mutant phenotypes, in which axons remain closer to the midline as they grow longitudinally (Rajagopalan et al., 2000a; Rajagopalan et al., 2000b; Simpson et al., 2000a; Simpson et al., 2000b). In vertebrates, Robo2 lacks the CC3 motif and is also localized to more lateral positions relative to the floor plate, and when Robo2 is removed, commissural axons generally tend to stay closer to the floor plate, consistent with the hypothesis that in wild type animals Robo2 drives commissural axons further away from the floor plate than does Robo1.

More Slit receptors in addition to Robo1 and Robo2

Commissural axon guidance phenotypes observed in the *Slit1;Slit2;Slit3* triple mutant embryos appear to be a combination of the phenotypes observed in the *Robo1* and *Robo2* single mutants, but with an increased severity. Indeed, stalled

axons are observed in the floor plates of both *Robo1* and *Slit1;Slit2;Slit3* triple mutants and a truncated lateral funiculus is observed in both *Robo2* and *Slit1;Slit2;Slit3* triple mutants. However, axons recrossing the floor plate are only observed in the *Slit1;Slit2;Slit3* triple mutants but in neither *Robo* single mutants. The similarity of phenotypes between the *Slit* mutants and that of their receptors indicates that, in vivo, Robos most likely mediate, at least partly, the response to Slit repellents in the floor plate.

However, several pieces of evidence indicate that there may be other receptors responsible for sensing Slits as repellents. First, since a large number of commissural axons express only Robo1, we would expect the *Robo1* single mutant to have a more severe phenotype than what we observe. Secondly, studies of the *Rig-1* phenotype have shown that in that mutant background commissural axons inappropriately become sensitive to Slits prior to crossing the floor plate (Sabatier et al., companion manuscript). Although Robo1 is the only Robo receptor co-expressed in Rig-1-positive commissural axons, crossing of the midline is not recovered to wildtype levels in *Robo1;Rig-1* double mutant embryos again suggesting that Rig-1-expressing commissural axons co-express another Slit receptor (Sabatier et al., companion manuscript). Together, these results suggest that there is likely to be another Slit receptor, although the precise contribution of this putative receptor to guidance can be determined only by analysis of *Robo1-/-;Robo2-/-* double mutants. If Robo1 and Robo2 are the sole sensors of Slit repellents on commissural axons, we expect the double

mutant phenotype to look identical to the *Slit1;Slit2;Slit3* triple mutant phenotype. However, if another Slit receptor is present, the double mutant phenotype might look like the sum of the two single mutant phenotypes, which would be significantly less severe than what is observed in the *Slit1;Slit2;Slit3* triple mutants. Unfortunately, due to the proximity of Robo1 and Robo2 on the same chromosome (1.8 Mb), this double mutant animal has proven difficult to generate.

Midline axon guidance in vertebrates and Drosophila

In this study, we have provided evidence that Slit repellents in the floor plate act through Robo1 and Robo2 to guide commissural axons in the spinal cord. However, despite the severity of the *Slit1;Slit2;Slit3* triple mutant phenotype, a significant number of commissural axons are observed that exhibit no obvious axon guidance phenotype. This is in contrast to what has previously been described in Drosophila, in which the removal of Slit leads to the collapse of both commissurally and longitudinally projecting axons into the ventral midline. This result suggests that in vertebrates other repulsion systems beside Slit/Robo are involved in guiding commissural axons out of the floor plate and beyond. One likely system is provided by Semaphorins acting through Neuropilin receptors. Whereas in Drosophila Semaphorins are not required for commissural axon guidance in the CNS (Yu et al., Neuron 1998), in vertebrates Sema3B is expressed by floor plate cells and has been implicated in expelling post-crossing commissural axons from the midline via Neuropilin-2 (Zou et al., 2000). Eph/Ephrin signaling may also contribute to guiding post-crossing axons, since

several EphrinB proteins are expressed in the floor plate and dorsal spinal cord, and B class Eph receptors are expressed in the post-crossing segment of commissural axons (Imondi et al., 2000). Despite this apparent redundancy between distinct repellent systems in the floor plate, removal of the Slit proteins is sufficient to severely disrupt midline axon guidance. The commissural axon phenotypes observed in the Slit triple mutant will serve as a useful baseline with which to compare future mutants where multiple repellent systems have been inactivated. These studies will be required to characterize the relative roles of each guidance system in directing commissural axons to leave the floor plate.

Experimental Procedures

Generation of *Robo1*-deficient Mice

We used a Robo1 specific cDNA probe to screen a BAC library (Incyte Genomics) and isolate genomic DNA containing portions of the Robo1 gene. BAC DNA was then used to generate the targeting vectors shown in Supplementary Figure 1A using standard recombinant DNA techniques. Southern blot and Rnase protection assays were performed using standard techniques. To identify targeting events, genomic DNA was digested with HindIII and hybridized with a DNA probe external to the targeting vector as noted in the targeting figures. ES cell culture and generation of mice was carried out as previously described (Mombaerts et al., 1996). For genotyping, a PCR-based screen was developed: a forward primer common to both wildtype and mutant alleles 5'- TGGCACGAAGGTATATGTGC-3'; a wildtype allele-specific reverse

primer 5'- GAAGGACTGGTGGTTTTGAG-3'; and a mutant allele specific reverse primer 5'- CCTCCGCAAACTCCTATTTC -3'. PCR was carried out using the same protocol previously described (Plump et al., 2002).

Immunohistochemistry

Embryos were fixed overnight at 4°C in 4% paraformaldehyde (PFA)/ phosphate-buffered saline (PBS), washed with PBS, incubated in 30% sucrose/PBS overnight, and embedded in OCT. Cryostat sections (20 μm) were collected on Superfrost Plus slides (Fisher) and kept at –80°C. Slides were blocked in PHT (PBS, 1% heat-inactivated goat serum, 0.1% Triton X-100) for 1h at room temperature (RT), incubated overnight at 4°C with the primary antibody diluted in PHT, washed 3 times for 15 min at RT in PHT, incubated for one hr at RT with the fluorescently-labeled secondary antibody diluted in PHT, washed 3 times for 15 min at RT in PHT and coverslip-mounted using Fluoromount G mounting media (Fisher). The TAG1 (clone 4D7, dilution 1:200), Neurofilament (clone 2H3, dilution 1:200) monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (University of Iowa). The rabbit polyclonal Robo1 and Robo2 antibodies (used at 5ng/ml and 10 ng/ml respectively) were generated in the lab of Fujio Murakami using Fc-tagged Robo ectodomains as antigens. The rat monoclonal L1 antibody (dilution 1:200) was from Chemicon.

In situ hybridization

Fluorescent in situ hybridization of mouse spinal cords was carried out as described in the TSA plus protocol (Perkin Elmer).

Lipophilic Dye Tracing

Spinal cords of E11.5 and E12.5 embryos were prepared in an open-book configuration, fixed with 4% paraformaldehyde, and injected with DiI (Molecular Probes) using iontophoresis into the dorsal region or just dorsal to the floor plate in the caudal part of the spinal cord. DiI was allowed to diffuse for two days to label commissural axons along their entire length, enabling their visualization by conventional fluorescence microscopy and confocal microscopy.

DiI tracing of commissural axons in transverse sections was conducted by injecting a small amount of DiI just dorsolateral to the floor plate in 100 μm thick vibratome sections of E12.5 embryos. The DiI was allowed to diffuse for two days. The extension of DiI into the ventral funiculus contralateral to the injection site was then visualized by fluorescence microscopy.

Figure Legends

Figure 2.1, Commissural axons are disorganized at the floor plate in *Slit 1,2,3* triple mutants.

Visualization of commissural axons in transverse sections of wildtype (A) or different combination of *Slit 1, 2, 3* knock-out mutant spinal cords (B-F). In wildtype and mutant spinal cords, TAG-1 labels commissural axons as they grow ventrally toward the floor plate and as they cross to the contralateral side. In E12.5 wild type animals, a tightly bundled commissure is formed at the floor plate (A), TAG-1 staining in the medial ventral funiculus is also seen (arrowhead in A). In *Slit 1, 2, 3* triple mutants, commissural axons appear disorganized and defasciculated at the floor plate (F), TAG-1 staining in the ventral funiculus is absent (arrowhead in F). *Slit1-/-;Slit3-/-* animals (B), *Slit1-/-; Slit2-/-* animals (C), and *Slit1-/-;Slit2+/-;Slit3-/-* (D) all appeared normal while the ventral commissure appeared thicker and TAG-1 staining at the ventral funiculus is reduced in *Slit1-/-;Slit2-/-;Slit3+/-* animals (E). (A') Confocal images of the floor plate in (A). (A") Confocal image of another wild type animal, showing the floor plate area. (F') Confocal image of the floor plate in (F). (F") Floor plate of another *Slit* triple mutant, showing consistent phenotype.

Scale bar, 200 μm (A-F)

Slit-1 -/-
Slit-2 +/+
Slit-3 +/+
Slit-1 -/-
Slit-2 +/+
Slit-3 -/-
Slit-1 -/-
Slit-2 -/-
Slit-3 +/+
Slit-1 -/-
Slit-2 +/-
Slit-3 -/-
Slit-1 -/-
Slit-2 -/-
Slit-3 +/-
Slit-1 -/-
Slit-2 -/-
Slit-3 -/-
A
B
C
D
E
F
A'
A''
F''
F'

Figure 2.2, Commissural axons remain disorganized beyond the floor plate in *Slit 1,2,3* triple mutants.

(A, B) Neurofilament staining of E12.5 mouse spinal cord in transverse sections. In Slit triple mutants axons project dorsally to the ventral ventricular zone (arrowhead in B), which is clear of axons in wild type animals (arrowhead in A). (C, D) L1 immunostaining of E12.5 mouse spinal cord in transverse sections. In Slit triple mutants (D), the commissure at the floor plate appears much thicker than in wild type animals (C), and the lateral funiculus (arrow in D) appears thinner than in wild type animals (arrow in C). (E,F) L1 immunostaining of E12.5 mouse spinal cord floor plate at a higher resolution. Arrowhead showing in mutants axons abnormally grow toward the ventral ventricular zone which is clear of axons in wild type animals. In mutants, L1 immunostaining is observed in precrossing segments of ventrally projecting axons appoaching the floor plate (asterisks in D and F). (G, H) Robo1 immunostaining of E12.5 mouse spinal cord in transverse sections. Robo1 staining is observed within the floor plate of Slit triple mutant animals whereas in controls the floor plate is clear of Robo1 antibody staining. The lateral funiculus appear thinner than control (arrow in G, H). (I, J) Robo1 immunostaining of the spinal cord floor plate at a higher resolution. Robo1 immunostaining is also observed in precrossing segments of some ventrally projecting axons in the Slit triple mutant (asterisks in H, J)

Scale bar, 200 μm (C, D, G, H). Scale bar, 100 μm (A, B, E, F, I, J).

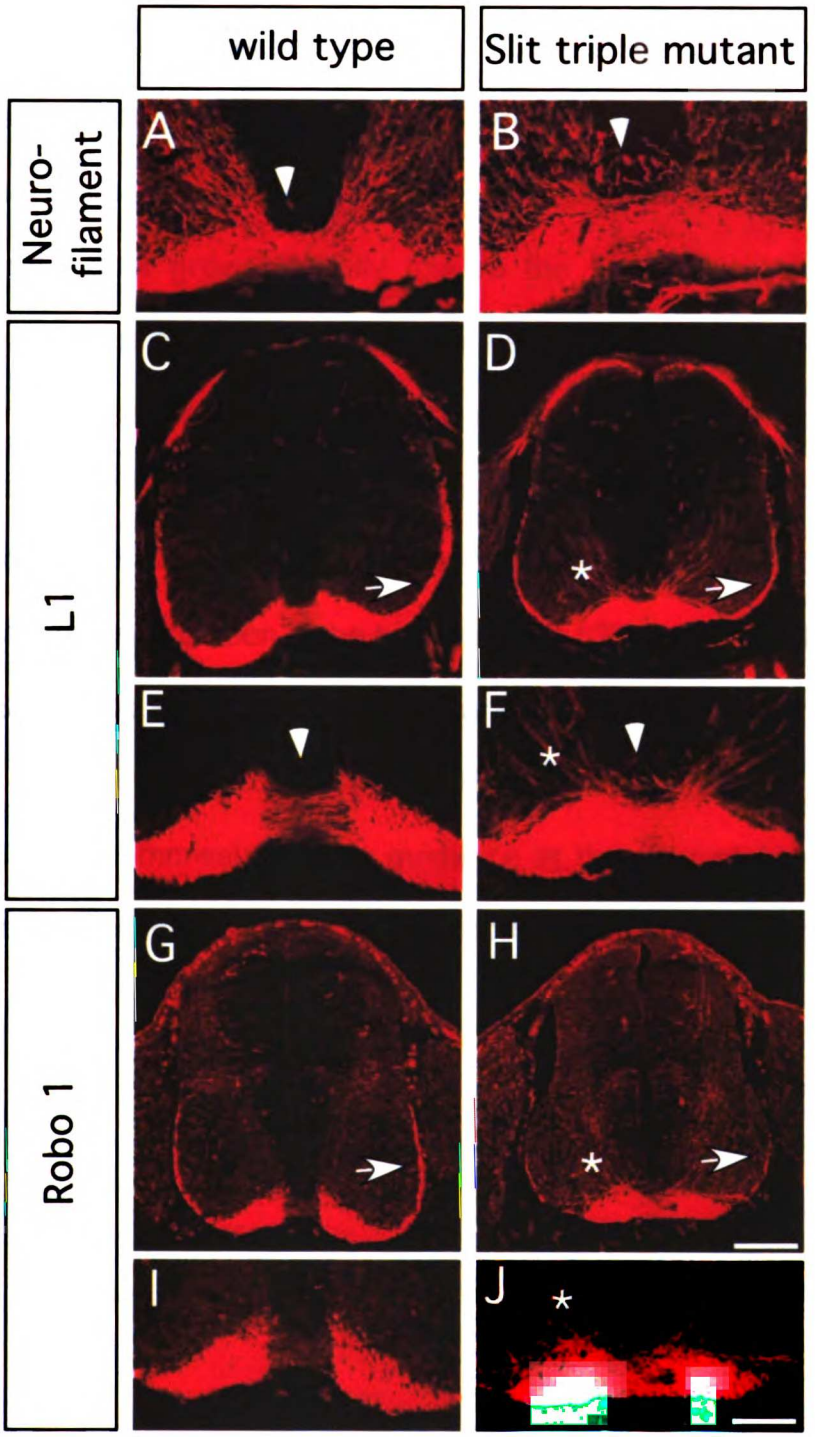
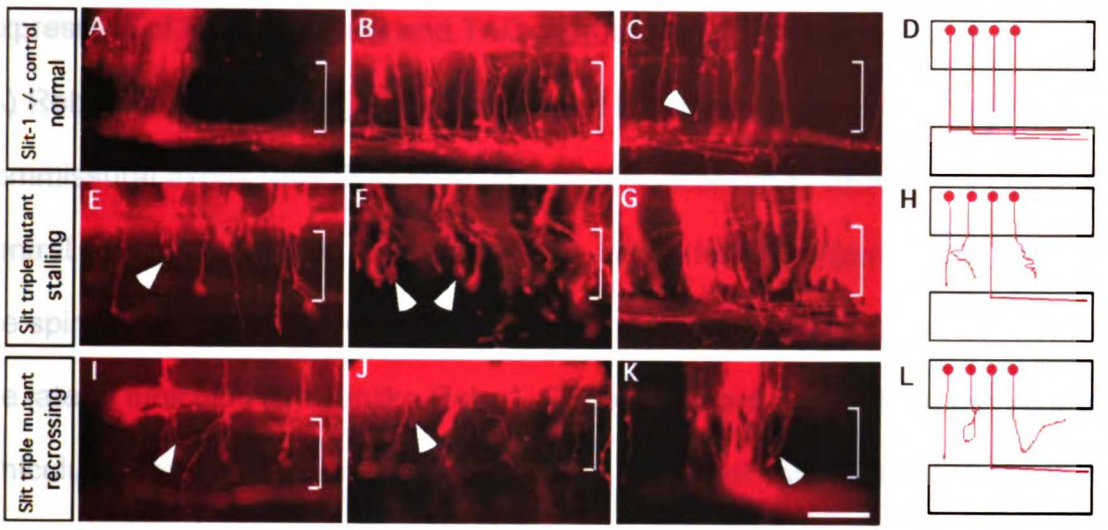

wild type
Slit triple mutant
Neuro-filament
L1
Robo 1
A
B
C
D
E
F
G
H
I
J

Figure 2.3, Commissural axons stall at the floor plate and recross it in the *Slit 1,2,3* triple mutants.

The lipophilic dye, DiI, was implanted into dorsal spinal cord of wildtype (A-C) and different combinations of *Slit* mutant animal open-book preparations. *Slit 1,2,3* triple mutants are shown in (E-G, I-K). In the wildtype spinal cord, commissural axons grow ventrally toward the floor plate, cross, and turn longitudinally on the contralateral side of the spinal cord, although stalling axons in the floor plate are seen at a low percentage (arrowhead in C). In *Slit 1,2,3* triple mutant spinal cords, many commissural axons stall in the floor plate (arrowhead in E, F), while others recross the floor plate (arrow in I-K), normal projecting axons are still seen in the triple mutants (G). Scale bar, 100 μm.

(D, H, L) Schematic drawings of commissural axon projections at the floor plate in wild type (D) and Slit triple mutants (H, L). (M) Histograms documenting abnormalities in commissural axon guidance at the floor plate. Bars showing percentage of injections where the corresponding phenotypes (stall or recross) are observed.


Slit-1 -/- control
normal
Slit triple mutant
stalling
Slit triple mutant
recrossing
A
B
C
D
E
F
G
H
I
J
K
L

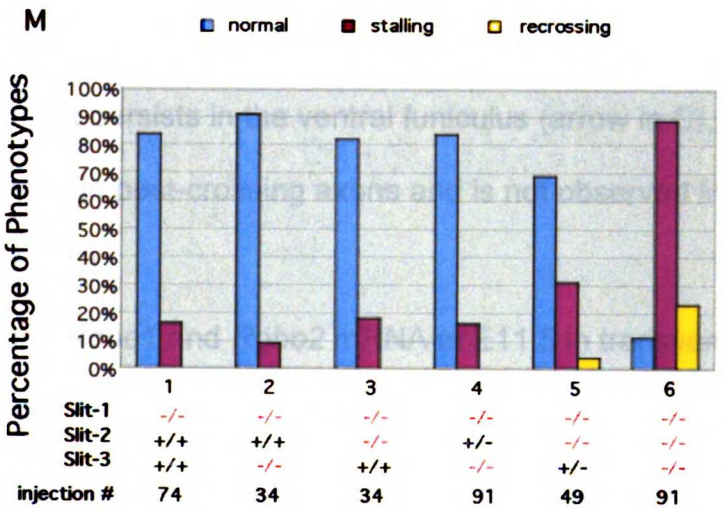

M
normal
stalling
recrossing
Percentage of Phenotypes
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1
2
3
4
5
6
Slit-1 -/- -/- -/- -/- -/- -/-
Slit-2 +/+ +/+ -/- +/- -/- -/-
Slit-3 +/+ -/- +/+ -/- +/- -/-
injection # 74 34 34 91 49 91

Figure 2.4, Robo receptors are expressed in subsets of commissural neurons and localized to distinct portions of their axons.

Expression of Robo1, Robo2 and TAG-1 protein on E11.5 commissural axons. (A) Robo1 protein, although it is observed at low levels in pre-crossing commissural axons (asterisks in A), is primarily localized to axons coursing in the ventral funiculus (arrow in A) as well as the lateral funiculus (arrowhead in A) in the spinal cord. Robo2 protein also appears to be primarily localized to axons in the lateral funiculus (B). Unlike Robo1, Robo2 appears absent from the ventral funiculus (arrow in B) but rather labels only a subset of commissural axons that are found in more dorso-lateral positions (arrowhead in B). In contrast, TAG1 protein is primarily localized to the pre-crossing portion of commissural axons (C) and, although it persists in the ventral funiculus (arrow in C), it appears rapidly downregulated in post-crossing axons and is not observed in the lateral funiculus (arrowhead in C).

Expression of *Robo1* and *Robo2* mRNA at E11.5 in transverse sections of the mouse spinal cord.

As previously reported (Brose et al., 1999), *Robo1* mRNA is expressed dorsally in regions corresponding to commissural and association neurons and ventrally in subpopulations of motor neurons in the E11.5 mouse spinal cord (D). *Robo2* mRNA is also expressed ventrally in the motor column as well as dorso-laterally in subsets of commissural and association neurons (E).

The spinal cord schematic (F) summarizes the data described in the previous panels. Robo1-expressing axons (blue) originate from more medial positions in

the dorsal spinal cord, express low levels of Robo1 as they grow ventrally toward and across the floor plate and adopt more medial positions in the contralateral spinal cord. Robo2-expressing axons (green) original from more lateral positions in the dorsal spinal cord and project to more lateral positions in the contralateral spinal cord, at which time the Robo2 protein is upregulated.

Scale bar, 200 μm.

protein
mRNA
Robo1
Robo2
TAG-1
A
B
C
D
E
F
Robo2
Robo1

Figure 2.5, Misguided commissural axons are observed in the floor plate of *Robo1* mutant but not *Robo2* mutant mice at E11.5.

The anti-Neurofilament antibody (2H3) labels more axons in the spinal cord. In wildtype animals, it labels a well-organized, compact fascicle under the floor plate (A,D). In Robo1 mutant animals, axons are observed invading the dorsal floor plate (B,E). These misprojecting axons are absent from adjacent sections labeled with the TAG-1 antibody (K), which labels commissural axons as they grow toward and through the floor plate. When transverse sections from another E11.5 Robo1-/- embryo were stained with anti-L1 antibody, the dorsally-projecting axons could also be visualized (H). L1 labels commissural axons only once they have entered the floor plate. No dorsally projecting axons are observed in the floor plate of Robo2 mutant animals with either Neurofilament (A,F), L1 (I) or TAG-1 (L) immunohistochemistry.

Scale bars, 200 μm (A-C) and 40 μm (D-L)

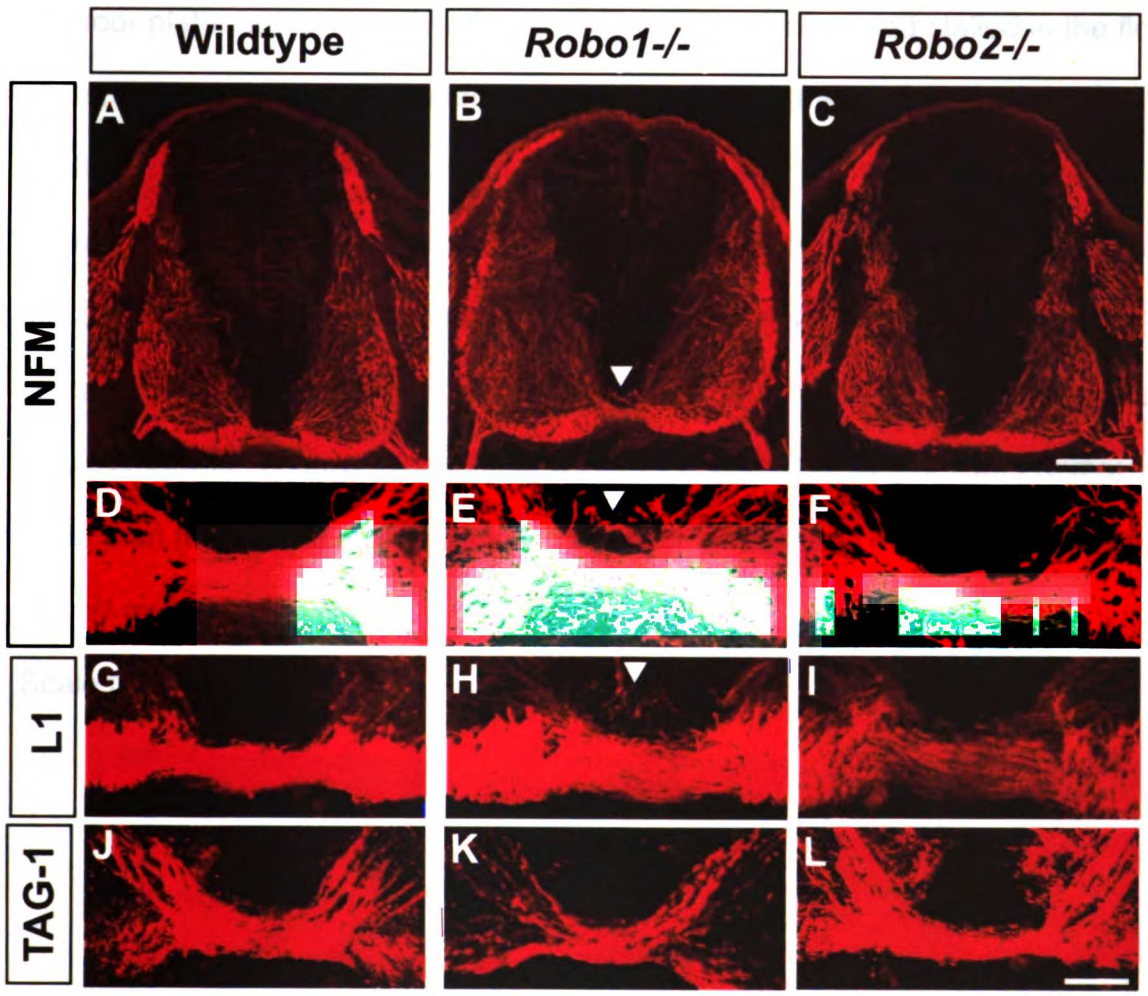

Wildtype
Robo1-/-
Robo2-/-
NFM
L1
TAG-1
A
B
C
D
E
F
G
H
I
J
K
L

Figure 2.6, An increased number of stalled axons are observed in the floor plate of *Robo1* mutant mice at E11.5.

The lipophilic dye, DiI, was used to label commissural axons in E11.5 spinal cord open-book preparations. At this age, not all commissural axons have crossed the floor plate, however, only a few growth cones are observed stalled in the floor plate (arrowheads in A) as determined in a z-series of the floor plate taken by confocal microscopy. However, in *Robo1* mutant animals, many more stalled growth cones are observed in the floor plate as visualized in individual stacks of the z-series (arrowheads in B) and these growth cones tend to be much more complex than what is typically observed in wildtype. These results are quantified in C. Whereas very few injections in wildtype or *Robo1+/-* spinal cords reveal more than five stalled growth cones in the floor plate, many more are observed in *Robo1* mutant spinal cords.

Scale bar, 20 μm

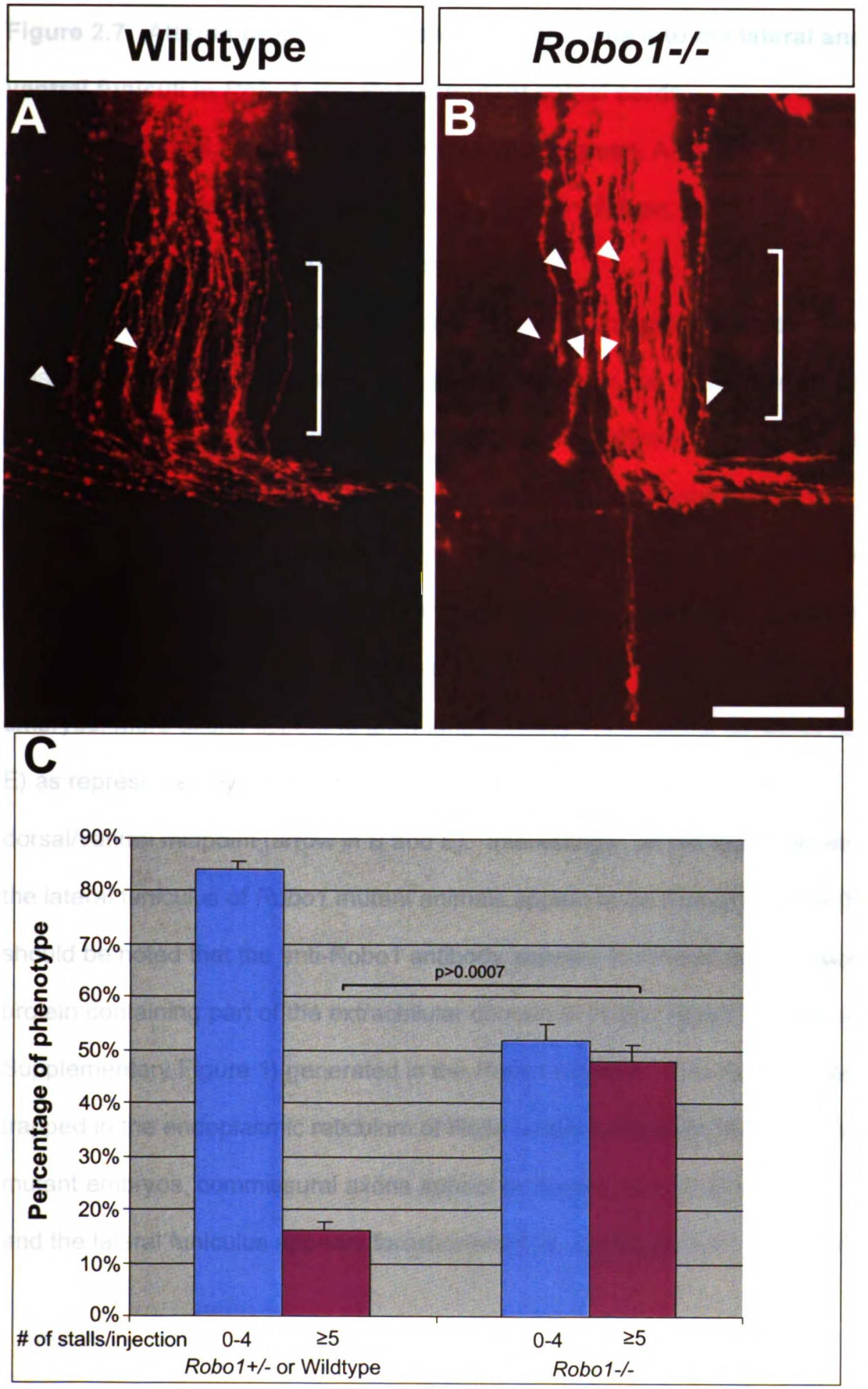

Wildtype
Robo1-/-
A
B
C
Percentage of phenotype
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
p>0.0007
# of stalls/injection
0-4
≥5
0-4
≥5
Robo1+/- or Wildtype
Robo1-/-

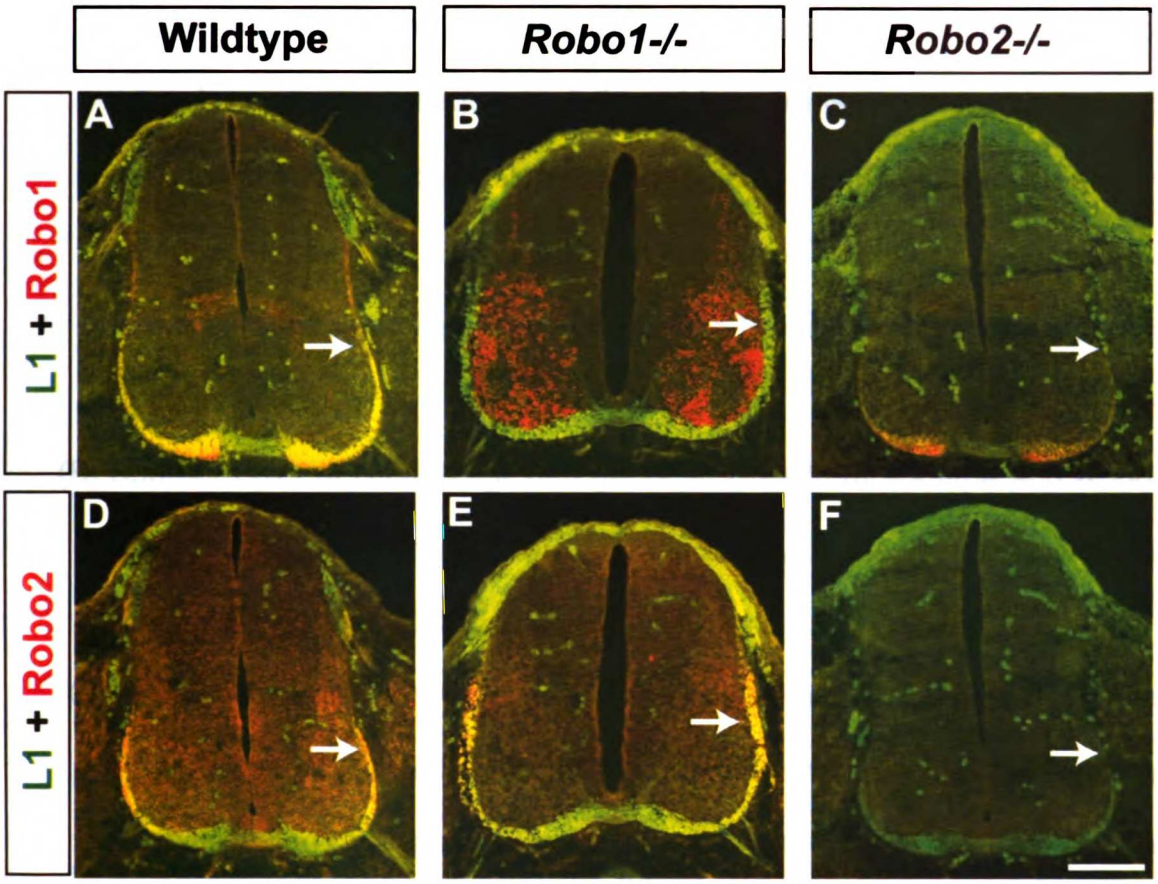
Figure 2.7. Altered projection of commissural axons into the lateral and ventral funiculi in *Robo1* and *Robo2* mutant spinal cords.

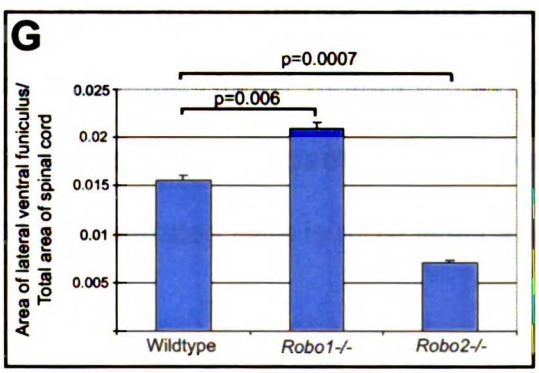
Analysis of ventral funiculus extension through L1 (green, A-F), Robo1 (red, A-C), and Robo2 (red, D-F) immunostaining of E11.5 wildtype, *Robo1-/-* or *Robo2-/-* mouse spinal cord transverse sections.

In wildtype animals, anti-L1 antibody labels commissural axons once they have crossed the floor plate and are growing longitudinally in the ventral and lateral funiculi (A and D). Similar to L1 immunostaining, an antibody directed against the ectodomain of Robo1 also labels both the ventral and lateral funiculi (A). However, an antibody against Robo2 labels primarily the lateral funiculus (D). In wildtype E11.5 spinal cords, the lateral funiculus extends dorsally to roughly the dorsal/ventral half of the spinal cord (arrow in A and D). In *Robo1* mutant embryos, more axons appear to grow longitudinally in the lateral funiculus (B and E) as represented by an increased density in L1-positive signal at the dorsal/ventral midpoint (arrow in B and E). Interestingly, all the axons growing in the lateral funiculus of *Robo1* mutant animals appear to be Robo2-positive (E). It should be noted that the anti-Robo1 antibody appears to recognize the fusion protein containing part of the extracellular domain of Robo1 fused to ß-geo (see Supplementary Figure 1) generated in the *Robo1* mutants. This fusion protein is trapped in the endoplasmic reticulum of *Robo1*-expressing cells (B). In *Robo2* mutant embryos, commissural axons appear to remain more medial (C and F) and the lateral funiculus appears foreshortened as assessed by L1 (arrow in C

and F) and Robo1 immunostaining (C). These observations are quantified in panel G.

Scale bar, 200 μm.


Wildtype
Robo1-/-
Robo2-/-
L1 + Robo1
L1 + Robo2
A
B
C
D
E
F

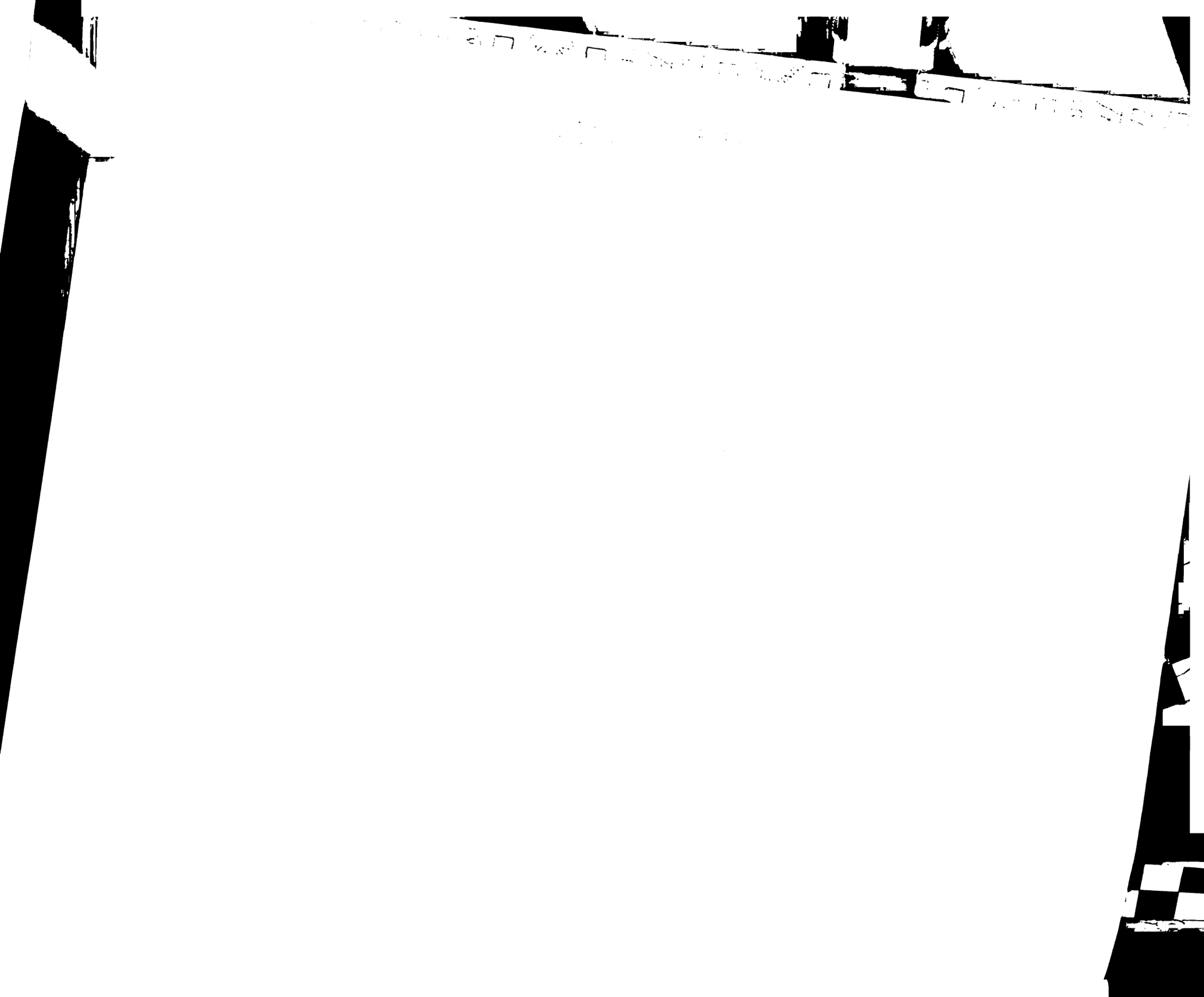

G
p=0.0007
p=0.006
Area of lateral ventral funiculus/
Total area of spinal cord
0.025
0.02
0.015
0.01
0.005
Wildtype
Robo1-/-
Robo2-/-

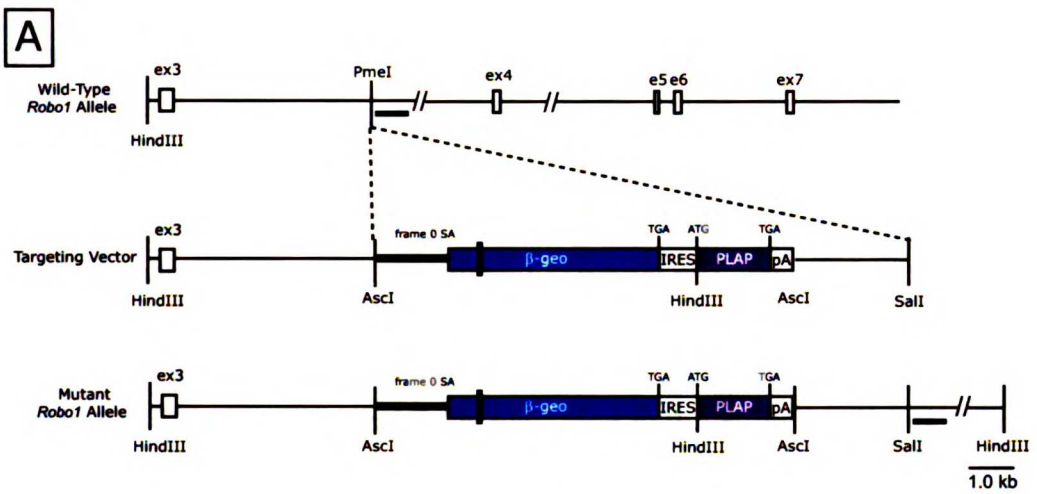
Supplementary Figure 2.1, Creation of *Robo1* deficient mice

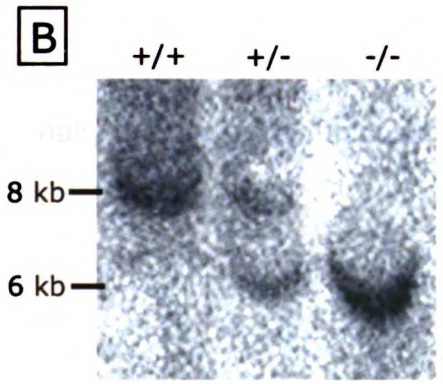
(A) Targeting strategy. The upper line shows the wild-type *Robo1* locus. The middle line shows the targeting vector. A cassette containing a splice acceptor consensus sequence followed by a transmembrane domain, β-galactosidase/neomycin fusion protein (β-geo), an internal ribosome entry site (IRES), placental alkaline phosphatase, and a polyA tail was targeted to the third intron of the *Robo1* gene. The lower line shows the correctly targeted locus.

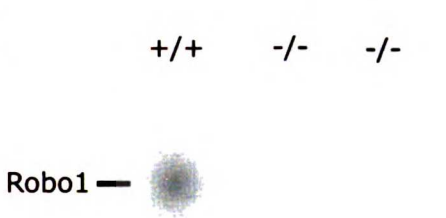
(B) Southern blot of HindIII-digested genomic DNA from wildtype and *Robo1* heterozygous and mutant mice hybridized with the 3' flanking probe shown in the schematic above.

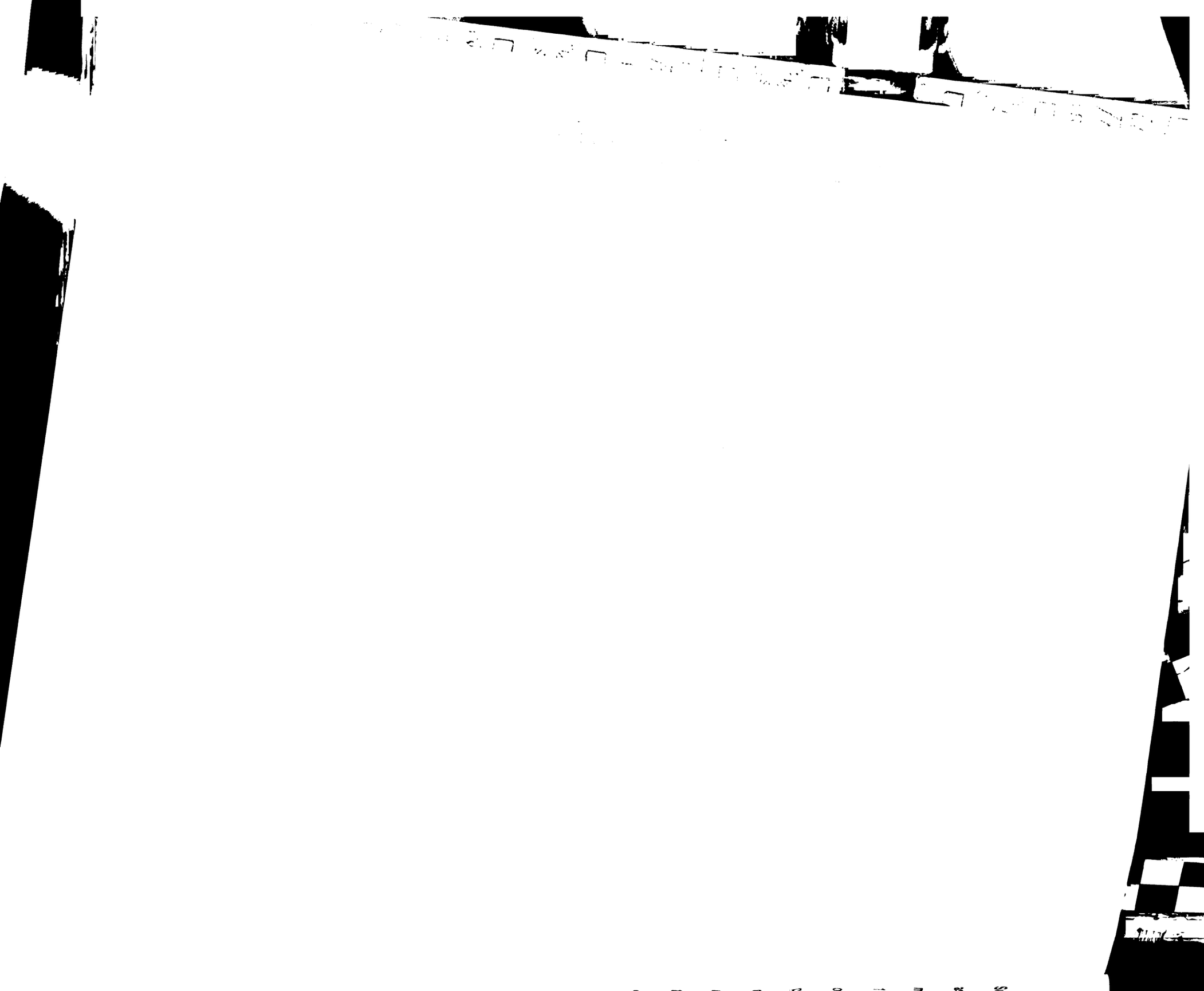
(C) RNA was isolated from the heads of wildtype and *Robo1* mutant E11.5 embryos and analyzed by RNase protection assay for the presence of wildtype *Robo1* transcript using a probe directed against a sequence in the very 3' region of the Robo1 mRNA. Some residual wildtype *Robo1* transcript is observed in the *Robo1* mutant embryos indicating that some splicing around the gene trap cassette does take place and that the *Robo1* mutant allele generated here is most likely a severe null.




A
Wild-Type
Robo1 Allele
ex3
PmeI
ex4
e5 e6
ex7
HindIII
Targeting Vector
frame 0 SA
β-geo
IRES
PLAP
pA
TGA
ATG
TGA
AscI
SalI
Mutant
Robo1 Allele
1.0 kb

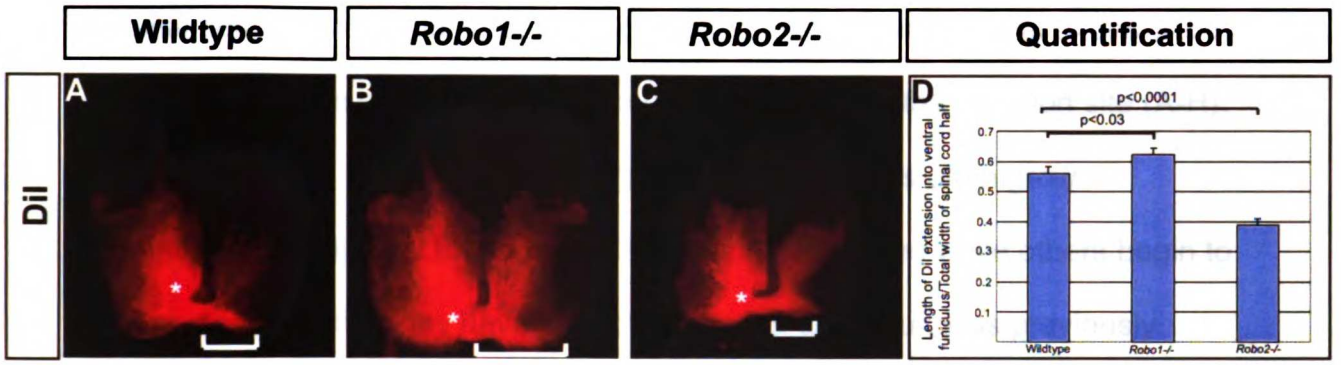

B
+/+
+/-
-/-
8 kb
6 kb

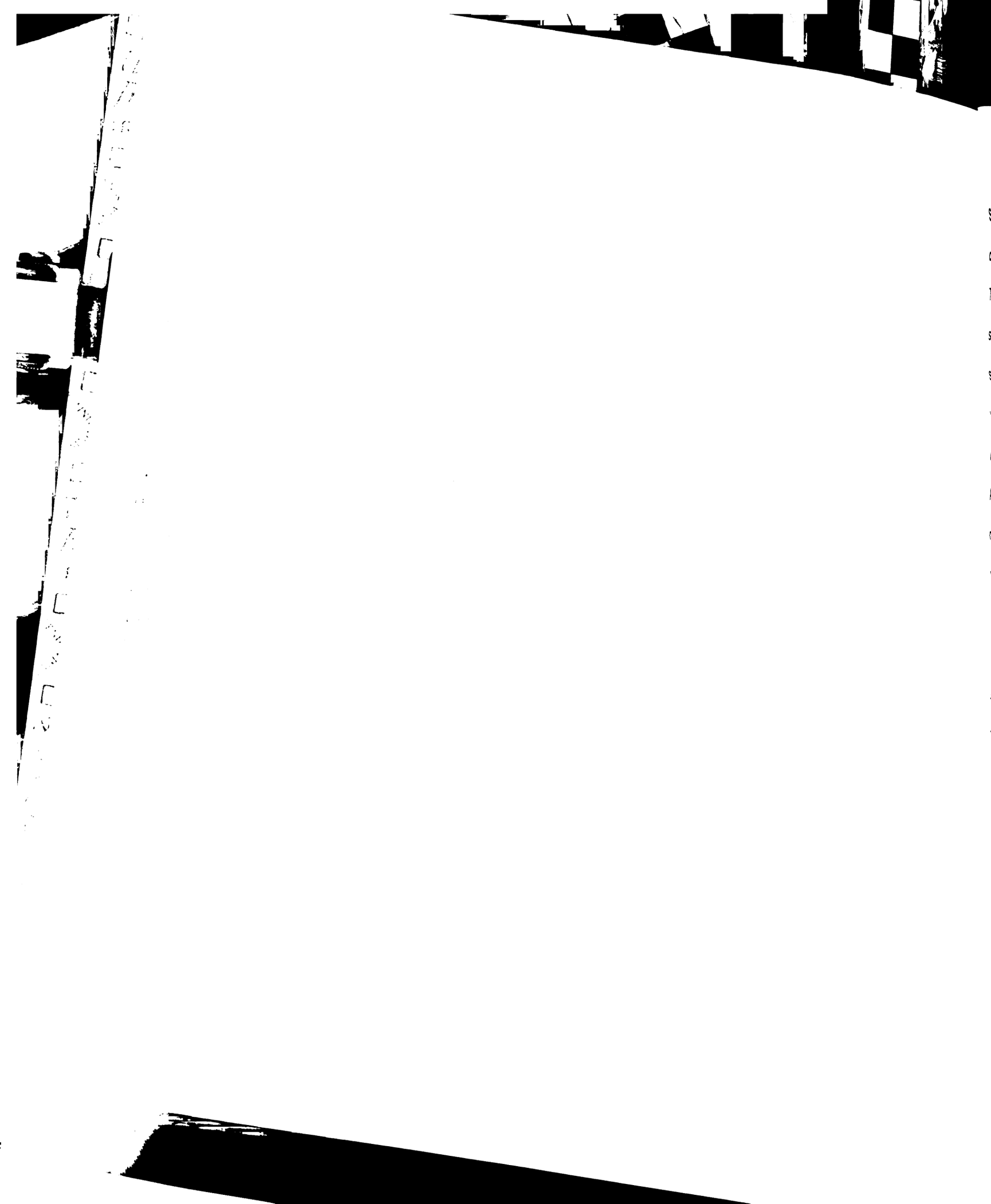

C
+/+
-/-
-/-
Robo1



Supplementary Figure 2.2, Extension of post-crossing commissural axons as assayed by DiI labeling in transverse sections of wildtype, *Robo1* mutant and *Robo2* mutant embryos.

100 μm vibratome sections of spinal cords were isolated from the hindlimb region of wildtype, Robo1-/- and Robo2-/- E12.5 embryos. A small amount of DiI was placed in the ventral spinal cord near the floor plate in an attempt to label the commissural axon fascicle just prior to its entering the floor plate (asterisks in A, B, and C). In wildtype animals, commissural axons are observed extending into the ventral funiculus in the side of the spinal cord contralateral to the injection site (bracket in A). This contralateral DiI is extended in the *Robo1* mutants (bracket in B) and truncated in *Robo2* mutants (bracket in C) indicating that commissural axons are adopting aberrant positions relative to the floor plate as they grow longitudinally in the ventral funiculus. A quantification of these effects is shown in D.

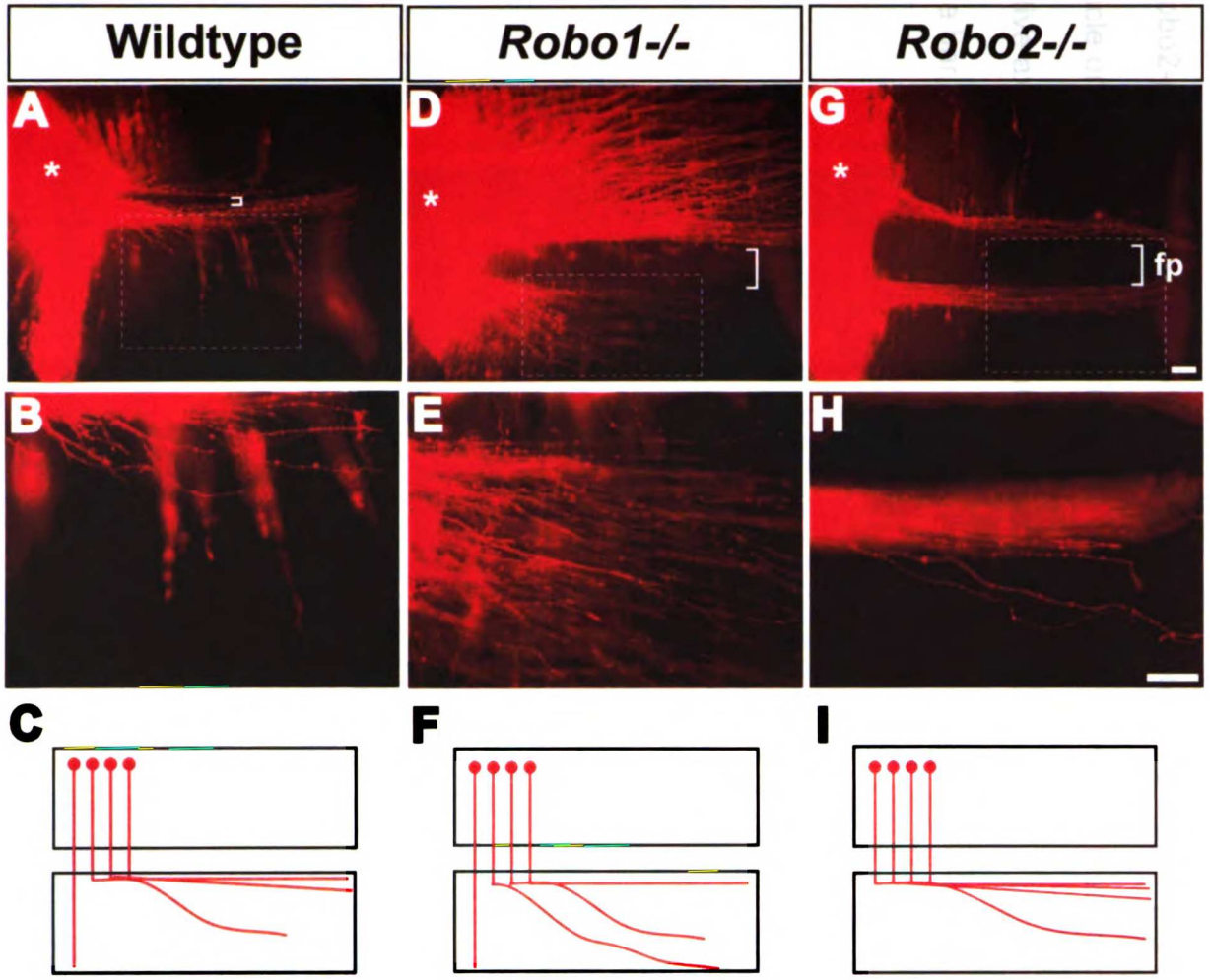

Wildtype
Robo1-/-
Robo2-/-
Quantification
DiI
A
B
C
D
p<0.0001
p<0.03
Length of DiI extension into ventral funiculus/Total width of spinal cord half
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Wildtype
Robo1-/-
Robo2-/-



Supplementary Figure 2.3, Robo1 and Robo2 are required to guide commissural axons away from the floor plate within the ventral funiculus.

To follow commissural axons as they grow longitudinally in the spinal cord, a small amount of DiI was placed adjacent to the floor plate in E12.5 open-book spinal cords (asterisk in A, D, and G). Post-crossing commissural axons were visualized on the side of the spinal cord contralateral to the injection site (A-H). In wiltype spinal cords, post-crossing commissural axons often grow longitudinally adjacent to the floor plate for long distances while others begin to move dorsally after they have turned in the ventral funiculus as previously reported (Imondi and Kaprielian, 2001). In *Robo1* mutant spinal cords, many more commissural axons appear to move dorsally relative to the floor plate in the contralateral side of the DiI injection site (D-F). Alternatively, in *Robo2* mutant spinal cords, the majority of commissural axons stay adjacent to the floor plate as they grow longitudinally (G-I).

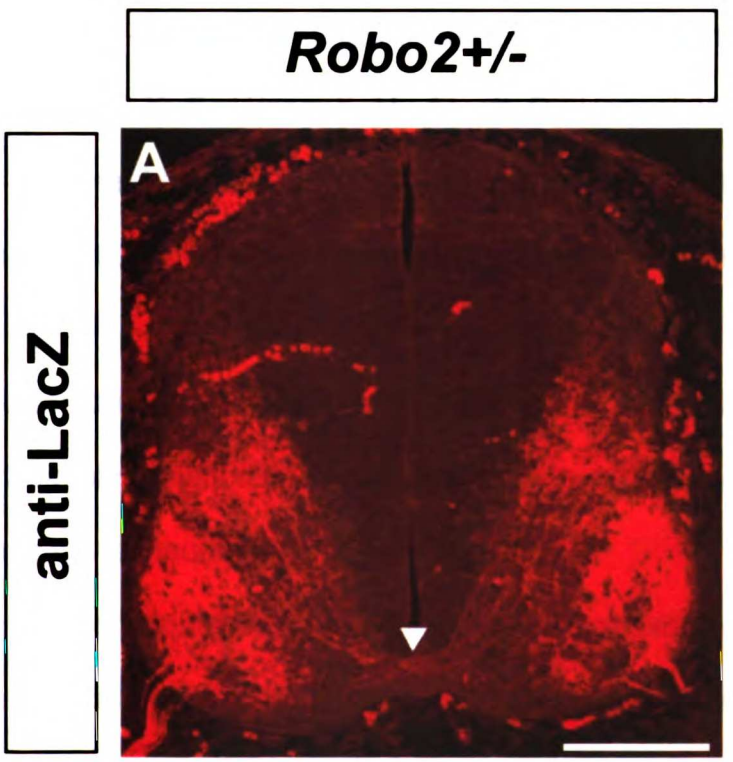
Scale bars, 100 μm.


Wildtype
Robo1-/-
Robo2-/-
A
B
C
D
E
F
G
H
I
*
*
*
fp

Supplementary Figure 2.4, *Robo2*-expressing axons cross the midline.

In generating the Robo2 mutant allele, the axonal marker tau-LacZ was inserted into the Robo2 locus (Grieshammer et al., manuscript in preparation). Therefore, in Robo2 heterozygous animals, this marker can be used to visualize the extent of Robo2-positive axons (A). Given the fact that we observe a LacZ-positive fascicle under the floor plate (arrowhead in A), we can conclude that Robo2-positive axons are at least in part commissural axons.

Scale bar, 200 μm.


Robo2+/-
anti-LacZ
A

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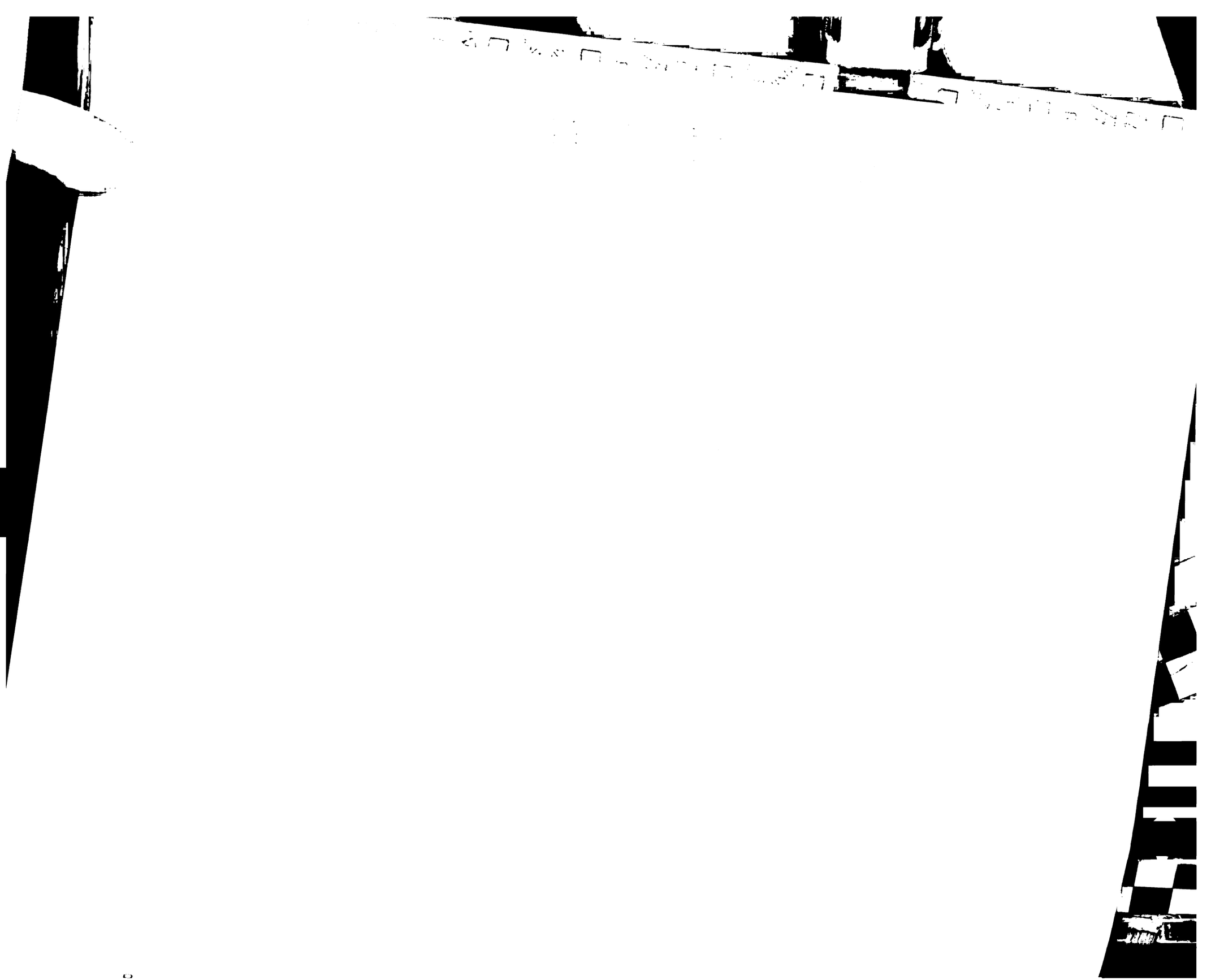
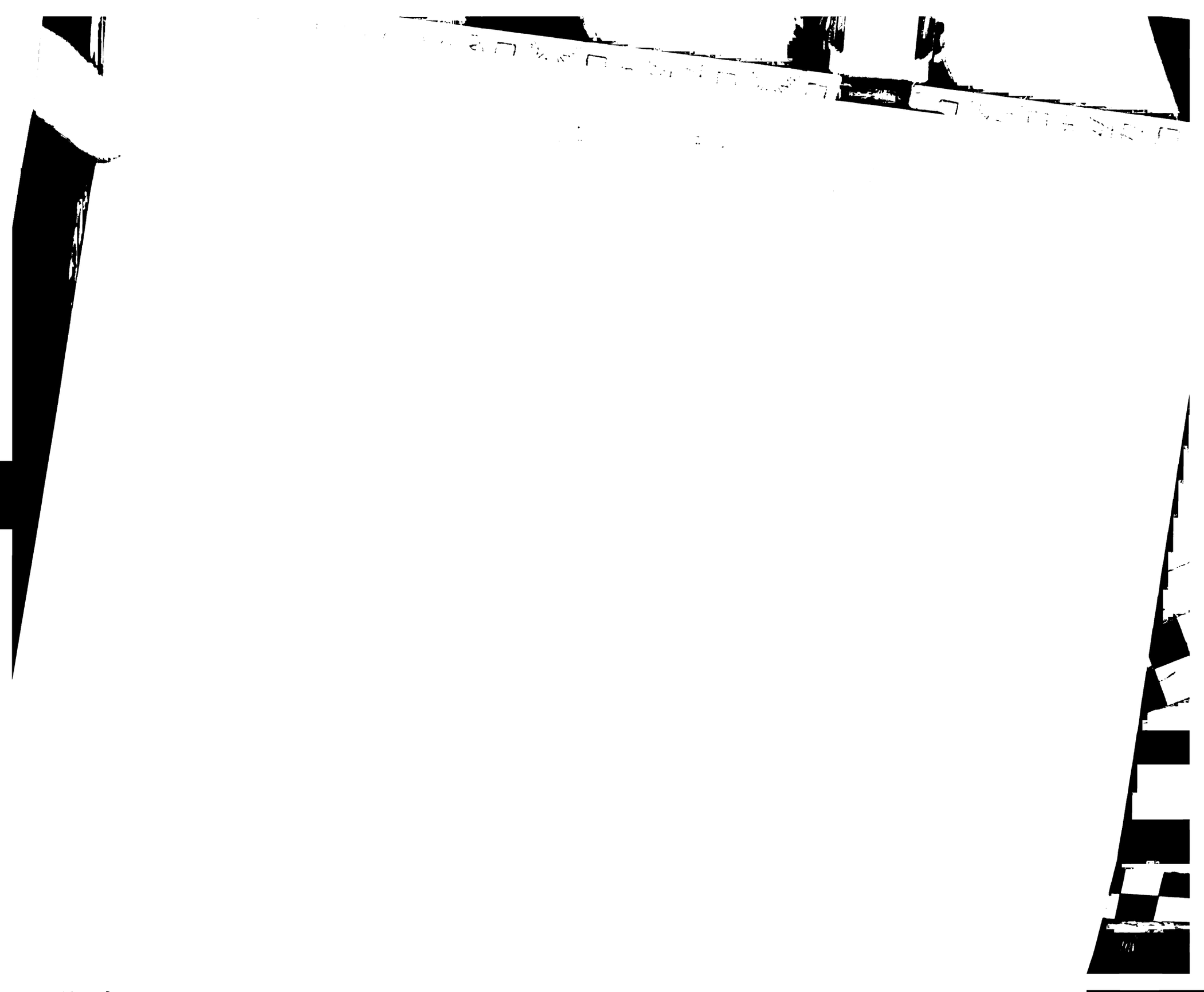


Chapter Three:

The Role of Rig-1 in Regulating the Response of Commissural Axons to Slits in the Floor Plate

Dr. Katja Brose initiated the Rig-1 project when she was a graduate student in the lab and generated the ^{35}S in situs shown in Figure 3.1. Dr. Andrew Plump generated the *Slit1*, *Slit2*, and *Robo1* mutant animals when he was a post-doctoral fellow in the lab. Dr. Le Ma, currently a post-doctoral fellow in the lab, generated the *Robo2* mutant mice. Dr. Eva Lee provided the *Rig-1* cDNA as well as antibodies directed against Rig-1. Dr. Fujio Murakami provided antibodies directed against the Robo1, Robo2, and Rig-1 ectodomains.



Abstract

Commissural axons in vertebrates and insects are initially attracted to the nervous system midline, but once they reach this intermediate target they undergo a dramatic switch, becoming responsive to repellent Slit proteins at the midline, which expel them onto the next leg of their trajectory. We have unexpectedly implicated a divergent member of the Robo family, Rig-1, in preventing premature Slit sensitivity in mammals. Expression of Rig-1 protein by commissural axons is inversely correlated with Slit sensitivity. Removal of Rig-1 results in a total failure of commissural axons to cross. Genetic and in vitro analyses indicate that Rig-1 functions to repress Slit responsiveness similarly to Commissureless (Comm) in *Drosophila*. Unlike Comm, however, Rig-1 does not produce its effect by downregulating Robo receptors on pre-crossing commissural axon membranes. These results identify a novel mechanism for regulating Slit repulsion that helps choreograph the precise switch from attraction to repulsion at a key intermediate axonal target.

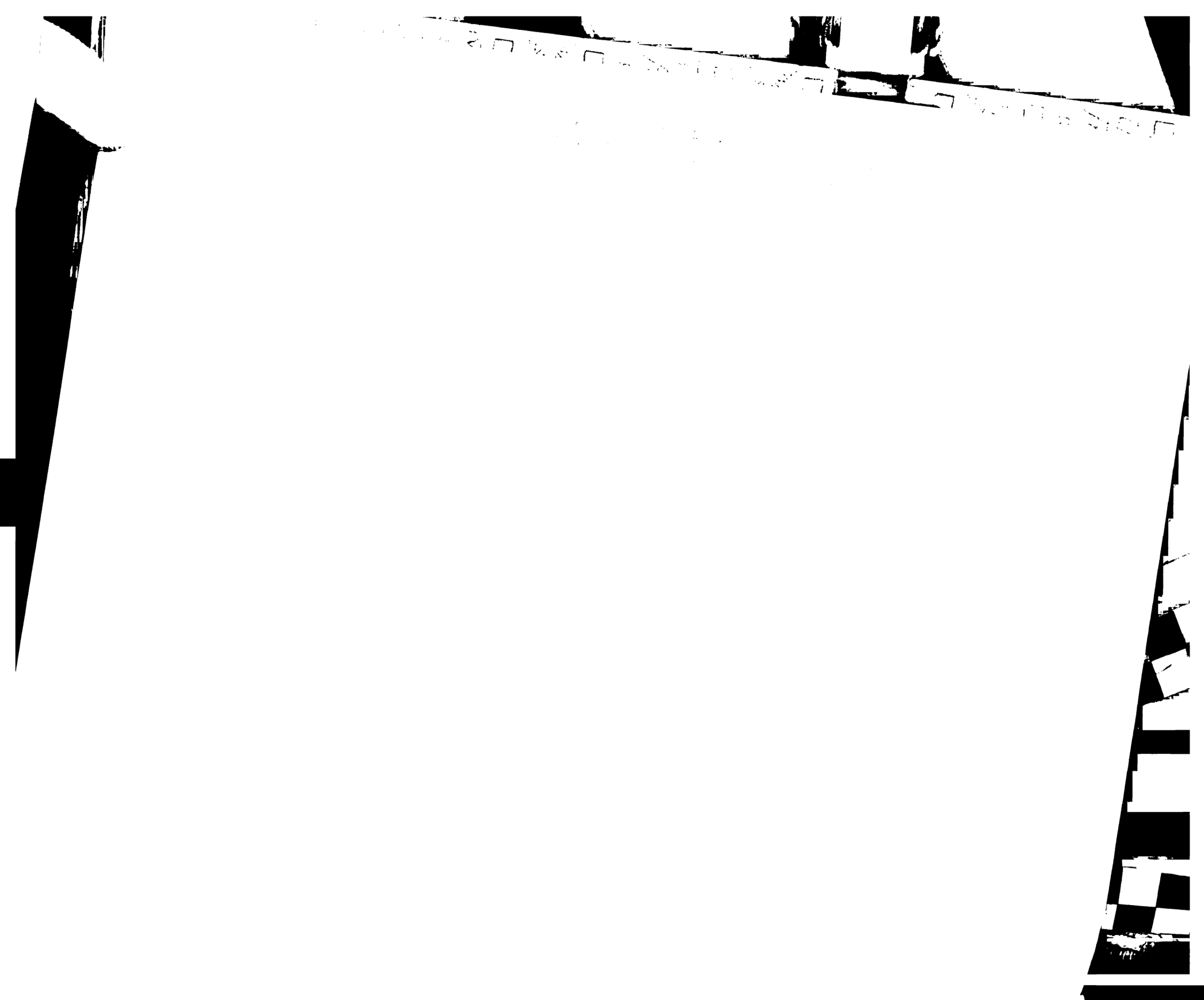
Introduction

As axons grow long distances over complex terrain in the developing embryo, they make use of intermediate targets to simplify their navigation into short, manageable segments (Tessier-Lavigne and Goodman, 1996). These intermediate targets produce both attractants and repellents, which axonal growth cones must recognize in sequential order to navigate properly. Thus,

after being initially attracted to their intermediate targets, growth cones must undergo a change in responsiveness to continue on their migratory route, losing responsiveness to the attractants that led them to their intermediate target and gaining responsiveness to repellents produced by that same target. This change must be tightly regulated – it must occur only after crossing, not before - so that growth cones can move on to the next stage in their trajectory only once they have passed through their intermediate target.

The ventral midline of the nervous system of both vertebrates and invertebrates has served as a model system for understanding the mechanisms by which axons interact with intermediate targets (Brose and Tessier-Lavigne, 2000; Kaprielian et al., 2001; Yu and Bargmann, 2001). Commissural neurons, a subset of interneurons, use the ventral midline as a key intermediate target on their way to their final targets in the contralateral half of the body. In vertebrates and insects, commissural axons are initially drawn to the midline by attractant proteins, which include members of the Netrin family. Upon crossing the midline and reaching the contralateral side, however, these growth cones turn longitudinally, lose responsiveness to Netrin-1 (Shirasaki et al., 1998), and become sensitive to repellents made by midline cells, which include Slit proteins (Brose et al., 1999; Kidd et al., 1999; Zou et al., 2000). This switch prevents commissural axons from recrossing the midline and allows them to move on toward their final targets.

In *Drosophila*, a single Slit protein is present and accounts for all midline repellent activity. Commissural axons become sensitive to Slit when its receptor, Roundabout (Robo), is upregulated on the membrane of commissural growth cones upon midline crossing (Kidd et al., 1998b). Robo expression prior to reaching the midline is repressed by the regulatory protein Commissureless (Comm), which keeps Robo in intracellular compartments away from the axonal surface. Upon crossing, this repressive action of Commissureless is lost (through still unidentified mechanisms), so that Robo surface expression and, concomitantly, Slit sensitivity, are upregulated, thereby expelling commissural axons from the midline and preventing them from ever recrossing (Keleman et al., 2002). Three mammalian homologs of *Drosophila* Slit (Slit1-3) and two homologues of Robo (Robo1, 2) were described, and their mRNAs were found to be expressed in structures analogous to those in which their homologues are expressed in *Drosophila* (midline floor plate cells for the three Slits, different subpopulations of commissural neurons for the two Robos) (Brose et al., 1999; Itoh et al., 1998; Kidd et al., 1998a; Li et al., 1999). This led to the hypothesis that this receptor-ligand system plays a similar role in vertebrate commissural axon guidance; support for this hypothesis has been obtained by our finding that spinal commissural axons become Slit responsive upon crossing the midline (Zou et al., 2000) and fail to be efficiently expelled from the midline floor plate in *Slit1, 2, 3* triple mutant embryos (Long et al., companion manuscript). However, no Comm homolog has yet been identified in vertebrates, raising the question of

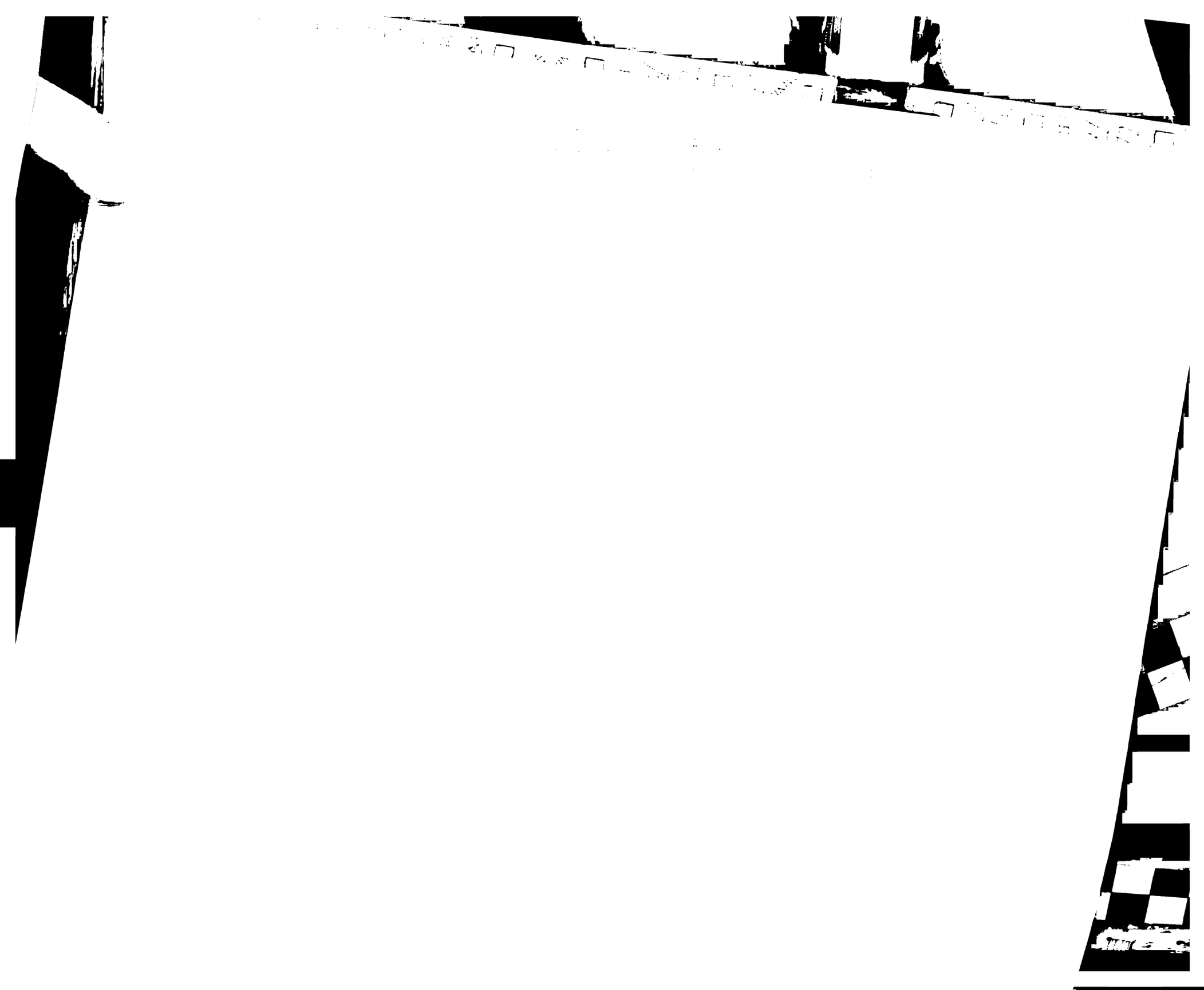


how commissural axons in vertebrates are prevented from becoming Slit responsive prior to crossing the midline.

A divergent member of the Robo subfamily, Rig-1, was identified as a gene that is upregulated in *Retinoblastoma (Rb)* mutant embryos (Yuan et al., 1999). Mouse Rig-1 (gene name: *Rbig1*) shares 40% amino acid identity with other vertebrate members of the Robo family, particularly in its extracellular domain, but is missing some important cytoplasmic motifs found in other Robo family members (see also Figure 1A). We found that Rig-1 is specifically expressed by commissural axons and we therefore hypothesized that Rig-1 might play a role in regulating Slit sensitivity. Unexpectedly for a Robo family member, however, Rig-1 is highly expressed before midline crossing and downregulated after crossing. Loss-of-function studies show that Rig-1 is required to allow commissural axons to enter the floor plate and cross to the contralateral side of the spinal cord. From in vitro and in vivo studies, we propose that Rig-1 normally functions to inhibit the ability of precrossing commissural axons to sense floor plate repellents of the Slit family through Robo receptors, thus allowing the axons to cross the midline.

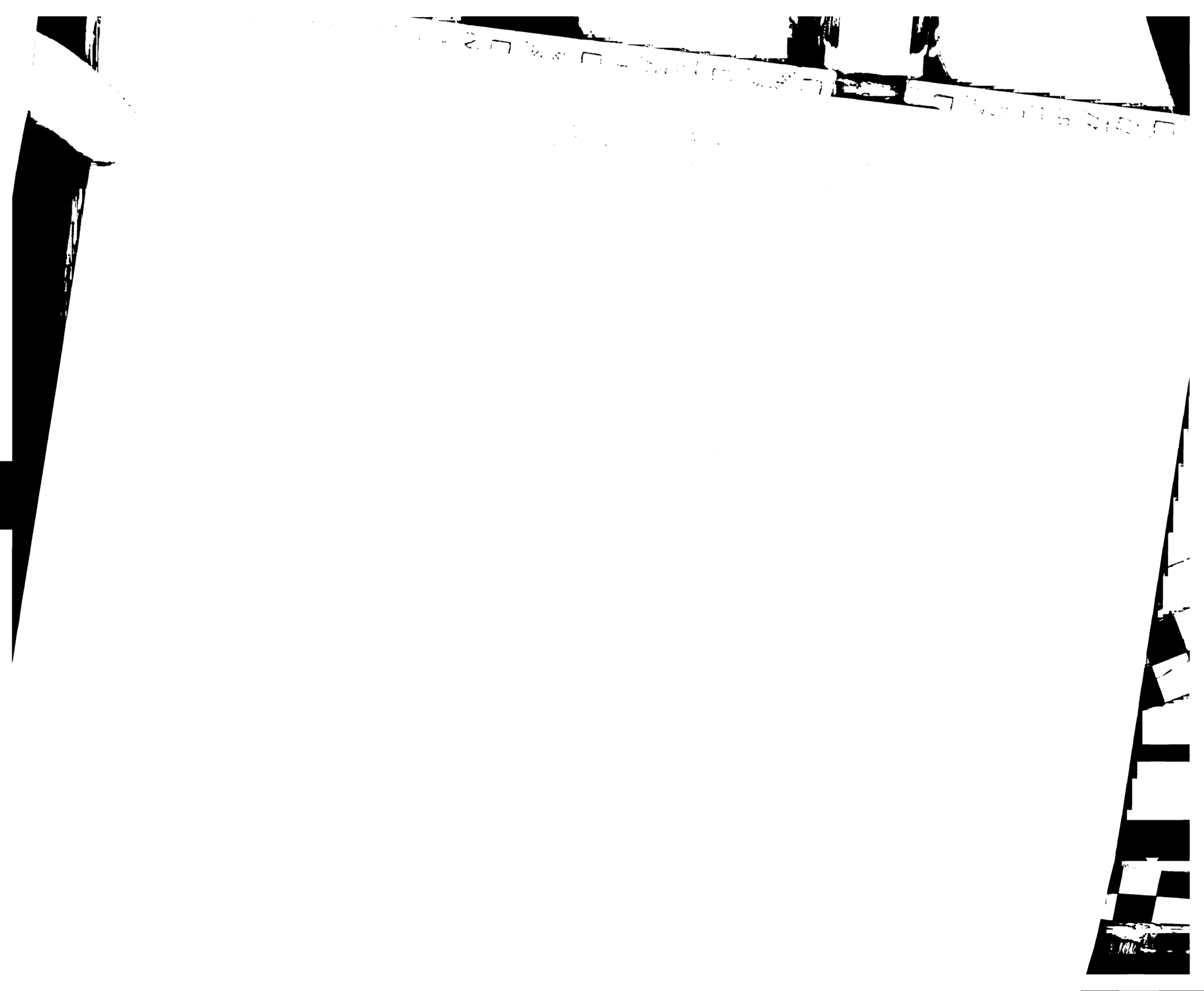
Results

Rig-1 binds Slit but is expressed on the precrossing portion of commissural axons



Since Rig-1 is a member of the Robo family, we asked whether it shares two properties of classic Robo proteins: the ability to bind Slit, and expression by commissural axons. To test for Slit binding, we performed a cell overlay binding assay. As previously described, a C-terminally myc-tagged human Slit2 protein (hSlit2) binds specifically to rRobo1 but not to DCC (Brose et al., 1999). Cells expressing mRig-1 also show significant binding of hSlit2 (Supplementary Figure 1); thus, the homology of the ectodomain of Rig-1 with those of other Slit-binding Robo family members is consistent with its ability to bind Slit proteins.

To determine whether Rig-1 might contribute to commissural axon guidance, we first examined the expression of *Rig-1* mRNA in the developing rat spinal cord at embryonic stages E11-13, the time when commissural axons are projecting to the midline (Altman and Bayer, 1984). At E11, when commissural axons begin their ventral migration (Figure 1B), commissural neurons express both *DCC* and *Robo1* (Kidd et al., 1998a), but *Rig-1* expression is not detectable (Figure 1E). By E12, when commissural axons are reaching the floor plate and a few pioneers have started to cross (Figure 1C), *Rig-1* expression is upregulated specifically in commissural neurons in the dorsal spinal cord as well as V3 interneurons located on either side of the floor plate (which also project across the midline) (Figure 1F). *Rig-1* mRNA expression in commissural neurons persists through E13 (Figure 1G), a time at which many commissural axons have crossed to the contralateral side of the spinal cord (Figure 1D).



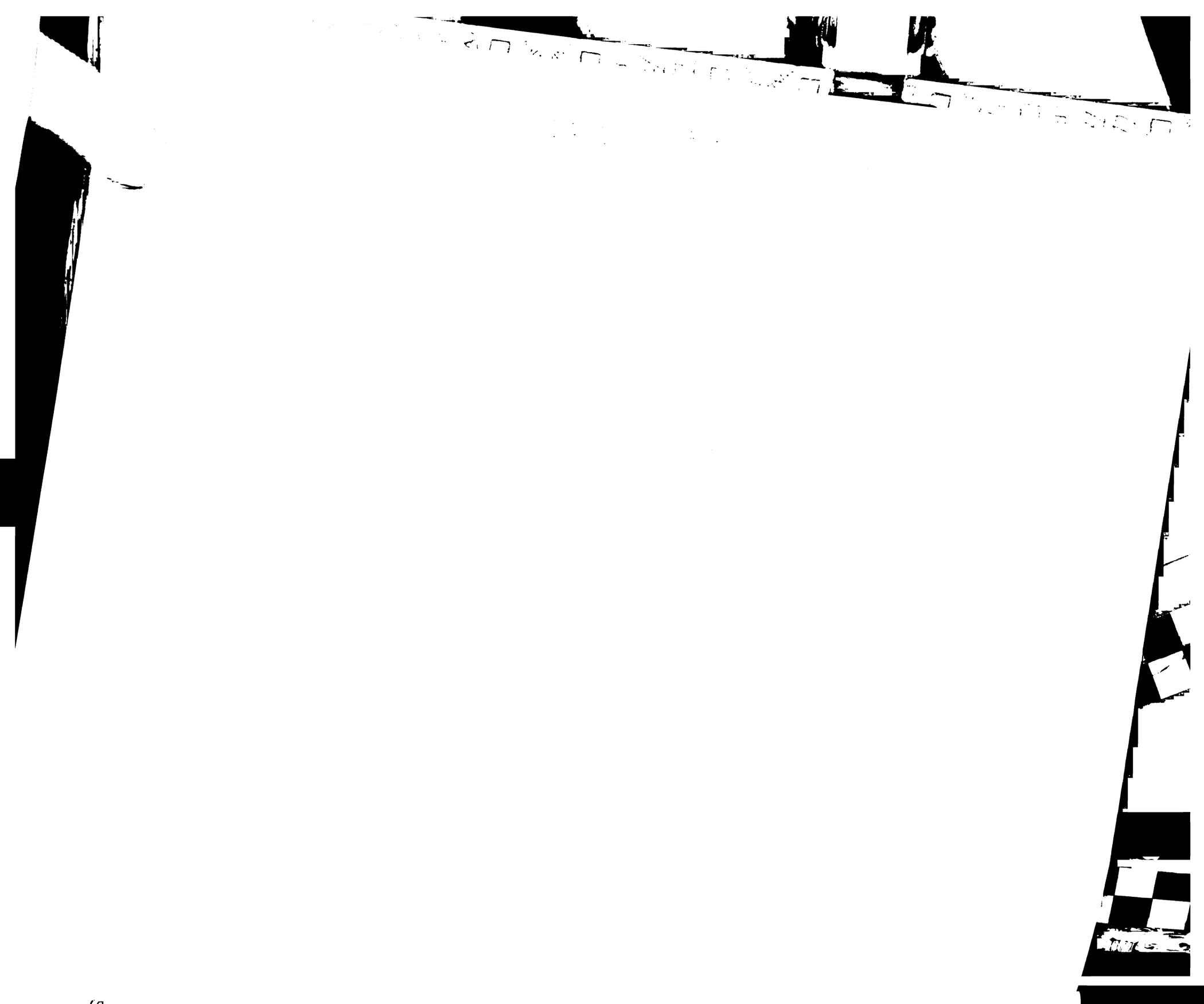
To determine to what extent the three vertebrate Robo receptors co-localize in neurons of the spinal cord, double fluorescent in situ hybridization was performed on E13 rat spinal cords. *Rig-1* expression overlaps significantly with that of *Robo1* in the dorsal spinal cord (Figures 1H-J). In the ventral spinal cord, co-localization is also observed in the V3 interneurons. *Robo1* is also expressed in motor neurons in the ventral spinal cord whereas *Rig-1* expression appears to be confined to commissural neurons. *Robo2* was previously described to be expressed by a lateral population of dorsal interneurons as well as motor neurons at E13 in the rat spinal cord (Brose et al., 1999). Interestingly, *Robo2* expression appears largely or completely non-overlapping with that of *Rig-1* (Figure 1K-M).

We next examined the expression of Rig-1 protein on commissural axons, using an antibody generated against the ectodomain of Rig-1 (Yuan et al., 1999). Surprisingly (for a Robo family protein), we found that Rig-1 is highly expressed on the precrossing portion of commissural axons, as visualized by immunohistochemistry on transverse sections of E11.5 mouse spinal cord (Figure 2B) (which corresponds developmentally to the E13 rat spinal cord). After midline crossing, Rig-1 initially continues to be expressed by commissural axons but then gets downregulated (Figure 2B, arrowhead). The expression of Rig-1 is similar to that of TAG-1, a cell surface protein also expressed on commissural axons that gets rapidly downregulated after midline crossing (Dodd et al., 1988). Rig-1 expression appears to persist longer than that of TAG-1 after

midline crossing (at least as assessed with these particular antibodies) (Figure 2A and 2B), but it is eventually lost, as assessed by labeling adjacent sections with a GFP marker driven from the *Rig-1* locus (Figure 2C) that labels the entire length of commissural axons (see Figure 3C below).

Generation of *Rig-1* mutant mice

The expression of Rig-1 before crossing and its downregulation after crossing were surprising, since Rig-1 is related to Robo proteins whose expression pattern shows the opposite regulation (Long et al., companion manuscript; see also Fig. 7). We therefore sought to determine the function of Rig-1 using gene targeting in embryonic stem (ES) cells to generate mice deficient in *Rig-1*. A targeting construct was generated using a 12 kilobase fragment of a bacterial artificial chromosome containing a portion of the Rig-1 locus that includes the first exon (see Experimental Procedures). A portion of this exon encoding the start ATG and the signal sequence was replaced with a cassette containing (in order from 5' to 3'): an internal ribosome entry site (IRES) element, a tau-GFP fusion protein, a loxP site, a PGK-1 promoter, a neomycin resistance gene, a PGK-1 polyA tail, and a second loxP site (abbreviated IRES-tauGFP-LNL and referenced in Rodriguez et al., 1999) (Supplementary Figure 2A). The IRES element was inserted to allow bicistronic expression of the tauGFP reporter from the *Rig-1* promoter.



ES cell colonies containing homologous integrants were isolated (Supplementary Figure 2B). These clones were used to generate chimeric male mice that were then mated to CD-1 or C57Bl6 females to generate germline transmissible *Rig-1*-deficient mice on either a CD-1/129Sv or a C57Bl6/129Sv genetic background. Resulting heterozygotes were crossed to generate homozygous deficient mice. Since initial experiments showed no difference in the phenotype between the two different backgrounds, most results discussed involved mutant mice on a CD-1 outbred genetic background. The homozygous deficient animals were born but lived no more than a few hours. *Rig-1* mutant pups never suckle, as they are never observed to have milk in their bellies. However, the reasons underlying the lethality remain unclear. To confirm that a null allele of *Rig-1* had indeed been generated, spinal cords from *Rig-1* mutant embryos were collected, lysed and probed for the presence of Rig-1 protein by Western blot. No Rig-1 protein was observed, confirming that the allele is likely a null (Supplementary Figure 2C).

Commissural axons fail to cross the floor plate in the *Rig-1* mutant spinal cord

The most immediately apparent phenotype of *Rig-1-/-* homozygous embryos was the inability of their spinal cords to stay attached at the ventral midline when dissected, indicating a thinned or fragile floor plate (data not shown). To further characterize this phenotype, we initially examined commissural axons in these hemicords by whole mount immunochemistry using an antibody against TAG-1. Surprisingly, the axons appear to project normally toward the floor plate, although

upon closer inspection, they seemed to wander slightly upon reaching the ventral side of the spinal cord (Supplementary Figures 3A and 3B). To examine the trajectory of commissural axons in more detail, we performed TAG-1 immunostaining of transverse sections through the spinal cord of E11.5 embryos. In wildtype embryos, commissural axons project ventrally near the edge of the spinal cord until they reach the level of the developing motor column, where they turn medially to head toward the floor plate. This normal projection was observed in both wildtype and heterozygous embryos (Figure 3A, 3B and 5A). Commissural axons in embryos homozygous for the *Rig-1* mutation, however, displayed a complete failure to cross the floor plate (Figure 3E and 3F). The initial trajectory appears normal until about the level of the motor columns, at which point commissural axons appear to veer away from the floor plate. This absence of axons crossing the floor plate was also observed in sections stained with an antibody to neurofilament, which labels all axons in these sections (data not shown) as well as in sections stained with an antibody to GFP, which is expressed under the control of the *Rig-1* promoter in these mutants (Figure 3F). Expression of the floor plate markers HNF3β (Ruiz i Altaba et al., 1993; Sasaki and Hogan, 1993) and Sonic hedgehog (Roelink, 1994) appeared normal (data not shown) indicating that the floor plate itself develops normally in the *Rig-1* mutants.

The complete absence of ventral commissures in the *Rig-1* mutant is observed throughout the spinal cord (this study) and hindbrain (Marillat et al., manuscript in

preparation) and presumably accounts for the fragility of the floor plate that is observed on removal of the spinal cord. The phenotype persists in older embryos (Figure 3G) until at least E14.5 (data not shown). To assess where commissural axons go when they remain on the ipsilateral side of the spinal cord, we injected DiI in the dorsal spinal cord of E13.5 embryos visualized in an open book preparation. In wild type embryos, by E13.5 most commissural axons have crossed the floor plate in a well-organized fashion and have turned sharply rostrally immediately upon exiting the floor plate (Bovolenta and Dodd, 1990). In E13.5 *Rig-1* mutant embryos, commissural axons have turned and grown longitudinally on the ipsilateral side of the floor plate. However, they are very disorganized in its vicinity. Some stall close to the floor plate and extend very large and complex growth cones. Many others, however, turn both rostrally and caudally; some actually appear to bifurcate and send projections in both directions. Most of the axons fail, however, to remain closely apposed to the floor plate, the way they normally are in wildtype embryos (Supplementary figure 3).

Commissural neurons from *Rig-1* mutant animals are prematurely Slit responsive

These studies have shown that Rig-1 is a Slit-binding protein expressed on the precrossing and crossing portion of the commissural axons that is required for these axons to enter the floor plate. At least two models could potentially account for all of these observations (Figure 4A). Model 1 postulates that Rig-1 is an attractive receptor required for the axons' response to an attractive ligand(s)

(perhaps Netrin-1?) that is required to enter the floor plate. Model 2 postulates that Rig-1 inhibits the axons' response to an inhibitory factor(s) secreted by the floor plate (perhaps the Slit proteins?) that prevents floor plate entry. To differentiate between these two models, we first set out to characterize the responsiveness of *Rig-1* mutant commissural axons to cues secreted by the floor plate in vitro, with the idea that we might be able to determine whether upon loss of Rig-1 they have lost an attractive response or gained a repulsive response to floor plate cells, when compared to wild-type commissural neurons. Commissural axons emanating from dorsal spinal cord (DSC) explants have previously been shown to be attracted to floor plate tissue (Tessier-Lavigne et al., 1988) and COS cells secreting Netrin proteins (Serafini et al., 1994) but are unresponsive to Slit2 until after they have crossed the floor plate (Zou et al., 2000). As a first step to characterizing the responsiveness of *Rig-1* mutant neurons, we cultured *Rig-1* mutant DSC explants in collagen in the presence of Netrin. The mutant explants respond normally to Netrin presented both as a point source (data not shown) and in the bath (Figures 4E and 4I, $p=0.06$). Thus, Rig-1 function is not required for commissural axon responsiveness to Netrin. However, when *Rig-1* mutant DSC explants were cocultured with floor plate tissue derived from wild-type embryos, which is a potent source of Netrin (Serafini et al., 1996), axons failed to grow out of the explant (Figure 4C and 4H, $p<0.001$). The fact that commissural axons from *Rig-1* mutant DSC explants cannot grow out into collagen in response to floor plate-derived netrin suggests that something else made by floor plate cells is antagonizing netrin's action. To

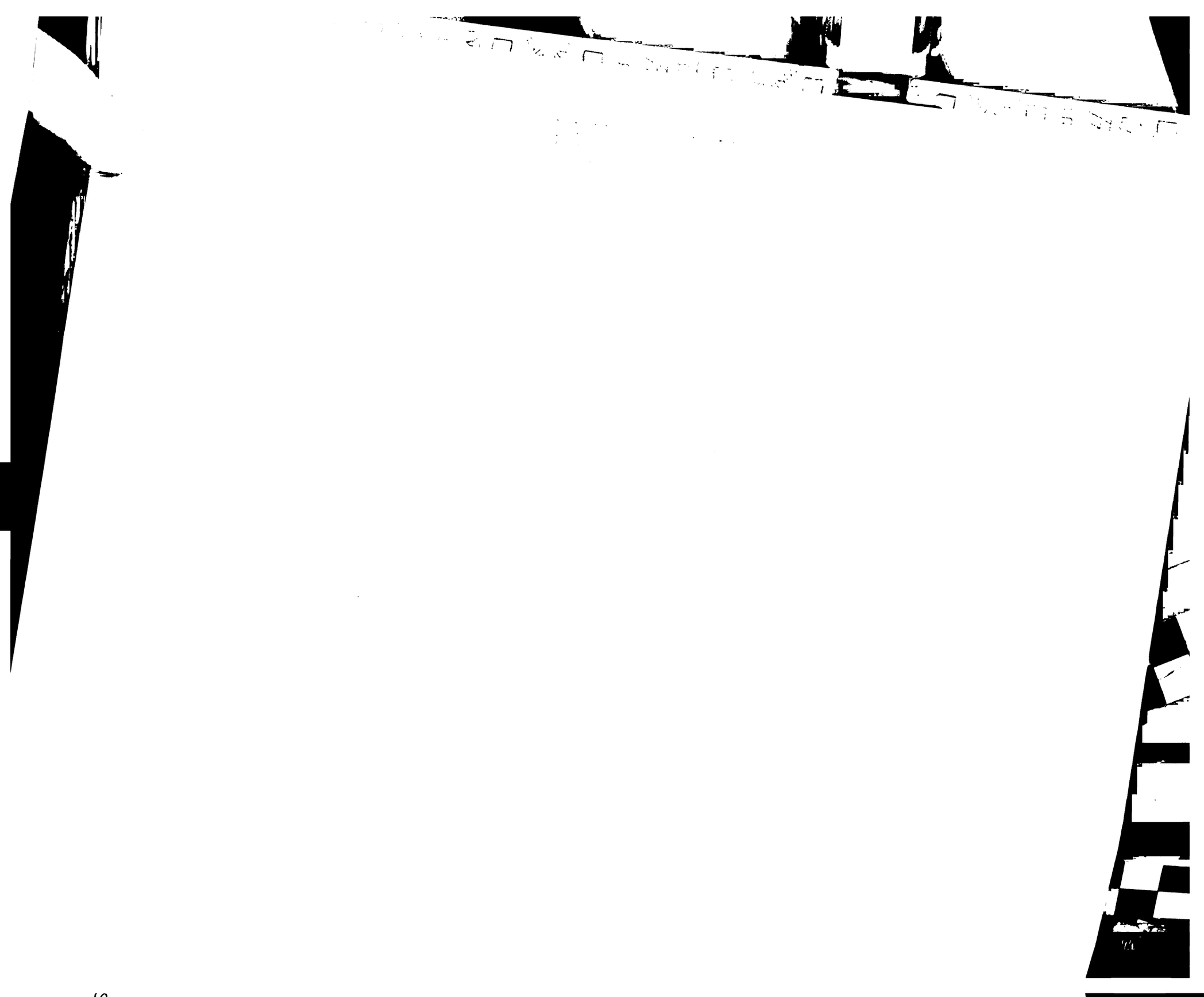
test whether Slit proteins derived from floor plate might be antagonizing the netrin outgrowth promoting effect, we co-cultured *Rig-1* mutant DSC explants with floor plate in the presence of an antagonist of Slit function provided by the ectodomain of Robo2 (fused to the Fc portion of the human IgG molecules). Remarkably, bath applied Robo2-Fc ectodomain rescued outgrowth of axons from *Rig-1* mutant DSC explants in the presence of floor plate (Figure 4D and Figure 4H); the amount of axon outgrowth from these mutant explants approached that seen with wild-type DSC explants in the presence of floor plate (as assessed by the total bundle length per explants; $p<0.001$).

These results suggested that Slit proteins derived from the floor plate were antagonizing the effect of floor plate-derived netrin on commissural axons from *Rig-1* mutant DSC explants. To test more directly whether these axons are prematurely responsive to Slit proteins, we cultured DSC explants with Netrin to elicit commissural axon outgrowth, and presented these axons with COS cells expressing the N-terminal cleavage product of Slit2 (Slit2-N), which has previously been shown to be a potent repellent of a variety of different axonal populations (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999). Axons emanating from wild type DSC explants are not repelled by Slit2-expressing COS cells unless they have already crossed the floor plate (Zou et al., 2000). In contrast, the growth of axons from *Rig-1* mutant DSC explants, which have not encountered floor plate, is strongly inhibited by the presence of Slit2-N-expressing COS cells (Figure 4G and 4I; $p<0.001$).

These in vitro results support a role for Rig-1 as an inhibitor of Slit signaling in commissural axons prior to crossing the floor plate rather than implicating Rig-1 in mediating the response to a floor plate attractant, and thus strongly favor model 2 over model 1 (Figure 4A). Interestingly, although the axons from *Rig-1-/-* DSC explants were prematurely responsive to Slit-2N, they were not inhibited by Sema3F (Figure 4F), a distinct floor plate-derived repellent known to inhibit post-crossing commissural axons (Zou et al., 2000). The fact that loss of Rig-1 does not result in loss of Netrin responsiveness or in premature responsiveness to Sema3F shows that loss of Rig-1 specifically causes the axons to become prematurely Slit responsive, rather than generally converting the axons to a post-crossing state.

Removal of *Slits* in a *Rig-1* mutant background leads to partial rescue of the crossing phenotype

Taken together, these results strongly suggested that the failure of axons to cross the midline in *Rig-1* mutant spinal cords results from the axons being prematurely responsive to midline Slit proteins, which block their entry into the midline. To test this hypothesis, we genetically removed individual Slits from the floor plate in the *Rig-1* mutant background. This was accomplished by crossing the *Slit1* and *Slit2* mutants with the *Rig-1* mutant. At the level of commissural axon crossing of the floor plate, neither *Slit1-/-*, *Slit2-/-* nor *Slit1-/-;Slit2-/-* mutants have any obvious phenotype (Plump et al., 2002; Long et al., companion manuscript), (although removal of all three Slits from the floor plate causes



stalling of these axons at the floor plate (Long et al., companion manuscript)). Since the loss of ventral commissures in the *Rig-1* mutant is so complete, we reasoned that any decrease in the Slit-dependent repulsion from the floor plate might be translated into a small recovery of crossing. *Slit1-/-*; *Rig-1-/-* embryos exhibit no crossing of the floor plate by commissural axons and look identical to the *Rig-1-/-* littermates (Figures 5C and 5D). On the other hand, *Slit2-/-*; *Rig-1-/-* embryos exhibit a small recovery of crossing of the floor plate by commissural axons (Figures 5E and 5F). In *Slit1-/-*; *Slit2-/-*; *Rig-1-/-* triple mutants, significant recovery of crossing is observed, although a majority of commissural axons still fail to cross (Figures 5G and 5H). Thus, as predicted, loss of Slit function can rescue, at least partly, the *Rig-1* mutant phenotype, consistent with the model that loss of Rig-1 function in vivo results in absence of midline crossing because commissural axons are prematurely Slit responsive.

Loss of *Robo1* but not *Robo2* partially suppresses the *Rig-1* mutant phenotype

Robo1 and Robo2 are the presumed Slit receptors mediating repulsive actions of Slits on different populations of commissural axons. In our companion paper, we report that loss of either *Robo1* or *Robo2* alone results in only a slight midline crossing phenotype (Long et al., companion manuscript), presumably due to redundancy between the two Robos, and/or to the functioning of other repulsive guidance systems at the midline, including the Semaphorin/Neuropilin signaling system (Zou et al., 2000). Whether or not signaling systems other than the Slit/Robo signaling system are normally involved in repelling commissural axons

out of the midline, however, we predict that removal of Robo function should suppress the phenotype of *Rig-1* mutant embryos, provided the Robo proteins contribute to signaling midline repulsion by Slit proteins in vivo. To test for such suppression, we examined the effects of mutating either Robo1 or Robo2 in the *Rig-1-/-* background. A dramatic effect of removing *Robo1* function was observed on the *Rig-1* mutant background. Indeed, significant crossing of the midline was observed in E11.5 *Rig-1-/-; Robo1-/-* double mutant embryos as visualized by TAG-1 staining (Figures 6E and F) and by neurofilament staining (data not shown). This partial suppression of the *Rig-1-/-* mutant phenotype appears to be specific to the Robo1 pathway as it is not observed in a *Rig-1-/-;Robo2-/-* double mutant (Figures 6C and 6D). This is presumably related to the fact that *Rig-1* expressing neurons in the spinal cord primarily express *Robo1* but not *Robo2* (Figure 1).

Robo1 and 2 proteins are localized primarily to the post-crossing axon in both wild type and *Rig-1-/-* embryos

The *Rig-1-/-* phenotype in the spinal cord is reminiscent of the *Drosophila commissureless (comm)* phenotype, in which commissural axons also fail to cross the CNS midline (Kidd et al., 1998b). Commissureless (Comm) has been shown to inhibit Robo signaling by preventing Robo1 from being targeted to the plasma membrane before and during midline crossing by commissural axons (Keleman et al., 2002). To test whether Rig-1 functions in a similar manner, we used antibodies generated against the ectodomains of Robo1 and Robo2 to determine their localization in commissural axons in both wildtype and *Rig-1-/-*

embryos. Robo1 and Robo2 are localized primarily to the post-crossing portion of the axon, although low levels of protein are observed pre-crossing (Long et al., companion manuscript) (see also Figures 7B and 7C). Unexpectedly, Robo1 and Robo2 immunoreactivity appears unchanged in *Rig-1-/-* embryos, i.e. low levels are observed prior to reaching the midline, and dramatic upregulation of expression is observed once the axons are coursing in the ventral funiculus, indicating that both receptors are confined to the longitudinal portion of commissural axons even though these axons have not crossed the floor plate (Figures 7E and 7F). This observation is distinct from what is observed in *Drosophila comm* mutants, in which Robo localization is disrupted, being expressed on precrossing commissural axons and leading to inappropriate repulsion of those axons by the midline prior to crossing. It also implies that the low levels of Robo protein expression observed prior to crossing must be sufficient to mediate Slit responsiveness in these axons.

Discussion

As axons grow toward their final targets, they interact in a highly regulated fashion with a series of intermediate targets that guide them using both attractants and repellents. Commissural axons in the spinal cord are initially attracted to the floor plate, but upon crossing it, they lose responsiveness to floor plate attractants (Shirasaki et al., 1998) and become responsive to floor plate repellents of the Slit and Semaphorin family (Zou et al., 2000). To be effective, this change in responsiveness to floor plate-derived guidance cues must be

tightly linked to crossing of that intermediate target. Our results support a model in which Rig-1, a member of the roundabout receptor family, keeps commissural axons from sensing ligands of the Slit family through their cognate receptor Robo1 as they grow toward the floor plate, allowing them to enter and cross the floor plate; on the contralateral side, downregulation of Rig-1 protein expression helps the axons to sense the floor plate as a repulsive environment, thus preventing them from recrossing the midline.

Rig-1 is required for commissural axons to cross the floor plate

Our finding that *Rig-1* mRNA is expressed highly and selectively in commissural neurons initially suggested that Rig-1, as a member of the Robo family, might play a role in preventing midline recrossing, similar to the role played by the Robo protein in *Drosophila*. We were therefore surprised by an unexpected phenotype in *Rig-1* mutants: a complete failure to enter the ventral midline region, reflected in the lack of ventral commissures throughout the spinal cord and hindbrain. This result indicated that Rig-1 plays a role in guiding commissural axons prior to crossing the midline. The pattern of expression of the Rig-1 protein, high prior to crossing and low post-crossing, fits with a role in guiding commissural axons before and/or during midline crossing. This expression pattern stands in contrast to those of Robo1 and Robo2, which are observed primarily post-crossing (although they are both also observed at low levels in pre-crossing and crossing commissural axons).

The *Rig-1* mutant phenotype superficially shares some features with the *Netrin* and *Dcc* mutant phenotypes, in which ventral commissures are also severely reduced in the spinal cord. There are, however, profound differences. In the *Netrin-1* or *Dcc* mutants, commissural axons are impaired in their ability to grow ventrally toward the floor plate, and few reach midline (Keino-Masu et al., 1996; Serafini et al., 1996). Thus, in those mutants, the reduced ventral commissure reflects a failure to reach the midline. In *Rig-1* mutant embryos, in contrast, commissural axons apparently grow normally, virtually all the way to the floor plate, deviating from their normal ventral migration only as they get close to it (Figure 3E). Furthermore, whereas in both *Netrin-1* and *Dcc* mutants a few commissural axons are observed crossing to the contralateral side through the floor plate, no axon was ever observed in the floor plate of *Rig-1* mutant spinal cords at any of the ages analyzed (Figure 3 and data not shown). These phenotypic distinctions suggest that Rig-1 is unlikely to be involved in sensing the attractant Netrin-1, an interpretation further supported by our in vitro experiments that show normal outgrowth in response to Netrin-1 of commissural axons from *Rig-1-/-* dorsal spinal cord explants (Figure 4F and I).

Rig-1 inhibits the responsiveness of commissural axons to Slit repellents

Thus, rather than being involved in commissural axon guidance to the midline, Rig-1 instead appears to be required specifically for midline crossing. We considered two possibilities (Figure 4): Rig-1 could be required to sense a floor plate attractant(s) required for crossing (model 1), or it could prevent axons from

sensing a floor plate repellent(s) until after crossing (model 2). Those repellents could include members of the Slit and/or Semaphorin families, to which the axons become responsive after crossing (Zou et al., 2000).

Our results strongly support model 2, more specifically that Rig-1 prevents commissural axons from becoming prematurely responsive to Slit repellents. In vitro, we found that commissural axons from *Rig-1* mutant dorsal spinal cord explants are repelled by COS cells secreting Slit2N, to which their wild-type counterparts are insensitive. This Slit sensitivity explains, we believe, why *Rig-1* mutant commissural axons failed to grow out of dorsal spinal cord explants in response to floor plate tissue: we interpret this failure to result from inhibition by floor plate-derived Slit overriding the outgrowth-stimulating effect of floor plate-derived Netrin, an interpretation supported by the finding that the outgrowth is restored when a soluble Robo-ectodomain is added to the culture medium, presumably blocking the effect of the Slit proteins. The in vivo counterpart of this in vitro experiment was to remove *Slit1* and *Slit2* in the *Rig-1* mutant background, which led to a partial rescue of commissural axon crossing, again consistent with the possibility that commissural axons fail to cross the midline in *Rig-1* mutants because of premature Slit sensitivity.

It could be argued that the partial suppression of the *Rig-1* midline crossing defect by removal of *Slit-1* and *Slit-2* might be due simply to the floor plate being a more generally attractive environment in the absence of Slits. Two lines of evidence argue against this alternative interpretation, however. First, no defect

in commissural axon guidance at the floor plate has been detected in the *Slit1;Slit2* double mutant embryos despite thorough analysis (Plump et al., 2002, and unpublished observations). In fact, recent results have shown that only when all three Slit proteins expressed by the floor plate are removed (in *Slit1;Slit2;Slit3* triple mutants) does a commissural axon guidance defect become apparent (H. Long et al, to be submitted). These observations imply that the balance of attractants and repellents in the floor plate is not significantly altered by removal of Slit1 and Slit2. Secondly, removal of Neuropilin-2 in the *Rig-1* mutant background fails to rescue crossing. Commissural axons in *Neuropilin-2* mutants stall out in the floor plate at high frequency, suggesting that upon crossing they sense the floor plate as a less repulsive environment than in wild-type animals (Zou et al., 2000). The fact that no rescue of the *Rig-1-/-* crossing phenotype is observed in the *Neuropilin-2; Rig-1* double mutants indicates that sensing fewer repellents in the floor plate *per se* is not sufficient to allow *Rig-1* mutant commissural axons to cross the floor plate, and that instead it is Slit repulsion specifically that must be lessened for the rescue of midline crossing. The failure of the *Neuropilin-2* mutation to suppress the *Rig-1* crossing defect is consistent with the fact that, in vitro, commissural axons from *Rig-1* mutant animals become prematurely responsive to Slit2N but not to Sema3F, a Neuropilin-2 ligand.

Thus, taken together, both our in vitro and in vivo results support model 2, in which *Rig-1* mutant commissural axons fail to cross the midline in vivo

specifically because they are prematurely responsive to Slit repellents. It should be noted, however, that while these observations strongly support model 2, they cannot rule out model 1 completely. Indeed, it remains possible that in addition to this inhibitory role, Rig-1 may also recognize an attractant in the floor plate that, unlike Netrin-1, is not responsible for drawing commissural axons ventrally but is important for crossing the floor plate. This issue may only be resolved if a putative attractive function of Rig-1 can be separated from its role in preventing repulsion, for example through structure-function studies. In addition, although model 1 is not completely excluded, it should also be noted that an inhibitory effect of Rig-1 on Slit responsiveness by commissural axons is nonetheless sufficient by itself to explain all of the observed *Rig-1* mutant phenotypes as well as our in vitro results.

To confirm the role of Rig-1 as an inhibitor of the Slit response, we assessed the effect of removing the receptors for Slit on the *Rig-1* mutant phenotype. Interestingly, whereas removal of Robo1 leads to significant rescue of commissural axon crossing in the *Rig-1-/-* background, removal of Robo2 has no effect on the *Rig-1-/-* phenotype (Figure 6). The lack of rescue in the *Robo2-/-* ;Rig-1 double mutant is presumably explained by the expression of *Robo2* mRNA in the spinal cord, which is almost entirely non-overlapping with that of *Rig-1* (Figure 1M). Therefore, the complete absence of both Robo1-positive and Robo2-positive fibers in the ventral commissures of the Rig-1 mutant spinal cord indicates that Robo2-expressing axons may be dependent on Rig-1 in a cell-non-

autonomous manner to cross the midline. For example, Robo1-expressing axons may pioneer floor plate crossing and Robo2-expressing axons may fasciculate onto the Robo1-positive pioneers. Consistent with this hypothesis, commissural axons are observed stalled in the floor plate of *Robo1* mutants at early time points, a phenotype that fits with a role for Robo1 in sensing Slits in the floor plate. On the other hand, Robo2 mutant commissural axons appear to cross the floor plate normally (H. Long et al., companion manuscript). Together these observations lead us to conclude that in the absence of Rig-1, Robo1-positive commissural axons fail to enter the floor plate because they are repelled by Slit proteins, and the Robo2-positive fibers then fasciculate with the misguided Robo1-positive axons, resulting in a failure to cross as well.

The lack of complete rescue of the *Rig-1* mutant phenotype by removal of Robo1 has several possible explanations. First, as already suggested by the subtle commissural axon guidance phenotype observed in the *Robo1* single mutants (Long et al., companion manuscript), Robo1 might not be the only receptor for Slit proteins on Rig-1 expressing commissural axons. Second, it remains possible that Rig-1 blocks premature responsiveness to yet other repellents in the floor plate beside Slits, although as argued above, such repellents would presumably not be ligands for Neuropilin-2 like Sema3B and Sema3F. Finally, there could be a small amount of residual Robo1 protein in the *Robo1* knockout, because of a small amount of splicing over the gene trap insertion, that results in presence of a small amount of wild-type *Robo1* mRNA in these animals (Long et

al., companion manuscript). Whatever the explanation, the significant rescue of midline crossing observed in *Robo1;Rig-1* double mutants strongly suggests that Rig-1 inhibits Slit signaling through Robo1 in commissural axons.

Crossing the midline in vertebrates and *Drosophila*

The lack of a ventral commissure in the spinal cord and hindbrain of *Rig-1* mutant mice is analogous to the complete absence of commissures in the CNS of *comm* mutants in *Drosophila* (Tear et al., 1996). Like Rig-1, Comm has been shown to inhibit Slit responsiveness in commissural axons prior to crossing (Kidd et al., 1999; Keleman et al., 2002). As commissural axons grow toward the midline, Comm interacts with *Drosophila* Robo1 (DRobo1) and prevents its localization to the axonal membrane. Once commissural axons have crossed the midline, Comm's inhibition of DRobo1 is relieved. In *comm* mutant embryos, DRobo1 is mislocalized to the axonal membrane as commissural axons approach the midline causing commissural axons to become sensitive to Slit prior to crossing. In mouse, as in *Drosophila*, Robo1 and Robo2 are primarily localized to the post-crossing portion of the axon (Figures 7B and 7C). However, in *Rig-1* mutants, Robo1 and Robo2 protein expression is not upregulated in commissural axons as they grow ventrally toward the floor plate. Rather, both proteins still appear to be confined to the "post-turning" commissural axons as they grow longitudinally – but in this case in the ipsi- rather than the contralateral spinal cord (Figures 7E and 7F). We conclude, therefore, that Rig-1 inhibits Slit responsiveness via a different mechanism than Comm. This conclusion is corroborated by

biochemical experiments in which we found that under conditions where Comm and DRobo1 interact in transfected COS cells (Keleman et al., 2002), Rig-1 and Robo1 do not show any biochemical interaction, whether or not Slit2 protein is added (data not shown). Similarly, whereas Comm has been shown to relocalize DRobo1 from the surface of transfected COS cells to intracellular compartments (Keleman et al., 2002), Rig-1 has no such effect on Robo1 in vitro (data not shown). Thus in mice, Rig1 appears to produce its effect not by affecting Robo protein expression, but rather by preventing Slit signaling via the small amount of Robo protein that is present on the axons precrossing; a corollary of this is that the small amount of Robo that is present must be enough to mediate a sufficient response to midline Slit proteins to prevent crossing in the absence of Rig-1.

Does this mean that flies and vertebrates have hit on completely different solutions to the problem of preventing premature Slit responsiveness? Not necessarily. First, in vertebrates, like flies, Robo protein expression is dramatically upregulated after midline crossing. It is possible that this regulation of protein localization uses a similar mechanism to that operating in flies, perhaps using a still to be discovered Comm-like protein. Second, although studies in *Drosophila* have focused on the role of Comm in regulating Robo protein expression, there is nonetheless some Robo expressed pre-crossing, and it is tempting to speculate that there must be some specific second mechanism to prevent that Robo from signaling sufficient repulsion to prevent crossing. Thus, flies and vertebrates might both have two mechanisms: one to regulate Robo

protein expression (involving Comm in flies and an unknown mechanism in vertebrates) and one to silence low level Robo protein precrossing (involving Rig-1 in vertebrates and an unknown mechanism in flies). It is important to note that a second mechanism in flies need not require a second molecule: Comm itself could, in principle, silence Robo pre-crossing independent of its effect on protein localization.

How does Rig1 inhibit the response to Slit through Robo1? Since we have shown that Rig-1 can directly bind Slit proteins (Supplementary Figure 1A), Rig-1 might behave as an endogenous dominant negative Robo receptor and bind Slit unproductively, sequestering it away from Robo1. Although the lack of a phenotype in Rig-1 heterozygous animals tends to argue against this hypothesis, it is possible that Rig-1 is present in such vast excess over Robo1 on pre-crossing commissural axons that a reduction of its levels by half would not affect its ability to inhibit Robo1. Alternatively, the divergence of the Rig-1 cytoplasmic domain compared to other Robo family members (Figure 1A) suggests that Rig-1 may signal differently in response to Slit than Robo1. Rig-1 signaling may, therefore, interfere with Robo1 downstream signals and thus inhibit repulsion by Slit. Since Abl is a negative regulator of Robo in flies (Bashaw et al., 2000), it is conceivable, for instance, that Rig-1 activation leads to Abl-dependent phosphorylation of Robo1. However, using available antibodies, we were unable to detect a change in tyrosine phosphorylation status of Robo1 in Rig1 mutants

(data not shown). Careful structure-function analysis of the Rig-1 receptor within the context of commissural axons will be required to distinguish among these possibilities.

Understanding the midline switch in commissural axon guidance

How does Rig1 fit in more broadly with the high fidelity switch from attraction to repulsion that occurs at the midline? As commissural axons grow ventrally toward the floor plate, they are attracted by Netrin-1 via the Netrin receptor DCC (Keino-Masu et al., 1996; Serafini et al., 1996). In vitro, Robo1 can silence Netrin responsiveness through its direct interaction with DCC in the presence of Slit (Stein and Tessier-Lavigne, 2001). The lack of inhibition of DCC in the pre-crossing commissural axon may be mediated by both the low levels of Robo1 present in commissural axons prior to crossing the floor plate and by the inhibition of Robo1 by Rig-1 in pre-crossing axons. Both of these phenomena together would ensure that commissural axon growth cones sense the floor plate as an overwhelmingly attractive environment as they grow ventrally in the spinal cord (Figure 9A). Once they have interacted with the floor plate and entered the contralateral side of the spinal cord, a cascade of events takes place that ultimately leads to a rapid change in direction from the dorsal-ventral axis to the anterior-posterior axis. The downregulation of Rig-1 and coincident upregulation of Robo1 would work together to ensure that commissural axons move past their intermediate target and stay on course in the contralateral ventral funiculus. Both of these events may be required to finely tune the switch at the midline. Rig-1 is

observed at low levels on the post-crossing portion of commissural axons but is by no means completely turned off immediately upon entering the contralateral spinal cord (Figure 2). Thus, the upregulation of Robo1 may be a means to overwhelm the Rig-1 inhibition prior to it being completely downregulated. Alternatively, Rig-1 function may be inhibited through other means besides protein downregulation so that tight control over the upregulation of the Slit response is achieved. Rig-1 downregulation and the subsequent disinhibition of Robo1 not only permits upregulation of Slit responsiveness, but may also make Robo1 available to bind DCC and thus silence Netrin-1 responsiveness. Together, these two events would convert commissural axons from sensing the floor plate as an attractive environment to sensing it as a repulsive environment, repelling them out of the midline and allowing them to move onto the next leg of their trajectory (Figure 9B).

There are of course many unanswered questions that remain regarding the molecular mechanisms that control the midline switch. How is Rig-1 switched off? How does Rig-1 inhibit Slit responsiveness? How is Robo1 upregulated specifically after the floor plate has been crossed? How is the response to other repellents such as Sema3F upregulated? Mechanisms for confining proteins to specific regions on commissural axons have recently been identified, including localized mRNA translation (Brittis et al., 2002) and the modulation of the activity of guidance receptors by metalloproteases (Galko and Tessier-Lavigne, 2000). Understanding these mechanisms together with the characterization of the

molecular components of the switch will be required to fully understand how growth cones can move on from their intermediate targets. The identification of Rig-1 provides an additional entry point that will make these studies possible.

Experimental Procedures

Generation of *Rig-1*-deficient Mice

Genomic DNA containing portions of the Rig-1 gene was isolated by screening a BAC library (Incyte Genomics) with a Rig-1 specific cDNA probe. BAC DNA was then used to generate the targeting vectors shown in Supplementary Figure 2A using standard recombinant DNA techniques. Southern blot and western blot analyses were performed using stardard techniques. To identify targeting events, genomic DNA was digested with PstI and hybridized with a DNA probe external to the targeting vector as noted in the targeting figures. ES cell culture and generation of mice was carried out as previously described (Mombaerts et al., 1996). For genotyping, a PCRbased screen was developed: wild-type allele-forward primer 5'- TACCAGCTACTTCCAGAGAG-3'; reverse primer 5'-CCAACATCGAGTGGTACAAG-3'; mutant allele forward-primer 5'-GATCTCTCGTGGGATCATTG-3'; reverse primer 5'-TACCAGCTACTTCCAGAGAG-3'. PCR was carried out using the same protocol previously described (Plump et al., 2002).

Commissural axon outgrowth assays

Explants of E11.5 mouse dorsal spinal cord were isolated and cultured as described for rat explants , except that they were cultured in 45% OptiMEM-1

(GIBCO BRL), 50% F12 (GIBCO BRL), 5% heat-inactivated horse serum, 40 mM glucose, 2 mM GlutaMAX I (GIBCO BRL), 100 µg/ml streptomycin sulfate and 100 U/ml penicillin G. Dorsal spinal cord explants were co-cultured with E11.5 mouse floor plate, or COS cell aggregates transfected with a control, Slit2-N or Sema3F-expression plasmid. When indicated, outgrowth of commissural axons was elicited by adding 125 ng/ml of purified Netrin-1 to the culture media. Explants were fixed and analyzed after 18-20h.

COS cell aggregates

For the production of transfected COS cell aggregates, COS cells were plated in a 6-well dish and transfected 16h later with 1 µg of DNA (pSecTagB, pSecTag-Slit2N, pSecTag-Sema3F) using 3 µl of FuGene 6, according to the manufacturer's protocol. 24h after transfection, cell layers were trypsinized, washed with DME-10% FBS, and resuspended in 150 µl DME-10% FBS. Drops of the cell suspension (20 µl) were placed onto the lids of 12-well dishes, which were inverted over dishes containing DME. These hanging drop cultures were incubated for 6-12 h and aggregates were trimmed with tungsten needles.

Immunohistochemistry

Embryos were fixed overnight at 4°C in 4% paraformaldehyde (PFA)/ phosphate-buffered saline (PBS), washed with PBS, incubated in 30% sucrose/PBS overnight, and embedded in OCT. Cryostat sections (20 µm) were collected on Superfrost Plus slides (Fisher) and kept at –80°C. Slides were blocked in PHT (PBS, 1% heat-inactivated goat serum, 0.1% Triton X-100) for 1h at room temperature (RT), incubated overnight at 4°C with the primary antibody diluted in

PHT, washed 3 times for 15 min at RT in PHT, incubated for one hr at RT with the fluorescently-labeled secondary antibody diluted in PHT, washed 3 times for 15 min at RT in PHT and coverslip-mounted using Fluoromount G mounting media (Fisher).

Whole-mount immunohistochemistry of spinal cord explants were done as described above for tissue-section immunohistochemistry, except that 6 one-hour washes were performed and that the fluorescently-labeled secondary antibody was incubated overnight at 4°C. The TAG1 (clone 4D7, dilution 1:200), Neurofilament (clone 2H3, dilution 1:200) monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (University of Iowa). The rabbit polyclonal GFP antibody (dilution 1:400) was from Molecular Probes. The rabbit polyclonal Rig-1, Robo1, and Robo2 antibodies (dilution 1:1000) were generated in the lab of Fujio Murakami using Fc-tagged ectodomains as antigens.

In situ hybridization

In situ hybridization of rat spinal cords was carried out essentially as described in Fan and Tessier-Lavigne (1994). Fluorescent in situ hybridization of mouse spinal cords was carried out as described in the TSA plus protocol (Perkin Elmer).

Binding Experiments

Conditioned media from cells transfected with C-myc-hSlit2 was used in cell overlay assays to detect binding to COS cells transiently transfected with control,

rRobo1, mRig-1 or rDCC expression vectors essentially as described in (Keino-Masu et al., 1996).

Lipophilic Dye Tracing

Spinal cords of E12.5 *Rig-1* mutant and wildtype embryos were prepared in an open-book configuration, fixed with 4% paraformaldehyde, and injected with DiI (Molecular Probes) using iontophoresis into the dorsal region. DiI was allowed to diffuse for two days to label commissural axons along their entire length, enabling their visualization by conventional fluorescence microscopy.

Figure Legends

Figure 3.1, Expression of *mRig-1* in the Developing Rat Spinal Cord and Co-Expression of *mRig-1* with *rRobo1* and *–2* in the Mouse Spinal Cord.

(A) Schematic representation of the Robo1 (red) and Rig-1 (blue) domain structure.

Expression of *Rig-1* at E11 (E), E12 (F) and E13 (G) in transverse sections of the rat spinal cord.

Rig-1 is not expressed in the E11 rat spinal cord (E) at time at which commissural axons have just begun their ventral migration toward the floor plate (B). By E12, when many commissural axons have reached the floor plate and a few pioneers have begun to cross (C), *Rig-1* is expressed at high levels in regions corresponding to dorsal commissural neurons as well as ventral V3 interneurons (F). *Rig-1* expression is maintained in commissural neurons at E13 in the rat (G), at which time many axons have crossed to the contralateral spinal cord and begun to grow longitudinally (D).

Co-expression of *Rig-1* with *Robo1* (H-J) and *Robo2* (K-M) in transverse sections of E11.5 mouse spinal cords.

As was shown for the rat spinal cord, *Rig-1* is expressed exclusively by commissural neurons in the E11.5 mouse spinal cord (H) and (K).

As described previously (Kidd et al., 1998; and Brose et al., 1999), *Robo1* is expressed dorsally in the region of the commissural and association neuron cell bodies and ventrally in subpopulations of motor neurons (I). Throughout the cord, the *Rig-1* pattern of expression appears coincident with that of *Robo1* (J).

As previously reported (Brose et al., 1999), *Robo2* is expressed in the motor column, in the dorsal root ganglia, and dorso-laterally along the edge of the spinal cord (L). *Robo2* expression is almost completely non-overlapping with that of *Rig-1* (M) in both the dorsal and ventral spinal cords.

Scale bar, 200 μm.

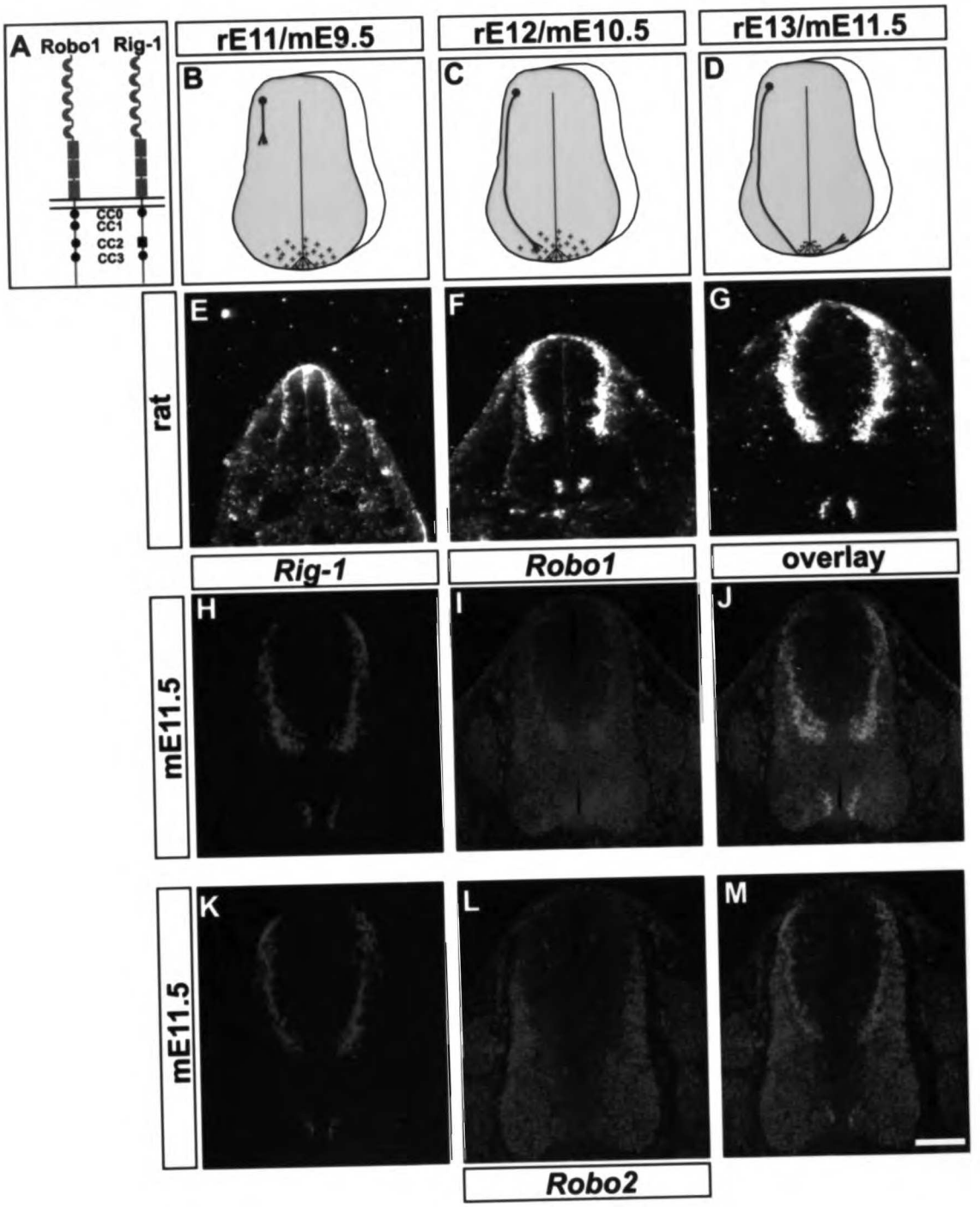
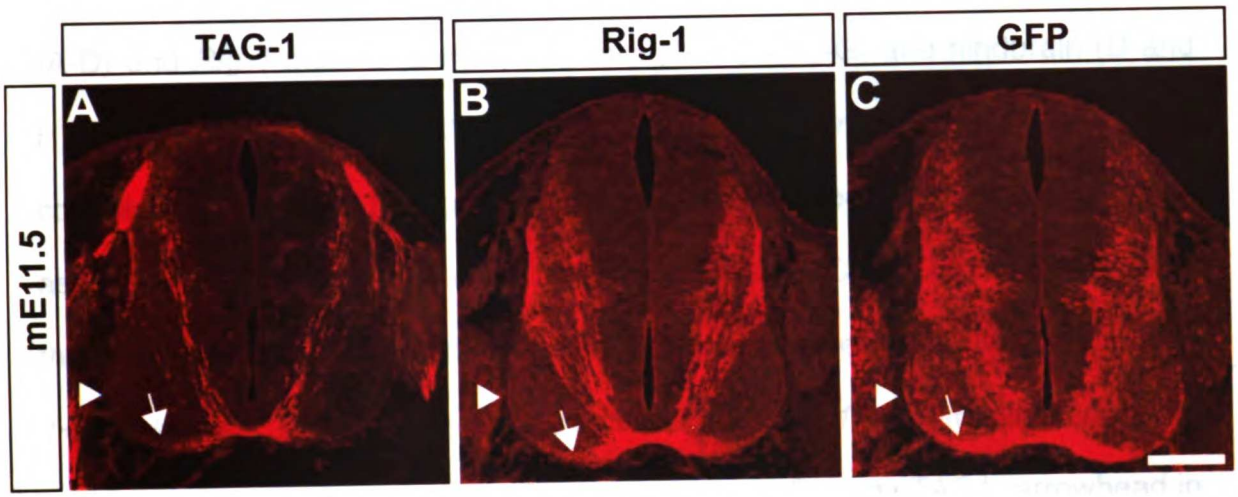

A Robo1 Rig-1
CC0
CC1
CC2
CC3
rE11/mE9.5
rE12/mE10.5
rE13/mE11.5
B
C
D
rat
E
F
G
Rig-1
Robo1
overlay
mE11.5
H
I
J
mE11.5
K
L
M
Robo2

Figure 3.2, Rig-1 Protein is Expressed on Commissural Axons Before and as They Cross the Floor Plate.

Adjacent transverse sections of E11.5 mouse spinal cords stained with TAG1 (A), Rig-1 (B) and GFP (C). TAG1 is a marker of commissural axons that is rapidly downregulated from post-crossing axons (arrow). Similarly, Rig-1 expression although strong on commissural axons as they course ventrally toward the floor plate also appears weaker once these axons have joined the ventral funiculus (B). This is in contrast to the GFP expression, in this case driven specifically in commissural axons by the Rig-1 promoter, which labels pre- and post-crossing commissural axons uniformly (C).

Scale bar, 200 μm.


TAG-1
Rig-1
GFP
mE11.5
A
B
C

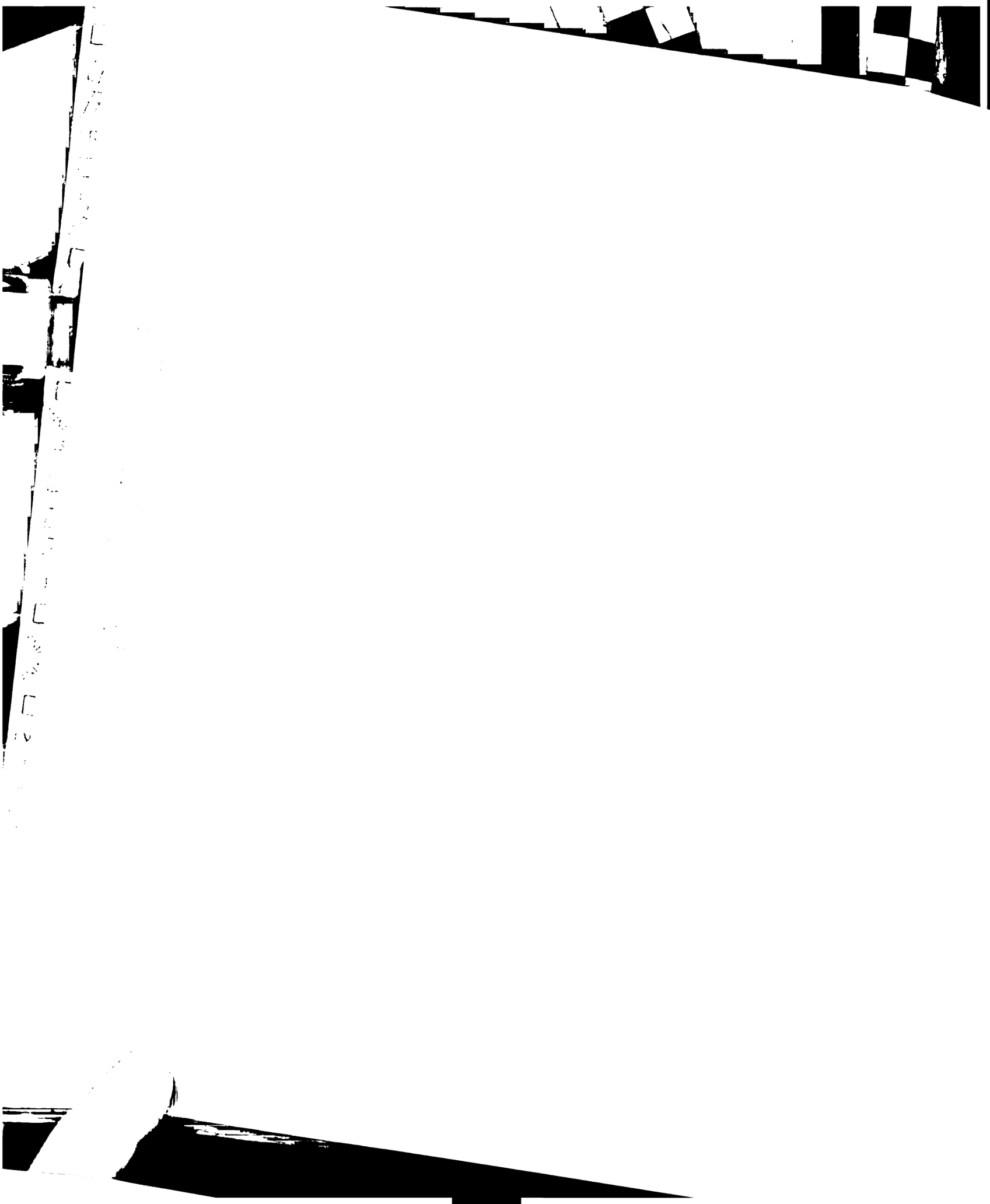
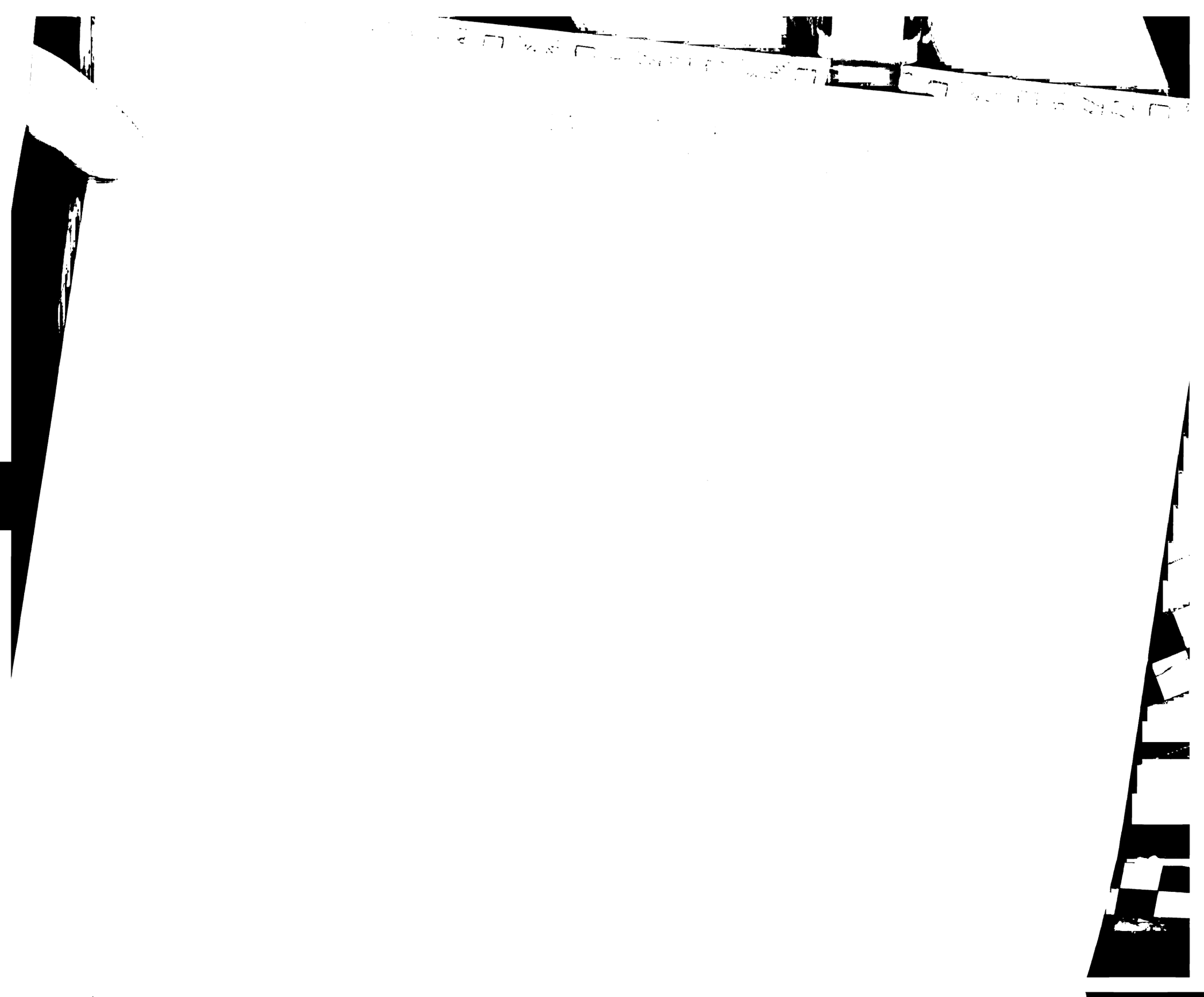


Figure 3.3, Lack of Commissural Axon Crossing of the Floor Plate in *Rig-1-/-* Mice.

Visualization of commissural axons in transverse sections of wildtype or *Rig-1+/-* (A-D) and *Rig-1* mutant (E-H) spinal cord (A-C and E-G) and hindbrain (D and H). In wildtype and *Rig-1+/-* spinal cords and hindbrains, TAG1 labels commissural axons as they grow ventrally toward the floor plate (arrow in A) and as they cross to the contralateral side (arrowhead in A, C, and D). GFP driven by the Rig-1 promoter labels commissural axons along their entire lengths in *Rig-1+/-* embryos (B). In transverse sections of E11.5 *Rig-1* mutant spinal cords, no axons are observed crossing the floor plate as visualized by TAG1 (arrowhead in E) or GFP (arrowhead in F) although commissural axons appear to grow normally toward the floor plate in the dorsal two-thirds of the spinal cord (arrow in E). The lack of floor plate crossing is observed throughout the spinal cord and hindbrain (arrowhead in H) and persists in E12.5 spinal cords (arrowhead in G) when most commissural axons have crossed the floor plate.

Scale bars, 200 μm.

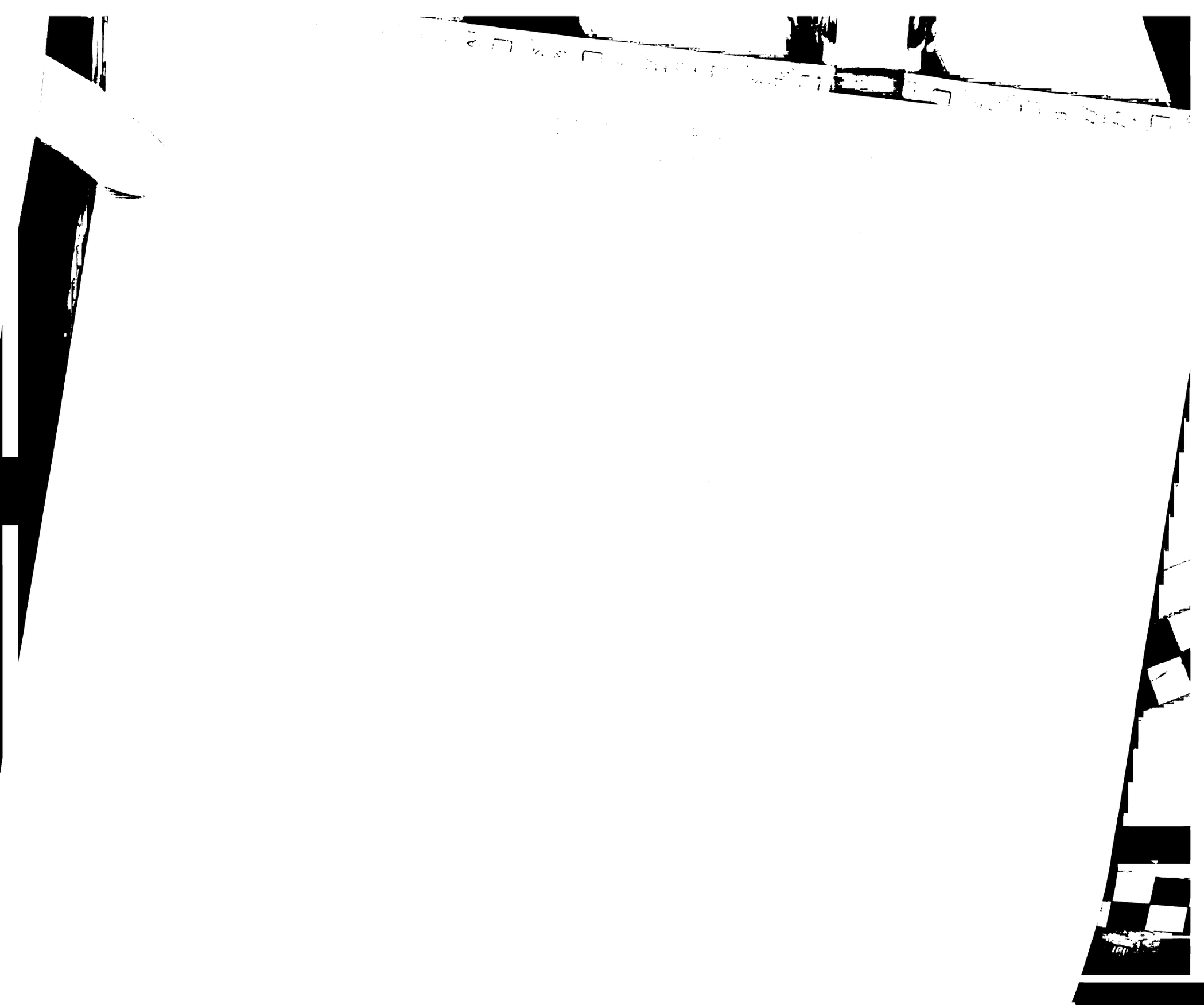


Wildtype or Rig-1+/-
Rig-1-/-
mE11.5 SC (TAG-1)
mE11.5 SC (GFP)
mE12.5 SC (TAG-1)
mE11.5 HB (TAG-1)
A
B
C
D
E
F
G
H

Figure 3.4, *Rig-1* mutant commissural axons are repelled by Slit protein in the floor plate prior to crossing

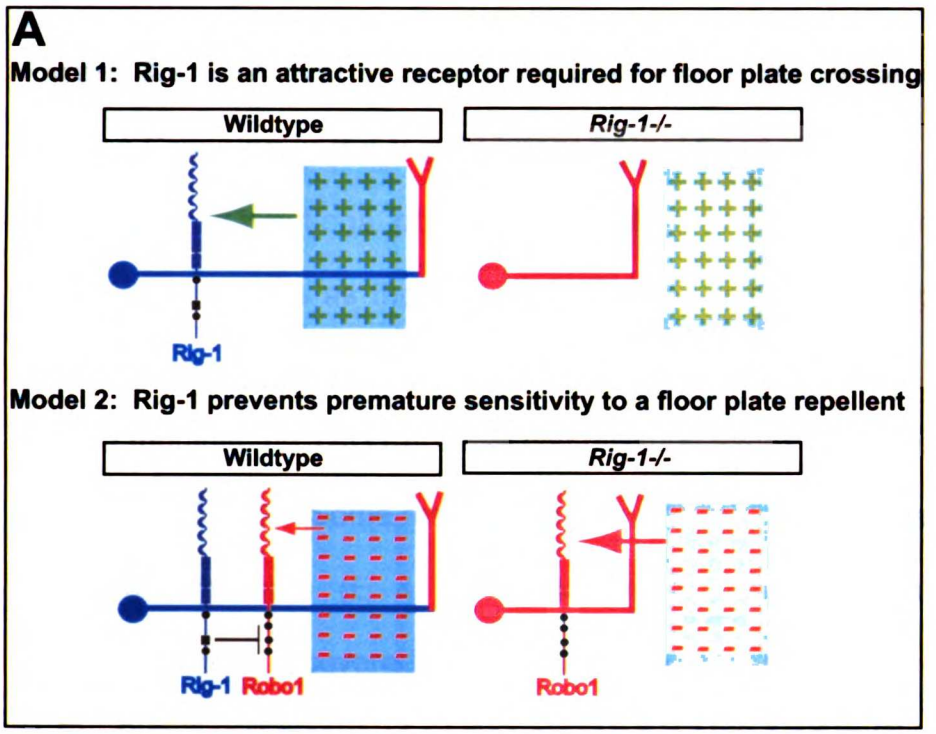
(A) Two models could account for the lack of midline crossing observed in the rig-1 mutant spinal cords. Model 1 postulates that Rig-1 may function as a receptor that responds to an attractant in the floor plate. In the absence of Rig-1, commissural axons fail to respond to this floor plate attractant and thus fail to cross to the contralateral side of the spinal cord. Model 2 postulates that Rig-1 inhibits the repulsive effects of a ligand expressed by the floor plate. For example, Rig-1 may prevent a repellent such as Slit from activating Robo1 on commissural axons prior to crossing. However, once commissural axons have crossed the floor plate, Rig-1 is downregulated and Slits in the floor plate become able to repel commissural axons. In the absence of Rig-1, Slits are able to activate Robo1 on commissural axons at any time and thus prevent commissural axons from ever crossing the floor plate.

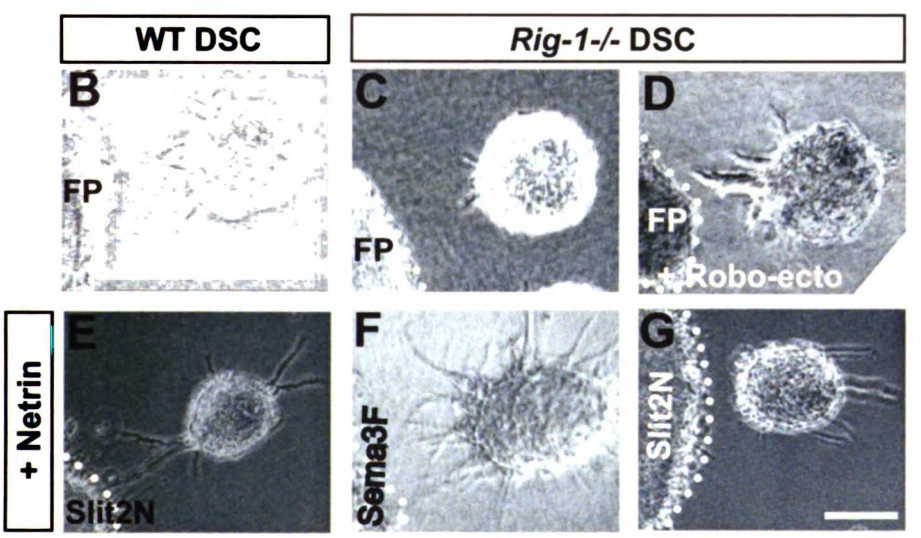
In vitro collagen cultures of DSC explants were used to distinguish between these two models (B-G). Commissural outgrowth was elicited from DSC explants by co-culturing with floor plate (FP) (B-D) or including 125 ng/ml purified cNetrin-1 in the bath (E-G). Commissural axons grow out of DSC explants from WT spinal cords in response to FP (B). However, *Rig-1-/-* commissural axons fail to grow out significantly when co-cultured with WT FP (C). The lack of commissural axon outgrowth from *Rig-1-/-* DSC in response to FP can be rescued by the inclusion of purified Robo2-ectodomain fused to

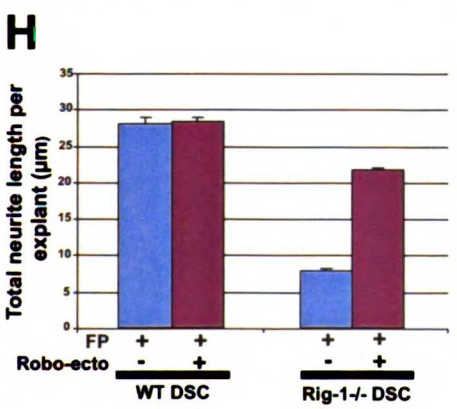


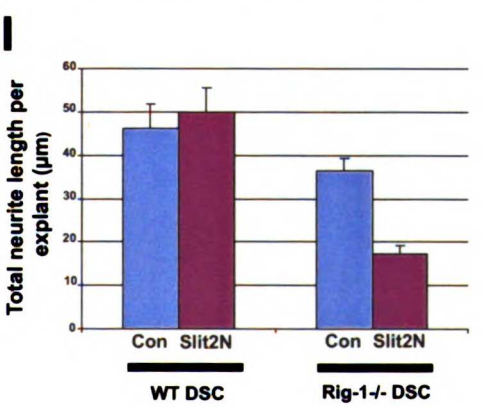
Fc in the culture medium (D). Commissural axons grow out into collagen from DSC explants in response to the presence of cNetrin-1 in the culture medium. When confronted with COS cells expressing the N-terminal fragment of hSlit2 (Slit2-N), Netrin-responsive commissural axons from WT DSC explants grow normally and are not repelled (E). However, commissural axons from *Rig-1-/-* DSC explants are strongly repelled by Slit2-N (G) but unaffected by another repellent found in the floor plate, Sema3F (F). The results of these in vitro experiments are quantified in panels H and I.

Scale bar, 100 μm.


A
Model 1: Rig-1 is an attractive receptor required for floor plate crossing
Wildtype
Rig-1-/-
Rig-1
Model 2: Rig-1 prevents premature sensitivity to a floor plate repellent
Wildtype
Rig-1-/-
Rig-1
Robo1
Robo1


WT DSC
Rig-1-/- DSC
B
FP
C
FP
D
FP
+Robo-ecto
+ Netrin
E
Slit2N
F
Sema3F
G
Slit2N


H
Total neurite length per explant (µm)
FP
Robo-ecto
WT DSC
Rig-1-/- DSC


I
Total neurite length per explant (µm)
Con
Slit2N
Con
Slit2N
WT DSC
Rig-1-/- DSC

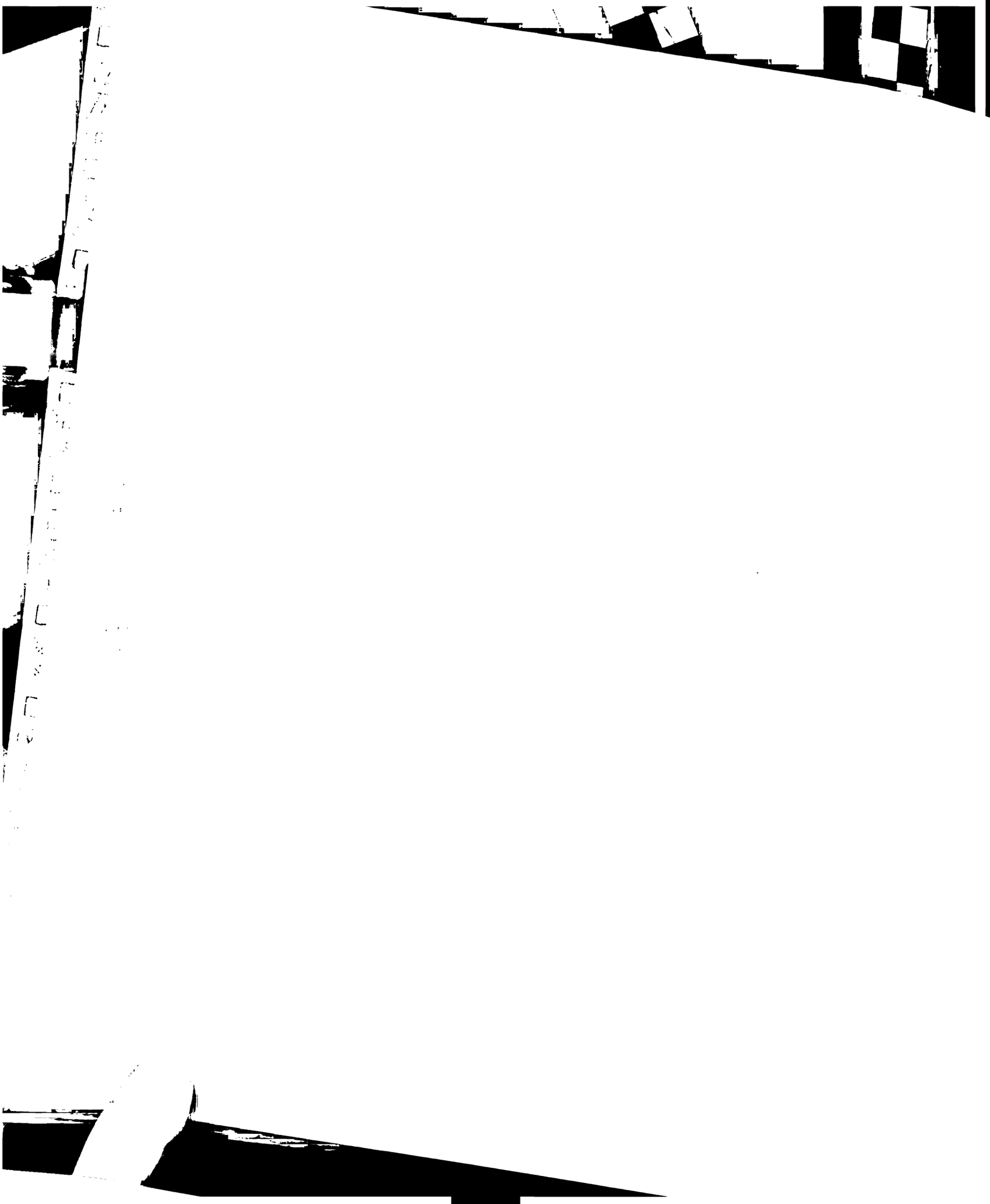
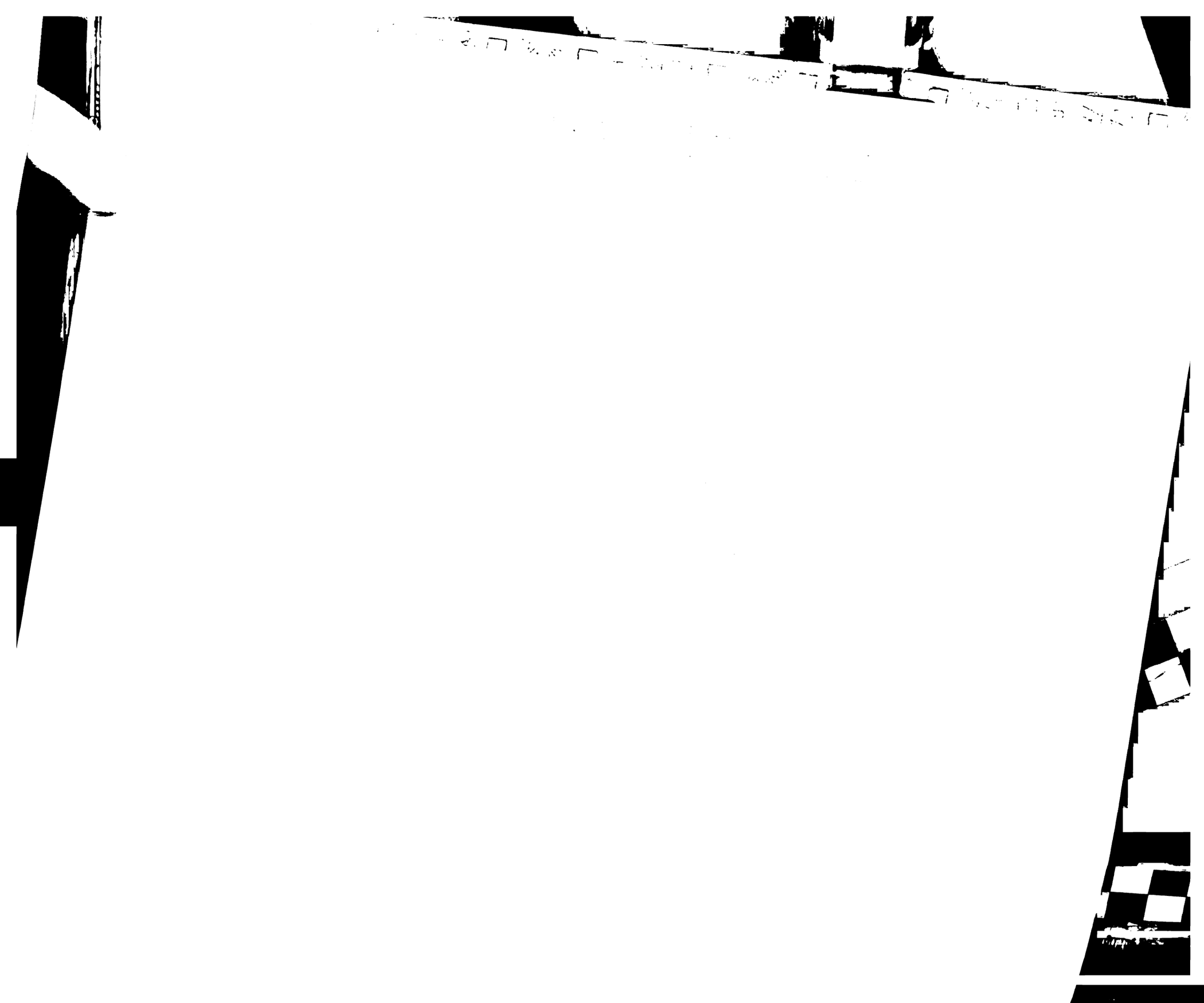


Figure 3.5, Removal of *Slits* from the Floor Plate Progressively Rescues Midline Crossing in the *Rig-1* Mutant Spinal Cord.

In transverse sections of wildtype or *Rig-1+/-* spinal cords, TAG1 labels commissural axons as they grow ventrally toward the floor plate and as they cross the floor plate in a thick bundle (A and B). In *Rig-1-/-* or *Rig-1-/-;Slit1-/-* double mutants, no TAG1-positive commissural axons are observed crossing the floor plate (C & D). Some rescue of midline crossing is observed in *Rig-1-/-;Slit2-/-* double mutants (E & F) as shown by the small amount of TAG1 immunoreactivity recovered in the floor plate of those double mutants (arrowhead in F). Significant rescue of midline crossing is observed in *Rig-1-/-;Slit1-/-;Slit2-/-* triple mutant spinal cords (G and H).

Scale bar, 200 μm (A, C, E, and G)

Scale bar, 100 μm (B, D, F, and H)



Rig-1+/-
Rig-1-/-;Slit1-/-
Rig-1-/-;Slit2-/-
Rig-1-/-;Slit1-/-;Slit2-/-
A
B
C
D
E
F
G
H

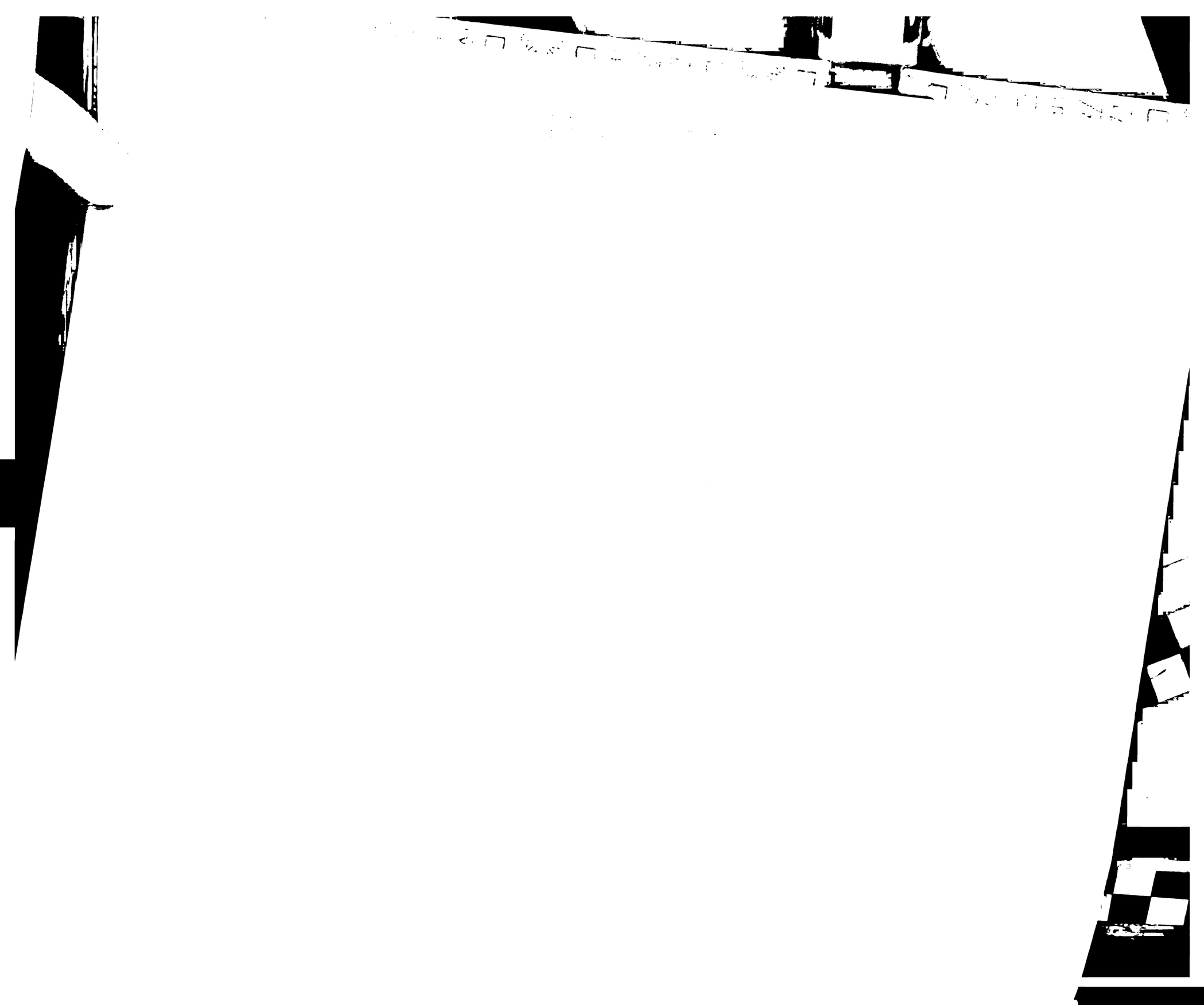
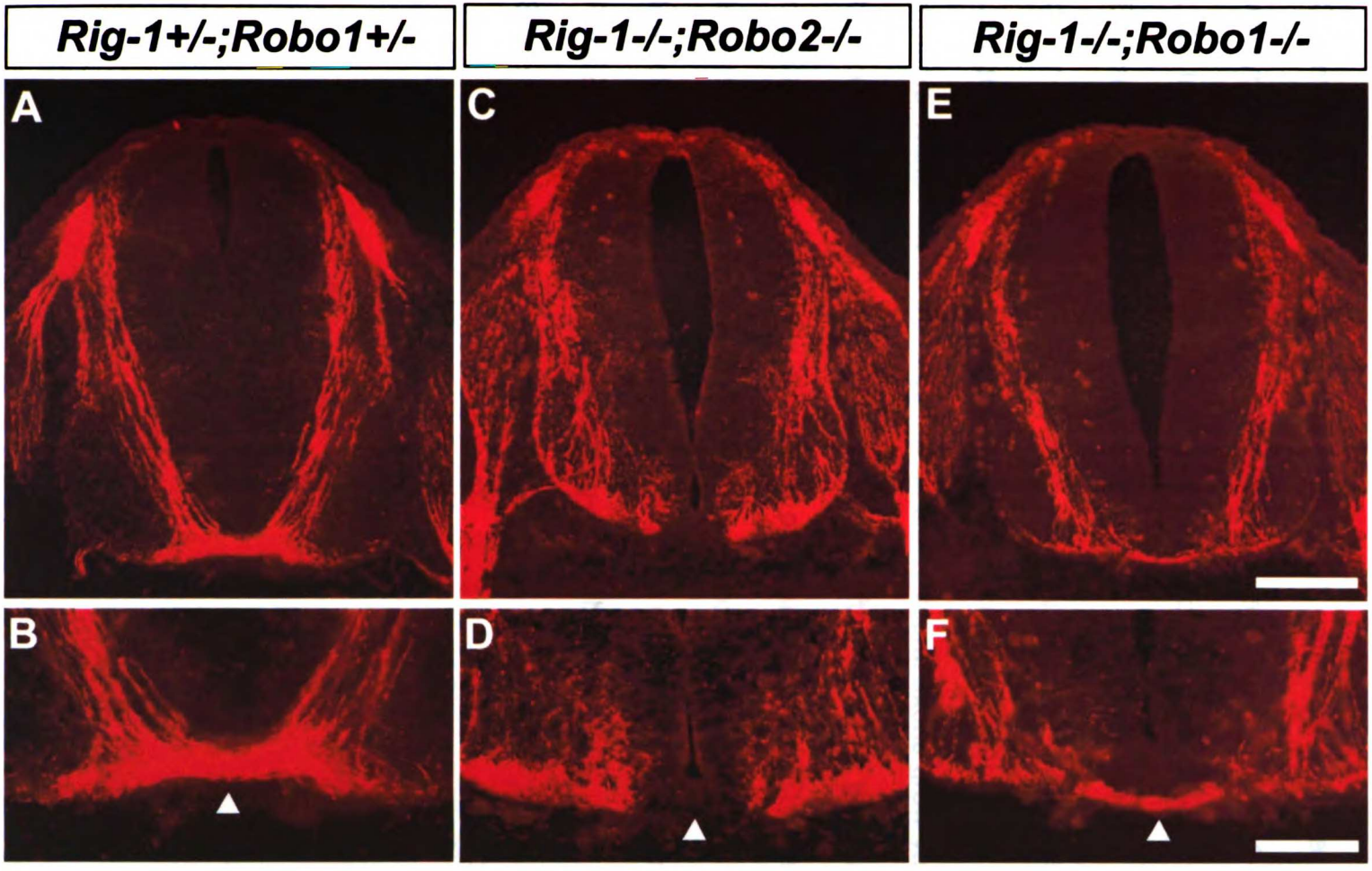


Figure 3.6, Removal of *Robo1* but not *Robo2* Leads to Rescue of Midline Crossing in *Rig-1* Mutants.

Anti-TAG1 immunostaining of transverse sections through *Rig-1-/-;Robo2-/-* double mutant embryos show no difference with *Rig-1-/-* single mutants, that is no crossing of commissural axons through the floor plate (C and D). Removal of Robo1 in the *Rig-1* mutant background, on the other hand, leads to significant rescue of midline crossing as assayed by TAG1 staining (E and F). However, the thickness of the commissural bundle in the floor plate is still not back to wildtype levels (A and B).

Scale bar, 200 μm (A, C, and E)

Scale bar, 100 μm (B, D, and F)


Rig-1+/-;Robo1+/-
Rig-1-/-;Robo2-/-
Rig-1-/-;Robo1-/-
A
C
E
B
D
F

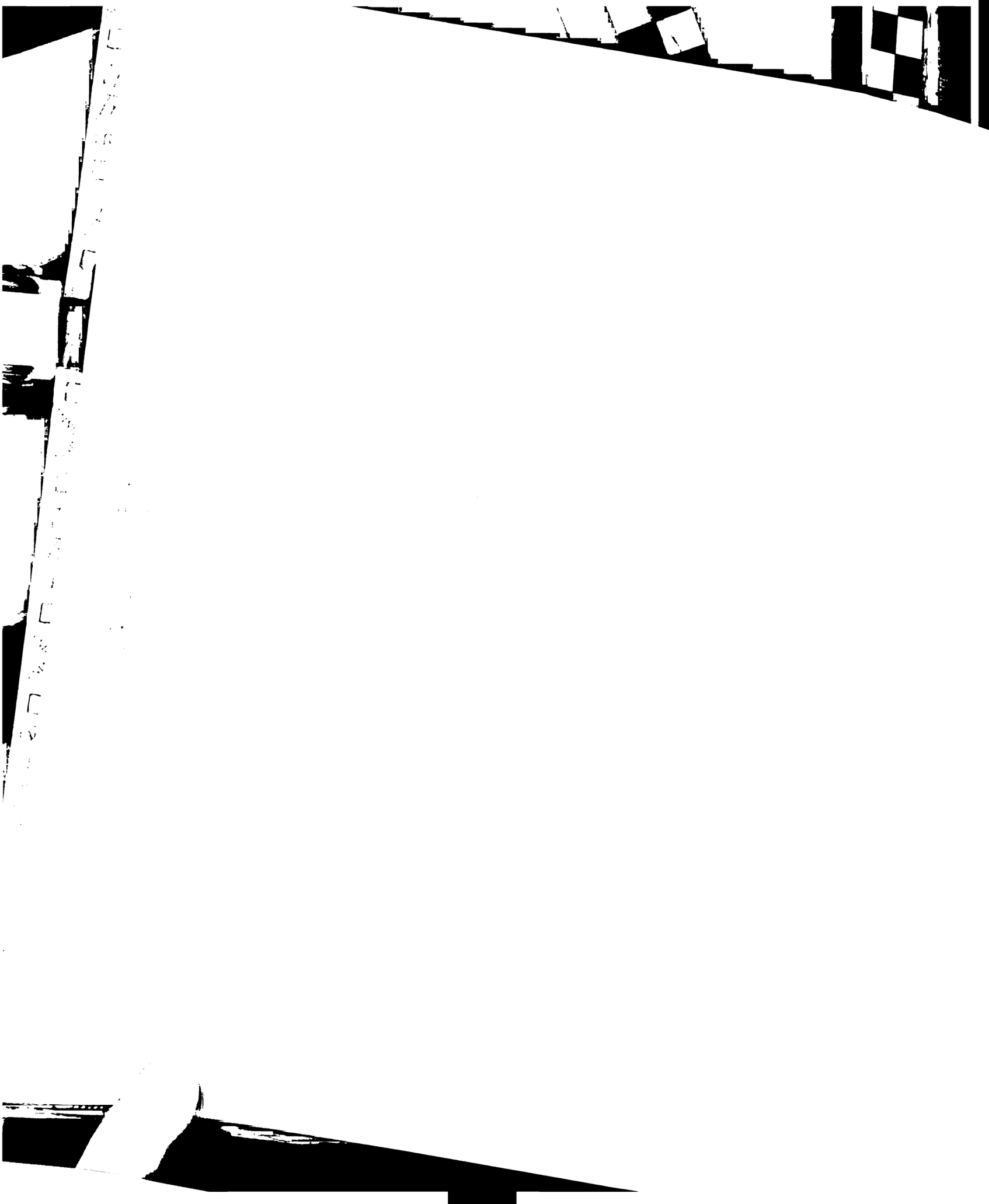


Figure 3.7, Robo1 and Robo2 localization is unchanged in *Rig-1* mutant spinal cords.

Commissural axons are labeled with TAG1 until they exit the floor plate in wildtype spinal cords (A) or as they course ventrally in the *Rig-1-/-* spinal cord (D). Immunohistochemistry against Robo1 protein labels pre-crossing (arrow in B) and crossing commissural axons at low levels (arrowhead in B). Once commissural axons enter the ventral funiculus, Robo1 levels are dramatically upregulated (B). Surprisingly, Robo1 localization appears unaltered in *Rig-1* mutants, Robo1 levels are kept low as commissural axons grow ventrally toward the floor plate (arrow in E) and become high after they enter the ventral funiculus despite the lack of crossing of the floor plate (arrowhead in E). Similarly, Robo2 is primarily expressed in a subset of axons growing in the ventral funiculus with low levels also observed in pre-crossing and crossing commissural axons (C). Robo2 localization is unchanged in *Rig-1-/-* embryos (F).

Scale bar, 200 μm.

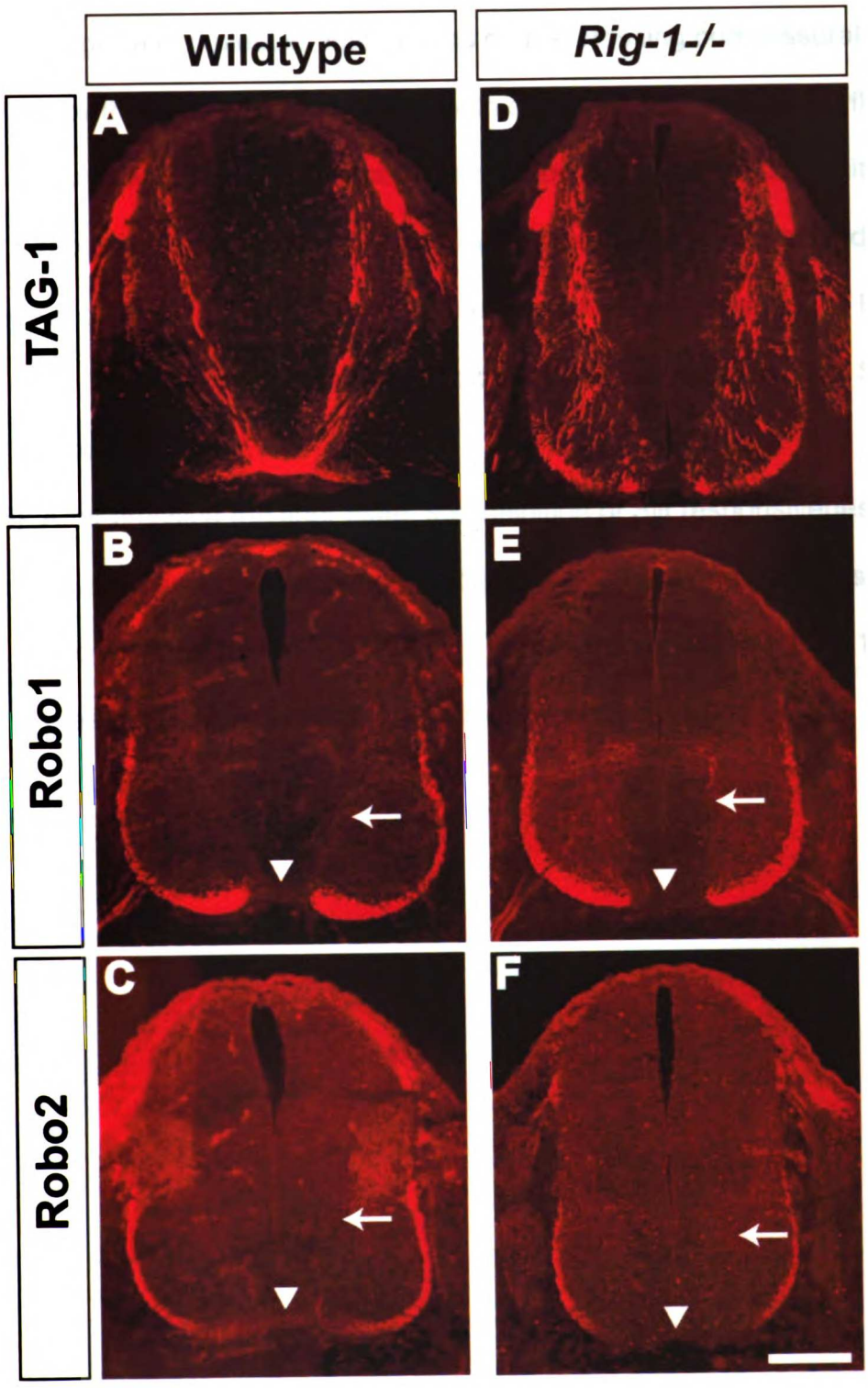
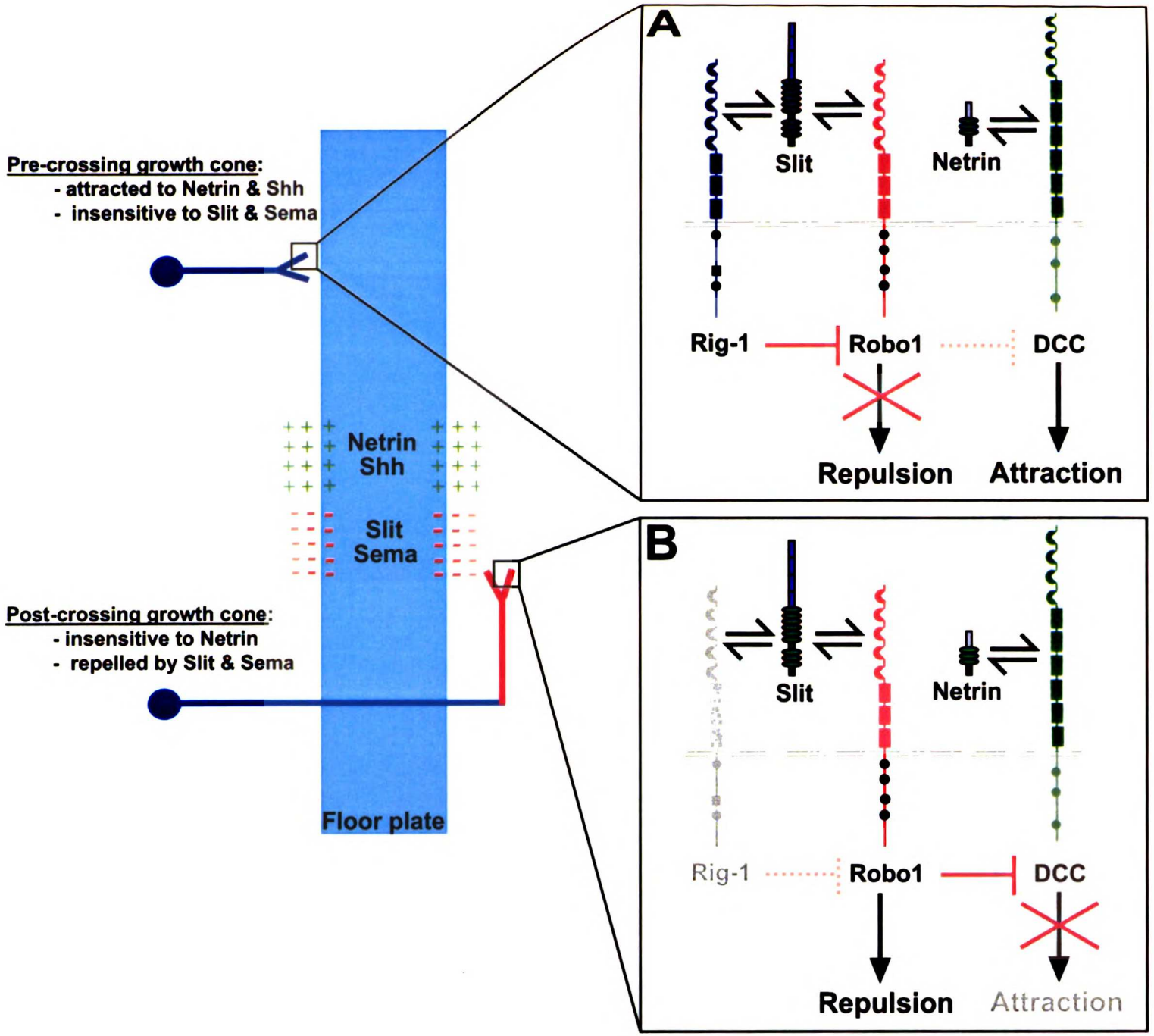

Wildtype
Rig-1-/-
TAG-1
Robo1
Robo2
A
B
C
D
E
F

Figure 3.8, Model of the Switch in Ligand Responsiveness as Commissural Axons Cross the Floor Plate

(A) Diagram of molecular interactions on pre-crossing commissural axon growth cones. Pre-crossing growth cones are attracted to the floor plate primarily through the effect of Netrin-1 on its receptor DCC. Slit, a repulsive ligand also expressed by the floor plate, is recognized by Robo1 on commissural axon growth cones, however, the presence of Rig-1 on the growth cone membrane inhibits Robo1 from responding to Slit as a repulsive ligand.

(B) After crossing the floor plate, the inhibition of Slit responsiveness is relieved due to the absence of Rig-1 on post-crossing commissural axons. This coincides with a loss of responsiveness to Netrin-1, presumably due to the interaction between Robo1 and DCC.


Pre-crossing growth cone:
- attracted to Netrin & Shh
- insensitive to Slit & Sema
Netrin
Shh
Slit
Sema
Post-crossing growth cone:
- insensitive to Netrin
- repelled by Slit & Sema
Floor plate
A
Slit
Netrin
Rig-1
Robo1
DCC
Repulsion
Attraction
B
Slit
Netrin
Rig-1
Robo1
DCC
Repulsion
Attraction

Supplemental Figure 3.1, Rig-1 Binds Slit2.

Supernatants from cells expressing hSlit2 were incubated with cells expressing either mRig-1 (A), rRobo1 (B) or a control protein, DCC (C), in the presence of 2 µg/ml heparin (which reduces background binding). Binding was detected using an antibody against the C-terminal myc tag on hSlit2 and corresponding Cy3-conjugated secondary antibodies. hSlit2 binds mRig-1 as well as rRobo1 but not DCC.

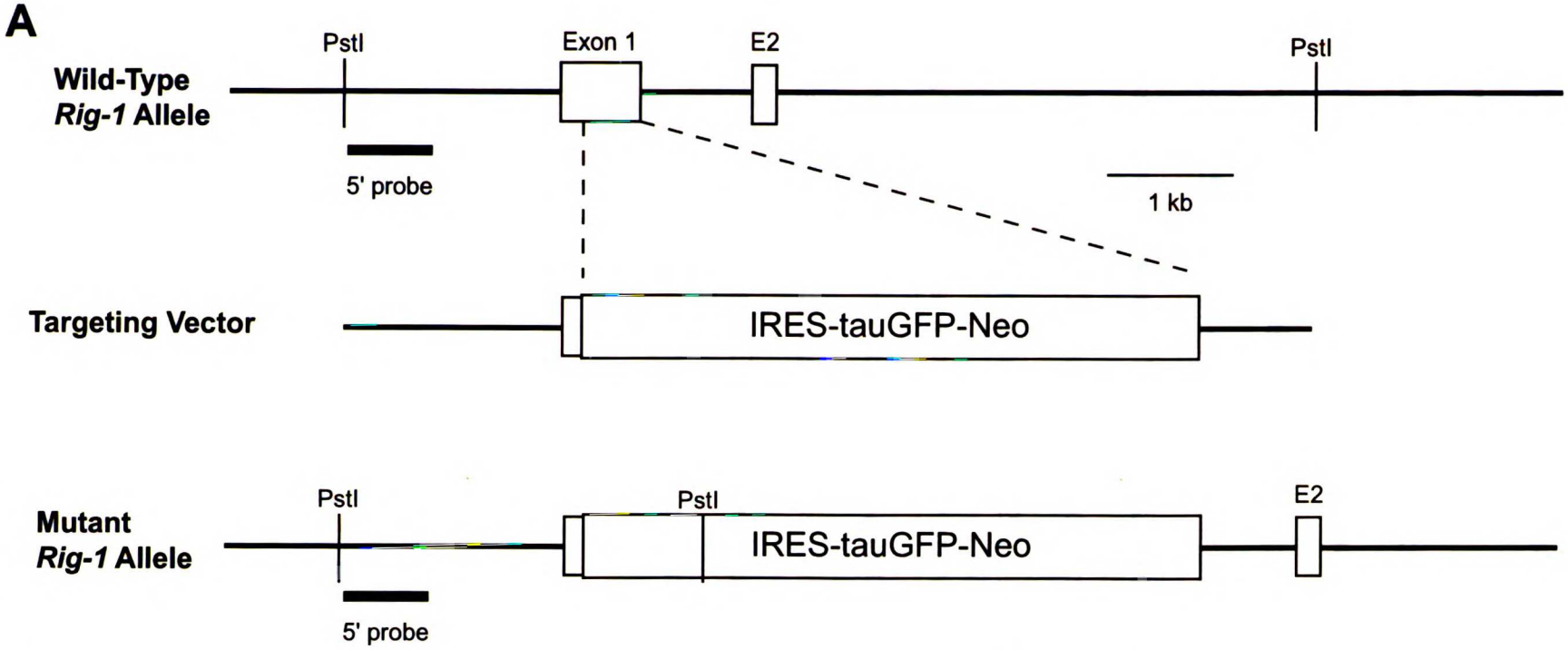
hSlit2
rRobo1
mRig-1
rDCC

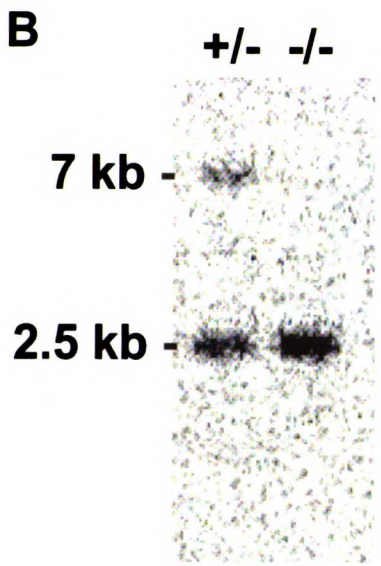
Supplemental Figure 3.2, Creation of *Rig-1*-deficient mice.

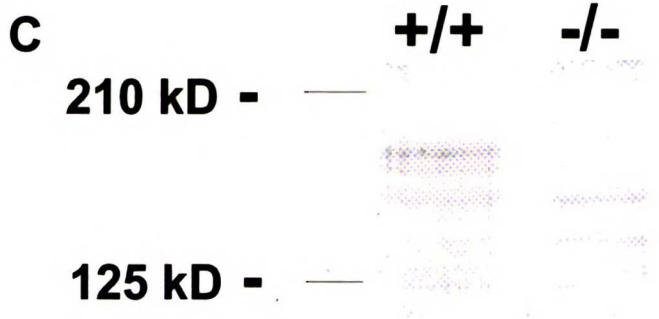
(A) Targeting strategy. The upper line shows the wild-type *Rig-1* locus. The middle line shows the targeting vector. A portion of the first *Rig-1* exon containing sequence encoding the start ATG and the signal sequence was replaced with a targeting cassette containing an internal ribosome entry site (IRES), a tauGFP fusion protein, and a neomycin resistance gene (neo) flanked by a pgk-1 promoter and a polyA tail, and by two loxP sites. The lower line shows the correctly targeted locus.

(B) Southern blot of PstI genomic DNA hybridized from *Rig-1* heterozygous and mutant mice hybridized with the 5' flanking probe shown in the schematic above.

(C) Western blot of lysed spinal cords from wildtype and *Rig-1* mutant mice probed with an antibody against the Rig-1 extracellular domain.


A
PstI
Exon 1
E2
PstI
Wild-Type
Rig-1 Allele
5' probe
1 kb
Targeting Vector
IRES-tauGFP-Neo
PstI
PstI
E2
Mutant
Rig-1 Allele
IRES-tauGFP-Neo
5' probe

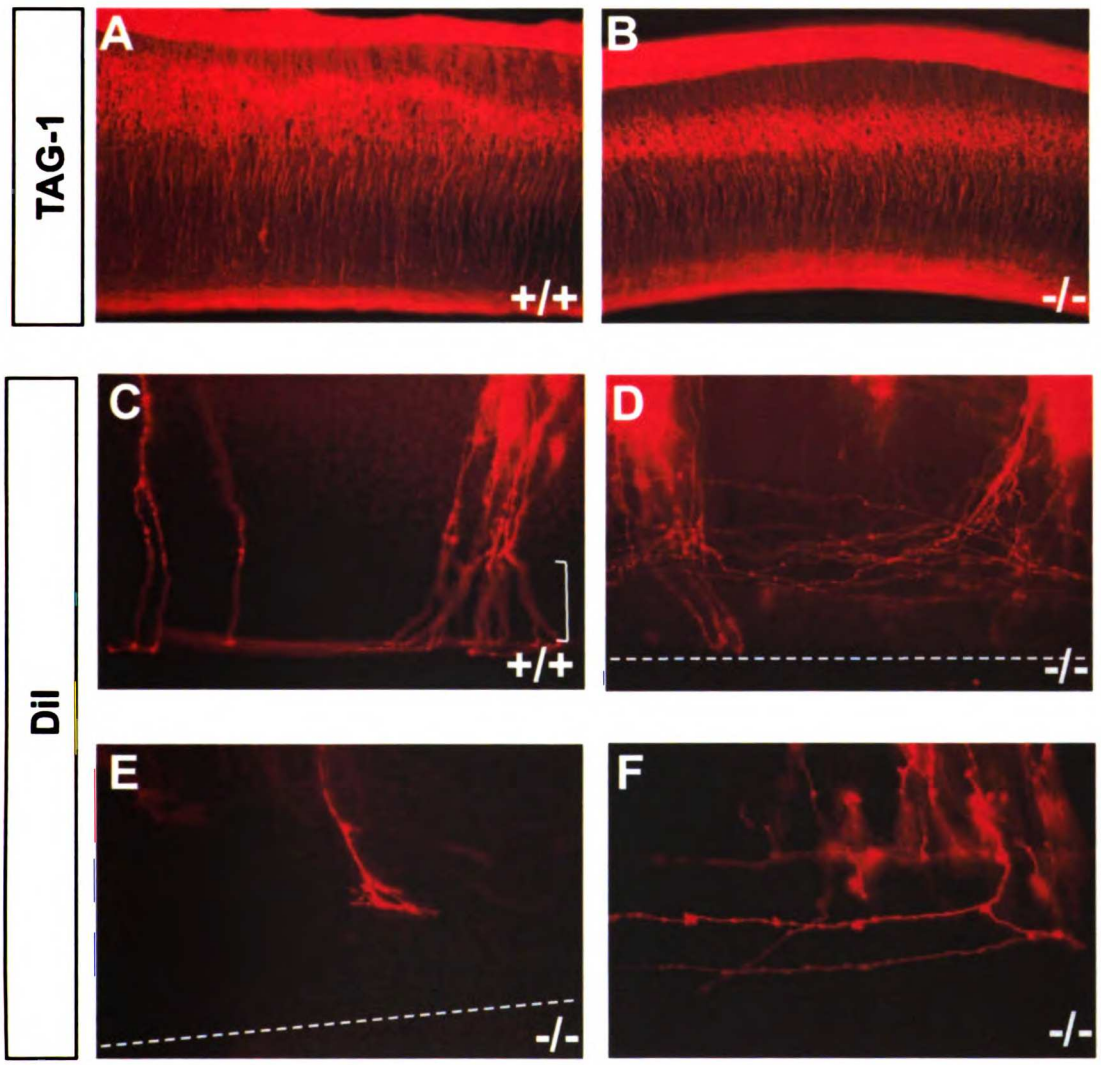

B
+/-
-/-
7 kb -
2.5 kb -

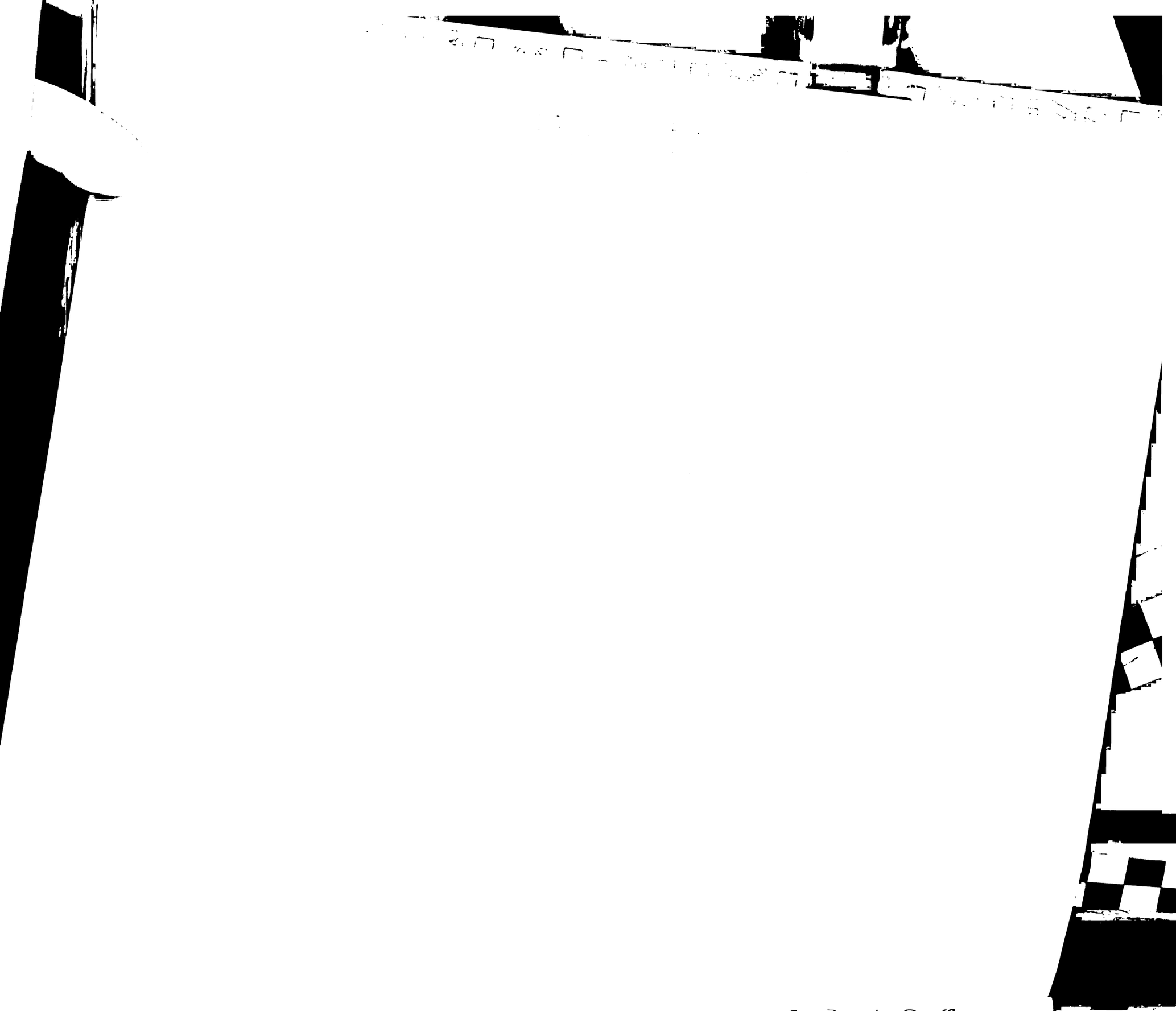

C
+/+
-/-
210 kD -
125 kD -

Supplemental Figure 3.3, Commissural Axons Appear Normal as They Grow Ventrally in the *Rig-1* Mutant.

Spinal cords of wildtype (A) and homozygous mutant (B) E11.5 embryos were fixed and subjected to wholemount immunohistochemistry with the anti-TAG1 antibody, 4D7. Commissural axons appear to grow normally through the ventral spinal cord toward the floor plate, although the staining appears more diffuse close to the floor plate in the *Rig-1* mutants.

The lipophilic dye, DiI, was implanted into the dorsal spinal cord of wildtype (C) and homozygous mutant (D-F) E12.5 embryos. In the wildtype spinal cord, commissural axons grow ventrally toward the floor plate, cross, and turn longitudinally on the contralateral side of the spinal cord. In Rig-1 mutant spinal cords, commissural axons fail to cross the floor plate. Many appear stalled close to the floor plate (D and E), while others turn longitudinally in the ipsilateral spinal cord (D and F) often bifurcating and growing both rostrally and caudally (F).

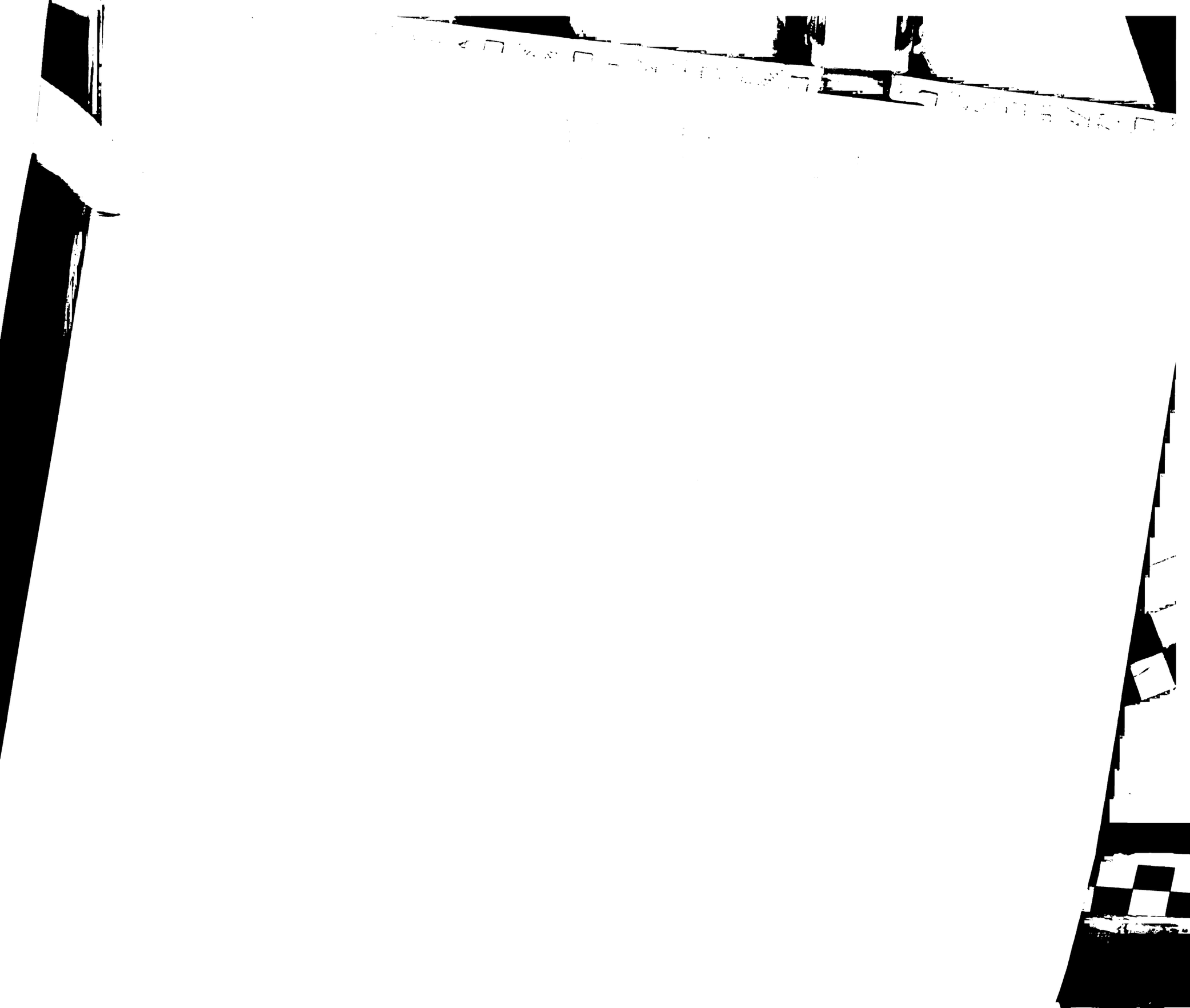

TAG-1
DiI
A
B
C
D
E
F
+/+
-/-
+/+
-/-
-/-



Supplemental Figure 3.4, Removal of *Neuropilin-2* Does Not Rescue Crossing in *Rig-1* Mutants.

Transverse sections of spinal cords from *Rig-1-/-;Neuropilin2-/-* double mutant mice were stained with anti-TAG1 antibody. No commissural axons are observed to cross the floor plate in these mice similarly to what is observed in the *Rig-1* single mutant mice.

Scale bars, 200 μm (A) and 100 μm (B).




Rig-1-/-;Neuropilin2-/-
A
B

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Chapter Four:

Conclusions and Perspectives

Summary

In this thesis, I describe the analysis of several mutant mice that have provided us with insight into the role of the axon guidance molecules of the Slit family and of their receptors of the Robo family in directing commissural axons across the midline and onto their final targets in the vertebrate spinal cord. Although the roles of Slit and Robos have been well-characterized in Drosophila, it was not clear what roles these molecule played in commissural axon guidance in the vertebrate spinal cord. Hua Long in the lab has shown that removal of all six alleles of Slit present in the vertebrate genome leads to severe axon guidance defects across the floor plate in the spinal cord. Hua's analysis of *Slit1;Slit2;Slit3* triple mutant mice correlated nicely with my analysis of *Robo1* and *Robo2* single mutant mice, which exhibit more subtle yet similar axon guidance phenotypes. Analysis of the *Robo1* and *Robo2* mutant mice also revealed a role for Slits in guiding commissural axons beyond the floor plate as they grow longitudinally in the ventral and lateral funiculi of the contralateral spinal cord. Like what has been previously observed in Drosophila, Robo1 and Robo2 mark different subsets of commissural axons that differ in the lateral position that they adopt relative to the floor plate in the contralateral spinal cord. Robo2-positive axons tend to grow in more lateral positions while Robo1-positive axons tend to stay more medial. The combinatorial code of Robo expression in commissural axons appears to dictate the sensitivity of these axons to Slits in the floor plate. Together our analyses of the *Robo1*, *Robo2* and triple *Slit* mutants indicate that

all three Slits expressed in the floor plate are involved in pushing commissural axons out of the midline and on to their next target.

Finally, my analysis of *Rig-1* mutant mice has revealed a novel role for a member of the Robo superfamily in inhibiting responsiveness to the ligand Slit. Rig-1, which is expressed primarily by Robo1-positive commissural axons, appears to be required to inhibit the ability of all commissural axons in the spinal cord and hindbrain to sense the repellent Slit in the floor plate prior to crossing. This role for Rig-1 is consistent with its expression on pre-crossing commissural axons and its apparent downregulation from commissural axons after they have crossed the floor plate. These studies have implicated a highly regulated response to Slit ligands in the floor plate as being crucial to proper commissural axon guidance, as well as raising many questions as to the details of the regulation of Slit responsiveness in commissural axons. In this section, I will discuss the outstanding questions that remain and show that the results discussed in this thesis can lead to the development of important tools that will be crucial to a more comprehensive understanding of the molecular events that take place as commissural axons interact with the floor plate before, while and after they cross.

Future Direction 1: Mechanism of Rig-1 inhibition of Slit Responsiveness

After reading through chapter three of this thesis, one is left wondering how Rig-1 could possibly be inhibiting Slit responsiveness in pre-crossing commissural axons. As discussed in that chapter, I have attempted to test all

obvious models primarily involving a direct effect on Robo1 in the presence of Rig-1, such as endocytosis or phosphorylation. The Robo inhibiting protein in Drosophila, Comm, has been shown to cause the relocalization of Robo to internal compartments both in vivo and in vitro (Keleman et al., 2002; Kidd et al., 1998). I have so far been unable to observe any change in the localization of Robo1 in the absence of Rig-1 either in vivo or when those two proteins are expressed in COS cells (see chapter 3 and appendix A). Although Robo1 and Rig-1 appear to co-localize when expressed in COS cells (Appendix A), I have not observed any direct interaction between Rig-1 and Robo1 through co-immunoprecipitation either from COS cells expressing both proteins or from spinal cord tissue (data not shown). Finally, since phosphorylation of Robo at the conserved motif, CC1, has been shown to regulate the ability of Robo1 to signal in response to Slit, I tested whether Rig-1 could influence the phosphorylation state of Robo1 both in vivo and in vitro. I did not observe any difference in the phosphorylation state of Robo1 in the absence or presence of Rig-1 (data not shown). These negative results lead me to conclude that Rig-1 does not function like Comm to inhibit Robo1 in vertebrate commissural axons and is unlikely to interact directly with Robo1 to mediate its inhibition of Slit responsiveness in pre-crossing commissural axons. Two other hypotheses as to the mechanism of Rig-1-dependent inhibition of Slit responsiveness remain to be tested. (1) Rig-1 binds directly to Slits secreted from the floor plate and keeps Slits from signaling through Robo1 and Robo2. (2) Rig-1 interferes with Slit signaling downstream of Robo1 either by directly competing for signaling partners or by signaling

through a different pathway that somehow inhibits repulsion downstream of Robo1. I will now discuss in detail each of these two possibilities and suggest experiments that may be able to distinguish between them.

Model 1: The Slit sequestration model

Like other members of the Robo family, Rig-1 binds Slits in vitro. However, the divergent cytoplasmic domain of Rig-1 may be unable to signal in response to Slit binding. It is plausible, therefore, that Rig-1 simply sequesters Slits and keeps it from interacting with Robo1 and Robo2 on pre-crossing axons. The large amount of Rig-1 expressed on pre-crossing commissural axons compared to the low levels of Robo1 and Robo2 proteins observed on pre-crossing axons would ensure that this sequestration takes place efficiently. Taking away one copy of *Rig-1*, for example, does not lead to any observable phenotype, indicating that half the amount of *Rig-1* in commissural neurons is sufficient to inhibit Slit signaling. Interestingly, this model would account for the fact that Robo2-expressing commissural axons, which do not coexpress Rig-1, are also unable to cross the midline in Rig-1 mutants. The Slit sequestration model would explain both the cell-autonomous and non-cell-autonomous effects of Rig-1 in commissural axons. One prediction from this model is that in the absence of a cytoplasmic domain, Rig-1 would still be able to inhibit Slit responsiveness in pre-crossing axons. One observation has been made, however, which does not favor this model. When a truncated form of Rig-1 (Rig-1ΔC) is overexpressed in chick commissural axons using the beta-actin promoter, some axons fail to cross the midline in a manner similar to what is

observed in the mouse Rig-1 mutants (Appendix C), a phenotype that is not observed upon overexpression of full-length Rig-1 in the chick spinal cord. The partial penetrance of this phenotype makes it difficult to interpret but it can be argued that if a truncated Rig-1 acts as a dominant negative, it is unlikely that full-length Rig-1 serves simply to sequester Slits away from Robo1 and Robo2 on pre-crossing axons.

Model 2: The signaling cross-talk model

The divergent cytoplasmic domain of Rig-1 may indicate a difference in the type of signaling in response to Slit rather than a lack of signaling as postulated above. In response to Slit binding, Rig-1 may usurp a subset of the signaling components required for a repulsive response to Slit, thus rendering the Robo1-dependent Slit repulsion ineffective. Alternatively, signaling downstream of Rig-1 may alter the environment inside the growth cone such that Robo1 no longer responds to Slits as repellents. As discussed extensively in the introduction, this may involve a change in cyclic nucleotide levels in the growth cone for example. Analysis of the Slit mutants indicates that no single Slit is absolutely required for Rig-1 function since none of the single or double Slit mutant combinations have a Rig-1-like phenotype. Overexpression of full-length Rig-1 in chick commissural neurons led to axons recrossing the midline (see appendix B). Immunostaining of the myc-tagged Rig-1 indicates that it is expressed in both pre- and post-crossing portions of commissural axons. These observations imply that misregulation of Rig-1, such that it remains expressed on post-crossing commissural axons, leads to downregulation of Slit responsiveness

and a phenotype similar to what is observed in the Slit triple mutants. The lack of recrossing in chick embryos overexpressing Rig-1ΔC also implies that the cytoplasmic domain of Rig-1 is required for its ability to downregulate Slit responsiveness. However, this model does not account for the non-cell-autonomous effects of Rig-1 on Robo2-expressing commissural axons. One possible explanation for this as discussed in chapter three is that Robo2-positive commissural axons are followers that must fasciculate onto Robo1-positive pioneering commissural axons in order to cross the floor plate. The lack of complete rescue of midline crossing in the Rig-1;Robo1 double mutant mice is inconsistent with this hypothesis unless another, as yet unidentified Slit receptor, is also regulated by Rig-1 in pre-crossing commissural axons. Analysis of the Robo1;Robo2 double mutant and the Rig-1;Robo1;Robo2 triple mutant mice could, in principle, provide evidence for the existence of this putative other Slit receptor.

Further structure-function analysis of the Rig-1 receptor along with the identification of potential interacting proteins will be required to determine the mechanism by which the Rig-1 cytoplasmic domain inhibits Slit responsiveness through Robo1. Although in ovo chick electroporation of Rig-1 constructs has proven informative, it is not the ideal system to ask these questions. This is due in part to the lack of controlled expression of transgenes in specific cell populations in the spinal cord as well as to the lack of complete penetrance of the phenotypes. Another possibility involves electroporating Rig-1 constructs in mouse embryos early in development when commissural axons are just growing

out and culturing these embryos in vitro until commissural axons have begun to cross the midline (Sturm and Tam, 1993). The ability to use mouse embryos will be useful not only for Rig-1 gain-of-function studies similar to those previously described in the chick, but will also allow for rescue experiments using the Rig-1 knock-out mice. Testing the ability of truncated Rig-1 constructs to rescue midline crossing in the Rig-1 mutants will provide information as to the domains required for Rig-1 function in vivo. The availability of specific promoters that are expressed in subsets of commissural neurons, including the Rig-1 promoter itself, will also simplify interpretation (Gowan et al., 2001; Yuan et al., 2002). Finally, RNAi experiments in this system can be used to determine the effect of knocking-down potential Rig-1 interactors on commissural axon crossing of the midline. Together with standard biochemical methods to identify Rig-1 interacting proteins, the manipulation of mouse embryos in whole embryo culture can prove a powerful tool to determining the mechanism by which Rig-1 interferes with Slit responsiveness.

Future Direction 2: Mechanism of Rig-1 downregulation

Beyond understanding how Rig-1 works, we also need to determine how the effects of Rig-1 are turned off upon crossing. As described previously, Rig-1 appears to be downregulated on post-crossing commissural axons. This phenomenon has been observed for another commissural axon-specific protein, TAG-1, which is preferentially shed from commissural axons once they have crossed the floor plate through an unknown mechanism. Shedding of the Netrin

receptor DCC, through the actions of a metalloprotease, has also been described, although it is unclear whether that is regulated by the floor plate(Galko and Tessier-Lavigne, 2000). Culturing of commissural neurons in vitro and testing for the presence of the Rig-1 ectodomain in the media bathing these neurons would determine whether Rig-1 undergoes a similar type of regulation as TAG-1. Similarly, examining Rig-1 localization in mice lacking a floor plate (Gli2-/- for example) would provide evidence for the floor plate playing an instructive role in regulating Rig-1 localization.

As described previously, electroporation of full-length Rig-1 under the control of the beta-actin promoter in the chick spinal cord led to mislocalization of the myc-tagged protein on the post-crossing portion of commissural axons. There are several explanations for this observation. (1) There may be fundamental differences between commissural axons in the mouse and the chick. The TAG-1 homolog Axonin-1, for example, is not localized to pre-crossing commissural axons (reference). (2) The strong expression from the beta-actin promoter may overwhelm the regulatory machinery required to keep Rig-1 from the post-crossing portion of the axons. (3) Finally, Rig-1 localization may not be regulated at the protein level but rather at the mRNA level. Whole mouse embryo culture experiments will help determine which of these possibilities are correct. The use of the Rig-1 promoter to drive Rig-1 expression in commissural neurons will ensure that normal levels of the protein are expressed and will help determine whether the pre-crossing localization of Rig-1 is regulated at the protein level.

Future Direction 3: Mechanism of Robo upregulation

While Rig-1 is downregulated from post-crossing commissural axons, Robo1 and Robo2 are upregulated dramatically. The localization of Robo1 and Robo2 in the vertebrate spinal cord is reminiscent of what has been observed in the Drosophila CNS, with Robo being expressed primarily on the longitudinally-projecting portion of commissural axons (Kidd et al., 1998). Since no vertebrate Comm homolog has yet been identified it is unclear how Robo1 and Robo2 proteins are specifically targeted or retained on the post-crossing portion of commissural axons. We do know, however, that Rig-1 is not involved in regulating Robo localization in vivo. Therefore, it is possible that a Comm-like mechanism does exist in vertebrates. The recent identification of specific domains in Comm that are responsible for binding to Robo and for its interaction with the ubiquitination machinery that is required to keep the Comm-Robo complex in internal compartments may make it easier to identify functional homologs to Comm in vertebrates (Keleman et al., 2002; Myat et al., 2002).

It is also possible that in vertebrates Robo1 and Robo2 are regulated via a completely different mechanism. As described in the introduction, the receptor EphA2 is specifically targeted to post-crossing commissural axons through local translation in growth cones only after the floor plate has been crossed in a mechanism that is controlled by the 3'UTR of the *EphA2* mRNA (Brittis et al., 2002). Characterization of the 3'UTR of the *Robo1* and *Robo2* mRNAs may shed some light into the mechanism by which these two receptors are regulated.

Finally, whole embryo culture experiments can once again be used to determine if the Robo1 and Robo2 proteins are expressed properly on their own or if they need the presence of a 3'UTR for their proper localization. Depending on the results of those experiments, structure-function analysis of the Robo1 and Robo2 receptors can indicate which regions are required for their localization.

Future Direction 4: Consequence of Robo1 and Robo2 upregulation on Netrin sensitivity

In vitro experiments using hindbrain explants have shown that commissural axons lose their ability to respond to Netrin as an attractant upon crossing the floor plate (Shirasaki et al, 1998). Later experiments using Xenopus spinal neuron cultures provided a possible mechanism for this loss of netrin-responsiveness by showing that in the presence of Slit, Robo1 silenced DCC signaling (Stein et al, 2001). These results provided a nice correlation between the upregulation of the Slit response in post-crossing commissural axons and the downregulation of the Netrin response in these same axons. With the *Robo1* and *Robo2* mutant mice, we now have a system in which to test whether Slit/Robo-dependent silencing of DCC takes place in vivo. Hindbrain explants from these mutant mice can be cultured in vitro and confronted with a heterologous source of Netrin (Netrin-expressing COS cells or floor plate). Commissural axons on one side of the explant are labeled with DiI and their response to the Netrin source on the contralateral side of the floor plate is assessed. If the silencing model is correct, we would predict that some axons would continue to be attracted to Netrin in either the *Robo1* or *Robo2* mutants.

The generation of the *Robo1;Robo2* double mutant mice might be required to assess the full implications of DCC silencing on commissural axon guidance in vivo.

Future Direction 5: Role of Robo1 and Robo2 in determining the lateral position of commissural axons after crossing the floor plate.

Like what has been observed in Drosophila, Robo1 and Robo2 appear to label distinct subsets of commissural neurons both when visualized by in situ hybridization and antibody immunostaining techniques. As described in chapter two of this thesis, analysis of the Robo1 and Robo2 single mutants indicates that these two receptors respond differentially to Slit emanating from the midline. Robo1-positive commissural axons appear to be less repelled by Slits and thus can grow closer to the floor plate in the ventral funiculus. Meanwhile, Robo2-positive commissural axons are observed primarily in the lateral funiculus, further away from the floor plate. This difference in sensitivity to Slits may have to do with a difference in signaling downstream of the two receptors. Robo2, for example, is lacking the conserved motif CC3, which has been shown to interact with Dock/Nck in response to Slit binding (Fan et al., 2003) as well as the kinase Abl (Bashaw et al., 2000). Phosphorylation of Robo by Abl has been shown to dampen the response of Robo to Slit as a repellent, such that a loss of Abl binding may lead to a hyperactive form of the receptor. Comparing the effectors downstream of both Robo1 and Robo2 will provide a way to explain the difference in responsiveness to the floor plate of commissural axons expressing either of these receptors.

The distinct levels of sensitivity to Slit by Robo1- and Robo2-positive commissural axons may also have to do with the expression of Slit itself by these two distinct populations. In addition to being expressed by the floor plate, Slit2 is also expressed by subpopulations of commissural neurons especially at later ages of development when commissural axons have crossed the midline (Brose et al., 1999). Although double in situs need to be done to confirm this, it appears as though the commissural neurons expressing Slit2 are likely to also express Robo1 but not Robo2. It has previously been shown that Ephrin ligand expression in retinal ganglion cells sharpens the response of the Eph receptors on these axons to Ephrin ligand in the superior coliculus (Feldheim et al., 2000). Therefore, it is possible that Slit2 expression on Robo1-positive commissural axons dampens the responsiveness of Robo1 to Slits emanating from the floor plate. This possibility can be tested in vitro by assessing whether Slit2 mutant commissural axons have a differential response to low levels of Slit. Similarly, we can assess whether Robo1-positive axons tend to grow further away from the floor plate in Slit2 mutants.

Future Direction 6: Identification of the Midline-derived signal(s) that orchestrates the switch in commissural axon responsiveness.

Finally, one large question remains unanswered in any system in which commissural axon guidance is studied. It is well-established that the floor plate plays an instructive role in regulating the switch that takes place within commissural growth cones as they cross the midline. In the absence of a floor plate, commissural axons make pathfinding errors on the contralateral side of the

neural tube. Several molecules known to be regulated in commissural axons relative to the floor plate are misregulated in mutants that lack floor plate cells. However, no specific molecular component has been identified that regulates any of these phenomena. It is possible that one molecular cue could govern all aspects of the switch or different pathways are activated or inhibited by distinct cues emanating from the floor plate. Analysis of Rig-1 mutant mice has revealed that several molecules such as TAG-1, L1, Robo1 and Robo2 are properly localized to either the pre-turning or post-turning portion of commissural axons. This may indicate that a transient interaction with the floor plate is sufficient to activate the mechanisms that regulate the localization of these proteins.

Conclusion

The analysis of the role that Robo receptors play in vertebrate commissural axon guidance answers some questions about the details of the switch in responsiveness that takes place as commissural axons cross the floor plate but it also raises many questions. Robo1, Robo2 and Rig-1 can now be used as tools to better understand this switch and more generally how pathways downstream of individual axon guidance cues are exquisitely regulated spatially and temporally as axons grow out during development.

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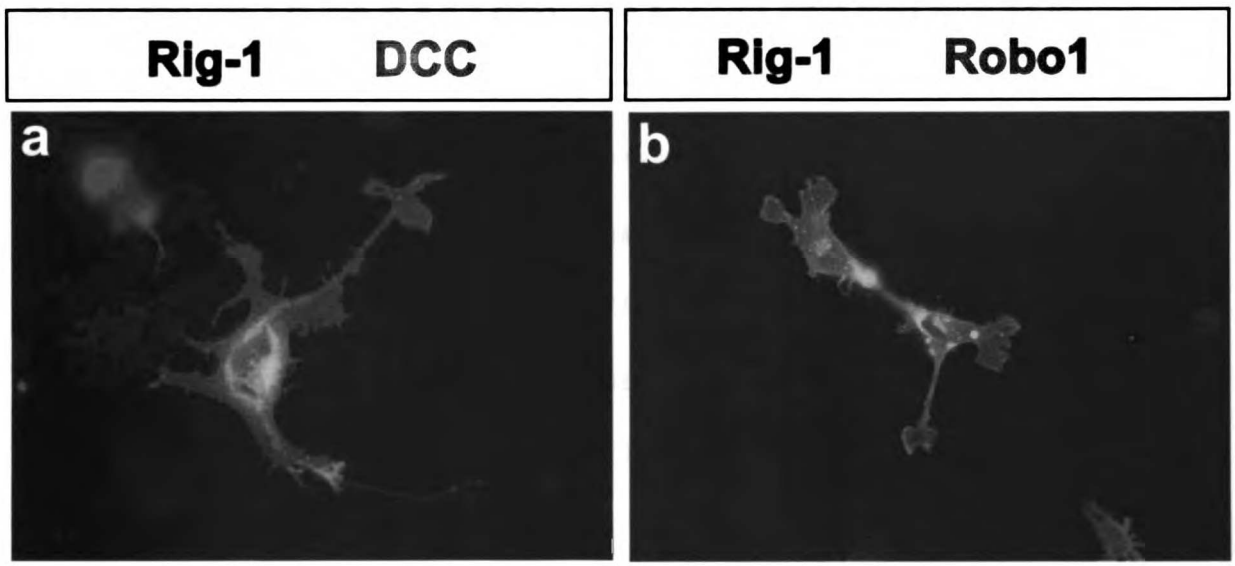
Appendix A: Rig-1 and Robo1 colocalize on the plasma membrane when expressed in COS cells.

Rig-1-myc (in green in both a and b) was expressed in COS cells along with DCC-HA (red in a) or Robo1-HA (red in b) and visualized with the appropriate antibodies.

(a) When Rig-1 and DCC are co-expressed in COS cells, very little overlap is observed with DCC being more prevalent on the plasma membrane and Rig-1 primarily in internal compartments (although some is observed on the plasma membrane). These patterns are not any different from what is observed when each protein is expressed on its own (data not shown).

(b) When Rig-1 and Robo1 are co-expressed in COS cells, significant overlap is observed between these two proteins on the plasma membrane as well as to some extent in internal compartments. Once again, these patterns are identical to what is observed when each protein is expressed on its own (data not shown). It should also be noted that co-expression of Robo1 with Rig-1 does not lead to the localization of Robo1 exclusively to intracellular compartments.


Rig-1 DCC
Rig-1 Robo1
a
b

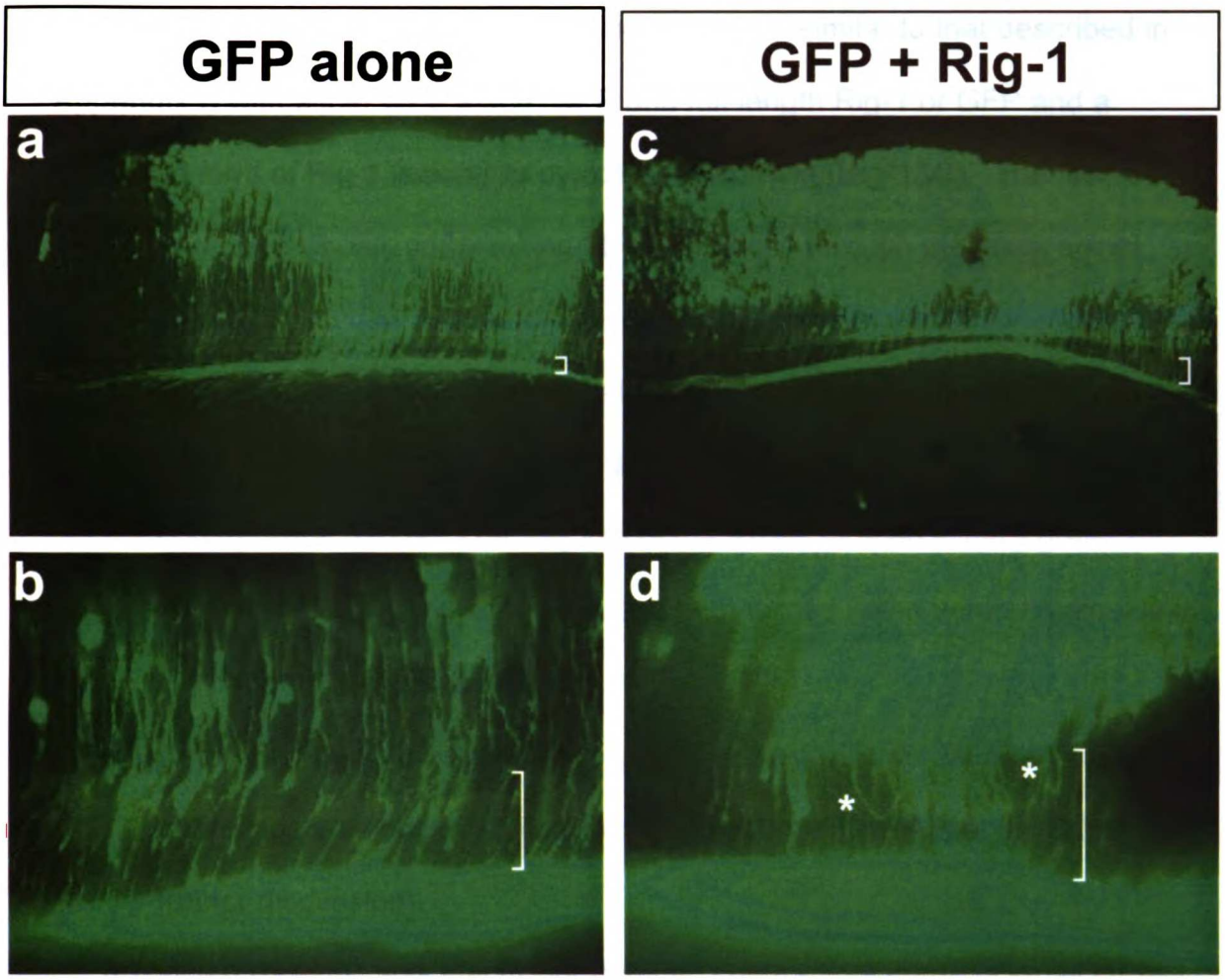
Appendix B: Overexpression of Rig-1 in the chick spinal cord causes commissural axons to recross the floor plate.

Stage 17 chick embryos were electroporated in ovo with either GFP cDNA alone or GFP and Rig-1 cDNA in a 5:1 ratio. The embryos were then allowed to develop to stage 25 at which time many commissural axons have crossed the floor plate. Then, the spinal cords were dissected out in an open book configuration and the electroporated axons were visualized by virtue of the GFP marker. Due to the inherent polarity of DNA, only one side of the spinal cord is electroporated in these experiments. Experiments not shown here had previously shown that the GFP marker faithfully reproduced the expression of the Rig-1 protein.

(a and b) When GFP was electroporated alone, the GFP-positive axons were observed to follow trajectories that had been previously ascribed to commissural axons. The majority of the axons labeled initially grew ventrally, crossed the floor plate parallel to each other and then turned in the contralateral spinal cord. Very few axons were observed growing close to the floor plate in the ipsilateral spinal cord (a) and no recrossing axons were ever observed in the floor plate (b).

(c and d) When Rig-1 was electroporated into chick spinal cords, a significant number of axons were observed growing close to the floor plate on the electroporated side of the embryo indicating that axons were either failing to cross or recrossing the midline. Upon closer inspection, many axons were

observed recrossing the floor plate in Rig-1 overexpressing embryos (asterisks in d).


GFP alone
GFP + Rig-1
a
c
b
d
*
*

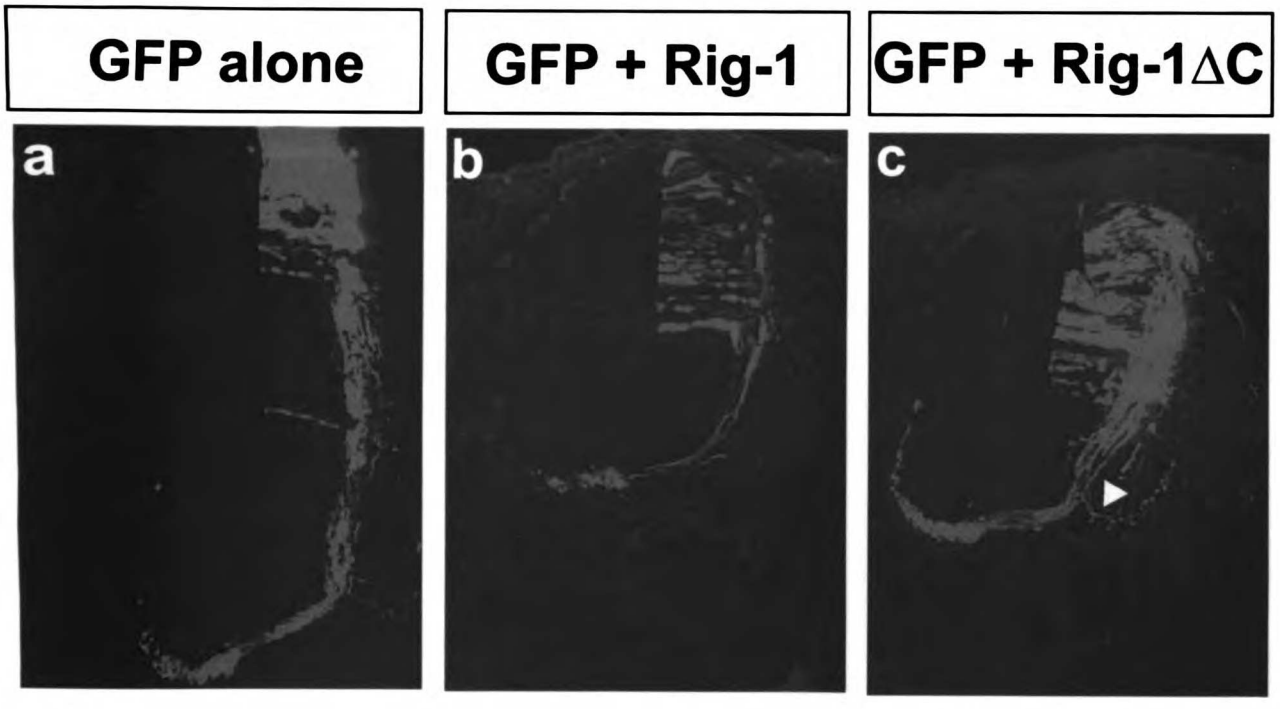
Appendix C: Overexpression of Rig-1ΔC in the chick spinal cord causes commissural axons to fail to cross the floor plate.

Chick embryos were electroporated using a protocol similar to that described in Appendix B with either GFP alone, GFP and full-length Rig-1 or GFP and a truncated form of Rig-1 lacking its cytoplasmic domain (Rig-1ΔC). The labeled axons were then visualized by taking 20 μm sections through the chick spinal cords.

(a) When GFP was electroporated alone, the GFP-positive axons were observed to follow trajectories that had been previously ascribed to commissural axons. The majority of the axons labeled initially grew ventrally, crossed the floor plate and then turned in the ventral funiculus in the contralateral spinal cord.

(b) When Rig-1 was electroporated into chick spinal cords, no significant phenotype was observed in transverse sections (see Appendix B for further discussion).

(c) When Rig-1ΔC was electroporated into chick spinal cords, a small subset of axons were observed growing ventrally but then veering away from the floor plate and heading toward the lateral side of the spinal cord perhaps to join axons in the lateral funiculus (arrowhead).


GFP alone
GFP + Rig-1
GFP + Rig-1ΔC
a
b
c