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Report

Alternative Splicing of the Robo3 Axon Guidance Receptor Governs the Midline Switch from Attraction to Repulsion

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SUMMARY

Alternative splicing provides a means to increase the complexity of gene function in numerous biological processes, including nervous system wiring. Navigating axons switch responses from attraction to repulsion at intermediate targets, allowing them to grow to each intermediate target and then to move on. The mechanisms underlying this switch remain poorly characterized. We previously showed that the Slit receptor Robo3 is required for spinal commissural axons to enter and cross the midline intermediate target. We report here the existence of two functionally antagonistic isoforms of Robo3 with distinct carboxy termini arising from alternative splicing. Robo3.1 is deployed on the precrossing and crossing portions of commissural axons and allows midline crossing by silencing Slit repulsion. Robo3.2 becomes expressed on the postcrossing portion and blocks midline recrossing, favoring Slit repulsion. The tight spatial regulation of opponent splice variants helps ensure high-fidelity transition of axonal responses from attraction to repulsion at the midline.

INTRODUCTION

Developing axons grow over long distances by navigating a series of intermediate targets along their trajectory to their final destination. Axons are attracted to intermediate targets, but upon arriving they must switch their responsiveness to cues at the targets so they are no longer attracted and instead are repelled away; this plasticity of responses allows axons to move on to the next leg of their trajectory (Dickson, 2002; Tessier-Lavigne and Goodman, 1996). Despite the importance of this switch for accurate long-range axon guidance, the mechanisms underlying it remain only partially understood. The midline of the central nervous system has provided a well-studied example of an intermediate target at which axons switch their responses. The axons of commissural interneurons grow toward the midline in response to attractants, including netrins and, in vertebrates, Sonic hedgehog (Charron et al., 2003; Dickson, 2002; Tessier-Lavigne and Goodman, 1996). These axons are initially insensitive to repellents also present at the midline, including Slit proteins; upon crossing the midline, a switch occurs such that these axons lose responsiveness to the attractants and instead become repelled by the repellents, so that the axons can leave the midline and then never recross (Kaprielian et al., 2001; Kidd et al., 1999; Long et al., 2004; Shirasaki et al., 1998; Zou et al., 2000).

The switch from attraction to repulsion requires coordinated control of the expression level as well as the activity of guidance receptors on the axon surface. For example, as axons approach the midline, receptors of the DCC family mediate the attractive action of netrins (Dickson, 2002), while the expression level of Robos, receptors for Slits, is kept low in order to minimize Slit repulsion (Kidd et al., 1998a; Long et al., 2004). Upon crossing, however, the axons upregulate Robo expression and become Slit responsive (Kidd et al., 1998a; Long et al., 2004), and Robo activity in turn contributes to switching off DCC attraction (Stein and Tessier-Lavigne, 2001).

A variety of mechanisms have been suggested to control the expression and activity of guidance receptors during midline crossing (reviewed in Garbe and Bashaw, 2004), including protein trafficking (e.g., in *Drosophila*, the Commissureless protein prevents Robo from localizing to the axon surface before midline crossing [Keleman et al., 2002; Kidd et al., 1998b]), local protein synthesis (e.g., the EphA2 receptor appears to be locally translated only in postcrossing axons [Brittis et al., 2002]), and the formation of receptor complexes (e.g., a Robo/DCC interaction in postcrossing axons can inhibit DCC activity [Stein and Tessier-Lavigne, 2001]).

Alternative pre-mRNA splicing provides an additional means to increase the complexity of gene function by producing protein products with a spectrum of properties from a single coding locus (Black, 2000; Graveley, 2001). This mechanism has been shown to diversify the functions of a number of genes regulating neural function and neural wiring (reviewed in Lipscombe, 2005; Li et al., 2007). For example, significant alternative splicing has been documented for neurexins and neuroligins, molecules implicated in establishing synaptic specificity (Craig and Kang, 2007). An extreme example of the molecular diversity generated

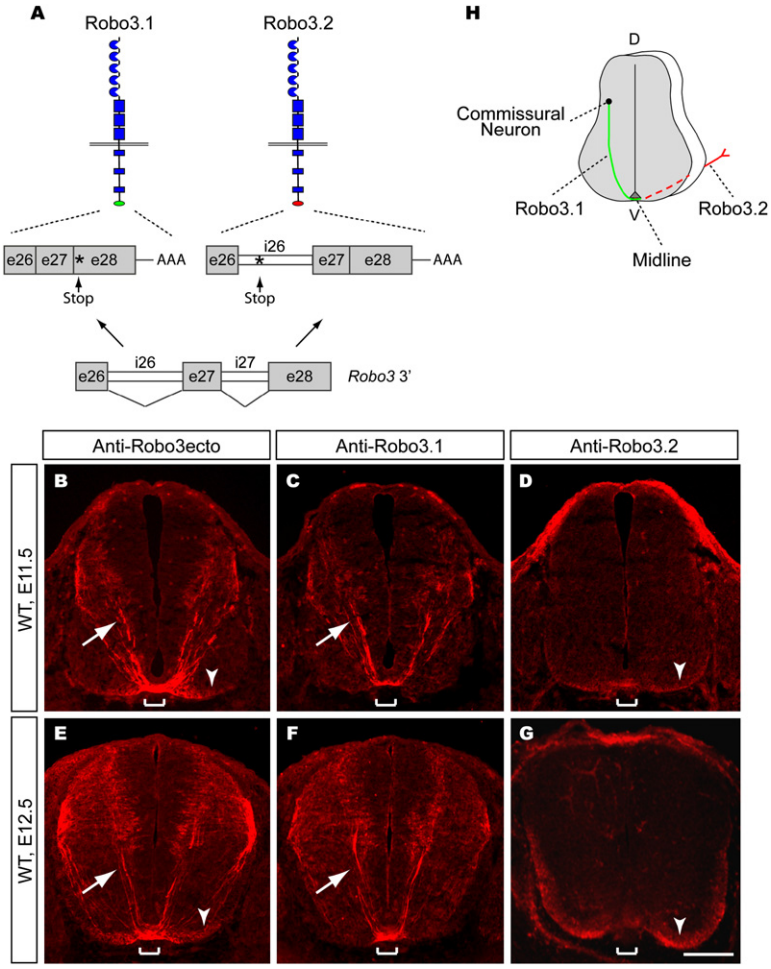

Figure 1. Alternative Splicing of *Robo3* Generates Two Isoforms with Distinct Expression Patterns in Commissural Axons

(A) Schematic of the structures of Robo3 isoforms and the alternative splicing of the 3′ coding sequence ("e" denotes an exon, "i" an intron, and "*" the stop codons utilized in the two transcripts).

(B–G) Immunohistochemistry was performed using three Robo3 antibodies on transverse sections of the wild-type spinal cord at E11.5 (B–D) and E12.5 (E–G). (B and E) An antibody against the extracellular domain of Robo3 (anti-Robo3ecto), which recognizes both Robo3 isoforms, detected epitopes in precrossing axonal domains (arrows), at the midline (brackets), and in the postcrossing ventral fiber tracts (arrowheads). (C and F) A Robo3.1-specific antibody detected epitopes in axonal domains that are precrossing and in the midline region. (D and G) A Robo3.2-specific antibody detected epitopes mostly on postcrossing axonal domains. Scale bar, 100 μm.

(H) Schematic showing the location of the cell body of a commissural neuron and the trajectory of its axon, as well as the axonal domains of Robo3 isoform expression, as seen in a transverse section view. D, dorsal; V, ventral.

by alternative splicing is provided by the neural recognition molecule Down's syndrome cell adhesion molecule (DSCAM), which, in *Drosophila*, can exist in tens of thousands of isoforms arising from alternative splicing (Zipursky et al., 2006). The evidence indicates that each isoform binds tightly to itself but not to the other isoforms, a remarkable degree of homophilic recognition that provides the basis for self-repulsion of the processes of individual neural cells (Hattori et al., 2007; Matthews et al., 2007; Wojtowicz et al., 2007). Whether alternative splicing is also used to diversify the function of guidance molecules at intermediate targets has not, however, been described.

Here, we report that alternative splicing provides a crucial level of control over the function of a guidance receptor used to navigate the midline intermediate target in the spinal cord. Previously, we described an unexpected role for the third mammalian Robo, Robo3 (also known as Rig1 [Yuan et al., 1999]), in regulating commissural axon guidance at the spinal cord midline. Contrary to the other two vertebrate Robos, Robo1 and Robo2, whose expression, as described above, is low before crossing and high after crossing (Long et al., 2004), Robo3 is expressed at high levels before crossing and is gradually downregulated after crossing (Sabatier et al., 2004; see also Figure 1B). Also contrasting with the other two Robos, Robo3 activity keeps commissural axons non-responsive to midline Slits before crossing, and it does so by silencing Slit repulsion that would otherwise be mediated by Robo1 and Robo2 (Sabatier et al., 2004). As a result, in *Robo3* knockout mice commissural axons are prematurely responsive to midline Slit repellents before midline crossing and are therefore totally unable to cross the midline (Marillat et al., 2004; Sabatier et al., 2004; also see Figure 2J). Mutations in human *Robo3*, discovered in patients with horizontal gaze palsy and progressive scoliosis syndrome (HGPPS), were similarly found to result in significant midline crossing defects (Jen et al., 2004).

Here, we report an unexpected complexity and sophistication in the function of Robo3 that arises from alternative splicing of its pre-mRNA. We found that two isoforms of Robo3, Robo3.1 and Robo3.2, are generated by differential intron retention, a less common form of alternative splicing. These isoforms have opposite functions: one favors, whereas the other blocks, midline crossing. In addition, they are tightly spatially regulated, with Robo3.1 expressed on the precrossing segment of commissural axons and Robo3.2 on the postcrossing segment. This abrupt transition helps ensure a high fidelity in the growth cone's switch from attraction to repulsion at the midline. The tight spatial localization of splice isoforms of a guidance receptor documented here thus provides another mechanism for the nervous system to control intricate guidance events, helping explain the accuracy of axon guidance required for proper brain wiring.

RESULTS

Alternative Splicing of the *Robo3* Coding Sequence Generates Two Isoforms

In the course of sequencing a collection of *Robo3* full-length cDNA clones from mouse dorsal spinal cord, we discovered that the 3′ end of the mouse *Robo3* mRNA can be alternatively

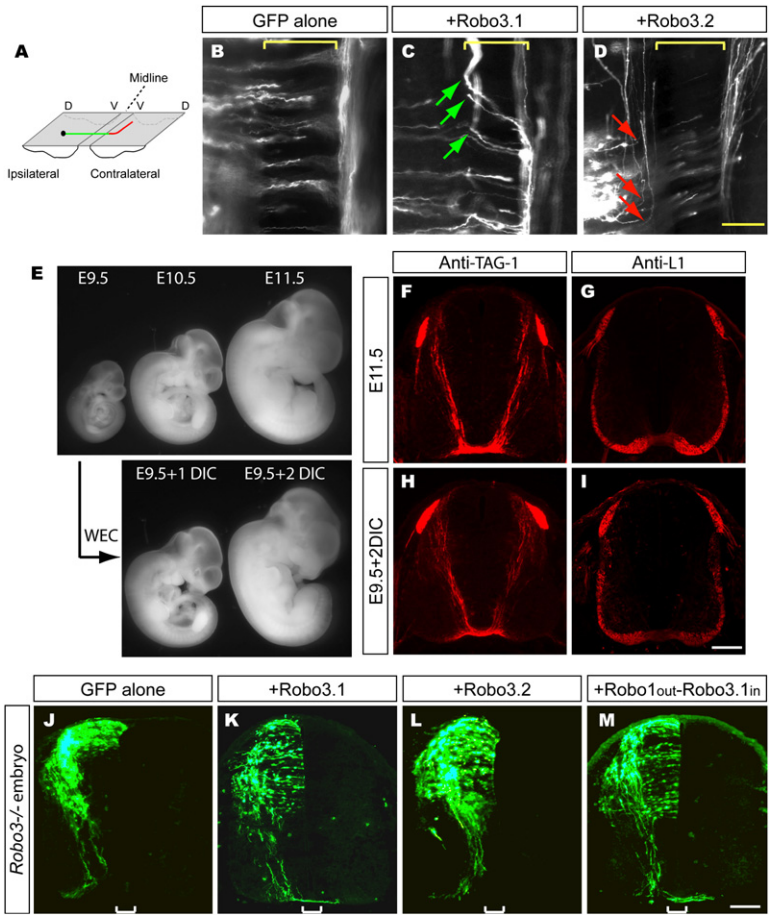

Figure 2. Forced Expression of Two Robo3 Isoforms Causes Distinct Commissural Axon Guidance Phenotypes

(A) Schematic showing an "open-book" preparation of the spinal cord. D, dorsal; V, ventral.

(B–D) The *Robo3* isoforms, together with *gfp*, were introduced into chick commissural axons by in ovo electroporation, and spinal cords were examined in an open-book view. In these and all the following open-book images, spinal cords were oriented with the ventral midline in the center, transfected neuronal cell bodies sitting to the left (not shown), and the rostral end of spinal cords pointing up. Brackets represent the span of the midline. All axons expressing GFP alone (B) appeared normal, entering and crossing the midline, then turning rostrally. When *Robo3.1* was overexpressed, all axons entered the midline normally but many then recrossed (green arrows in [C]). Ectopic expression of *Robo3.2*, however, led to some axons turning away from the midline before entering (red arrows in [D]). Scale bar, 20 μm.

(E–I) A modified whole-embryo culture (WEC) method was used to express *Robo3* coding sequences. The cultured mouse embryos (bottom panel of [E], [H], and [I]) were comparable to those that developed normally in vivo (top panel of [E]–[G]) in their gross morphology, size, and axonal marker expression after 1 or 2 days in culture (DIC). Scale bar, 100 μm.

(J–M) Rescue of the commissural axon guidance defect seen in *Robo3*$^{-/-}$ embryos by exogenous Robo3 proteins. GFP alone (J) or Robo3.2 (L) did not restore midline crossing in *Robo3*$^{-/-}$ embryos. In contrast, many *Robo3* mutant axons were able to project to the contralateral side in embryos transfected with cDNAs for *Robo3.1* (K) or *Robo1*$_{out}$-*Robo3.1*$_{in}$ (M). Scale bar, 100 μm.

spliced to generate a second isoform that is distinct in its carboxy terminus from the previously published cDNA ([Yuan et al., 1999]; NCBI accession number AF060570; Figure 1A). We term the new isoform Robo3.1 and the original one Robo3.2 (based on their expression: see below). The novel *Robo3.1* transcript arises from splicing of exon 26 to exon 27 and then to exon 28 (an analogous splicing event is seen for the human *Robo3* locus [Jen et al., 2004]); the resulting 78 amino acid peptide is highly enriched in arginine/serine dipeptide repeats (see Figure S1 available online). The previously described *Robo3.2* transcript, in contrast, arises from retention of the intron between exons 26 and 27. The intronic sequence contains a stop codon and encodes a 43 amino acid peptide that is quite distinct from the Robo3.1 carboxy terminus but shows conservation between rodents and humans (Figure 1A and Figure S1).

Switching of Robo3 Isoform Expression at the Midline

The mRNAs for the two isoforms are both expressed in the spinal cord during the period of commissural axon growth to and across the midline, i.e., from embryonic day 9.5 (E9.5) to 12.5 (E12.5) (see below). Immunohistochemistry with isoform-specific antibodies on spinal cord sections revealed strikingly distinct localization patterns (Figures 1B–1G and Figures S1 and S2A–S2F; the signals were specific, because they were absent in the *Robo3* mutant, which loses both isoforms [Figures S2G–S2I]). Robo3.1 is highly expressed on commissural axons before and during midline crossing (Figure 1C, Figure S2B, and data not shown). Its expression is rapidly switched off after midline crossing because it was not seen at all in ventral fiber tracts (Figure 1C and Figure S2B). In contrast, Robo3.2 expression was detectable only at a low level at E11.5 and increased in expression at E12.5, and at both ages was seen exclusively on the portion of commissural axons distal to the midline (Figure 1D and Figure S2C and Figure 1G and Figure S2F). The expression patterns of these two isoforms together appear to account to a considerable extent, and perhaps entirely, for the pattern we visualized previously using a pan-Robo3 antibody, which showed high expression before crossing (attributed to Robo3.1) and expression after crossing that was eventually downregulated (attributed to Robo3.2) (Sabatier et al., 2004; Figures 1B and 1E and Figures S2A and S2D). The immunoreactivity in the ventral fiber tracts seen with the pan-Robo3 antibody appeared slightly more extensive than with the Robo3.2 antibody, suggesting either that there is an additional Robo3 isoform expressed after crossing or that the pan-Robo3 antibody is more sensitive. Nonetheless, there is a precise switch in expression on commissural axons from Robo3.1 before and during midline crossing to Robo3.2 after crossing (Figure 1H).

Robo3 Isoforms Exhibit Opposite Activities in Commissural Axon Guidance

To functionally characterize the two isoforms, we ectopically expressed them individually in chick commissural neurons by in ovo electroporation (see Experimental Procedures). Unlike the endogenous Robo3 in mice, Robo3 expressed from the transgene (possessing the β-actin promoter and the β-globin 3′UTR) was expressed throughout the length of chick commissural axons (Figures S3A and S3B). When Robo3.1 is persistently expressed before and after crossing, all GFP-expressing axons entered the midline normally, but many inappropriately recrossed (Figure 2C and Figure S6; 13/13 embryos). This result supports our model (Sabatier et al., 2004) that Robo3—more specifically, the Robo3.1 isoform—can repress midline repulsion. Remarkably, overexpression of Robo3.2 had the opposite effect: many axons failed to enter the midline and remained on the ipsilateral side, whereas those that did cross did not recross (Figure 1D and Figure S6; 14/14 embryos; the same phenotype was also observed with a full-length *Robo1* cDNA [data not shown]). These axons appear to be misrouted commissural axons rather than ipsilateral projection axons, as the latter assume a longitudinal trajectory at more dorsal positions, far from the midline.

To further test the activities of the two Robo3 isoforms, we investigated their abilities to rescue the murine *Robo3/Rig1* knockout phenotype by introducing each of them back into the mutant. This was achieved by electroporating *Robo3.1* and *Robo3.2* constructs into the spinal cord of embryonic day 9.5 (E9.5) mouse embryos and culturing the embryos in vitro for 2–3 days, to a developmental stage equivalent to E11.5–E12.5, using a modification of existing whole-embryo culture methods (Figures 2E–2I; for detailed description and protocol, see Experimental Procedures). Plasmids introduced in this way were expressed in commissural neurons on the electroporated side during the period of growth to and across the midline. For example, a *gfp* plasmid introduced into *Robo3* mutant embryos revealed the previously described noncrossing phenotype (Figure 2J). *Robo3.1* cDNA could restore midline crossing in the mutant to a large extent (Figure 2K and Table S1). In contrast, no axons were seen at the midline or on the contralateral side when *Robo3.2* was introduced back into the mutant (Figure 2L and Table S1), even though the protein, like Robo3.1, was expressed precrossing (Figures S3C and S3D). These data together support the idea that Robo3.1 (but not Robo3.2) can repress premature repulsion during commissural axon midline crossing.

Further support was obtained by repeating in wild-type mouse embryos the forced expression experiment that we had performed in chicks, using the mouse whole-embryo culture technique, with similar results (Figures S4 and S6). We also found that a chimeric protein comprising the cytoplasmic domain of Robo3.1 fused to the extracellular and transmembrane regions of Robo1 ($Robo1_{out}$-$Robo3.1_{in}$) was able to rescue midline crossing when introduced into *Robo3* mutants (Figure 2M and Table S1), thus focusing attention on the cytoplasmic domain of Robo3.1 in mediating its function.

Requirement of Robo3.1 for Midline Crossing

The expression patterns of Robo3.1 and Robo3.2 and their activities in forced expression and rescue experiments suggest a model in which Robo3.1 expressed on commissural axons before and during midline crossing serves to suppress Slit repulsion, allowing them to enter the midline (Figure 5A), whereas Robo3.2 expressed on the axons after crossing may contribute to preventing midline crossing, either by directly mediating Slit repulsion, by interfering with residual Robo3.1 on the axons, or by some other means (Figure 5B). To test this model further, we performed isoform-specific knockdown in vivo using small interfering RNA (siRNA) oligonucleotides in the whole-embryo culture system. For *Robo3.2*, we used an siRNA targeting a sequence in intron 26, which is not found in *Robo3.1* (Figure 3A). We had less flexibility for *Robo3.1* because all sequences in *Robo3.1* are also found in *Robo3.2*. We reasoned, however, that an siRNA specifically targeting the junction between exons 26 and 27 might inhibit *Robo3.1* expression without affecting *Robo3.2* mRNA (Figure 3A). A positive control was provided by a pan-*Robo3* siRNA targeting a sequence in the common exon 9, and a negative control by introducing five mismatch mutations into this pan-*Robo3* siRNA ($Robo3_{mismatch}$). In control experiments using transfected COS cells, pan-*Robo3* siRNA caused a complete absence of both isoforms (but did not affect Robo1, DCC [the netrin receptor], or the surface proteins TAG-1 and L1 [Figure 3B and data not shown]), whereas $Robo3_{mismatch}$ had no effect on any of these proteins (Figure 3B and data not shown). *Robo3.1* and *Robo3.2* siRNAs were able to selectively reduce the levels of their respective targets with no detectable reduction of the other, although the *Robo3.1* siRNA was only partially effective (Figure 3B).

We tested these siRNAs in vivo by electroporating into E9.5 embryos followed by whole-embryo culture. The pan-*Robo3* siRNA caused commissural axons to steer away from the midline before entering and to navigate on the ipsilateral side (Figures 3C and 3G), phenocopying the effect of genetic removal of *Robo3* (Sabatier et al., 2004), whereas the negative control siRNA ($Robo3_{mismatch}$) did not cause any detectable phenotype (Figures 3D and 3G). The *Robo3.1*-specific siRNA led to the same midline crossing defect as did pan-*Robo3* siRNA, but to a lesser degree, as a few axons crossed (Figures 3E and 3G); this partial penetrance may reflect the fact that this siRNA was not completely effective in suppressing Robo3.1 (Figure 3B). Although *Robo3.2* siRNA was able to efficiently knock down Robo3.2 in COS cells, it did not affect the ability of the axons to enter and cross the midline (Figures 3F and 3G), consistent with our model (Figure 5).

Because the transfected axons were intermingled with an excess of untransfected axons, we could not ascertain by immunohistochemistry whether the proteins targeted by the siRNAs were downregulated, which we expect to be occurring given the phenotypes. To test the specificity of the siRNA effect, we therefore performed rescue experiments. The midline crossing defect seen with the pan-*Robo3* siRNA was reversed by coelectroporating with a *Robo3.1* cDNA mutated in the siRNA target sequence such that its transcript is resistant to the siRNA (Figures S5B and S5E) but not by the wild-type construct whose transcript is sensitive (Figures S5A and S5E) nor by a *Robo3.2* construct engineered so that its transcript is resistant to the pan-*Robo3* siRNA (Figures S5C and S5E). A construct encoding the $Robo1_{out}$-$Robo3.1_{in}$ chimera, which does not contain the

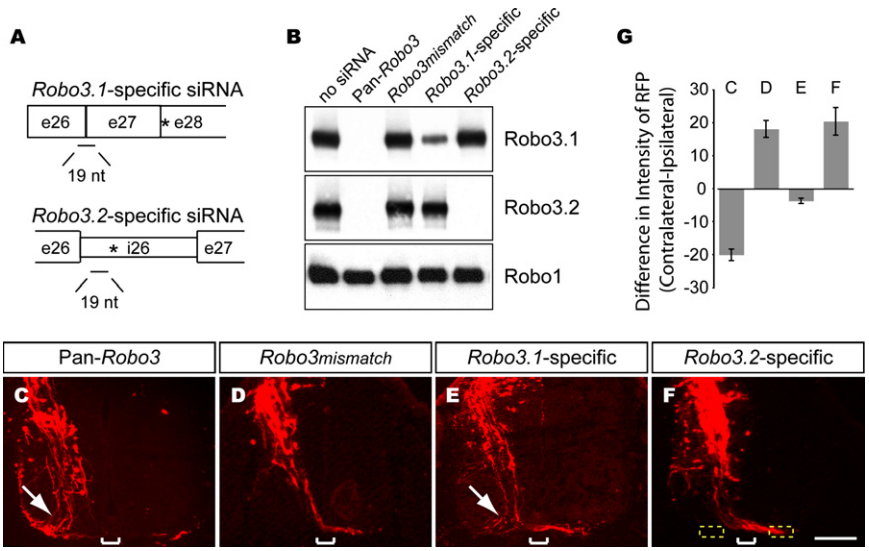

Figure 3. siRNA-Mediated Knockdown Implicates Robo3.1 in Allowing Midline Crossing

(A) Targets of *Robo3.1*- and *Robo3.2*-specific siRNAs. The *Robo3.1* target sequence corresponds to 10 nt in exon 26 and 9 nt in exon 27 at the exon-exon junction. The *Robo3.2* target sequence corresponds to 19 nt in intron 26.

(B) siRNA-mediated knockdown of Robo3 isoforms in COS-1 cells, assessed by western blotting of COS cell extracts.

(C–F) siRNA-mediated knockdown of Robo3 isoforms in commissural neurons. siRNAs were introduced into one-half of the spinal cord by electroporation at E9.5, and embryos were cultured using the whole-embryo culture technique. Pan-*Robo3* siRNA caused premature turning of the axons (RFP labeled) from the midline (C), whereas $Robo3_{mismatch}$-treated axons appeared normal (D). *Robo3.1* siRNA had a similar effect to that of pan-*Robo3*, although to a lesser degree (E). *Robo3.2* siRNA treatment did not affect midline entry of commissural axons (F). Arrows point to axons that remained abnormally on the ipsilateral side. Boxed areas in (F) indicate regions used for quantification in (G). Scale bar, 100 μm.

(G) Quantification of data in (C)–(F). For each condition, the intensity of RFP expression from the contralateral axons (those that had crossed the midline) was compared to that from the ipsilateral axons (those that failed to cross). The difference, plotted on the y axis in arbitrary units, is shown as mean ± SEM.

pan-*Robo3* siRNA target sequence, was also able to rescue the RNAi effect (Figures S5D and S5E), as predicted.

These results indicate that siRNA-mediated knockdown of Robo3 in vivo was efficient and specific and that Robo3.1 appears to account for most or all of the Robo3 activity that is necessary for inhibiting premature repulsion from the midline.

Robo3.2 Helps Expel Postcrossing Commissural Axons

Given its expression pattern and overexpression phenotype, we considered the possibility that Robo3.2 contributes to repelling commissural axons out of the midline. Previous studies had, in fact, suggested that Robo1 and Robo2 may not account fully for the repulsion of postcrossing commissural axons by Slits, because only a small fraction of axons stalls within the midline in *Robo1* or *Robo2* single mutants, whereas more extensive stalling was seen in *Slit1;Slit2;Slit3* triple mutants (lacking all midline Slits) (Long et al., 2004). We generated a double mutant lacking both *Robo1* and *Robo2* (which are linked on chromosome 16; see Experimental Procedures) but found that the amount of stalling in the double mutants (Figures 4B and 4E and data now shown) was that expected from additive effects of removing *Robo1* and *Robo2* and still considerably less than in the *Slit* triple mutant (Long et al., 2004). Despite this, when we bred the *Robo1;Robo2* double mutant to the *Robo3* mutant, the failure of midline crossing seen in the *Robo3* mutant (Figure 4C) was rescued to a considerable extent (Figure 4D), supporting the model that the low levels of Robo1 and Robo2 on precrossing axons contribute significantly to mediating the premature repulsion by Slits in the *Robo3* mutant.

The modest stalling phenotype of the *Robo1;Robo2* double mutant compared to the *Slit1;Slit2;Slit3* triple mutant supports the existence of another Slit receptor(s) that mediates midline repulsion. We therefore tested the possibility that Robo3.2 collaborates with Robo1 and Robo2 in this function. To knock down *Robo3.2* without affecting *Robo3.1* expression, we carried out RNAi against *Robo3.2* in the *Robo1;Robo2* double mutant embryos (which were electroporated at E9.5 and cultured for 3 days). In these embryos, some axons were seen to recross the midline (Figure 4G and Figure S6, phenotype seen consistently in 4/5 embryos examined); this phenotype was not seen with the $Robo3_{mismatch}$ siRNA (Figure 4F and Figure S6, 6 embryos studied). No recrossing was observed, either, when the *Robo3.2* siRNA was electroporated into wild-type embryos, in which Robo1 and Robo2 function were intact (data not shown). The recrossing phenotype was also observed in some axons in the triple *Robo* mutant (Figures 4D and 4E) and in the triple *Slit* mutant (Long et al., 2004). Taken together, these results support the model that Robo3.2 contributes to expelling axons from the midline in collaboration with Robo1 and Robo2.

DISCUSSION

Alternative splicing plays an important role in regulating many processes in neural development, including cell fate determination, neuronal recognition, and synaptogenesis (Li et al., 2007), and defects in splicing are being increasingly implicated in various neurological disorders (Licatalosi and Darnell, 2006). Our results indicate that alternative splicing of axon guidance receptors provides a level of sophisticated regulation that can be utilized to ensure the fidelity of growth cone switching from attraction to repulsion at intermediate targets.

Distinct Spatial Localization of Opponent Splice Isoforms

We found that alternative splicing gives rise to Robo3 isoforms with opposite functions. Robo3.1 favors midline crossing, and genetic analysis (Sabatier et al., 2004 and this study) indicates that it does so by suppressing Slit repulsion mediated by

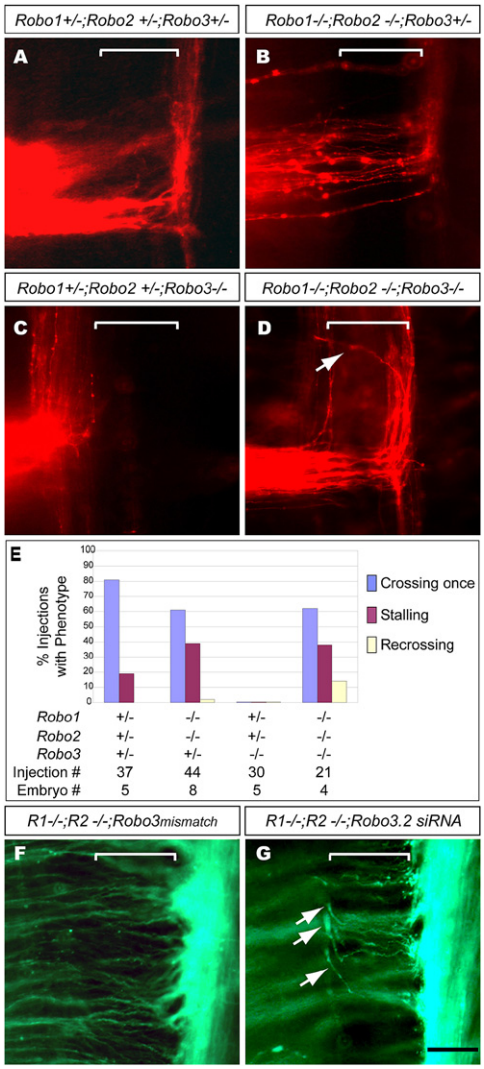

Figure 4. Robo3.2 Also Contributes to Commissural Axon Guidance

(A–D) DiI tracing of commissural axons in compound *Robo* spinal cords (shown in an "open-book" view). In *Robo1*$^{+/-}$;*Robo2*$^{+/-}$;*Robo3*$^{+/-}$ embryos (A), which served as controls, virtually all commissural axons crossed the midline. In *Robo1*$^{-/-}$;*Robo2*$^{-/-}$;*Robo3*$^{+/-}$ mutants (B), a few axons stalled within the midline (quantified in [E]; note that in [B] the stalling was best seen by focusing up and down and that the stalled profiles are not readily seen in the single plane of focus that is displayed). In *Robo1*$^{+/-}$;*Robo2*$^{+/-}$;*Robo3*$^{-/-}$ mutant spinal cords (C), no axons were seen within the midline or on the contralateral side. However, when all six *Robo* alleles were mutant, many axons were able to enter the midline (D), though many failed to exit (E) and some recrossed the midline ([E], indicated with arrow in [D]).

(E) Quantification of phenotypes in (A)–(D).

(F and G) In *Robo1;Robo2* double mutants, electroporation of the *Robo3.2* siRNA caused some commissural axons (GFP labeled) to recross the midline (indicated with arrows in [G]), whereas the control siRNA, *Robo3*$_{mismatch}$, had no effect (F). Scale bar, 20 μm.

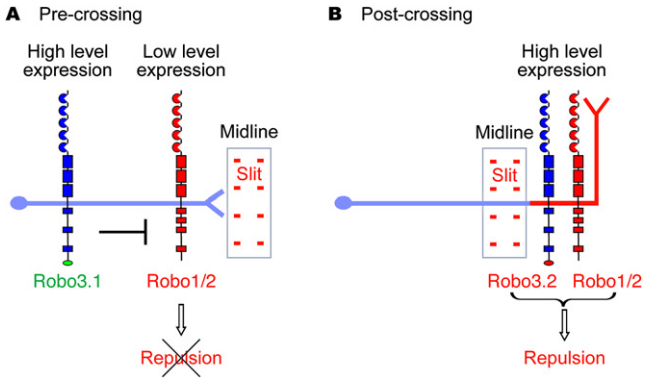

Figure 5. Model of Robo3 Activities in Commissural Axon Guidance at the Midline

(A) Before axons reach the midline, Robo3.1 is highly expressed on axons, and its activity prevents the activation of Robo1 and Robo2, present at low levels on the axons. As a result, axons are unresponsive to Slit repellents and thus enter the midline.

(B) Upon entry, a switch in Robo3 expression occurs: Robo3.2 expression is switched on, but the protein is restricted to axonal regions distal to the midline, where Robo3.1 is excluded. Consequently, Robo1, Robo2, and Robo3.2 act in concert to help expel axons to the contralateral side.

Robo1 and Robo2. In contrast, Robo3.2 blocks midline crossing (in gain-of-function experiments) and normally collaborates with Robo1 and Robo2 to block midline recrossing (as shown in loss-of-function experiments), thus favoring Slit repulsion (Figure 5). It remains to be determined whether Robo3.2 produces its effect by functioning directly as a repellent Slit receptor or by some other means (e.g., blocking residual Robo3.1 on postcrossing axons, or potentiating Robo1 or -2 function). Importantly, our results also demonstrate a remarkable and unexpectedly tight level of spatial localization of the two splice isoforms, with Robo3.1 expressed precrossing and Robo3.2 postcrossing, consistent with their functional roles in enabling midline crossing and blocking midline recrossing, respectively.

These findings extend to midline guidance themes highlighted in previous studies of alternative splicing in the nervous system. Several other neuronal genes have been documented to undergo regulated splicing to produce functionally distinct isoforms. For example, two splice variants of the EphA7 receptor, which differ in their C termini, exhibit distinct activities in regulating cell adhesion during neural tube closure (Holmberg et al., 2000). Alternative splicing to yield isoforms with distinctive subcellular localization properties has also been documented. For example, alternative splicing of the NR1 subunit of NMDA receptors generates two isoforms with distinct carboxy termini that accumulate differentially at the synapse in an activity-dependent manner (Mu et al., 2003). The splicing of the potassium channel Kv3.1 pre-mRNA gives rise to two isoforms, with Kv3.1a prominently expressed in axons within certain neuronal populations and Kv3.1b mostly expressed in the soma and dendrites of these neurons (Ozaita et al., 2002). It will be of interest to examine whether discrete spatial localization of splice isoforms contributes to the function of other axon guidance receptors implicated in switching from attraction to repulsion at intermediate targets.

Regulation of Expression of the Robo3 Splice Isoforms

It is intriguing to consider how the two Robo3 isoforms are confined to appropriate axonal segments. The simplest mechanism would be for there to be a temporal switch in splicing, with the

Robo3.1 transcript made first, followed at an appropriate time by the *Robo3.2* transcript. However, by quantitative RT-PCR, we found a roughly constant ratio of the two transcripts in the spinal cord during the period of commissural axon growth to and across the midline (Figure S7A). In situ hybridization studies similarly suggest that the two transcripts appear in parallel in commissural neurons (Figure S7B–S7K), arguing against a temporal switch.

Alternative mechanisms to a temporal switch include one or more of the following: (1) constitutive translation of the two transcripts followed by selective trafficking to (or removal from) the two axon segments, (2) a temporal switch in translation of the two transcripts in the cell bodies (with *Robo3.1* translated while the axon is extending to the midline and *Robo3.2* after it has crossed), (3) transport of the transcripts into axons followed by selective translation of *Robo3.1* in the precrossing axon segment and *Robo3.2* in the postcrossing segment. As a variation on the third possibility, the *Robo3.2* transcript could be transported down the axons but spliced selectively in the precrossing portion to yield *Robo3.1*, followed by local translation (this mechanism would require splicing within the axons, which has not so far been demonstrated). At present, we cannot distinguish fully between these mechanisms, although the absence of detectable transcripts in the axons (Figures S7B–S7K) tends to argue against models involving local translation.

Additional Splicing Events

Previous studies have suggested the existence of other splice variants of Robos and Slits (Challa et al., 2005; Clark et al., 2002; Dalkic et al., 2006; Little et al., 2002; Tanno et al., 2004), raising the possibility that alternative splicing might be widely involved in regulating Slit/Robo signaling. Additional splicing events in the *Robo3* pre-mRNA have been previously described (Camurri et al., 2005; Yuan et al., 1999), but their functional consequences are largely undefined. One particularly interesting splicing event in the *Robo3* 5′ coding sequence has been shown to generate two different amino termini (the Robo3A and Robo3B isoforms) with distinct Slit-binding activities (Camurri et al., 2005). Our immunolocalization and loss-of-function experiments did not distinguish between A and B isoforms. In the gain-of-function and rescue experiments, the cDNAs we used encoded the A isoforms, which, strictly speaking, should be referred to as *Robo3A.1* and *Robo3A.2*. The finding in human patients with the HGPPS syndrome of a familial missense mutation in the signal sequence of Robo3A (Jen et al., 2004) supports the functional importance of the Robo3A isoforms in both mice and humans. The relative contributions of A and B isoforms remain to be defined.

Contribution of Slit/Robo Signaling to Leaving the Midline and Mechanism of Action of Robo3

Our results, particularly using the newly derived *Robo1;Robo2* double knockout, also help flesh out the specific contribution of Slit/Robo signaling to entering and leaving the midline. As we have shown, removal of *Robo3.1*, either by gene knockout or by siRNA knockdown, prevents midline crossing. The finding in the triple *Robo* knockout that removal of both *Robo1* and *Robo2* can largely reverse the inability of commissural axons to enter the midline in the *Robo3* knockout, supports the theory that Robo3.1 functions to suppress Slit repulsion mediated by low levels of Robo1 and Robo2. After midline crossing, Robo3.2 collaborates with Robo1 and Robo2 to prevent midline recrossing. Our results (Figure 4) also suggest, however, that these three Robos do not account fully for midline repulsion and that additional Slit receptor(s) and distinct repulsive signaling pathways may collaborate with them to mediate expulsion from the midline; indeed, there is already evidence for a contribution from semaphorin3B/neuropilin-2 and ephrin/Eph signaling (Kadison et al., 2006; Zou et al., 2000).

The molecular basis for the divergent actions of the two Robo3 isoforms also remains to be elucidated. Robo3.1 could interfere with Robo1 and Robo2 function through direct physical interaction. However, we have not been able to detect such an interaction in transfected COS cells expressing Robo3.1 with either Robo1 or Robo2, whether or not Slit2 protein is added (data not shown). Alternatively, Robo3.1 may interfere with Robo1 and Robo2 signaling further downstream in the signaling pathway. Thus, further studies will be required to fully define the complement of receptors and signaling pathways that expel commissural axons from the midline.

EXPERIMENTAL PROCEDURES

See the Supplemental Data available online.

SUPPLEMENTAL DATA

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/58/3/325/DC1/.

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